UNBC UNIVERSITY OF NORTHERN BRITISH COLUMBIA

Natural Resources & Environmental Studies institute

"Our environment is our future"

BIODIVERSITY MONITORING AND ASSESSMENT PROGRAM 2013–2015

Final Report

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Cecil Creek. Photo credit: Mark Shrimpton

Contents

Executive Summary

The Biodiversity Monitoring and Assessment (BMAP) program was initiated in 2012 through independent research teams at UNBC, Archipelago Marine Research Ltd., and the Smithsonian Institution — all supported by Pacific Trail Pipelines Limited Partnership (PTPLP) and Kitimat LNG (the Project). A Contribution Agreement to fund the implementation of UNBC's contribution to the BMAP was signed in June 2013.

The BMAP was originally designed to respond to research questions before and during construction of the Pacific Trail Pipeline and the Kitimat LNG facility, and to allow the incorporation of findings into construction and operational practices for these and other development projects. The BMAP was intended to make a complementary contribution to the Project by monitoring and evaluating the status and trends of ecological units and habitats within the Project footprint and areas of influence. The BMAP was also intended to contribute to the knowledge and understanding of the biodiversity in the region as the Project transects through four ecological units: marine, coastal, mountain, and interior. Because of delays in the final investment decision (FID), the program was suspended at the end of 2015, and only those portions of the research protocols that could be conducted before construction have been completed.

This final report summarizes UNBC's contributions to the BMAP before construction for six UNBC-led Protocols: the Aquatic Communities Protocol, the Tailed Frog as a Model for Understanding Connectivity within Aquatic, Riparian, and Terrestrial Ecosystems Protocol, the Soil Integrity and Revegetation Protocol, the Anadromous Movement and Estuarine Habitat Use of Cutthroat Trout Protocol, the Animal Movement Restoration Protocol, and the High Elevation Terrestrial Invertebrate and Lichen Restoration Protocol. For each of these Protocols, many of the main contributions related to the testing of alternate (usually DNA based or environmental DNA based) techniques for the assessment of species biodiversity.

Overall, our studies suggest diverse aquatic and terrestrial ecosystems in the vicinity of the proposed pipeline and LNG facility. Within the six protocols, we uncovered numerous indicators of ecosystem function that could be used during pipeline construction and restoration. In the Aquatic Communities Protocol, we identified benthic invertebrate and fish

biodiversity using both traditional sampling methods and taxonomic identification as well as using novel DNA-based sampling methods. In the Coastal Tailed Frog Protocol, we evaluated standard tailed frog sampling methods and used DNA-based water sampling to identify coastal tailed frog distribution along the pipeline right-of-way. In addition, the Tailed Frog Protocol group described coastal tailed frog population genetics in order to effectively use this species as an indicator of ecosystem connectivity after disturbance. The Soil Integrity and Restoration Protocol group used a microcosm experiment in combination with field sampling to describe and identify the components of the soil microbiome as indicators of soil resilience and function. The Cutthroat Trout Protocol looked at the movement patterns and population genetics of cutthroat trout in the vicinity of the natural gas terminal as an indicator of aquatic ecosystem function near the plant construction site. The majority of the Animal Movement and Restoration Protocol was designed to evaluate post-construction animal movement and restoration, but the pre-construction work focused on the development of DNA-based methods to assess small mammal movements across the right-of-way, and to assess the optimal time for using DNA to assess populations of larger mammalian herbivores. Finally, the High Elevation Protocol outlined baseline invertebrate biodiversity along the pipeline route over Nimbus Mountain and explored promising techniques to restore high elevation ecosystem function through restoration of biocrust communities above the treeline.

Chapter 1 – UNBC Biodiversity Monitoring and Assessment Program Overview

Tatsunai Creek. Photo credit: Mark Shrimpton

1.1. BMAP origins

The Biodiversity Monitoring and Assessment Program (BMAP) was designed to be a state-of-the-art monitoring and assessment program developed in collaboration with Kitimat LNG and Pacific Trails Pipeline Partnership and three partner organizations: the University of Northern British Columbia (UNBC) in Prince George BC, Archipelago Marine Research Ltd. (Archipelago) in Victoria BC, and the Center for Conservation Education and Sustainability of the Smithsonian Conservation Biology Institute (Smithsonian) in Washington DC. The BMAP was initiated at UNBC by Apache Canada Ltd. on behalf of Kitimat Liquid Natural Gas (KLNG) terminal and the Pacific Trail Pipeline (PTP) projects in late 2012. In 2013, Chevron Canada Ltd. joined Apache Canada in the ownership of Kitimat LNG terminal and the PTP pipeline projects,

collectively called the Kitimat LNG Operating General Partnership (hereafter the Project), and joined the newly formed BMAP steering committee. Chevron ultimately took on sole representation at the BMAP steering committee on behalf of Kitimat LNG Operating General Partnership.

The original BMAP was planned as a long-term continuing research program to inform ongoing best-practices in project development, construction, and management, but because of delays leading to FID by the Kitimat LNG Operating General Partnership for the pipeline and natural gas terminal in northern British Columbia (BC), the BMAP was discontinued in early 2015. Archipelago finalized their projects at the end of 2014 and the Smithsonian Institution finalized their projects in January 2015. Kitimat LNG Operating General Partnership, represented by Chevron, extended the Contribution Agreement with UNBC until December 31, 2015 in order to finish the research projects that were ongoing. This report, therefore, covers UNBC's scientific contributions to the goals and objectives set out in the original BMAP proposal, including any progress up to December 31, 2015.

1.2. BMAP vision

From the outset, the BMAP vision was to develop a collaborative research program that would complement, but not overlap with, any legal or accommodation requirements of the Project. As such, the BMAP was intended to address potential applied research questions from the Project (both during construction and operation), as well as to provide a complimentary process to collect and report scientific information to be used for the development and application of best practices associated with pipeline construction and management. Across all of the original BMAP partners, the BMAP was developed to provide a contribution to protecting and managing the environment by monitoring and evaluating the status and trends of habitats, ecosystems, and species within the pipeline footprint and to provide management recommendations for maintaining ecosystem function — again above and beyond any legal requirements of the Project. The BMAP also aimed to contribute to the knowledge and understanding of the biodiversity in the pipeline project area as it transects through the marine, coastal, mountain, and interior ecological units.

The BMAP was initiated to provide additional insight through long-term monitoring and assessment in order to investigate, through a biodiversity lens, how current construction and operational techniques were working and what new techniques might be applied. The BMAP was developed to addresses critical questions that link Project activities to an understanding of the surrounding environment through research. The research was conducted according to Protocols — cohesive projects that were independently proposed by partner organizations and scientifically peer reviewed. UNBC proposed, and contributed to, six research protocols.

1.3. UNBC BMAP protocols

For each of the six UNBC-led BMAP protocols, we addressed the protocol goals and objectives (as outlined in our 2013 proposal) as far as was possible before pipeline construction and installation — in some cases significant portions of some protocol involved post-construction research and monitoring, but those have not been implemented. In this final report, we describe the research contributions within the six protocols developed and implemented by UNBC: the Aquatic Communities Protocol, the Tailed Frog as a Model for Understanding Connectivity within Aquatic, Riparian, and Terrestrial Ecosystems Protocol, the Soil Integrity and Revegetation Protocol, the Anadromous Movement and Estuarine Habitat Use of Cutthroat Trout Protocol, the Animal Movement Restoration Protocol, and the High Elevation Terrestrial Invertebrate and Lichen Restoration Protocol. Although dealt with in detail in subsequent chapters, here we provide an overview of the six protocols.

Aquatic Communities Protocol (Chapter 2)

The Aquatic Communities Protocol used several approaches to assess ecosystem function for a subset of streams that will be crossed during pipeline construction and installation in order to recommend restoration methods for different types of stream crossings. We used behavioral, ecological, physiological, and genetic methods to establish baseline biodiversity and to monitor aquatic invertebrate and vertebrate biodiversity. This Protocol combined in-stream monitoring of physical parameters with biological sampling to assess species biodiversity, food-web structure, species movement, and connectivity. In addition to

traditional taxonomic measures for biodiversity, this Protocol developed a suite of tools that allowed for rapid assessment of aquatic ecosystem function using molecular genetic techniques, including environmental DNA sampling (eDNA).

Tailed Frog as a Model for Understanding Connectivity within Aquatic, Riparian, and Terrestrial Ecosystems Protocol (Chapter 3)

One of the potential effects of landscape-level linear features is as a barrier to connectivity, particularly in riparian habitats and for small terrestrial vertebrate species that are not highly mobile. A combination of slow life history and habitat requirements suggests that the coastal tailed frog (*Ascaphus truei*) is sensitive to anthropogenic effects that reduce forest and riparian structure and alter stream hydrology. Given the sensitivity of tailed frogs to environmental change and their use of a range of aquatic and terrestrial communities, this species is a good indicator of changes in habitat connectivity for wet montane ecosystems.

The Tailed Frog Protocol included two integrated components designed to monitor and understand changes in connectivity of aquatic and terrestrial systems. In the first component, we investigated the movements of post-metamorphic tailed frogs at three existing Experimental Watersheds south of Terrace, BC and evaluated the efficacy and cost effectiveness of a range of monitoring methods. In the second component, we monitored the distribution of tailed frogs at locations where the pipeline bisects streams with known populations of frogs. This work applied a number of monitoring approaches for the post-metamorphic and larval life stages of tailed frog and included a novel approach that measures environmental DNA within the water course (i.e., environmental DNA). From the samples collected on and near the right-of-way (RoW), we analysed the population genetics of this species in its northern range in order to assess the effects of RoW construction on population connectivity. Finally, we assessed the habitat features associated with tailed frog populations in order to recommend effective riparian restoration practices after pipeline construction.

Soil Integrity and Revegetation Protocol (Chapter 4)

Soils are the naturally occurring materials that result from the interactions among parent geologic material, biota, climate, topography and time. They provide numerous

important ecosystem functions including: a medium for plant growth; habitat for soil organisms (soil microflora and fauna); a natural filter for water; a recycler of nutrients; and sources of unique biochemical compounds and genetic materials. The soil environment has a high level of biodiversity, but soils are sensitive to disturbance. A disturbed soil environment cannot be completely restored to its pre-construction state, but ecosystem services provided by the soil can be the target of restoration.

The Soil Integrity and Revegetation Protocol aimed to identify ways to safeguard and monitor the ecosystem functions of soils through construction and operation of the pipeline. Initially, the research team set up a microcosm experiment in the Enhanced Forestry Laboratory at UNBC, using soil collected from the pipeline route that examined the influence of soil type and disturbance on microbial community structure using environmental DNA analysis, environmental RNA analysis, and functional soil enzyme assays. In addition, the team investigated key soil properties and processes (i.e., chemical, physical and biological) that are indicative of soil health through a series of small-scale experimental disturbances applied to a field plot on the pipeline RoW near Summit Lake, north of Prince George, BC.

Anadromous Movement and Estuarine Habitat Use of Coastal Cutthroat Trout Protocol (Chapter 5)

Estuarine residency is an important life-history stage for most anadromous salmonids. The coastal cutthroat trout (*Oncorhynchus clarkii clarkii*) is an anadromous fish with an extended nearshore marine residency period, and one known to be sensitive to anthropogenic activities affecting freshwater habitat quality.

This Protocol assessed ecosystem function in the Bish Creek estuary and adjacent near-shore and marine environments by monitoring coastal cutthroat trout. We captured and tagged cutthroat trout in the vicinity of the Bish Creek estuary and near-shore environments adjacent to the KLNG plant construction site. Using two complementary datasets (acoustic telemetry and population genetic analyses), we monitored the physical movement and habitat use for tagged fish as well as the cutthroat trout population genetics structure in the wider Kitimat Arm. This

information provided insight into the potential effects of the LNG facility on this sensitive and understudied indicator species.

Animal Movement Restoration Protocol (Chapter 6)

Linear corridors have the potential to both fragment habitats and to create pathways for the movement of invasive species — the fragmentation of natural habitats has long been recognized as a threat to biodiversity. In order to test the potential effects of a linear corridor and to recommend effective mitigation actions, the Animal Movement Restoration Protocol group tested methods that could be used to track animal movement including the extraction DNA from non-invasive hair and pellet samples. In anticipation of proposed post-construction monitoring, the research team compiled annotated bibliographies on: the application of large mammal fecal DNA collection for animal movement assessment; and the efficacy of exclosure use to monitor vegetation recovery and the effects of herbivores on restoration activities.

High Elevation Terrestrial Invertebrates and Lichen Restoration Protocol (Chapter 7)

The flora and fauna of high-elevation environments are potentially particularly sensitive to disturbance whether natural or related to anthropogenic in origin. Most of the high-elevation pipeline route is covered with lichens and slow-growing alpine plants. In addition, this area is at risk for invasion by non-endemic species via temporary access roads being built during construction. Restoration of the vegetative cover on the high-elevation RoW, work areas, and roads will be a challenging and necessary task, during construction. The High Elevation Protocol research team, therefore, conducted initial surveys of the terrestrial macroinvertebrates as indicators of pre-construction ecosystem function. The group also developed methods to restore biological soil crusts and lichens on disturbed substrate by using both greenhouse experiments and field experiments on Nimbus Mountain.

1.4. Report structure

Each of the UNBC protocols is addressed individually in chapters 2–7. Each chapter begins with an introduction to the protocol and then is followed with a section that summarizes

the research approach and contributions. In Chapter 2, the Aquatic Communities Protocol Chapter, is preceded by a general description of study streams that are referred to in the subsequent sections of that Chapter. One of the strengths of the BMAP program was its focus on ensuring the scientific rigor of the research — an outcome that is ultimately judged through peer-reviewed publications. Therefore the main findings from each protocol are reported on in separate Sections, each eventually intended for submission to scientific journals. Each chapter concludes with a literature cited section specific to that chapter.

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We gratefully acknowledge the valuable support and feedback from Sheryl Maruca, Lynn McBrien, and Maria Hartley at Chevron Corporation throughout the Biodiversity Monitoring and Assessment Program. We would also like to acknowledge our collaborators and colleagues at Apache Corporation, Archipelago Marine Research Ltd., and the Smithsonian Institution for their contributions and feedback during the program.

1.5. Literature cited

Gillingham MP. 2013. Biodiversity Monitoring and Assessment Program Phase 1: Completion Report. Natural Resources and Environmental Studies Institute, UNBC. June 2013, 153pp.

Chapter 2 – Aquatic Communities Protocol

Principal Investigators: Dezene Huber, Mark Shrimpton, Daniel Erasmus, Brent Murray, Lisa Poirier, Allan Costello, Jeanne Robert

A thriving aquatic community in Cecil Creek. Photo credit: Mark Shrimpton

2.1. Introduction

Rivers and streams are complex open-transport systems with physical properties that vary over time and space (Knighton 1984). Riparian zones at the boundary of the terrestrial and aquatic systems have dynamic physical properties that change in response to fluvial and non-fluvial disturbances (Gregory et al. 1991). Small headwater streams lack the energy to determine their own path, but stream morphology and the riparian zone is determined by roughness elements, valley gradient and hydrology. As streams become larger, the energy

within the system is greater and capable of creating floodplains and modifying the landscape through which they flow (Knighton 1984; Church 1992). The same processes also affect morphology of the riparian zone. Small high-gradient streams have riparian zones composed of large and rough sediments, mostly of non-alluvial origin (Church 1992). Further downstream, riparian zones are predominantly composed of sediments deposited from the alluvial channel (Knighton 1984).

Figure 2-1 – The riparian zone of Chist creek along the proposed pipeline right-of-way. Photo credit: Mark Shrimpton.

Riparian zones are important for ecosystem function as they provide important physical and biological features needed for productivity and aquatic biodiversity (Figure 2-1). Many species found in small streams depend on the energy that is provided by organic matter from

the riparian zone, but also the habitat complexity that is created from physical inputs such as large woody debris. Larger river systems are less dependent on riparian inputs for productivity, but the riparian zone plays an important role in stabilizing fluvial processes in river systems. Construction of the 463-km long Pacific Trails Pipeline (PTP) will affect riparian areas where the right-of-way (RoW) runs adjacent to rivers and streams and where it crosses these aquatic systems. We, therefore, proposed to evaluate the potential effect of the PTP on the aquatic community function at sites where the pipeline crosses rivers and streams. Because of delays in pipeline construction beyond the UNBC BMAP, we focused on method development and baseline biodiversity assessment in order to predict best practices for ensuring aquatic ecosystem resilience during and after construction, when it occurs.

Baseline biodiversity in temperate streams includes the aquatic invertebrates and fish that are intimately associated with bottom substrates. Many benthic invertebrates depend on gravel and cobble of stream beds to provide spaces for attachment, protection, feeding, and the interstitial flow of water for oxygen consumption (Wood and Armitage 1997). There is also a link to vertebrate fauna as many invertebrate species that reside on coarse substrates are preferred prey for fish (Figure 2-2).

Environmental impact assessments on freshwater systems rely in large part on assessments of shifts in aquatic invertebrate populations and diversity. Benthic macroinvertebrates, in particular, are useful for assessing site-specific effects: they tend to stay in a small area, are relatively easy to identify to Family level or beyond, and species assemblages include multiple trophic levels and tolerances, permitting assessment of cumulative effects (Barbour et al. 1999). Aquatic invertebrates, particularly the immature stages of certain groups of insects, are also very responsive to changes in water quality such as levels of particulate matter or dissolved oxygen.

Figure 2-2 – An example of benthic substrates in Cecil Creek that form the foundation for aquatic community development. Photo credit: Mark Shrimpton

Traditional impact assessments make use of what is commonly referred to as "EPT" (i.e., Ephemeroptera [mayflies], Plecoptera [stoneflies], and Trichoptera [caddisflies]). These three groups are important members of stream ecosystems and food webs, but are also susceptible to a variety of environmental effects and disturbances. In some assessment methods, fly larvae (Diptera) are also included. Various count and ratio calculations of EPT and Diptera Families or species are considered to be accurate measures of overall stream health (Hilsenhoff 1988; Reif 2002). Much of the reason for relying on EPT ratios, beyond their known sensitivity to conditions, is the fact that traditional assessment methods have relied on highly trained personnel with taxonomical knowledge of various species groups combined with labor- and time-intensive work at a microscope. As such, detailed data collection on individual species is often not conducted, relying instead on gross counts and comparisons of large groups at the

Family or Order taxonomic levels. Although this has proven adequate, recent advances in the use of environmental DNA (eDNA) combined with massive taxonomic DNA barcoding efforts (e.g., the Barcode of Life project, www.barcodeoflife.org) and the growth of species-specific DNA sequence databases have allowed us to develop genetic methods for stream biodiversity assessment.

2.2. Research approach and outline of contributions

The Aquatic Communities Protocol implemented several approaches to study aquatic invertebrates and vertebrates around proposed pipeline stream crossings using behavioral, ecological, physiological and genetic methods. This Protocol combined in-stream monitoring of physical variables with biological sampling to assess species biodiversity, food-web structure, species movement, and connectivity. In addition to traditional taxonomic measures for biodiversity, we developed a suite of tools that allow for rapid assessment of aquatic ecosystem function using molecular genetic techniques, including eDNA.

In this Chapter, Section 2.3 describes the general physical and biological characteristics of the stream systems where we focused the sampling for the Aquatic Communities Protocol. This Section illustrates the unique characteristics of the sampled streams using tabulated summary data on measures such as benthic substrate, representative biological taxa, and stream water measures (i.e., average temperature, dissolved oxygen content, etc.).

Section 2.4 outlines a study that used the morphological identification of benthic invertebrates to establish baseline biodiversity and reference stream conditions at proposed RoW crossings for a subset of the study streams. Benthic invertebrates exhibit a wide array of responses to various types of environmental changes and are often used to assess stream quality and monitor responses to different types of disturbances such as infrastructure development (e.g., roads), forestry-related activities, and pollution. In this study, benthic invertebrate samples were processed, and specimens identified, according to industry standards. In addition, instream monitoring of physical attributes was conducted in concert with benthic invertebrate sampling to determine seasonal patterns in benthic invertebrate

populations. The objectives of Section 2.4 were to establish baseline benthic macroinvertebrate diversity and determine preliminary stream conditions of the study streams. Based on traditional measures of stream quality, such as the Hilsenhoff Biotic Index (HBI), study streams were found to be in very good condition.

Section 2.5 provides an in-depth description of previously unknown moth fly (Diptera: Psychodidae) in the streams along the pipeline RoW. Little is known of moth fly larvae taxonomy, life history and distribution in BC despite their use in stream quality assessments. Larvae collected during 2013 and 2014 were identified as belonging to a Genus called *Pnuemia*, which is currently only known from the Palearctic region. Due to the absence of *Pneumia spp.* in North American literature, specimens collected throughout North America have been incorrectly identified as species of the Genus *Pericoma.* These misidentifications arise from a lack of knowledge of moth flies in the Nearctic region and from the standard use of taxonomic keys found in secondary sources. After consulting with an expert on psychodid taxonomy, the true identity of the psychodid specimens collected in the study area was revealed. Specimens were sent for DNA barcoding to confirm its identity as *Pneumia spp.*, but a species-level match could not be made; the closest matches were identified only to Family (Psychodidae) or incorrectly to the Genus *Pericoma*. Elucidation of the species identity for this aquatic moth fly resolves previous taxonomic errors, rapidly permits the correct identification of the hundreds of misidentified specimens in DNA sequence databases, and provides a solid foundation for future studies of the life history, distribution range and sensitivity to disturbance of this familiar but unrecognized group of moth flies.

Not only can DNA barcoding be used to identify a single new species of moth fly in the study region, it is a molecular method that is particularly useful for benthic invertebrates in general because they often lack the morphological characters for distinguishing among dominant stream taxa (i.e., mayflies, caddisflies, stoneflies, and flies) and few keys available for the aquatic larval stage. In Section 2.6, DNA barcoding was, therefore, used to obtain species-level identifications for taxa present in study streams crossed by the RoW, confirm morphological identifications, and provide a reference library of cytochrome oxidase subunit 1 (COI) sequences (DNA barcodes) for comparison with the high-throughput sequencing results

generated by subsequent Aquatic Communities Protocol projects. A total of 221 species or barcode clusters were identified from 647 reference specimens sequenced, most of which could not be matched with species already present in BOLD. Species divergences in the study area were greater than average for North American benthic invertebrates, possibly due to geographic isolation from southern and eastern North American populations. DNA barcoding contributed significantly to establishing species-level baseline biodiversity in the study area.

Beyond single species barcoding, metabarcoding is a rapidly advancing field in environmental monitoring that incorporates next-generation sequencing (NGS) technology of the COI barcoding region to allow for the identification of hundreds to thousands of mixed taxa in bulk samples with far greater taxonomic resolution than is possible based on traditional methods that rely on morphological identification of specimens. In Section 2.7, DNA was extracted from the ethanol used to preserve bulk Surber samples of benthic invertebrates taken from nine systems where pipeline construction is planned. The COI sequences from the ethanol were amplified using primers that target a 157 basepair (bp) section of the standard barcode region, and were sequenced using the Ion Torrent NGS platform. Results revealed that metabarcoding is a superior alternative to traditional morphological assessments of benthic invertebrate communities and provides greater information on taxa richness, not only of benthic invertebrates but also other taxa not detected by traditional methods. Metabarcoding also provided insight into the baseline biodiversity of benthic invertebrates in study streams in a shorter period of time than would be possible using traditional morphological taxonomic approaches.

In Section 2.8, fish were captured from the Fraser River and Kitimat River watersheds in a subset of study streams to assess species composition, size of fish, and abundance. For tributary streams in both watersheds, the majority of fish caught were juvenile Salmonidae — most from a single species, *Oncorhynchus mykiss*. Cyprinidae were found in the larger rivers and creeks sampled, but were absent from the smallest systems sampled. Despite their wide geographic distribution, Cottidae were found in only four of 14 systems sampled. A few individuals from the Families Gadidae and Petromyzontidae were also caught in our sampling program. All fish caught were small and for Salmonidae were mostly young-of-the-year or one-

year-old fish. Cottidae were older; the youngest sculpin was four years old. Other Families were not aged. Abundance varied with sample date and among the streams sampled, but moderate numbers of fish were caught in all systems. Sizes and ages of fish caught indicate that the streams sampled represent important rearing habitat for young age classes and also smaller species.

In Section 2.9, we used three approaches to assess diets of fishes from a central BC stream; a traditional taxonomic method, sequencing of individual specimens, and next generation sequencing of homogenized gut contents. Fish diets were determined for rainbow trout (*Oncorhynchus mykiss*) and prickly sculpin (*Cottus asper*). Morphological identification of invertebrates identified prey items to Order for count and biomass data. Both species preyed on a wide range of aquatic invertebrates; however, trout also ate terrestrial invertebrates. Genetic analysis of ingested prey provided better resolution of specific taxa, but the next generation sequencing approach identified more diversity in the prey for both fish species. This result was likely due to our development of a new reverse primer for aquatic invertebrates in central BC. We have demonstrated an efficient approach for both prey analysis and biodiversity assessment using genetic analysis that complemented the traditional taxonomic method — which is still useful for assessing count and mass of prey items, ecological information that cannot be obtained through genetic analysis alone.

In Section 2.10, NGS was used to compare the presence of aquatic invertebrate taxa in environmental DNA samples from water and ethanol preservative from Surber samples from a central BC coastal stream, Chist Creek. We also examined the stomach contents of three fish: rainbow trout, Dolly Varden, and coastrange sculpin sampled in the creek. The greatest diversity of taxa was found in the water samples that also detected terrestrial species. Fish preyed on a subset of relatively few taxa identified in Chist Creek. The exception was Ephemeroptera, where over 50% of potential taxa identified in the eDNA were also found to be prey. Although Salmonidae and Cottidae typically occupy different depths in the water column, juvenile Salmonidae from Chist Creek appear to exploit benthic-dwelling invertebrates and eat a similar diet to Cottidae. The next step in our analysis will be to use vertebrate primers to assess the aquatic community and potential fish prey species, but also include samples from the

brief 2015 field season in order to understand potential inter-annual variation in aquatic invertebrate biodiversity and fish diet.

In Section 2.11, we used NGS to assess biodiversity of aquatic invertebrates from stream water samples. We validated our NGS technique using a mock community of 20 different aquatic invertebrates and were able to use NGS to detect the diversity of organisms present in the mock community. We then compared diversity of central and coastal BC streams invertebrates that were identified morphologically to environmental DNA collected from filtered water samples. Both methods indicated a diverse range of taxa from the streams, but a greater number of taxa were identified from sequencing stream water eDNA samples compared to morphological identification of invertebrate specimens collected. The size of stream and the number of replicate samples collected from each stream were important factors affecting how completely the sampling of DNA used for next-generation sequencing captured the full diversity of taxa present; our sampling approach was more effective for small streams, but multiple sampling events improved our ability to assess biodiversity, even in larger streams.

Section 2.12 outlines our molecular genetic approaches for assessing biodiversity using primer sequences that amplify regions of DNA that are variable among local species of fish, yet consistent within species. We developed primers for two wide-ranging Families of fish, the Salmonidae and the Cottidae, that amplify a portion of the cytochrome oxidase subunit 1 (COI) gene. Primers developed for the Family Salmonidae, SAL_1, amplified a variable region of COI that was useful for discriminating species within the Genus *Oncorhynchus*, amplified a conserved region for the Genus *Salvelinus*, but did not amplify the Genus *Prosopium*. Primers developed for the Family Cottidae, COT_2, amplified a variable region of COI that was useful for discriminating all species within the Genus Cottus that were tested. These primers can be used to determine the presence of species within these two important Families of freshwater fish within environmental DNA extracted from water samples.

Finally, in Section 2.13, we used elemental signatures of otoliths to establish movement patterns from species of fish belonging to two wide-ranging Families, Salmonidae and Cottidae. Examining otolith microchemistry allowed us to trace the extent and patterns of movement

over the life history of individuals that reflect distinctive freshwater chemistries in the study streams. Patterns of movement were highly variable within both Families of fish among the rivers examined. Patterns included movement from the ocean into streams in the Kitimat River watershed for spawning, movement among major river systems, use of smaller tributaries within a river, but also some individuals exhibited limited movement. There was a tendency for less variability in elemental signatures during the older life stages of fish within the Family Cottidae, but greater range of elemental signatures within the Family Salmonidae with age of the fish. The life-history information gained in this study demonstrates that extensive movements occur and there is considerable connectivity with the larger mainstem rivers for streams that will be intersected by the proposed pipeline.

2.3. Stream system descriptions

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UNNAMED CHANNEL 9.8 km

Unnamed Channel 9.8 km is located in the Coastal Mountains of west coast BC. This stream is located in the Coastal Western Hemlock biogeoclimatic zone dominated by second growth forests. This perennial stream flows into Goose Creek and is part of the Kitimat River watershed. Sampling for benthic invertebrates was conducted on 3 May, 3 June, and 27 July in 2014. Sampling for fish was conducted on 4 June and 7 August in 2014.

Table 2-1 – Sample site and Universal Transverse Mercater (UTM) coordinates for the three sampling locations in Unnamed Channel 9.8 km.

Site	UTM
+200	09U 0521042 5995959
0	09U 0521003 5995845
-200	09U 0520977 5995730

Figure 2-3 – Representative photographs of Unnamed Channel 9.8 km.

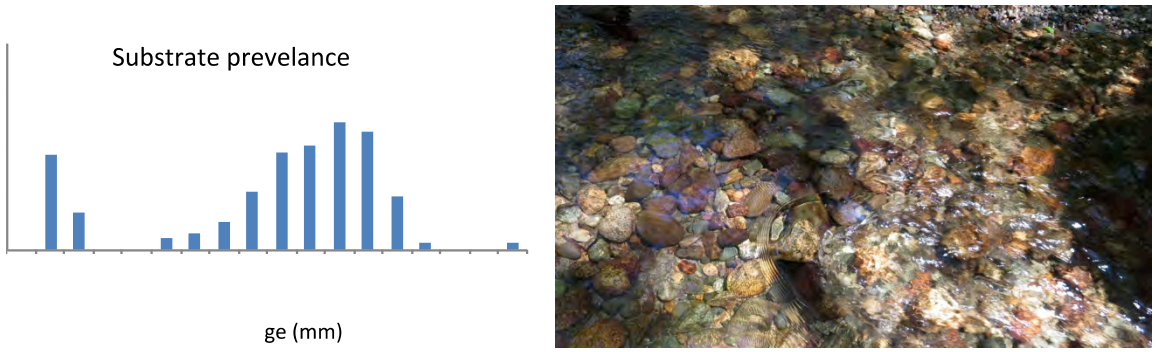

Figure 2-4 – Substrate composition histogram and representative photograph of substrate type.

Figure 2-5 – Representative fauna collected in Unnamed Channel 9.8 km; (left) Trombidiformes and (right) rough skinned newt (Family Salamandridae).

Table 2-2 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Coastal Cutthroat Trout (Salmonidae)
	Coho Salmon (Salmonidae)
	Chinook Salmon (Salmonidae)
	Dolly Varden (Salmonidae)
	Lamprey ammocoete (Petromyzontidae)
Aquatic invertebrates	Chironomidae – 49.8% (Order Diptera)
	Chloroperlidae – 14.3% (Order Ephemeroptera)
	Simuliidae – 10.3% (Order Diptera)
	Heptageniidae – 10.0% (Order Ephemeroptera)

Table 2-3 – Physical measurements collected for Unnamed Channel 9.8 km at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	July
-200	Temp (˚C)	6.9	–	6.9
	pH	6.8	–	6.9
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	29.5	–	28.9
	Dissolved O_2 ($mg \times L^{-1}$)	13.3	–	9.8
	Flow ($m \times s^{-1}$)	0.3	0.6	0.2
	Channel width	3.8	3.4	3.6
	Wetted width	2.8	2.9	2.6
	Channel depth	–	0.6	0.3
	Wetted depth	–	0.2	0.1
0	Temp (˚C)	7.1	8.4	8.6
	pH	6.9	6.5	6.5
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	29.5	33.3	28.4
	Dissolved O_2 ($mg \times L^{-1}$)	13.4	13.7	9.5
	Flow ($m \times s^{-1}$)	0.2	0.4	0.1
	Channel width	4.0	3.9	4.1
	Wetted width	3.3	2.5	3.0
	Channel depth	-	0.4	0.3
	Wetted depth	-	0.2	0.1
+200	Temp (˚C)	7.6	5.9	8.3
	pH	6.9	6.4	6.4
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	29.9	23.8	27.1
	Dissolved O_2 ($mg \times L^{-1}$)	13.0	13.8	9.2
	Flow ($m \times s^{-1}$)	0.6	0.4	0.3
	Channel width	5.2	4.8	4.7
	Wetted width	3.1	2.8	4.5
	Channel depth	–	0.6	0.3
	Wetted depth	–	0.1	0.2

UNNAMED CHANNEL 10 km

Unnamed Channel 10 km is located in the Coastal Mountains of west coast BC. This stream is located in the Coastal Western Hemlock biogeoclimatic zone dominated by second growth forests. This perennial stream flows into Unnamed Channel 9.8 km and is part of the Kitimat River watershed. Sampling for benthic invertebrates was conducted on 6 May, 5 June, and 27 July in 2014. Sampling for fish was conducted on 11 June and 7 August in 2014.

Table 2-4 – Sample site and UTM coordinates for the three sampling locations in Unnamed Channel 10 km.

Site	UTM
+200	09U 0520355 5996044
0	09U 0520938 5996048
-200	09U 0521043 5995994

Figure 2-6 – Representative photographs of Unnamed Channel 10 km.

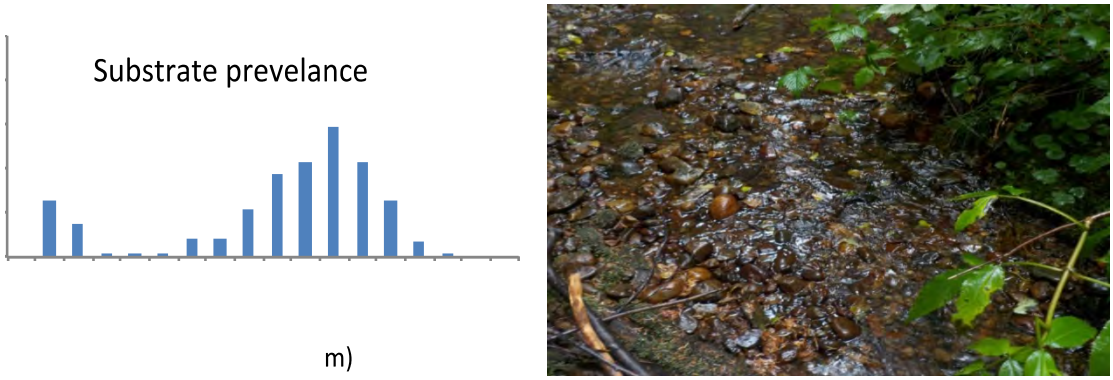

Figure 2-7 – Substrate composition histogram and representative photograph of substrate type.

Figure 2-8 – Representative fauna collected in Unnamed Channel 10 km; (left) Nematoda and (right) Dolly Varden (Salmonidae).

Table 2-5 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Coastal Cutthroat Trout (Salmonidae)
	Coho Salmon (Salmonidae)
	Dolly Varden (Salmonidae)
	Lamprey ammocoete (Petromyzontidae)

Table 2-6 – Physical measurements collected for Unnamed Channel 10 km at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	July
-200	Temp (˚C)	6.2	7.3	8.0
	pH	7.1	6.6	7.0
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	31.2	33.1	22.4
	Dissolved O_2 ($mg \times L^{-1}$)	10.0	13.9	8.3
	Flow ($m \times s^{-1}$)	0.4	0.4	0.3
	Channel width	4.6	2.5	3.6
	Wetted width	2.5	3.1	2.1
	Channel depth	–	0.2	0.2
	Wetted depth	–	0.1	0.1
0	Temp (˚C)	6.4	7.3	7.8
	pH	7.2	6.5	7.0
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	31.4	33.3	27.4
	Dissolved O_2 ($mg \times L^{-1}$)	11.4	13.1	7.8
	Flow ($m \times s^{-1}$)	0.1	0.9	0.2
	Channel width	4.1	3.0	4.0
	Wetted width	2.9	3.0	2.4
	Channel depth	–	0.4	0.3
	Wetted depth	–	0.4	0.1
+200	Temp (˚C)	7.4	8.2	7.7
	pH	7.0	6.5	6.8
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	32.7	34.3	28.9
	Dissolved O_2 ($mg \times L^{-1}$)	12.1	12.7	8.0
	Flow ($m \times s^{-1}$)	0.2	0.4	0.3
	Channel width	3.6	1.8	2.0
	Wetted width	2.4	1.5	1.8
	Channel depth	–	0.2	0.2
	Wetted depth	–	0.1	0.1

TROUT CREEK

Trout Creek is located in the Coastal Mountain range on the west coast of BC dominated by the Coastal Western Hemlock biogeoclimatic zone. Trout Creek is a perennial 3rd order stream that drains into the Little Weedeene River and is part of the Kitimat River watershed. The forests surrounding Trout Creek are mainly mature second growth forests. The stream was sampled for benthic invertebrates on 2 May, 12 June, and 28 July in 2014. Sampling for fish was conducted on 11 June and 6 August in 2014.

Table 2-7 – Sample site and UTM coordinates for the three sampling locations in Trout Creek.

Site	UTM
+200	09U 0520317 6000197
0	09U 0520522 6000186
-200	09U 0520624 6000175

Figure 2-9 – Representative photographs of Trout Creek.

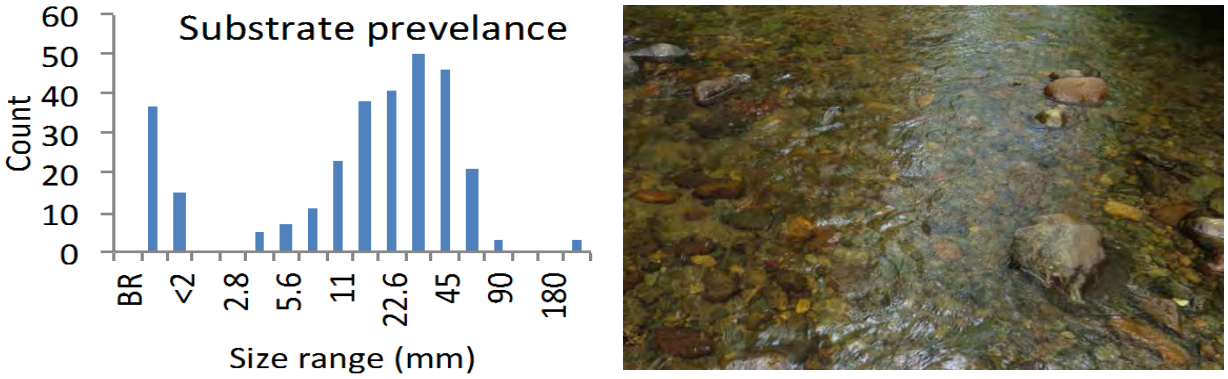

Figure 2-10 – Substrate composition histogram and representative photograph of substrate type.

Table 3 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Coastal Cutthroat Trout (Salmonidae)
	Coastal Tailed Frog (Ascaphidae)
Aquatic invertebrates	Heptageniidae – 28.9 % (Order Ephemeroptera)
	Chironomidae – 28.2 % (Order Diptera)
	Chloroperlidae – 9.7 % (Order Plecoptera)
	Baetidae – 8.6 % (Order Ephemeroptera)

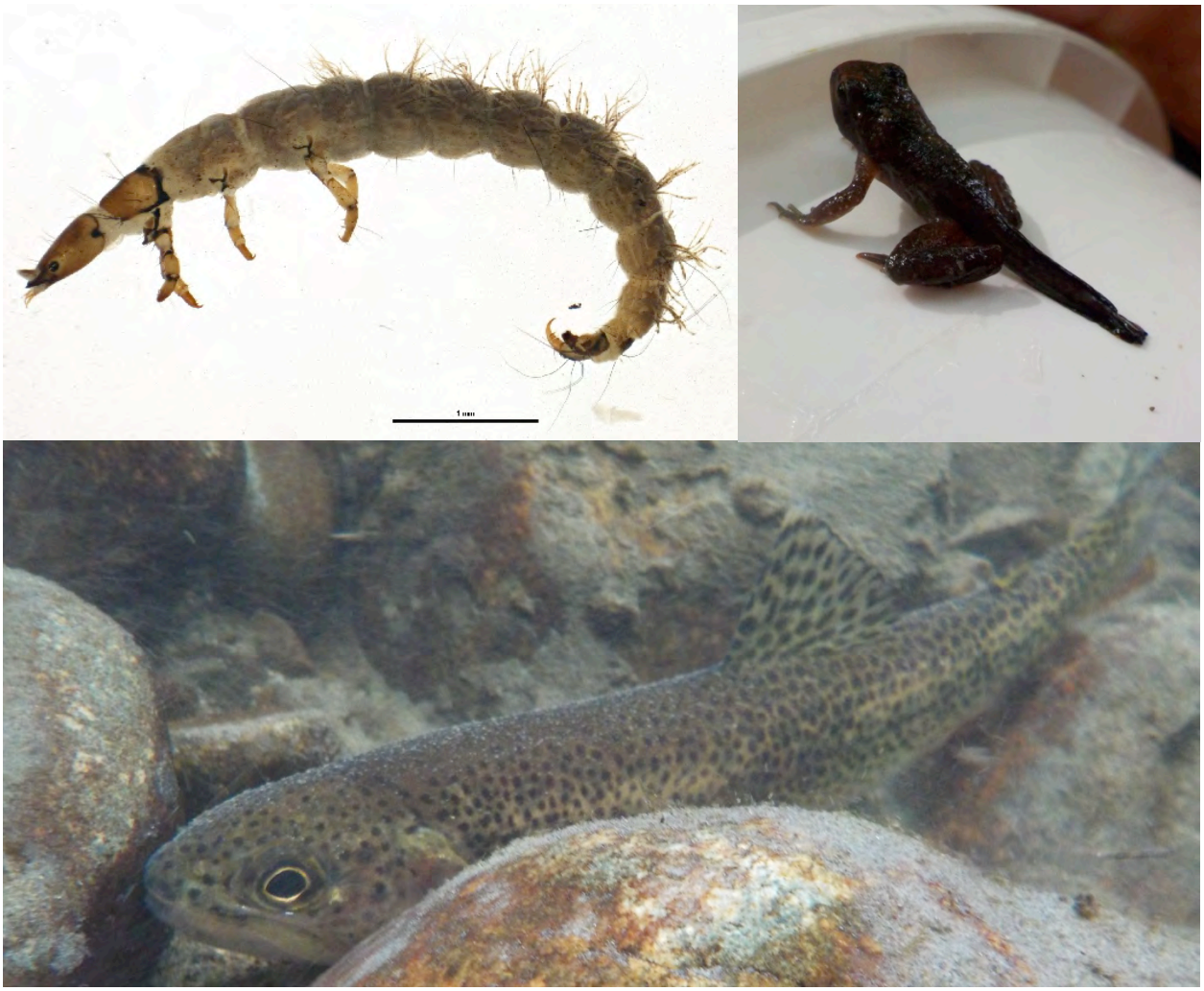

Figure 2-11 – Representative fauna collected in Trout Creek; (top left) Trichoptera, Rhyacophylidae, (top right) Coast tailed frog (Ascaphidae), and (bottom) Coastal Cutthroat Trout (Salmonidae).

Table 2-8 – Physical measurements collected for Trout Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	July
-200	Temp (˚C)	5.8	10.1	12.2
	pH	6.2	6.8	7.0
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	22.5	59.3	59.0
	Dissolved O_2 ($mg \times L^{-1}$)	15.3	13.1	7.7
	Flow ($m \times s^{-1}$)	0.7	0.9	0.6
	Channel width	10.2	7.4	5.7
	Wetted width	9.0	6.1	4.6
	Channel depth	–	0.5	0.3
	Wetted depth	–	0.3	0.1
0	Temp (˚C)	6.0	10.3	12.2
	pH	6.3	7.0	7.1
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	22.8	59.9	59.2
	Dissolved O_2 ($mg \times L^{-1}$)	14.0	13.0	7.1
	Flow ($m \times s^{-1}$)	–	0.3	0.3
	Channel width	7.6	9.6	7.2
	Wetted width	4.1	6.8	5.5
	Channel depth	–	0.3	0.3
	Wetted depth	4.93	2.17	2.44
+200	Temp (˚C)	6.4	10.6	12.3
	pH	6.5	7.3	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	23.3	60.4	59.2
	Dissolved O_2 ($mg \times L^{-1}$)	14.4	12.6	8.8
	Flow ($m \times s^{-1}$)	–	0.3	0.2
	Channel width	9.4	8.2	7.5
	Wetted width	5.7	4.8	3.9
	Channel depth	–	0.6	0.6
	Wetted depth	–	0.4	0.2

CECIL CREEK

Cecil Creek is located in the Coastal Mountains of BC's west coast. This stream is part of the Coastal Western Hemlock biogeoclimatic zone located in an area of second growth forests. Cecil Creek is a perennial 4th order stream that flows into the Kitimat River. The stream was sampled for benthic invertebrates on 7 May, 8 June, and 26 July in 2014. Sampling for fish was conducted on 13 June and 24 July in 2014.

Table 2-9 – Sample site and Universal Transverse Mercater (UTM) coordinates for the three sampling locations in Cecil Creek.

Site	UTM
+200	09U 0523971 6014079
0	09U 0524107 6014068
-200	09U 0524163 6013935

Figure 2-12 – Representative photographs of Cecil Creek.

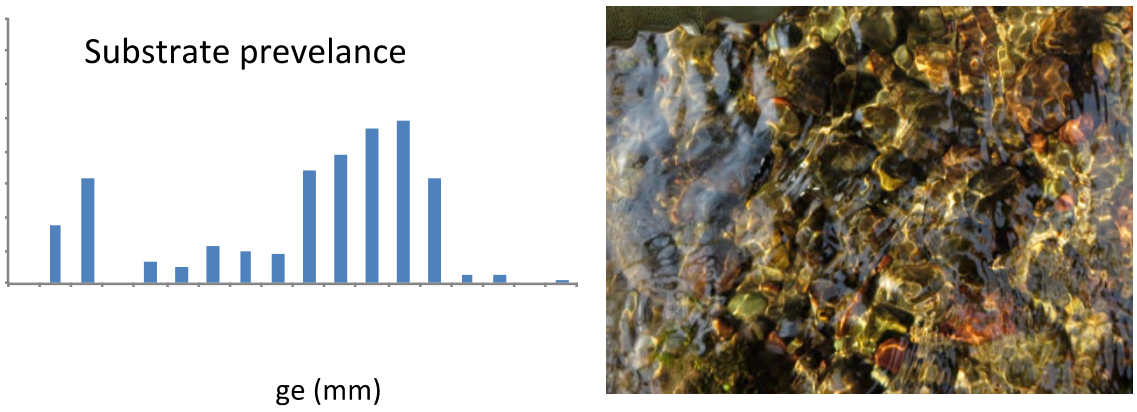

Figure 2-13 – Substrate composition histogram and representative photograph of substrate type.

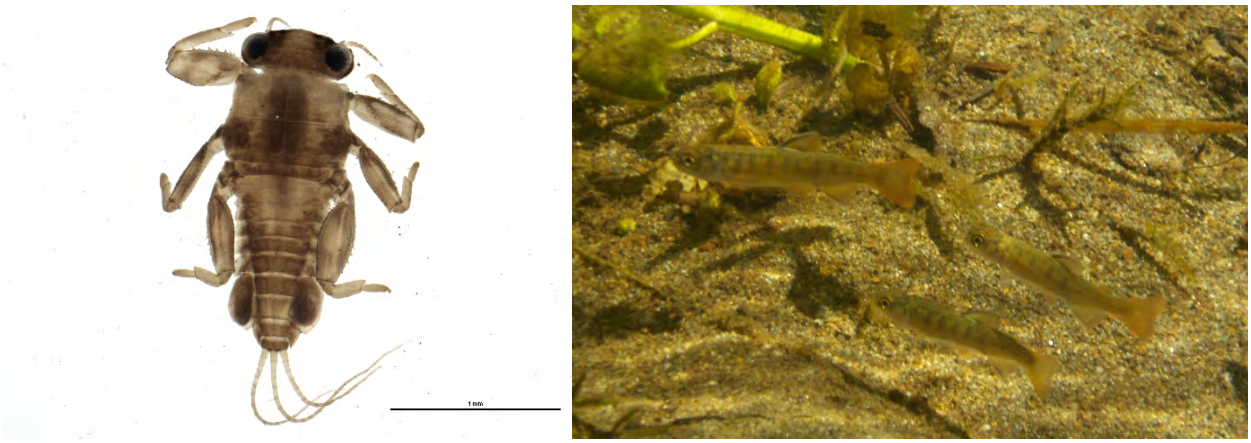

Figure 2-14 – Representative fauna collected in Cecil Creek; (left) Ephemeroptera, Heptageniidae and (right) Coho Salmon (Salmonidae).

Table 2-10 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Coastal Cutthroat Trout (Salmonidae)
	Dolly Varden (Salmonidae)
	Coastal Tailed Frog larvae (Ascaphidae)
Aquatic invertebrates	Chironomidae – 34.4 % (Order Diptera)
	Heptageniidae – 26.8 % (Order Ephemeroptera)
	Baetidae – 12.0 % (Order Ephemeroptera)
	Chloroperlidae – 8.3 % (Order Plecoptera)

Table 2-11 – Physical measurements collected for Cecil Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	May	June	July
-200	Temp (˚C)	4.8	7.8	8.0
	pH	7.1	6.1	6.9
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	21.5	30.2	52.5
	Dissolved O_2 ($mg \times L^{-1}$)	14.7	13.1	11.1
	Flow ($m \times s^{-1}$)	0.4	0.2	0.2
	Channel width	15.0	13.0	7.6
	Wetted width	9.6	10.0	5.1
	Channel depth	N/A	0.5	0.3
	Wetted depth	0.5	0.4	0.1
0	Temp (˚C)	5.7	8.0	8.1
	pH	7.8	6.7	7.0
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	21.9	28.8	50.6
	Dissolved O_2 ($mg \times L^{-1}$)	14.5	13.4	12.0
	Flow ($m \times s^{-1}$)	0.6	0.5	0.6
	Channel width	16.0	13.0	14.0
	Wetted width	9.4	13.0	9.0
	Channel depth	1.0	0.5	0.4
	Wetted depth	N/A	0.3	0.2
+200	Temp (˚C)	5.9	8.1	8.2
	pH	7.4	6.7	7.1
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	21.8	28.5	50.1
	Dissolved O_2 ($mg \times L^{-1}$)	14.4	13.5	12.3
	Flow ($m \times s^{-1}$)	0.6	0.5	0.1
	Channel width	11.0	9.3	12.0
	Wetted width	10.0	7.8	8.8
	Channel depth	0.9	0.5	0.4
	Wetted depth	N/A	0.3	0.2

CHIST CREEK

Chist Creek Chist Creek is located in the Coastal Mountains of BC's west coast. This stream is part of the Coastal Western Hemlock biogeoclimatic zone located in an area dominated by old growth forests. Chist Creek is a large perennial 4th-order stream that drains into the Kitimat River. The stream was sampled for benthic invertebrates on 4 May, 7 June, and 29 July in 2014. Sampling for fish was conducted on 7 June and 29 July in 2014. During freshet water levels for Chist Creek were too high and made wading conditions dangerous, consequently channel widths and wetted widths were not measured during the first two benthic invertebrate sampling periods.

Table 2-12 – Sample site and UTM coordinates for the three sampling locations in Chist Creek.

Site	UTM
+200	09U 0532215 6014618
0	09U 0532055 6014514
-200	09U 1531892 6014454

Figure 1 – Representative photographs of Chist Creek.

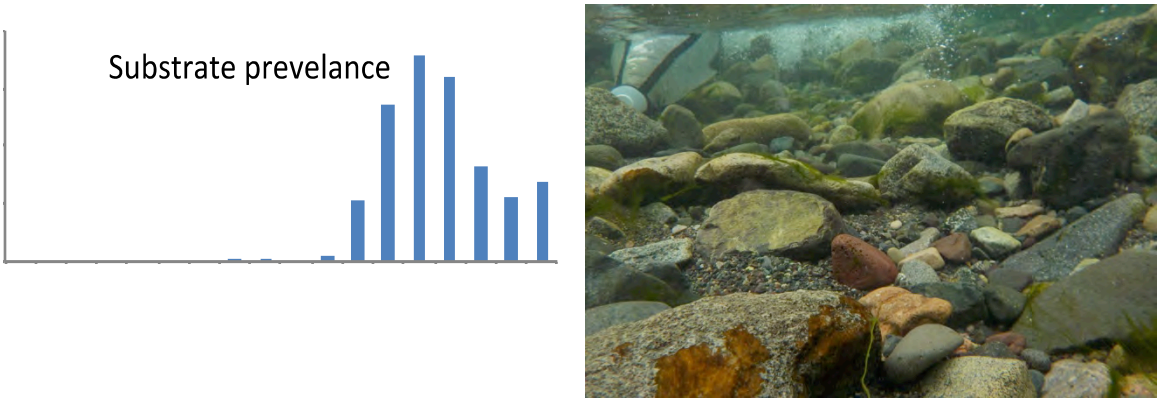

Figure 2-15 – Substrate composition histogram and representative photograph of substrate type.

Table 2-13 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)
	Dolly Varden (Salmonidae)
	Coho Salmon (Salmonidae)
	Chinook Salmon (Salmonidae)
	Coastrange Sculpin (Cottidae)
Aquatic invertebrates	Heptageniidae – 36.5 % (Order Ephemeroptera)
	Chironomidae – 32.7 % (Order Diptera)
	Baetidae – 12.9% (Order Ephemeroptera)
	Ephemerellidae – 6.8 % (Order Ephemeroptera)

Figure 2-16 – Representative fauna collected in Chist Creek; (top left) Ephemeroptera, Heptageniidae, (top right) Coastrange Sculpin (Cottidae), and (bottom) Rainbow Trout (Salmonidae).

Table 2-14 – Physical measurements collected for Chist Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	July
-200	Temp (˚C)	6.5	6.5	10.2
	pH	7.3	6.6	7.6
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	25.0	23.3	30.5
	Dissolved O_2 ($mg \times L^{-1}$)	14.1	14.5	6.7
	Flow ($m \times s^{-1}$)	1.0	0.6	0.4
	Channel width	–	–	43.9
	Wetted width	–	–	23.6
	Channel depth	–	0.6	0.9
	Wetted depth	–	0.3	0.2
0	Temp (˚C)	6.4	6.6	10.3
	pH	7.3	7.1	7.0
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	25.5	23.9	31.0
	Dissolved O_2 ($mg \times L^{-1}$)	13.7	14.5	8.5
	Flow ($m \times s^{-1}$)	0.8	0.9	0.3
	Channel width	–	–	40.3
	Wetted width	–	–	20.5
	Channel depth	–	0.6	0.7
	Wetted depth	–	0.4	0.4
+200	Temp (˚C)	.5	6.7	10.5
	pH	7.2	7.0	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	25.5	23.7	30.7
	Dissolved O_2 ($mg \times L^{-1}$)	13.7	14.5	8.0
	Flow ($m \times s^{-1}$)	0.6	1.0	0.4
	Channel width	–	–	32.9
	Wetted width	–	–	29.7
	Channel depth	–	0.6	0.9
	Wetted depth	–	0.4	0.5

UNNAMED CHANNEL 45.1 KM

Unnamed Channel 41.5 km is located in the Coastal Mountains of BC's west coast. This stream is part of the Coastal Western Hemlock biogeoclimatic zone located in an area of old growth forests. This small stream drains into Chist Creek and is part of the Kitimat River watershed. This stream was sampled for benthic invertebrates on 8 May and 8 June in 2014. Sampling for fish was conducted on 6 June 2014. Because this stream was found to be ephemeral the full sampling regime could not be conducted. Due to time constraints benthic samples were not processed.

Table 2-15 – Sample site and UTM coordinates for the three sampling locations in Unnamed Channel 45.1 km.

Site	UTM
+200	09U 0533226 6012098
0	09U 0533105 6011984
-200	09U 0532983 6011909

Figure 2-17 – Representative photographs of Unnamed Channel 45.1 km.

Figure 2-18 – Representative fauna collected in Unnamed Channel 45.1 km; (left) Coastal Tailed Frog larvae (Ascaphidae) and (right) Rainbow Trout (Salmonidae).

Table 2-16 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae) Dolly Varden (Salmonidae) Coho Salmon (Salmonidae) Chinook Salmon (Salmonidae)

Table 2-17 – Physical measurements collected for Unnamed Channel 45.1 km at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June
-200	Temp (˚C)	6	8.6
	pH	6.6	6.4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	15.9	20.6
	Dissolved O_2 ($mg \times L^{-1}$)	13.7	13.5
	Flow ($m \times s^{-1}$)	0.4	0.3
	Channel width	4.4	3.6
	Wetted width	2.5	2.8
	Channel depth	–	0.3
	Wetted depth	–	0.2
0	Temp (˚C)	6.1	8.6
	pH	6.9	6.3
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	16	20.8
	Dissolved O_2 ($mg \times L^{-1}$)	14.0	13.3
	Flow ($m \times s^{-1}$)	0.8	0.2
	Channel width	5.0	3.6
	Wetted width	2.4	2.3
	Channel depth	–	0.5
	Wetted depth	–	0.2
+200	Temp (˚C)	6.3	8.4
	pH	6.9	6.4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	17.8	23.2
	Dissolved O_2 ($mg \times L^{-1}$)	13.4	12.7
	Flow ($m \times s^{-1}$)	0.6	0.3
	Channel width	4.4	2.3
	Wetted width	1.8	1.7
	Channel depth	–	0.2
	Wetted depth	–	0.1

UNNAMED CHANNEL 57.1 KM

Unnamed Channel 57.1 km is located in the Coastal Mountains of BC's west coast. This stream is part of the Coastal Western Hemlock biogeoclimatic zone located in an area of old growth forests. This high gradient stream flows into the Kitimat River. The stream was sampled for benthic invertebrates on 5 May, 14 June, and 25 July in 2014. Sampling for fish was conducted on 10 June and 9 August in 2014.

Table 2-18 – Sample site and UTM coordinates for the three sampling locations in Unnamed Channel 57.1 km.

Site	UTM
+200	09U 0547077 6011609
0	09U 0547055 6011449
-200	09U 0546966 6011366

Figure 2-19 – Representative photographs of Unnamed Channel 57.1 km.

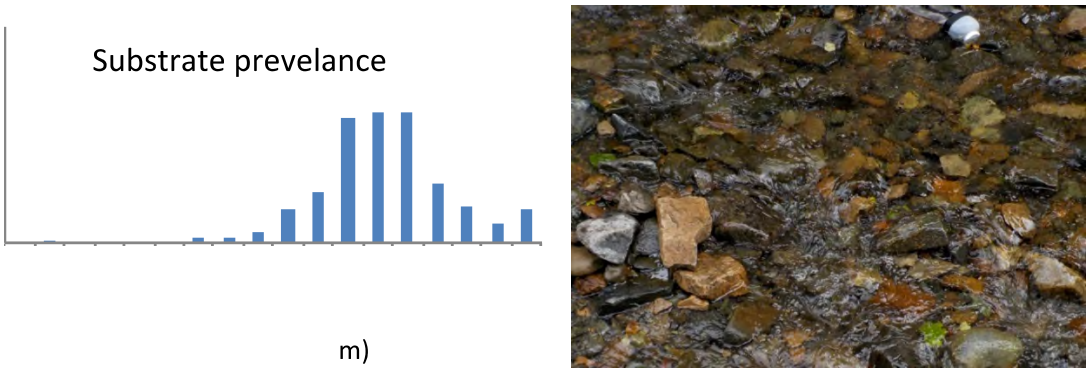

Figure 2-20 – Substrate composition histogram and representative photograph of substrate type.

Figure 2-21 – Representative fauna collected in Unnamed Channel 57.1 km; (left) Diptera, Ceratopogonidae and (right) Coastal Cutthroat Trout (Salmonidae).

Table 2-19 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Coastal Cutthroat Trout (Salmonidae)
	Coho Salmon (Salmonidae)
	Dolly Varden (Salmonidae)
	Coastrange Sculpin (Cottidae)
	Prickly Sculpin (Cottidae)
	Coastal Tailed Frog (Ascaphidae)
Aquatic invertebrates	Chironomidae – 34.1 % (Order Diptera)
	Heptageniidae – 33.2 % (Order Ephemeroptera)
	Baetidae – 13.6 % (Order Ephemeroptera)
	Chloroperlidae – 6.2 % (Order Plecoptera)

Table 2-20 – Physical measurements collected for Unnamed Channel 57.1 km at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	July
-200	Temp (˚C)	4.3	6.2	11.4
	pH	7.3	6.7	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	33.9	25.9	88.0
	Dissolved O_2 ($mg \times L^{-1}$)	14.1	14.7	11.8
	Flow ($m \times s^{-1}$)	0.4	0.9	0.1
	Channel width	6.6	5.5	6.4
	Wetted width	4.2	5.1	3.4
	Channel depth	0.4	0.5	0.3
	Wetted depth	0.2	0.4	0.1
0	Temp (˚C)	4.1	6.2	11.3
	pH	7.9	6.8	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	33.7	26.3	85.7
	Dissolved O_2 ($mg \times L^{-1}$)	14.0	15.6	11.9
	Flow ($m \times s^{-1}$)	0.9	0.8	0.4
	Channel width	8.9	6.5	5.5
	Wetted width	4.3	5.5.	3.6
	Channel depth	0.7	0.4	0.3
	Wetted depth	0.3	0.3	0.2
+200	Temp (˚C)	4.5	6.2	11.4
	pH	7.3	7.0	7.5
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	34.5	26.6	88.4
	Dissolved O_2 ($mg \times L^{-1}$)	13.5	14.6	11.8
	Flow ($m \times s^{-1}$)	1.1	1.2	0.5
	Channel width	11.4	5.8	5.3
	Wetted width	3.9	5.1	3.3
	Channel depth	–	0.4	0.4
	Wetted depth	0.9	0.2	0.1

TCHESINKUT CREEK

Tchesinkut Creek is located in the interior of BC near Burns Lake, dominated by the Sub-Boreal Spruce biogeoclimatic zone. This perennial streams flows into Francois Lake and is part of the Francois Lake watershed. The stream was sampled on 9 July and 25 August in 2014. Sampling for fish was conducted on 26 August 2014.

Table 2-21 – Sample site and UTM coordinates for the three sampling locations in Tchesinkut Creek.

Site	UTM
+200	10U 0341474 5996211
0	10U 0341598 5996347
-200	10U 0341716 5996417

Figure 2-22 – Representative photographs of Tchesinkut Creek.

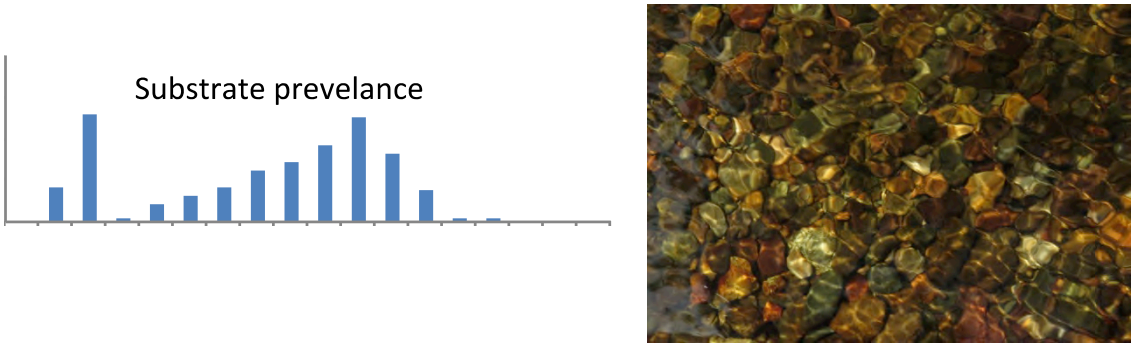

Figure 2-23 – Substrate composition histogram and representative photograph of substrate type.

Table 2-22 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae) Mountain Whitefish (Salmonidae) Burbot (Gadidae) Longnose Dace (Cyprinidae) Northern Pikeminnow (Cyprinidae) Redside Shiner (Cyprinidae) Peamouth (Cyprinidae)

Figure 2-24 – Representative fauna collected in Tchesinkut Creek; (above left) Clitellata, Oligochaete, (above right) redside shiner (Cyprinidae), and (below) mountain whitefish (Salmonidae).

Table 2-23 – Physical measurements collected for Tchesinkut Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	July	August
-200	Temp (˚C)	14.2	11.0
	pH	7.1	7.6
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	88.4	117.2
	Dissolved O_2 ($mg \times L^{-1}$)	9.7	7.8
	Flow ($m \times s^{-1}$)	0.8	0.2
	Channel width	9.2	7.7
	Wetted width	8.4	4.6
	Channel depth	0.8	1.0
	Wetted depth	0.7	0.2
0	Temp (˚C)	14.2	9.4
	pH	7.3	7.5
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	88.6	112.0
	Dissolved O_2 ($mg \times L^{-1}$)	9.9	6.2
	Flow ($m \times s^{-1}$)	0.4	N/a
	Channel width	8.4	9.5
	Wetted width	7.5	3.4
	Channel depth	0.7	1.1
	Wetted depth	0.6	0.2
+200	Temp (˚C)	14.4	10.6
	pH	7.5	8.0
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	89.4	115.9
	Dissolved O_2 ($mg \times L^{-1}$)	9.8	4.0
	Flow ($m \times s^{-1}$)	0.2	N/A
	Channel width	10.1	7.3
	Wetted width	8.3	4.0
	Channel depth	0.4	0.3
	Wetted depth	0.3	0.1

ORMOND CREEK

Ormond Creek is located in the interior of BC near Vanderhoof and Fort Fraser dominated by the Sub-Boreal Spruce biogeoclimatic zone. This perennial stream is a 4th order stream that flows into Fraser Lake, and is part of the Francois Lake watershed. Due to high freshet water levels, this stream was only sampled twice. The stream was sampled for benthic invertebrates on 24 June and 9 September in 2014. Sampling for fish was conducted on 23 June and 8 September in 2014.

Table 2-24 – Sample site and UTM coordinates for the three sampling locations in Ormond Creek.

Site	UTM
+200	10U 0384436 5997856
0	10U 0384509 5997663
-200	10U 0384611 5997500

Figure 2-25 – Representative photographs of Ormond Creek.

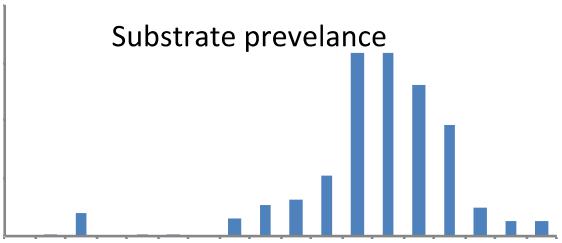

Figure 2-26 – Substrate composition histogram and representative photograph of substrate type.

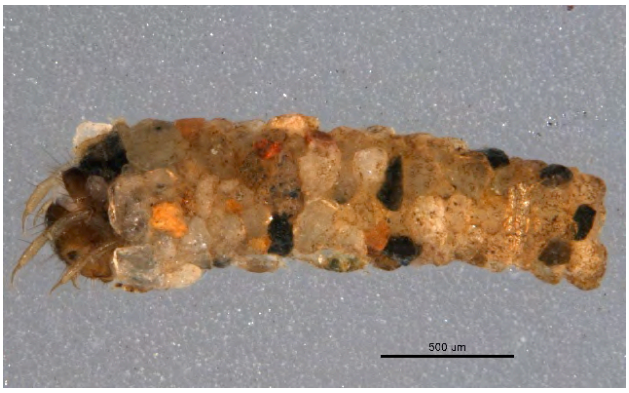

Figure 2-27 – Representative fauna collected in Ormond Creek; (left) Trichoptera, Brachycentridae and (right) Rainbow Trout (Salmonidae).

Table 2-25 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)
	Longnose Dace (Cyprinidae)
	Prickly Sculpin (Cottidae)
Aquatic invertebrates	Chironomidae – 46.0 % (Diptera)
	Heptageniidae – 22.7 % (Ephemeroptera)
	Leptophlebiidae – 9.5 % (Ephemeroptera)
	Chloroperlidae – 4.9 % (Plecoptera)

Table 2-26 – Physical measurements collected for Ormond Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	June	September
-200	Temp (˚C)	16.8	8.2
	pH	7.3	7.3
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	61.9	75.4
	Dissolved O_2 ($mg \times L^{-1}$)	9.4	11.2
	Flow ($m \times s^{-1}$)	0.9	0.1
	Channel width	21.0	13.5
	Wetted width	7.9	5.9
	Channel depth	0.9	0.5
	Wetted depth	0.3	0.1
0	Temp (˚C)	17.5	8.7
	pH	7.5	7.4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	62.7	76.4
	Dissolved O_2 ($mg \times L^{-1}$)	9.1	12.0
	Flow ($m \times s^{-1}$)	0.7	0.2
	Channel width	22.7	20.4
	Wetted width	7.5	4.0
	Channel depth	0.9	0.8
	Wetted depth	0.4	0.2
+200	Temp (˚C)	18/3	8/9
	pH	7/6	7/4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	63.5	76.9
	Dissolved O_2 ($mg \times L^{-1}$)	9.3	11.9
	Flow ($m \times s^{-1}$)	0.9	0.2
	Channel width	14.6	15.2
	Wetted width	11.7	6.2
	Channel depth	0.6	0.6
	Wetted depth	0.3	0.1

DOG CREEK

Dog Creek is located in the interior of BC near Vanderhoof and Fort Fraser dominated by the Sub-Boreal Spruce biogeoclimatic zone. This relatively small stream flows into the Nechako River. Sampling for benthic invertebrates was conducted on 8 July and 21 August in 2014. Sampling for fish was conducted on 10 July and 20 August in 2014.

Table 2-27 – Sample site and UTM coordinates for the three sampling locations in Dog Creek.

Site	UTM
+200	10U 396022 5997808
0	10U 396103 5997795
-200	10U 396162 5997600

Figure 2-28 – Representative photographs of Dog Creek.

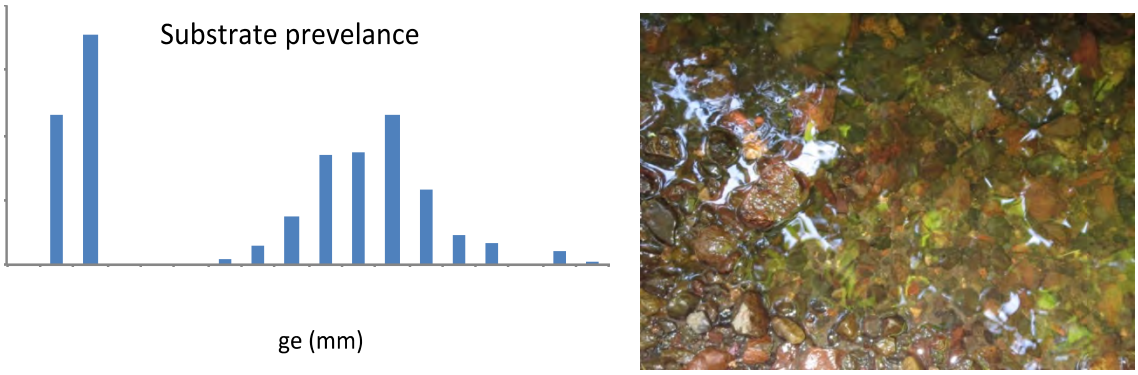

Figure 2-29 – Substrate composition histogram and representative photograph of substrate type.

Table 2-28 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)

Table 2-29 – Physical measurements collected for Dog Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	July	August
-200	Temp (˚C)	12.7	9.3
	pH	7.2	7.4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	83.0	138.6
	Dissolved O_2 ($mg \times L^{-1}$)	9.5	5.3
	Flow ($m \times s^{-1}$)	0.1	0.1
	Channel width	2.3	2.3
	Wetted width	0.9	1.0
	Channel depth	0.2	0.2
	Wetted depth	0.1	0.0
0	Temp (˚C)	13.1	9.6
	pH	7.5	7.5
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	117.6	142.5
	Dissolved O_2 ($mg \times L^{-1}$)	9.7	6.1
	Flow ($m \times s^{-1}$)	0.1	0.2
	Channel width	1.6	2.0
	Wetted width	1.0	1.1
	Channel depth	0.2	0.2
	Wetted depth	0.1	0.0
+200	Temp (˚C)	13.2	9.5
	pH	7.5	7.4
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	118.8	142.5
	Dissolved O_2 ($mg \times L^{-1}$)	9.0	6.6
	Flow ($m \times s^{-1}$)	0.1	0.2
	Channel width	1.5	1.7
	Wetted width	1.4	0.9
	Channel depth	0.1	0.4
	Wetted depth	0.0	0.1

TATSUNAI CREEK

Tatsunai Creek is located in the interior of BC near Vanderhoof and Fort Fraser, dominated by the Sub-Boreal Spruce biogeoclimatic zone. This stream is classified as a 4th order stream flowing into the Nechako River. The stream was sampled for benthic invertebrates on 2 July and 27 August in 2014. Sampling for fish was conducted on 27 June and 28 August in 2014.

Table 2-30 – Sample site and UTM coordinates for the three sampling locations in Tatsutnai Creek.

Site	UTM
+200	10U 0398499 5999452
0	10U 0398575 5999333
-200	10U 0398697 5999239

Figure 2-30 – Representative photographs of Tatsutnai Creek.

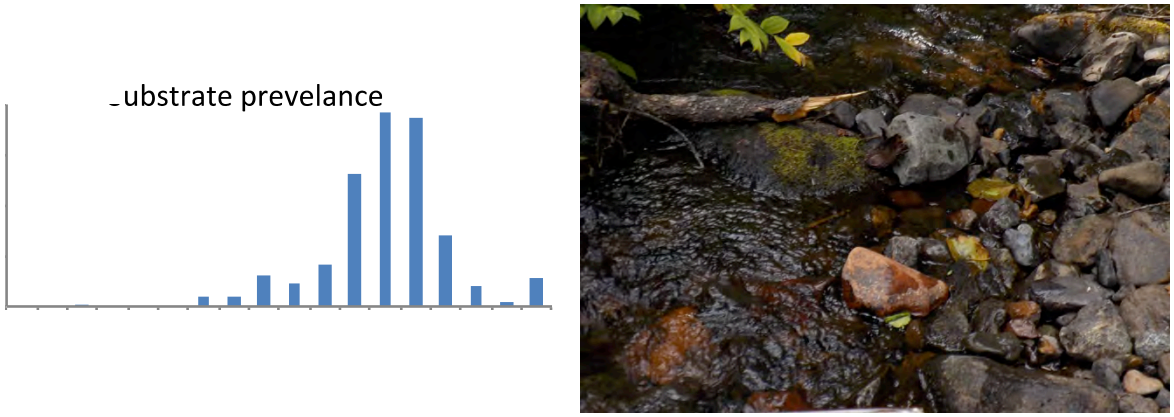

Figure 2-31 – Substrate composition histogram and representative photograph of substrate type.

Figure 2-32 – Representative fauna collected in Tatsutnai Creek; (left) Trichoptera, Hydropsychidae and (right) Rainbow Trout (Salmonidae).

Table 2-31 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)
Aquatic invertebrates	Chironomidae – 35.0 % (Diptera)
	Baetidae – 14.4 % (Ephemeroptera)
	Heptageniidae – 13.6 % (Ephemeroptera)
	Simuliidae – 5.7 % (Diptera)

Table 2-32 – Physical measurements collected for Tatsutnai Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	June	August
-200	Temp (˚C)	14.2	11.0
	pH	7.1	7.6
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	88.4	117.2
	Dissolved O_2 ($mg \times L^{-1}$)	9.7	7.8
	Flow ($m \times s^{-1}$)	0.8	0.2
	Channel width	5.1	7.1
	Wetted width	3.6	3.0
	Channel depth	0.2	0.6
	Wetted depth	0.1	0.2
0	Temp (˚C)	14.2	9.4
	pH	7.3	7.5
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	88.6	112.0
	Dissolved O_2 ($mg \times L^{-1}$)	9.9	6.2
	Flow ($m \times s^{-1}$)	0.4	N/a
	Channel width	6.0	4.5
	Wetted width	4.9	2.8
	Channel depth	0.5	0.3
	Wetted depth	0.2	0.2
+200	Temp (˚C)	14.4	10.6
	pH	7.5	8.0
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	89.4	115.9
	Dissolved O_2 ($mg \times L^{-1}$)	9.8	4.0
	Flow ($m \times s^{-1}$)	0.2	N/A
	Channel width	4.8	4.6
	Wetted width	4.1	2.2
	Channel depth	0.4	0.4
	Wetted depth	0.0	0.1

NINE MILE CREEK

Nine Mile Creek is located in the interior of BC near Vanderhoof and Fort Fraser, dominated by the Sub-Boreal Spruce biogeoclimatic zone. This perennial stream is a 3rd-order stream that drains into the Nechako River. Sampling for benthic invertebrates was conducted on 23 May, 26 June, and 5 September in 2014. Sampling for fish was conducted 24 June and 4 September in 2014.

Table 2-33 – Sample site and Universal Transverse Mercater (UTM) coordinates for the three sampling locations in Nine Mile Creek.

Site	UTM
+200	10U 0403845 6000690
0	10U 0403769 6000796
-200	10U 0403714 6000935

Figure 2-33 – Representative photographs of Nine Mile Creek.

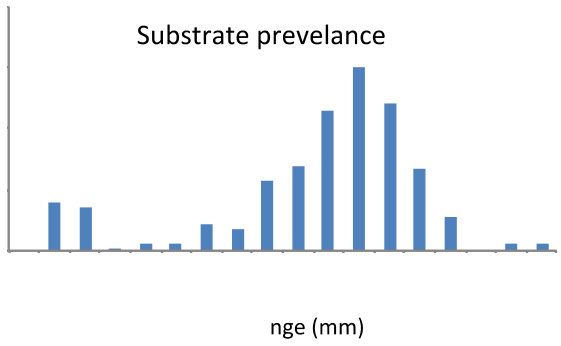

Figure 2-34 – Substrate composition histogram and representative photograph of substrate type.

Figure 3 – Representative fauna collected in Nine Mile Creek; (left) Coleoptera, Elmidae and (right) Rainbow Trout (Salmonidae).

Table 2-34 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)
Aquatic invertebrates	Heptageniidae – 27.1 % (Ephemeroptera)
	Chironomidae – 26.6 % (Diptera)
	Baetidae – 12.3 % (Ephemeroptera)
	Chloroperlidae – 5.5 % (Plecoptera)

Table 2-35 – Physical measurements collected for Nine Mile Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements. "–" indicates no measurement was taken.

Site	Variable	May	June	September
-200	Temp (˚C)	–	10.5	7.2
	pH	–	7.6	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	–	98.6	127.0
	Dissolved O_2 ($mg \times L^{-1}$)	–	8.88	9.11
	Flow ($m \times s^{-1}$)	0.7	0.1	0.3
	Channel width	4.8	4.6	4.9
	Wetted width	4.2	3.1	2.7
	Channel depth	0.4	0.4	0.4
	Wetted depth	0.3	0.3	0.2
0	Temp (˚C)	–	11.1	8.1
	pH	–	7.7	7.6
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	–	96.2	130.3
	Dissolved O_2 ($mg \times L^{-1}$)	–	10.9	2.3
	Flow ($m \times s^{-1}$)	0.4	0.4	0.4
	Channel width	7.2	6.5	7.2
	Wetted width	4.9	2.1	2.4
	Channel depth	0.3	0.6	0.5
	Wetted depth	0.3	0.3	0.2
+200	Temp (˚C)	–	11.4	9.0
	pH	–	7.7	7.6
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	–	101.5	133.3
	Dissolved O_2 ($mg \times L^{-1}$)	–	11.1	8.1
	Flow ($m \times s^{-1}$)	0.7	0.6	0.3
	Channel width	4.9	4.2	4.4
	Wetted width	4.1	2.9	3.3
	Channel depth	0.4	0.4	0.5
	Wetted depth	0.3	0.2	0.1

KLUK CREEK

Kluk Creek is located in the interior of BC near Vanderhoof and Fort St. James, dominated by the Sub-Boreal Spruce biogeoclimatic zone. This 3rd-order stream flows into the Nechako River. Sampling for benthic invertebrates was conducted on 29 May, 3 July and 18 August in 2014. Sampling for fish was conducted on 4 July and 19 August in 2014.

Table 2-36 – Sample Sample site and UTM coordinates for the three sampling locations in Kluk Creek.

Site	UTM
+200	10U 0410487 6001364
0	10U 0410369 6001326
-200	10U 0410190 6001209

Figure 2-35 – Representative photographs of Kluk Creek.

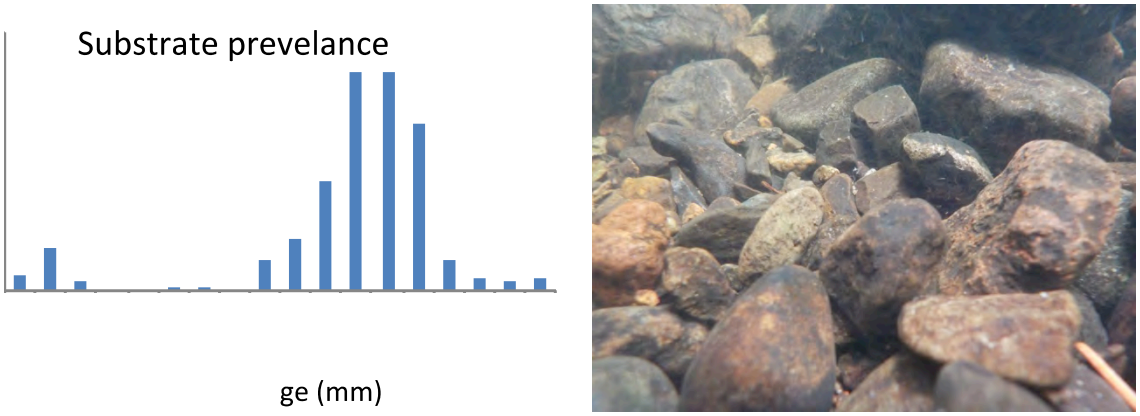

Figure 2-36 – Substrate composition histogram and representative photograph of substrate type.

Figure 2-37 – Representative fauna collected in Kluk Creek; (left) Plecoptera, Perlodidae and (right) Rainbow Trout (Salmonidae).

Table 2-37 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Rainbow Trout (Salmonidae)

Table 2-38 – Physical measurements collected for Kluk Creek at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	May	July	August
-200	Temp (˚C)	11.0	12.3	13.5
	pH	7.3	7.2	7.2
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	49.5	87.2	123.5
	Dissolved O_2 ($mg \times L^{-1}$)	11.5	9.7	8.7
	Flow ($m \times s^{-1}$)	0.6	0.1	0.3
	Channel width	4.9	2.9	2.4
	Wetted width	2.8	1.5	1.2
	Channel depth	0.6	0.2	0.2
	Wetted depth	0.2	0.2	0.1
0	Temp (˚C)	12.0	12.7	13.9
	pH	7.4	7.2	7.3
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	50.8	90.2	128.7
	Dissolved O_2 ($mg \times L^{-1}$)	11.9	9.7	12.6
	Flow ($m \times s^{-1}$)	0.5	0.2	0.2
	Channel width	4.0	3.5	3.0
	Wetted width	2.7	1.5	1.2
	Channel depth	0.6	0.2	0.2
	Wetted depth	0.2	0.1	0.1
+200	Temp (˚C)	13.0	13.4	14.3
	pH	7.5	7.4	7.5
	Turbidity	C	C	C
	Conductivity ($\mu S \times cm^{-1}$)	51.9	81.0	130.4
	Dissolved O_2 ($mg \times L^{-1}$)	10.4	9.6	2.7
	Flow ($m \times s^{-1}$)	0.5	0.3	0.2
	Channel width	5.4	3.2	3.6
	Wetted width	4.2	1.9	1.8
	Channel depth	0.3	0.3	0.2
	Wetted depth	0.2	0.3	0.1

SALMON RIVER

Salmon River is located in the interior of BC north of Prince George dominated by the Sub-Boreal Spruce biogeoclimatic zone. The Salmon River is a 6th order stream that flows into the Fraser River. Due to high freshet water levels, this stream was only sampled twice for benthic invertebrates and twice for fish. The River was sampled for benthic invertebrates on 14 July and 2 September in 2014. Sampling for fish was conducted on 15 July and 3 September in 2014.

Table 2-39 – Sample site and UTM coordinates for the three sampling locations in Salmon River.

Site	UTM
+200	10U 0496349 6006285
0	10U 0496605 6006298
-200	10U 0341716 5996417

Figure 2-38 – Representative photographs of Salmon River.

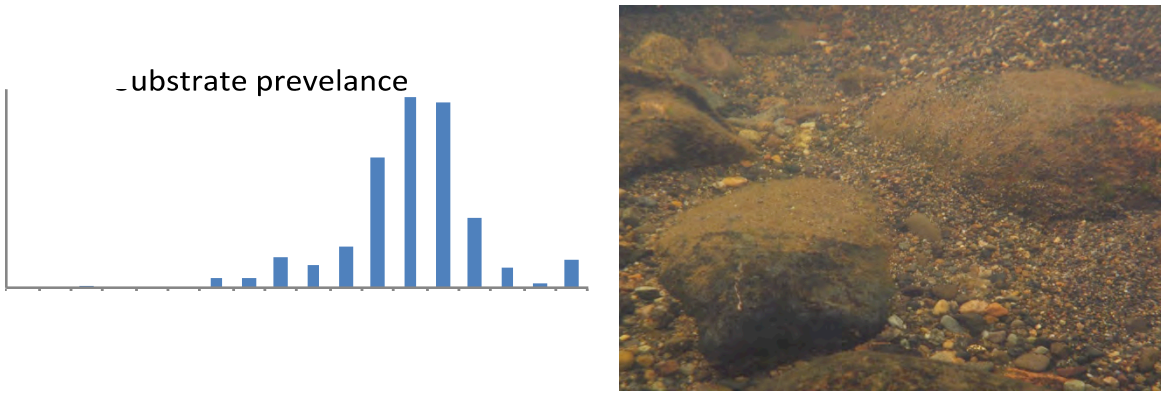

Figure 2-39 – Substrate composition histogram and representative photograph of substrate type.

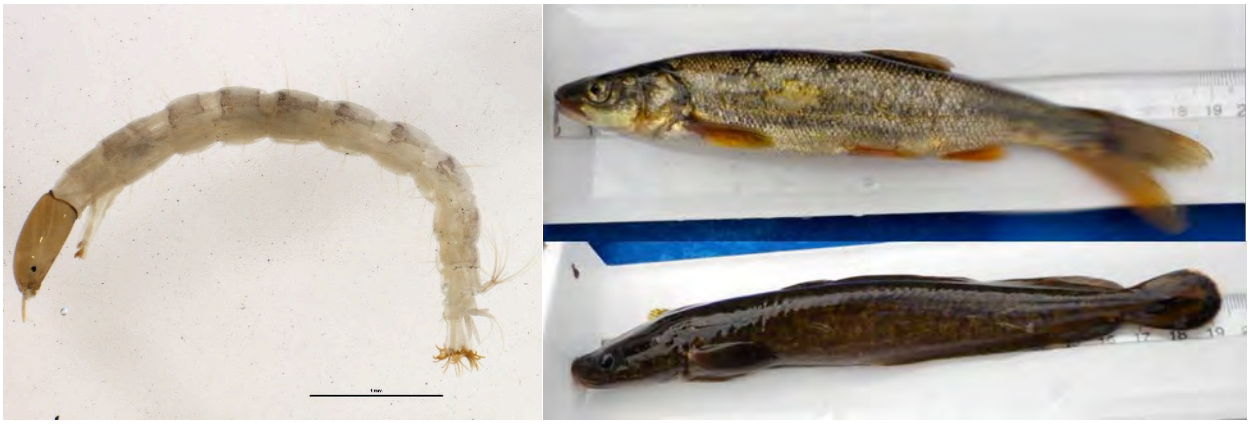

Figure 2-40 – Representative fauna collected in Salmon River; (left) Diptera, Ceratopogonidae, (top right) burbot (Gadidae), and (bottom right) northern pikeminnow (Cyprinidae).

Table 2-40 – List of aquatic vertebrates collected electrofishing and summary of the most common aquatic invertebrate Families present in the Surber sample as a percentage.

	Taxonomic group
Aquatic vertebrates	Burbot (Gadidae)
	Longnose Dace (Cyprinidae)
	Leopard Dace (Cyprinidae)
	Redside Shiner (Cyprinidae)
	Northern Pikeminnow (Cyprinidae)
	Sculpin (Cottidae)
Aquatic invertebrates	Chironomidae – 50.8 % (Diptera)
	Hydropsychidae – 15.9 % (Trichoptera)
	Baetidae – 7.7 % (Ephemeroptera)
	Heptageniidae – 7.3 % (Ephemeroptera)

Table 2-41 – Physical measurements collected for Salmon River at the location of the right of way (RoW) for the proposed Pacific Trails Pipeline (0), 200 m above the RoW (+200), and 200 m below the RoW (-200). Turbidity was assessed visually; C = clear. Width and depth was an average of six measurements.

Site	Variable	July	September
-200	Temp (˚C)	22.0	13.1
	pH	7.9	7.9
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	148.8	144.5
	Dissolved O_2 ($mg \times L^{-1}$)	8.4	6.9
	Flow ($m \times s^{-1}$)	0.7	0.2
	Channel width	47.0	40.1
	Wetted width	33.7	27.8
	Channel depth	1.0	0.7
	Wetted depth	0.5	0.3
0	Temp (˚C)	22.8	13.5
	pH	7.9	8.0
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	152.0	146.2
	Dissolved O_2 ($mg \times L^{-1}$)	8.9	3.6
	Flow ($m \times s^{-1}$)	0.4	0.5
	Channel width	46.1	42.9
	Wetted width	39.8	50.9
	Channel depth	0.7	0.7
	Wetted depth	0.3	0.2
+200	Temp (˚C)	24	13.5
	pH	7.4	8.0
	Turbidity	C	C
	Conductivity ($\mu S \times cm^{-1}$)	156.2	144.1
	Dissolved O_2 ($mg \times L^{-1}$)	10.1	2.4
	Flow ($m \times s^{-1}$)	0.5	0.8
	Channel width	51.8	54.1
	Wetted width	29.4	24.2
	Channel depth	1.5	0.8
	Wetted depth	0.6	0.2

2.4. Morphological identification of benthic invertebrates to establish baseline biodiversity and reference stream conditions at proposed right-of-way crossings

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Introduction

Benthic invertebrate communities in freshwater streams play an important role in ecosystem function and stream integrity. Invertebrates help drive energy cycling and nutrient flow throughout stream systems by providing essential linkages in freshwater food webs (Wallace and Webster 1996; Covich et al. 1999). Assemblages vary both temporally and spatially (Johnson et al. 2012), and are affected by the abiotic characteristics of the systems (Corkum1989; Reece and Richardson 2000). Ecosystem productivity and stability is largely determined by species diversity and loss of diversity is known to be a significant driver of environmental change (Tilman et al. 2014).

A common approach for water quality assessment is monitoring so-called indicator species present in benthic macroinvertebrate communities (Barbour et al. 1999; Rosenberg et al. 1999; Relyea et al. 2000). Some species are known to be tolerant of changes in water quality, while other species are far less tolerable. A shift from less-tolerant to more-tolerant species following a disturbance can be used as an indication of deteriorating water quality (Relyea et al. 2000). Conversely, ecosystem recovery might also be indicated by a shift back to species that are less tolerant of poor water quality. There are limitations to such an approach. Tolerances must be assessed *a priori* for a wide range of species in similar ecosystems, habitats, seasons, and geographic areas, and baseline data on species composition must be recorded. The responses of organisms may vary with the type of disturbance, for example, an increase in sedimentation might cause a different shift in species than organic pollution (Carlisle et al. 2007). The reliability of species as bioindicators can be influenced by factors such as their tendency to drift, their distribution patterns, and their rarity within a stream (Barbour et al. 1999; Rosenberg et al. 1999). For these reasons, it is important to consider the stream-species assemblage as a whole, rather than focusing on one, or even a few, indicator species.

When taxonomic resolution to species level is not feasible, however, Family-level bioindicators provide indications of stream health (Hilsenhoff 1988). Common measurements derived from benthic macroinvertebrates samples include taxa richness (i.e., number of taxa present), diversity indices such as the Shannon and Simpson Indices, the percent of mayflies, stoneflies, and caddisflies (Ephemeroptera, Plecoptera, and Trichoptera, or %EPT; Lenat 1988), and the Hilsenhoff Biotic Index (Hilsenhoff 1982), which incorporates assignments of tolerance values from known sensitivities to environmental changes. These biotic indices are based on the knowledge that mayflies, stoneflies, and caddisflies are particularly sensitive to environmental changes and decreases in taxa richness are correlated with reduced stream quality.

Studies of benthic invertebrates from freshwater streams in central BC are limited. Relevant studies have focused more on southern regions, including a similar study conducted in interior and coastal streams of southern BC (Reece and Richardson 2000) and one on Vancouver Island (Halwas et al. 2005). Others focused on specific taxa, such as a study on mayfly taxa in streams located near Bella Coola (Wigle and Thommason 1990), or on specific studies of macroinvertebrate responses to environmental changes, such as the Perrin and Richardson (1997) study that investigated the response of periphyton and invertebrate communities to the addition of nitrogen of phosphorus to streams mecocosms in the Nechako River in central BC. Corkum (1989) conducted a biodiversity study of western Canada, by single-sampling a large number of sites, but did not account for variation over time or within the sampled stream. Baseline assessment of current benthic invertebrate diversity across time and space is crucial to understanding the current state of stream ecosystem function so that potential responses to changes associated with resource management and activities can be monitored and assessed, and restoration activities initiated when necessary.

To establish benthic invertebrate community structure of streams, benthic invertebrates were collected from streams located along the proposed RoW in coastal and interior regions of BC and identified morphologically to the Family level. Biological metrics such as taxa richness as well as the Shannon Diversity and Hilsenhoff Biotic Indices were calculated and used to characterize community structure in order to assess stream quality. In addition, patterns

between biological metrics and abiotic measurements were assessed to provide a greater understanding of benthic invertebrate biodiversity and distribution in the study area. The results of this study confirmed the expected relatively good conditions of the streams located in this previously understudied and undisturbed region of BC, and furthered the objectives of establishing baseline diversity of benthic invertebrates in study streams.

Methods

Study area

Study streams were located in coastal and interior ecosystems of central BC (Figure 2-41). The interior ecosystem unit (EU) is located in section 2 of the proposed RoW and encompasses the regions surrounding Prince George, Vanderhoof and Fort Fraser. These streams are part of the Nechako River, Fraser River, and Francois Lake watersheds (BC Ministry of Environment 2015). This region is predominantly in the sub-boreal spruce biogeoclimatic zone, dominated by subalpine fir, white spruce and lodge pole pine. Extreme seasonal temperature characterizes the climate in this region; winters are long and cold while summers are short but hot (Alldritt-McDowell 1998). The coastal EU streams were located in section 4 of the proposed RoW, in the Kitimat valley, and are part of the Kitimat River watershed (BC Ministry of Environment 2015). This area is located in the Coastal Western Hemlock biogeoclimatic zone and dominated by western hemlock and western red cedar. This region is part of the coastal temperate rainforest and characterized by milder temperature and high levels of precipitation throughout the year. In both EUs, small-to medium-sized streams were selected from those that were fish bearing with permanent flow.

2013 benthic invertebrate sampling

In 2013, a pilot study was conducted to test the sampling methods and assess the suitability of potential study streams. Ten streams were sampled in late August to early October, four streams located in the coastal EU (section 4) and six streams in the interior EU (section 2). Benthic invertebrate samples were collected from three sampling sites in each stream (Figure 2-42), one along the proposed RoW (0 m), one located 200 m upstream (+200 m), and one located 200m downstream (-200 m).

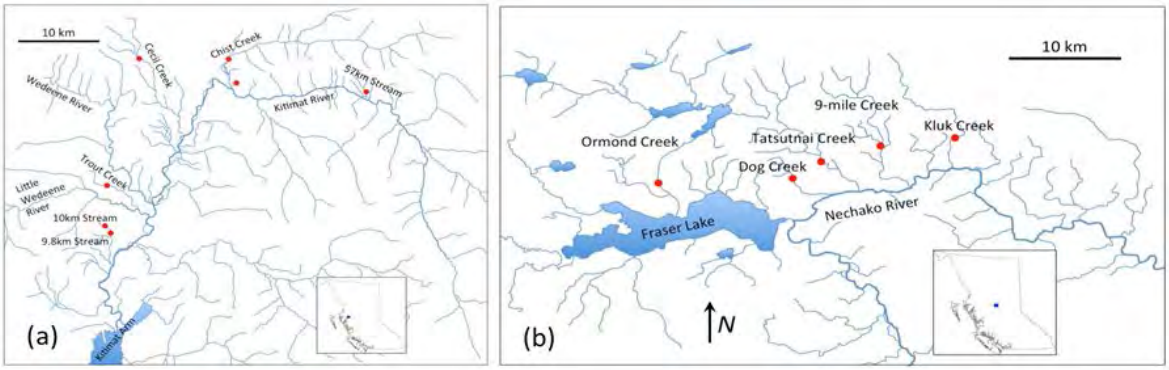

Figure 2-41 – Maps of study areas (location indicated by blue squares in BC maps, inset). (a) Coastal ecosystem unit (EU) streams in section 4. (b) Interior EU streams in section 2.

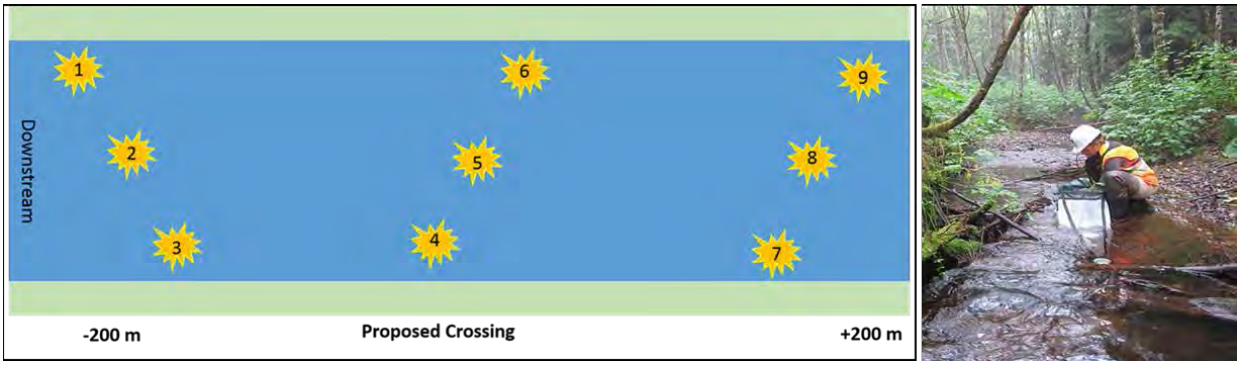

Figure 2-42 – Sampling design for each stream (left) using a Surber sampler (right).

Three benthic invertebrate sample replicates were collected from riffles located obliquely across the stream. Due to time and accessibility constraints in 2013, all study streams could not be sampled thoroughly, with some streams sampled at a single site within a stream only, or only two replicate samples taken per site (Table 2-42).

Table 2-42 – Summary of Surber samples obtained in 2013 including ecosystem unit (EU), stream name, sampling date, number of sites per stream, number of replicate per site, and total number of samples collected per stream and among all streams.

EU	Stream	Date Collected	Sites	Reps.	Total samples
Interior	Nine Mile	27-Aug-13	3	2	6
	Salmon	29-Aug-13	3	3	9
	Tatsunai	28-Aug-13	1	2	2
	Dog	30-Aug-13	3	2	6
	Ormond	28-Aug-13	1	2	2
Coastal	Clear	26-Sep-13	1	2	2
	Unnamed 9.8 km	22-Aug-13	3	2	6
	Unnamed 57.1 km	04-Oct-13	3	2	6
	Cecil	21-Aug-13	1	3	3
	Trout	05-Oct-13	3	2	6
				Total:	**48**

Benthic invertebrate sampling methods were adapted from protocols developed for streams in the Greater Vancouver Regional District (Page et al. 2008). At each sample replicate location within a site, benthic invertebrates were sampled using a Surber sampler with a 30- × 30-cm frame (0.09 m^2) and 250-micron mesh size. The metal frame was securely placed into the substrate with the net flowing downstream to catch invertebrates released by disturbing the substrate within the framed area. Each Surber sample was obtained from one, three-min sampling event within a riffle section of the stream, with three sample replicates taken per site, for a total of nine min of sampling performed at three locations within each site.

First, large rocks within the sampling frame were rubbed causing invertebrates to be dislodged downstream into the mesh and then removed from the sampling area. Once large rocks were removed, the gravel and sand was stirred up to displace invertebrates found deeper in the substrate allowing the current to wash them into the net as well. At the end of the three-min interval, five elutriation steps were performed to concentrate the organic material and discard the inorganic debris such as rocks and sand. A sterilized 5-L pail was used to swirl the sample and suspend the organic material, which was then poured back into the funnel of the Surber sampler. After the inorganic debris was rinsed five times, it was discarded. The organic material was transferred to a 1-L sterile Nalgene bottle, and the sample, including any remaining debris, was then covered with 95 % ethanol to preserve invertebrate specimens for subsequent morphological analyses.

During this sampling season, we also tested the feasibility of using aquarium nets for invertebrate sampling. For each Surber sample placement we also took an invertebrate sample using 25- × 25-cm, 0.09- × 0.09-mesh size aquarium net. These samples were collected in the same manner as the Surber samples, except instead of elutriating samples, the net was cut and placed with its contents into a 1-L Nalgene bottle and covered with 95 % ethanol.

In each EU, an additional two aquarium nets were used to control for potential invertebrate contamination between streams. Samples with these control nets were collected following the same protocol as the Surber samples. In the interior EU, the control nets were used to sample two locations at each of the three sites in Nine Mile Creek, Salmon River, and Dog Creek. In the coastal EU, two control nets were used to sample two locations at each site in Trout Creek and Unnamed 57.1 km.

2014 benthic invertebrate sampling

Based on preliminary results from 2013 data, 14 streams (seven from the interior EU and seven from the coastal EU) were chosen to sample multiple times throughout the 2014 field season. Sampling methods were revised to exclude all aquarium net sampling, and limited Surber sampling to three sites (+200 m, 0 m, -200 m), allowing for subsequent analyses of spatial and temporal variation in benthic invertebrate communities in the study area.

Surber sampling effort was also increased in 2014. Each Surber sample replicate was a composite of three, three-min placements within one or more riffles depending on stream size for a total of nine min of sampling performed for each Surber sample. Three replicate Surber samples were taken at each site within a stream for a total of nine samples taken from three sites within one stream (Table 2-43).

In all coastal streams except Unnamed 41.5 km, benthic invertebrates were sampled three times, once in early spring, once in early summer, and again in mid summer. During the first sampling period, Unnamed 41.5 km was very small; therefore only two replicates were taken at each site and it was not sampled during the third sampling period due to a dry streambed. High freshet water levels in interior streams prevented their sampling in early spring, except Nine Mile Creek and Kluk Creek, which were sampled on May 23 and 29,

respectively. Most interior streams were sampled twice, once in early summer and again in late summer (Table 2-43).

Table 2-43 – Summary of samples obtained in 2014 including ecosystem unit (EU), stream name, sampling dates, and the total number of samples collected per stream and from all streams. Asterisk (*) indicates that two replicates instead of three were taken at each sampling site due to small stream size

Section	Stream	Date Collected	Date Collected	Date Collected	Total no. samples
2	Nine Mile	23-May-14	26-Jun-14	05-Sep-14	27
	Salmon		14-Jul-14	02-Sep-14	18
	Tatsunai		02-Jul-14	27-Aug-14	18
	Dog		08-Jul-14		9
	Ormond		24-Jun-14	09-Sep-14	18
	Kluk	29-May-14	03-Jul-14	18-Aug-14	27
	Tchesinkut		09-Jul-14	25-Aug-15	18
	Unnamed 9.8 km	03-May-14	03-Jun-14	27-Jul-14	27
	Unnamed 57.1 km	05-May-14	14-Jun-14	25-Jul-14	27
4	Cecil	07-May-14	08-Jun-14	26-Jul-14	27
	Chist	04-May-14	07-Jun-14	29-Jul-14	27
	Unnamed 41.5 km	08-May-14*	08-Jun-14		15
	Unnamed 10 km	06-May-14	05-Jun-14	27-Jul-14	27
	Trout	02-May-14	12-Jun-14	28-Jul-14	27
				TOTAL:	**309**

Measurement of environmental parameters

Environmental and physical data were collected at the same time as invertebrate sampling. Channel width and wetted width were measured using a 50-m measuring tape, while channel depth, wetted depth, and velocity were measured using a current velocity meter (Model 2100, Swoffer Instruments Inc.). The physical stream characteristics, including pH, conductivity, dissolved oxygen and temperature, were measured at each site using a Professional Plus multiparameter water quality instrument (SKU 605596, YSI Inc.). Discharge was calculated using average measurements of wetted depth, wetted width and velocity measured over the course of the summer (Table 2-44 and Table 2-45).

Morphological identification of specimens

Invertebrate samples were processed following the Canadian Aquatic Biomonitoring Network (CABIN) sub-sampling protocol (Reynoldson et al. 2001). Large samples that were estimated to contain >600 individuals were subsampled using either a grid tray with 25 cells or

a Marchant box with 100 cells. Specimens found in individual, randomly selected cells were sorted to one of six groups (Ephemeroptera, Plecoptera, Trichoptera, Diptera, Other Insects, and Non-Insects) until at least 300 individuals were selected for further morphological identification. If samples contained <600 specimens, the entire sample was processed. The benthic invertebrates included in the specimen count were composed of insects and water mites (Hydrachnida: Trombidiformes; Figure 2-43).

Table 2-44 – Physical and environmental characteristics for study streams in the interior ecosystem unit (EU). Ranges are given to those attributes that varied throughout the sampling period and are based on the measurements taken while sampling. The streams are part of the Nechako River (NECR), lower Salmon River (LSAL), and Francois Lake (FRAN) watershed.

	Nine Mile	Ormond	Salmon	Tatsunai	Tchesinkut	Kluk	Dog
	10U	10U	10U	10U	10U	10U	10u
UTM	0403760 6000823	0384516 5997656	0496605 6006298	0398575 5999333	0341621 5996361	0410369 6001326	0396112 5997776
Elevation (m)	780	740	725	775	710	825	845
Watershed	NECR	FRAN	LSAL	NECR	FRAN	NECR	NECR
Channel width (m)	4.6–4.9	13.5–21.0	40.1–54.0	4.6–7.1	7.3–10.1	4.9–2.4	1.5–2.3
Mean depth (m)	0.23	0.19	0.39	0.1	0.35	0.14	0.04
Max. Velocity (ms^{-1})	0.65	0.89	0.82	0.77	0.29	0.56	0.16
Discharge (m^3s^{-1})	0.33	0.76	6.08	0.12	0.40	0.09	0.01
Temperature (°C)	7.2–11.4	8.2–18.3	13.1–24	10.6–14.4	14.5–17.1	11.0–14.3	9.3–13.2
pH	7.2–7.7	7.3–7.8	7.4–8.0	7.1–8.0	7.4–7.7	7.2–7.5	7.2–7.5
Conductivity (µS at 25 °C)	98.6–133.3	61.9–76.9	144.1–156.2	88.4–117.2	109.1–136.4	49.5–130.4	83.0–142.5
Dissolved O_2	8.12–11.12	9.28–11.96	2.38–10.06	4.02–9.82	4.6–9.3	2.73–11.9	5.3–9.7

Table 2-45 – Physical and environmental characteristics for study streams in the coastal ecosystem unit (EU). Ranges are given to those attributes that varied throughout the sampling period and are based on the measurements taken while sampling. The streams are part of the Kitimat River (KITR) watershed.

	Cecil	Chist	Trout	57.1 km	9.8 km	10 km	41.5 km
UTM	09U 0524093 6014068	09U 0532066 6014515	09U 0520509 6000189	09U 0547054 6011453	09U 0520992 5995851	09U 0520938 5996048	09U 0533105 6011984
Elevation (m)	175	165	120	990	55	75	135
Watershed	KITR	KITR	KITR	KITR	KITR	KITR	KITR
Channel width (m)	7.6–14.8	32.9–43.9	5.74–10.15	5.3–6.6	3.3–5.2	1.8–3.9	2.8–4.3
Mean depth (m)	0.28	0.34	0.23	0.38	0.15	0.15	0.17
Max. Velocity (ms^{-1})	0.62	0.99	0.85	1.19	0.58	0.99	0.58
Discharge (m^3s^{-1})	0.79	6.01	0.67	1.1	0.19	0.12	0.17
Temperature (°C)	4.8–8.2	6.4–10.5	5.8–12.3	4.3–11.4	5.9–8.7	6.2–8.2	6.0–8.6
pH	6.1–7.2	6.6–7.3	6.2–7.2	6.7–7.5	6.4–6.9	6.5–7.2	6.3–6.9
Conductivity (µS at 25 °C)	21.5–52.5	23.3–30.7	22.5–60.4	25.9–88.4	23.8–29.9	22.4–34.3	15.9–20.8
Dissolved O_2	11.14–14.7	6.67–14.49	7.65–15.28	11.77–14.68	9.20–13.75	7.8–13.8	12.8–13.9

Figure 2-43 – Images of representative specimens of each of the dominant Orders included in the specimen count and identified to Family including: (a) mayflies (Ephemeroptera), (b) stoneflies (Plecoptera), (c) caddisflies (Trichoptera), (d) flies (Diptera), (e) riffle beetles (Coleoptera), and (f) water mites (Trombidiformes).

Other invertebrates identified, but not included in the specimen count, were worms (Annelida, Nematoda and Nematomopha), sponges (Porifera), ostracods (Ostracoda), copepods (Copepoda), amphipods (Amphipoda), as well as algae, vertebrate eggs, and unknown organisms when present (Figure 2-44). The total abundance for individuals of each Family was extrapolated to the whole sample using the CABIN approach (Reynoldson et al. 2001). Benthic invertebrates were morphologically identified to at least Family level using available taxonomic keys including Clifford (1991), Needham (1996), Wiggins (1996), Stewart and Osgood (2006), and Merritt et al. (2008). Some samples and some particularly large and well-preserved invertebrates from both 2013 and 2014 field seasons were further identified to Genus and species.

Because this study assessed the impacts of pipeline stream crossings, sedimentation was thought to be the most likely and apparent impact on stream water quality. Benthic macroinvertebrates identified to the Genus or species level were assigned a suspended sediment tolerance index value. Higher values, on a scale of 0–10, were assigned to organisms considered more tolerant of poor water quality (i.e., higher suspended sediment levels). Tolerance index values were extracted from the literature (Barbour et al. 1999; Relyea et al. 2000; Rosenberg et al. 2000; Carlisle et al. 2007). Organisms without available tolerance values were excluded from the summary.

In 2013, benthic invertebrates from all Surber samples collected were identified (n = 48). The remaining samples were stored in clearly labeled containers preserved in 95 % ethanol for potential future morphological and molecular analyses. Laboratory work then focused on the identification of specimens obtained during the 2014 field season to identify taxa collected earlier in the season in spring when many common stream taxa are known to be most abundant.

Due to the considerably greater number of samples collected in 2014 and limited resources, morphological identification of specimens focused on samples from the three upstream (+200 m) replicate samples and three downstream (-200 m) replicate samples from nine study streams. Four of the study streams were located in the interior EU (section 2):

Ormond, Nine Mile, Salmon, and Tatsunai, and five study streams were located in the coastal EU (section 4): Cecil, Chist, Trout, Unnamed 57.1, and Unnamed 9.8 km.

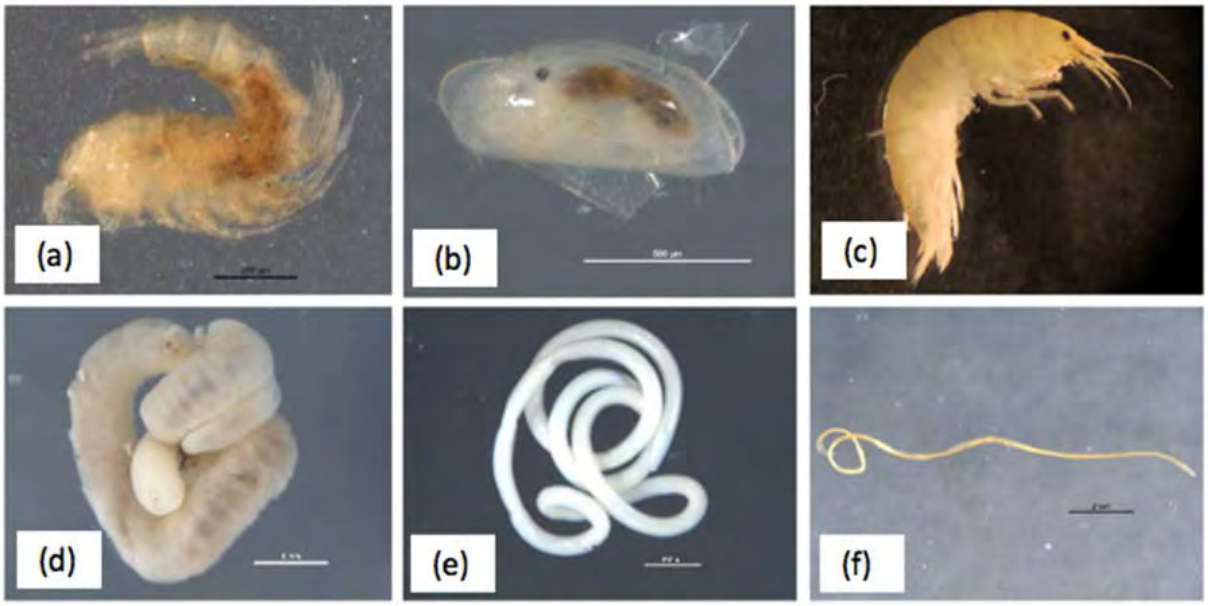

Figure 2-44 – Images of representative specimens of other common taxa in Surber sample collected but not included in the specimen count and identified to Family including: (a) copepods (Copopoda), (b) ostracods (Ostracoda), (c) amphipods (Amphipoda), (d) annelid worms (Clitellata), (e) nematode worms (Nematoda), and (f) horsehair worms.

Statistical analyses

Further statistical analyses were conducted on the three downstream (-200 m) replicate samples and the three upstream (+200 m) replicate samples collected from the nine study streams. Abundance data for taxa collected from the three replicate samples at each site were pooled to give a value for each sampling site. From the pooled samples, eight biotic parameters were calculated according to Reynoldson et al. (2001): (1) total invertebrate abundance; (2) taxa richness; (3) Shannon Diversity; (4) Evenness; (5) % EPT, % Hydropsychidae, % Baetidae; and (6) the Hilsenhoff Biotic Index or HBI (Hilsenhoff 1988; Sponseller et al. 2001; Buss and Salles 2007; Sola and Prat 2006).

Rarefaction curves for the study streams were calculated using AccuCurve 1.0 (Drozd and Novotny 2010). Each sample replicate was treated as an independent sample and added

consecutively. Four curves for each study stream were generated including maximum, minimum and average rarefaction curves and the number of singleton taxa identified calculated over 1,000 iterations.

Due to incomplete sampling during the first sampling period in 2014, all of the samples from the first sampling period were dropped from further statistical analyses. Three environmental variables (wetted width, dissolved oxygen and temperature) were chosen for further analysis based on their influence on invertebrate community, representation of time and reliability as a measurement. Two different models were tested to determine how the biological metrics are influenced by abiotic variables measured over time. First a repeated measures design was implemented to determine how time covaried with the environmental measures for each biotic measurement. Secondly, a nested model was tested which nested sampling time within stream and investigated the effects of environmental measurements on the biotic measures at each time point.

Results

2013 morphological identifications

From Surber samples obtained in 2013, a total of 16,011 specimens were processed from 48 samples, and from 13,999 individuals a total of 30 Families were identified. Chironomidae (Diptera) and Heptageniidae (Ephemeroptera) were among the four most abundant Families present in all streams except Unnamed 9.8 km in section 4 and Clear Creek in section 2 (Table 2-46). Also dominant overall in both interior and coastal streams was Baetidae (Ephemeroptera). Next most dominant in interior streams was Hydropsychidae (Trichoptera) and Chloroperlidae (Plecoptera) in coastal streams.

Taxon rarefaction curves revealed that sampling effort in 2013 was insufficient to detect all Families likely present in most study streams. Among the interior streams, the slope of the curves for Ormond and Tatsutnai Creeks do not begin to decrease or approach asymptote (the point on the curve where slope equals zero), indicating that the low number of samples collected from those streams was insufficient to detect all benthic invertebrate Families present in the study area (Figure 2-45). The slope of the rarefaction curve for Nine Mile Creek begins to

decrease with three samples collected and reaches asymptote by six samples, suggesting that sampling was sufficient to detect most of the Families present in this stream. Because the slope of the curve for Salmon River does not begin to reach asymptote by nine samples, however, sampling effort was insufficient to detect all Families likely present in Salmon, and indicates that taxa richness is considerably higher in Salmon than in Nine Mile.

Table 2-46 – Four most abundant taxa and their relative percent abundance (%) based on taxa identified from Surber samples (n) collected at each stream in late 2013. The total abundances for each ecosystem units were calculated by including counts from all samples within the respective ecosystem unit.

Stream	n	Dominant 1	%	Dominant 2	%	Dominant 3	%	Dominant 4	%
Nine Mile	6	Heptageniidae	17.3	Chironomidae	14.7	Psychodidae	13.4	Hydropsychidae	9.0
Ormond	2	Heptageniidae	18.1	Chironomidae	16.1	Psychodidae	10.7	Hydropsychidae	8.9
Salmon	9	Hydropsychidae	41.2	Chironomidae	30.6	Heptageniidae	4.7	Tipulidae	3.9
Tatsutnai	2	Hydropsychidae	23.0	Heptageniidae	13.6	Baetidae	13.6	Chironomidae	10.3
Dog	6	Chironomidae	19.7	Glossosomatidae	16.3	Chloroperlidae	13.5	Heptageniidae	12.4
Clear	2	Chironomidae	71.9	Baetidae	9.1	Brachycentridae	6.4	Simuliidae	6.0
Interior	**27**	**Chironomidae**	**23.7**	**Hydropsychidae**	**16.9**	**Heptageniidae**	**11.5**	**Baetidae**	**7.1**
Cecil	3	Chironomidae	19.4	Glossosomatidae	16.9	Heptageniidae	16.0	Baetidae	8.8
Trout	6	Heptageniidae	22.3	Chironomidae	20.5	Hydropsychidae	16.0	Baetidae	8.7
57.1 km	6	Heptageniidae	28.6	Baetidae	14.9	Chironomidae	10.4	Glossosomatidae	8.2
9.8 km	6	Chironomidae	30.3	Simuliidae	26.0	Nemouridae	10.5	Baetidae	7.8
Coastal	**21**	**Chironomidae**	**19.4**	**Heptageniidae**	**19.2**	**Baetidae**	**10.6**	**Simuliidae**	**8.2**

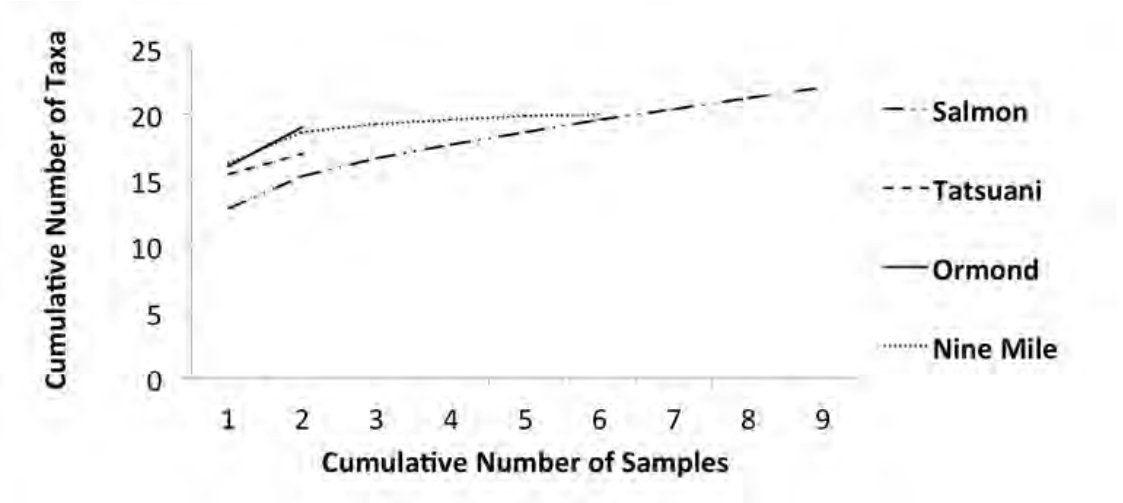

Figure 2-45 – Family rarefaction curves for Surber samples collected from interior study streams from August to October 2013, calculated over 1,000 iterations.

Of the coastal streams, the slopes of Family rarefaction curves for Unnamed Creeks 9.8 and 57.1 km and Trout Creek began to decrease by six Surber samples but did not reach asymptote, indicating that sampling effort was insufficient to detect all Families likely present in these three streams (Figure 2-46). Only three Surber samples collected from Cecil Creek were identified and the slope of the curve began to decrease with three samples but also did not reach asymptote, suggesting more Families are present in Cecil Creek than were detected with sampling effort.

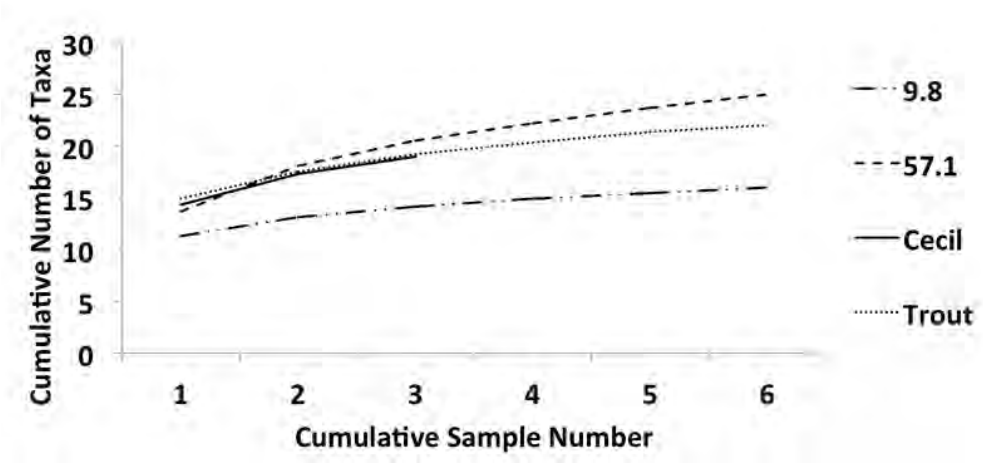

Figure 2-46 – Family rarefaction curves for Surber samples collected from coastal study streams from May to September 2014, calculated over 1,000 iterations.

2014 morphological identifications

From Surber samples collected in 2014, a total of 53,724 benthic invertebrate specimens were sorted from 144 samples and from 42,484 individuals 35 Families were identified. Similar to 2013 data, Chironomidae (Diptera) and Heptageniidae (Ephemeroptera) were among the four most abundant taxa for all of the streams investigated. In both the interior and coastal EU, Chironomidae (Diptera), Heptageniidae (Ephemeroptera), and Baetidae (Ephemeroptera) were the top three dominant Families. The fourth dominant Family was Hyropsychidae (Trichoptera) in the interior EU and Chloroperlidae (Plecoptera) in the coastal EU (Table 2-47).

Taxon rarefaction curves revealed that the increased sampling effort conducted in study streams during the 2014 field season contributed to the detection of a greater proportion of all Families likely present in the study streams, indicating that sampling methods in 2014 were more efficiently capturing stream diversity. With 12 samples per stream, the slopes of the Family rarefaction curves for all four interior streams decrease and begin to reach asymptote (Figure 2-47), indicating that most of the Families present in these streams were detected with the amount of sampling conducted. Even with 12 samples, however, the total numbers of Families per stream were still accumulating, which suggests that more Families may still be identified from the interior study streams with increased sampling effort (Figure 2-47).

Table 2-47 – Four most abundant taxa and their relative percent abundance (%) based on taxa identified from Surber samples (n) collected at study streams during 2014 field season.

Stream	n	Dominant 1	%	Dominant 2	%	Dominant 3	%	Dominant 4	%
Nine Mile	27	Heptageniidae	27.1	Chironomidae	26.6	Baetidae	12.3	Choloroperlidae	5.5
Ormond	18	Chironomidae	46.0	Heptageniidae	22.7	Leptophlebiidae	9.5	Chloroperlidae	4.9
Salmon	18	Chironomidae	50.8	Hydropsychidae	15.9	Baetidae	7.7	Heptageniidae	7.3
Tatsunai	18	Chironomidae	35.0	Baetidae	14.4	Heptageniidae	13.6	Simuliidae	5.7
Interior	**81**	**Chironomidae**	**38.3**	**Heptageniidae**	**19.2**	**Baetidae**	**9.2**	**Hydropsychidae**	**5.0**
Cecil	27	Chironomidae	34.4	Heptageniidae	26.8	Baetidae	12.0	Chloroperlidae	8.3
Chist	27	Heptageniidae	36.5	Chironomidae	32.7	Baetidae	12.9	Ephemerellidae	6.8
Trout	27	Heptageniidae	28.9	Chironomidae	28.2	Chloroperlidae	9.7	Baetidae	8.6
57.1 km	27	Chironomidae	34.1	Heptageniidae	33.2	Baetidae	13.6	Chloroperlidae	6.2
9.8 km	27	Chironomidae	49.8	Chloroperlidae	14.3	Simuliidae	10.3	Heptageniidae	10.0
Coastal	**135**	**Chironomidae**	**36.3**	**Heptageniidae**	**26.8**	**Baetidae**	**10.1**	**Chloroperlidae**	**8.8**

Family rarefaction curves for coastal streams were similar to those for interior streams, with the slopes of the curves for all five streams decreasing with approximately nine Surber samples identified per stream and those for Unnamed 9.8 km, Trout and Cecil Creeks reaching asymptote with 18 samples identified (Figure 2-48), indicating that sampling effort was sufficient to identified most Families present in these three streams during the sampling period (early June to late July). The slopes of the curves for Chist and Unnamed 57.1 km did not reach asymptote with 18 samples identified per stream, indicating that increased sampling would be required to capture all Families present in these two streams.

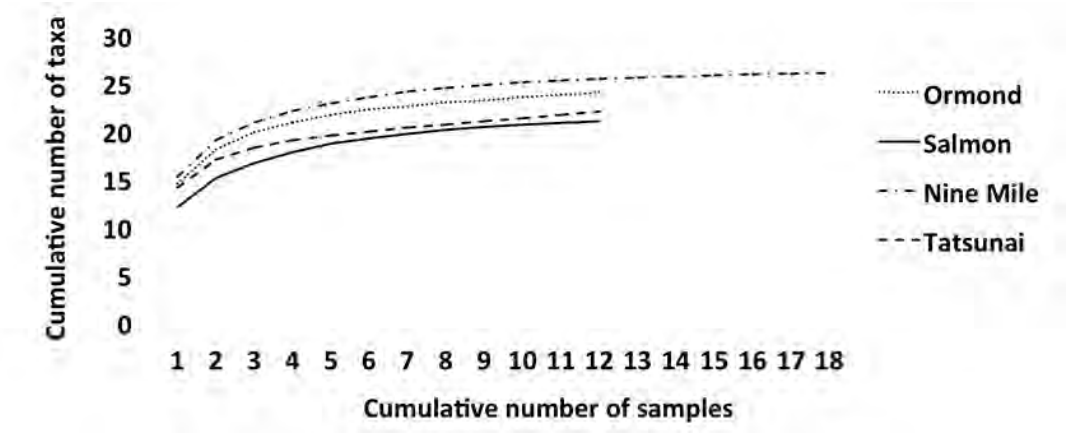

Figure 2-47 – Family rarefaction curves for Surber samples obtained from interior streams between late June and early September in 2014, calculated with 1,000 iterations.

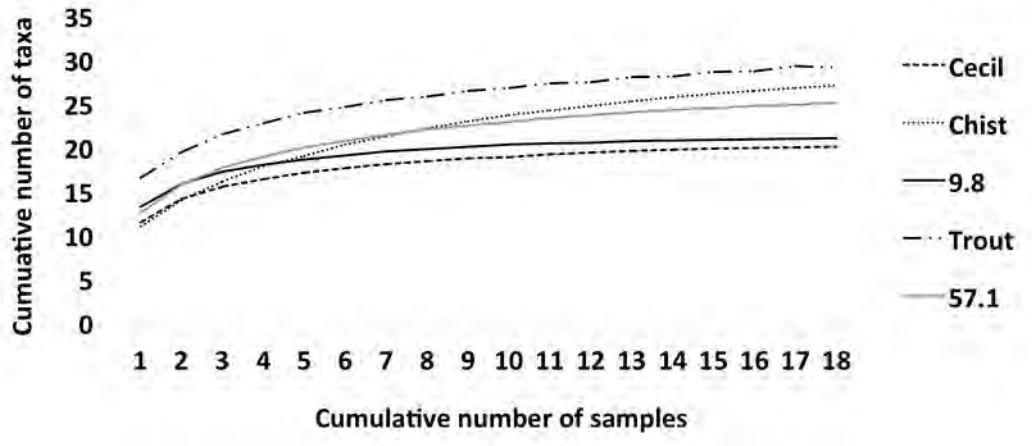

Figure 2-48 – Family rarefaction curves for Surber samples obtained from coastal streams between early June and late July in 2014, calculated with 1,000 iterations.

Comparison of 2013 and 2014 morphological identifications

When morphological identifications of benthic invertebrates obtained in 2013 and 2014 were compared, differences between the Families obtained in each year were found (Table 2-48). Three Families were identified only from 2013 samples: the stonefly Family Peltoperlidae and the caddisfly Family Philopotamidae. Six Families were identified only from samples collected in 2014: caddisfly Families Leptoceridae and Psychomyiidae and fly Families

Blephariceridae, Empididae, Sciomyiidae and Stratiomyidae. Individuals of most Families that were specific to one year were found in low numbers (less than 10), but 15 Peltoperlidae specimens were identified in samples collected from five streams in 2013 (two coastal streams and three interior streams) and 28 Stratiomyidae specimens were identified in samples collected from seven streams in 2014 (all five coastal streams and two interior streams).

In 2014, some Families were identified in samples collected from only coastal or interior streams and some only from larger study streams, which includes Chist Creek in section 4 and Salmon River in section 2 (Table 2-49). Most notably, the stonefly Family Leuctridae was obtained exclusively in samples collected from coastal streams and the caddisfly Hydroptilidae was present only in samples obtained from interior streams. In the coastal ecosystem unit, fly Families Empididae and Athericidae were found only in the largest stream, Chist Creek but were also found in streams located in the interior ecosystem. The caddisfly Family Leptoceridae and stonefly Family Pteronarcyidae were found exclusively in the largest interior stream, Salmon River, and were not found in any coastal streams.

Table 2-48 – Invertebrate Families that were identified exclusively in 2013 or in 2014 and the streams from which they were collected. Number of identified individuals is indicated in parentheses.

Year	Taxa	Streams from which exclusive taxa were collected
2013	Peltoperlidae (15):	57.1 km (2), Trout (1), Nine Mile (1), Ormond (9), Dog (2)
	Philopotamidae (9):	57.1 km (9)
2014	Leptoceridae (2):	Salmon (2)
	Psychomyiidae (3):	Trout (2), Tatsutnai (1)
	Blephariceridae (4):	57.1 km (3), Chist (1)
	Empididae (8):	Chist (5), Ormond (3)
	Sciomyzidae (1):	Nine Mile (1)
	Stratiomyidae (28):	57.1 (11), Trout (4), Chist (4), 9.8 km (5), Cecil (2), Salmon (1), Ormond (1)

2014 biological metrics

Biotic indices were calculated for each stream based on three Surber sample replicates obtained from two sites within each of four interior and five coastal streams of interest (Table 2-50). Mean abundance tended to be higher among streams from the interior ecosystem unit;

however, the standard error was also much larger. Family taxa richness varied between individual streams; no ecosystem unit had visibly higher richness than the other. Diversity and evenness both varied from stream to stream, however higher diversity was associated with higher evenness. Percent EPT and percent Baetidae were generally high among all streams, while percent Hydropsychidae tended to be higher within interior streams. Hydropsychidae were not found in two of the coastal streams. HBI values were high among all streams, putting all streams within "very good" and "good" stream health categories according to the Hilsenhoff Family-level stream health classifications (Hilsenhoff 1988).

Table 2-49 – Families identified exclusively in one ecosystem unit (coastal or interior) or from the largest stream within the ecosystem unit. Number of individuals of each Family identified is indicated in parentheses. Invertebrates were collected in 2014.

Coastal	Interior	Large Coastal	Large interior
Leuctridae (36)	Pteronarcyidae (2) Hydroptilidae (32) Leptoceridae (2) Sciomyzidae (1)	Empididae (8) Athericidae (3)	Leptoceridae (2) Pteronarcyidae (2)

2013 and 2014 species-level bioindicators

Organisms in both coastal (Table 2-51) and interior (Table 2-52) streams had sediment tolerance index values that ranged from 0–6. In both sample years, streams in both ecosystems were dominated by genera and species that were relatively intolerant of high levels of suspended sediment.

2014 environmental data analyses

The repeated measures design suggested that taxa richness is significantly affected by temperature ($F_{1,16} = 6.54$, $p < 0.05$) and percent Baetidae was significantly affected by temperature ($F_{1,16} = 9.44$, $p < 0.05$) and a covariate of time ($F_{1,14} = 33.27$, $p < 0.001$). The second model provided another approach to interpret the data. During the second sampling period percent Baetidae was significantly affected by temperature ($Z = 4.55$, $p < 0.001$), and percent Hydropsychidae was significantly affected by dissolved oxygen ($Z = -2.50$, $p < 0.05$) and wetted width ($Z = -2.15$, $p < 0.05$). More significant effects were found during the third sampling

period. Taxa richness was significantly affected by ecotype ($Z = 4.93$, $p < 0.001$), dissolved oxygen ($Z = 3.68$, $p < 0.001$) and wetted width ($Z = -4.67$, $p < 0.001$). Percent EPT was affected significantly by wetted width ($Z = -2.11$, $p < 0.05$). Percent Baetidae was significantly affected by temperature ($Z = -2.33$, $p < 0.05$), ecotype ($Z = 3.29$, $p < 0.001$), and wetted width ($Z = 5.01$, $p < 0.001$), while percent Hydropsychidae was significantly affected by temperature ($Z = 2.31$, $p < 0.05$).

Table 2-50 – The mean number of invertebrates (±1SE) found at each stream from pooled (three replicates of 3 min composite Surber samples) samples taken from two study sites within each stream over two or three sampling dates (n = 6 per stream, except for Ormond, Salmon, and Tatsunai which were only sampled twice at both study sites; n = 4). Family level taxa richness, Shannon diversity, Pielous' evenness, % EPT, % Baetidae, % Hydropsychidae, and Hilsenhoff biodiversity index (HBI) represents all taxa found at each stream over all sampling dates. A pooled sample consisted of three replicated 3 min composite Surber samples.

Stream	Mean inverts. (±SE)	Taxa rich.	Shannon diversity	Pielous' evenness	% EPT	% Baetidae	% Hydrops.	HBI
Interior								
Nine Mile	4301(±1065)	27	2.38	1.09	63.30	25.74	36.90	4.19
Ormond	11852(±5674)	25	2.07	0.95	50.18	6.40	27.56	4.42
Salmon	7299(±840)	22	1.96	0.90	43.76	37.63	91.63	4.62
Tatsunai	6039(±1949)	23	2.30	1.05	55.41	43.64	34.93	4.36
Cecil	5438(±2240)	21	1.91	0.88	60.97	29.23	0.00	4.19
Coastal								
Chist	4530(±785)	26	1.74	0.80	62.46	25.26	8.33	4.23
Trout	1693(±531)	27	2.20	1.01	62.96	24.33	47.56	3.94
57.1 km	2782(±774)	26	1.84	0.84	60.18	33.51	5.92	4.36
9.8 km	3662(±809)	21	1.76	0.81	38.01	20.69	0.00	4.47

Table 2-51 – Species-level sediment tolerance levels and number of each macroinvertebrate species found in coastal streams in 2013 and 2014. Tolerance level (Tol.) of 0 indicates lowest tolerance; E = Ephemeroptera, P = Plecoptera, T = Trichoptera, D = Diptera.

			Cecil		Chist		Trout		Unmd 9.8		Unmd 57.1		Unmd 41.5		Unmd 10	
	Taxon	Tol.	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
E	*Ameletus sp.*	0				7		7						3		3
E	*Drunella doddsi*	0	49			4	25	43			8					
E	*Drunella sp.*	0	1	35		12		5	1							5
E	*Epeorus sp.*	0	8	102		231		2	1					2		7
E	*Rithrogena sp.*	0	7	37		29	76	73			20					24
P	*Suwallia sp.*	0				39										
P	*Visoka sp.*	0						3			32					
T	*Psychoglypha sp.*	0				1										
T	*Rhyacophila sp.*	0	20	79		1	39	42	19		90			1		5
E	*Caudatella sp.*	1		2												
E	*Ephemerella sp.*	1		4		19										
P	*Paraperla sp.*	1					4									42
P	*Sweltsa sp.*	1	1			11			1		4			1		
T	*Arctopsyche sp.*	1				1										
T	*Brachycentrus sp.*	1	1													
T	*Glossosoma sp.*	1	117	15			78		1		77			1		2
T	*Lepidostoma sp.*	1						1								
T	*Micrasema sp.*	1	3	58			1	3			1			1		
E	*Serratella sp.*	2	1	37		22			2							
P	*Kogotus sp.*	2				1										
P	*Taenionema sp.*	2						1								
P	*Zapada sp.*	2	58	1		14	21		111		25			1		2
T	*Ecclisomyia sp.*	2				3					49					
D	*Dicranota sp.*	3		8		2	2				5					
E	*Heptagenia sp.*	3					1									
D	*Pericoma sp.*	4					3	1								
E	*Cinygmula sp.*	4	25	367		131	29	74	5		28			18		85
E	*Ironodes sp.*	4					12	1	4							
E	*Paraleptophlebia sp.*	4	10	1			5	1	22		18			7		8
T	*Hydropsyche sp.*	4					118	23								
E	*Baetis sp.*	5				53										
E	*Stenonema sp.*	5														1
D	*Forcipomyia sp.*	6									1					

Table 2-52 – Species-level sediment tolerance levels and number of each macroinvertebrate species found in coastal streams in 2013 and 2014. Tolerance level (Tol.) of 0 indicates lowest tolerance; E = Ephemeroptera, P = Plecoptera, T = Trichoptera, D = Diptera.

			Nine Mile		Ormond		Salmon		Tatsunai		Clear		Dog	
Taxon		Tol.	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
E	*Ameletus* sp.	0	27		3		3							
E	*Drunella doddsi*	0											21	
E	*Drunella* sp.	0	38		2				2	17			4	
E	*Epeorus* sp.	0	87							48				
E	*Rithrogena* sp.	0			48		1							
P	*Pteronarcella* sp.	0											4	
T	*Rhyacophila* sp.	0	79						19	4	5		69	
E	*Ephemerella* sp.	1							1				4	
P	*Paraperla* sp.	1	4											
P	*Sweltsa* sp.	1	4		7					1			24	
T	*Brachycentrus* sp.	1	28											
T	*Glossosoma* sp.	1	29				30	10	23	3			21	
T	*Micrasema* sp.	1								12	6			
E	*Serratella* sp.	2	4					7		4			1	
P	*Skwala* sp.	2	5		1				1					
P	*Zapada* sp.	2	102		18				54	26			32	
T	*Parapsyche* sp.	2	1						1					
D	*Dicranota* sp.	3	9		2				1				4	
E	*Heptagenia* sp.	3		2										
T	*Ceraclea* sp.	3						2						
D	*Pericoma* sp.	4	201		11				44				6	
E	*Cinygmula* sp.	4	32		6					13				
E	*Ironodes* sp.	4	6							6			5	
E	*Paraleptophlebia* sp.	4	28		102		6		9				36	
T	*Hydropsyche* sp.	4	102		31		25		116	15			2	
E	*Baetis* sp.	5	20		8									
D	*Simulium* sp.	6	3											
T	*Hydroptila* sp.	6					4							

Discussion

The pilot study conducted in 2013 provided an overview of the stream present in both the coastal and interior ecosystem and enabled us to focus on nine study streams based on suitability and accessibility. Additionally, the pilot study enabled us to refine the sampling design to efficiently capture greater stream invertebrate diversity in 2014.

Rarefaction curves representing both ecosystems in 2013 show that greater taxonomic diversity was captured among streams with higher sample numbers (Figure 2-45). Generally

streams with six samples seemed to approach a plateau state suggesting the taxonomic richness is sufficiently represented at that sampling time. Sampling over different time periods, however, would require more samples to capture the taxa diversity present over a temporal range. Salmon River was one system that had relatively large number of samples (n = 9), yet the rarefaction curve did not approach asymptote (Figure 2-45). This could be a result of the large size of this system and three sample replicates at each site, which would potentially cover more microhabitats that contain different invertebrate Families. The taxa rarefaction curve results from the 2013 sampling season allowed the sampling design to be adapted to include three replicate samples at each site with increased sampling effort from three min to nine min per replicate.

In 2014, rarefaction curves show that the sampling design was sufficient to capture the diversity at most streams. There were exceptions to this trend, however. For example, the rarefaction curve for Chist Creek did not reach an asymptote. Chist Creek did have a relatively large width and high taxa richness and samples collected in this stream were spread over a larger distance and likely would have included more microhabitats, each adding additional taxa. Overall the rarefaction curves indicate that our level of sampling described most of the diversity within the sampled streams, however, individual stream size and physical characteristics should be taken into account when designing a benthic invertebrate biodiversity assessment to ensure that highly diverse streams are adequately sampled.

As was expected, greater taxa diversity was found in 2014, resulting from more intensive sampling regimes collected over a larger temporal range. The intensified sampling regime likely increased the detection of rarer taxa. Other taxa were found in abundance and consistently throughout each sample for all streams. Both Chironomidae and Heptageniidae were found in all streams at high abundances. Even though Genera and species within these Families may be specialists, combined at the Family level they can be found in many habitats which accounts for their large percentage abundance with respect to other invertebrate Families present in samples (Reece and Richardson 2000).

Certain taxa were found exclusively in 2013 or in 2014. These taxa were identified in relatively small abundances and generally only found in one or two streams (Table 2-49).

Fifteen Peltoperlidae individuals were identified exclusively in 2013 in five streams from both the interior and coastal ecosystem. This Family generally has a semivoltine life history meaning that one generation extends over more than one year (O'Hop et al. 1984; Schultheis et al. 2002). Generally eggs laid in late summer hatch in fall and grow through different instars the first year then emerge as adults in early spring of the second year (O'Hop et al. 1984; Schultheis et al. 2002). If the cohorts of Peltoperlidae are synchronized as O'Hop and others (1984) describes for the species *Peltoperla maria*, there would be a gap from spring until fall with no larval stages present in streams. This could explain the absence of Peltoperlidae in 2014 samples. Additionally, 28 individuals from Stratiomyidae Family were identified exclusively from 2014 samples in seven streams located in both the interior and coastal streams. Individuals from the Stratiomyidae Family have a univoltine life cycle, meaning that they live one year (Merrit et al. 2008). Samples collected in 2013 may have occurred too late in the season to capture larval stages of Stratiomyidae. Because sampling was intensified in 2014, taxa found exclusively in 2014 could be an artifact from greater sampling effort and samples collected over larger temporal range.

We identified a difference in the taxonomic community composition between streams samples in the coastal and interior ecosystem units. Most notably, Hydroptilidae were found in relatively large numbers exclusively in the interior ecosystem and Leuctridae in the coastal ecosystem. Little is known of Hydroptilidae ecology, generally they have a univoltine life cycle and are difficult to identify until they reach fifth instar stage when they construct their characteristic case (Wiggins 1996; Thorp and Covich 2010). Their difficulty to be identified could have contributed to finding them in only a few streams, however, there may be an unknown ecological explanation as to why this Family was only found in the interior EU. In contrast, the ecology of the Leuctridae Family has been studied more; they have a semivoltine life history and prefer stream habitats with cool summer temperatures (Elliot 1987; Stewart and Oswood 2006). Elliot (1987) studied the impacts of increasing stream temperature on Leuctridae and found that they turn to a univoltine life cycle at the increased cost of survival and egg production. Since the temperatures of coastal streams were generally lower than interior streams, the taxa may have been abundant in the coastal were only found in the coastal EU.

The remaining Families exclusive to the EUs were found in an abundance of 8 or fewer individuals. Ecological habitat difference between the two ecosystems may be the explanatory factor, however, taxonomic misidentification or missed specimens during sub-sampling may possibly account for the difference as well.

Greater invertebrate abundance present in the interior EU versus the coastal EU may be a result of the difference in land uses and discharge regimes in the respective watersheds. At the time of sampling, mature forests predominantly surrounded the coastal streams with minimal impact from land development. In contrast, active logging and open forests used for cattle pasturelands surrounded the interior streams. Run off in interior ecosystem unit could influence the amount of phosphorus and nitrogen in interior streams leading to increased primary production and increased invertebrate abundance (Howarth and Fisher 1976; Binkley and Brown 1993). The interior streams, however, would also be subjected to heavier disturbances by logging activity and cattle. Discharge regimes are much more sever in headwater streams, such as those present in the coastal EU. These are subject to frequent high-flow events after heavy rainstorms, which can impact the invertebrate abundance and taxa types able to exist (Kiffney 2003).

Taxa richness, diversity and evenness patterns varied between streams. Streams with higher taxa richness were also found to have higher associated diversity and evenness values. Evenness describes how different taxa are spread throughout an ecosystem, and in the past, has been found to decrease with increased diversity (Death and Winterbourn 1995). Death and Winterbourn attributed this effect to competition between taxonomic groups, limiting certain taxa to a particular microhabitat and decreasing the evenness measure. Our study could be limited by taxonomic resolution obscuring the true richness, diversity and evenness measures. In addition only Families from Ephemeroptera, Plecoptera, Trichoptera, Diptera, Coleoptera and Trombidiformes were included.

EPT richness, percent Baetidae, percent Hydropsychidae and HBI values were all relatively high, suggesting high stream quality (Reynoldson et al. 2001). For each stream percent EPT was greater than 35 % indicating that EPT richness is not being compromised by disturbances (Reynoldson et al. 2001). Percent Baetidae identified was high and consistent for

all streams except Nine Mile, which had a low percentage. This suggests that Baetidae is the dominant Ephemeroptera Family present in these ecosystems. Nine Mile Creek may have unique stream conditions that reduce Baetidae survival, or perhaps this Family is being outcompeted by other taxa. Percentage of Hydropsychidae among interior streams was very high, but this Family was absent from two coastal streams. This indicates that Hydropsychidae is a poor indicator taxa for coastal streams, whereas Baetidae is better represented on the coast and is more useful as an indicator taxa.

Species-level indicators present in both ecosystems were dominated by Genera and species that were relatively intolerant of high levels of suspended sediment. A few species in each ecosystem were moderately tolerant (Table 2-51 and Table 2-52), suggesting there may have been some sediment load in some streams, possibly from existing linear or forestry disturbances. Caution must be used, however, in interpreting these results. A species that has been assigned a high tolerance index value is not necessarily restricted to poor quality water; the high value simply means that the species can tolerate more sediment in the water than other species with lower values. In addition, some of the tolerance values used were drawn from sources outside central BC. Differences in ecosystems, habitats, seasons, and geographic areas could have affected the sediment tolerance index value assigned to any given Genus or species. Finally, tendency to drift, distribution pattern and rarity were not assessed for these potential indicator species. All of these factors influence the suitability of a taxon as a bioindicator. Despite these limitations, the Genera and species listed in Table 2-51 and Table 2-52 provide a baseline for assessing changes in water quality in coastal and interior BC streams, respectively, using the biotic index approach.

Typically environmental conditions of an ecosystem define the biotic distribution (Corkum 1989; Reece and Richardson 2000). Both analyses we conducted found that temperature was the main effector on benthic communities; in particular the Baetidae Family was affected by temperature. Past research shows that Baetidae emergence corresponds to warmer temperatures (Ide 1935; Corkum and Pointing 1979). Ide (1935) demonstrated that Baetidae were more likely to be found along the edges of streams and downstream where temperature where generally higher. Biotic parameters were most affected by wetted width

during the third sampling period, which is likely due to water levels significantly receding in late summer compared to water levels throughout spring and into mid summer. We also found that the EU affected biotic parameters in the third sampling period. Water levels were found to recede to a greater extent in the interior EU compared to the coastal EU, which could further explain why ecosystem unit had significant effects on biotic parameters.

Due to low degrees of freedom, however, these statistical tests are not very robust. Unforeseen events such as extremely high freshet water flow and low water levels at some streams limited the data that could be analyzed. For more robust statistical analysis, future sampling should be more frequent with a more rigorous time schedule.

Conclusions

We found that the EU is influential on the invertebrate abundance and Shannon diversity present in the streams. Appropriate biological indicator species also depended on the ecosystem unit. Baetidae were more uniformly found in the coastal EU while Hydropsychidae were found in all interior streams but not all coastal streams. The different stream health measures (i.e., Shannon diversity, %EPT, HBI) indicated that the overall health of the study streams was good to excellent. Even though patterns are beginning to appear, more frequent and consistent sampling regimes are required over multiple years to achieve a sufficient sampling effort to permit more rigorous statistical analyses of the stream ecology. However, these data provide a valuable preliminary picture of the biodiversity and stream health present in these ecosystems and can be used as a base to measure post project mitigation effectiveness.

2.5. The familiar but unrecognized Genus *Pnuemia* (Diptera: Psychodidae): the challenging taxonomic identification of a common aquatic larval moth fly species in Canada

Aynsley Thielman and Dezene Huber

Introduction

During processing of benthic invertebrates samples collected from streams located in the study areas, aquatic dipteran larvae of the Family Psychodidae were identified using standard taxonomic keys (Merritt et al. 2008) as either *Pericoma* or *Telmatoscopus,* indicating that characters to distinguish these two Genera were absent or not yet described. In the attempt to determine the correct Genus or species identity of the specimens, we searched the primary literature pertaining to moth flies in North America. Among other studies pertaining to benthic invertebrates in the Pacific Northwest and adjacent regions (Culp and Davies 1982; Clifford et al. 1991, Reece and Richardson 2000; Huebner and Vinson 2004), psychodid specimens are typically identified as *Pericoma* with no explanation of how they were distinguished from *Telmatoscopus.* Given that benthic invertebrate assemblages have routinely been studied as bioindicators of stream quality and environmental changes for decades, and tolerance values of their sensitivity to disturbance for *Pericoma* species have been reported (Merritt et al. 2008), the accurate identification of the species or Genus present in the study area (Figure 2-49) proved to be much more challenging than expected.

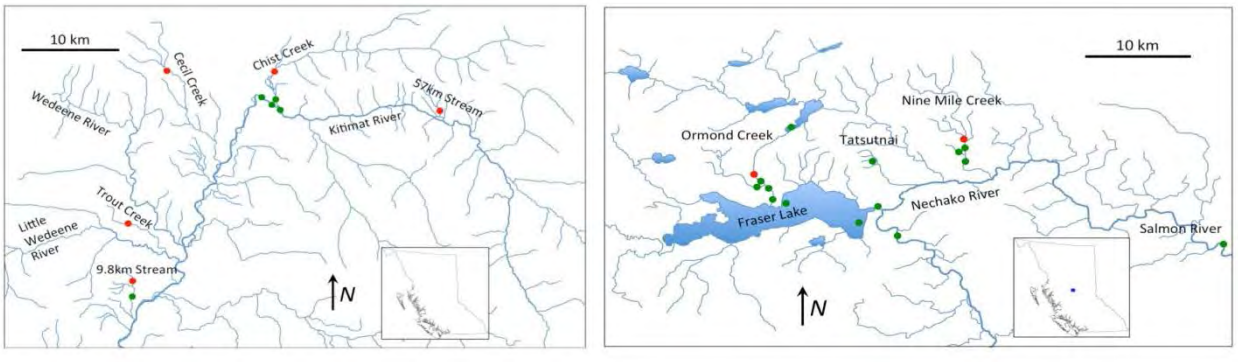

Figure 2-49 – Location of study streams sampled during 2013 and 2014.

Much of what is known about psychodids in North America has remained unchanged since the revision of the Family by Quate (1955), in which aquatic moth fly larvae are incorrectly named *Pericoma*, the larvae of which are now known to occur in only terrestrial habitats (G. Curler, *pers. comm.*). Quate (1955) noted the opportunity for discovery of new species of moth flies (Diptera: Psychodidae), but dipterists still have yet to focus their attention on this Family. Thus, taxonomic understanding of this group is convoluted and incomplete, and primary sources such as the Manual of Nearctic Diptera (Teskey 1981) remain inaccurate with respect to Psychodidae.

Taxonomic resolution is important in biomonitoring, and while Family level identification of most taxa has been found to be a sufficient measure of determining biological richness in many cases (Bailey et al. 2001), increasing taxonomic resolution and accuracy can only help contribute to better measures of stream quality (Lenat and Resh 2001). Moth flies, at the Family level, occur in a wide variety of aquatic and terrestrial habitats depending on their Genus and species, and consequently are differentially sensitive to disturbance. Moth flies therefore hold great potential as bioindicators of stream quality, if the taxonomy of this group is better resolved.

Taxonomic information for aquatic psychodid species generally states that most psychodid larvae known from Canada occur in moist to peripherally aquatic (e.g., wetted zones near streams) habitats, and the only fully aquatic species belong to the Genus *Maurina,* which occur in streams and waterfalls (Teskey 1981; Scudder and Cannings 2006).Unlike the specimens collected from within-stream substrates in this study, however, larvae of members of the Genus *Maurina* have suctorial discs ventrally on the abdomen and are known to occur in fast-moving streams and in the splash zone of waterfalls (Quate and Vockeroth 1981). In the Manual of Nearctic Diptera (Teskey 1981), Quate and Vockeroth provide a key for larvae to Subfamily only, descriptions of only some of the Nearctic Genera, and illustrations of a *Pericoma spp.* larva, which appear similar to the psychodid larvae present in samples collected in the study area (Figure 2-50). This has contributed to the incorrect identification of aquatic moth fly larvae as either *Pericoma spp*. or *Telmatoscopus spp*. by North American taxonomists, despite that fact that larvae of both Genera are not known to reside in streams.

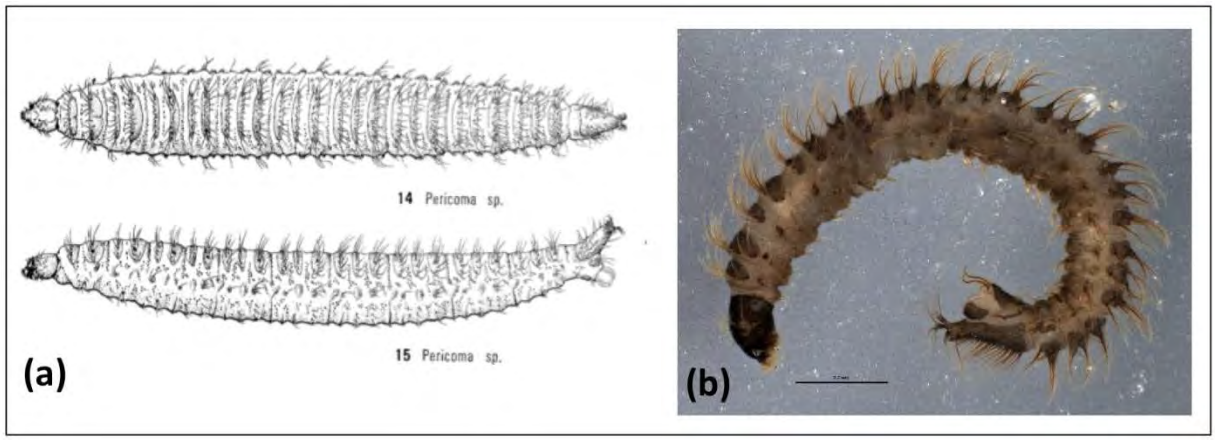

Figure 2-50 – Larvae of Psychodidae. (a) Illustration of *Pericoma sp.* from Manual of Nearctic Diptera (Teskey 1981), (b) specimen collected through current study.

Quate (1955) recognized the incorrect placement of many Nearctic psychodid species into the Genera *Psychoda* and *Pericoma*, as well as inconsistencies in descriptions and identifications between American and European workers. He transferred many species to the Genus *Telmatoscopus* but provided keys, descriptions, and distribution records for adults only. He stated that *Pericoma californica* was the only species known from BC and is suspected to be a synonym of *P. canescens* (Quate 1955). As current studies have started delving deeper into the taxonomy of this dipteran Family, new records and new species of *Pneumia* and other Psychodinae Genera have been described from other regions (Wagner and Masteller 1996; Ježek and van Harten 2005; Ibáñez-Bernal 2009; Kvifte and Andersen 2012; Oboña and Ježek 2012; Omelková and Ježek 2012,). Specifically, Omelková and Ježek (2012) have revised psychodid taxonomy in the Palearctic region and described the separation of *Pneumia* species from those of *Pericoma* and *Telmatoscopus*, which are now considered to be partly taxonomic dumping grounds for unknown moth fly taxa (G. Curler, *pers. comm.*).

DNA barcoding, the sequencing of an approximately 650-bp fragment of the cytochrome *c* oxidase subunit I (COI) gene, has become a reliable molecular method of species identification (Hebert et al. 2003). It can be used to accurately identify specimens unidentifiable by other means — including lack of morphological keys, relatively unstudied life stages, and damaged

specimens — and can be used determine species-level identifications when sequences from accurately identified specimens are present in the BOLD database (www.boldsystems.org).

Therefore, in order to determine the correct Genus or species for the specimens collected in our study as well as contributing to a greater understanding of baseline biodiversity and better accuracy in aquatic monitoring in the study area, we examined: a) literature from the Palearctic region; b) used DNA barcoding of select specimens; and c) consulted with an expert psychodid taxonomist, Dr. Greg Curler.

Methods

All available literature pertaining to Palearctic psychodids was examined in the context of the collected specimens, including Vaillant's series (1973), Von Freuerborn-Kiel (1923), and Omelková and Ježek (2012). In addition, 39 specimens identified as Psychodidae were sent to the Canadian Centre for DNA Barcoding at the University of Guelph for standard sequencing and imaging services. DNA was extracted, PCR-amplified and sequenced according to standard protocols (Ivanova et al. 2006; Ratnasingham and Hebert 2007; DeWaard et al. 2008).

Resulting barcode (COI) sequences were checked against BOLD (www.boldsystems.org) and Genbank (http://www.ncbi.nlm.nih.gov/genbank/) databases and the identity of the specimens were determined to the lowest possible taxonomic level. Using the BOLD Workbench (http://www.boldsystems.org), the Kimura-2-Parameter Model using pairwise-deletion (Kimura 1980) was used to calculate the percent sequence divergence among specimens and to generate distance summary histograms and neighbor-joining trees. Voucher specimens that were barcoded are currently held at the Canadian Centre for DNA Barcoding at the University of Guelph.

In addition, Psychodid expert, Dr. Greg Curler, was consulted. He provided assistance with the Palearctic literature pertaining to *Pneumia spp.* and his expert opinion on the most likely identity of the specimens from our study areas. In addition, 12 larvae collected through this study were recently sent to Dr. Curler for his examination using slide-mounted specimens and comparison with known specimens in his collection.

Results

A total of 654 psychodid specimens were identified from Surber samples collected primarily during August and September of 2013 and 2014 (Table 2-53). The majority of larvae were obtained from samples taken late in the season (August to October) and from interior streams (n = 647); however, a few specimens were collected early in the season (May) and from coastal streams (n = 7).

Vaillant (1973) provided detailed taxonomic information for Palearctic psychodids. Although these works are known to contain many errors and incorrectly propose the name *Satchelliella* for the Genus *Pnuemia*, they include detailed descriptions, life-history information, and illustrations for larvae. These descriptions include the Genus *Satchelliella*, known from aquatic habitats, and the Genera *Pericoma* and *Telmatoscopus,* described as primarily residing in moist or terrestrial habitats (Vaillant 1973). *Satchelliella californica* and *Pericoma californica*, and potentially also the Palearctic species *Pnuemia canescens* are synonyms commonly used in older literature (G. Curler, *pers. comm.*).

Of 39 psychodid specimens examined using DNA barcoding, 38 of the resulting sequences were found to diverge by <2 %, the standard threshold used to delineate insect species (Hebert et al. 2003). All but one of the resulting barcode sequences formed a single cluster in a neighbor-joining tree (Figure 2-51), suggesting that all specimens except for one belong to the same species. Although all sequences matched with 100 % certainty to dozens of previous records in the BOLD database, those specimens had been identified only as Psychodidae or seemingly incorrectly as *Pericoma* (n = 115). The nearest neighbor (i.e., most closely related species) in both BOLD and Genbank databases was *Pnuemia stammeri*, collected from Norway and described from the Palearctic Region (and included in Vaillant 1973).

Among the specimens in BOLD that were identified as Psychodidae or *Pericoma*, intraspecies sequence divergence was found to be <2% and interspecies >2% compared to nearest neighbor *Pneumia stammeri* (Figure 2-52).

Table 2-53 – Surber sample identification number and date and location of collection of aquatic psychodid larvae obtained from study streams in along the pipeline route.

Sample ID	Date Collected	Stream name	GPS	Number
I13-02	2013-08-21	Cecil Creek	09u 5022768 6014901	2
I13-01	2013-08-21	Cecil Creek	09u 5022768 6014901	1
I13-43	2013-10-05	Trout Creek	09u 520622 6000161	1
I13-44	2013-10-05	Trout Creek	09u 520622 6000161	2
I14-07	2014-05-02	Trout Creek	09u 520324 6000214	1
I13-29	2013-08-30	Dog Creek	10u 396162 5997601	1
I13-31	2013-08-30	Dog Creek	10u 396112 5997776	11
I13-34	2013-09-06	Dog Creek	10u 396030 5997807	1
I13-32	2013-09-06	Dog Creek	10u 396112 5997776	4
I13-33	2013-09-06	Dog Creek	10u 396030 5997807	3
I13-11	2013-08-27	Nine Mile Creek	10u 403847 6000693	14
I13-14	2013-08-27	Nine Mile Creek	10u 403734 6000949	15
I13-15	2013-08-27	Nine Mile Creek	10u 403734 6000949	31
I13-12	2013-08-27	Nine Mile Creek	10u 403743 6000840	66
I13-13	2013-08-27	Nine Mile Creek	10u 403743 6000840	75
I13-10	2013-08-27	Nine Mile Creek	10u 403847 6000693	9
I14-299	2014-09-05	Nine Mile Creek	10u 403719 6000950	6
I14-298	2014-09-05	Nine Mile Creek	10u 403720 6000945	14
I14-300	2014-09-05	Nine Mile Creek	10u 403723 6000956	15
I14-294	2014-09-05	Nine Mile Creek	10u 403825 6000705	26
I14-293	2014-09-05	Nine Mile Creek	10u 403828 6000704	39
I14-292	2014-09-05	Nine Mile Creek	10u 403831 6000699	59
I13-17	2013-08-28	Ormond Creek	10u 385848 5995737	10
I14-302	2014-09-09	Ormond Creek	10u 384611 5997500	8
I14-307	2014-09-09	Ormond Creek	10u 38444 5997851	11
I14-309	2014-09-09	Ormond Creek	10u 38404 5997858	13
I14-301	2014-09-09	Ormond Creek	10u 384643 5997490	15
I14-303	2014-09-09	Ormond Creek	10u 384606 5997504	27
I14-308	2014-09-09	Ormond Creek	10u 38436 5997856	28
I14-276	2014-08-27	Tatsutnai Creek	10u 398691 5999254	1
I14-282	2014-08-27	Tatsutnai Creek	10u 398483 5999435	9
I14-275	2014-08-27	Tatsutnai Creek	10u 398594 5999236	22
I14-274	2014-08-27	Tatsutnai Creek	10u 398717 5999244	23
I14-281	2014-08-27	Tatsutnai Creek	10u 398490 5999442	23
I14-280	2014-08-27	Tatsutnai Creek	10u 398472 5999429	24
I13-19	2013-08-28	Tatsutnai Creek	10u 400078 5998601	12
I13-18	2013-08-28	Tatsutnai Creek	10u 400078 5998601	32

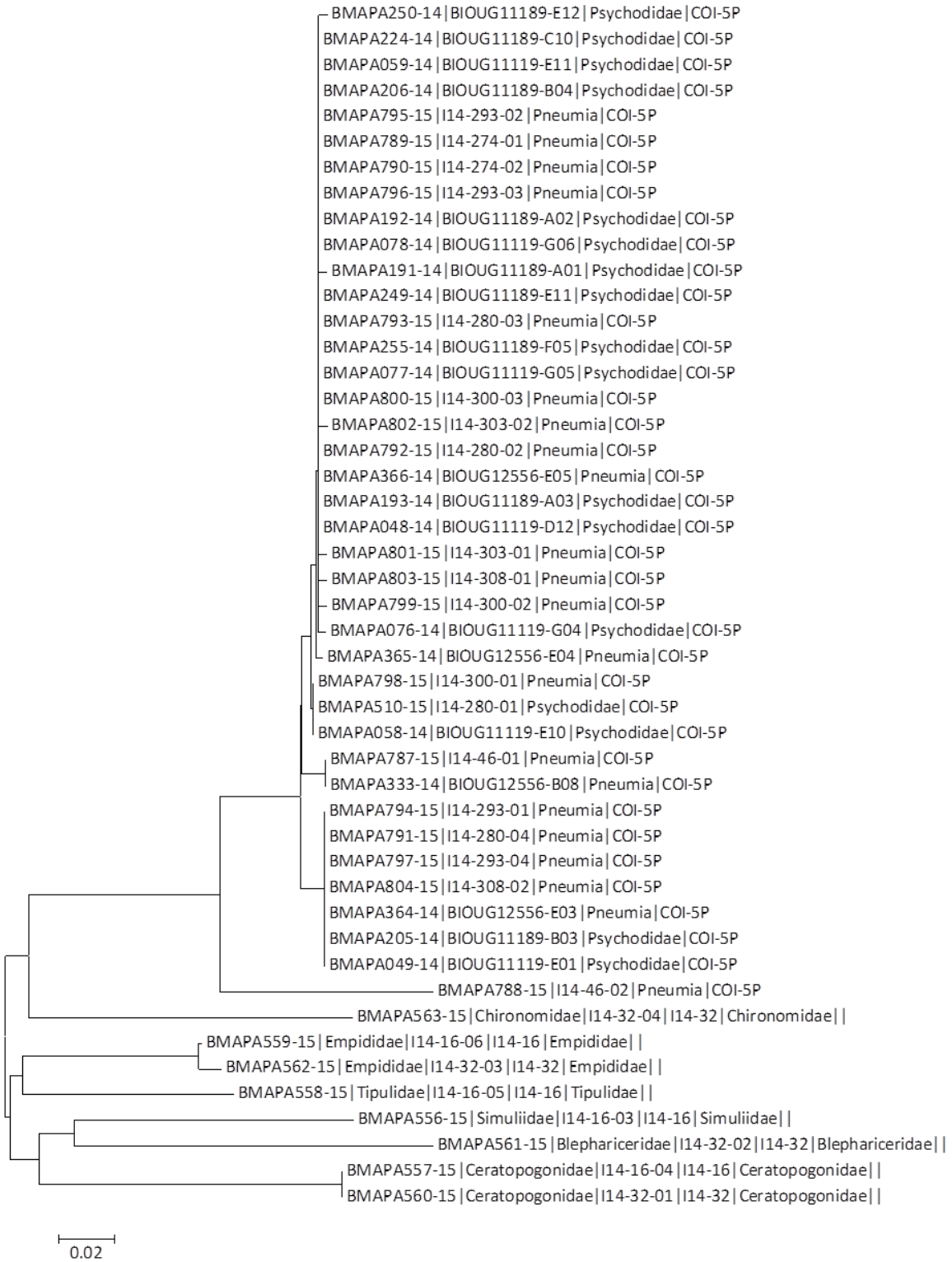

Figure 2-51 – Neighbor-joining tree of psychodid larvae collected from study streams including other dipteran larvae for comparison.

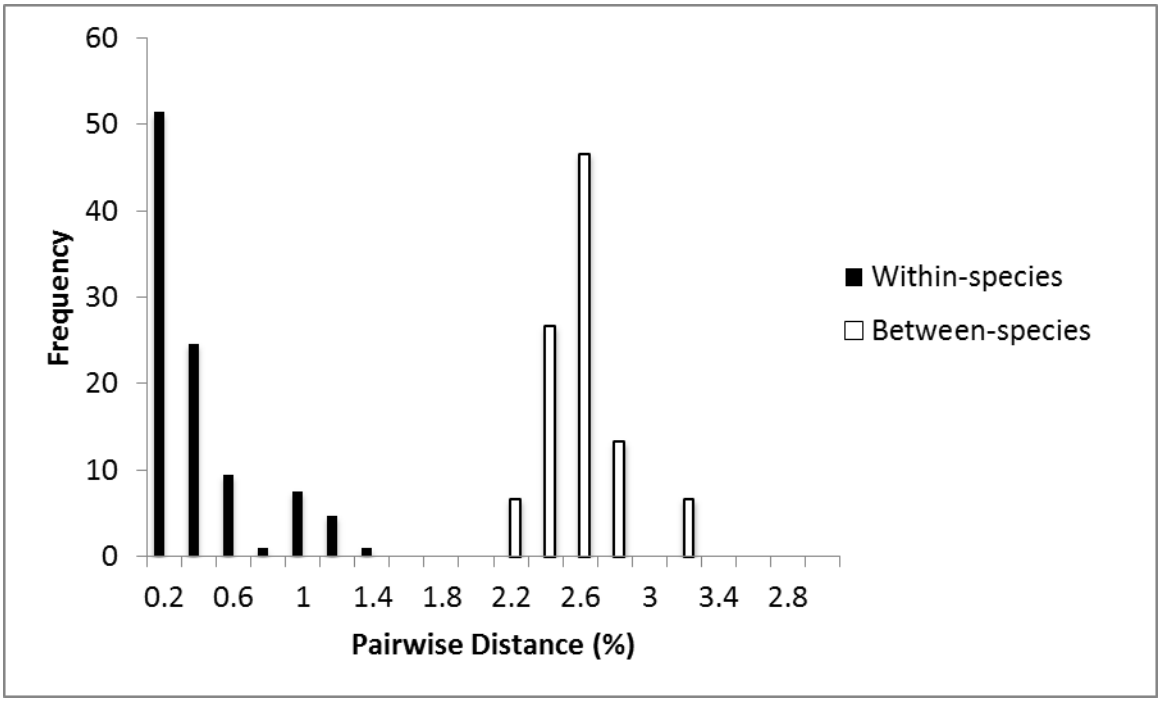

Figure 2-52 – Pairwise distance (%) distribution of Psychodidae (or *Pericoma spp.*) specimens in the BIN BOLD:AAU4662 and nearest neighbor *Pnuemia stammeri*.

The correct Genus level identification of these specimens as *Pneumia* was only possible after consulting an expert in this dipteran Family. Based on Curler's preliminary examination of photos of specimens collected through this study and known distributions of psychodids in North America, the most likely identity of the species present in the study area was determined to be *Pneumia californica*. Unfortunately, because barcode sequence data for *P. californica* or *P. canescens*, suspected synonym of *P. californica*, is not yet available molecular, confirmation of this identification was not possible.

Discussion

Initial attempts based on examination of primary literature pertaining to North American psychodids to resolve the species identity of the moth fly larvae collected in the study area were unsuccessful due to inconsistencies and errors in taxonomic information. Further attempts using DNA barcoding to determine the correct Genus or species were also unsuccessful due the absence of previously barcoded and accurately identified material

available in Genbank and BOLD. Following consultation with an expert psychodid taxonomist, recent revisions based on Palearctic material was revealed and the correct Genus of the specimens in question, *Pneumia*, was determined. Curler has published a number of articles pertaining to Nearctic Psychodidae, including phylogenetic analyses and morphological descriptions of new species (Curler and Moulton 2010, 2012a, 2012b), and information on the recent updating of psychodid taxonomy by Omelková and Ježek (2012) and synonymy of *Pneumia* with previously described Genera *Pericoma* and *Satchelliella* in the Palearctic region. The confusion surrounding the identification of aquatic psychodid larvae commonly reported in studies of benthic invertebrates in western Canada is understandable considering the inconsistencies among early taxonomic work conducted primarily in the Palearctic region.

Interestingly, unlike a similar study in southern BC in which psychodid larvae were collected from interior streams but not from coastal streams (Reece and Richardson 2002), seven specimens in our study were obtained from samples collected in coastal streams.

Although sampling effort was not consistent across seasons in 2013 and 2014, making conclusions about seasonal activity impossible, the collection of one specimen in May of 2014 suggests that this psychodid species overwinters in the larval stage, with adults emerging in the spring, followed by egg-laying by females and an increasing larvae populations as the season progresses, in agreement with Vaillant (1973) who notes the ability of aquatic larvae to freeze solid during winter months and become active again in spring.

Many researchers (Ward 2009) accept 2 % dissimilarity in the barcoding region of the DNA as evidence that two taxa belong to separate species. All, except possibly one of the sequences, likely belong to one species because they are within this 2 % sequence similarity cutoff. Our samples showed greater than 2 % pairwise dissimilarity to, providing evidence that these specimens belong in the Genus *Pneumia* but are distinct from Palearctic species *P. stammeri*. DNA barcoding of the adult type material used to delineate species by psychodid taxonomist would contribute greatly to the correct identification of larvae from the same species and permit a greater understanding impetus for study of their larval habitats and ecological role. Confirmation of the correct identity of our specimens will allow for the correction of BOLD records and the accurate identification of this taxon through public BOLD

databases in the future. This case highlights the value of combining rigorous morphological and other taxonomic identification efforts with DNA barcoding in the development of databases such as BOLD – helping to improve a powerful tool for aiding in accurate species level identification and to move towards monitoring of biodiversity both locally and globally.

2.6. DNA barcoding to establish baseline benthic invertebrate diversity in freshwater streams of central interior and coastal regions of British Columbia

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Introduction

Aquatics invertebrates have been used in environmental monitoring and assessment projects for decades due to their suitability as bioindicators of stream quality resulting from the wide variety of responses to disturbance that they exhibit. The presence of many taxa is known to be effected by changes in stream quality such resulting from alterations such as water level, pH, sediment quantity, and organic pollution (Hodkinson and Jackson 2005; Bonada et al. 2006). Benthic invertebrate studies typically identify specimens morphologically only to Family or Genus level because identification to the species level is often difficult to impossible and previous studies have shown that closely related taxa often share similar life-history traits and exhibit similar responses to environmental changes (Bailey et al. 2001).

DNA barcoding is a molecular method of species identification based on the cytochrome *c* oxidase subunit I (COI) gene. DNA sequence-based identification overcomes many of the challenges associated with traditional taxonomy and has contributed immensely to the discovery of previously unrecognized taxa and the elucidation of cryptic species (Hebert et al. 2003). DNA barcoding can be used to identify immature or damaged specimens, and has been shown to be particularly useful in studies of benthic invertebrates (Zhou et al. 2009; Zhou et al. 2010; Sweeney et al. 2011). Therefore, to contribute to the establishment of baseline invertebrate biodiversity in study streams, DNA barcoding was used to identify reference specimens to the lowest taxonomic level possible and provide a more accurate assessment of the local fauna.

In most studies of North American benthic invertebrates, specimens are morphologically identified using secondary sources of taxonomic information such as Merritt et al. (2008), which provides identification keys for the Families and Genera of most taxa present in the study area, but keys for their identification to the species level are not readily available. Mayflies (Ephemeroptera), stoneflies (Plecoptera), caddisflies (Trichoptera), flies (Diptera), and midges

(the dipteran Family Chironomidae) are the dominant invertebrates present in stream habitats but pertinent taxonomic knowledge varies widely among groups. Comprehensive species-level information and identification keys are available for stoneflies of the Pacific Northwest but only Family- or Subfamily-level information and identification keys available for midges despite their diversity, abundance, and varying sensitivity to disturbance. Scudder et al. (2001) provide a summary of taxonomic knowledge of aquatic insects in BC, as well as bibliographies for each known Order, but keys to local species are scattered across primary literature and are often difficult to access or do not include all taxa known from BC.

Needham (1996) provides illustrated keys to the Families and Genera of mayflies (Ephemeroptera) in BC, and Edmunds et al. (1976) provides Family- and Genus-level keys for North American mayflies, as well as information about nymphal habitat, life history and distribution. Keys for most mayfly species, however, are largely unpublished and not available for all species present in the study area. Stewart and Osgood (2006) provide keys to the Families and Genera of stoneflies (Plecoptera) of BC and Alaska, and keys and illustrations for all species for which distinguishing characters have been described, as well as life history and distribution ranges. Wiggins (1996) provides keys to the Families and Genera of North American caddisflies (Trichoptera) and includes illustrations of representative species of each Genus and information on life history, retreat descriptions, and distribution ranges. Keys to the Families and larvae of true flies (Diptera), illustrations and brief descriptions of the characteristics and biology of each Family are available in Teskey (1981).

Lists of species known from BC are available for mayflies, stoneflies and caddisflies on the E-Fauna website (http://ibis.geog.ubc.ca/biodiversity/efauna/) but those for Diptera and other taxa obtained through this study were not readily available. Due to the labor-intensive and difficult nature of morphologically identifying benthic invertebrates, however, secondary literature such as Merritt et al. (2008) is standard practice in academic, governmental and industrial assessments of stream quality. Therefore, much of what is known about diversity remains at the Family or Genus level. Keys to species or even Genera are not available for many aquatic invertebrates commonly collected from streams including riffle beetles (Coleoptera),

water mites (Hydrachnida: Trombidiformes), worms (Clitellata), nematodes (Nematoda), and copepods (Copapoda).

Although the development of diversity indices, such as the Hilsenhoff Biodex index based upon the known tolerance of benthic invertebrate to various types of potential disturbances (Hilsenhoff 1988), has proven effective for assessing stream quality and monitoring changes due to disturbance, knowledge of species-level processes has the potential to provide a greater understanding of community composition and ecosystem functioning (Sweeney et al. 2011; Stein et al. 2014). In particular, recent studies have revealed an immense potential for chironomids to be used as bioindicators of stream health and environmental changes (Nicacio and Juen 2015); they are the most diverse, abundant and difficult to identify group present in stream habitats and vary widely in their sensitivity to various types of disturbances (Lindegaard 1995). Their incredible diversity is beginning to understood as chironomids taxonomy begin to use DNA barcoding to confirm morphological identification at the species level (Carew et al. 2013), which requires slide mounting of mouth parts or other structures and compound microscope and therefore often beyond the scope of traditional studies.

The search for available literature pertaining to invertebrate biodiversity in the study area revealed a distinct lack of easily accessible information, species lists or regional identification keys for the central interior and coastal regions of BC. For example, a recent study of mayflies in North America (Webb et al. 2012) examined morphologically and using DNA barcoding over 4000 mayfly specimens collected from locations throughout most of North America but did not include any specimens from sites north of southern BC. Therefore, to enable a more accurate determination of baseline biodiversity in study streams, DNA barcoding was used to complement morphological analyses and provide species-level identifications for local taxa. Representative specimens were selected for barcode sequence analysis during processing of Surber samples obtained during the 2013 and 2014 field seasons. Our objectives were to: 1) identify as many taxa as possible that were present in study streams to the species level; 2) verify morphological identifications; and 3) contribute to the development a reference

library of DNA barcodes (COI sequences) for use in associated next-generation sequencing projects.

Methods

Specimens were identified to the lowest taxonomic level possible using available morphological keys (Clifford 1991; Needham 1996; Wiggins 1996; Stewart and Osgood 2006;Merritt et al. 2008) and sent to the Canadian Centre for DNA Barcoding at the University of Guelph for standard sequencing and imaging services. DNA was extracted, PCR-amplified and sequenced according to standard protocols (Ivanova et al. 2006; Ratnasingham and Hebert 2007; DeWaard et al. 2008).

Resulting barcode (COI) sequences were checked against the BOLD database (www.boldsystems.org), and the identity of the specimen determined to the lowest taxonomic level possible. Species names were assigned to barcode sequences using the standard 2 % sequence divergence threshold for invertebrates. When sequence divergence was 2–4 % from database sequences, species were named "near closest species" (e.g., *Rhyacophila nr. narvae*). If a species name could not be assigned due to the absence of similar species in the database (i.e., the BMAP reference specimen represents the first of its species to be added to BOLD Barcode Index Number, or BIN, then they were named *BMAP species 1, BMAP species 2*, etc. (e.g., *Baetis BMAP sp. 1,* Psychodidae *BMAP sp. 2*). The BOLD Workbench (www.boldsystems.org) was used to generate distance summary histograms, Neighbor-joining trees, and taxon rarefaction curves using the Kimura-2-Parameter Model with pairwise deletion (Kimura 1980).

Results

A total of 647 specimens COI sequences were obtained from 692 specimens sent for barcoding and imaging, which were comprised of 647 insects (Insecta), 31 water mites (Arachnida), three worms (Clitellata), two ostracods (Ostracoda), one copepod (Copapoda), and one amphipod (Maxillopoda).

Ephemeroptera

Of 119 mayfly specimens sequenced, 34 species from 15 Genera and five Families were identified, of which 22 did not have a species match in the BOLD database (Table 2-54). The high proportion (22 of 34) barcode clusters that did not have BOLD species match is likely because many species known from BC have not been included in previous barcoding studies. DNA barcoding detected 50 % of the number of mayfly Families (n = 10), 42 % of the number of Genera (n = 31) and 36 % of the number of species (n = 94) known from BC. Most of the 34 resulting mayfly barcode clusters had fewer than five other sequences in the same BOLD BIN (Table 2-54), further evidence of the lack of coverage of British Columbian taxa in previous studies. Some barcode clusters had sequence divergence levels of 2–4 % from known species, suggesting that intraspecific variation of these taxa in the study area exceeds the standard threshold of 2 %.

Genera identified morphologically, but not represented in barcoded specimens, include: *Diphetor* (Baetidae), *Caudatella* and *Serratella* (Ephemerellidae), *Cinygma* and *Heptagenia* (Heptageniidae). Genera known from BC but detected by morphological or molecular methods include *Callibaetis, Centroptilum* and *Procleon* (Baetidae), *Eurylophella* and *Timpanago* (Ephemerellidae), *Leptophlebia* (Leptophlebiidae), and *McCaffertium* (Heptageniidae). Families known from BC but detected by morphological or molecular methods include Siphlonuridae (*Parameletus* and *Siphlonurus*), Metretopodidae (*Metretopus*), Leptohyphidae (*Tricorythrodes*), Caenidae (*Caenis*), and Ephemeridae (*Ephemera*).

Mean sequence divergence within cluster/species was <1 % and the minimum sequence divergence between species 17 % (Figure 2-53a), providing evidence that DNA barcode sequences can successfully identify unique mayfly species in the study area. The rarefaction curves of mayfly taxa differed for Genera, species and barcode clusters (Figure 2-53b). The slopes of the barcode cluster and species curves decreased but did not reach asymptote with a sample of 120 specimens, whereas the Genus curve reached asymptote with 100 specimens. This indicates that sampling effort in the current study was sufficient to identify most Genera, but insufficient to identify most species and barcode clusters, in the study area. The greater number of barcode clusters recovered compared to species reveals that a greater number of

mayfly taxa are present in the study area than could be matched to verified species identifications in the BOLD database.

Table 2-54 – Molecular species identifications of mayfly (Ephemeroptera) species grouped by Family, including number of sequences per species, presence (Y) or absence (N) of a match in BOLD, and number of other specimens sharing the same barcode cluster or BOLD BIN.

Species	Number of Sequences	Species Match?	Number in BIN
Ameletidae			
Ameletus celer	1	Y	99
Ameletus vernalis	1	Y	4
Ameletus nr. validus	3	N	2
Baetidae			
Acentrella insignificans	1	Y	-
Acentrella turbida	2	Y	3
Baetis bicaudatus	5	Y	59
Baetis BMAP sp. 1	1	N	3
Baetis nr. tricaudatus	2	N	2
Baetis nr. tricaudatus 2	3	N	4
Baetis nr. tricaudatus 3	3	N	3
Baetis tricaudatus	14	N	32
Plauditus BMAP sp. 1	1	N	1
Ephemerellidae			
Attenella margarita	1	Y	4
Drunella coloradensis	4	Y	3
Drunella grandis	3	Y	83
Drunella nr. doddsii	15	N	14
Ephemerella nr. aurivillii	2	N	2
Ephemerella tibialis	10	N	2
Heptageniidae			
Cinygmula BMAP sp. 1	6	N	4
Cinygmula BMAP sp. 2	1	N	3
Cinygmula BMAP sp. 3	5	N	11
Cinygmula nr. mimus 1	2	N	4
Cinygmula nr. mimus 2	4	N	3
Ecdyonurus simplicioides	1	Y	23
Epeorus deceptivus	9	Y	98
Epeorus longimanus	3	Y	97
Epeorus nr. longimanus	2	N	2
Ironodes sp. BMAP sp. 1	1	N	-
Rhithrogena BMAP sp. 1	1	N	4
Rhithrogena BMAP sp. 2	4	N	4
Rhithrogena BMAP sp. 3	1	N	2
Leptophlebiidae			
Paraleptophlebia heteronea	2	Y	70
Paraleptophlebia nr. vaciva	2	N	2
Paraleptophlebia nr. sp. JMW1	3	N	3

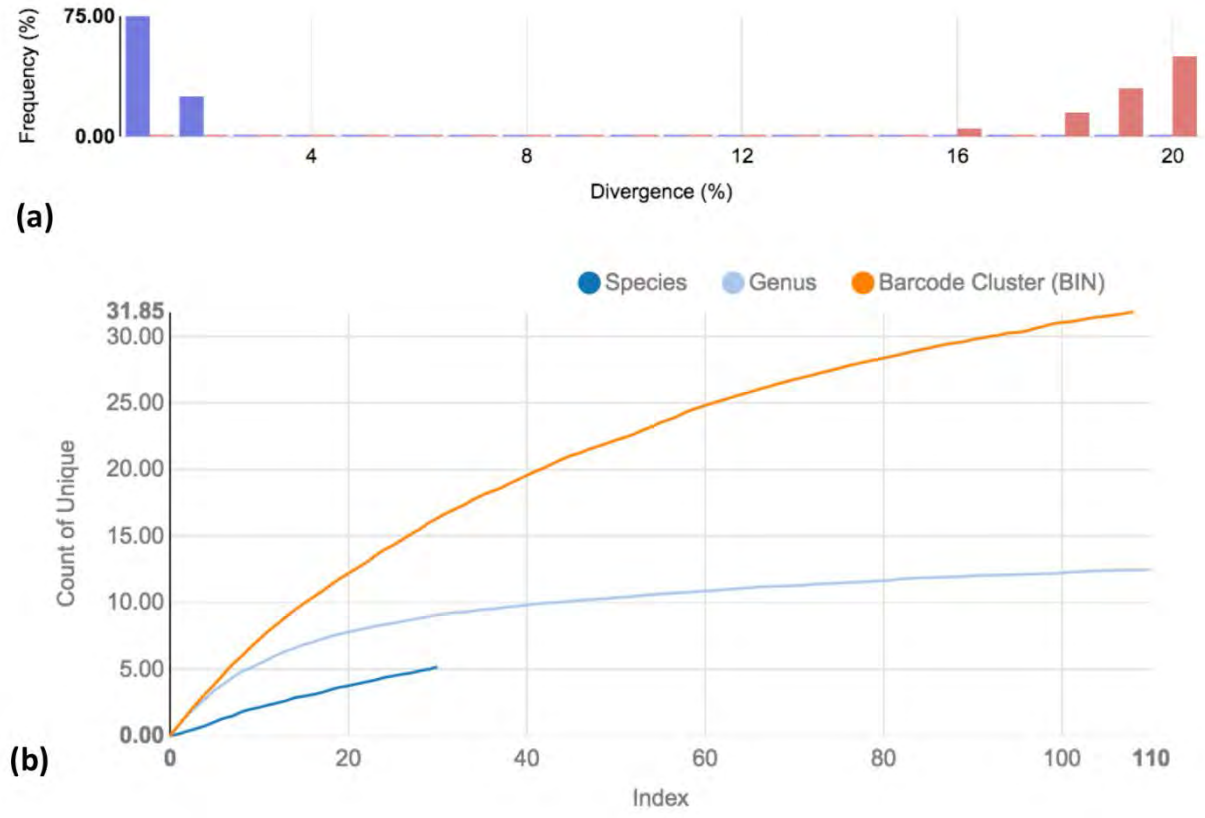

Figure 2-53 – (a) Distribution of sequence divergence (%) within mayfly species (pink) and within Genera (green), normalized to reduce sampling bias at the species level; (b) Randomized rarefaction curves based on DNA barcode sequence data for Genera, species and barcode clusters, constructed in BOLD using 100 random replicates.

Plecoptera

From 93 stonefly sequences, a total of 26 species in 15 Genera and eight Families were identified (Table 2-55), of which only seven did not have a match in the BOLD database. The low proportion of barcode clusters that did not have BOLD species match (27 %) is likely because keys for the identification of most stonefly nymphs are available in Stewart and Osgood (2006). DNA barcoding detected 88 % (8 of 9) of the number of stonefly Families, 29 % (15 of 51) of the number of Genera, and 20 % (26 of 133) of the number of species known from BC. Most of the 26 resulting mayfly barcode clusters had >10 other sequences in the same BOLD BIN (Table

2-55), illustrating the depth of coverage of British Columbian taxa in previous studies. Some barcode clusters had intraspecific sequence divergence levels up to 4 % from known species, suggesting that intraspecific variation of stonefly taxa in the study area also exceeds the standard threshold of 2 %. Only one Genus, *Yoraperla* (Peltoperlidae), was identified morphologically but not represented in barcoded specimens. All Families known from BC were detected by morphological or molecular methods, but 71 % Genera (n = 36) were not.

Mean sequence divergence within clusters/species was less than 2.88 % and the minimum sequence divergence between species 8.16 % (Figure 2-54a), providing evidence that DNA barcode sequences can successfully identify stonefly species in the study area. The rarefaction curves of stonefly taxa differed for Genera, species and barcode clusters (Figure 2-54b). The slope of the barcode cluster curves begins to decrease with a sample of 92 specimens, whereas the asymptotes are almost reached for the Genus and species curves. This indicates that sampling effort in the current study was sufficient to identify most Genera and species, but insufficient to identify most barcode clusters, in the study area. The greater number of barcode clusters recovered compared to species (Figure 2-54b) reveals that a greater number of stonefly taxa may be present in the study area than could be matched to verified species identifications in the BOLD database, possibly due to the presence of morphologically cryptic species.

Trichoptera

From 93 caddisfly sequences, a total of 33 species in 17 Genera and 8 Families were identified (Table 2-56), of which only seven did not have a match in the BOLD database. The low proportion (27 %) barcode clusters that did not have BOLD species match is likely because keys to species for most Genera have not yet been published (Wiggins 1996). DNA barcoding detected 44 % (8 of 18) of the number of caddisfly Families, 23 % (17 of 72) of the number of Genera and 12 % (33 of 275) of the number of species known from BC. Most of the 33 resulting caddisfly barcode clusters had >10 other sequences in the same BOLD BIN (Table 2-56), illustrating the depth of coverage in previous studies of caddisfly taxa in BC. Most barcode clusters had intraspecific sequence divergence levels <2 %, but some had intraspecific sequence divergence levels much higher than the standard threshold of 2 %.

Table 2-55 – Molecular species identifications of stonefly (Plecoptera) species, including number of sequences per species, the presence (Y) or absence (N) of a match in BOLD, and number of other specimens in the BOLD BIN.

Species	Number of Sequences	Species Match?	Number in BIN
Capniidae			
Eucapnosis brevicauda	1	N	3
Chloroperlidae			
Kathroperla perdita	2	Y	1
Suwallia sp. BIOUG02	2	Y	5
Suwallia sp. B BG	1	Y	24
Sweltsa BMAP sp. 1	1	N	1
Sweltsa borealis	10	Y	144
Sweltsa coloradensis	2	Y	73
Sweltsa nr. pacifica	2	N	2
Sweltsa sp. XZ02	2	Y	–
Triznaka signata	1	Y	29
Leutricidae			
Paraleuctra occidentalis	8	Y	21
Nemouridae			
Zapada cinctipes	16	Y	97
Zapada nr. oregonensis	2	N	19
Perlidae			
Hesperoperla pacifica	5	Y	132
Perlodidae			
Isoperla fulva	2	Y	58
Isoperla nr. quinquepunctata	1	N	7
Isoperla petersoni	1	Y	42
Isoperla sobria	1	Y	26
Isoperla transmarina	2	Y	–
Kogotus nr. modestus	14	N	14
Megarcys nr. watertoni	1	N	1
Megarcys signata	1	Y	22
Skwala americana	7	Y	40
Pteronarcyidae			
Pteronarcys princeps	3	Y	6
Teaniopterygidae			
Doddsia occidentalis	2	Y	6
Taenionema pallidum	2	Y	10

Three caddisfly Families were identified morphologically but not represented in barcoded specimens, including Polycentropodidae, Philopotamidae, and Uenoidae. There are eight Families known from BC that were not detected by morphological or molecular methods, including Calamoceratidae, Apataniidae, Molannidae, Goeridae, Helicopsychidae, Rossianidae, and Phryganeidae, possibly only occurring in more southern parts of the province.

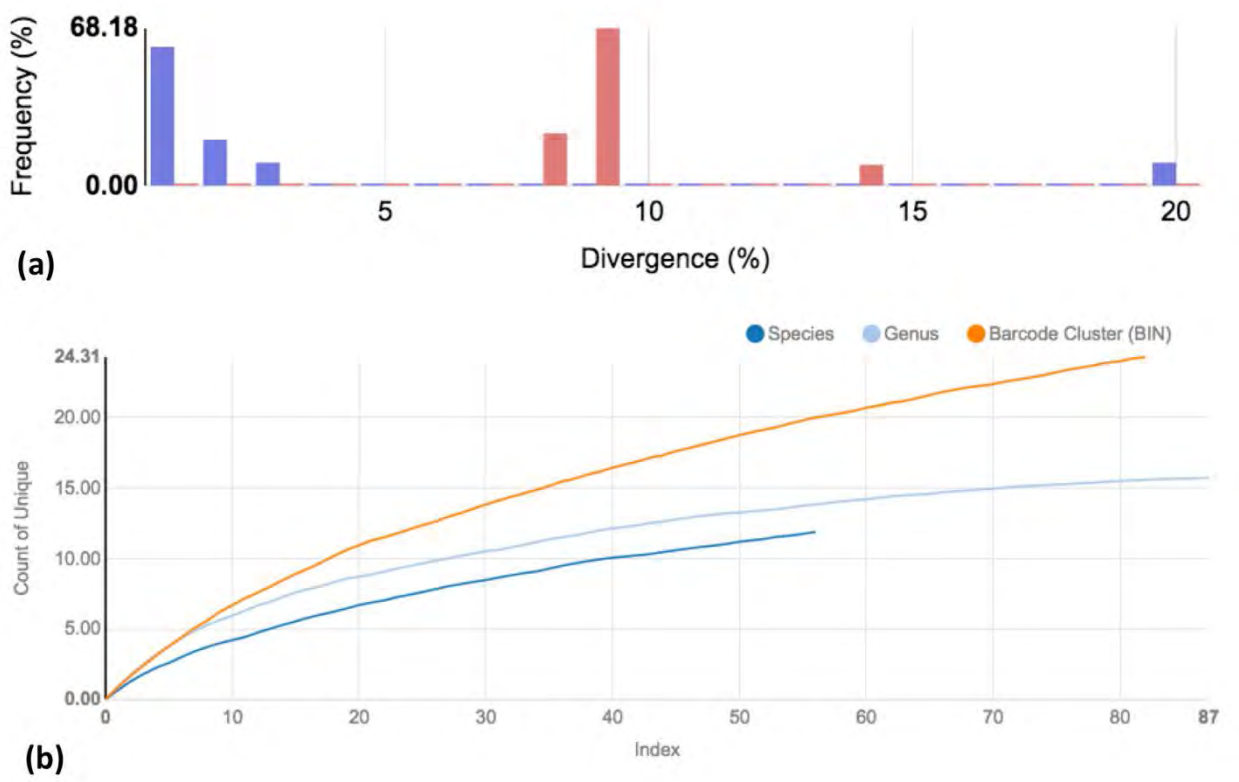

Figure 2-54 – (a) Distribution of sequence divergence (%) within stonefly species (pink) and within Genera (green), normalized to reduce sampling bias at the species level; (b) Randomized rarefaction curves based on DNA barcode sequence data for Genera, species and barcode clusters, constructed in BOLD using 100 random replicates.

Mean sequence divergence within clusters/species was 0.9 %, although intraspecific divergence levels were much higher, up to 11 %, for some barcode clusters/species (Figure 2-55a), suggesting that morphologically cryptic species may be present in the study area. The minimum sequence divergence between species was 8.44 %, <11 % exhibited by some of the barcode clusters/species in this study, indicating that DNA barcode sequences may not be able to successfully distinguish among all caddisfly species present in the study area. The slope of the rarefaction curve for caddisfly Genera begins to reach asymptote with a sample of 92 specimens, but the asymptotes of species and barcode cluster curves do not begin to approach

Table 2-56 – Molecular species identifications of caddisfly (Trichoptera) species, including number of sequences per species, the presence (Y) or absence (N) of a match in BOLD, and number of other specimens in the BOLD BIN.

Species	Number of Sequences	Species Match	Number in BIN
Brachycentridae			
Amiocentrus nr. aspilus	1	N	1
Brachycentrus americanus	3	Y	61
Micrasema bactro	3	Y	24
Micrasema nr. bactro	3	N	4
Glossosomatidae			
Anagapetus bernea	1	Y	9
Glossosoma nr. pyroxum	1	N	24
Glossosoma penitum	13	Y	20
Glossosoma verdonum	9	Y	10
Hydropsychidae			
Arctopsyche grandis	1	Y	57
Cheumatopsyche nr. campyla	1	N	23
Hydropsyche occidentalis	1	Y	36
Hydropsyche oslari	2	Y	184
Hydropsyche tana	12	Y	55
Parapsyche almota	1	Y	4
Parapsyche elsis	2	Y	32
Hydroptilidae			
Hydroptila rono	1	Y	-
Hydroptila wyomia	2	Y	14
Lepidostomatidae			
Lepidostoma BMAP sp. 1	1	N	2
Lepidostoma rayneri	1	Y	23
Lepidostoma unicolor	1	Y	56
Leptoceridae			
Oecetis nr. avara	2	Y	4
Limnephilidae			
Dicosmoecus atripes	1	Y	21
Ecclisomyia conspersa	1	Y	1
Onocosmoecus unicolor	3	Y	27
Psychoglypha subborealis	1	Y	11
Rhyacophilidae			
Rhyacophila angelita	1	Y	136
Rhyacophila BMAP sp. 1	3	N	12
Rhyacophila BMAP sp. 2	3	N	6
Rhyacophila brunnea	1	Y	-
Rhyacophila grandis	1	Y	6
Rhyacophila narvae	4	Y	9
Rhyacophila nr. vaccua	9	N	2
Rhyacophila vao	8	Y	64

asymptote by 102 specimens (Figure 2-55b). This indicates that sampling effort in the current study was insufficient to identify most barcode clusters and species, but sufficient to identify

the Genera present in the study area. The greater number of barcode clusters recovered compared to species (Figure 2-55b) suggests that a greater number of caddisfly taxa may be present in the study area than could be matched to verified species identifications in the BOLD database, or high levels genetic variation within species.

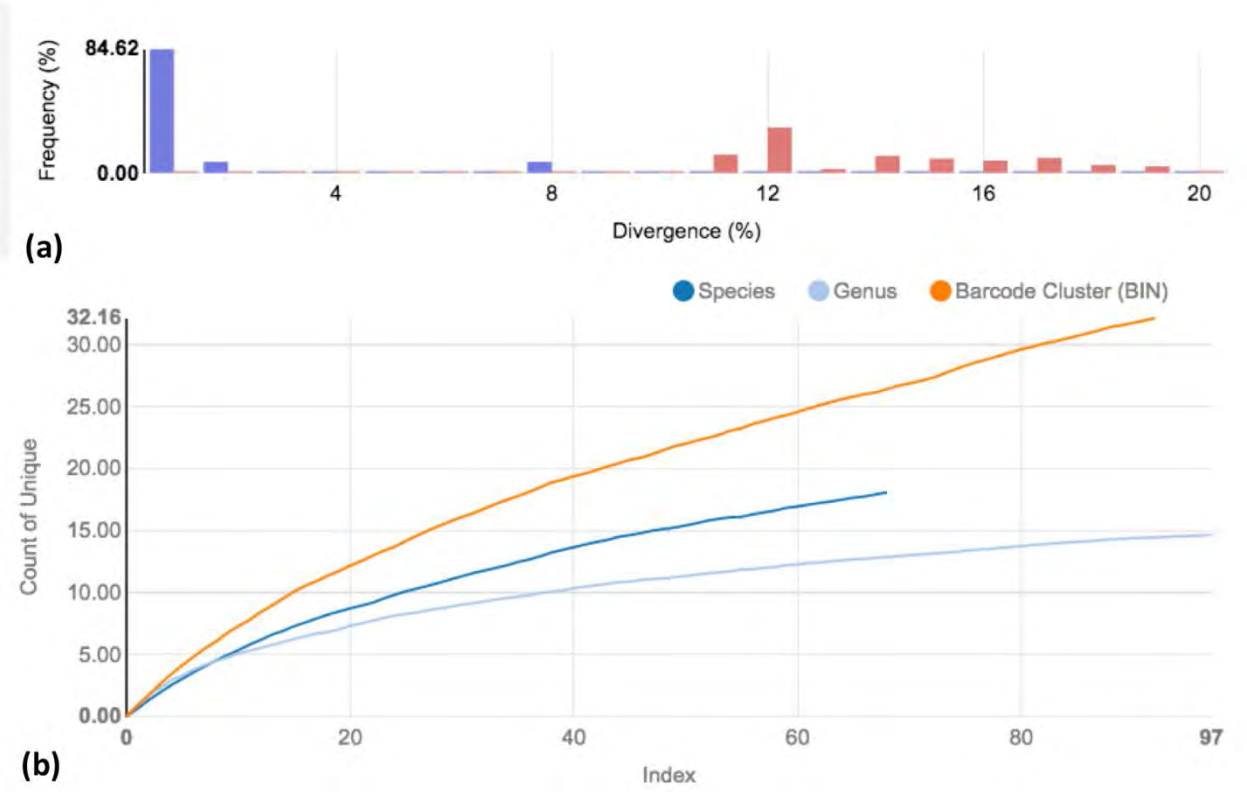

Figure 2-55 – (a) Distribution of sequence divergence (%) within caddisfly species (pink) and within caddisfly Genera (green), normalized to reduce sampling bias at the species level; (b) Randomized rarefaction curves based on DNA barcode sequence data for Genera, species and barcode clusters, constructed in BOLD using 100 random replicates.

Diptera (non-Chironomidae)

From 155 true fly sequences, not including those of the Chironomidae Family because their abundance and diversity warrants their separate analysis, a total of 35 species in 16 Genera and nine Families were identified (Table 2-57), of which only 24 did not have a species-

level match in the BOLD database. A high proportion of the barcode clusters (69 %) did not have BOLD species match, but there are many more Families, Genera and species of flies, and their larvae much more difficult to identify based on morphological characters, compared to mayflies, stoneflies, and caddisflies. Most of the 35 non-chironomid fly sequences had fewer than five other members in the barcode clusters BOLD BINS (Table 2-57), which indicates that local fly taxa have not been well studied using DNA barcoding, except for black flies (Simuliidae) for which comprehensive barcoding studies have been published that included local taxa. Species of three Families could not be identified based on barcode sequences beyond Family level including Blephariceridae (net-winged midges), Ceratopogonidae (no-see-ums) and Deuterophlebiidae (mountain midges) due to the lack of previous morphological and DNA barcoding studies on these groups. Because most barcode clusters could not be identified beyond the Family or Genus level, analysis of intraspecific and interspecific sequence divergence levels was not warranted.

The slope of the rarefaction curve for fly Genera (excluding chironomids) approached asymptote with a sample of 155 specimens (Figure 2-56), indicating that sampling effort was sufficient to identify most Genera present in study streams. The slope of the barcode cluster curve, however, did not begin to reach asymptote, suggesting that many more barcode clusters or species likely occur in study streams than were detected using DNA barcoding.

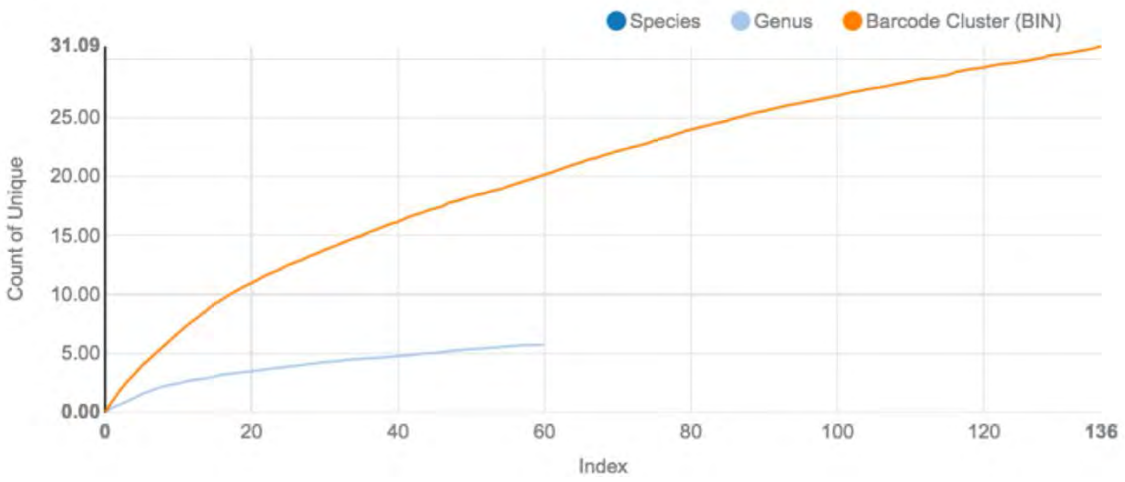

Figure 2-56 – Randomized rarefaction curves based on DNA barcode sequence data for non-chironomid fly Genera and barcode clusters, constructed in BOLD using 100 random replicates.

Table 2-57 – Molecular species identifications of true fly (Diptera, non-Chironomidae) species, including number of sequences per species, the presence (Y) or absence (N) of a match in BOLD, and number of other specimens in the BOLD BIN.

Species	Number of Sequences	Species Match	Number in BIN
Anthiomyiidae			
Egle atomaria	1	Y	19
Athericidae (snipe fly)			
Atherix nr. verigatum	1	N	2
Blepharceridae (mountain midge)			
Philorus BMAP sp. 1	1	N	1
Blephariceridae BMAP sp. 1	2	N	2
Ceratopogonidae (no-see-ums)			
Ceratopogonidae BMAP sp. 1.	20	N	18
Palpomyia nr. nigripes	3	N	2
Deuterophlebiidae			
Deuterophlebia nr. coloradensis	1	N	1
Deuterophlebia sp.	1	N	-
Empididae (dance fly)			
Clinocera nr. disjucta	1	N	2
Metachela collusor	1	Y	2
Oreogeton scopifer	3	Y	9
Trichoclinocera comata	1	Y	7
Weidemannia nr. lepida	1	N	-
Tipulidae (Limoniidae) (crane fly)			
Antocha BMAP sp. 1	6	N	14
Hexatoma BMAP sp. 1	1	N	1
Hexatoma BMAP sp. 2	4	N	26
Hexatoma BMAP sp. 4	6	N	4
Hexatoma BMAP sp. 5	5	N	3
Tipulidae (Pediciidae) (crane fly)			
Dicranota BMAP sp. 1	1	N	2
Dicranota BMAP sp. 2	4	N	4
Dicranota BMAP sp. 3	2	N	2
Dicranota BMAP sp. 4	5	N	5
Dicranota BMAP sp. 5	1	N	1
Dicranota BMAP sp. 6	1	N	1
Dicranota BMAP sp. 7	1	N	1
Dicranota BMAP sp. 8	1	N	1
Psychodidae (moth fly)			
Pneumia BMAP sp. 1	38	N	121
Pneumia BMAP sp. 2	1	N	8
Simuliidae (black fly)			
Helodon onychodactylus complex	5	Y	15
Prosimulium travesi	3	Y	423
Simulium arcticum complex	13	Y	324
Simulium balteatum	3	Y	-
Simulium hunteri	1	Y	-
Simulium turbosum complex	2	Y	220
Simulium venustum complex	1	Y	896

Chironomidae

Non-biting midges (or chironomids) belong the true fly Family Chironomidae are often the most abundant and diverse group of benthic invertebrates collected from streams but their identification beyond Family level is difficult and labor-intensive and usually not attempted in standard assessments of stream quality. From 112 chironomid sequences, DNA barcoding allowed the identification of 54 barcode clusters from 23 Genera (Table 2-58), only four of which had species matches in the BOLD database. Some barcode clusters were the first members of their BOLD BINs, while others had many similar sequences represented in the database, but still could not be matched to a species because of the lack of previous morphological and DNA barcoding studies that include local taxa.

Table 2-58 – Molecular species identifications of non-biting midge (Chironomidae) species, including number of sequences per species, the presence (Y) or absence (N) of a match in BOLD, and number of other specimens in the BOLD BIN.

Species	Number of Sequences	Species Match	Number in BIN
Chironomidae *BMAP sp. 1*	1	N	-
Chironomidae *BMAP sp. 2*	3	N	3
Subfamily Orthocladiinae *BMAP sp. 1*	7	N	9
Subfamily Orthocladiinae *BMAP sp. 2*	1	N	3
Subfamily Orthocladiinae *BMAP sp. 3*	2	N	1
Subfamily Orthocladiinae *BMAP sp. 4*	3	N	3
Subfamily Orthocladiinae *BMAP sp. 5*	4	N	1
Brilla BMAP sp. 1	1	N	31
Conchapelopia nr. pallens 1	1	N	1
Conchapelopia nr. pallens 2	1	N	13
Conchapelopia nr. telema	1	N	5
Conchapelopia pallens	5	Y	41
Corynoneura BMAP sp. 1	2	N	26
Corynoneura BMAP sp. 2	1	N	14
Coryoneura BMAP sp. 3	3	N	5
Cricotopus BMAP sp. 1	1	N	1
Cricotopus BMAP sp. 2	5	N	5
Cricotopus nr. annulator complex	1	N	2
Cricotopus nr. draysoni	2	N	6
Diamesa nr. nr. hyperborea	3	N	10
Eukiefferiella claripennis	3	Y	420
Eukiefferiella nr. devonica	1	N	2
Gymnometriocnemus BMAP sp. 3	7	N	16
Hydrobanaenus nr. conformis	1	N	1
Limnophyes BMAP sp. 1	1	N	84
Limnophyes BMAP sp. 2	2	N	36
Metriocnemus BMAP sp. 1	1	N	-

Species	Number of Sequences	Species Match	Number in BIN
Micropsectra BMAP sp. 1	1	N	125
Micropsectra BMAP sp. 2	1	N	38
Micropsectra BMAP sp. 3	1	N	7
Micropsectra BMAP sp. 4	9	N	1
Micropsectra BMAP sp. 5	1	N	2
Micropsectra nr. peniculata	1	N	-
Micropsectra nr. recurvata	2	N	11
Nilotanypus fimbriatus	1	Y	51
nr. Gymnometriocnemus BMAP sp. 1	3	Y	3
nr. Gymnometriocnemus BMAP sp. 2	1	N	14
nr. Tvetenia BMAP sp. 1	1	N	1
Orthocladius BMAP sp. 1	3	N	8
Orthocladius BMAP sp. 2	2	N	36
Orthocladius nr. dorenus	2	N	10
Orthocladius nr. rivulorum	3	N	2
Pagastia nr. orthogonia	1	N	9
Parametriocnemus BMAP sp. 1	2	N	2
Parametriocnemus BMAP sp. 2	2	N	-
Polypedilum BMAP sp. 1	3	N	-
Rheocricotopus nr. robacki	1	N	9
Rheopelopia ornata	1	Y	-
Rheotanytarsus BMAP sp. 1	1	N	65
Smittia BMAP sp. 1	1	N	1
Stempellinella BMAP sp. 1	1	N	5
Thienemannimyia BMAP sp. 1	2	N	1
Thienemannimyia BMAP sp. 2	1	N	-

The slope of the rarefaction curves for chironomid Genera and species reached asymptote with samples of 20 and 40 specimens respectively (Figure 2-57), suggesting that sampling effort was sufficient to obtain most of the previously described Genera and species that area present in study area. The slope of the barcode cluster curve, however, barely begins to decrease with a sample of 112 specimens, indicating that many more barcode clusters or unknown species likely occur in study streams. This large discrepancy of sampling sufficiency between species and barcode clusters is due to the considerable difficulty in morphological identification of chironomids beyond the Family level.

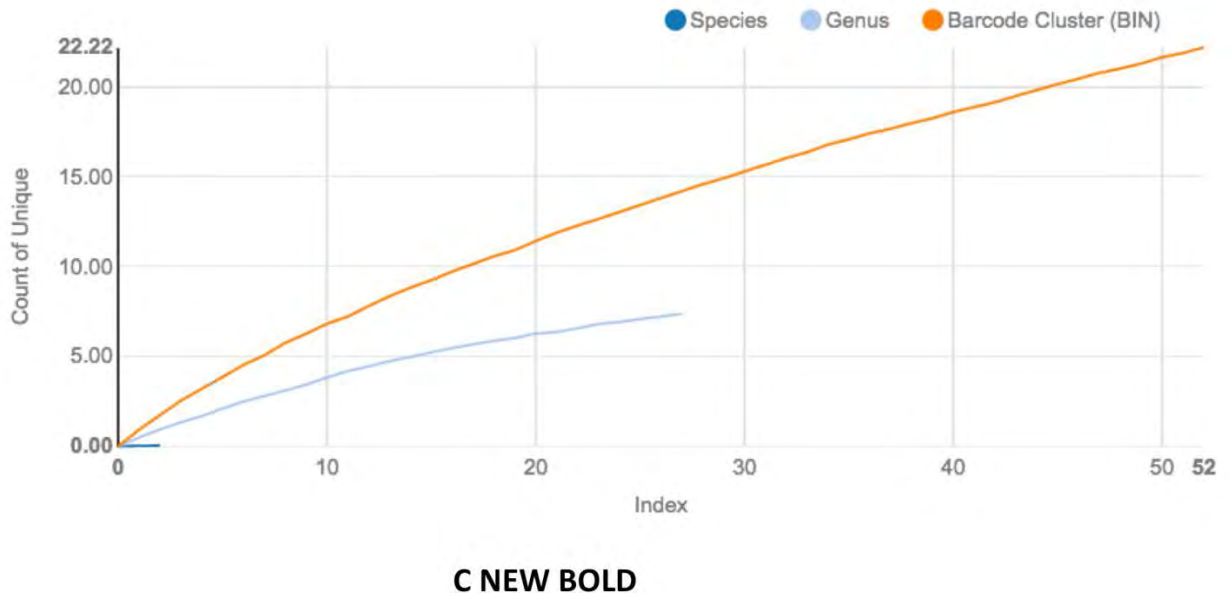

Figure 2-57 – Randomized rarefaction curves based on DNA barcode sequence data for chironomid Genera and barcode clusters, constructed in BOLD using 100 random replicates.

Other taxa

Barcode sequences were obtained for a total of other 94 taxa including 22 riffle beetles (Coleoptera), 30 water mites (Trombidiformes), one copapod (Harpacticoida), two ostracods (Podocopida), three annelid worms (Haplotaxida), three nematode worms (Nematoda), and three terrestrial taxa, aphids (Aphidae). Riffle beetles are the only other insect taxa commonly obtained in Surber samples, and three barcode clusters from three separate Genera were identified from the resulting 22 sequences, one of which has a species match in BOLD, one was identifiable to the Genus level only, and one that was identifiable only to the Family level (Table 2-59).

Also often collected by Surber sampling are water mites, which are rarely identified in studies of benthic invertebrate assemblages beyond the level of Class despite their common occurrence and apparent significant diversity in stream habitats. From 30 specimens barcoded, 18 barcode clusters were identified comprised of eight Families and at least eight Genera; none were identifiable to the species level based on barcode sequences, 26 were identifiable to the Genus level and four were identifiable only to the Family level (Table 2-59). The high proportion

Table 2-59 – Molecular species identifications of other invertebrate taxa, including number of sequences per species, the presence (Y) or absence (N) of a match in BOLD, and number of other specimens in the BOLD BIN.

Class	Order	Species	No. Seqs.	Spp. Match?	No. in BIN
Insecta	Coleoptera	**Elmidae**			
		Elmidae *BMAP sp. 1*	15	N	23
		Optioservus BMAP sp. 1	4	N	64
		Ordobrevia nubifera	3	Y	41
Insecta	Hemiptera	**Aphidae**			
		Aphis salicariae	1	Y	64
		Chaitophorus pusillus	1	Y	16
		Prociphilus xylostei	1	Y	32
Maxillopoda	Harpacticoida	Harpacticoida *BMAP sp. 1*	1	N	1
Ostracoda	Podocopida	Cyprididae *BMAP sp. 1*	2	N	1
Hydrachnida	Trombidiformes	**Arrenuridae**			
		Arrenurus BMAP sp. 1	1	N	6
		Arrenurus BMAP sp. 2	1	N	1
		Aturidae			
		Aturus BMAP sp. 2	4	N	-
		Aturus BMAP sp. 1	1	N	1
		Hygrobatidae			
		Atractides BMAP sp. 1	3	N	3
		Atractides BMAP sp. 2	1	N	1
		Lebertiidae			
		Lebertia BMAP sp. 1	2	N	3
		Lebertia BMAP sp. 2	1	N	1
		Lebertia BMAP sp. 3	1	N	3
		Pionidae			
		Pionidae *BMAP sp. 1*	1	N	1
		Protziidae			
		Protzia BMAP sp. 1	1	N	3
		Protzia BMAP sp. 2	1	N	1
		Sperchontidae			
		Sperchontidae *BMAP sp. 1*	1	N	1
		Sperchontidae *BMAP sp. 2*	2	N	2
		Sperchon BMAP sp. 1	1	N	4
		Sperchonopsis BMAP sp. 1	1	N	1
		Torrenticolidae			
		Testudacarus BMAP sp. 1	6	N	6
		Torrenticola BMAP sp. 1	1	N	-
Clitellata	Haplotaxida	**Naididae**			
		Naididae *BMAP sp. 1*	1	N	1
		Naididae *BMAP sp. 2*	2	N	10
		Nematoda *BMAP sp. 1*	1	N	1
		Nematoda *BMAP sp. 2*	1	N	1
		Nematoda *BMAP sp. 3*	1	N	1

of unique barcode clusters resulting from 30 specimens (60%) and low number of other members in the associated BOLD BINs indicates that water mites are a very diverse but understudied group of stream invertebrates.

Of the three aphid specimens, all had matches in the database and were identifiable to the species level (Table 2-59). The copepod specimen could not be identified beyond Order and was the first member in its BIN to be submitted to BOLD. Both ostracod specimens formed the same barcode cluster and did not have a species match the BOLD, but were identifiable as a species belonging to the Family Cyprididae. Barcode sequences of the three annelid worms resulted in two clusters that did not have matches in BOLD and were identifiable only to the Family level. Sequences from the three nematode worms resulted in three barcode clusters that did not have matches in BOLD and were not identifiable beyond the Phylum level (Table 2-59).

All taxa combined

Mean sequence divergence of all barcoded taxa combined (n = 638) was 1.5 % and minimum sequence divergence between species was 8.2 % (Figure 2-58a), illustrating the ability of DNA barcoding to reliably distinguish among most species obtained in Surber samples. The rarefaction curves of all taxa combined differed for Genera, species and barcode clusters (Figure 2-58b). The slopes of the Genera, species and barcode cluster curves did not reach asymptote with sequences from all 638 specimens (Figure 2-58b), indicating that more Genera, species, and barcode clusters are present in samples collected from study streams than were represented in specimens submitted for DNA barcoding analysis. The greater number of barcode clusters recovered compared to species revealed that a greater number of benthic invertebrate taxa is present in the study area than could be matched to verified species identifications in the BOLD database.

Discussion

DNA barcoding was used to obtain species-level identifications of reference specimens selected while processing Surber samples collected from streams in the study area. Although keys to species based on morphological characters are not available for many of the taxa

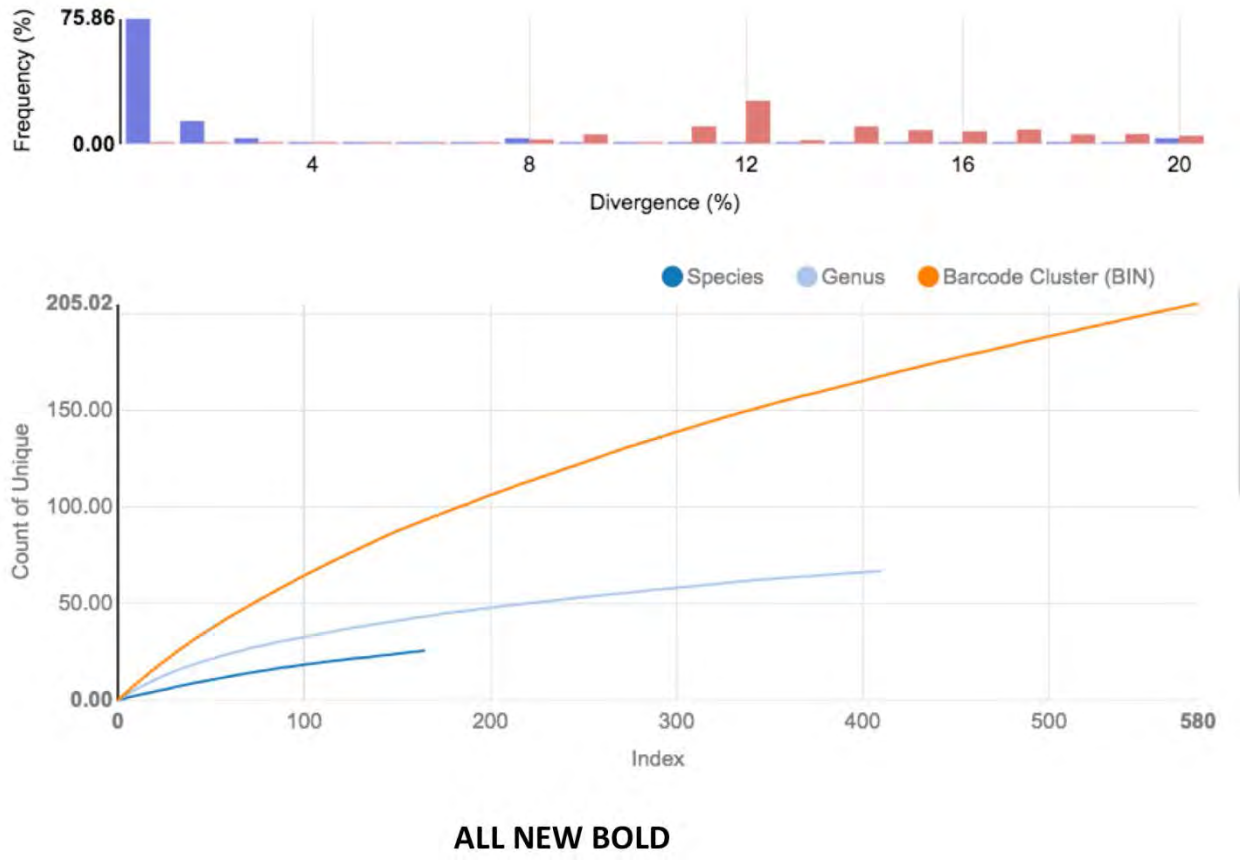

Figure 2-58 – Distance summary and rarefactions curves for all barcoded taxa. (a) Randomized taxon rarefaction curves based on DNA barcode sequence data. Curves were constructed in BOLD using 40 random replicates and default settings, (b) Randomized taxon rarefaction curves based on DNA barcode sequence data. Curves were constructed in BOLD using 80 random replicates and default settings.

common to stream habitats, many of the taxa that we barcoded could be identified to the species level because sequence data from accurately identified adult material were present in the BOLD database. DNA barcoding proved an effective method of establishing baseline biodiversity in the study area in addition to confirming the morphological identifications that were made to the Family or Genus level. Family-, and particularly, Genus-level identifications are generally reasonably adequate for ecosystem service or functional diversity considerations (Bailey et al. 2001), because pollution tolerance values, functional feeding groups, etc. often

arbitrarily assigned to species based on knowledge of natural history of the Genus. DNA barcoding also provided identification of common but understudied groups of taxa (e.g., water mites and chironomids) to a much lower taxonomic level than was reasonably possible based morphological characters.

Mayflies, caddisflies and stoneflies (EPT) are common inhabitants of stream ecosystems and are the primary taxa used as indicators of stream quality because many species are known to be sensitive to environmental disturbance (Bonada et al. 2006). DNA barcoding enabled the identification of 34 mayfly species, 26 stonefly species and 33 caddisfly species, many of which would not have been possible based on morphological characters because they were immature or keys to species level are not available. Intraspecific divergence levels in barcode sequences ranged from 0–4%, considerably higher than the 2 % threshold reported in similar studies (Zhou et al. 2009; Sweeney et al. 2011; Stein et al. 2014). Many EPT taxa collected through this study formed distinct clusters with greater than 2 % divergence from valid species known from BC and were named *near* (nr.) *species*, but they likely represent genetically-distinct populations of valid species known from other regions, possibly associated with their isolated geographic location in the Pacific Northwest surrounded by various mountain ranges which may act as reproduction barriers with other populations.

True flies are another dominant group of invertebrates present in stream ecosystems and included in assessments of stream quality. In general, the proportion of fly larvae are known to increase as stream quality decreases relative to the proportion of EPT taxa, but this is largely due to the high tolerance of certain chironomid species to pollution and other types of environmental disturbance. Thirty-five species of non-chironomid fly larvae were identified based on barcode sequences, most of which were not identifiable beyond Genus or Family level, mainly because fly larvae are more difficult to identify morphologically, some of which require painstaking dissection of mouthparts and microscope magnification greater than possible using typical microscopes. Black flies (Simuliidae) and crane flies (Limoniidae and Pediciidae) were the most diverse Families present in stream samples. Snipe flies (Athericidae), mountain midges (Deuterophlebiidae) and net-winged midges (Blephariceridae) were rare in Surber samples, all of which are Families known to contain relatively few Genera and species

and to occur in undisturbed habitats. Dance fly larvae (Empididae) were the taxa morphologically misidentified most often, having been initially confused with crane fly larvae.

Previous DNA barcoding studies of chironomids have revealed surprising levels of diversity relative to what was known based on morphological studies and higher mean intraspecific sequence divergence levels, approximately 4 %, compared to other insect taxa (Sweeney et al. 2011; Nicacio and Juen 2015). DNA barcoding revealed 54 putative midge species, but most could not be identified beyond the Genus level due to a lack of species-level identifications available in BOLD. Studies of chironomids have shown that they often exhibit species-level responses to environmental changes (Lenat 1983; Carew et al. 2013), which is often masked when Family-level assessments are required due to limitations on resources or taxonomic expertise. Thus, DNA barcoding of chironomids, particularly when combined with novel techniques involving metabarcoding of bulk invertebrate and environmental DNA samples, hold great potential for their use as indicators of stream quality.

Of the other taxa analysed using DNA barcoding, riffle beetles (Insecta: Coleoptera) and water mites (Hydrachnida: Trombidiformes) are the most common taxa identified from Surber samples after mayflies, caddisflies, stoneflies, and flies, and are often included in taxa richness estimates of benthic invertebrate diversity although identified only to the Family or Genus level. Three species of riffle beetles and 17 species of water mites were identified based on barcode sequences, which would not have been feasible based on morphology due the absence of available keys.

DNA barcoding provided insight into the richness and diversity of benthic invertebrates in the study area by increasing the taxonomic resolution of their identification compared to morphological identifications. The results of this study revealed where gaps in taxonomic knowledge of pertaining to local taxa existed, such as the omission from a recent study of North American mayflies of specimens collected from locations BC north of the Lower Mainland (Webb et al. 2012). In a mayfly study conducted in streams of the Bella Coola and Owikeno watersheds located south of Terrace, Wigle and Thommasen (1990) identified 26 mayfly species from 11 Genera and five Families, similar to results of the current study, but the study

was based on morphological characters and did not include DNA barcoding analyses for comparison with our specimens.

The generation of DNA barcodes for local reference specimens will also contribute to the development of a DNA barcode library for use in associated projects involving next-generation sequencing of fish gut content, environmental DNA (eDNA) and Surber sample preservative ethanol samples. Next-generation sequencing (NGS) technology combined with DNA barcoding, known as metabarcoding, allows the simultaneous identification of all taxa present in samples composed of hundreds or thousands of specimens from hundreds of samples. While still a relatively new field of environmental science, metabarcoding has revealed an immense potential for identifying taxa present in bulk samples much more rapidly and with greater taxonomic resolution compared to morphological identification and without the need for the expensive expertise of a skilled taxonomist. Early results of metabarcoding studies suggest the existence of higher levels of undescribed biodiversity than have been previously estimated based on morphological data. In addition, metabarcoding has the potential to contribute to a far greater understanding of aquatic invertebrate responses to changing environments when conducted over longer time scales to allow the detection of changes in community composition.

In conclusion, DNA barcoding furthered the BMAP objective of establishing baseline diversity of benthic invertebrates from study streams by providing more accurate estimates of species richness. It confirmed researchers' ability to morphologically identify the dominant Families and Genera present in the study area, as well as contributed to improving future accuracy in morphological identifications by highlighting troublesome groups that require more careful consideration. This study also generated reference barcode sequences for development of a local library for use in NGS projects, revealed where gaps in taxonomic knowledge of local fauna would benefit from future investigations of morphological and DNA barcoding analysis, and confirmed central BC as hotspot for unexplored biodiversity.

2.7. Metabarcoding benthic invertebrate communities using DNA extracted from Surber sample preservative ethanol

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Introduction

Freshwater aquatic ecosystems face many anthropogenic alterations, including forestry in and around riparian regions, agricultural run-off, oil and gas exploration and associated pipeline construction, and climate change (Dudgeon et al. 2006). Biological monitoring has long been used to determine the ecological condition of aquatic ecosystems, to identify levels of biological degradation, and to assess the success of remediation (Buss et al. 2015). Benthic invertebrate communities are particularly important for biomonitoring due to their abundance, species richness, mostly sedentary lifestyles, and taxon-specific sensitivities to aquatic pollution or degradation (Bonada et al. 2006). Many countries have developed standardized protocols to facilitate collection and comparison of long-term data over wide geographic areas; however, these protocols are often not standardized internationally or even consistently applied within the designated regions (Buss et al. 2015).

The ideal benthic invertebrate biomonitoring protocol would be cost-effective, rapid, easy to use, and sensitive to impacts (Buss et al. 2015); however, there are conflicts between these criteria. For example, increasing replication of samples improves the ability of researchers to distinguish anthropogenic impacts from the background “noise” resulting from sampling diverse communities in heterogeneous habitats, yet simultaneously increases costs of collecting and processing samples. Morphological identification of specimens is time-consuming and requires high levels of expertise (Gibson et al. 2015). For this reason, in most biomonitoring protocols, specimens are only identified to higher taxonomic levels (Genus, or more commonly, Family; Buss et al. 2015). As well, often only more easily identifiable taxa are included; even when specimens are identified to Genus or species, efforts may focus on Ephemeroptera, Plecoptera, and Trichoptera (EPT) but exclude Diptera, even though it forms a dominant component of the benthic fauna (Carew et al. 2013). The utility of higher-level identifications

for biomonitoring has been much discussed (Hewlett 2000; Jones 2008; Lenat and Resh 2001); however, at least some studies indicate that lack of taxonomic resolution may limit the fine-grained detail needed for biomonitoring (Lenat and Resh 2001; Timms et al. 2013).

DNA barcoding, wherein a standardized region of the cytochrome c oxidase gene is sequenced and compared to online databases, has been proposed as a method for increasing ease of identifications (Hebert et al. 2003). Barcoding has successfully been used for freshwater invertebrates, greatly increasing the taxonomic resolution and identifying a much larger number of species in a sample, as well as improving the statistical power of comparative metrics (Zhou et al. 2010; Sweeney et al. 2011; Gill et al. 2014; Jackson et al. 2014; Stein et al. 2014). It remains expensive and time-consuming, however, to sequence every specimen in a sample, or even a representative subset. More recently, the field of metabarcoding has taken advantage of the increasing capacity and decreasing cost of next-generation sequencing technologies to develop protocols for sequencing entire communities simultaneously (Taberlet et al. 2012; Ji et al. 2013; Cristescu 2014). This has the potential to revolutionize biodiversity studies of arthropods, and has been referred to as “Biomonitoring 2.0” (Baird and Hajibabaei 2012). Most metabarcoding studies of arthropods have been based on the homogenization of samples (Yu et al. 2012; Gibson et al. 2014; Gibson et al. 2015). Although this approach can provide robust estimates of diversity and other biodiversity metrics, it does now allow the vouchering of specimens and their retention for subsequent morphological analyses (Hajibabaei et al. 2012). Barcode libraries are still highly incomplete and mismatches between barcode sequences and species identity are not uncommon (Virgilio et al. 2010; Bergsten et al. 2012; Trebitz et al. 2015); vouchers are thus essential for validating identifications and for future taxonomic research.

Non-destructive metabarcoding from preservative ethanol is a newly developed technique that may allow both objectives (rapid biodiversity assessment and maintenance of a voucher collection for morphological and taxonomic identification) to be met. Shokralla et al. (2010) first demonstrated that specimen DNA could be amplified from preservative ethanol, and the technique was used for freshwater benthic invertebrates by Hajibabaei et al. (2012). Both studies, however, were proof-of-concept, and to the best of our knowledge, the technique

has not yet been tested in an applied setting, using benthic samples collected according to standardized protocols for biodiversity monitoring.

Benthic invertebrates from streams in central BC have received relatively little ecological or taxonomic attention. The region is increasingly under pressure from the construction of linear features (e.g., roads, power lines, pipelines) (Levesque and Dube 2007; Cott et al. 2015), however, added to a longer history of agriculture and forestry. Disturbance of streams and adjacent riparian zones, such as through the construction of pipelines, has been shown to affect stream processes by changing substrate conditions, flow patterns, and riparian inputs (Levesque and Dube 2007). There is thus a strong need for baseline data that can be used to assess the resilience of these ecosystems to increasing industrial development in the region, and for methods that can facilitate and standardize the acquisition of these data.

The goal of this project was to extract and sequence DNA from preservative ethanol used to store benthic invertebrate samples collected from streams in central BC, to compare the biodiversity data with that obtained from traditional morphological identifications of the same samples.

Methods

In 2014, benthic invertebrate samples were collected from fish-bearing streams located along the proposed pipeline RoW in interior (section 2 of the pipeline RoW) and coastal (section 4 of the pipeline RoW) regions of central BC. Surber sampling was conducted in seven streams in section 4 (Figure 2-59) and seven streams in section 2 (Figure 2-60), of which nine were selected for metabarcoding analysis (Table 2-60).

Benthic invertebrate collection

At each stream, Surber samples were collected from three sites: at the proposed pipeline crossing (0 m), 200 m upstream of the crossing (+200 m), and 200 m downstream of the crossing (-200 m). Field sampling followed a general Before-After-Control-Impact (BACI) study design (Smith 2002), including sampling upstream (control) and downstream (impact) throughout both “before” and “after” construction of the pipeline. Sample collection started downstream and proceeded upstream to minimize disturbance and avoid contamination

between sites, with three replicate Surber samples collected at each of the three sites (-200 m, 0 m, +200 m) within each stream, for a total of nine benthic invertebrate samples obtained per stream (Figure 2-42) per sampling date. Benthic invertebrates were sampled using a Surber net (Dynamic Aqua Supply, Surrey, BC, Catalog No. SBN250) with a 30- × 30-cm frame (0.09 m^2) and 250-micron mesh size, using methods adapted from the Canadian Aquatic Benthic Invertebrate Network (CABIN) Field Manual (Reynoldson et al. 2003).

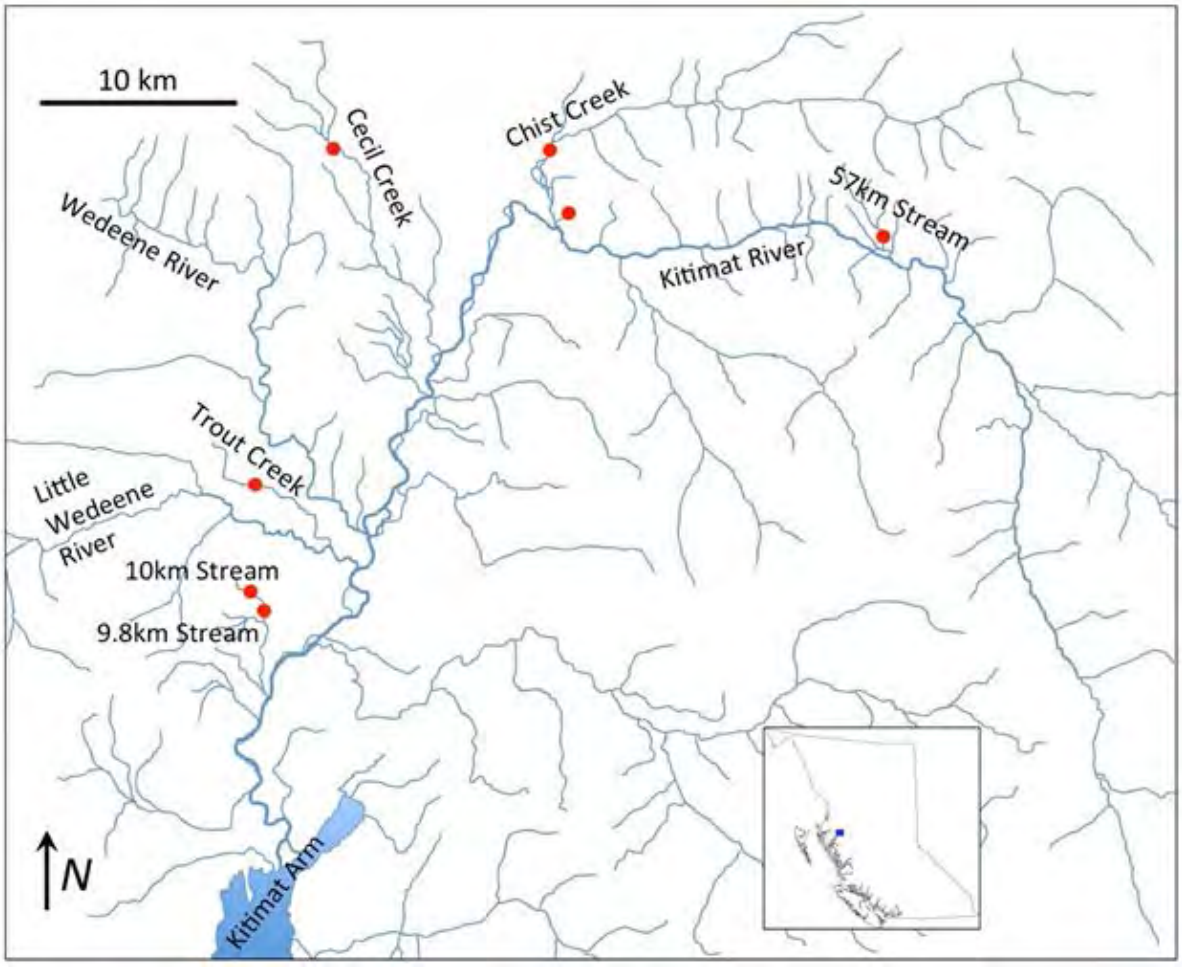

Figure 2-59 – Map of the Kitimat River watershed; approximate locations where samples were collected indicated by red circles (●). Inset map shows location of section 4 streams in BC.

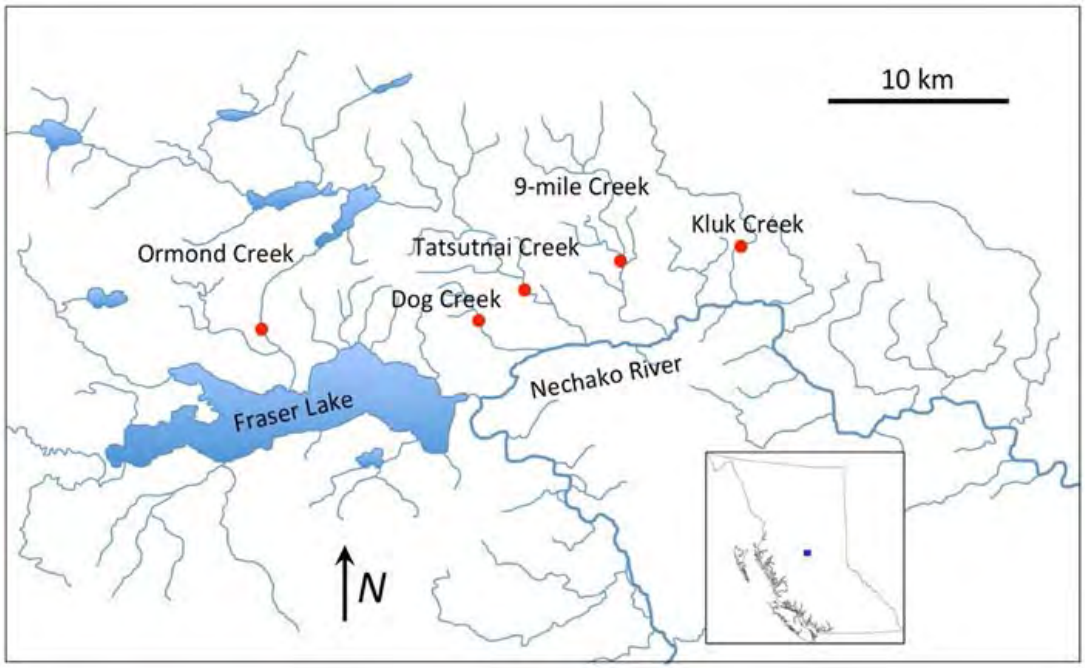

Figure 2-60 – Map of the Nechako River watershed and Fraser Lake; approximate locations where samples were collected indicated by red circles (●). Sampling locations not shown include Salmon River, approximately 200 km east of Fraser Lake, and Tchesinkut Creek, approximately 90 km west of Fraser Lake. Inset map shows location of section 2 streams in BC.

Table 2-60 – Benthic invertebrate samples analysed using metabarcoding, including stream name, collection location (UTM), site, sample code (Sample ID) and date of collection.

Section	Stream Name	Location (UTM)	Site	Sample ID	Date Collected
2	Nine Mile Creek	10U 0403828 6000704	-200	I14-293	05-Sep-14
	Nine Mile Creek	10U 0403723 6000956	+200	I14-300	05-Sep-14
	Ormond Creek	10U 0384606 5997504	-200	I14-303	09-Sep-14
	Ormond Creek	10U 038436 5997856	+200	I14-308	09-Sep-14
	Salmon River	10U 0496808 6006249	-200	I14-284	02-Sep-14
	Salmon River	10U 0496287 6006280	+200	I14-291	02-Sep-14
	Tatsutnai Creek	10U 0398490 5999442	-200	I14-281	27-Aug-14
4	Cecil Creek	9U 0524148 6013967	-200	I14-213	26-Jul-14
	Chist Creek	9U 0531924 6014489	-200	I14-249	29-Jul-14
	Chist Creek	9U 0532211 6014617	+200	I14-253	29-Jul-14
	Trout Creek	9U 0520528 6000162	-200	I14-240	28-Jul-14
	Unnamed 57.1 km	9U 0546970 6011379	-200	I14-204	25-Jul-14
	Unnamed 57.1 km	9U 0547078 6011609	+200	I14-209	25-Jul-14
	Unnamed 9.8 km	9U 0521045 5996024	-200	I14-231	27-Jul-14
	Unnamed 9.8 km	9U 0520852 5996112	+200	I14-236	27-Jul-14

Each Surber sample was a composite of three, 3-min placements within riffle sections of the stream, working sequentially upstream for each sample. During each placement, the large rocks within the sampling frame are picked up and rubbed, displacing invertebrates that are carried by the current downstream into the mesh collecting net, then removing the rock from the sampling area. Once large rocks were removed, the gravel within the sampling frame is swirled to stir up to allow the remaining invertebrates found deeper within the substrate to be carried into the mesh net. After the first 3-min interval, the Surber sampler was moved a few meters upstream to an undisturbed area in the same riffle, or a separate riffle when riffle size was too small for three placements, for the second and third 3-min placements to complete the composite Surber sample.

After each sample was collected, five elutriation steps were performed to concentrate the organic material and discard inorganic debris such as rocks and sand. A sterilized 5-L bucket was used to swirl sample contents and suspend the organic material, which was then poured back into the funnel of the Surber sampler. After the inorganic debris was rinsed five times, it was discarded. The organic material was then transferred to a 1-L plastic bottle and the specimens preserved in 95 % ethanol. To main a high concentration of ethanol and ensure preservation of DNA for genetic analyses, preservative ethanol from each sample was replaced once shortly after collection with fresh 95 % ethanol using clean, sterile funnels and 250-micron mesh netting. Upon return to the lab, samples were stored at -20 °C until processing.

Processing of benthic invertebrate samples

Before processing, benthic invertebrate samples were removed from the freezer and allowed to warm on the bench while a clean, sterile 250-mL bottle and 20-mL glass scintillation vials and pre-printed sample and specimen identification labels were prepared. The Surber sample was then inverted ten times to ensure contents were well-mixed and approximately 250 mL of the preservative ethanol poured into the 250-mL bottle a using clean, sterile funnel and 250-micron mesh netting. The Surber ethanol sample was then stored at -80 °C until subsequent metabarcoding analysis.

Benthic invertebrate specimens were identified following CABIN Laboratory Manual protocols (Reynoldson et al. 2003). Sample contents were spread out in a large tray and

examined under a dissecting microscope and large samples estimated to contain more than 600 invertebrate specimens were placed in gridded trays, the cells of which were then subsampled randomly (using a random number generator) until at least 300 specimens were sorted from the sample for identification, and the total number of invertebrates estimated based on the number of cells sorted of the total number of cells in the tray. If the sample contained less than 600 individuals, the entire sample was sorted. All aquatic insect taxa (Insecta) were included in the specimen count, mainly the Orders dominant in stream habitats: Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies), Diptera (true flies), and Coleoptera (riffle beetles), as well as water mites (Hydrachnida: Trombidiformes). Other invertebrates were identified but not included in the count, such as worms, copepods, ostracods, terrestrial fall-in (e.g., spiders and aphids), which can represent a substantial food source for some fish.

Invertebrates were identified to the Family level (or to Order level for very immature or damaged specimens) using taxonomic keys, primarily those of McCafferty (1983), Clifford (1991), Needham (1996), Stewart and Osgood (2006), and Merritt et al. (2008).

DNA extraction from Surber sample ethanol

To prevent contamination of Surber ethanol samples with foreign DNA, DNA extractions were performed using laminar flow hood in a room dedicated to low-quality DNA sources. All equipment was sterilized using a 10 % bleach solution, treated with RNase Away (to destroy potential enzymes that could degrade DNA, and exposed to ultraviolet (UV) light for approximately 30 min. To monitor for possible accidental contamination, a negative centrifuge control consisting of 40 mL of 95 % ethanol was included in all subsequent DNA extraction and PCR amplifications for each set of DNA extractions (three samples from the +200 m sites and three samples from the -200 m site from one stream sampling event).

Surber ethanol samples were removed from the -80 °C freezer and allowed to equilibrate to room temperature while seven 50-mL clean, sterile centrifuge tubes (Thermo Scientific, Rochester, New York, 3119-0050) were clearly labeled with sample and negative control identification labels. Samples were inverted ten times to ensure DNA was re-suspended evenly in solution, and 40 mL or 80 mL of preservative ethanol transferred into centrifuge tubes using a funnel and 250-micron mesh netting. Surber ethanol and negative centrifuge control

samples were centrifuged at 13,000 rpm for 1 h at 4 °C to recover cellular material and free-floating DNA. Taking care not to disturb the formed pellet, 38 mL of supernatant was then removed using clean, sterile pipettes and the remaining 2 mL of ethanol gently swirled to re-suspend the pellet transferred to a sterile 2 mL microcentrifuge tube and centrifuged at 13,000 rpm for 30 min at 4 °C. Without disturbing the resulting pelleted material, as much of the remaining supernatant as possible was removed and samples left in the laminar flow hood overnight with caps open to evaporate any remaining ethanol, which can interfere with subsequent DNA extraction steps.

The following morning, DNA was extracted from each Surber ethanol and negative centrifuge control sample, as well as a negative extraction control, using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Toronto, ON, Catalog No. 69506), following the manufacturer's instructions with the following modifications: The first two steps involving tissue lysis were omitted since starting material is already in cellular form, and the final elution step (recovering DNA bound to the spin-column membrane) was modified to include three 50 µl elutions using Buffer AE and incubating the samples at room temperature for 5 min prior to centrifugation in order to increase quantity of DNA recovered from samples. DNA concentration of each sample and negative control was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay, Life Technologies, Burlington, ON, Catalog No. Q32854) and then stored at -20 °C until PCR amplification.

Selection of universal invertebrate primers

To target a 157bp fragment located at the 5' end of the barcoding gene cytochrome c oxidase subunit I, DNA was amplified using a modified version of the general invertebrate COI primers published by Zeale et al. (2011), hereafter referred to as ZBJ forward (ZBJ F) and ZBJ reverse (ZBJ R). These primers were designed to capture a broad range of arthropod taxa, and successfully amplified single bands at the expected size in preliminary trials using mixed samples such as Surber ethanol, fish gut contents and eDNA samples. The use of DNA extracted from a single specimen of the taxa most abundant in stream habitats, including mayflies (E), stoneflies (P), caddisflies (T), and flies (D), however, revealed that the ZBJ primers did not

consistently amplify representative specimens selected from various EPTD Families, which was being masked by amplification of other taxa in mixed-DNA samples.

To improve amplification of the target sequence from EPTD taxa obtained in Surber samples, an additional reverse primer was designed using COI sequences of 12 EPTD Families commonly identified from study streams. It was chosen by aligning sequences from 12 common Families (Baetidae, Ephemerellidae, Heptageniidae, Perlodidae, Chloroperlidae, Nemouridae, Rhyacophilidae, Hydropsychidae, Glossosomatidae, Simuliidae, Tipulidae, and Psychodidae) and manually selecting bases in the ZBJ R-binding region that were more similar to the aligned sequences. Combined with ZBJ F, this primer (EPT reverse, EPT R) targets the same 157-bp amplicon as the ZBJ primers, located within at the 5' end of standard COI barcode region (Hebert et al. 2003).

PCR amplification

Surber ethanol samples and negative and positive controls were prepared for unidirectional sequencing on a PGM Ion Torrent Sequencer using a two-step PCR amplification method that incorporates fusion primers designed by the Heath Lab at the University of Windsor in Ontario (Dan Heath, *pers. comm*.). Two tailed-end sequences, UniA and UniB, were added to the end of primers sequences used in the current study: UniA onto the forward primer (ZBJ F) and UniB onto reverse primer EPT R (Figure 2-61). These "universal" tailed-end sequences are used in the second PCR step to incorporate the Ion Torrent adaptor sequences into the final amplicon sequence: an IonA adaptor sequence and a unique 10-base pair identifier sequence (used to identify the individual samples sent for sequencing) bind to UniA sequence, and the P1 adaptor sequence used to initiate the sequencing reaction during an Ion Torrent sequencing run binds to the UniB sequence. The P1 adaptor sequence is required for correct attachment of prepared amplicons to the Ion Spheres, which rest in the wells of the semi-conductor chip and the IonA adaptor the priming site for the sequencing reaction on the Ion Torrent chip (D. Heath, *pers. comm*.).

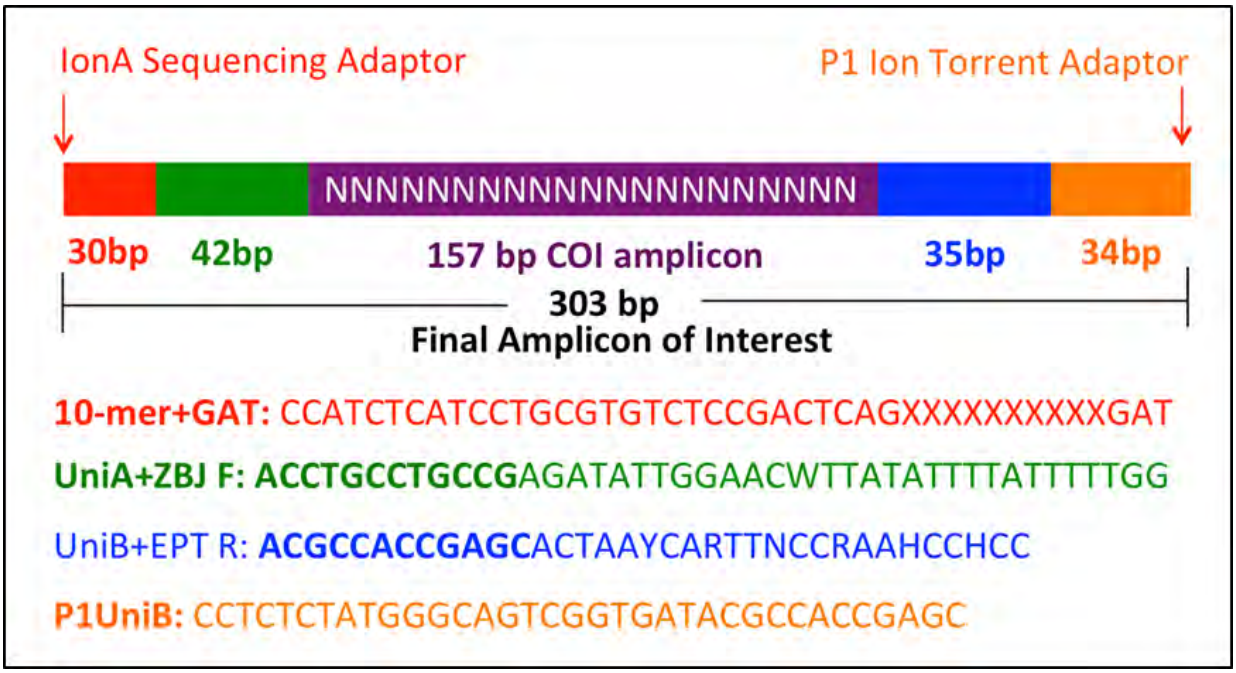

Figure 2-61 – Two-tailed Amplicon Fusion Primer Design. PCR1 amplifies the 157-bp target sequence of the COI gene plus primer (ZBJ F and EPT R) and adaptor (UniA and UniB) sequences. PCR2 incorporates the additional IonA and P1 sequencing adaptors and the unique 10-mer sample identification sequence.

During the first PCR step (PCR1), the target 157-bp COI sequence plus ZBJ F, EPT R and UniA and UniB sequences was amplified in 35-µL reaction volumes using a Qiagen Multiplex PCR kit (Toronto, ON, Catalog No. 206143). Each reaction contained 17.8 µL Multiplex MasterMix, 0.75 µL UniA-tailed ZBJ F (10 µM) and 0.75 µL UniB-tailed EPT R (10 µM) (IDT Technologies, Coralville, IA, custom order), and 15.0 µL of nuclease-free water and extracted DNA, which were combined for a total mass of 20–60 ng of template DNA per sample, or 15.0 µL of nuclease-free water for PCR negative controls. Each Surber ethanol sample, negative and positive controls were amplified in triplicate. PCR positive controls consisted of a mock community prepared using DNA extracted from 20 benthic invertebrate specimens (Table 2-61) obtained from study streams and representing taxa common in the study area. DNA from each specimen was extracted from one to three legs of nymphs depending on size of specimen, or from the thoracic region of legless specimens (e.g., dipteran larvae), using Qiagen DNeasy Blood and Tissue Kits according to manufacturer protocols, quantified with QUBIT® 2.0 Fluorometer and pooled in equimolar concentrations to normalize each specimen to 10ng of DNA.

PCR reactions were amplified using the following thermal cycling conditions: 95 °C for 15 min, 30 cycles of 94 °C for 30s, 46 °C for 60s and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR products were visualized on 2 % TBE agarose gels stained with ethidium bromide to verify the presence of single bands at the expected size (235 bp) for the COI amplicon (157 bp) plus UniA+ZBJ F (42 bp) and UniB+EPT R (35 bp) sequences for Surber ethanol samples and mock community positive controls and the absence of bands in centrifuge, extraction and PCR negative controls. PCR1 products were purified using Agencourt AMPure XP purification beads (Beckman and Coulter, Mississauga, ON, Catalog No. A63881). DNA concentrations of purified PCR1 products were quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

Table 2-61 – Identification of specimens used to prepare the Mock Community including DNA concentration (conc.) and volumes required for normalization to 10ng DNA per specimen (*except for the spider due to very low concentration of extracted DNA).

Order	Family	DNA conc. (ng/µL)	Vol. for 10ng	Mass DNA (ng)
Ephemeroptera	Ephemerellidae	4.98	2.01	10.00
Ephemeroptera	Heptageniidae	1.61	6.21	10.00
Ephemeroptera	Baetidae	0.40	25.06	10.00
Ephemeroptera	Leptophlebiidae	4.43	2.26	10.00
Plecoptera	Chloroperlidae	0.84	11.90	10.00
Plecoptera	Perlidae	1.10	9.09	10.00
Plecoptera	Perlodidae	0.97	10.35	10.00
Plecoptera	Nemouridae	1.57	6.37	10.00
Trichoptera	Hydropsychidae	1.02	9.80	10.00
Trichoptera	Glossosomatidae	25.80	0.39	10.00
Trichoptera	Rhyacophilidae	8.34	1.20	10.00
Trichoptera	Brachycentridae	0.59	16.89	10.00
Diptera	Ceratopogonidae	0.77	13.04	10.00
Diptera	Chironomidae	4.48	2.23	10.00
Diptera	Tipulidae	11.30	0.88	10.00
Diptera	Psychodidae	0.29	34.60	10.00
Coleoptera	Elmidae	4.48	2.23	10.00
Hydrachnida	Trombidiformes	5.05	1.98	10.00
Arachnida	Lycosidae*	0.04	34.60	1.21
Hymenoptera	Apidae	0.65	15.31	10.00
TOTAL	**191.20**	**206.42**	**1.08**	

The second PCR step (PCR2) was performed to attach the IonA plus unique identifier and P1 adaptor sequences to the amplicons produced during PCR1 (Figure 2-61). Total volume

of PCR2 reactions was 22.5 µL and contained 3.6 µL 10 × Reaction Buffer, 1.5 µL $MgCl_2$ (25 mM), and 0.15 µL Platinum Taq polymerase (5 Units × μL^{-1}) (all from Invitrogen, Burlington, ON, Catalog No. 1096634), 0.75 µL dNTPs mix (10 mM) (New England BioLabs, Whitby, Ontario, N0446S), 0.75 µL IonA-UniA primer (10 µM) (IDT Technologies, Coralville, IA, custom order), which contains a single unique 10-mer identifier sequence for each sample and positive/negative control (to allow sample identification in downstream data analyses),and 0.75 µL P1-UniB primer (10 µM) IDT Technologies, Coralville, IA, custom order). The volume of purified PCR1 product added to the reaction varied depending on concentration, for a total mass of 10–30 ng of purified PCR1 product per reaction, adjusting the volume of nuclease-free water accordingly for a total volume of 15 µL. PCR2 reactions were amplified at 94 °C for 2 min, 5 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec, followed by a final extension at 72 °C for 5 min.

A subset of PCR2 products were analysed using an Experion DNA 1K Analysis kit (Bio-Rad, Mississauga, ON, Catalog No. 7007107) in order to verify that unique identifier and PI adaptor sequences were attached correctly to PCR1 amplicons. Successful PCR2 reactions produce three bands per Surber ethanol sample or PCR positive control: (1) PCR1 product plus both the IonX-unique identifier and P1 adaptor sequences (~299 bp); (2) PCR1 product plus the IonX-unique identifier sequence only (278 bp); and (3) PCR1 product plus the P1 adaptor sequence only (258 bp). Following confirmation that PCR2 reactions were successful, the triplicates of each sample, negative and positive control were then pooled (~60 µL), purified with Agencourt AMPure XP purification beads, and final DNA concentration quantified using QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

Preparation of final COI amplicons for ion torrent sequencing

Samples and positive controls were normalized by pooling equimolar ratios of each sample, as well as 5 µL of each negative control, which had DNA concentrations too low to be read by QUBIT® 2.0 Fluorometer (or significantly lower than that of Surber ethanol samples or positive controls) as expected. Two 40-µL aliquots of the final normalized COI amplicon library (all samples and positive/negatives controls) were then run on 1.5% TAE agarose gels stained with ethidium bromide at ~200 v for 1.5 h to allow sufficient separation of the 295-bp bands

(amplicons plus all required adaptor sequences) from those containing amplicons with only one adaptor sequence attached (255 and 275 bp). The 295-bp fragment was extracted from the gel by visualizing the bands on a UV-light box using a clean, sterile scalpel and the DNA purified using Qiagen MinElute Gel Extraction Kits (Toronto, ON, Catalog No. 28606). Finally, the purified DNA was analyzed using the Experion DNA 1K Analysis Kit to verify successful excision of the correct band from the gel and determine the concentration of the resulting COI amplicon library for submission to the Great Lakes Institute for Environmental Research (GLIER) Lab at the University of Windsor in Ontario where they were sequenced using a PGM Ion Torrent Sequencer using an Ion Torrent 318 chip.

Data analysis

Bioinformatics analyses were performed on the Rocks server using the UNBC High Performance Computing Lab. A python script was used to strip non-biologically informative sequences (10-mer identifier, UniA and primer sequences) from the raw reads (raw sequences generated by Ion Torrent PGM Sequencer), and to relabel reads with the unique identifier label (Sample ID) associated with each read. USEARCH (http://drive5.com/usearch/) (Edgar 2010; Edgar et al. 2011; Edgar 2013) was used to filter the resulting sequences for quality using a maximum error rate of 0.5 (allows one error for every 200 bp) and to truncate sequences to the expected amplicon length (157 bp). Sequences were sorted by size using a minimum size of two sequences to remove singletons, and then clustered into operational taxonomic units (OTUs) using a sequence identity of 97% (i.e., sequences that are 97% to similar to each are clustered around a central sequence), with the resulting sequences re-labelled with sequential OTU numbers, and a fasta file containing all OTUs generated. OTUs were then mapped back onto the original reads (COI sequences labeled with unique sample identifiers), including singletons, and a taxonomy table generated that includes the number of all sequences for each OTUs contained in each Surber ethanol samples and positive controls, as well as negative controls if present.

COI sequences of all OTUs were analysed using MegaBLAST (www.ncbi.nlm.nih.gov) to assign taxonomy to the resulting OTU sequences. The BLASTn file containing all OTUs and their top hits in the GenBank database (i.e., taxonomic identity of the organism with closest

sequence identity to the queried OTU) and the USEARCH OTU fasta file were imported into MEGAN5 (Huson et al. 2007) to allow the taxonomic assignments for each OTU to be exported as a tab separated value (tsv) file for manipulation in Excel. Taxonomic assignments were added to the OTU table containing all OTUs present in each sample/control and the data analysed in Excel to determine which taxa were detected in each of the nine streams analysed. These results were then compared with the results of the morphological identifications of the specimens contained in each Surber ethanol sample.

Results

Mock community (positive controls)

To verify that the primers selected in this study could successfully amplify the targeted region of the COI gene for the dominant taxa present in Surber samples obtained in study streams, mock communities (prepared by combining equimolar concentrations of DNA extracted from 20 individual specimens representing a broad range of common taxa) were analysed using the same methods as those for Surber ethanol. Eighteen of the 20 species comprising the mock community were detected by Ion Torrent sequencing, including all 16 EPTDs, as well as the water mite (Trombidiformes) and ground beetle (Coleoptera: Carabidae) (Table 2-62). Not detected using these primers were the spider (Araneae: Lycosidae), and the bee (Hymenoptera: Apidae). The crane fly (Diptera: Tipulidae) was detected in only one of the three replicates, represented by only one sequence. Except for Tipulidae, all other EPTD taxa were detected all three replicates and were represented by between 78 – 43282 sequences.

Coastal streams (section 4)

COI sequences were successfully generated by Ion Torrent sequencing for eight Surber ethanol samples collected from five streams located in section 4 — one from each of the -200 m and +200 m sites at Chist and Unnamed Creeks 9.8 km and 57.1, and one from the -200 site at Cecil and Trout Creeks (Table 2-63).

Table 2-62 – Morphological identifications of specimens comprising the mock community used as a PCR positive control in metabarcoding analyses, and the number of replicates (Reps.) and sequences of each taxon present in mock community positive controls.

Morphological identification		Metabarcoding identification	
Order	Family	No. Reps.	No. Sequences
Ephemeroptera	Ephemerellidae	3	1729
Ephemeroptera	Heptageniidae	3	1611
Ephemeroptera	Baetidae	3	1140
Ephemeroptera	Leptophlebiidae	3	102
Plecoptera	Chloroperlidae	3	1605
Plecoptera	Perlidae	3	78
Plecoptera	Leuctridae	3	14733
Plecoptera	Nemouridae	3	651
Trichoptera	Hydropsychidae	3	1552
Trichoptera	Glossosomatidae	3	220
Trichoptera	Rhyacophilidae	3	149
Trichoptera	Brachycentridae	3	43282
Diptera	Ceratopogonidae	3	4068
Diptera	Chironomidae	3	2310
Diptera	Tipulidae	1	1
Diptera	Psychodidae	3	375
Coleoptera	Carabidae	3	61
Trombidiformes	-	3	42
Araneae	Lycosidae	0	0
Hymenoptera	Apidae	0	0

Of the samples analyzed from Unnamed Creek 57.1 km, 13 Families were identified based on morphological data and 28 based on COI sequence data. Ion Torrent (IT) sequencing detected all Families identified morphologically from both samples, except for the stonefly Family Perlodidae, the caddisfly Family Glossosomatidae, and the Order Trombidiformes (water mites) in sample I14-209 (Table 2-63). All three Families were detected by IT sequencing in sample I14-204, however, indicating that the primers are able to amplify the target sequence for these taxa and that other factors likely contributed to this incongruence between samples. In addition to taxa identified morphologically, IT sequencing also detected one mayfly Family (Ameletidae), two stonefly Families (Capniidae and Leuctridae), five caddisfly Families (Goeridae, Lepidostomatidae, Limnephilidae, Polycentropodidae, and Uenoidae), and five fly Families (Blephariceridae, Cecidomyidae, Phoridae, Pipunculidae, and Psychodidae). The greater taxonomic richness detected by IT sequencing compared to morphological identifications is likely due to the ability of IT sequencing to identify immature specimens —

Table 2-63 – Comparison of taxa identified morphologically (M) or by Ion Torrent sequencing (IT) – to Family for EPTD and beetles and to the Hydrachnidiae (unranked taxonomic level) for water mites – from Surber samples obtained from coastal streams in section 4.

Sample ID	I14-204		I14-204		I14-209		I14-209		I14-213		I14-213		I14-231		I14-231		I14-236		I14-236		I14-240		I14-240		I14-249		I14-249		I14-253		I14-253	
Stream	km 57.1		km 57.1		km 57.1		km 57.1		Cecil		Cecil		km 9.8		km 9.8		km 9.8		km 9.8		Trout		Trout		Chist		Chist		Chist		Chist	
Site	-200		-200		+200		+200		-200		-200		-200		-200		+200		+200		-200		-200		-200		-200		+200		+200	
Date	25-Jul-14		25-Jul-14		25-Jul-14		25-Jul-14		26-Jul-14		26-Jul-14		27-Jul-14		27-Jul-14		27-Jul-14		27-Jul-14		28-Jul-14		28-Jul-14		29-Jul-14		29-Jul-14		29-Jul-14		29-Jul-14	
Run	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT
Ephemeroptera																																
Ameletidae																																
Baetidae																																
Ephemerellidae																																
Heptageniidae																																
Leptophlebiidae																																
Plecoptera																																
Capniidae																																
Chloroperlidae																																
Leutricidae																																
Nemouridae																																
Perlidae																																
Perlodidae																																
Trichoptera																																
Brachycentridae																																
Georidae																																
Glossosomatidae																																
Hydropsychidae																																
Lepidostomatidae																																
Leptoceridae																																
Limnephilidae																																
Polycentropodidae																																
Rhyacophilidae																																
Uenoidae																																
Diptera																																
Blephariceridae																																
Cecidomyiidae																																
Ceratopogonidae																																
Chironomidae																																
Culicidae																																
Empididae																																
Muscidae																																
Phoridae																																
Pipunculidae																																
Psychodidae																																
Scaridiae																																
Simuliidae																																
Tipulidae																																
Coleoptera: Elmidae																																
Hydrachnida																																

first and second instars that have not yet developed the characters used to distinguish among different Families (i.e., those identified to Order only) — as well as comparatively rare specimens present in large samples (greater than 600 specimens) for which only 300 specimens were sorted and identified.

Eleven Families were identified from the Cecil Creek sample (I14-213) based on morphological data and 13 based on COI sequence data. Two caddisfly Families (Nemouridae and Perlidae) and one fly Family (Simuliidae) were detected by morphological identification but not by IT sequencing (Table 2-63). IT sequencing identified five Families not identified morphologically including Leptophlebiidae (caddisfly), Perlodidae (stonefly), Glossosomatidae and Limnephilidae (caddisflies), and Simuliidae (black flies).

Fourteen Families were identified from the Unnamed Creek 9.8 km samples (I14-231 and I14-236) based on morphological data and 23 Families based on COI sequence data. The stonefly Family Nemouridae and fly Family Ceratopogonidae were identified morphologically from both samples but was only detected in I14-231 by IT sequencing, whereas the crane fly Family Tipulidae and water mites (Trombidiformes) were also identified morphologically in both samples but was only detected in I14-236 by IT sequencing (Table 2-63). The stonefly Family Perlodidae was identified morphologically in both samples but not detected in either sample based on COI sequence data. In addition to the taxonomic identifications based on morphological data, IT sequencing revealed the presence of one mayfly Family and four caddisfly Families (Ameletidae, Lepidostomatidae, Leptoceridae Limnephilidae and Polycentropodidae) in Unnamed Creek 9.8 km.

Fifteen Families were identified based on morphological data and only 10 based on COI sequence data from the sample analyzed from Trout Creek (I14-240). Not detected by IT sequencing were stonefly Families Nemouridae and Perlodidae, Brachycentridae (caddisfly), Tipulidae (crane flies), riffle beetles (Coleoptera: Elmidae) and water mites (Trombidiformes) (Table 2-63). Families not identified morphologically but detected by IT sequencing were Ameletidae (mayflies), Culicidae (mosquitoes), and Muscidae (house flies).

Two samples were analysed from Chist Creek (I14-249 and I14-253), the largest stream sampled in section 4, with 18 Families detected by IT sequencing and only nine based on

morphological data. All Families identified morphologically were also identified based on COI sequence data. Detected only by IT sequencing and not morphological analyses were Ameletidae (mayflies); Perlodidae (stoneflies); the caddisfly Families Goeridae, Glossosomatidae, Hydropsychidae, Lepidostomatidae and Limnephilidae; and fly Families Empididae (dance flies) and Muscidae (house flies).

There were three Families that were identified based on both morphological and COI sequence data including the mayfly Families Baetidae, Ephemerellidae, and Heptageniidae and fly Family Chironomidae (midges). The latter was often the most abundant and diverse group of invertebrates present in Surber samples. The mayfly Family Ameletidae was detected all samples by IT sequencing but morphologically identified in only one sample (I14-209 obtained from Unnamed Creek 57.1 km). Sixteen Families were identified from coastal streams based on COI sequence data only including stonefly Families Capniidae and Leuctridae; caddisfly Families Goeridae, Lepidostomatidae, Leptoceridae, Limnephilidae, Polycentropodidae and Uenoidae; and fly Families Blephariceridae (mountain midges), Cecidomyiidae (gall midges), Culicidae (mosquitoes), Empididae (dance flies), Muscidae (house flies), Phoridae (phorid flies), Pipunculidae (big-eyed flies), Psychodidae (moth flies), and Sciaridae (fungus gnats). Of these, unlike many of the Dipteran Families, the stonefly and caddisfly Families were common in morphological identifications of Surber samples collected in the study area. Cecidomyiidae, Culicidae, Muscidae, Phoridae, Pipunculidae, Phoridae, and Sciaridae were not been identified morphologically from Surber samples collected in the study, suggesting that the larvae from these fly Families could have been mistaken for other taxa during sample processing, or specimens were present in the adult stage, which were identified only as “terrestrial fall-in”. Among all nine samples, many mayfly, stonefly and caddisfly specimens were morphologically identifiable to the Order level only. Conversely, very few samples contained COI sequences of these common stream Families that could not identified beyond Order.

Interior streams (section 2)

COI sequences were successfully generated by Ion Torrent sequencing for seven Surber ethanol samples collected from four streams located in section 4 — one from each of the -200 m and +200 m sites at Nine Mile and Ormond Creek and Salmon River, and one from the +200 m site at Tatsutnai (Table 2-64 and Table 2-65). Of the samples analyzed from Nine Mile Creek, 21 Families were identified based on morphological data and 23 based on COI sequence data. Two Families were morphologically identified but not detected by IT sequencing, riffle beetles (Coleoptera: Elmidae) and the relatively rare caddisfly Family Hydroptilidae. Hydroptilid nymphs are completely encased by their purse-like cases made from tiny pebbles, making them difficult to find amongst inorganic debris present in Surber samples, but riffle beetles are distinct, not easily missed during sample processing and were detected by IT sequencing in sample I14-284. Two other Families, Limnephilidae (caddisflies) and Tipulidae (crane flies) were morphologically identified but not detected by IT sequencing in one sample but morphologically identified and detected by IT sequencing in the other sample, possibly due to misidentifications or sufficient damage to specimens during collection to make identification to Family level impossible. Four Families were identified by IT sequencing: Siphlonuridae (mayfly); Pteronarcyidae and Taeniopterygidae (stonefly); and Simuliidae (black flies). Simuliidae, Pteronarcyidae and Taeniopterygidae are easily distinguished from other fly larvae and stonefly nymphs, suggesting that IT sequencing likely detected specimens present in the sample but not identified during sample processing due to the subsampling procedure commonly used by workers for samples with more than 600 specimens and emulated in our study.

Invertebrate Family composition of the Ormond Creek samples was similar to that of Nine Mile Creek, with 21 Families identified morphologically and 24 by Ion Torrent sequencing. Six Families were identified based on morphological data but not detected based on COI sequence data including Nemouridae and Perlidae (stoneflies); Brachycentridae and Hydroptilidae (caddisflies); Ceratopogonidae (no-see-ums); and Elmidae (riffle beetles) (Table 2-64). Ten Families were detected by IT sequencing but not identified morphologically: Capniidae, Leuctridae and Pteronarcyidae (stoneflies); Limnephilidae and Rhyacophilidae (caddisflies); and the fly Families Athericidae, Culicidae, Dolichopodidae, and Sciomyzidae. Of

these, the stonefly and caddisfly Families are taxa commonly identified from Surber samples in the study area but sometimes in low numbers, suggesting they may have been present in the Ormond Creek samples but not identified during processing due to subsampling procedure. Larvae of Athericidae (snipe flies) and Dolichopodidae (long-legged flies) were relatively rare in Surber samples but well known from stream habitats were also likely present in samples but missed due to subsampling. Larvae of the other fly Families detected by IT sequencing but not by morphological analysis — Culicidae, Sciomyzidae, and Sphaeroceridae — are not known from stream habitats and may have been present in the adult stage and identified only as "terrestrial fall-in".

From the two samples analysed from Salmon River (I14-284 and I14-291), 19 Families were identified morphologically and 25 based on COI sequence data. There were nine Families that were present in both samples and detected by both methods: Baetidae, Heptageniidae and Leptophlebiidae (mayflies); Perlodidae (stoneflies); Hydropsychidae and Lepidostomatidae (caddisflies); Chironomidae (midges); Tipulidae (crane flies); and water mites (Trombidiformes) (Table 2-65). Five Families were identified based morphological data only, Chloroperlidae and Pteronarcyidae (stoneflies); Brachycentridae (caddisflies); Ceratopogonidae (no-see-ums); Stratiomyidae (soldier flies); and Elmidae (riffle beetles) — although Chloroperlidae and Pteronarcyidae were detected by IT sequencing in the second Salmon River sample. Detected in Surber samples by IT sequencing only were three stonefly Families (Capniidae, Nemouridae and Taeniopterygidae), three caddisfly Families (Limnephilidae, Psychomyiidae and Rhyacophilidae), and six fly Families (Anthomyzidae, Athericidae, Empididae, Hybotidae, Sciomyzidae and Sphaeroceridae). The stonefly Families, Athericidae, Empididae and Psychomyiidae are rare in Surber samples, Limnephilidae and Rhyacophilidae more common, but all are known from the study area, and likely were present but missed during sample processing due to the subsampling protocol. The fly Families Hybotidae, Sciomyzidae and Sphaeroceridae have not been identified based on morphological data through this study and possibly were present in the adult stage and identified only as "terrestrial fall-in".

Table 2-64 – Comparison of EPT taxa (Ephemeroptera, Plecoptera, Trichoptera) identified morphologically (M) or by Ion Torrent sequencing (IT) from Surber samples obtained from interior streams (section 2).

Sample ID	I14-293	I14-293	I14-300	I14-300	I14-303	I14-303	I14-308	I14-308	I14-284	I14-284	I14-291	I14-291	I14-281	I14-281
Stream	Nine Mile	Nine Mile	Nine Mile	Nine Mile	Ormond	Ormond	Ormond	Ormond	Salmon	Salmon	Salmon	Salmon	Tatsuntnai	Tatsuntnai
Site	-200	-200	+200	+200	-200	-200	+200	+200	-200	-200	+200	+200	+200	+200
Date	5-Sep-14	5-Sep-14	5-Sep-14	5-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	27-Aug-14	27-Aug-14
ID Method	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT
Ephemeroptera														
Ameletidae														
Baetidae														
Ephemerellidae														
Heptageniidae														
Leptophlebiidae														
Siphlonuridae														
Plecoptera														
Capniidae														
Chloroperlidae														
Leutricidae														
Nemouridae														
Perlidae														
Perlodidae														
Pteronarcyidae														
Taeniopterygidae														
Trichoptera														
Brachycentridae														
Glossosomatidae														
Goeridae														
Hydropsychidae														
Hydroptillidae														
Lepidostomatidae														
Leptoceridae														
Limnephilidae														
Psychomyidae														
Rhyacophilidae														

Table 2-65 – Comparison of Diptera, Coleoptera, and Trombidiformes taxa identified morphologically (M) or by Ion Torrent sequencing (IT) from Surber samples obtained from interior streams (section 2).

Sample ID	I14-293	I14-293	I14-300	I14-300	I14-303	I14-303	I14-308	I14-308	I14-284	I14-284	I14-291	I14-291	I14-281	I14-281
Stream	Nine Mile	Nine Mile	Nine Mile	Nine Mile	Ormond	Ormond	Ormond	Ormond	Salmon	Salmon	Salmon	Salmon	Tatsuntnai	Tatsuntnai
Site	-200	-200	+200	+200	-200	-200	+200	+200	-200	-200	+200	+200	+200	+200
Date	5-Sep-14	5-Sep-14	5-Sep-14	5-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	27-Aug-14	27-Aug-14
ID Method	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT	M	IT
Diptera														
Anthomyzidae														
Athericidae														
Cecidomyiidae														
Ceratopogonidae														
Chironomidae														
Culicidae														
Doliochopodidae														
Empididae														
Hybotidae														
Pipunculidae														
Psychodidae														
Sciomyidae														
Schizophora														
Simuliidae														
Sphaeroceridae														
Stratiomyidae														
Tipulidae														
Coleoptera: Elmidae														
Hydrachnida														

Only one sample was analysed from the fourth stream located in section 2, I14-281 from Tatsutnai, from which 12 Families were identified morphologically and 24 based on COI sequence data. Three Families were identified morphologically but not detected by IT sequencing, including Hydropsychidae (caddisfly), Simuliidae (black flies), and Trombidiformes

(water mites (Table 2-64)). Identified by both methods were three mayfly Families (Baetidae, Heptageniidae and Leptophlebiidae), three stonefly Families (Chloroperlidae, Nemouridae and Perlidae), one caddisfly Family (Brachycentridae), and two Families (Chironomidae and Psychodidae). There were 15 Families identified by IT sequencing but not detected morphologically: Ameletidae and Ephemerellidae (mayflies); Capniidae, Leuctridae, Perlodidae and Taeniopterygidae (stoneflies); Glossosomatidae, Goeridae, Rhyacophilidae (caddisflies); Ceratopogonidae, Culicidae, Empididae, Pipunculidae, and Sphaeroceridae (flies); and Elmidae (riffle beetles).

Overall, among all samples and streams analysed, 25 Families were identified based on morphological data, 47 Families based on COI sequence data, and a total of 48 for both methods combined, indicating IT sequencing was able to detect 98% of all taxa present in the eighteen Surber samples examined. Only one Family, Stratiomyidae (soldier flies) was identified morphologically but not detected by IT sequencing. IT sequencing detected 22 Families not found based on morphological data including one mayfly Family (Siphlonuridae), two stonefly Families (Leuctridae and Taeniopterygidae), five caddisfly Families (Goeridae, Leptoceridae, Polycentropodidae, Psychomyiidae, and Uenoidae), and 14 fly Families (Anthomyzidae, Athericidae, Blephariceridae, Cecidomyidae, Culicidae, Dolichopodidae, Empididae, Hybotidae, Muscidae, Phoridae, Pipunculidae, Sciomyzidae, Sciaridae, and Sphaeroceridae).

Of the Families identified by IT sequencing only, most are known from study streams and have been identified based on morphological data throughout this study, suggesting that they were likely in the Surber samples in which they were detected but not processed due to subsampling, and are not the result of contamination or incorrect taxonomic assignments using the NCBI database and MEGAN software. Combining morphological and IT sequencing data, 43 Families were identified from interior streams (section 2) and 35 from coastal streams (section 4). Five Families were found only in coastal streams including Polycentropodidae, Blephariceridae, Muscidae, Phoridae and Sciaridae; while nine Families were present only in interior streams including Siphlonuridae, Pteronarcyidae, Taeniopterygidae, Hydroptilidae, Anthomyzidae, Athericidae, Sphaeroceridae, and Stratiomyidae.

Taxonomic resolution

Not only was IT sequencing able to detect more Families present in Surber samples than were morphologically identified, it also provided significantly greater taxonomic resolution based upon matches with previous annotations in databases. Most of the COI sequences generated from Surber ethanol samples were identifiable to Genus or species level, providing far greater insight into baseline biodiversity in the study area than was possible using traditional methods. Based on COI sequence data, a total of 287 taxa were identified: 111 species in 71 Genera and 37 Families from coastal streams (section 4), and 194 species in 106 Genera and 42 Families from interior streams (section 2).

Additional taxa identified by IT sequencing

In addition to the dominant stream taxa present in Surber samples — mayflies (Ephemeroptera), stoneflies (Plecoptera), caddisflies (Trichoptera), and flies (Diptera) — many additional taxa were identified by IT sequencing. A Neighbor-joining tree illustrating phylogenetic relationships among all taxa from the nine study streams, identified based on COI sequence data, is provided in Figure 2-62. Many unicellular taxa were detected by IT sequencing including Bacteria, Alveolata (e.g., ciliates, dinoflagellates, and other protozoans), Amoebozoa (freshwater and marine amoebas), Apusozoa (flagellate protozoans), Cryptophyta (freshwater and marine algae), Glaucocystophyceae (rare freshwater algae), Haptophyceae (algae), and Malawimonadidae (protists). Multicellular taxa detected include Viridiplantae (green algae and land plants), Stramenopiles (algae and oomycetes, parasites of plants and fish), Rhodophyta (red algae), Nucleariidae (freshwater amoeba), Porifera (sponges), Cnidaria (hydrozoans, pararsites, etc.), Rotifera (rotifers), Mollusca (slugs and snails), Gastrotricha (hairybacks), and Annelida (earthworm-type worms).

Taxa more closely related to aquatic insects that were detected in Surber ethanol samples include Scalidophora (ecdysozoans such as horsehair worms and other worm-like animals), Tardigrada (water bears), Nematoda (nematode worms), Chelicerata (crabs and spiders), Myriapoda (millipedes and centipedes), Crustacea (clams, ostracods, and copepods), and Ellipura (springtails and proturans). In addition to Ellipura, other hexapod taxa detected by IT sequencing included other insects not commonly obtained or identified in Surber samples

(Figure 2-63), such as moths and butterflies (Lepidoptera), ground beetles (Coleoptera), grasshoppers and crickets (Orthoptera), ants, bees and wasps (Hymenoptera), lacewings (Neuroptera), aphids and other true bugs (Hemiptera), barklice (Psocoptera), and thrips (Thysanura). These taxa, if processed, would have likely been identified only as "terrestrial fall-in".

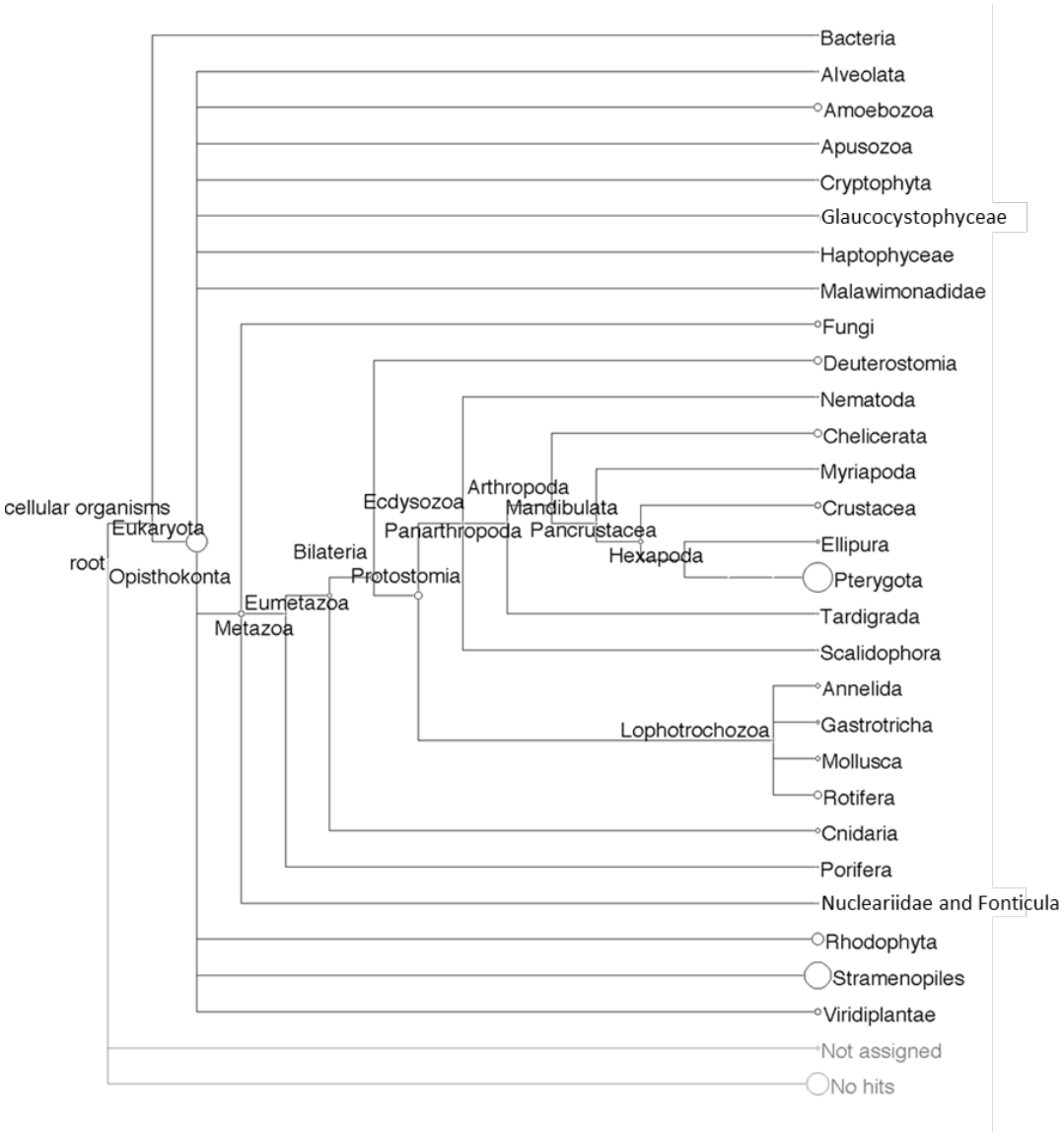

Figure 2-62 – Neighbor-joining phylogenetic tree of all COI sequences produced by IT sequencing of Surber ethanol samples obtained from the nine study streams located in sections 2 and 4. Circles at the axis of the branches the number of reads assigned to that taxonomic group.

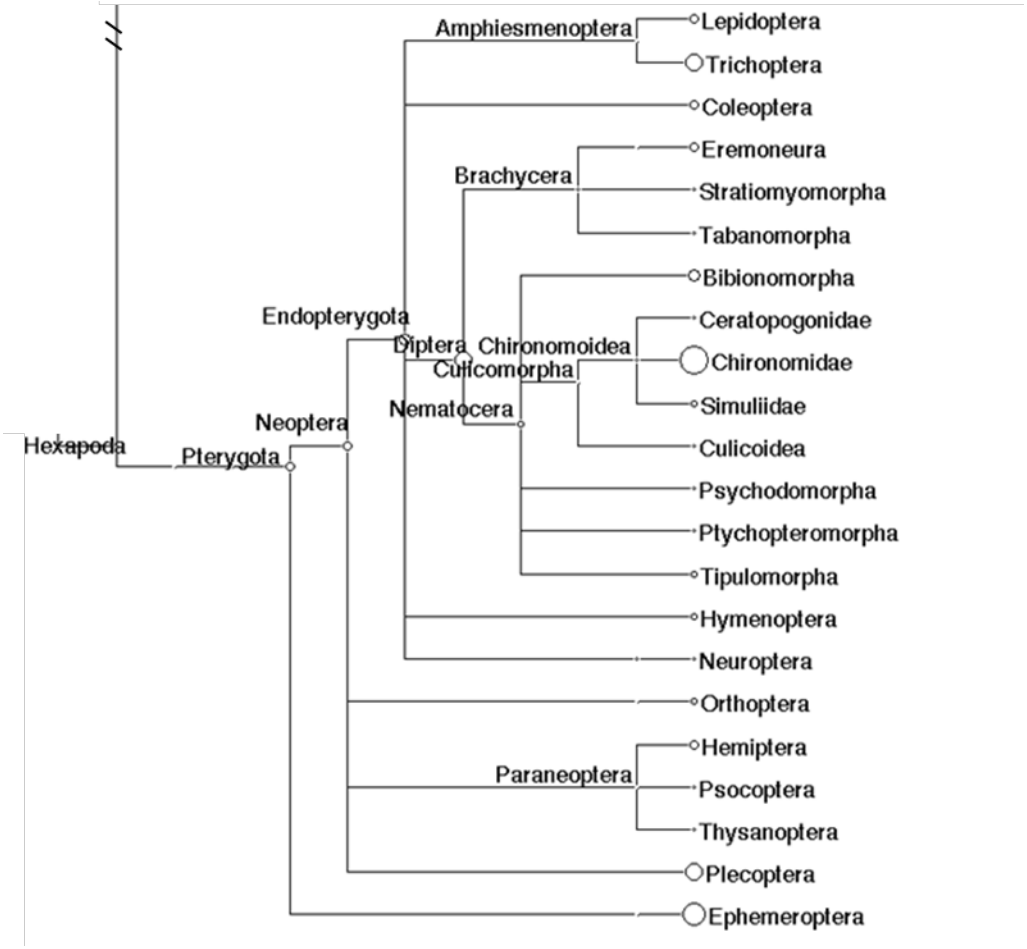

Figure 2-63 – Expanded neighbor-joining phylogenetic tree of insect COI sequences produced by IT sequencing of Surber ethanol samples obtained from study streams. Circles at the axis of the branches the number of reads assigned to that taxonomic group.

Ion Torrent high-throughput DNA sequencing technology when combined with metabarcoding techniques has the potential to vastly increase our understanding of taxonomic richness and community structure by allowing the simultaneous molecular identification of specimens present in bulk samples with greater taxonomic resolution than is possible based on more time-consuming morphological methods. This study revealed that IT sequencing of the ethanol used to preserve benthic invertebrate samples can be used to reliably identify not only the dominant EPTD taxa present in stream habitats, but other taxa as well: 1) “terrestrial fall-

in", which can comprise a large proportion of the diet of certain fish species; 2) other taxa not normally identified in traditional assessments of stream quality (i.e., CABIN protocols) but that may be as important to or useful in assessing stream quality, such as clitellatan worms, nematodes, sponges, ostracods and copepods; and 3) zooplankton and phytoplankton (e.g., diatoms, rotifers, amoeba, and algae), which are also known to play a large role ecosystem function and to be affected by environmental stressors (Griffith et al. 2005). This level of information would not be possible using traditional methods based only on morphological data without substantial increases in the expense, amount of time, and taxonomic expertise, and equipment required to process samples.

IT sequencing using the single 157-bp amplicon produced by ZBJ F and EPT R primers failed to detect only one Family present in stream samples, Stratiomyidae (soldier flies), which are typically rare in Surber samples (represented by a single specimens in only one of the 18 samples analysed). Those insects also have a toughened, leathery exoskeleton that may reduce cellular degradation and thus the amount DNA present in the preservative ethanol. In almost all samples identified morphologically, many of the 300 specimens processed from large samples were either too immature or damaged to be identified beyond the Order level, whereas very few taxa were identifiable to Order only based on COI sequence data. In addition, some EPTD Families were detected in samples that were not represented in the morphological identifications, which, since they are known in low numbers from other samples identified through the associated morphological study (see Morphology Report for details), were likely overlooked during sample processing due the subsampling protocol used for large samples.

There were individual instances of specimens identified morphologically but not detected by IT sequencing (Table 2-64 and Table 2-65), but because those taxa were detected by IT sequencing in other samples, it is possible that those morphological identifications were incorrect, or that competition among COI sequences present in Surber ethanol samples for the ZBJ F and EPT R primers resulted in PCR bias and preferential amplification of certain taxa over others present in some samples. Both of these possibilities could be overcome. For instance one benefit of using only the ethanol used to preserve samples is that intact specimens are available for subsequent morphological analyses to verify taxonomic identification. In addition

PCR biases can be reduced through the use of additional primer sets, such as the multiplexed use of both ZBJ F ZBJ R and ZBJ F/EPT R primers sets or those that target other regions of the DNA (e.g., 16S rDNA sequences). Recent metabarcoding studies have revealed that a greater number of primer sets targeting more regions of the genome can improve the accuracy of taxonomic identifications of specimens present in bulk samples (for examples, see Hajibabaei et al. 2011; Carew et al. 2013; and Clarke et al. 2014).

Conclusions

IT sequencing proved to be an efficient and accurate method of identifying benthic invertebrate and other taxa present in Surber samples collected from study streams and provided far greater taxonomic resolution than is possible based on morphological methods, particularly for specimens too immature or damaged to allow identification beyond the Order level and those specimens present but not processed in large samples due to subsampling. By providing more accurate estimates of taxonomic richness and community composition present in study streams, metabarcoding of Surber ethanol samples contributes to a greater understanding of ecosystem function and potential responses to disturbances such as those created by pipeline construction. Identification of benthic invertebrates through metabarcoding of the ethanol used to preserve Surber samples allows retention of specimens for future morphological analyses to confirm identifications based on COI sequence data. This method holds great promise for elucidating responses of stream invertebrate taxa to disturbance and the discovery of bioindicators for use in stream quality monitoring and assessment projects.

2.8. Demographics of fishes from British Columbia streams intersected by the proposed Pacific Trails Pipeline

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Introduction

Biodiversity in headwater streams is important for ecological function of river systems and their riparian network (Vannote et al. 1980). Tremendous variation in physical, chemical, and biotic attributes exists among small streams even if they are close geographically — providing potential habitat for a wide range of species that may be permanent residents or migrants that travel to headwaters at particular seasons or life stages. Headwaters streams influence the rivers downstream by providing specific habitat that meets the requirements of not only resident individuals, but also migratory individuals that may transiently use the habitat. Headwater streams offer refuge from temperature extremes (Power et al. 1999; Hawkshaw et al. 2014) and flow extremes (Dieterich and Anderson 2000), competitors (Moyle and Cech 2004), predators (Erman and Hawthorne 1976), and provide spawning sites (Bahr and Shrimpton 2004; Shrimpton et al. 2008; McRae et al. 2012) and rearing areas (Shrimpton et al. 2014; Clarke et al. 2015). Degradation and loss of headwaters and their connectivity to ecosystems downstream threaten the biological integrity of entire river networks. Understanding habitat requirements for fish in natural systems, therefore, is important for effective management — particularly in areas affected by anthropogenic disturbance.

Alterations to the environment can result in aquatic community changes over time. In response to habitat changes, fish can be displaced from, or avoid, disturbed areas that are no longer suitable. Fish abundance has been found to decrease downstream following pipeline installation (Anderson et al. 1998). The response appears to be related to higher levels of suspended sediment. Lloyd et al. (1987) found reduced abundance of zooplankton, macroinvertebrates, and *Thymallus arcticus* in naturally and artificially turbid aquatic systems, but recovery of abundance occurred when higher flows removed disturbed sediments from the stream-bed. By identifying habitat conditions important for stream fishes, management efforts

can be focused on specific practices that protect and potentially enhance critical habitat — but it is essential to identify the assemblage of species and their abundance before construction activities to properly assess the response of the fish community. Our objective was to monitor baseline fish biodiversity to compare potentially affected areas (at crossing points and downstream from those points) to areas that should remain free from any effects from construction (e.g., above crossing points).

Methods

This study was conducted within tributaries of the Kitimat River (Figure 2-64) and Fraser River watersheds in central BC. Most of the sampling locations in the Fraser River watershed were small streams that flowed into the Nechako River, one of the largest tributaries flowing into the Upper Fraser River. Five of the tributaries sampled are shown in Figure 2-65. We also sampled Tchesinkut Creek, 50 km west of Ormond Creek, and Salmon River, 25 km north or Prince George, BC. The Salmon River flows directly into the Fraser River.

Sample sites were chosen from rivers and creeks that were characterized as fish bearing. Population composition and density estimates for fish were assessed using single-pass electrofishing in the selected study streams. Fish were captured using a Smith Root L24 backpack electrofisher (Vancouver, WA) during the summers of 2013, 2014, and 2015. Because of the negative effects of electrofishing on fish physiology, we limited our effort on any given sample date to minimize stress. We also limited sampling to no more than two times each season to allow fish to recover fully from the effect of electrofishing. Estimates of fish species richness that use single-pass electrofishing increase significantly with a decrease in stream width (Meador et al. 2003), although single-pass electrofishing may underestimate abundance (Mitro and Zale 2000). For small first-order streams, a single-pass sampling design repeated with temporal separation was considered to be appropriate for detecting fish diversity. Additionally, such a sampling design should also provide a relative index of abundance. Peterson et al. (2004) showed that capture efficiency was low for the first pass (28 %) and decreased considerably with successive passes, suggesting that fish responded to the electrofishing procedures. Consequently, the first pass through a reach should provide the best

opportunity to collect fish while they are still naive to sampling (Mesa and Schreck 1989). We used this approach to provide a better estimate of relative abundance than a depletion approach, especially if some species are more likely to develop avoidance behaviours than others (Edwards et al. 2003). We selected portions of streams below and above the proposed pipeline crossing for electrofishing on each stream. Below the proposed pipeline crossing RoW we fished a representative portion of the reach that was approximately 100 m long that corresponded to approximately -250 – -150 m. Above the proposed crossing RoW we fished a representative portion of the reach of a similar length that corresponded to approximately +150 – +250 m. Population composition and density estimates were determined for fish in each study stream.

Fish captured were transferred to a bucket containing 100 mg $\times$ mL^{-1} tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate as an anesthetic. When fish had ceased movement, they were removed, photographed, and then measured for fork length (or total length depending on species) to 0.1 cm and weighed to the nearest 0.1 g and a tissue sample collected for genetic analysis. Following handling, fish were transferred to a bucket of fresh stream water to recover. Vertical orientation and response to stimuli (active avoidance) were used to assess recovery from anesthesia. Following recovery fish were returned to the location of capture. Some of the fish were lethally sampled (see also Section 2.9, Section 2.10, and Section 2.13). For lethal sampling, fish were handled once ventilation movements have ceased and measurements were collected as described above. Additionally, muscle, gill, and liver samples were collected from these fish for physiological analysis. The stomach was also removed to assess prey items and sagittal otoliths removed for microchemistry analysis. Species collected in both watersheds are listed in Table 2-66. Condition factor was calculated as $100 \times W \times L^{-3}$, where W is the fish weight in grams and L is the fork length, the distance from the tip of the nose to the fork in the tail.

Information on growth of fish can be obtained from length data. Species with restricted spawning times — most temperate freshwater fish — will show limited range of lengths within a cohort. For younger age-classes, the range of lengths is often small and the length frequency distributions show clear modes. For older fish, the overlap in ranges of length is greater and the

modes are often difficult to distinguish. Consequently, age for young fish captured in our study was determined from length frequency analysis. Due to the low number of larger, and putatively older, fish caught in the study streams — it was not possible to determine age from analysis of length frequencies. Annual variation in otoliths was used to estimate age for larger fish. Zinc oscillations were used to estimate age and annuli zonation for each fish. Halden et al. (2000) showed that zinc fluctuations in otoliths provide temporal information consistent with the annual cycle in the biology of the fish. Age of approximate annuli zonation was confirmed by counting annuli of each sectioned otolith using light microscope images (Section 2.13, this volume).

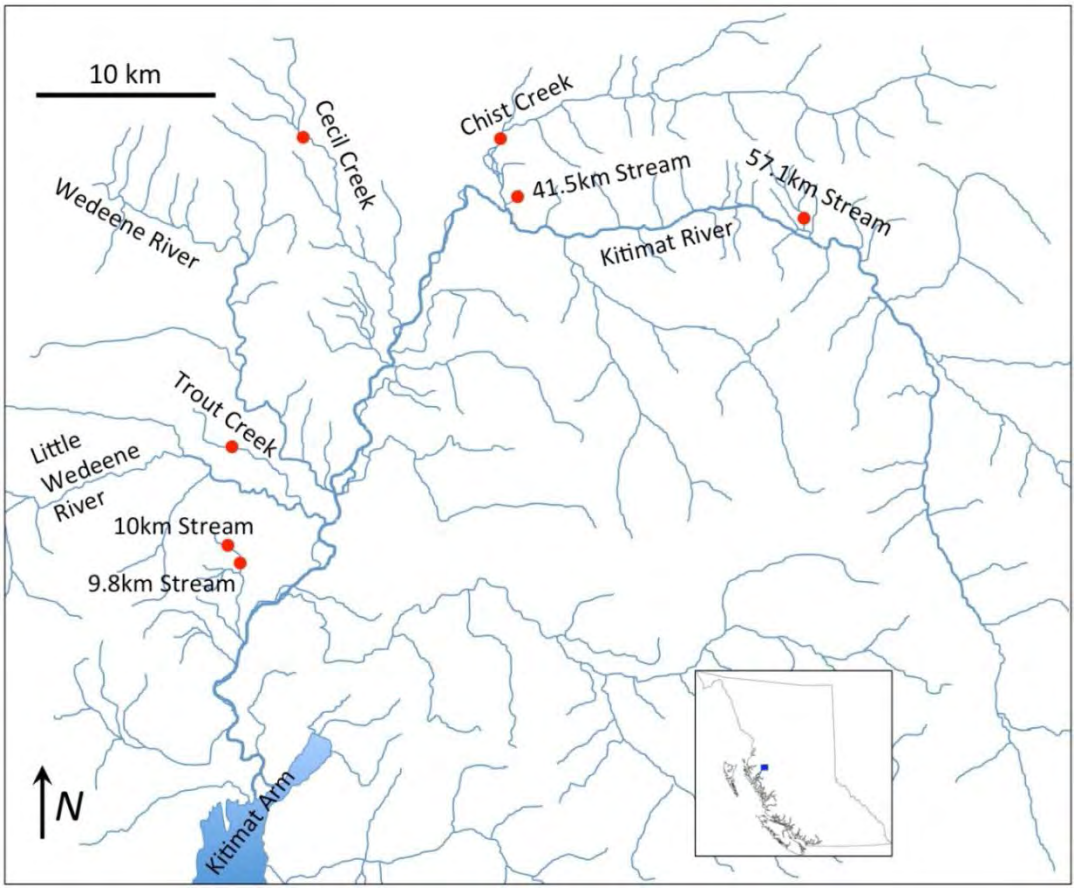

Figure 2-64 – Map of the Kitimat River watershed showing the locations of rivers where water samples were collected and fish were captured. Approximate locations where fish were captured are shown with red circles (●); electrofishing was conducted from approximately -250 to -150 m below the RoW and from approximately +150 to +250 m above the RoW. Inset map shows the region of BC where the study was located.

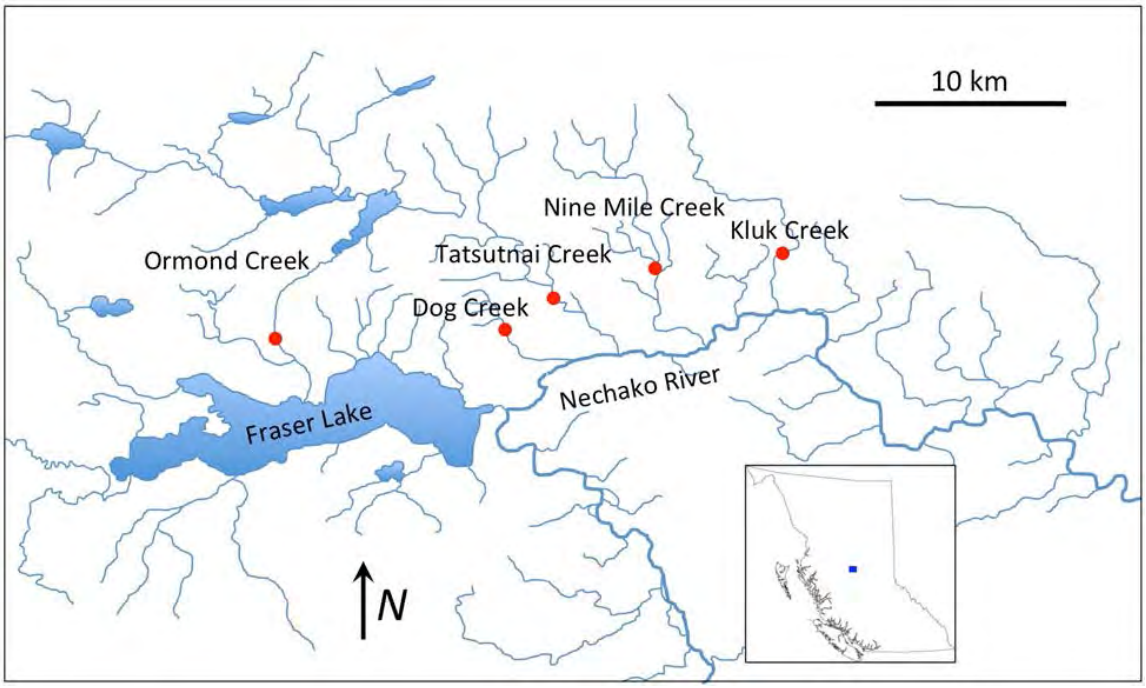

Figure 2-65 – Map of the Nechako River watershed and Fraser Lake showing the locations of rivers where water samples were collected and fish were captured. Approximate locations where fish were captured are shown with red circles (●); electrofishing was conducted from approximately -250 – -150 m below the RoW and from approximately +150 – +250 m above the RoW. Inset map shows the region of BC where the study was located.

An indirect measure of abundance, catch-per-unit effort (CPUE), was calculated for each of the study streams. Distance electrofished was measured using GPS and the total duration of electrofishing was recorded as effort for each study stream. Changes in the catch-per-unit effort were used to infer changes in abundance.

Results

Of the 1783 fish caught in the three years of our study, 1424 belonged to the Family Salmonidae. Within this Family, a single species, *Oncorhynchus mykiss*, dominated the catch with 677 individuals; caught in eight of the 14 watersheds sampled (Table 2-66). The second most frequently caught species also belonged to the Family Salmonidae; *O. clarkii*. The Families Cyprinidae and Cottidae were the next most abundant, with 216 and 124 individuals captured,

respectively. In Ormond Creek *Rhinicthys cataractae* (Family Cyprinidae) and in Chist Creek *Cottus aleuticus* (Family Cottidae) were the only non-salmonids that were almost as abundant as the Family Salmonidae.

Length frequency

Small individuals of a species or small species dominated the assemblage of fish captured for each of the study sites, both above and below the RoW. For many of our study streams, species composition was similar below and above the proposed pipeline crossing locations. For streams inhabited by a single species, abundance and size classes differed between sample locations related to habitat availability and life-history characteristics for each species. Similarly, for some streams with multiple species of fish, abundance and species composition did vary below and above the proposed pipeline crossing location.

Three species of fish were caught in two small tributaries to the Kitimat River, 9.8 km Stream and 10 km Stream, *O. clarkii*, *Salvelinus malma* and *Lampetra richardsoni* were caught in both streams, although the number of lamprey caught was low. Catch composition in both streams were similar above and below the RoW in June (Figure 2-66 A and B), but sampling in August captured very small *O. clarkii* at locations below the RoW in both streams (Table 2-66; Figure 2-66C and D).

Table 2-66 – List of species found in each of the study streams, with number caught (n), length (cm), and condition factor (100 × g × cm^{-3}). Study streams were located in the Kitimat River and Nechako River watersheds. Fish were caught by electrofishing or minnow trapping above (+), below (-), or at the crossing site (=) of the proposed Pacific Trails Pipeline. See Figures 1 and 2 for stream location. Data is shown as means ± SD with range (minimum - maximum value).

Stream		Species	n	Length (cm)	Condition Factor
9.8 km Stream (9.8 – Kitimat River watershed)					
4-Jun-2014	+	*Oncorhynchus clarkii*	13	8.3 ± 2.6 (5.7–14.1)	1.00 ± 0.10 (0.84–1.15)
	+	*Salvelinus malma*	3	6.7 ± 1.3 (5.2–7.7)	1.10 ± 0.07 (1.06–1.18)
	+	*Lampetra richardsoni*	3	14.2 ± 0.9 (13.2–14.8)	0.17 ± 0.05 (0.12–0.22)
4-Jun-2014	-	*Oncorhynchus clarkii*	18	8.1 ± 2.4 (5.4–13.4)	1.18 ± 0.14 (0.93–1.52)
	-	*Salvelinus malma*	9	7.3 ± 0.8 (6.3–8.6)	1.08 ± 0.09 (0.95–1.19)
		Lampetra richardsoni	2	12.8 ± 2.6 (10.9–14.6)	0.21 ± 0.02 (0.20–0.22)
7-Aug-2014	+	*Oncorhynchus clarkii*	13	8.8 ± 1.9 (6.8–13.0)	0.98 ± 0.08 (0.88–1.14)
	+	*Salvelinus malma*	3	8.0 ± 1.6 (7.1–9.8)	0.96 ± 0.03 (0.92–0.99)
7-Aug-2014	-	*Oncorhynchus clarkii*	24	6.4 ± 2.7 (3.8–14.1)	0.93 ± 0.05 (0.89–1.09)
		Salvelinus malma	5	8.1 ± 0.5 (7.5–8.8)	0.97 ± 0.08 (0.88–1.05)

Stream		Species	n	Length (cm)	Condition Factor
5-Aug-2015	+	*Oncorhynchus clarkii*	8	10.0 ± 3.2 (7.4–16.4)	1.00 ± 0.10 (0.93–1.19)
5-Aug-2015	-	*Oncorhynchus clarkii*	8	8.6 ± 0.7 (7.6–9.8)	0.98 ± 0.08 (0.91–1.14)
10 km Stream (10 – Kitimat River watershed)					
11-Jun-2014	+	*Oncorhynchus clarkii*	5	7.9 ± 0.6 (7.1–8.6)	1.10 ± 0.07 (1.05–1.21)
	+	*Salvelinus malma*	3	7.1 ± 2.1 (5.4–9.4)	0.99 ± 0.04 (0.95–1.03)
11-Jun-2014	-	*Oncorhynchus clarkii*	14	8.9 ± 3.1 (5.8–18.4)	1.01 ± 0.11 (0.82–1.19)
	-	*Salvelinus malma*	11	7.1 ± 0.9 (6.2–9.1)	1.02 ± 0.09 (0.84–1.17)
	-	*Lampetra richardsoni*	1	13.8	0.17
7-Aug-2014	+	*Oncorhynchus clarkii*	6	10.9 ± 3.3 (8.5–17.2)	1.03 ± 0.10 (0.94–1.24)
	+	*Salvelinus malma*	1	8.3	1.16
7-Aug-2014	-	*Oncorhynchus clarkii*	12	7.4 ± 2.7 (2.5–11.1)	0.99 ± 0.07 (0.85–1.09)
	-	*Salvelinus malma*	6	7.5 ± 0.4 (7.1–8.1)	0.98 ± 0.07 (0.84–1.03)
Trout Creek (TR – Kitimat River watershed)					
4-Oct-2013	-	*Oncorhynchus clarkii*	11	10.9 ± 3.7 (5.8–18.4)	1.02 ± 0.06 (0.94–1.13)
11-Jun-2014	+	*Oncorhynchus clarkii*	26	9.7 ± 3.3 (5.9–18.4)	0.98 ± 0.12 (0.80–1.28)
11-Jun-2014	-	*Oncorhynchus clarkii*	28	10.2 ± 3.5 (5.2–18.5)	1.00 ± 0.10 (0.86–1.32)
6-Aug-2014	+	*Oncorhynchus clarkii*	32	10.4 ± 3.3 (6.8–19.2)	0.98 ± 0.06 (0.85–1.11)
6-Aug-2014	-	*Oncorhynchus clarkii*	33	9.7 ± 2.5 (6.4–18.3)	0.96 ± 0.06 (0.83–1.08)
5-Aug-2015	+	*Oncorhynchus clarkii*	8	9.9 ± 1.2 (7.9–11.6)	1.07 ± 0.09 (0.97–1.25)
Trout Creek (TR – Kitimat River watershed)					
5-Aug-2015	-	*Oncorhynchus clarkii*	8	9.8 ± 0.7 (8.9–10.9)	1.04 ± 0.07 (0.92–1.15)
Cecil Creek (CE – Kitimat River watershed)					
13-Jun-2014	+	*Oncorhynchus clarkii*	6	8.9 ± 4.1 (5.8–16.5)	1.09 ± 0.17 (0.84–1.28)
	+	*Oncorhynchus kisutch*	12	4.9 ± 1.5 (3.8–7.8)	0.96 ± 0.07 (0.91–1.14)
	+	*Salvelinus malma*	24	7.8 ± 2.7 (6.0–17.7)	1.04 ± 0.09 (0.86–1.20)
13-Jun-2014	-	*Oncorhynchus clarkii*	4	7.3 ± 3.1 (5.5–12.0)	0.86 ± 0.10 (0.78–1.00)
	-	*Oncorhynchus kisutch*	92	4.1 ± 0.9 (3.3–7.6)	0.93 ± 0.07 (0.76–1.19)
	-	*Salvelinus malma*	14	6.6 ± 1.1 (4.9–9.6)	0.94 ± 0.12 (0.72–1.13)
24-Jul-2014	+	*Oncorhynchus clarkii*	9	7.6 ± 0.7 (6.7–9.1)	1.01 ± 0.07 (0.88–1.11)
	+	*Oncorhynchus kisutch*	8	5.6 ± 1.2 (4.2–7.3)	1.05 ± 0.05 (0.99–1.13)
	+	*Salvelinus malma*	15	7.6 ± 1.3 (6.4–11.5)	0.98 ± 0.11 (0.65–1.07)
24-Jul-2014	-	*Oncorhynchus clarkii*	3	7.9 ± 1.0 (7.2–9.1)	0.98 ± 0.07 (0.91–1.05)
	-	*Oncorhynchus kisutch*	21	5.6 ± 1.7 (4.1–9.7)	1.07 ± 0.07 (0.93–1.15)
	-	*Salvelinus malma*	6	7.1 ± 0.3 (6.8–7.5)	0.98 ± 0.05 (0.93–1.07)
7-Aug-2015	+	*Oncorhynchus clarkii*	11	9.4 ± 1.6 (7.0–11.6)	1.06 ± 0.12 (0.93–1.31)
7-Aug-2015	-	*Oncorhynchus clarkii*	5	8.2 ± 1.1 (6.9–9.3)	1.00 ± 0.05 (0.93–1.05)
Chist Creek (CH – Kitimat River watershed)					
6-Jun-2014	+	*Oncorhynchus mykiss*	10	9.0 ± 2.8 (6.1–14.3)	1.10 ± 0.09 (1.00–1.29)
	+	*Oncorhynchus kisutch*	6	6.9 ± 1.6 (4.2–8.8)	1.04 ± 0.10 (0.88–1.13)
	+	*Salvelinus malma*	3	13.0 ± 0.9 (11.9–13.7)	0.85 ± 0.06 (0.79–0.88)
	+	*Cottus aleuticus*	11	8.4 ± 1.7 (6.1–11.0)	1.15 ± 0.11 (0.97–1.30)
6-Jun-2014	-	*Oncorhynchus mykiss*	15	7.4 ± 0.9 (4.6–8.7)	0.93 ± 0.12 (0.73–1.20)
	-	*Oncorhynchus kisutch*	3	5.0 ± 1.3 (4.0–6.4)	0.86 ± 0.15 (0.77–1.03)
	-	*Salvelinus malma*	2	11.9 ± 2.8 (9.9–13.9)	0.85 ± 0.01 (0.85–0.86)
	-	*Cottus aleuticus*	26	7.9 ± 1.1 (6.1–9.7)	1.13 ± 0.13 (0.95–1.42)
23-Jul-2014	+	*Oncorhynchus mykiss*	14	8.1 ± 0.8 (7.0–9.4)	0.94 ± 0.09 (0.78–1.07)
	+	*Oncorhynchus kisutch*	1	5.9	1.05
	+	*Oncorhynchus tshawytscha*	2	5.8 ± 0.4 (5.5–6.0)	1.01 ± 0.03 (0.99–1.03)
	+	*Salvelinus malma*	3	13.6 ± 0.3 (13.3–13.9)	0.82 ± 0.07 (0.74–0.86)
	+	*Cottus aleuticus*	17	7.8 ± 1.2 (5.8–9.6)	0.99 ± 0.11 (0.83–1.18)

Stream		Species	n	Length (cm)	Condition Factor
23-Jul-2014	-	*Oncorhynchus mykiss*	5	8.9 ± 1.5 (7.2–11.0)	1.04 ± 0.16 (0.92–1.31)
	-	*Oncorhynchus kisutch*	3	4.5 ± 0.6 (4.0–5.1)	1.10 ± 0.05 (1.05–1.15)
	-	*Oncorhynchus tshawytscha*	3	5.6 ± 0.7 (4.8–6.1)	1.08 ± 0.09 (0.98–1.16)
	-	*Salvelinus malma*	4	11.0 ± 3.8 (7.1–14.6)	0.93 ± 0.06 (0.84–0.99)
	-	*Cottus aleuticus*	26	7.4 ± 1.0 (5.7–9.3)	1.03 ± 0.12 (0.84–1.27)
Chist Creek (CH – Kitimat River watershed)					
6-Aug-2015	+	*Oncorhynchus mykiss*	9	10.4 ± 2.7 (7.6–15.1)	1.07 ± 0.16 (0.91–1.36)
	+	*Cottus aleuticus*	8	9.0 ± 1.0 (7.3–10.1)	0.99 ± 0.10 (0.87–1.16)
6-Aug-2015	-	*Oncorhynchus mykiss*	7	9.2 ± 1.4 (7.9–11.8)	1.01 ± 0.03 (0.97–1.05)
	-	*Salvelinus malma*	1	9.4	1.05
	-	*Cottus aleuticus*	8	8.6 ± 0.7 (7.1–9.5)	1.01 ± 0.05 (0.91–1.08)
41.5 km Stream (41 – Kitimat River watershed)					
6-Jun-2014	+	*Oncorhynchus clarkii*	2	15.5 ± 7.8 (9.9–21.0)	0.93 ± 0.1 (0.86–1.00)
	+	*Oncorhynchus kisutch*	1	9.2	1.00
	+	*Salvelinus malma*	1	11.6	0.97
6-Jun-2014	-	*Oncorhynchus clarkii*	2	12.8 ± 3.7 (10.1–15.4)	1.04 ± 0.02 (1.02–1.06)
	-	*Oncorhynchus kisutch*	36	6.5 ± 1.6 (3.2–9.4)	1.01 ± 0.11 (0.82–1.26)
	-	*Cottus aleuticus*	2	7.3 ± 0.5 (6.9–7.6)	1.12 ± 0.01 (1.12–1.13)
57.1 km Stream (57 – Kitimat River watershed)					
10-Jun-2014	+	*Oncorhynchus clarkii*	18	9.0 ± 3.2 (4.9–14.7)	0.98 ± 0.08 (0.89–1.21)
10-Jun-2014	-	*Oncorhynchus clarkii*	3	7.8 ± 2.8 (6.1–11.0)	0.91 ± 0.09 (0.84–1.01)
	-	*Oncorhynchus mykiss*	1	7.0	0.99
	-	*Oncorhynchus kisutch*	1	6.1	1.01
	-	*Salvelinus malma*	12	9.5 ± 4.9 (6.2–19.0)	0.95 ± 0.06 (0.86–1.04)
	-	*Cottus aleuticus*	1	10.5	1.07
9-Aug-2014	+	*Oncorhynchus clarkii*	36	10.0 ± 3.0 (3.2–17.6)	0.98 ± 0.05 (0.90–1.08)
9-Aug-2014	-	*Oncorhynchus clarkii*	4	8.3 ± 0.9 (7.4–9.3)	0.94 ± 0.04 (0.89–0.99)
	-	*Oncorhynchus kisutch*	15	7.3 ± 1.0 (6.4–9.2)	1.04 ± 0.09 (0.79–1.18)
	-	*Salvelinus malma*	29	9.0 ± 3.6 (5.0–17.2)	0.96 ± 0.08 (0.81–1.18)
	-	*Cottus asper*	2	11.6 ± 1.6 (10.5–12.7)	1.21 ± 0.00 (1.21–1.21)
6-Aug-2015	+	*Oncorhynchus clarkii*	8	9.9 ± 2.6 (6.8–14.4)	0.97 ± 0.03 (0.92–1.03)
6-Aug-2015	-	*Oncorhynchus clarkii*	8	9.4 ± 2.7 (7.4–15.7)	1.04 ± 0.08 (0.96–1.14)
Tchesinkut Creek (TC – Nechako River watershed)					
26-Aug-2014	+	*Oncorhynchus mykiss*	8	8.3 ± 2.9 (4.7–11.6)	1.03 ± 0.10 (0.92–1.20)
	+	*Oncorhynchus tshawytscha*	1	7.6	1.18
	+	*Prosopium williamsoni*	1	8.3	0.89
	+	*Rhinichthys cataractae*	3	7.1 ± 1.3 (5.9–8.4)	1.10 ± 0.02 (1.08–1.12)
	+	*Rhinichthys falcatus*	1	7.6	1.05
	+	*Richardsonius balteatus*	31	6.9 ± 1.2 (4.9–8.6)	1.28 ± 0.19 (0.84–1.75)
	+	*Ptycheilus oregonensis*	1	9.5	1.08
	+	*Mylocheilus caurinus*	2	9.3 ± 0.3 (9.1–9.5)	1.11 ± 0.01 (1.11–1.11)
	+	*Catostomus spp.*	18	9.9 ± 0.9 (8.2–11.4)	1.11 ± 0.08 (0.94–1.26)
	+	*Lota lota*	4	18.2 ± 1.9 (16.8–21.0)	0.55 ± 0.08 (0.44–0.61)
26-Aug-2014	-	*Oncorhynchus mykiss*	5	7.5 ± 3.0 (5.1–12.4)	1.12 ± 0.14 (0.99–1.36)
	-	*Rhinichthys cataractae*	5	8.9 ± 1.9 (6.1–11.1)	1.13 ± 0.13 (0.93–1.26)
	-	*Richardsonius balteatus*	6	7.0 ± 1.3 (5.0–8.5)	1.29 ± 0.05 (1.21–1.37)
	-	*Mylocheilus caurinus*	15	8.4 ± 2.9 (5.8–16.6)	1.12 ± 0.11 (0.95–1.34)
	-	*Catostomus spp.*	19	10.3 ± 1.2 (8.9–13.8)	1.11 ± 0.10 (0.96–1.32)
	-	*Lota lota*	2	19.1 ± 0.0 (19.1–19.1)	0.58 ± 0.02 (0.56–0.59)
Ormond Creek (OR – Nechako River watershed)					
20-Sep-2013	-	*Oncorhynchus mykiss*	23	7.4 ± 3.2 (4.7–15.6)	0.98 ± 0.06 (0.87–1.10)
	-	*Richardsonius balteatus*	1	7.9	1.12

Stream		Species	n	Length (cm)	Condition Factor
	-	*Lota lota*	2	10.2 ± 0.3 (10.0–10.4)	0.62 ± 0.01 (0.61–0.62)
	-	*Cottus asper*	21	5.7 ± 2.2 (3.8–12.7)	1.12 ± 0.14 (0.77–1.41)
23-Jun-2014	+	*Rhinichthys cataractae*	12	6.9 ± 1.5 (4.6–9.3)	1.03 ± 0.11 (0.92–1.28)
23-Jun-2014	-	*Oncorhynchus mykiss*	2	9.2 ± 0.0 (9.2–9.2)	1.16 ± 0.06 (1.12–1.21)
		Rhinichthys cataractae	3	6.1 ± 0.6 (5.4–6.5)	1.02 ± 0.17 (0.83–1.14)
8-Sep-2014	+	*Oncorhynchus mykiss*	109	5.8 ± 2.0 (3.9–17.4)	na
	+	*Rhinichthys cataractae*	18	5.2 ± 2.2 (3.6–9.9)	na
8-Sep-2014	-	*Oncorhynchus mykiss*	31	5.8 ± 1.7 (4.3–10.9)	na
		Rhinichthys cataractae	13	3.7 ± 0.3 (3.3–4.3)	na
Ormond Creek (OR – Nechako River watershed)					
20-Sep-2013	-	*Oncorhynchus mykiss*	23	7.4 ± 3.2 (4.7–15.6)	0.98 ± 0.06 (0.87–1.10)
	-	*Richardsonius balteatus*	1	7.9	1.12
	-	*Lota lota*	2	10.2 ± 0.3 (10.0–10.4)	0.62 ± 0.01 (0.61–0.62)
	-	*Cottus asper*	21	5.7 ± 2.2 (3.8–12.7)	1.12 ± 0.14 (0.77–1.41)
23-Jun-2014	+	*Rhinichthys cataractae*	12	6.9 ± 1.5 (4.6–9.3)	1.03 ± 0.11 (0.92–1.28)
23-Jun-2014	-	*Oncorhynchus mykiss*	2	9.2 ± 0.0 (9.2–9.2)	1.16 ± 0.06 (1.12–1.21)
		Rhinichthys cataractae	3	6.1 ± 0.6 (5.4–6.5)	1.02 ± 0.17 (0.83–1.14)
8-Sep-2014	+	*Oncorhynchus mykiss*	109	5.8 ± 2.0 (3.9–17.4)	na
	+	*Rhinichthys cataractae*	18	5.2 ± 2.2 (3.6–9.9)	na
8-Sep-2014	-	*Oncorhynchus mykiss*	31	5.8 ± 1.7 (4.3–10.9)	na
		Rhinichthys cataractae	13	3.7 ± 0.3 (3.3–4.3)	na
Dog Creek (DG – Nechako River watershed)					
27-Sep-2013	=	*Oncorhynchus mykiss*	6	7.2 ± 2.3 (4.6–10.6)	1.05 ± 0.12 (0.90–1.23)
10-Jul-2014	+	*Oncorhynchus mykiss*	13	9.1 ± 2.0 (6.4–12.1)	1.07 ± 0.05 (0.99–1.14)
10-Jul-2014	-	*Oncorhynchus mykiss*	12	8.1 ± 2.4 (6.1–12.4)	0.95 ± 0.12 (0.76–1.20)
20-Aug-2014	+	*Oncorhynchus mykiss*	19	9.5 ± 2.2 (6.6–14.0)	1.03 ± 0.05 (0.90–1.12)
20-Aug-2014	-	*Oncorhynchus mykiss*	12	8.1 ± 1.2 (6.2–10.3)	1.09 ± 0.10 (0.90–1.23)
Tatsutnai Creek (TA – Nechako River watershed)					
20-Sep-2013	=	*Oncorhynchus mykiss*	21	6.2 ± 2.4 (4.1–12.4)	0.98 ± 0.07 (0.87–1.17)
27-Jun-2014	+	*Oncorhynchus mykiss*	10	8.3 ± 2.7 (5.7–14.3)	1.07 ± 0.08 (0.97–1.22)
27-Jun-2014	-	*Oncorhynchus mykiss*	16	8.1 ± 1.4 (6.4–10.6)	1.12 ± 0.07 (0.97–1.24)
28-Aug-2014	+	*Oncorhynchus mykiss*	35	6.8 ± 2.3 (4.6–11.3)	1.00 ± 0.07 (0.92–1.14)
28-Aug-2014	-	*Oncorhynchus mykiss*	40	6.2 ± 1.7 (4.2–11.8)	1.01 ± 0.08 (0.88–1.19)
Nine Mile Creek (9-m – Nechako River watershed)					
27-Sep-2013	+	*Oncorhynchus mykiss*	35	7.4 ± 2.3 (5.0–12.5)	0.99 ± 0.06 (0.84–1.11)
27-Sep-2013	=	*Oncorhynchus mykiss*	28	6.6 ± 2.1 (4.6–12.7)	0.98 ± 0.09 (0.75–1.13)
27-Sep-2013	-	*Oncorhynchus mykiss*	27	6.5 ± 1.9 (4.4–12.3)	0.97 ± 0.10 (0.77–1.17)
24-Jun-2014	+	*Oncorhynchus mykiss*	22	7.6 ± 0.9 (6.0–9.6)	1.05 ± 0.10 (0.74–1.16)
24-Jun-2014	-	*Oncorhynchus mykiss*	19	8.3 ± 2.1 (6.1–13.4)	1.04 ± 0.06 (0.94–1.14)
4-Sep-2014	+	*Oncorhynchus mykiss*	25	7.4 ± 2.4 (4.0–13.2)	1.00 ± 0.08 (0.84–1.16)
4-Sep-2014	-	*Oncorhynchus mykiss*	13	6.8 ± 1.9 (4.7–11.5)	1.05 ± 0.10 (0.96–1.32)
Kluk Creek (KL – Nechako River watershed)					
4-Jul-2014	+	*Oncorhynchus mykiss*	21	8.4 ± 3.0 (2.5–14.6)	1.10 ± 0.09 (0.96–1.30)
4-Jul-2014	-	*Oncorhynchus mykiss*	22	8.2 ± 1.7 (6.5–12.3)	1.11 ± 0.12 (0.93–1.42)
19-Aug-2014	+	*Oncorhynchus mykiss*	13	7.6 ± 2.5 (5.6–12.4)	1.07 ± 0.04 (0.98–1.14)
19-Aug-2014	-	*Oncorhynchus mykiss*	29	8.5 ± 1.6 (6.6–12.3)	1.05 ± 0.08 (0.95–1.26)
Salmon River (SA – Nechako River watershed)					
15-Jul-2014	+	*Rhinichthys cataractae*	7	4.2 ± 0.9 (3.4–5.6)	1.04 ± 0.07 (0.95–1.17)
	+	*Rhinichthys falcatus*	2	5.0 ± 1.1 (4.2–5.8)	1.13 ± 0.07 (1.08–1.17)
	+	*Richardsonius balteatus*	1	8.9	1.15

Stream		Species	n	Length (cm)	Condition Factor
15-Jul-2014	-	*Rhinichthys cataractae*	12	4.9 ± 1.0 (3.4–6.6)	0.96 ± 0.11 (0.70–1.09)
	-	*Rhinichthys falcatus*	3	4.5 ± 0.4 (4.2–4.9)	1.04 ± 0.04 (1.01–1.08)
	-	*Ptycheilus oregonensis*	1	4.7	0.96
	-	*Lota lota*	3	12.5 ± 0.8 (11.6–13.2)	0.60 ± 0.08 (0.53–0.68)
3-Sep-2014	+	*Rhinichthys cataractae*	7	7.3 ± 2.1 (6.2–12.1)	1.25 ± 0.12 (1.02–1.38)
	+	*Rhinichthys falcatus*	3	5.9 ± 1.4 (4.5–7.2)	1.12 ± 0.07 (1.07–1.20)
	+	*Ptycheilus oregonensis*	5	5.7 ± 1.1 (4.4–6.7)	0.97 ± 0.13 (0.84–1.18)
3-Sep-2014	-	*Rhinichthys cataractae*	6	7.4 ± 3.0 (3.9–12.1)	1.01 ± 0.07 (0.92–1.07)
	-	*Rhinichthys falcatus*	1	4.3	1.01
	-	*Richardsonius balteatus*	6	5.4 ± 1.3 (4.0–7.3)	1.13 ± 0.26 (0.80–1.48)
	-	*Ptycheilus oregonensis*	11	4.6 ± 0.6 (3.8–5.6)	1.08 ± 0.14 (0.92–1.35)
	-	*Catostomus spp.*	3	6.0 ± 1.7 (4.3–7.7)	1.01 ± 0.04 (0.97–1.05)
	-	*Lota lota*	2	12.8 ± 0.4 (12.5–13.0)	0.61 ± 0.06 (0.57–0.66)
	-	*Cottus cognatus*	2	6.2 ± 0.6 (5.7–6.6)	0.97 ± 0.15 (0.86–1.08)

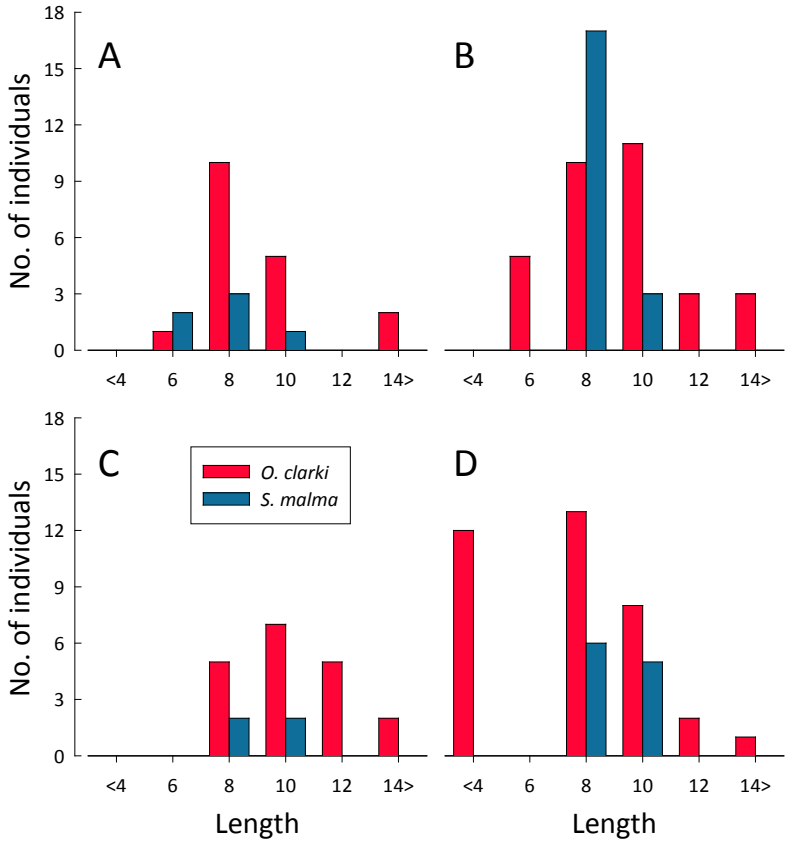

Figure 2-66 – Length frequencies for fish captured in 9.8 km and 10 km Streams combined. Fish were caught above (A) and below (B) the RoW on 4-Jun-2014 and above (C) and below (D) the RoW on 7-Aug-2014. The two most common species collected in these two streams were plotted, however, *Lampetra richardsoni* were also found in both streams. Length was measured in cm.

Trout Creek is larger than 9.8 km and 10 km Streams, although only a single species of fish was found. The analyses of the length-frequency distribution of *O. clarkii* collected on June 11 and August 6, 2014 show that multiple age classes reside in the stream (Figure 2-67). Multiple age classes found during the early summer and early fall suggest that Trout Creek is an important stream for the various life stages of this fish species.

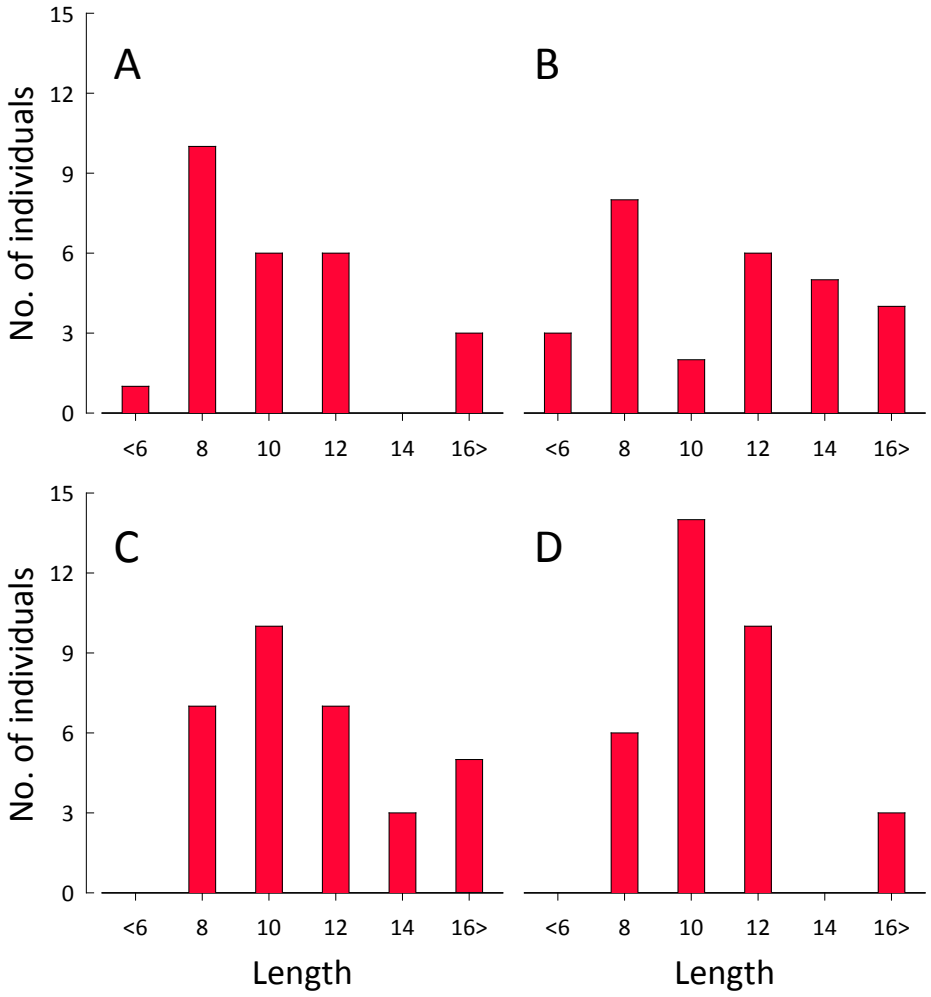

Figure 2-67 – Length frequencies for fish captured in Trout Creek. Fish were caught above (A) and below (B) the RoW on 11-Jun-2014 and above (C) and below (D) the RoW on 6-Aug-2014. *Oncorhynchus clarkii* was the only species captured in Trout Creek. Length was measured in cm.

Cecil Creek is similar to Trout Creek in size, but slightly lower gradient and substrate composition tends to be finer. Cecil Creek also has a large spawning run of anadromous *O. kisutch*, so it was not surprising that the most abundant species caught was juvenile *O. clarkii* — particularly the smallest size classes less than 4 cm in June and less than 6 cm in late July (Figure 2-68). The modal distribution in Cecil Creek also suggests that one-year old *O. kisutch* are also found in the Cecil Creek, but they are less abundant. A range of sizes of other Salmonidae were also found in Cecil Creek; *O. clarkii* and *S. malma* were the largest individuals captured.

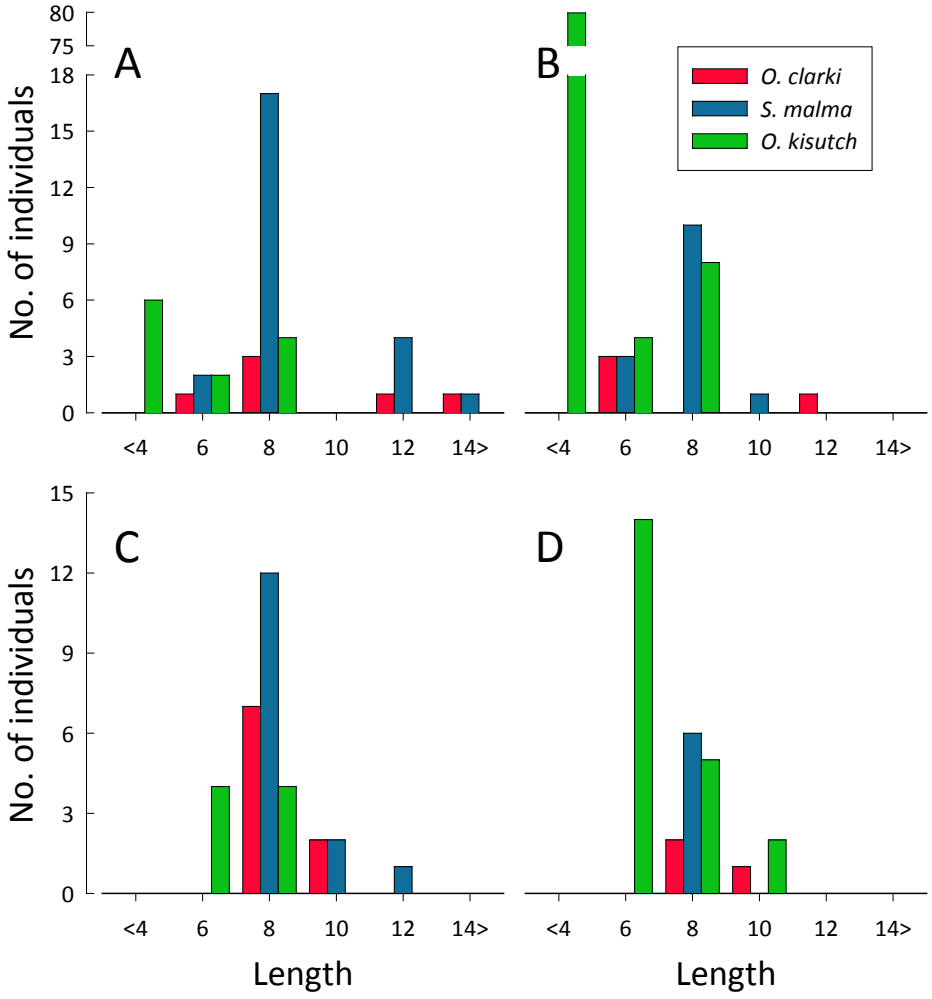

Figure 2-68 – Cecil Length frequencies for fish captured in Cecil Creek. Fish were caught above (A) and below (B) the RoW on 13-Jun-2014 and above (C) and below (D) the RoW on 24-Jul-2014. Only Salmonidae were captured in Cecil Creek. Length was measured in cm.

Chist Creek was the largest stream we sampled in the Kitimat River watershed. We caught a considerable diversity of fish species in Chist Creek (Table 2-66). Due to the size of the stream and also mid-channel depth, we were only able to sample Chist Creek along the margins. The lack of access to the deepest sections of the Creek and fastest areas of flow likely biased our sampling effort. Consequently it is likely that we only sampled the smaller size classes for both *O. mykiss* and *S. malma* — a range of sizes that also included juveniles of anadromous salmon, both *O. kisutch* and *O. tshawytscha* (Figure 2-69). The high catch number of *C. aleuticus* enabled us to calculate the length-frequency distribution for this species of fish (Figure 2-69). For both sampling dates, more sculpin were caught below the proposed pipeline crossing location than above. Very few small *C. aleuticus* were caught in Chist Creek; based on size, most fish were likely older than two years. For our otolith microchemistry work (Section 2.13), fish used in that study were at least four-years old. Consequently, we did not catch any YOY *C. aleuticus*.

A striking example of differences in species composition along the length of a stream was found for 57.1 km Stream (Figure 2-70). There was a large range in sizes for fish caught in this system, suggesting multiple age classes for the species caught. The species composition differed below and above the proposed pipeline crossing location. Only cutthroat trout were found above the pipeline crossing. The absence of other species may be due to differences in gradient as the stream flows through much steeper terrain above the proposed location for the pipeline. Below the pipeline crossing, the creek has a low gradient and is composed of multiple pools and slow-moving riffles. Above the pipeline crossing, the creek has a high gradient and is fast-flowing with deeper pools than the lower section.

Tchesinkut Creek is the most westerly system we sampled in the Nechako River watershed. It is a shallow gradient system with deep pools that made it unsuitable to sample with the backpack electrofisher. Consequently we used minnow traps to sample for fish in Tchesinkut Creek. We caught 11 different species in Tchesinkut Creek; five species from the Family Cyprinidae. Although, the Family Cyprinidae dominated the Creek, we also caught species from the Families Salmonidae (three species), Catastomidae (*Catostomus spp.*

contained at least two species, *Catostomus catostomus* and *Catostomus columbianus*, although we could not differentiate the smallest individuals in the field), and Gadidae (one species).

Ormond Creek is an intermediate sized stream that flows into Fraser Lake (Figure 2-65). Multiple species of fish were caught in Ormond Creek (Table 2-66). Although considerable diversity of fish was found for Ormond Creek, the catch was dominated by *O. mykiss*. For example, on September 8, 2014, of the 171 fish caught in Ormond Creek, 140 were *O. mykiss*. Length-frequency distribution for the *O. mykiss* caught revealed that multiple age classes were

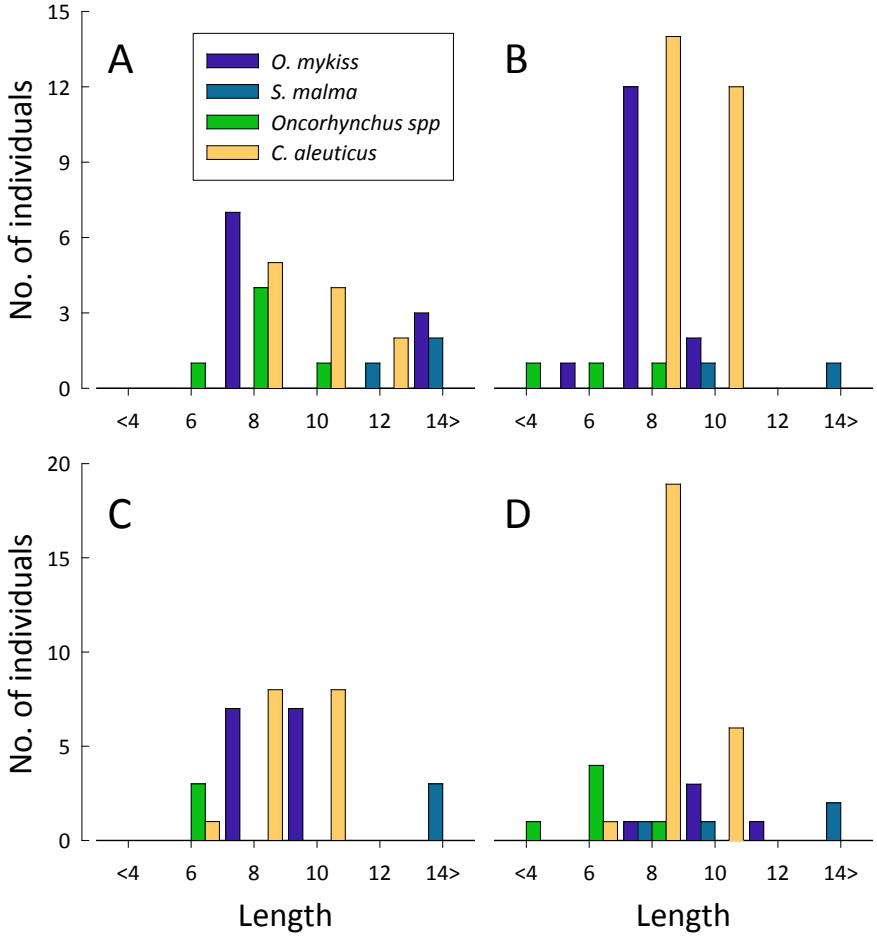

Figure 2-69 – Length frequencies for fish captured in Chist Creek. Fish were caught above (A) and below (B) the RoW on 6-Jun-2014 and above (C) and below (D) the RoW on 23-Jul-2014. Five species were caught in Chist Creek; anadromous *Oncorhynchus kisutch* and *O. tshawytscha* are combined for presentation. Length was measured in cm.

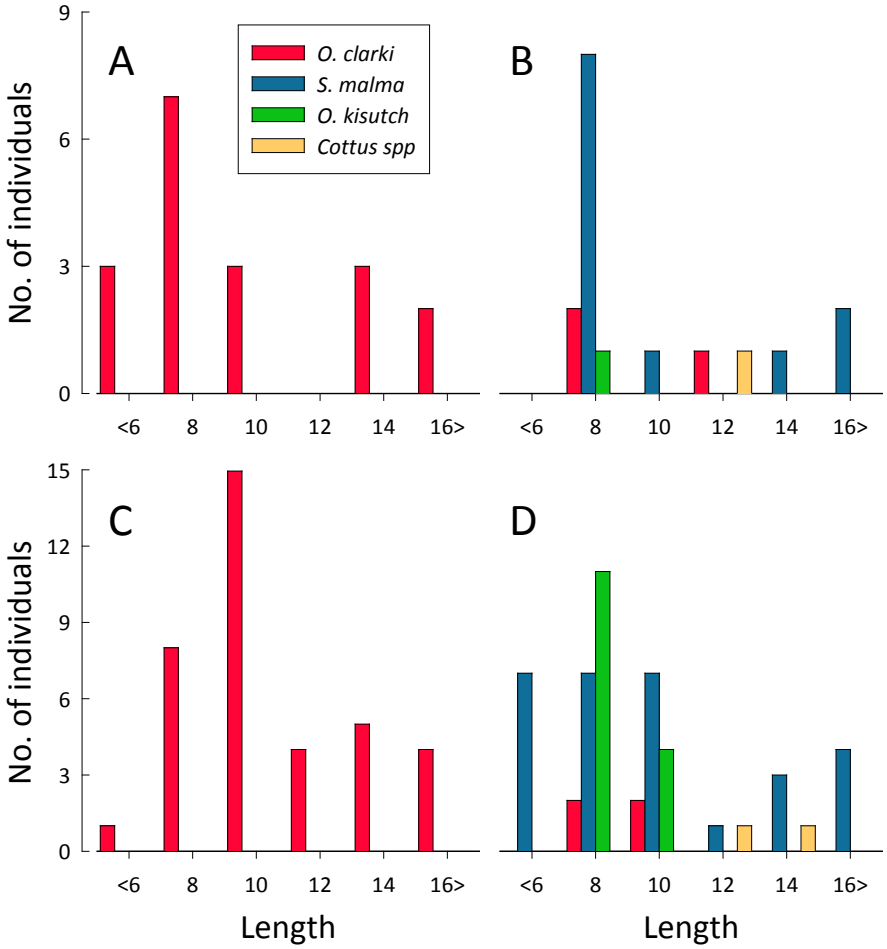

Figure 2-70 – Length frequencies for fish captured in 57.1 km Stream. Fish were caught above (A) and below (B) the RoW on 10-Jun-2014 and above (C) and below (D) the RoW on 9-Aug-2014. Five species were caught in 57.1 km Stream; *Cottus aleuticus* and *C. asper* are combined for presentation. Length was measured in cm.

present in the stream on this date (Figure 2-71). The high catch rate of smaller fish suggests that Ormond Creek is an important stream for YOY *O. mykiss* (length 4–6 cm). Fish in their second year were 9–11 cm in length and one individual was captured from an older age class. The lower catch rate of larger fish in the system indicates that a large proportion of the older fish are likely moving downstream of our study locations and potentially in to a different system — probably to overwinter.

The remaining tributaries to the Nechako River that we sampled were exclusively inhabited by a single species of fish; *O. mykiss*. Dog Creek is a very small system with low gradient, but extensive large woody debris in the channel. Although some small fish were captured (Table 2-66), the majority of the *O. mykiss* were greater than 6 cm in length (Figure 2-72 A and B) — suggesting few YOY fish near the RoW for Dog Creek and potentially spawning areas further away as dispersal tends to increase with age.

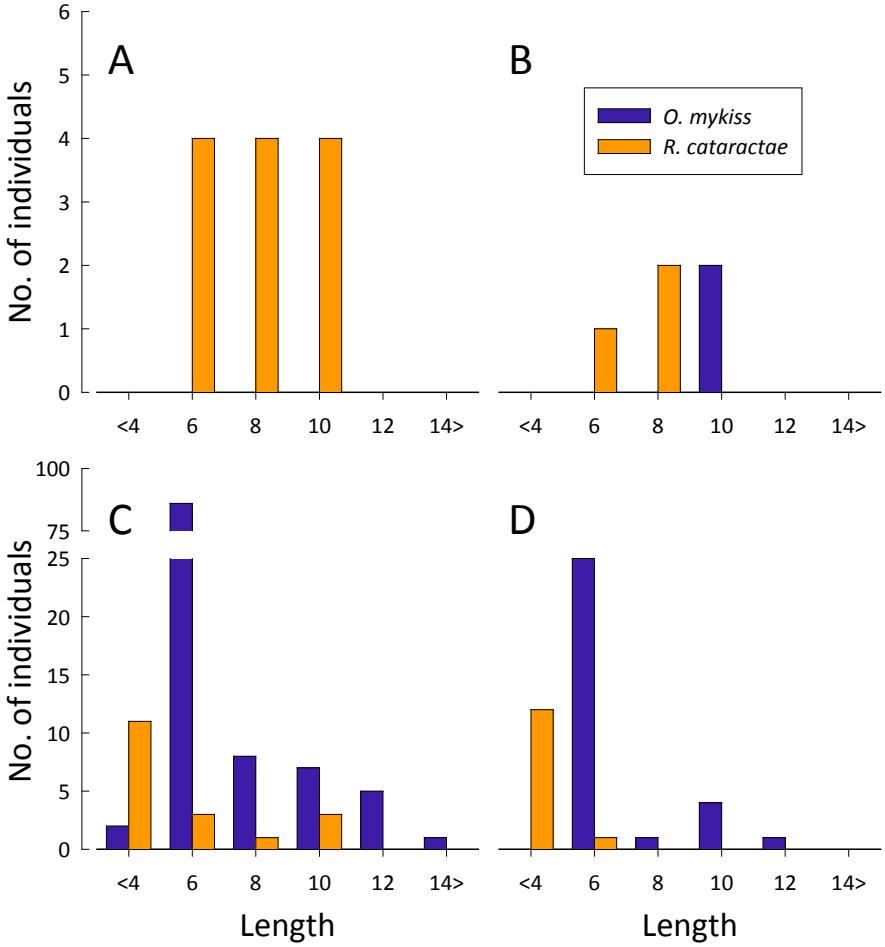

Figure 2-71 – Length frequencies for fish captured in Ormond Creek. Fish were caught above (A) and below (B) the RoW on 23-Jun-2014 and above (C) and below (D) the RoW on 8-Sep-2014. Although only *Oncorhynchus mykiss* and *Rhinicthys cataractae* were caught in 2014, at the site we sampled in 2013 three more species were present; *Richardsonius balteatus*, *Lota lota*, and *Cottus asper*. Length was measured in cm.

Tatsutnai Creek (Figure 2-72 C and D) and Nine Mile Creek (Figure 2-73 A and B) were also sampled twice in 2014 and only a single species of fish was found residing in the both creeks. A total of 101 and 79 *O. mykiss* were collected from Tatsutnai Creek and Nine Mile Creek, respectively, during the summer of 2014 (Table 2-66). The number of rainbow trout caught above and below the proposed pipeline crossing was similar for both months. In June, the length-frequency distribution revealed that a single age class dominated the assemblage of fish in both creeks. This finding also indicates that rainbow trout likely overwinter in Tatsutnai Creek and Nine Mile Creek, as there was a number of fish that based on size were at least one-year old. Larger and older fish caught in the system (length 7–10 cm) were likely two-year-old fish. Interestingly, the majority of fish caught during the second sampling period in September were smaller than the fish caught in June. The dominant size class of fish sampled in late August (Tatsutnai Creek) and early September (Nine Mile Creek) were less than 6 cm and appeared to represent a single age class of YOY. There were also a few larger fish representing cohorts from previous years. This suggests that many of the older fish are moving out of the systems in the summer months and that both creeks are important habitat for spawning and the production and development of young *O. mykiss*. Multiple size classes of *O. mykiss* were also caught in Kluk Creek (Figure 2-73 C and D). More small YOY, however, were caught in June than later in the summer and may indicate that the proposed RoW is closer in proximity to the spawning areas in Kluk Creek.

Salmon River was the largest and furthest east of the systems we sampled in the Fraser River watershed. We caught eight different species in Salmon River; four species from the Family Cyprinidae. Although, Cyprinidae dominated the river, we also caught species of Catastomidae (*Catostomus spp.* contained at least two species, *Catostomus catostomus* and *Catostomus commersoni*, although we could not differentiate the smallest individuals in the field), and Gadidae (1 species), but no Salmonidae.

Age of fish sampled

Figure 2-74 (Kitimat River watershed) and Figure 2-75 (Nechako River watershed) reveal that young Salmonidae represented the majority of fish sampled in each of the creeks. One-year and two-year old fish were the most abundant age-class for fish caught in tributaries of the

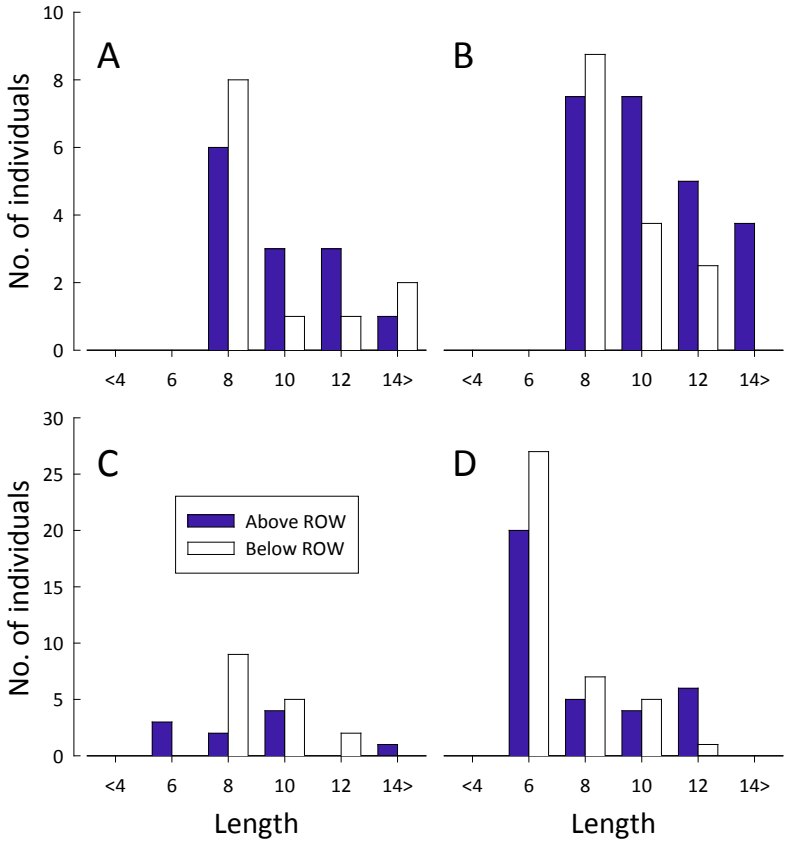

Figure 2-72 – Length frequencies for *Oncorhynchus mykiss* captured in Dog and Tatsutnai Creeks. *O. mykiss* was the only species caught in both creeks. Fish were caught above and below the proposed RoW crossing on (A) 10-Jul-2014 and (B) 20-Aug-2014 in Dog Creek. Fish were also caught above and below the proposed RoW crossing on (C) 27-Jun-2014 and (D) 28-Aug-2014 in Tatsutnai Creek. Length was measured in cm.

Kitimat River and two of the Nechako River tributaries; Dog Creek (data not shown) and Kluk Creek (Figure 2-75D). In contrast, YOY were the most abundant age-class for three of the creeks sampled in the Nechako River watershed; Ormond Creek (Figure 2-75A), Tatsutnai Creek (Figure 2-75B), and Nine Mile Creek (Figure 2-75C). For the sample sites where we captured sufficient numbers of fish to conduct the length frequency analysis, there was little difference in sizes between fish sampled above and below the road within a species. Growth opportunities are likely similar for fish rearing in habitat above and below the proposed RoW for all the study sites sampled. Another indication of growth potential for each of the streams sampled is condition factor, a ratio of weight to length. Values for Salmonidae were consistently close to 1.0 for all streams sampled. Variation in condition factor for many of the other species was due

to differences in shape. *Lampetra* and *Lota* are long and thin with low condition factor. *Cottus* tend to be more robust with age and correspondingly showed higher condition factor for larger fish.

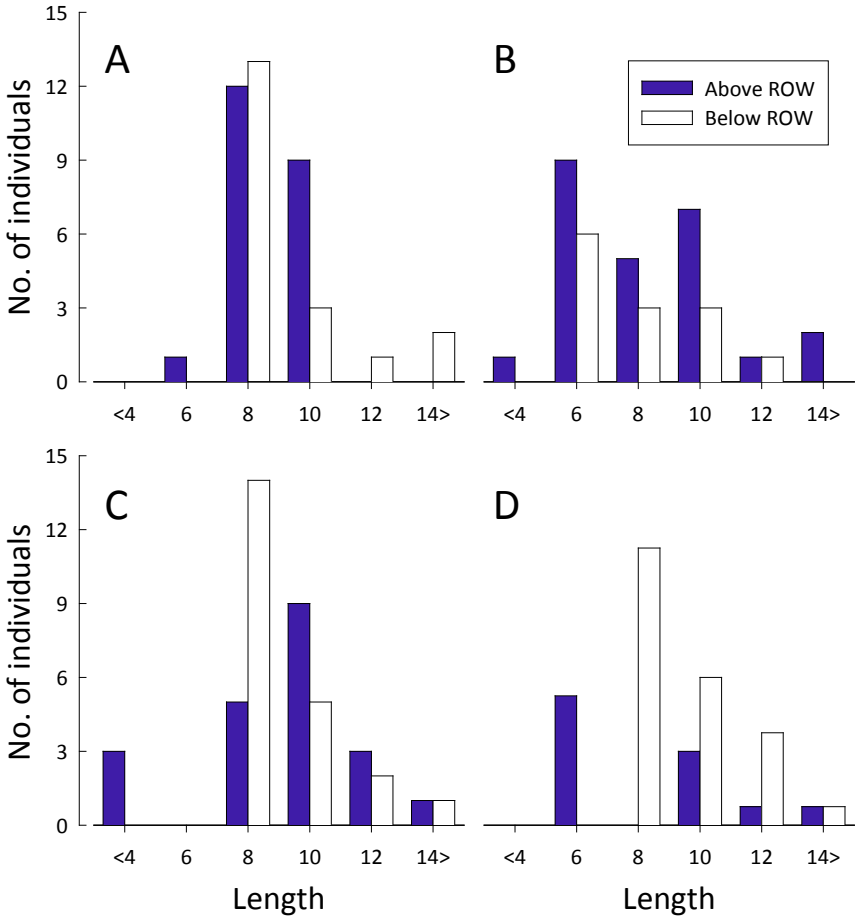

Figure 2-73 – Length frequencies for *Oncorhynchus mykiss* captured in Nine Mile and Kluk Creeks. *O. mykiss* was the only species caught in both creeks. Fish were caught above and below the proposed RoW crossing on (A) 24-Jun-2014 and (B) 4-Sep-2014 in Nine Mile Creek. Fish were also caught above and below the proposed RoW crossing on (C) 4-Jul-2014 and (D) 19-Aug-2014 in Tatsutnai Creek. Length was measured in cm.

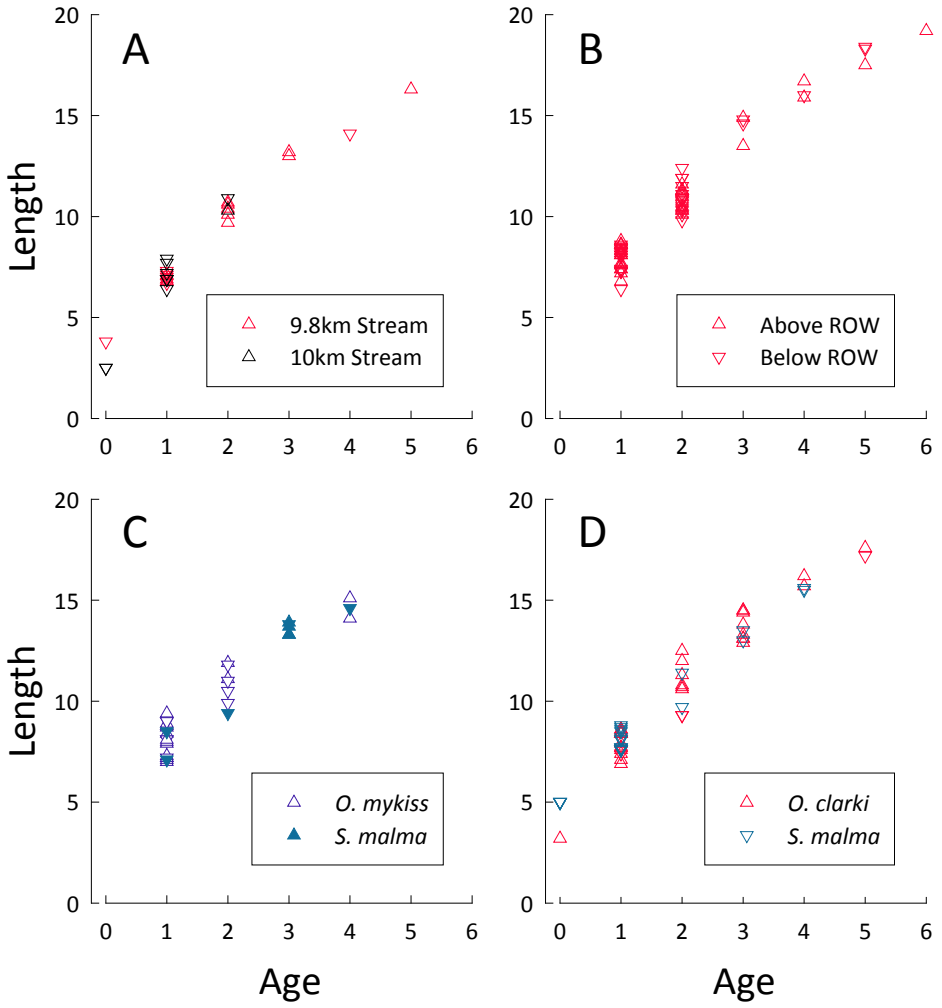

Figure 2-74 – Length as a function of age for fish caught in (A) 9.8 km and 10 km Streams, (B) Trout Creek, (C) Chist Creek, and (D) 57.1 km Stream. Age was determined by oscillations in Zn:Ca ratios from laser ablation inductively coupled plasma mass spectrometry for older fish and from length frequency analysis for younger fish.

Catch-per-unit effort

Catch-per-unit effort as assessed by the number of fish caught per 100 sec of electrofishing was remarkably consistent among the study streams above (Figure 2-72A) and below (Figure 2-72B) the proposed pipeline RoW. There were some patterns; CPUE tended to be lower in the larger streams such as Ormond Creek and Chist Creek and higher in smaller streams such as 9.8 km Stream, Cecil Creek and Tatsutnai Creek. An exception was above the RoW for 10 km Stream — a small creek, but low abundance of fish. Another exception was

above the RoW in Ormond Creek; this value was inflated by a large number of YOY captured on 8-Sep-2014.

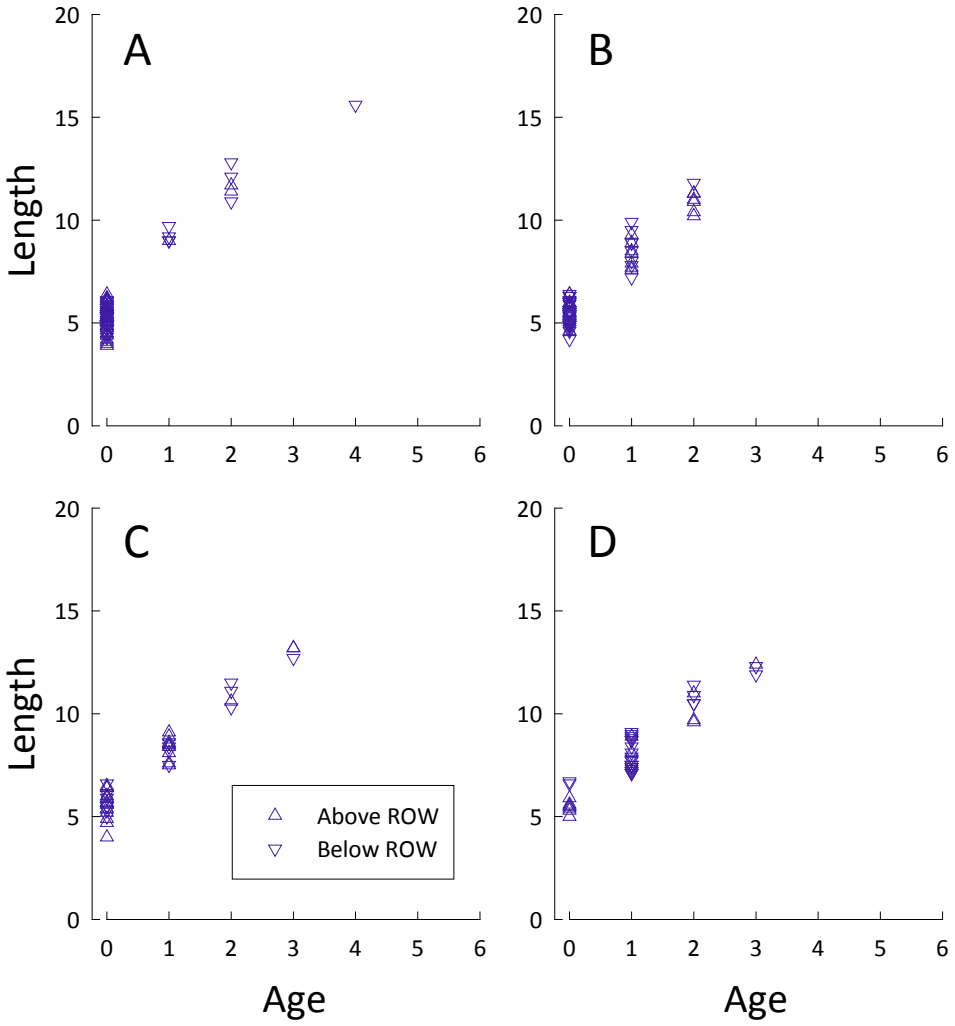

Figure 2-75 – Length as a function of age for fish caught in (A) Ormond Creek, (B) Tatsutnai Creek, (C) Nine Mile Creek, and (D) Kluk Creek. Age was determined by oscillations in Zn:Ca ratios from laser ablation inductively coupled plasma mass spectrometry for older fish and from length frequency analysis for younger fish. For Tatsutnai Creek and Kluk Creek, only length frequency analysis was used to determine age.

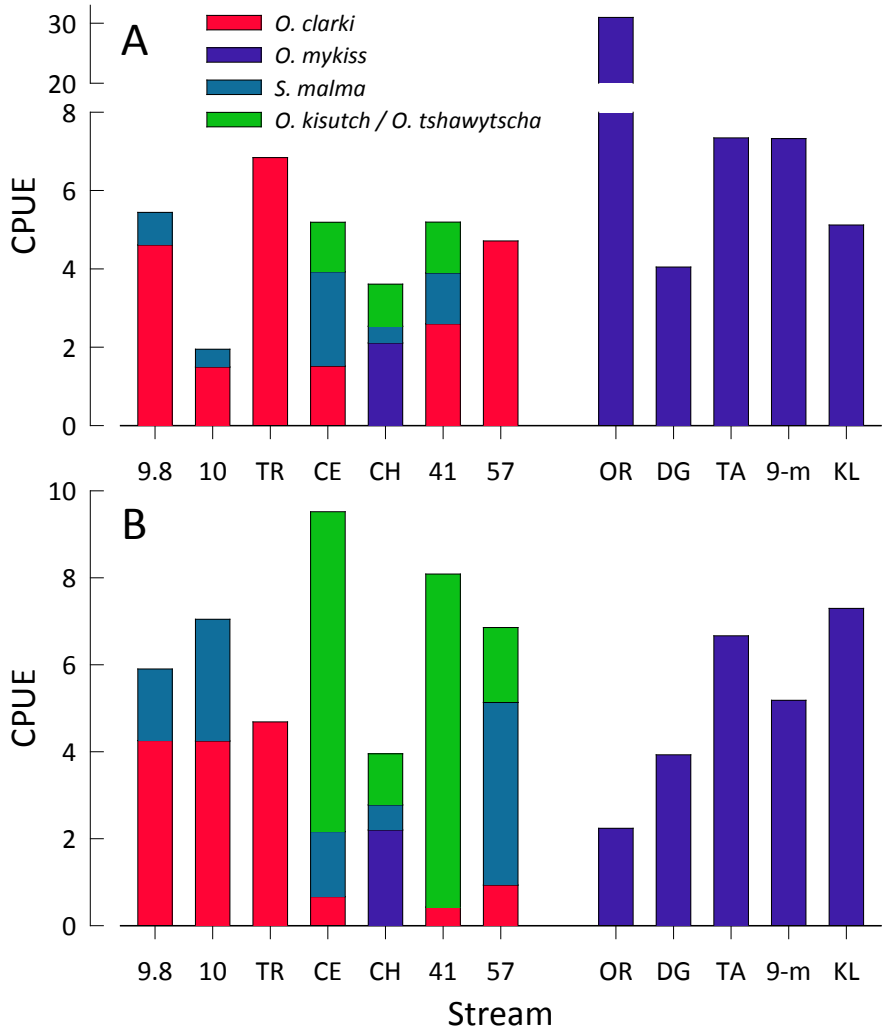

Figure 2-76 – Catch-per-unit effort (CPUE) determined by the number of fish caught per 100 sec of electrofishing. *O. kisutch* and *O. tshawytscha* are grouped for display. Species distribution is shown in Table 2-66. Data are presented as means of sampling effort for between 1 and 4 surveys per stream sampled over approximately a 100 m length of stream (number caught 100 m^{-1}) (A) above and (B) below the proposed Pacific Trails Pipeline crossing. Stream locations are shown in Figure 2-64 and Figure 2-65; 9.8 km Stream (9.8), 10 km Stream (10), Trout Creek (TR), Cecil Creek (CE), Chist Creek (CH), 41.5km Stream (41), 57.1 km Stream (57), Ormond Creek (OR), Dog Creek (DG), Tatsutnai Creek (TA), Nine Mile Creek (9-m), and Kluk Creek (KL).

Discussion

The rivers and streams that we sampled that are intersected by the PTP RoW are clearly important habitats for small fish; both younger individuals within a species and smaller species.

Based on the length-frequency among the most abundant species, YOY and fish less than two years dominated our catch from the Family Salmonidae — particularly in the smaller streams where often only a single species of Salmonidae was found. Within the larger streams and rivers sampled, individuals from the Family Cyprinidae dominated the assemblage — often several species from this Family. The finding is not surprising as juvenile Salmonidae habitat preference is cool well-oxygenated headwater streams during the first few years before moving into larger streams and rivers at older ages — a finding supported by our otolith microchemistry study (Section 2.13). In contrast, Cyprinidae prefer habitat that is warmer with lower gradient and deeper pools characteristic of larger fluvial systems (Hoar 1983; McPhail 2007). The Family Cottidae was also abundant in some of the systems sampled, mainly intermediate sized streams with moderate gradient — habitat where species from this Family are common (Brown et al. 1995; White and Harvey 1999).

The large number of YOY Salmonidae sampled in our study suggests that spawning habitat may be close to the pipeline crossing location for many of the study streams. Salmonidae show considerable fidelity to spawning locations (Bahr and Shrimpton 2004) due to specific characteristics of the stream, but also the hyporheic environment (McRae et al. 2012). It is likely, therefore, that spawners migrate annually to locations within the study streams. The area of suitable spawning habitat within a river system has been positively linked to the effective population size (Shrimpton and Heath 2003) — a genetic measure that is important for defining resiliency of a breeding group of animals to stochastic events. Protection of such habitat, therefore, is important — but so is maintaining connectivity between spawning habitat in the tributaries and other parts of the watershed where individuals move throughout their life history. Movement within river systems is important and common for individuals within the Family Salmonidae. Such patterns of movement have been shown to contribute to gene flow, which is important for genetic stability and effective population size. Low straying levels favour local adaptation, but even low levels of gene flow can elevate effective population size and preserve genetic variability (Walter et al. 2009, 2010). Even when connectivity to potential spawning habitat in tributary streams has been lost for decades, restoration efforts have been

shown to be successful with *S. confluentus* spawners exploiting habitat when barriers have been removed within months of the instream work (Shrimpton et al. 2008).

The most commonly caught species in the Kitimat River watershed was *O. clarkii*, whereas *O. mykiss* were found most commonly in streams of the Nechako River watershed. *O. mykiss*, however, were captured in Chist Creek in the Kitimat River watershed. An anadromous strain of *O. mykiss* from the Kitimat River population were stocked in Chist Creek on 12-Oct-1982; a total of 26,500 fry with a mean weight of 1.5 g were stocked into Chist Creek in 1982 (Fisheries Information Summary System, Province of British Columbia). It would appear, therefore, that the stocking program into Chist Creek was successful — although no information is provided on whether anadromous *O. mykiss* were present within Chist Creek before the stocking program. Anadromous *O. mykiss* were also stocked into Cecil Creek on 14-May-1986; 2934 *O. mykiss* with a mean weight of 61.3 g were stocked into Cecil Creek in 1986 (Fisheries Information Summary System, Province of British Columbia). During our sampling in 2014 and 2015, however, no *O. mykiss* were captured suggesting that the stocking program was not successful at establishing a self-sustaining population in Cecil Creek. This does not appear to be due to lack of appropriate habitat as *O. clarkii* were common in Cecil Creek and there is considerable overlap in habitat requirements for these two species (McPhail 2007).

Cottidae were found in four of the streams and rivers sampled in our study — but only in the lower reaches of three of the study systems. Additionally very few small sculpins were caught in our study; based on the size of fish and otolith annuli the youngest fish were older than two years. Our findings can be interpreted in two ways. First, there may be sampling bias: YOY Cottidae are difficult to catch by electrofishing since they bury under the rocks and do not float when they are stunned. The second interpretation is that YOY Cottidae move downstream to develop in a different location, before moving back into the stream when they are larger. Dispersal of YOY *C. cognatus* was recently shown by Clarke et al. (2015) for streams in the Williston Watershed in north central BC using otolith microchemistry. A similar pattern of dispersal as juveniles and then residency for older fish is suggested by the otolith microchemistry for Cottidae caught in the present study (Section 2.13). Movement of young

Cottidae may be a strategy for smaller fish to increase growth potential in larger river systems (Neverman and Wurtsbaugh 1994).

Although different species of *Cottus* are known to occur in sympatry, use of distinct habitats within a watershed has been shown previously. White and Harvey (1999) found that *C. aleuticus* used shallower habitat with faster water velocity than *C. asper*. Such a pattern may account for the higher number of *C. aleuticus* caught in our study compared to *C. asper* as we sampled primarily smaller high gradient streams — habitat preferred by *C. aleuticus*. *C. asper* were only caught in Ormond Creek in 2013 in a section well below the RoW — suggesting that this species was not present in the upper reaches of Ormond Creek. *C. cognatus* were only captured in Salmon River, which was the largest system sampled.

Conclusions

Physical and biological habitat changes have been documented following pipeline stream- and river-crossing construction in Canada (Lévesque and Dubé 2007). Water quality, physical habitat, benthic invertebrate community, and fish behavior and physiology have shown measurable changes. Such changes may alter the integrity of the physical and chemical nature of the aquatic ecosystem during construction, but also may persist later due to alterations to the riparian zone by the RoW. Natural variations in physical and biological variables, however, may vary seasonally or annually and it is important, therefore, to identify whether effects noted due to the pipeline instream works are masked or additive to natural changes.

Commonly environmental impact assessments use a before-after control-impact (BACI) study design. Our sampling 'before' has shown variation among sampling dates; our sampling for 'control' has shown tremendous variation among and within the study streams. With our sampling in 2013, 2014 and 2015, we do not know whether we have captured all potential temporal variation. Due to the variation that exists among the streams sampled, identification of proposed 'control' sites is also problematic. Every system is different — not only do the creeks differ in physical characteristics, but also biological diversity and abundance — making spatial replication difficult. Our sampling program to assess the effect of the Pacific Trails Pipeline project on stream crossings proposed to use upstream sections as controls for

assessing downstream changes due to the construction of the pipeline. Frequent differences between the biological diversity and abundance above and below the potential RoW make using the upstream section as a control section problematic. Consequently, the proposed BACI study design will be almost impossible to implement due to the lack of representative control sites. Such a finding indicates that temporal sampling is extremely important to characterize natural variation in the stream sections below the RoW to assess whether construction creates any measurable change in biological composition of the intersected streams.

2.9. Determining diets for fish from a small interior British Columbia stream: A comparison of morphological and genetic approaches

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Introduction

Construction of linear developments presents special concern for freshwater watercourses and the high level of biodiversity they sustain. Headwater streams are the benefactors of large amounts of allochthonous inputs, which help sustain aquatic communities throughout the larger downstream watercourses (Vannote 1980). Linear developments crossing small streams typically require changes and potential excavation of the channel bed. Such construction can not only affect the riparian habitat, but also cause increased sedimentation within the stream, both potentially harming aquatic organisms (Levesque and Dube 2007). With an increasing level of industrial development in central BC, it is essential that ecological interactions be monitored and assessed to our best ability.

Trophic interactions have been a primary focus in ecology as community and species level relationships can illuminate nutrient and energy fluctuations and competitive associations (Carreon-Martinez et al. 2010; Woodward et al. 2010). Predator-prey interactions also play an important role in these competitive associations and understanding them is essential to describe relationships between communities and their environment (Reiriz et al. 1998; Nakano et al. 1999; Symondson 2002; Sheppard and Harwood 2005). Prey analysis is complex and influenced by many factors, such as prey type, digestion rate, accurate identification, as well as spatial and temporal factors which contribute to the contents within the stomach of predators. Similarly, data on fish diet can be used to describe many ecological processes, such as prey selection, niche partitioning, competition, and nutrient and energy flow.

Traditional fish prey analysis has relied on morphological identification of stomach contents (Nakano et al. 1999; Carreon-Martinez et al. 2011; Merz 2002). This approach provides data on prey abundance, relative proportion, as well as biomass (Smock 1980), but can also describe qualitative aspects such as prey life stage, colour, and predatory behaviour (Kislalioglu

and Gibson 1976; Utne-Palm 1999). Morphological identification, however, can be labour-intensive and introduce observational bias. In particular the data are biased towards organisms with identifiable hard structures (bones, exoskeletons), whereas more soft-bodied or quickly digested organisms are overlooked (Symondson 2002; Carreon-Martinez et al. 2011; Pompanon et al. 2012). As well, for insectivorous predators, data are further limited by the challenging taxonomy of the prey taxa. Even when prey specimens are complete, many invertebrate taxa, such as Chironomidae and other Dipteran Families, cannot be identified below the Family level without high-level expertise (Kerans and Karr 1994).

For DNA barcoding, a standardized region of the Cytochrome C Oxidase subunit I (COI) gene is sequenced and compared to online databases (Hebert et al. 2003). Barcoding has successfully been used for freshwater invertebrates, greatly increasing the taxonomic resolution and identifying a much larger number of species in a sample (Zhou et al. 2010; Sweeney et al. 2011; Jackson et al. 2014; Stein et al. 2014). Barcoding has also been a successful technique in the analysis of diet, where greater taxonomic diversity and resolution are obtainable (Deagle et al. 2013; De Barba et al. 2014). Sequencing DNA can allow for identification of even highly degraded prey items (Sheppard and Harwood 2005; Pompanon et al. 2012). More recently, the field of metabarcoding has taken advantage of the increasing capacity and decreasing cost of next generation sequencing (NGS) technologies to develop protocols for sequencing entire communities at once (Taberlet et al. 2012; Ji et al. 2013; Cristescu 2014). These technologies have rapidly been adopted in diet studies, as they are cost-effective and can provide a more complete list of prey items than can be obtained through morphological identifications (Pompanon et al. 2012; Quemere et al. 2013; De Barba et al. 2014; Lopes et al. 2015). Despite the advantages of this approach, many issues remain that need to be tested, such as data biases due to PCR taxonomic bias (Deagle et al. 2014; Brandon-Mong et al. 2015; Elbrecht and Leese 2015) and whether quantitative data, rather than presence-absence, can be obtained (Deagle et al. 2013).

Our objective was to examine the effectiveness and the ability of NGS of homogenized stomach content samples to describe the diet of fish from a representative stream in central BC. This was accomplished by comparing NGS of homogenized samples with two more common

methods to identify fish prey; morphological identification and individual prey DNA barcoding. Prey within each fish were removed and morphologically identified, then each prey specimen was isolated and its DNA was sequenced. The remaining stomach contents were homogenized into a single sample, representing an individual fish, and DNA was isolated and sequenced on a NGS Ion Torrent platform. Taxonomic diversity, abundance and resolution of prey were compared between each of the three methods.

Methods

Ormond Creek is a 7405-m long, 4th-order stream that drains into Fraser Lake in central interior BC (BC Ministry of Environment 2013; Figure 2-77). It is located in section 2 of the proposed Pacific Trails Pipeline RoW. In the summer of 2013, Ormond Creek was sampled at a single location for benthic invertebrates and fish (10U 385848 5995678).

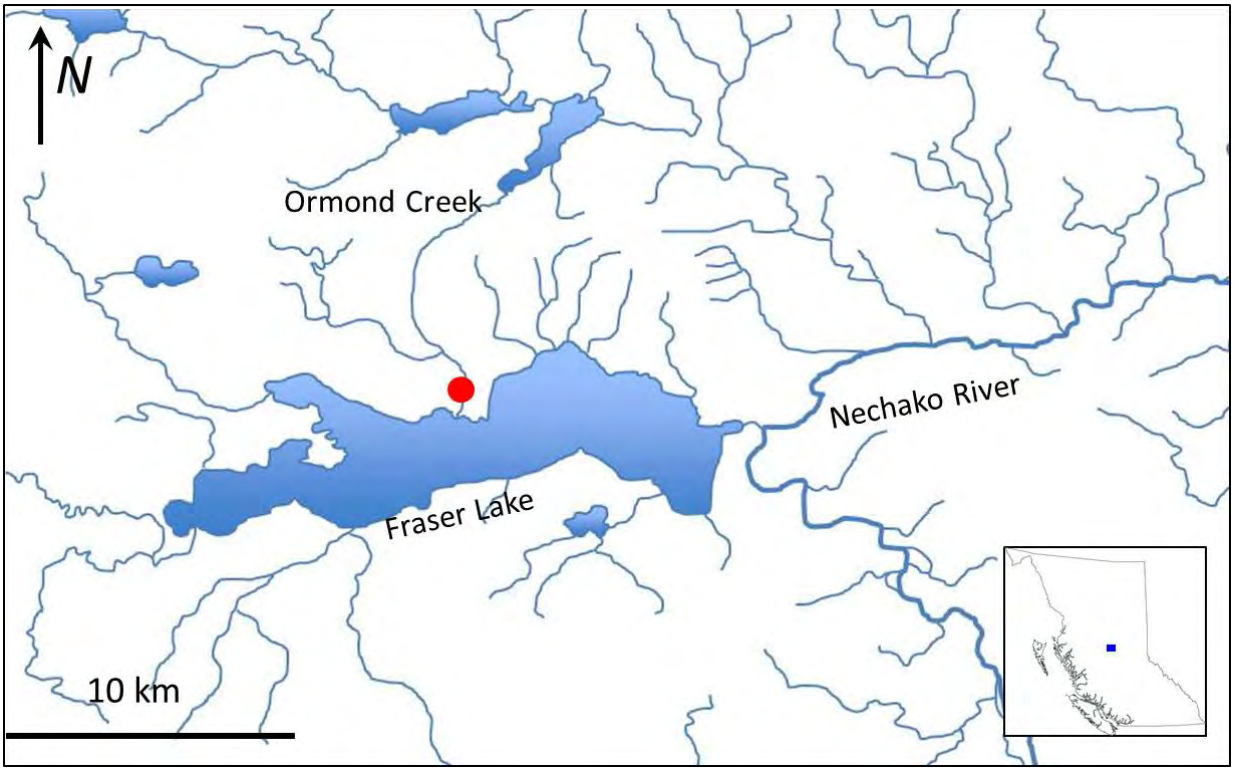

Figure 2-77 – Ormond Creek, located in central BC, with the 2013 sampling location indicated by a red dot.

Fish collection

Fish collection was conducted on September 20, 2013 along a 278-m section using a backpack electrofishing unit (Model LR-24, Smith-Root Inc., Vancouver, WA). Fish were anesthetized in a bucket containing 200 mg × L^{-1} MS-222 (tricaine methanesulfonate) buffered with 400 mg × L^{-1} sodium bicarbonate. Once fish were anesthetized, fork length to the nearest mm and weight to the nearest 0.1 g were recorded for each fish, stomachs removed, then preserved in 95 % ethanol, and stored at -20 °C until processing.

Morphological identification of prey items

Ten fish were analysed for diet: five rainbow trout (*Oncorhynchus mykiss*) and five prickly sculpin (*Cottus asper*). Using sterile techniques, the stomachs were removed from the 95 % ethanol and weighed to the nearest 0.1 g. Subsequently, each gastrointestinal (GI) tract was opened with a clean scalpel and forceps, and the contents were flushed into a sterile petri dish using 95 % ethanol. To avoid scraping cells from the predator stomach lining, either flushing with 95 % ethanol or physical removal with tweezers carefully removed the stomach contents.

Sorting of stomach contents focused on isolating and identifying invertebrate heads as this standardized the quantification for the presence of prey. Heads were morphologically identified to the lowest possible taxonomic level using the keys in Clifford (1991), measured on a dissecting microscope with an ocular micrometer, photographed, and individually stored in 96-well plates with 95 % ethanol at -20 °C. All remaining partially digested invertebrate prey matter were combined into a 15 mL tube (bulk stomach content samples) and stored in 95 % ethanol at -20 °C until DNA analysis.

Complete biomass for individual invertebrates was calculated from head width measurements using published head width or biomass regressions for each of the Orders (Smock 1980). Adult Dipterans were excluded, as published regressions were not available. Total biomass of each Order was determined for each fish and standardized for the total fish weight. Average biomass was then calculated for each Order of each fish species. A two-way analysis of variance (ANOVA) was used to compare fish species, invertebrate Order, and fish species by Order effect on count and biomass exclusively (SPSS v.21). When effects were

significant, a Bonferroni post-hoc test was used to identify differences among treatment groups. Significance was accepted for levels of $p < 0.05$.

Sequencing of individual prey items

A total of 251 invertebrate heads were isolated for DNA analysis from the ten dissected fish stomachs. DNA extractions were conducted using DNeasy Blood and Tissue Kit spin-column protocol (Qiagen, Toronto, ON, Catalog No. 69506). To avoid contamination, all extractions were performed in a room dedicated to low-quality DNA sources. No DNA samples from other species were handled in this room and separate laboratory coats, pipets, pipette filter tips and laboratory equipment were used to perform the extractions at that location. Additionally, all equipment was first sterilized using a 10% bleach solution and exposed to UV light for a minimum of 15 min. To monitor for contamination, a negative control was included for each set of DNA extractions and no contamination was found.

Invertebrate heads that were stored in 96-well plates were removed and air-dried for approximately two min to remove the residual ethanol. Once the ethanol evaporated, each individual head was placed into the appropriate well of a new sterile genogrinder 96-well block plate that contained 180 µL Buffer ATL and 20 µL Proteinase K. Two 5/32'' steel grinding beads (Ops Diagnostics, Lebanon, NJ, Catalog No. GBSS 156-5000-01) were added to each well and shaken in the Genogrinder 2000 (BT&C Inc., Burlington, ON) at 1,000 oscillations × min^{-1} for 1 min. Samples were then incubated at 56 °C for 2 h until the tissue completely lysed. The plate was centrifuged for 5 min at 20,000 × g and then samples transferred into new sterile 1.5 mL labelled tubes. Hereafter, the samples were subjected to a classical DNA extraction for the remaining steps with the Qiagen DNeasy Blood and Tissue Kit (manufacturer's protocol), with the modification of using 2 × 75 µL AE Buffer for each sample for the final elution step.
DNA was amplified using primers designed by Zeale et al. (2011), which targets a 157-bp amplicon located within the standard COI barcode region for a broad range of arthropod taxa (Table 2-67). Samples were amplified in 10 µL reaction volumes in triplicate using the Qiagen Multiplex PCR kit (Toronto, ON, Catalog No. 206143). Each PCR reaction contained 5 µL Multiplex MasterMix, 0.2 µL forward primer (10 µM), 0.2 µL reverse primer (10 µM), 1.6 µL of nuclease-free water and 3 µL of extracted DNA. A negative extraction control and a negative

PCR control were also included in triplicate and did not reveal any contamination. Samples were amplified at 95 °C for 15 min, 39 cycles of 94 °C for 30s, 52 °C for 60s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. PCR products were verified on 2 % agarose gels and purified using Agencourt AMPure XP purification beads (Beckman and Coulter, Mississauga, ON, Catalog No. A63881). The concentration of the purified PCR product was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay, Life Technologies, Burlington, ON, Catalog No. Q32854) and stored at -20 °C until sequencing.

PCR products were sequenced on an ABI 3130xl genetic analyzer (Burlington, ON). Sequences were visualized on CLC Main Workbench 7.6 and edited if ambiguous bases were discernable through chromatogram interpretation. To determine species similarities for sequences, all forward and reverse sequences were compared with publicly available databases GenBank (http://www.ncbi.nlm.nih.gov) and Biodiversity of Life Database (BOLD) version 3 (Ratnasingham and Hebert 2007). Similarities larger than 95 % were considered to be successful for species identification.

Table 2-67 – List of primers used to amplify extracted DNA from individual invertebrate samples, ZBJ, from Zeale et al. (2011) and primers designed for the next generation sequencing analysis used to amplify DNA extracted from stomach homogenates, EPT. UniA and UniB sequences included within Zeale et al. (2011) and EPT primers (shown in bold).

Source	Primer	Sequence (5' to 3')	Amplicon Size
ZBJ	UniA_ZBJ-ArtF1c	**ACCTGCCTGCCG**AGATATTGGAACWTTATATTTTATTTTTGG	235 bp
	UniB_ZBJ-ArtR2c	**ACGCCACCGAGC**WACTAATCAATTWCCAAATCCTCC	
EPT	UniA_ZBJ-ArtF1c	**ACCTGCCTGCCG**AGATATTGGAACWTTATATTTTATTTTTGG	235 bp
	UniB_ZBJ-EPTD4d	**ACGCCACCGAGC**ACTAAYCARTTNCCRAAHCCHCC	
PCR2 (Ion Torrent)	P1UniB	CCTCTCTATGGGCAGTCGGTGAT**ACGCCACCGAGC**	

Next generation sequencing of homogenized stomach samples

Stomach content samples from five rainbow trout and five prickly sculpin were used in the analysis. To avoid contamination, all extractions were performed in a room dedicated to low-quality DNA sources. No DNA from other species was handled in this room with separate laboratory coats, pipets, pipette filter tips and laboratory equipment used to perform the extractions. Additionally, all equipment was first sterilized using a 10% bleach solution and

exposed to UV light for a minimum of 15 min. To monitor for contamination a negative control was included for each set of stomach content DNA extractions — no contamination was revealed.

Samples were centrifuged for 1 min at 20,000 × g (14,000 rpm) to collect the prey items at the bottom of the tube. Excess ethanol was removed and the stomach contents were transferred into new sterile 2-mL centrifuge tubes using sterile techniques. Two 5/32'' steel grinding beads were then added to each 2-mL tube and shaken in the Genogrinder 2000 at 1500 oscillations × min^{-1} for 5 min. Grinding beads were removed with sterile forceps and approximately 200 µL of homogenized contents were transferred into a pre-weighed 1.5-mL centrifuge tube. Tubes were left open overnight at 37 °C to evaporate remaining ethanol. Hereafter the stomach content samples were subjected to a classical DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (spin-column protocol), with the slight modification of using 2 × 75 µL AE Buffer for the final elution steps. DNA concentration of each bulk sample was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay) and stored at -20 °C until PCR amplification.

We amplified DNA using a modified version of the general invertebrate COI primers published by Zeale et al. (2011). Because these primers were designed to capture a broad range of arthropod taxa, we designed an additional reverse primer to target the specific Orders found in our study streams, including Ephemeroptera, Plecoptera, Trichoptera, and Diptera. These primers targeted the same 157-bp amplicon located within the COI barcode region and were designed by aligning sequences from 12 dominant Families (Baetidae, Ephemerellidae, Heptageniidae, Perlodidae, Chloroperlidae, Nemouridae, Rhyacophilidae, Hydropsychidae, Glossosomatidae, Simuliidae, Tipulidae, and Psychodidae) and manually selecting bases in the ZBJ-ArtR2c primer-binding region that were more similar to the sequences of the dominant stream taxa.

Stomach homogenates samples were prepared for unidirectional sequencing on a PGM Ion Torrent Sequencer (Burlington, ON) using a two-step PCR amplification method that incorporates fusion primers designed at the University of Windsor (D. Heath, *pers. comm.*). Two tailed-end sequences, UniA and UniB, were added to the end of primers sequences used in the

current study: UniA onto the forward primer (ZBJ F) and UniB onto the reverse primer, EPT R (Table 2-67). These "universal" tailed-end sequences were used in the second PCR step to incorporate the Ion Torrent adaptor sequences into the final amplicon sequence: an IonA adaptor sequence and a unique 10-bp identifier sequence (used to identify the individual samples sent for sequencing) bind to UniA sequence, and the P1 adaptor sequence used to initiate the sequencing reaction during an Ion Torrent sequencing run binds to the UniB sequence. The P1 adaptor sequence was required for correct attachment of prepared amplicons to the Ion Spheres, which rest in the wells of the semi-conductor chip and the IonA adaptor the priming site for the sequencing reaction on the Ion Torrent chip (D. Heath, *pers. comm.*).

During the first PCR step (PCR1), the target 157-bp COI sequence (plus UniA and UniB adaptor sequences) was amplified in 35-µL reaction volumes in triplicate using a Qiagen Multiplex PCR kit (Toronto, ON, Catalog No. 206143). Each reaction contained 17.8 µL Multiplex MasterMix, 0.75 µL UniA-tailed ZBJ forward primer (10 µM), 0.75 µL UniB-tailed EPT reverse primer (10 µM), and 15.0 µL of nuclease-free water and extracted DNA which combined for a total of 20-60 ng of template DNA per reaction. Also included were a negative extraction control, a negative PCR control, and a positive control (mock community), each in 35-µL reaction volumes and in triplicate.

PCR reactions were amplified at 95 °C for 15 min, 30 cycles of 94 °C for 30 sec, 46 °C for 60 sec and 72 °C for 60 sec, followed by a final extension at 72 °C for 10 min. PCR products were visualized on 2 % TBE agarose gels stained with ethidium bromide to verify the presence of single bands at the expected size (235 bp) for the COI amplicon plus UniA+ZBJ F and UniB+EPT sequences for stomach sample homogenates and the absence of bands in centrifuge, extraction and PCR negative controls. PCR1 products were purified using Agencourt AMPure XP purification beads (Beckman and Coulter, Mississauga, ON, Catalog No. A63881) and the resulting DNA concentrations quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

The second PCR step (PCR2) was performed to attach the IonA adaptor plus the unique identifier and the P1 adaptor sequences to the amplicons produced during PCR1. Total volume of PCR2 reactions was 22.5 µL and contained 3.6 µL 10 × Reaction Buffer, 1.5 µL $MgCl_2$ (25 mM),

and 0.15 µL Platinum Taq polymerase (5 Units × μL^{-1}) (all from Invitrogen, Burlington, ON, Catalog No. 1096634), 0.75 µL dNTPs mix (10 mM) (New England Biolabs, Ipswich, Massachusetts, Catalog No. N0446s), 0.75 µL IonA-UniA primer (10 µM) (IDT Technologies), which contains a single unique identifier sequence for each sample or control (to allow sample identification in downstream data analyses), and 0.75 µL P1-UniB primer (10 µM) (IDT Technologies). The amount of purified PCR1 product added to the reaction varied depending on concentration, for a total mass of 30-50 ng of purified PCR1 product per reaction, volume of nuclease-free water adjusted to a total volume of 15 µL. PCR2 reactions were amplified at 94 °C for 2 min, 5 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec, followed by a final extension at 72 °C for 5 min.

Samples were normalized by pooling equimolar ratios of each sample, as well as approximately 5 µL of each negative control, which had DNA concentrations too low to be read by QUBIT® 2.0 Fluorometer (or significantly lower than that of stomach samples) as expected. Two 40-µL aliquots of the final normalized COI amplicon library (all samples and positive/negatives controls) were then run on 1.5% TAE agarose gels stained with ethidium bromide at ~200 v for 1.5 h to allow sufficient separation of the 295-bp bands (amplicons plus all required adaptor sequences) from those containing amplicons with only one adaptor sequence attached (255 and 275 bp). The 295-bp fragment was extracted from the gel by visualizing the bands on a UV-light box using a clean, sterile scalpel and the DNA purified using Qiagen MinElute Gel Extraction Kits (Toronto, ON, Catalog No. 28606). Finally, the purified DNA was analyzed using the Experion DNA 1K Analysis Kit to verify successful excision of the correct band from the gel and determine the concentration of the resulting COI amplicon library for submission to the Great Lakes Institute for Environmental Research (GLIER) Lab at the University of Windsor in Ontario where they were sequenced using a PGM Ion Torrent Sequencer using an Ion Torrent 318 chip.

Results

A total of 47 fish were sampled from Ormond Creek, 23 rainbow trout (*Oncorhynchus mykiss*), 21 prickly sculpin (*Cottus asper*), two burbot (*Lota lota*), and one redside shiner (*Richardsonius balteatus*). The average length and weight of the five rainbow trout used for analysis was 6.4 cm and 7.6 g, respectively. For the five prickly sculpin used for analysis, average length and weight were 8.0 cm and 7.3 g, respectively.

Morphological identification

A total of 251 invertebrate prey were collected and morphologically identified from the five rainbow trout (n = 179) and five prickly sculpin (n = 72) GI tracts. Proportional comparison of count data between Orders showed prickly sculpin to feed mainly on Trichoptera (43 %) and Ephemeroptera (39 %), with the remaining 18% consisting of Diptera (larvae) (12 %) and Plecoptera (6 %). Rainbow trout diet consisted of similar Orders with Trichoptera (56 %) being most abundant. Diptera (23 %) and Ephemeroptera (13 %) contributed over a third of the total, with Plecoptera (6 %), Hemiptera (2 %), and Coleoptera (<1 %) completing the prey.

Statistical analysis of invertebrate count data for diet composition was found to vary significantly by Order ($p < 0.05$) and fish species ($p = 0.011$), while no significant effect of Order × fish species effect was detected ($p = 0.176$; Figure 2-78A). A Bonferroni post-hoc test found Trichoptera was significantly greater than Coleoptera ($p = 0.032$). Ephemeroptera also differed significantly from Plecoptera ($p = 0.006$), Coleoptera ($p < 0.001$), and Hemiptera ($p = 0.010$). Diptera count differed significantly with Coleoptera ($p = 0.049$). Analysis of biomass data also revealed significant differences between Orders ($p < 0.05$), but no effect of fish species ($p = 0.593$) and no interaction ($p = 0.967$; Figure 2-78B). Post hoc test for biomass found Ephemeroptera to be significantly greater than Plecoptera ($p = 0.037$) and Diptera ($p = 0.032$).

Individual sequencing of prey items

Sequence analysis for 250 of the morphologically identified prey items resulted in 110 sequence matches from online taxonomic databases which aligned with the morphological identification (Figure 2-79A). The remaining samples provided sequences, which once compared to online databases did not match the morphological identification (n = 97) or did

not match any known sequences (n = 43). Correct sequence matches were obtained for 46 % of the prey items from rainbow trout and 40% of prickly sculpin.

A proportional comparison of the 110 sequences which matched their corresponding morphological identification shows subtle difference between rainbow trout and prickly sculpin (Figure 2-79B). Trichoptera (40 %) was the most common among the successful sequences for prickly sculpin, followed by Ephemeroptera (35 %) and Diptera (25 %). Diptera (41 %) was the most common within rainbow trout, followed by Ephemeroptera (28 %), Trichoptera (27 %), and Hemiptera (4 %). None of the samples of Plecoptera identified in the stomachs of rainbow trout and prickly sculpin sequenced successfully.

Next generation sequences from stomach samples

Next generation sequencing of the homogenized contents from the fish stomachs produced 378 000 sequences, 57 % from rainbow trout and 43 % from prickly sculpin, belonging to 362 unique operational taxonomic units (OTUs). The 362 OTUs were condensed to 162 unique taxa by pooling all OTUs and corresponding sequences which were assigned identical taxonomy.

Rainbow trout stomachs produced 215,644 sequences among 90 unique taxa, with almost half (44) having more than 100 sequences. The most abundant sequences for a given taxa in rainbow trout prey items were *Paraleptophlebia heteronea* (68,419; 32 %), *Ameletus validus* (32,944; 15 %), *Bibio longipes* (22,792; 11 %), Chironomidae (16,143; 7 %), and *Procladius* (16,003; 7 %). The most common taxa based on frequency of occurrence in rainbow trout stomach contents were *Paraleptophlebia heteronea*, Chironomidae, and *Cricotopus sp*. (1,398; <1 %), which were present within all five stomachs; Nematocera (4,298; <2 %), Diptera (133; <1 %) and Aphidini (59; <1 %) were each found in four of the five fish stomach contents. Prickly sculpin stomachs produced 162,701 sequences among 83 unique taxa, with 24 having more than 100 sequences. The most abundant prickly sculpin prey sequences for each taxon were *Arcynopteryx compacta* (60,352; 37 %), *Paraleptophlebia heteronea* (43,437; 27 %), *Drunella grandis* (12,246; 8 %), *Ameletus vernalis* (9,150; 6 %), and *Ameletus validus* (7,374; 5 %). The most common taxa based on frequency of occurrence were *Paraleptophlebia heteronea* and *Acentrella turbida* (25; <1 %), which were present in all five stomachs. *Arcynopteryx*

compacta, *Ameletus validus*, Capniidae (1,292; <1 %), *Cinygmula* (1,079; <1 %), and *Paraleptophlebia memoralis* (792; <1 %) were found in four of the five sculpin stomachs.

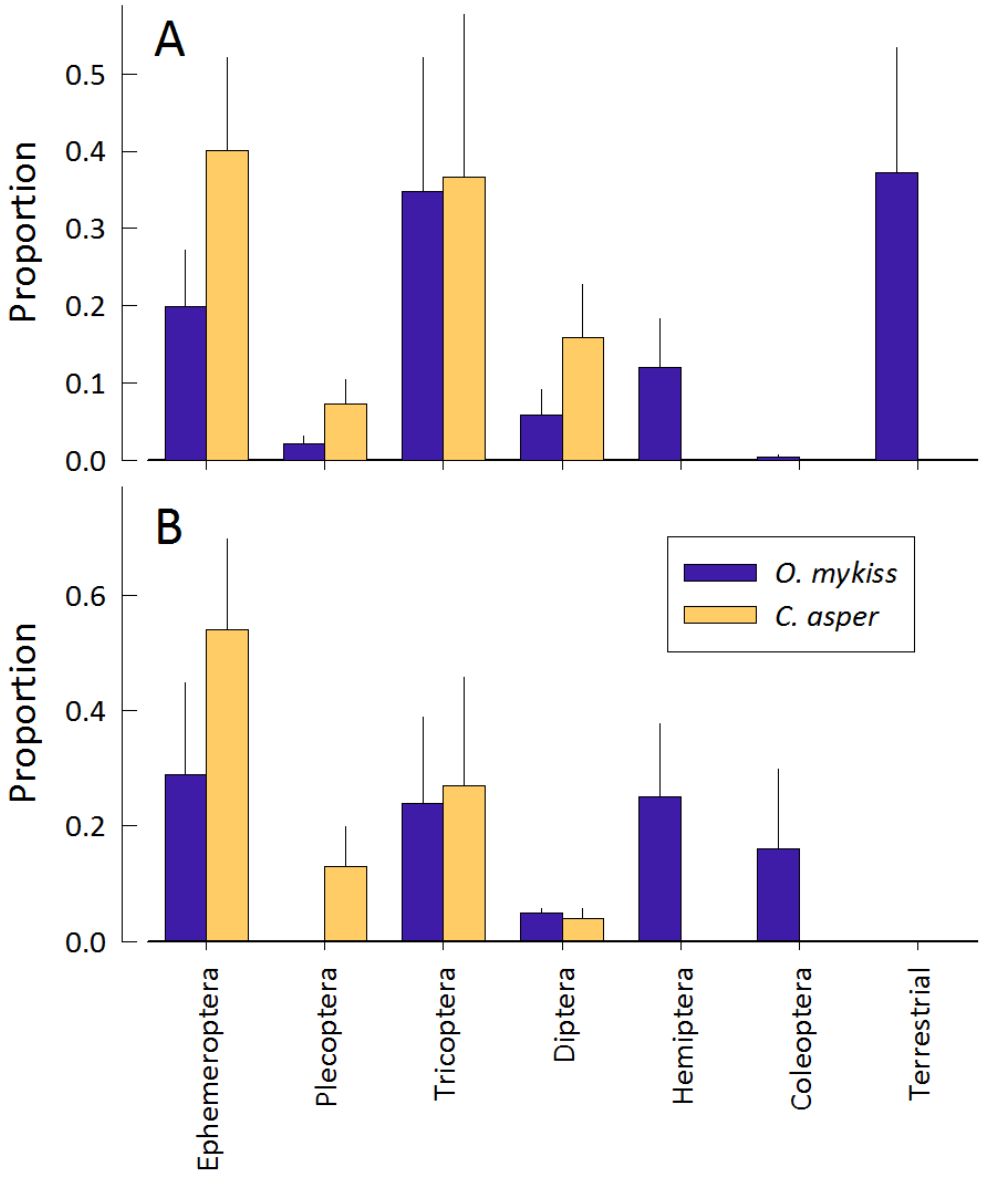

Figure 2-78 – Proportional contribution of (A) count data and (B) mass data for different invertebrate Orders sampled from rainbow trout (*Oncorhynchus mykiss*) and prickly sculpin (*Cottus asper*) caught in Ormond Creek, BC. No regressions were available for adult Diptera, so they are not included in the calculations for Figure 2-78B.

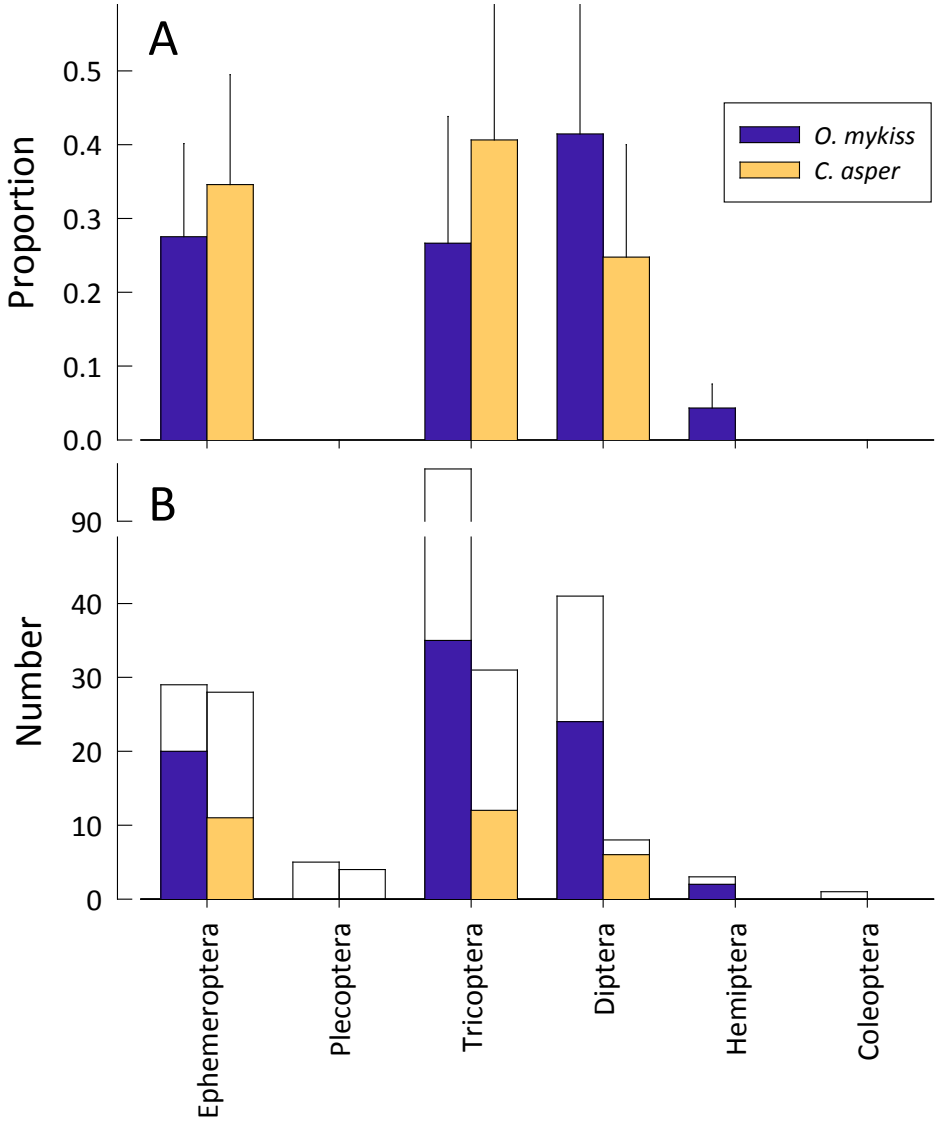

Figure 2-79 – (A) Proportional comparison of individual prey from rainbow trout (*Oncorhynchus mykiss*) and prickly sculpin (*Cottus asper*) which produced a barcoding sequence that correctly matched their morphological identification (n = 110). (B) Number of samples extracted for DNA (open bars) and successfully sequenced (closed bars) for rainbow trout and prickly sculpin using ZBJ primers from Zeale et al. (2011).

Comparison of approaches

Using classic morphological methods to identify taxa from the stomach samples, most specimens (214 of 251) were resolved to the level of Order, some of the more distinctive organisms to Family, and a few to Genus for both rainbow trout (Table 2-68) and prickly sculpin

(Table 2-69). Less than 10 % were classified to Genus and none to species. In comparison, identification to the level of Genus and species was possible for barcoded samples from individual specimens within the Orders Ephemeroptera, Trichoptera, Diptera and Hemiptera — but not Plecoptera. The highest number of Genera and species, however, was identified from the stomach homogenates that were analyzed using Next Generation Sequencing. Use of the EPT R primer also provided successful sequences for Genera and species within the Order Plecoptera (Table 2-68 and Table 2-69).

From the stomach homogenates 123 unique OTU signatures were identified — 68% were resolved to at least the level of Genus (Figure 2-80A). Comparison of the number of sequences for each Order of aquatic invertebrates to the proportional contribution of count data and mass data for the different invertebrate Orders revealed little relationship for rainbow trout and prickly sculpin (Figure 2-80B) — consequently amplification or sequence count data did not appear to be quantitative for the number or mass of specimens in the samples.

Discussion

Rainbow trout fed on aquatic and terrestrial invertebrates, whereas prickly sculpin diet consisted entirely of larval aquatic invertebrates. Our morphological comparison of diet between the two fish species focused on Orders of aquatic invertebrates. Having taken this approach, the inclusion of terrestrial specimens as an independent category was not possible, and ultimately there was no difference in diet detected. Prickly sculpin are a benthic dwelling species whose diet has been found to consist largely of invertebrate larvae (Brown et al. 1995; Merz 2002; McPhail 2007), while rainbow trout inhabit a wide variety of lotic habitats, throughout the water column and forage primarily on terrestrial invertebrates (Angradi and Griffith 1990; McPhail 2007). Although habitat alone would indicate species whose diet should differ, these feeding habits appear to explain two different foraging approaches. Having experienced an apparent overlap in diet between rainbow trout and prickly sculpin, an increase in sample size would benefit the analysis and potentially reduce the error associated with small sample size, thereby increasing the power to resolve differences in diet. Terrestrial

Table 2-68 – Aquatic invertebrates from gut contents of five Rainbow Trout identified visually (Morphological ID), by DNA sequencing of individual heads (Barcode Sequence ID), or DNA sequencing of homogenized gut contents (Next Generation Sequence ID). The lowest taxonomic classification determined for specimens within the different Orders are listed for each method — light blue shading for Genus and species; dark blue shading for Order and Family.

	Family	Morph. ID	Barcode Sequence ID	Next-Gen. Sequence ID
Ephemeroptera	Leptophlebiidae	*Paraleptophlebia*	*Paraleptophlebia memorialis*	*Paraleptophlebia memorialis*
	Leptophlebiidae		*Paraleptophlebia heteronea*	*Paraleptophlebia heteronea*
	Leptophlebiidae			*Leptophlebia cupida*
	Heptageniidae	Heptageniidae		*Cinygmula sp.*
	Baetidae		*Diphetor hageni*	*Diphetor hageni*
	Baetidae			*Acentrella turbida*
	Baetidae			*Baetis sp.*
	Ephemerellidae			*Ephemerella dorothea infrequens*
	Ephemerellidae			*Drunella doddsi*
	Ephemerellidae			*Drunella grandis*
	Siphlonuridae			*Ameletus validus*
Plecopt	Capniidae	Plecoptera		*Utacapnia trava*
	Perlodidae			*Arcynopteryx compacta*
	Perlodidae			*Isoperla fulva*
	Nemouridae			*Zapada cinctipes*
Tricop	Lepidostomatidae	Tricoptera	*Lepidostoma rayneri*	*Lepidostoma rayneri*
	Lepidostomatidae			*Lepidostoma sp.*
	Limnephilidae			*Ecclisomyia conspersa*
Diptera	Chironomidae	Chironomidae	*Procladius culiciformis*	*Procladius sp.*
	Chironomidae		*Procladius denticulatus*	
	Chironomidae		*Ironopolia sp.*	
	Chironomidae		*Thienemanniella xena*	*Thienemanniella sp.*
	Chironomidae			*Conchapelopia sp.*
	Chironomidae			*Cricotopus sp.*
	Chironomidae			*Eukiefferiella sp.*
	Chironomidae			*Heterotrissocladius sp.*
	Chironomidae			*Micropsectra sp.*
	Chironomidae			*Micropsectra subletteorum*
	Chironomidae			*Orthocladius oliveri*
	Chironomidae			*Paracladopelma sp.*
	Chironomidae			*Psectrocladius*
	Chironomidae			*Spilogona sp.*
	Chironomidae			*Synorthocladius sp.*
	Chironomidae			*Tanytarsus lestagei*
	Bibionidae		*Bibio longipes*	*Bibio longipes*
	Simuliidae		*Simulium arcticum*	*Simulium sp.*
	Simuliidae		*Simulium argus*	
Hemipt	Cicadeliidae	Cicadeliidae		*Idiocerus delongi*
	Aphidae		*Aphis salicariae*	*Aphis sp.*
	Aphidae			*Euceraphis sp.*
	Anthocoridae			*Anthocoris sp.*

invertebrates contributed to a large portion of rainbow trout diet and appear to be a vital component.

Table 2-69 – Aquatic invertebrates from gut contents of five Prickly Sculpin identified visually (Morphological ID), by DNA sequencing of individual heads (Barcode Sequence ID), or DNA sequencing of homogenized gut contents (Next Generation Sequence ID). The lowest taxonomic classification determined for specimens within the different Orders are listed for each method — light blue shading for Genus and species; dark blue shading for Order and Family.

	Family	Morph. ID	Barcode Sequence ID	Next-Gen. Sequence ID
Ephemeroptera	Leptophlebiidae	*Paraleptophlebia*	*Paraleptophlebia memorialis*	*Paraleptophlebia memorialis*
	Leptophlebiidae		*Paraleptophlebia heteronea*	*Paraleptophlebia heteronea*
	Ephemerellidae	*Drunella*		*Drunella grandis*
	Ephemerellidae			*Ephemerella aurivillii*
	Siphlonuridae			*Ameletus vernalis*
	Siphlonuridae			*Ameletus validus*
	Heptageniidae			*Cinygmula sp.*
	Heptageniidae			*Epeorus albertae*
	Baetidae			*Accentrella turbida*
	Baetidae			*Baetis sp.*
	Baetidae			*Diphetor hageni*
Plecoptera	Perlodidae	Plecoptera		*Arcynopteryx compacta*
	Chloroperlidae			*Haploperla sp.*
	Capniidae			*Utacapnia trava*
	Capniidae			*Capnia sp.*
	Nemouridae			*Zapada cinctipes*
Tricoptera	Lepidostomatidae	Tricoptera	*Lepidostoma rayneri*	*Lepidostoma rayneri*
	Lepidostomatidae		*Lepidostoma ormeum*	*Lepidostoma sp.*
	Polycentropodidae		*Plectrocnemia variegata*	*Polycentropus variegatus*
	Leptoceridae		*Oecetis disjuncta*	*Oecetis sp.*
	Glossosomatidae			*Glossosoma sp.*
Diptera	Chironomidae	Chironomidae	*Synorthocladius semivirens*	*Synorthocladius sp.*
	Chironomidae		*Tokunagaia tonollii*	
	Chironomidae			*Micropsectra subletteorum*
	Chironomidae			*Orthocladius*
	Pediciidae		*Ula kiushiuensis*	
	Culicidae		*Culex spiculosus*	

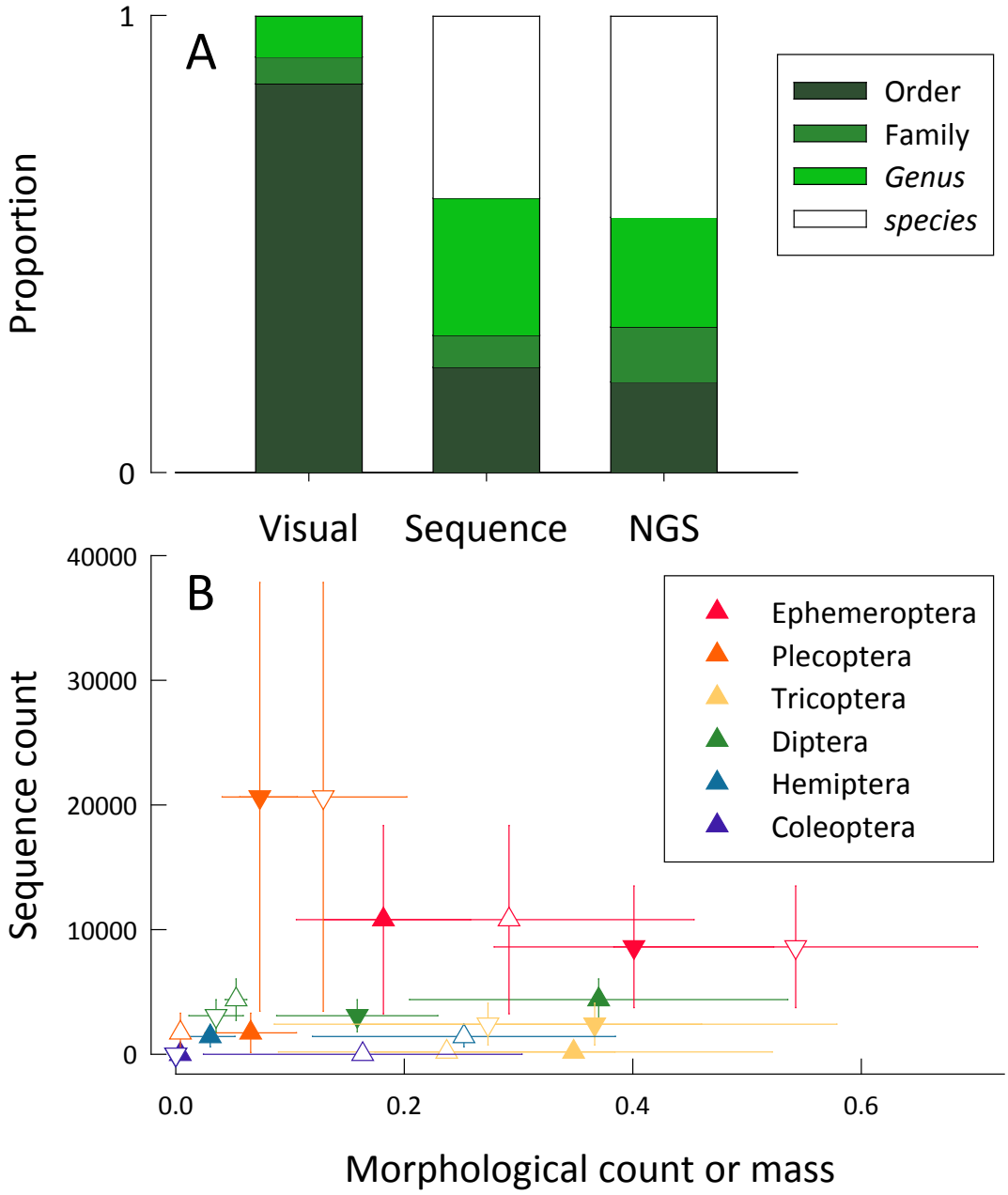

Figure 2-80 – (A) Proportional taxonomic resolution of samples from stomach contents of fish determined visually by morphological analysis, barcode sequencing of individual prey, and Next Generation Sequences (NGS) from homogenized stomach contents. (B) Number of sequences for each prey species within each Order for aquatic invertebrates as a function of their proportional contribution of count data (closed symbols) and mass data (open symbols) from rainbow trout (*Oncorhynchus mykiss*, upright triangles) and prickly sculpin (*Cottus asper*, upside down triangles) caught in Ormond Creek, BC. Data are plotted as means ± SD.

Species-level classification of invertebrate fragments from the rainbow trout and prickly sculpin stomachs was possible following amplification and sequencing. Traditional diet analysis of fish requires a considerable amount of time and experience to accurately identify organisms to the species level (Lenat and Resh 2001). Use of morphological identification and sequencing of intact invertebrate prey heads identified samples to the level of Order, Family, Genus and for some to the species level, however, this approach fails to identify the highly digested or soft bodied prey items (Symondson 2002; Carreon-Martinez et al. 2011). With the ability to accurately classify prey to the species level through DNA analysis, determination of specific prey items is much greater when compared to traditional methods, but may also prove to be a more efficient method for species identification. Further, DNA analysis and next generation sequencing of a single sample containing all the unrecognisable stomach contents allowed us to identify a much greater number of prey items ingested regardless of species degradation — although relative comparisons between species did not correlate with abundance. To assess quantities of different taxa and relative proportions of organisms in the diet, visual examination of stomach contents is required. A combination of visual identification and sequencing of individual specimens can provide a good estimate of what fish are eating and the relative quantity. To gain an understanding of the biodiversity of organisms that contribute to the diets of fish, next generation sequencing was effective and necessary if we want to know all organisms that contribute to the diet of fish.

Traditional sampling and identification methods should remain an important aspect to complement DNA analysis, as data such as morphological features, biomass, and an overall exposure to the ecology of the system or specimen are important and cannot be obtained through genetic analysis alone. Examination of the DNA within a system or animal can provide detailed results which are difficult to obtain otherwise, and can therefore broaden sampling efficiencies to studies which are exposed to time, experience or data quality restraints. The scope of potential eDNA studies is large and will no doubt contribute to methods similar to our approach, but also to areas and species of conservation, extensive biodiversity or population assessments, presence or absence surveys, and a multitude of wildlife and fisheries

management implications (Ficetola et al. 2008; Goldberg et al. 2011; Jerde et al. 2011; Dejean et al. 2012; Lodge et al. 2012; Thomsen et al. 2012).

The general Arthropod primers of Zeale et al. (2011) did not amplify some of the taxa common in our study streams — particularly the Order Plecoptera, but also Tricoptera and Coleoptera. Consequently, our combination of morphological identification and sequencing individual samples did not provide us with a very high proportion of successful Genus or species level taxonomic identifications for prey items in either fish species sampled. The EPT reverse primer designed using sequences from central BC stream macroinvertebrates resulted in a much greater number of Genus and species level sequence matches for all Orders, suggesting that next generation sequencing was a better approach. Likely this was due to the novel primer set used and the EPT reverse primer would have improved our resolution of taxa when sequencing individual samples.

Our study represents an initial attempt to use molecular tools to assess biodiversity and ecosystem function in a small interior stream, but our findings have potential to also characterize effects of anthropogenic change. Stream crossings have the potential to influence the aquatic community through a number of aspects during and post construction. Sediment released during installation of the pipe can have detrimental effects to both fish and invertebrates (Newcombe and MacDonald 1991; Berry et al. 2003; Cover et al. 2008, Levy 2009), while the removal of riparian vegetation can alter abiotic conditions within the stream and result in changes to the aquatic community (Vannote et al. 1980; Levesque and Dube 2007; Coe et al. 2013). The removal of riparian vegetation along the pipeline RoW also has effects on the terrestrial dwelling invertebrates. Inputs of terrestrial invertebrates are highest when riparian forests are dominated by deciduous plants (Baxter et al. 2005), while old growth coniferous forests have been linked with low abundance (Wipfli 1997). Kawaguchi and Nakano (2001), however, found heavily forested reaches contribute more terrestrial invertebrates when compared to open grassland sections. With the removal of riparian vegetation during pipeline installation, terrestrial invertebrate inputs into streams may be reduced, limiting one of the main contributions to rainbow trout diet. Although the RoW is typically short relative to stream lengths, the cumulative impacts experienced from all linear features affecting a given

stream may greatly impact the stream community. Tools that allow rapid assessment of aquatic ecosystem function may make an important contribution for the assessment of biodiversity. Molecular methods in addition to traditional morphological approaches, therefore, show tremendous potential for assessing changes associated with linear developments in small interior streams.

Conclusions

Morphological, barcoding and NGS approaches to examine stomach contents provided complementary information on fish prey. Quantitative information was realized using the morphological identification — barcoding individual specimens should enhance this approach. A qualitative assessment of diversity of prey taxa, however, was best realized using the NGS approach. The selection of primers, therefore, is vital for both genetic approaches and use of ZBJ-F and EPT-R should improve barcoding success. It is more labour intensive than the NGS approach, but the greater diversity of invertebrate taxa may represent genetic signatures from the gut contents of fish prey.

2.10. Aquatic biodiversity in a coastal British Columbia Stream: the relationship between prey availability and fish diet

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Introduction

An understanding of food web dynamics informs ecological theory regarding predator-prey relationships, population dynamics, and nutrient and energy flow through ecosystems (Paine 1980). An accurate understanding of food webs can be applied to fields as diverse as conservation biology, agroecology, fisheries science, and the study of invasive species (Pompanon et al. 2012). Food webs, however, are incredibly complex and most studies have, by necessity, focused on just a subset of potential interactions. Developing accurate food-webs for generalist species has proven particularly challenging, due to the wide range of prey consumed (Pompanon et al. 2012).

Traditionally fish diet studies have relied on morphological identification of prey items (Nakano et al. 1999; Merz 2002; Carreon-Martinez et al. 2011). This type of work provided a great deal of useful information; however, it is both labor-intensive and subject to observational bias. In particular the data are biased towards organisms with identifiable hard structures (e.g., bones, exoskeletons), whereas more soft-bodied or quickly digested organisms are overlooked (Symondson 2002; Carreon-Martinez et al. 2011; Pompanon et al. 2012; Paquin et al. 2014). As well, for insectivorous predators, data are further limited by the challenging taxonomy of the prey taxa; due to the "taxonomic impediment" even undigested specimens are often not identifiable to the species-level (Kerans and Karr 1994; Relyea et al. 2012). DNA barcoding, wherein a standardized region of the cytochrome c oxidase subunit 1 gene (COI) is sequenced and compared to online databases, has been proposed as a method for increasing ease of identifications (Hebert et al. 2003). Barcoding has successfully been used for freshwater invertebrates, greatly increasing the taxonomic resolution and identifying a much larger number of species in a sample (Zhou et al. 2010; Sweeney et al. 2011; Gill et al. 2014; Jackson et

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al. 2014; Stein et al. 2014). Sequencing DNA can also allow for identification of even highly degraded prey items (Sheppard and Harwood 2005; Pompanon et al. 2012). More recently, the field of metabarcoding has taken advantage of the increased capacity and decreased cost of next generation sequencing (NGS) technologies to develop protocols for sequencing entire communities at once (Taberlet et al. 2012b; Ji et al. 2013; Cristescu 2014). These technologies have been rapidly adopted in diet studies, as they are cost-effective and can provide a more complete list of prey items than obtained through morphological identifications (Pompanon et al. 2012; Quéméré et al. 2013; De Barba et al. 2014; Lopes et al. 2015). Despite the advantages of this approach, many issues remain that need to be tested, such as data biases due to PCR taxonomic bias (Deagle et al. 2014; Brandon-Mong et al. 2015; Elbrecht and Leese 2015) and whether quantitative data, rather than presence-absence, can be obtained (Deagle et al. 2013).

Aquatic food-webs are complex, influenced by both allochthonous and autochthonous inputs (Soluk 1993; Nakano et al. 1999; Kawaguchi and Nakano 2001; Kawaguchi et al. 2003). Benthic communities influence fish populations (Ramezani et al. 2014), while selective predation by fish can also affect benthic communities (Dahl and Greenberg 1996). Changes in riparian inputs have the potential to affect fish populations, especially for surface-feeding fish such as salmonids (Wipfli 1997; Allan et al. 2003). Anthropogenic disturbances can greatly impact fish and stream ecosystems, especially when multiple stressors are present (Fausch et al. 2010; McHugh et al. 2010; Stendera et al. 2012). In northern Canada, aquatic ecosystems have long been affected by forestry (Pike et al. 2010), and are increasingly influenced by linear features (e.g., roads, power lines, pipelines) associated with industrial activities (Lévesque and Dubé 2007). Such disturbances often increase sedimentation which has a strong negative impact on fish, both directly and due to changes in the invertebrate community prey base (Newcombe and Macdonald 1991; Trombulak and Frissell 2000; Suttle et al. 2004; Ramezani et al. 2014; Cott et al. 2015).

To understand, predict, and mitigate impacts of increasing industrialization on economically and ecologically important fish it is first necessary to accurately determine prey use. Along with identifying what fish are eating, it is important to develop an accurate understanding of the available prey base, thus permitting analysis of prey selection and

potential diet shifts in response to disturbance. Traditionally, prey availability has been determined through targeted collecting (Hasegawa et al. 2012; Domagala et al. 2015); however, this has the potential to miss prey sources that are more difficult to collect or have a patchy distribution. Since species of fish forage differently (e.g., bottom-feeders vs. surface-feeders), determining the entire prey base for a given habitat is challenging. Furthermore, morphological identification of specimens is time-consuming and requires high levels of expertise (Gibson et al. 2015). Due to these challenges, metabarcoding techniques can be used to provide a snapshot of the biodiversity potentially available as fish forage. Environmental DNA (eDNA) is DNA that can be obtained directly from environmental samples (e.g., water or soil samples) that may or may not include the physical bodies of the organisms present (Taberlet et al. 2012a; Rees et al. 2014). Two sources of eDNA show particular promise for quickly and efficiently determining the available prey in aquatic ecosystems: filtered water samples and preservative ethanol from bulk invertebrate samples. Environmental DNA from water samples has been used to successfully detect vertebrate communities (Thomsen et al. 2012; Valentini et al. 2015), but has only more recently been applied to invertebrates (Mächler et al. 2014; Section 2.11). Non-destructive metabarcoding from preservative ethanol is another newly developed technique that has the potential to identify freshwater benthic invertebrates using DNA extracted from the ethanol in which they are stored (Hajibabaei et al. 2012; Section 2.7).

Our goal was to elucidate the aquatic food web of a representative creek on the central coastal BC. We used multiple data sources to provide a substantial and potentially novel picture of a single aquatic food web; eDNA from water samples and from preservative ethanol to identify potential prey items, and molecular identification of prey from three species of fish. Our study aimed to develop a toolkit of methods that can be used to assess and predict the effects of anthropogenic changes on aquatic environments.

Methods

Chist Creek is a 5th-order stream belonging to the Kitimat River watershed of coastal BC. It is one of the larger tributaries of the Kitimat River with a length of 31.8 km and a wetted width near our sampling locations which ranged from 18.4–28.9 m. It is located in section 4 of

the proposed pipeline RoW and is crossed by the Upper Kitimat Forest Service Road at ~ 3.5 km (Figure 2-81 and Figure 2-82). Chist Creek was sampled at two sites for environmental DNA (eDNA) and benthic invertebrates: at 200 m upstream of the RoW (+200 m), and at 200 m downstream of RoW (-200 m). Sample collection started at the -200m site and proceeded upstream to minimize disturbance and avoid contamination.

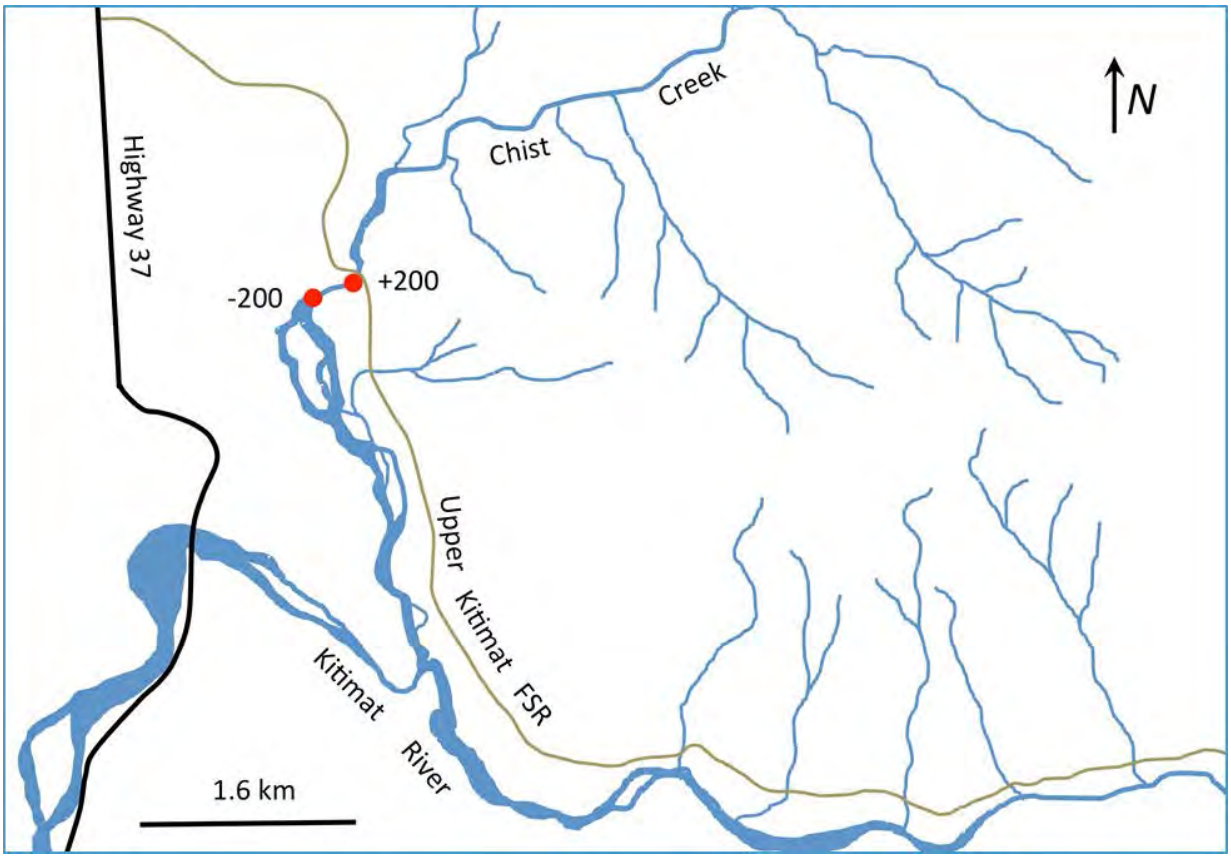


Figure 2-81 – Map of the sampling locations (●) for eDNA, invertebrates and fish in Chist Creek

eDNA collection

On July 29, 2014 three, 2-L water samples were collected at each site in sterile Nalgene bottles from the upper 5 cm of flowing surface water (in ripples). When sampling, we collected upstream from our standing location to reduce potential contamination by clothing or equipment. Immediately after collection, 1 L steam-water samples were vacuum filtered through 47 mm diameter, 0.45 µm pore size, mixed-cellulose filters (Cole-Palmer, Montreal,

QC, Catalog No. A045A047A) for a total of two filter papers per 2-L water sample. Each filter was then preserved in 95 % ethanol and stored at -20 °C until extracted for DNA. In total, six water samples were taken from Chist Creek for this analysis. To monitor for contamination from field and laboratory equipment, a 1-L Nalgene bottle filled with distilled water was taken to each stream and subjected to the same filtering process using the sterilized equipment.

Figure 2-82 – Chist Creek looking south at a section approximately 200 m above the RoW. Photograph was taken from the bridge on the Upper Kitimat FSR.

Benthic invertebrate collection

Immediately following water sample collection, benthic invertebrates were sampled from sites above and below the RoW. Three replicates were collected at each site, each from a different nearby riffle. Sampling methods were adapted from protocols developed for streams in the Greater Vancouver Regional District (Page et al. 2008). At each replicate location, benthic invertebrate sampling was conducted using a Surber sampler (Dynamic Aqua Supply, Surrey, BC, Catalog No. SBN250) with a 30 × 30 cm frame (0.09 m^2) and 250-µm mesh size. One replicate Surber sample was a composite of three 3-min placements within one riffle working

sequentially upstream from each sampling incident. During a placement, large rocks within the sampling frame were rubbed and removed causing invertebrates to float downstream into the mesh, and then gravel and sand were mixed for invertebrates to be carried into the mesh. At the end of the 3-min interval the sampler was moved upstream to an undisturbed area in the same riffle for another 3-min placement. A third placement in the same riffle, upstream of the previous samples, completed the composite Surber sample.

After the sample was collected, five elutriation steps were performed to concentrate the organic material and discard inorganic debris. A sterilized 5-L bucket was used to swirl the sample and suspend the organic material, which was then poured back into the funnel of the Surber sampler. After the inorganic debris was rinsed five times, it was discarded. The organic material was then transferred to a 500 mL plastic bottle and preserved in 95 % ethanol. In the laboratory, ethanol from each sample was replaced with clean 95 % ethanol and samples were stored at -20 °C. The ethanol used to originally preserve the invertebrates (ethanol-based DNA) was then used as a source target of DNA for genetic analysis.

Fish collection and processing

Fish collection was conducted on July 23, 2014 using a backpack electrofisher (Model LR-24, Smith-Root Inc., Vancouver, WA). Fish were captured in two 100-m sections; downstream from approximately -250 – -150 m and upstream from approximately +150 – +250 m. After capture, fish were anesthetized in a bucket containing 200 mg × L^{-1} MS-222 (tricaine methanesulfonate) buffered with 400 mg × L^{-1} sodium bicarbonate. Once fish were anesthetized, fork length and weight were determined to the nearest 1 mm and 0.1 g for each fish, stomachs removed, preserved in 95 % ethanol, and stored at -20 °C until processing. Using sterile techniques in the laboratory, stomachs were removed from the 95 % ethanol and weighed to the nearest 0.1 g. Subsequently, each gastrointestinal (GI) tract was opened with a clean scalpel and forceps, and contents were flushed into a sterile petri dish using 95 % ethanol. To avoid scraping cells from the predator stomach lining, the stomach contents were carefully removed by either flushing with 95 % ethanol or physical removal with tweezers. Sorting of stomach contents focused on isolating and identifying invertebrate heads, as this standardized the quantification of prey presence for future analysis. The remaining partially digested

invertebrate prey matter was combined into a 15-mL tube (bulk stomach content samples) and stored in 95 % ethanol at -20 °C until DNA extraction.

eDNA extraction

DNA was extracted from the filters using bead beating and the DNeasy Blood and Tissue Kit: spin-column protocol (Qiagen, Toronto, ON, Catalog No. 69506). To avoid contamination, all extractions were performed in a room dedicated to low-quality DNA sources. No DNA samples from other species were handled in this room and separate laboratory coats, pipets, pipette filter tips and laboratory equipment were used to perform the extractions at that location. Additionally, all equipment was first sterilized using a 10 % bleach solution and exposed to UV light for a minimum of 15 min prior to each set of extractions. To monitor for contamination, a negative control was included for each set of water filter DNA extractions.

Filters were removed from the ethanol and cut into ca. 1-mm slices using sterile forceps and tweezers. The filter pieces were placed into 2-mL tubes that contained two 5/32'' steel grinding beads (Ops Diagnostics, Lebanon, NJ, Catalog No. GBSS 156-5000-01) and left to air-dry overnight. Filter pieces were then shaken in the Genogrinder 2000 (BT&C Inc., Burlington, ON) at 1500 oscillations × min^{-1} for 90 s. To prevent contamination, the filter particles were spun for 1 min at 6000 g before opening the tube and adding 870 µL Buffer ATL and 30 µL Proteinase K solution. The tubes were incubated at 56 °C for 30 min with 150 rpm agitation, then shaken in the Genogrinder at 1,000 oscillations × min^{-1} for 60 s. Samples were incubated once again at 56 °C with agitation for 1.5 h. After the final incubation step, samples were vortexed for 15 secs and spun for 3 min at 10,000 × g. The supernatant (~600 µL) from each tube was transferred into a new, labelled 2-mL tube without disturbing the filter paper pellet. Because multiple filters were required to filter each 2-L water sample, the supernatants from the appropriate samples were combined before proceeding with the wash steps. Hereafter the supernatant was subjected to a classical DNA extraction for the remaining steps with the Qiagen DNeasy Blood and Tissue Kit (manufacturer's protocol) using the following modifications; 600 µL Buffer AL, 600-µL 95 % ethanol, and final elution steps of 2 × 50 µL AE Buffer for each sample. The DNA concentration of each eDNA extract was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS

Assay, Life Technologies, Burlington, ON, Catalog No. Q32854) and stored at -20 °C until PCR amplification.

DNA extraction: ethanol from Surber samples

To correspond with the eDNA analysis, ethanol-based DNA analysis was performed on Surber samples collected at the two sites upstream and downstream of the RoW. All extractions were done in a dedicated low-quality DNA room. To monitor for contamination a negative control was included for each set of ethanol DNA extractions.

Each sample was inverted ten times to mix and re-suspend the DNA contents in the ethanol. Approximately 40 mL of the preserved ethanol was transferred into a clean, sterile 50-mL centrifuge tube(s) and centrifuged at 18,000 × g for 1 h at 4 °C to recover precipitated DNA. Taking care not to disturb the formed pellet, approximately 38 mL of supernatant was removed. The remaining ethanol (1.5 – 2 mL) was gently swirled to re-suspend the pellet. With a clean, sterile disposable pipette, the pellet was transferred to a sterile 2-mL microcentrifuge tube and centrifuged at 18,000 × g for 30 min at 4 °C; ethanol was removed without disturbing the pellet and samples left overnight to evaporate any remaining supernatant. For the remaining steps, the ethanol-based DNA was subjected to a classical DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (spin-column protocol) beginning at Step 3. Additionally, 3 × 50 µL AE Buffer was used for the final elution step. The DNA concentration of each ethanol-based DNA sample was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay) and stored at -20 °C until PCR amplification.

DNA extraction: invertebrates from stomach contents

Bulk stomach content samples from five Dolly Varden (*Salvelinus malma*), five rainbow trout (*Oncorhynchus mykiss*), and five coastrange sculpin (*Cottus aleuticus*) were used for the analysis. To avoid contamination, all extractions were performed in a dedicated low-quality DNA room. To monitor for contamination a negative control was included for each set of stomach content DNA extractions.

Samples were centrifuged for 1 min at 20,000 × g to collect the prey items at the bottom of the tube. Excess ethanol was removed and the stomach contents were transferred into new sterile 2-mL centrifuge tubes using sterile techniques. Two 5/32'' steel grinding beads were

then added to each 2 mL tube and shaken in the Genogrinder at 1500 oscillations × min^{-1} for 5 min. The grinding beads were removed with sterile forceps and approximately 200 µL of homogenized contents were then transferred into a pre-weighed 1.5-mL centrifuge tube. Tubes were opened and left overnight at 37 °C to evaporate remaining ethanol. Stomach content samples were subjected to the classical DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (spin-column protocol), with the modification of using 2 × 75 µL AE Buffer for the final elution steps. DNA concentration of each bulk sample was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay) and stored at -20 °C until PCR amplification.

Selection of universal invertebrate primers

To target a 157-bp fragment located at the 5' end of the barcoding gene cytochrome c oxidase subunit I (COI), DNA was amplified using the general invertebrate COI forward primer (ZBJ F) published by Zeale et al. (2011) (Table 2-70). To improve amplification of the target sequence a reverse primer was designed using COI sequences obtained through the DNA Barcoding project (Section 2.6). This primer, EPT reverse (EPT R; Table 2-67) targets the same 157-bp amplicon located within the 5' end of standard COI barcode region (Hebert et al. 2003) amplified using the ZBJ primers.

Table 2-70 – List of primers used to amplify extracted DNA for next generation sequencing. The forward ZBJ forward primer (ZBJ-F) is from Zeale et al. (2011) and the reverse EPT primer (EPT-R) was specifically designed as a degenerate primer to amplify the COI gene for common aquatic invertebrates in central BC. Base pair Y = CT; R = AG; H = ACT; and N = ACGT. UniA and UniB sequences included within the ZBJ-F and EPT-R primer (shown in bold). Primer P1UniB was attached to the amplified product in the PCR2 step.

Name	Sequence	Amplicon length
ZBJ-F: UniA_ZBJ-ArtF1c	**ACCTGCCTGCCG**AGATATTGGAACWTTATATTTTATTTTTGG	157
EPT-R: UniB_EPTD4d	**ACGCCACCGAGC**ACTAAYCARTTNCCRAAHCCHCC	
P1UniB	CCTCTCTATGGGCAGTCGGTGAT**ACGCCACCGAGC**	-

PCR amplification

We prepared the samples for unidirectional sequencing on a PGM Ion Torrent Sequencer (Burlington, ON) using a two-step PCR amplification method that incorporates fusion

primers designed at the University of Windsor (D. Heath, *pers. comm.*). Two tailed-end sequences, UniA and UniB, were added to the end of primers sequences used in the current study: UniA onto the forward primer (ZBJ F) and UniB onto both reverse primers, ZBJ R and EPT R (Table 2-67). These "universal" tailed-end sequences are used in the second PCR step to incorporate the Ion Torrent adaptor sequences into the final amplicon sequence: an IonA adaptor sequence and a unique 10-bp identifier sequence (used to identify the individual samples sent for sequencing) bind to UniA sequence, and the P1 adaptor sequence used to initiate the sequencing reaction during an Ion Torrent sequencing run binds to the UniB sequence. The P1 adaptor sequence is required for correct attachment of prepared amplicons to the Ion Spheres, which rest in the wells of the semi-conductor chip and the IonA adaptor the priming site for the sequencing reaction on the Ion Torrent chip (data not shown).

During the first PCR step (PCR1), each sample was amplified in 35 µL reaction volumes in triplicate using a Qiagen Multiplex PCR kit (Toronto, ON, Catalog No. 206143). Each reaction contained 17.8 µL Multiplex MasterMix, 0.75 µL UniA-tailed forward primer (10 µM), 0.75 µL UniB-tailed reverse primer (10 µM), and 15.0 µL of nuclease-free water and extracted DNA which combined for a total of 20-60 ng of template DNA per reaction. Also included were a negative extraction control, a negative PCR control, and a positive control (mock community), each in 35 µL reaction volumes and in triplicate.

The second PCR step (PCR2) attached the IonA and P1 adaptors to prepared amplicons; P1 to the UniB primed end and IonA and the unique identifier to the UniA primed end (Table 2-70). Each PCR reaction contained 3.6 µL 10 x reaction buffer, 1.5 µL $MgCl_2$ (25 mM), 0.75 µL dNTPs mix (10 mM), 0.75 µL IonX-UniA primer (10 µM), 0.75 µL P1-UniB primer (10 µM), and 0.15 µL Platinum Taq polymerase (5 Units × μL^{-1}) (Invitrogen, Burlington, ON, Catalog No. 1096634). The amount of purified PCR1 product added to the reaction varied depending on the concentration. As a standard, we added approximately 30–50 ng of purified PCR1 template to each reaction and adjusted the amount of nuclease free water accordingly for a total volume of 15 µL. Samples were amplified at 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 75 °C for 5 min.

A subset of samples were confirmed using Experion DNA 1K Analysis kit (Bio-Rad, Mississauga, ON, Catalog No. 7007107) to verify that the barcodes attached correctly to the PCR1 amplicons. The triplicates of each sample were then pooled (~60 µL), purified with Agencourt AMPure XP purification beads, and quantified using QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

Preparation of COI amplicons for Ion torrent sequencing

Samples were normalized by pooling equimolar ratios of each sample, as well as 5 µL of each negative control, which had DNA concentrations too low to be read by QUBIT® 2.0 Fluorometer (or significantly lower than that of Surber ethanol samples or positive controls) as expected. Two 40-µL aliquots of the final normalized COI amplicon library (all samples and negatives controls) were then run on 1.5 % TAE agarose gels stained with ethidium bromide at 200 v for 1.5 h to allow sufficient separation of the 295-bp bands (amplicons plus all required adaptor sequences) from those containing amplicons with only one adaptor sequence attached (255 and 275 bp). The desired amplicon was extracted from the gel by visualizing the bands on a UV-light box using a clean, sterile scalpel and the DNA purified using Qiagen MinElute Gel Extraction Kits (Toronto, ON, Catalog No. 28606). Finally, the purified DNA was analyzed using the Experion DNA 1K Analysis Kit to verify successful excision of the correct band from the gel and determine the concentration of the resulting COI amplicon library for submission to the Great Lakes Institute for Environmental Research (GLIER) Lab at the University of Windsor, ON, and sequenced using a PGM Ion Torrent Sequencer using an Ion Torrent 318 chip.

Data analysis

Bioinformatic analyses were performed on the Rocks server using the UNBC High Performance Computing Lab. A python script was used to strip non-biologically informative sequences (primer and UniA sequences) from the raw reads and relabel reads with the unique identifier label (Sample ID) associated with each read. USEARCH (Edgar 2005) was used to filter the resulting sequences for quality using a maximum error rate of 0.5 (allows one error for every 200 bp) and to truncate sequences to the expected amplicon length (157 bp). Sequences were sorted by size using a minimum size of two sequences to remove singletons, and then clustered into operational taxonomic units (OTUs) using a sequence identity of 97%, with the

resulting sequences re-labelled with sequential OTU numbers, and a fasta file containing all OTUs generated. OTUs were then mapped back onto the original reads (COI sequences labeled with unique sample identifiers), including singletons, and a taxonomy table generated that includes the number of all sequences for each OTUs contained in each sample and negative controls if present.

MegaBLAST (www.ncbi.nlm.nih.gov) was used to assign taxonomy to the resulting OTU sequences. The BLASTn file containing all OTUs and their top hits in the GenBank database (i.e., taxonomic identity of the organism with closest sequence identity to the queried OTU) and the USEARCH OTU fasta file were imported into MEGAN to allow the taxonomic assignments for each OTU to be exported as a tab separated value (.tsv) file for manipulation in Excel. Taxonomic assignments were added to the OTU table containing all OTUs present in each sample and control and the data analysed in Excel to determine which taxa were detected in each sample analysed.

Results

Amplification and sequencing

DNA was successfully isolated from all samples, with PCR1 amplifying the desired 157-bp length of the COI gene region. PCR2 was performed on the purified PCR1 product and produced the desired approximately 300-bp length amplicons (Figure 2-83), indicating successful bonding with the Ion Torrent specific UniA and UniB sequences. Experion results following PCR2 show three congregated peaks, each representing a different binding outcome of PCR1 product with the UniA and UniB sequences (Figure 2-84). The larger 303-bp fragment indicates the desired outcome of having both UniA and UniB sequences being attached to the PCR1 product. The largest fragment isolated and submitted for sequencing was also produced in the highest concentration.

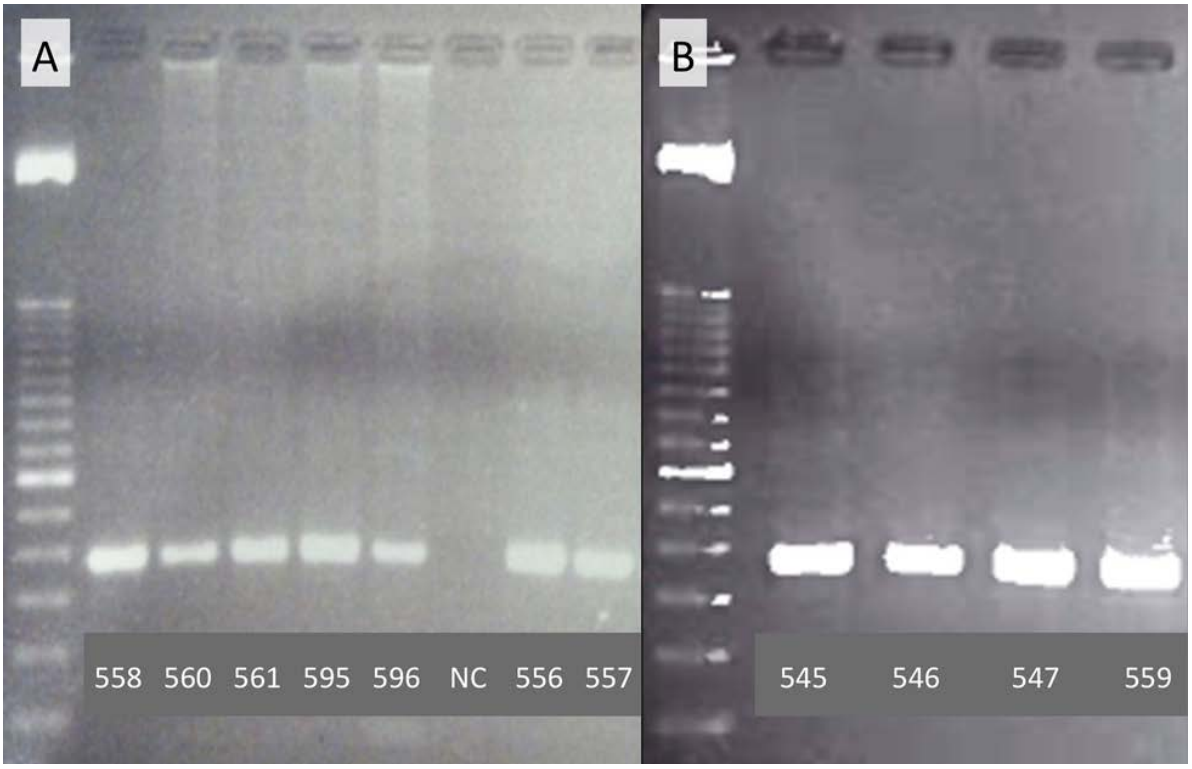
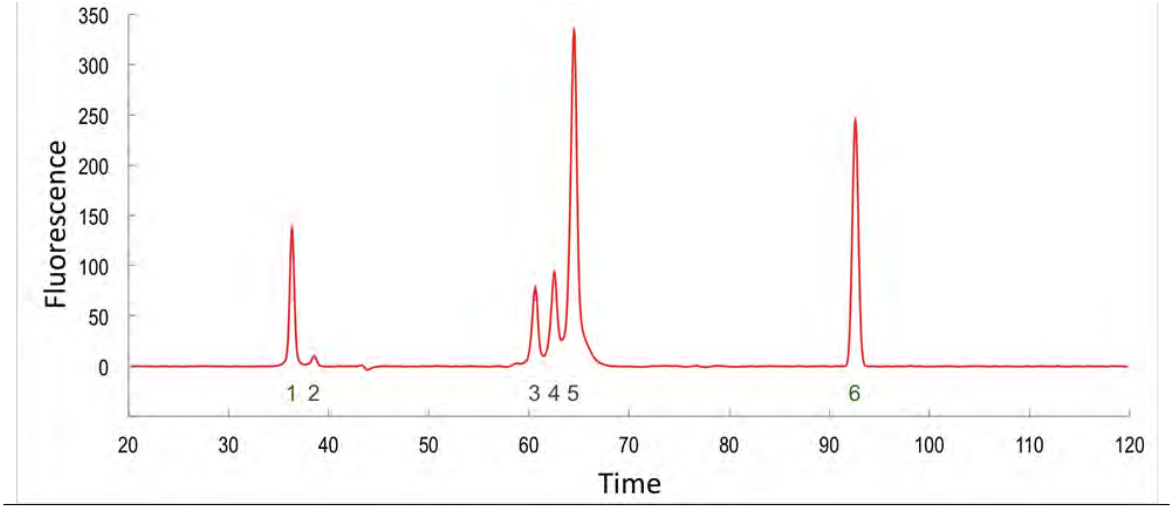


Figure 2-83 – Representative agarose gels of the COI amplicons for (A) Dolly Varden (*Salvelinus malma*; 557, 558, 560, 595, 596), rainbow trout (*Oncorhynchus mykiss*; 556, 561), and (B) coastrange sculpin (*Cottus aleuticus*; 545, 546, 547, 559) stomach content samples after the initial PCR amplification with ZBJ-F and EPT-R primers. Amplification was performed in triplicate for each sample. NC is a negative control. Ladder (100 bp) shown in the left lane of each gel.

Species composition

Genetic analysis identified 108 OTUs for invertebrates from four Orders — Ephemeroptera, Plecoptera, Trichoptera, and Diptera — to the level of *Genus* or *species*, belonging to 30 different Families. Ephemeroptera had the most unique species level matches with 28, followed by Trichoptera with 16, Plecoptera and Diptera both had nine, while Diptera taxa were largely identified only to the level of *Genus* (Figure 2-85). Diptera and Ephemeroptera were the most abundant of the total taxa with 37 and 34, respectively. Trichoptera contained the most Families with 11 (Table 2-71).



Peak Number	Size (bp)	Concentration (ng · µl^{-1})	Observation
1	15		Lower Marker
2	25	0.4	
3	260	1.7	
4	281	2.1	
5	303	8.1	
6	1500		Upper Marker

Figure 2-84 – Experion output indicating the COI fragment sizes and their corresponding concentrations following the PCR2 amplification step for a single Coastrange sculpin stomach content sample. The desired amplicon size of approximately 300 bp is shown in bold.

The majority of OTUs were found in the eDNA (85.2 %) and EtOH (69.4 %) samples. Only 16 of the total 108 taxa were not present within eDNA samples; 33 taxa were not identified in the EtOH samples. eDNA was able to detect 33 taxa not picked up by EtOH while EtOH detected 15 taxa which eluded eDNA. Of the 51 unique taxa found within the fish stomach contents, eDNA failed to detect four — *Acentrella*, *Ephemerella dorothea infrequens*, *Ceratopsyche amblis*, and *Phaenopsectra*.; although the Baetidae *Genus Acentrella* was detected as *A. tubida* in the eDNA sample. EtOH samples did not detect five taxa found in fish stomachs; *Phaenopsectra*, *Rhyacophila vao*, *Heterotrissocladius*, *Limonia nubeculosa*, and *Glossosoma intermedium*.

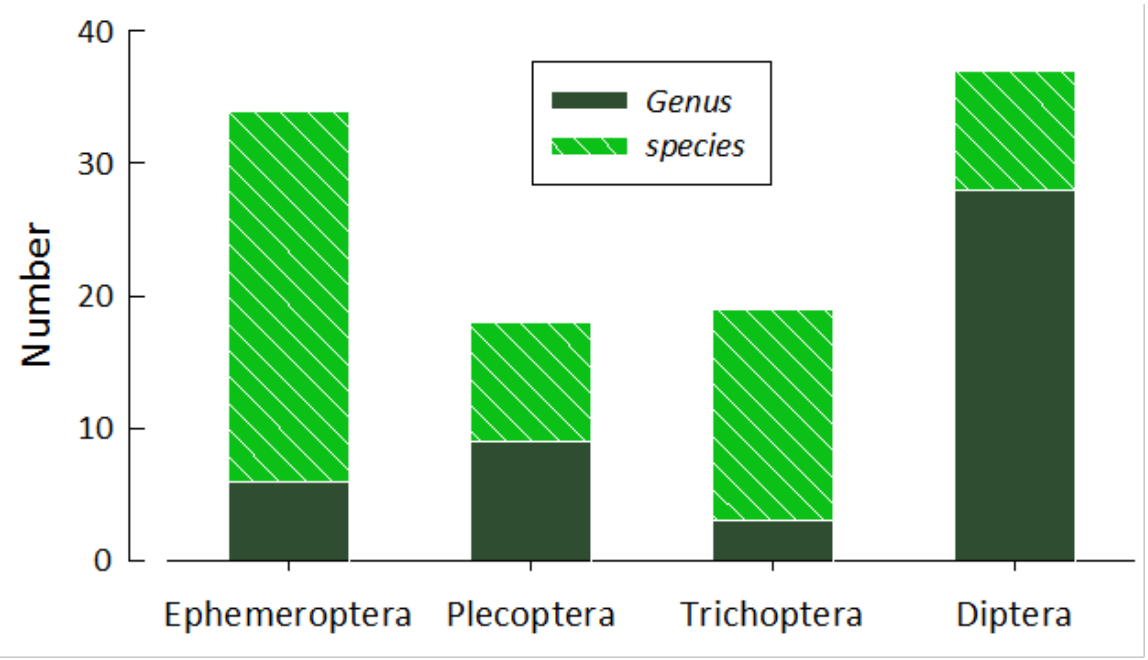


Figure 2-85 – The number of Operational Taxonomic Unit (OTU) sequences assigned to the level of *Genus* and *species* for the major Orders of aquatic invertebrates detected in Chist Creek.

In addition to the 51 taxa identified from fish GI contents, eDNA and EtOH detected 57 additional taxa in the Chist Creek samples; 46 and 29 for eDNA and EtOH, respectively. These 57 taxa were from 13 Families which were not present the diets of the three fish species — six Trichoptera, five Diptera, and two Plecoptera — with eDNA picking up all but one of these Families and EtOH picking up five (Table 2-72, Table 2-73, Table 2-74 and Table 2-75).

Table 2-71 – Number of different Families, Genera, and species detected from the next generation sequencing analysis for the four most abundant Orders of aquatic invertebrates present in filtered water samples, ethanol from Surber samples, and the stomachs of rainbow trout, Dolly Varden, and coastrange sculpin.

Order	Families	Genera	species
Ephemeroptera	5	13	28
Plecoptera	5	14	9
Trichoptera	11	14	16
Diptera	9	33	9

Ephemeroptera

The majority of Genus and species level OTUs for Ephemeroptera were found in the eDNA and EtOH samples. Thirty-four taxa from Ephemeroptera were identified within all samples; 21 were detected as prey items of fish. There was considerable overlap of Ephemeroptera prey among the three species of fish. Twenty of the 21 OTUs were found in prickly sculpin stomachs, whereas 19 of 21 were found in both rainbow trout and Dolly Varden. *Paraleptophlebia* was found in all samples; to the level of Genus in all samples, but the species *P. heteronea* was not detected in rainbow trout (Table 2-72).

Environmental DNA detected all but two taxa identified in fish gut contents while also accounting for 10 additional species level taxa, six which were exclusive to eDNA. EtOH detected all taxa in the fish with seven additional species level identifications, three only found in EtOH. These two methods combined to detect 13 taxa, which were absent from the fish, belonging to nine different *Genera*. Of the nine *Genera* containing 13 unique *species* level identifications from eDNA and EtOH, three appeared in the fish as a Genus level match — *Baetis*, *Ephemerella*, and *Paraleptophlebia* — which accounted for five of these 13 unique species. In total eDNA did not detect five OTUs, two of which were present in the fish. EtOH did not detect six taxa (Table 2-72).

Plecoptera

Similar to the Order Ephemeroptera, the majority of OTUs within the Order Plecoptera were detected in eDNA and EtOH samples (Table 2-73). There were 18 Plecoptera OTUs identified in all samples; only seven were found in fish gut contents, but all belonged to different *Genera*. Three of the taxa were present in each fish, with rainbow trout and coastrange sculpin consuming five taxa each, and Dolly Varden consuming four (Table 2-73). All OTUs detected as prey in the fish were also detected in the eDNA samples from the water and the EtOH from the Surber samples. Eleven additional OTUs were found in the eDNA and EtOH samples; although only three of the OTUs were common to both eDNA and EtOH samples. Two Families, Capniidae and Leuctridae, were detected in eDNA or EtOH, but were not prey items for any of the fish species (Table 2-73).

Table 2-72 – Summary of next generation sequencing results indicating Families, Genera and species from the Order Ephemeroptera identified in filtered water samples (eDNA), ethanol from Surber samples of aquatic invertebrates (EtOH), homogenized stomach contents from rainbow trout (RBT), Dolly Varden (DV), and coastrange sculpin (CAL).

Family	*Genus species*	eDNA	EtOH	RBT	DV	CAL
Baetidae	*Acentrella turbida*	✓	✓	✓	✓	✓
Ephemerellidae	*Drunella doddsi*	✓	✓	✓	✓	✓
Baetidae	*Baetis tricaudatus*	✓	✓	✓	✓	✓
Heptageniidae	*Epeorus longimanus A BG*	✓	✓	✓	✓	✓
Ameletidae	*Ameletus cooki*	✓	✓	✓	✓	✓
Heptageniidae	*Epeorus deceptivus*	✓	✓	✓	✓	✓
Ephemerellidae	*Drunella coloradensis*	✓	✓	✓	✓	✓
Ephemerellidae	*Ephemerella tibialis*	✓	✓	✓	✓	✓
Ameletidae	*Ameletus validus*	✓	✓	✓	✓	✓
Ephemerellidae	*Ephemerella*	✓	✓	✓	✓	✓
Heptageniidae	*Cinygmula sp. A BG*	✓	✓	✓	✓	✓
Baetidae	*Baetis*	✓	✓	✓	✓	✓
Heptageniidae	*Rhithrogena robusta*	✓	✓	✓	✓	✓
Heptageniidae	*Cinygmula*	✓	✓	✓	✓	✓
Heptageniidae	*Epeorus*	✓	✓	✓	✓	✓
Baetidae	*Acentrella*		✓	✓	✓	✓
Leptophlebiidae	*Paraleptophlebia heteronea*	✓	✓		✓	✓
Heptageniidae	*Epeorus longimanus B BG*	✓	✓		✓	✓
Ameletidae	*Ameletus pritchardi*	✓	✓	✓		✓
Leptophlebiidae	*Paraleptophlebia*	✓	✓	✓		✓
Ephemerellidae	*Ephemerella dorothea infrequens*		✓	✓	✓	
Baetidae	*Baetis bicaudatus*	✓	✓			
Leptophlebiidae	*Paraleptophlebia vaciva*	✓	✓			
Ephemerellidae	*Drunella grandis*	✓	✓			
Ephemerellidae	*Ephemerella aurivillii*	✓	✓			
Leptophlebiidae	*Paraleptophlebia memorialis*		✓			
Ephemerellidae	*Attenella margarita*		✓			
Baetidae	*Diphetor hageni*		✓			
Heptageniidae	*Ecdyonurus corsicus*	✓				
Ameletidae	*Ameletus suffusus*	✓				
Ameletidae	*Ameletus similior*	✓				
Leptophlebiidae	*Paraleptophlebia debilis*	✓				
Heptageniidae	*Maccaffertium terminatum*	✓				
Ameletidae	*Ameletus celer*	✓				

Table 2-73 – Summary of next generation sequencing results indicating Families, Genera and species from the Order Plecoptera identified in filtered water samples (eDNA), ethanol from Surber samples of aquatic invertebrates (EtOH), homogenized stomach contents from rainbow trout (RBT), Dolly Varden (DV), and coastrange sculpin (CAL).

Family	*Genus species*	eDNA	EtOH	RBT	DV	CAL
Perlodidae	*Steatoda*	✓	✓	✓	✓	✓
Perlodidae	*Kogotus*	✓	✓	✓	✓	✓
Chloroperlidae	*Suwallia*	✓	✓	✓	✓	✓
Chloroperlidae	*Sweltsa*	✓	✓	✓		✓
Perlodidae	*Arcynopteryx compacta*	✓	✓			✓
Nemouridae	*Zapada*	✓	✓		✓	
Perlodidae	*Isoperla petersoni*	✓	✓	✓		
Capniidae	*Eucapnopsis brevicauda*	✓	✓			
Chloroperlidae	*Plumiperla diversa*	✓	✓			
Nemouridae	*Zapada cinctipes*	✓	✓			
Chloroperlidae	*Haploperla*		✓			
Chloroperlidae	*Sweltsa coloradensis*		✓			
Capniidae	*Capnia*		✓			
Perlodidae	*Isoperla fulva*		✓			
Chloroperlidae	*Alloperla*	✓				
Perlodidae	*Megarcys signata*	✓				
Perlodidae	*Megarcys*	✓				
Leuctridae	*Paraleuctra occidentalis*	✓				

Trichoptera

Although 19 OTUs from the Order Trichoptera were identified within the eDNA or EtOH samples, only five were detected as prey for fish — just three OTUs for rainbow trout and Dolly Varden and only two OTUs for coastrange sculpin (Table 2-74). OTUs for the *Genera Lepidostoma and Rhyacophila* were identified in eDNA only, but present to the species level in fish gut contents, *L. cascadense* and *R. vao*. Only one species of Trichoptera was present within all samples, *Dicosmoecus atripes*. Some of the OTUs detected as prey items were not observed in the eDNA samples (1) or the EtOH samples (2) (Table 2-74).

Diptera

There were 37 total Diptera taxa identified within all samples representing the Order of aquatic invertebrates with the greatest diversity; 18 different OTUs were prey items for the fish (Table 2-75). Six of the 18 taxa were present in all fish. Dolly Varden contained 15 taxa, rainbow trout 11, and coastrange sculpin 8. Only three species level OTUS, however, were present

within the fish. The *Genus Phaenopsectra* was the only taxa among all Orders to be a prey item for fish, but was not detected in either the eDNA or EtOH samples.

Table 2-74 – Summary of next generation sequencing results indicating Families, Genera and species from the Order Trichoptera identified in filtered water samples (eDNA), ethanol from Surber samples of aquatic invertebrates (EtOH), homogenized stomach contents from rainbow trout (RBT), Dolly Varden (DV), and coastrange sculpin (CAL).

Family	*Genus species*	eDNA	EtOH	RBT	DV	CAL
Limnephilidae	*Dicosmoecus atripes*	✓	✓	✓	✓	✓
Lepidostomatidae	*Lepidostoma cascadense*	✓	✓	✓	✓	
Glossosomatidae	*Glossosoma intermedium*	✓				✓
Rhyacophilidae	*Rhyacophila vao*	✓			✓	
Ceratopsyche	*Ceratopsyche amblis*		✓	✓		
Goeridae	*Goerita semata*	✓	✓			
Limnephilidae	*Onocosmoecus unicolor*	✓	✓			
Glossosomatidae	*Glossosoma penitus*	✓	✓			
Rhyacophilidae	*Rhyacophila vaccua*	✓	✓			
Arctopsyche	*Arctopsyche grandis*	✓	✓			
Leptoceridae	*Ceraclea annulicornis*	✓	✓			
Lepidostomatidae	*Lepidostomatinae*		✓			
Glossosomatidae	*Anagapetus*		✓			
Rhyacophilidae	*Rhyacophila*	✓				
Lepidostomatidae	*Lepidostoma*	✓				
Helicophidae	*Helicopha einap*	✓				
Uenoidae	*Oligophlebodes sierra*	✓				
Rhyacophilidae	*Rhyacophila bifila*	✓				
Parapsyche	*Parapsyche elsis*	✓				
Limnephilidae	*Pycnopsyche virginica*	✓				

eDNA and EtOH accounted for 19 additional taxa that were not detected in the stomach contents of fish. Sixteen taxa were identified in the eDNA samples, but were not present in any of the fish stomach content samples; 11 OTUs were unique to eDNA. Not all taxa, however, were detected in the eDNA; three OTUS were found in the EtOH samples, but not eDNA. The greatest diversity of taxa for the Order Diptera was found in the eDNA samples; 14 OTUs were identified in eDNA, but not EtOH. Interestingly, three of these OTUs were identified as prey items for fish. In addition to the three Families identified in the fish, eDNA and EtOH also detected five more unique Families. Of the taxa representing the five additional Families, however, three are solely terrestrial Dipterans, *Dilophus femoratus, Bicellaria,* and *Siphona*

flavipes; the remaining two taxa belong to *Oreogeton*, which was present in Chist Creek Surber samples, and *Spilogona,* which could be aquatic or terrestrial (Table 2-75).

Table 2-75 – Summary of next generation sequencing results indicating Families, Genera and species from the Order Diptera identified in filtered water samples (eDNA), ethanol from Surber samples of aquatic invertebrates (EtOH), homogenized stomach contents from rainbow trout (RBT), Dolly Varden (DV), and coastrange sculpin (CAL).

Family	*Genus Species*	eDNA	EtOH	RBT	DV	CAL
Chironomidae	*Stempellinella*	✓	✓	✓	✓	✓
Chironomidae	*Barbadocladius*	✓	✓	✓	✓	✓
Chironomidae	*Orthocladius*	✓	✓	✓	✓	✓
Simuliidae	*Simulium tuberosum*	✓	✓	✓	✓	✓
Simuliidae	*Simulium*	✓	✓	✓	✓	✓
Chironomidae	*Pagastia*	✓	✓	✓	✓	✓
Chironomidae	*Eukiefferiella*	✓	✓		✓	✓
Chironomidae	*Parametriocnemus*	✓	✓	✓	✓	
Chironomidae	*Polypedilum*	✓	✓	✓	✓	
Chironomidae	*Orthocladius dorenus*	✓	✓	✓	✓	
Chironomidae	*Corynoneura*	✓	✓			✓
Chironomidae	*Thienemannimyia*	✓	✓		✓	
Chironomidae	*Zavrelimyia*	✓	✓		✓	
Chironomidae	*Diamesa*	✓	✓		✓	
Chironomidae	*Micropsectra*	✓	✓	✓		
Chironomidae	*Heterotrissocladius*	✓			✓	
Tipulidae	*Limonia nubeculosa*	✓			✓	
Simuliidae	*Prosimulium*	✓	✓			
Chironomidae	*Tanytarsus*	✓	✓			
Chironomidae	*Brillia*	✓	✓			
Chironomidae	*Cricotopus*	✓	✓			
Chironomidae	*Micropsectra lacustris*	✓	✓			
Chironomidae	*Phaenopsectra*			✓		
Muscidae	*Spilogona*		✓			
Chironomidae	*Orthocladius oliveri*		✓			
Chironomidae	*Thienemanniella*		✓			
Tipulidae	*Pterelachisus*	✓				
Chironomidae	*Parachironomus arcuatus*	✓				
Chironomidae	*Gymnometriocnemus brumalis*	✓				
Empididae	*Oreogeton*	✓				
Hybotidae	*Bicellaria*	✓				
Bibionidae	*Dilophus femoratus*	✓				
Tipulidae	*Tipula*	✓				
Chironomidae	*Conchapelopia*	✓				
Chironomidae	*Paratrichocladius*	✓				
Chironomidae	*Psectrocladius*	✓				
Tachinidae	*Siphona flavipes*	✓				

Discussion

Considerable diversity of invertebrate taxa was identified in Chist Creek using environmental DNA from both filtered water and ethanol preservative from Surber sampling. There was a strong concordance between visual determination of what was in the Surber samples and the ethanol preservative using NGS sequencing. In Section 2.7, we found that genetic analysis of Surber EtOH identified a greater number of taxa and improved resolution compared to morphological identification of Surber samples — consequently metabarcoding represents a superior alternative to traditional morphological assessments of benthic invertebrate communities. Our analysis of the ethanol preservative from Surber samples therefore provided a qualitative assessment of the biodiversity of benthic invertebrates in Chist Creek. Genetic analysis of eDNA provided an even greater scope of the invertebrate community within Chist Creek compared with the benthic invertebrate sampling protocol (Section 2.11). Similarly, we found diversity of the major aquatic invertebrate taxa (Ephemeroptera, Plecoptera, Trichoptera, and Diptera) was greatest from eDNA samples compared to EtOH. Such a result was not surprising as taxa from the EtOH samples were derived from a sampling method designed to target benthic invertebrates in flowing water which tend to be larvae or nymphs that are relatively sedentary. Consequently, Surber sampling may have missed invertebrates that use alternate stream habitats such as pools, deep or very shallow sections of the stream, or finer substrate.

The taxonomic diversity obtained from the water eDNA samples was larger than from Surber ethanol, partly due to the inclusion of three terrestrial taxa, all belonging to Diptera. Interestingly, a fourth taxa from the Order Diptera which may be terrestrial was detected in the EtOH samples, *Spilogona*. The presence of the three terrestrial taxa only in the eDNA samples exemplifies the ability and power of eDNA to give a more complete picture of the aquatic invertebrate community. Further, the increased breadth of invertebrate taxa including terrestrial invertebrates provides a more comprehensive analysis of biodiversity and potential prey availability for fish diet.

Each fish contained only a subset of the available prey identified in the water and Surber preservative eDNA — suggesting selectivity of prey from the overall invertebrate community. It

is also possible that fish may be preying on the most numerous invertebrates in the stream. This would imply a quantitative aspect for stream taxa abundance and that those detected within the fish are a dominant component of the invertebrate community. Considerable overlap in taxa consumed was exhibited for most Orders among the three species of fish sampled and was most apparent with the Order Ephemeroptera. This was an unexpected result as coastrange sculpin are mainly benthic feeders (Brown et al. 1995), whereas salmonids predominantly feed on drift prey in the water column (Johansen et al. 2010) or on terrestrial inputs on the surface (Nakano et al. 1999). Juvenile salmonids, such as those sampled in the present study, may exhibit a different feeding behaviour. Domagala et al. (2015) demonstrated that juvenile salmonids exploited benthic invertebrates in addition to other taxa as prey items, suggesting that juvenile salmonid diet may be similar to coastrange sculpin — consistent with the findings of the present study and our work on a central interior stream comparing rainbow trout and prickly sculpin (*Cottus asper*; Section 2.9).

Next generation sequencing resulted in good taxonomic resolution for the identified OTUs; 62 of the 108 OTUs were identified to the level of species. Identification to the level of species was prominent for the Orders Ephemeroptera, Plecoptera, and Trichoptera, but for Diptera Genus level identifications were most frequent. Diptera is one of the largest Orders of Arthropoda, with Chironomidae being one of the largest Families within the Order Diptera. In addition to their abundance and diversity, identifications of Chironomidae to the level of species are very difficult (Ekrem et al. 2010) — contributing to the paucity of sequences and genetic barcodes identified for specific species within this Family. This knowledge gap may be a significant reason for the lack of species level identification of Diptera in our study.

The Chironomidae *Genus Phaenopsectra* was the only taxa detected in a fish stomach, rainbow trout, and not detected in either eDNA of water or EtOH. It was only present in one of the rainbow trout stomachs with 117 sequences. *Phaenopsectra* is present in BOLD and Genbank databases and has been documented across Canada, including locations in British Columbia. We are confident in our identification of the taxa as the DNA sequence producing the taxonomic match was 98 % similarity to Genbank (HQ938422.1) and 98.1 % similarity to BOLD (CFWIA038-10) for *Phaenopsectra*. The published article associated with this BOLD result,

CFWIA038-10, implies this *Genus* is an aquatic Chironomidae (Stein et al. 2013). This suggests that although being present in only one fish stomach, its occurrence is plausible in our stream and the match with known taxa to online databases is sufficient to verify its identity.

Why *Phaenopsectra* was absent from our eDNA water and EtOH samples is not clear, but this *Genus* may be present in low numbers in Chist Creek and though it was consumed by one rainbow trout from our study, its low abundance lead to an inability of eDNA to detect its presence. Ecological processes may also explain this outcome. It is possible that *Phaenopsectra* pupae in Chist Creek emerged into adults simultaneously resulting in little aquatic presence until the adults return to the water surface days later to lay eggs. Meanwhile the presence of *Phaenopsectra* DNA in the stomach of one rainbow trout may be remnant from emergence, or an adult that was eaten at the water surface. It is also possible that the result may be an error associated with the genetic procedures.

Error during PCR amplification can occur as amplicons are artificially created through nucleotide base substitution (Kobayashi et al. 1999). The potential, therefore, exists for an incorrect base to be assigned during amplification, resulting in one or more bases to be mismatched with the original DNA. If this occurred the final sequence compared with online databases may not match the actual taxa or may align with incorrect taxa. DNA degradation can also affect DNA amplification. Degradation of environmental DNA can occur rapidly, depending on various factors, such as pH, ultraviolet radiation, and temperature (Strickler et al. 2015). Lastly, it is possible that species exist in Chist Creek which have not been formally identified elsewhere; therefore, no sequence data is available for comparison when we assign the OTUs.

Conclusions

Rainbow trout, Dolly Varden and coastrange sculpin from Chist Creek in the Kitimat River watershed consumed a wide range of aquatic invertebrates — but just a subset of the potential prey items available. We also found little difference in prey consumed between the three species suggesting considerable overlap in diet among species that putatively exploit quite different habitats. Our findings, therefore, suggest that there may be considerable overlap in exploitation of prey items by juvenile Salmonidae and Cottidae in Chist Creek. It is

also possible that we have missed key taxonomic groups of potential prey. The ZBJ-F (Zeale et al. 2011) and EPT-R (Section 2.7) primers were not designed to amplify DNA from species of vertebrate. Vertebrate primers such as those in Section 2.12 should be used to identify potential vertebrate sources of prey and whether they contribute to the diet. Juvenile fish and eggs from anadromous salmon were visually identified in the stomach of juvenile rainbow trout and Dolly Varden; NGS should reveal vertebrate species that contribute to the diet. Increasing sample size for the three species of fish sampled from Chist Creek and also including fish, eDNA, and ethanol samples from the subsequent year will increase the sample size and lead to a more thorough assessment of biodiversity and fish diet analysis, but also potentially an assessment of annual variation.

2.11. Environmental DNA extracted from filtered water samples: a novel approach for sampling stream biodiversity

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Introduction

Biodiversity forms the basis of resilient and stable ecosystems, yet it is being lost at unprecedented rates, both locally and globally (Tilman et al. 2014). Biodiversity in freshwater ecosystems is particularly threatened due to anthropogenic impacts, including forestry, agriculture, industrial activities, invasive species, and climate change (Sala 2000; Dudgeon et al. 2006; Hambler et al. 2011;). To predict and mitigate the effects of these threats, it is essential to obtain detailed taxonomic data for freshwater systems, and to elucidate the relationships between diversity, species composition, and ecosystem function (Giangrande 2003; Tilman et al. 2014).

Biological monitoring has long been used to determine the ecological condition of aquatic ecosystems, to identify levels of biological degradation, and to assess the success of remediation (Buss et al. 2015). Benthic invertebrate communities are particularly important for biomonitoring due to their abundance, species richness, mostly sedentary lifestyles, and taxon-specific sensitivities to aquatic pollution or degradation (Bonada et al. 2006). Standardized protocols for collecting and identifying benthic invertebrates have been developed by many governmental and non-governmental organizations world-wide (Bonada et al. 2006). For most biomonitoring protocols, however, specimens are only identified to higher taxonomic levels [Family or Order; Buss et al. (2015)], as morphological identification of specimens is time-consuming and requires high levels of expertise (Gibson et al. 2015). When specimens are identified to lower taxonomic levels (Genus or species), efforts often focus on more easily identifiable Orders, such as Ephemeroptera, Plecoptera, and Trichoptera (EPT) but exclude challenging Orders such as Diptera (D), even though they may form a dominant component of the benthic fauna (Carew et al. 2013). Other exclusions include often-unidentifiable immature

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and damaged specimens. Although the utility of higher-level identifications for biomonitoring has been much discussed (Hewlett 2000; Lenat and Resh 2001; Jones 2008), at least some studies indicate that lack of taxonomic resolution may limit the fine-grained detail needed for biomonitoring (Lenat and Resh 2001; Timms et al. 2013).

DNA barcoding, wherein a standardized region of the cytochrome c oxidase (COI) gene is sequenced and compared to online databases, has been proposed as a method for increasing ease of identifications (Hebert et al. 2003). Barcoding has been successfully used for freshwater invertebrates, greatly increasing the taxonomic resolution and identifying a much larger number of species in a sample, as well as improving the statistical power of comparative metrics (Zhou et al. 2010; Sweeney et al. 2011; Gill et al. 2014; Jackson et al. 2014; Stein et al. 2014). It remains expensive and time-consuming, however, to sequence every specimen in a sample, or even a representative subset. More recently, the field of metabarcoding has taken advantage of the increasing capacity to develop protocols for sequencing entire communities all together (Taberlet et al. 2012; Ji et al. 2013; Cristescu 2014). This has the potential to revolutionize biodiversity studies of arthropods, and has been referred to as “Biomonitoring 2.0” (Baird and Hajibabaei 2012). This technique has been used successfully for identifying soil beetle communities (Andujar et al. 2015), arthropod diversity in pitcher plants (Bittleston et al. 2015), arthropods from Malaise traps (Yu et al. 2012; Gibson et al. 2014;), marine meiofauna (Leray and Knowlton 2015), and nematodes (Porazinska et al. 2009; Porazinska et al. 2010). In aquatic environments this technique has shown promise for successfully identifying benthic invertebrates (Hajibabaei et al. 2011; Gibson et al. 2015;), including taxa that are typically left out of biomonitoring studies (Carew et al. 2013).

In addition to the taxonomic impediment for species identification, there are collection biases with regards to aquatic biomonitoring. Species are not evenly distributed in aquatic habitats either spatially or temporally. Therefore, to fully sample the amount of species present in a given water body, it is necessary to sample all micro-habitats throughout the year, an effort that would quickly result in an insurmountable amount of samples, even if it were logistically practical in the field. For this reason, aquatic biomonitoring protocols attempt to use standardized sampling methods; however sampling methods will be biased towards some

species, while missing others, and different protocols (e.g., from different countries) can hinder direct comparisons between studies (Bonada et al. 2006).

Environmental DNA (eDNA) is DNA that is obtained directly from environmental samples (i.e., water, soil, faeces, etc.) that may or may not include the physical bodies of the organisms (Bohmann et al. 2014). In aquatic ecosystems, eDNA has most commonly been used to detect fish and amphibians, including endangered and invasive species (Ficetola et al. 2008; Jerde et al. 2011; Minamoto et al. 2011; Pilliod et al. 2013; Janosik and Johnston 2015). A few studies have successfully detected macroinvertebrates in freshwater samples using species-specific primers (Thomsen et al. 2012; Mächler et al. 2014;), including specimens that were rarely captured in traditional kick-net samples collected from the same sites (Mächler et al. 2014). If it is possible to use universal primers to similarly detect invertebrate communities, it would greatly facilitate biomonitoring studies by both increasing the taxonomic resolution and decreasing the sampling bias associated with traditional collections of invertebrates (Bohmann et al. 2014). In addition, it would greatly reduce costs of biomonitoring, while facilitating standardization among protocols.

The use of eDNA for biological monitoring, however, still requires extensive testing by comparison to morphological methods. For example, there is debate as to whether any measure of abundance data can be obtained through this method, with several studies concluding that only presence-absence data can be obtained (Mächler et al. 2014; Elbrecht and Leese 2015;). Other areas under active investigation include the effect of PCR bias and primer design (Brandon-Mong et al. 2015; Pinol et al. 2015), the fine-tuning of bioinformatics pipelines (Brandon-Mong et al. 2015), and the trade-off between numbers of replicates and sequencing depth in order to improve accuracy (Smith and Peay 2014; Ficetola et al. 2015;).

The goal of this study is to determine the composition of aquatic communities, specifically key indicator species of EPTD, from small streams in central BC, Canada using eDNA and next generation sequencing techniques. Environmental DNA extracted from water samples was compared to standard assessments of benthic arthropod samples that were collected and was identified following standardized protocols (Environment Canada 2014) and the implications of each method for ecological and taxonomic analyses are discussed.

Methods

Sampling locations

From May – September 2014, water samples for eDNA and benthic invertebrates were collected from 14 fish-bearing streams situated along the route of the proposed pipeline RoW in interior and coastal central BC; seven streams in section 4 and seven streams in section 2. Of the 14 streams, eight were selected for metabarcoding analysis (Table 2-76). At each stream, samples were collected from three sites: the proposed pipeline crossing (0 m), 200 m upstream of the crossing (+200 m), and 200 m downstream of the crossing (-200m). Sample collection started downstream and then proceeded upstream to minimize disturbance and avoid contamination.

Table 2-76 – Benthic invertebrate samples analysed using metabarcoding, including stream name, GPS coordinates (UTM), site, sample code (Sample ID) for corresponding invertebrate and eDNA samples, and date of collection. For eDNA analysis of Chist Creek, three 2 L replicates were used for the -200 and +200 sampling sites.

Section	Stream Name	Location (UTM)	Site	Sample ID	eDNA	Date Collected
2	Nine Mile Creek	10U 0403828 6000704	-200	I14-293	E14-132	05-Sep-14
2	Nine Mile Creek	10U 0403723 6000956	+200	I14-300	E14-134	05-Sep-14
2	Ormond Creek	10U 0384606 5997504	-200	I14-303	E14-143	09-Sep-14
2	Ormond Creek	10U 038436 5997856	+200	I14-308	E14-146	09-Sep-14
2	Salmon River	10U 0496808 6006249	-200	I14-284	E14-156	02-Sep-14
2	Salmon River	10U 0496287 6006280	+200	I14-291	E14-159	02-Sep-14
2	Tatsutnai Creek	10U 0398490 5999442	-200	I14-276	E14-169	27-Aug-14
2	Tatsutnai Creek	10U 0398514 5999827	+200	I14-281	E14-172	27-Aug-14
4	Chist Creek	9U 0531924 6014489	-200	I14-249	E14-49-51	29-Jul-14
4	Chist Creek	9U 0532211 6014617	+200	I14-253	E14-52-54	29-Jul-14
4	Trout Creek	9U 0520528 6000162	-200	I14-240	E14-70	28-Jul-14
4	Unnamed 57.1 km	9U 0546970 6011379	-200	I14-204	E14-93	25-Jul-14
4	Unnamed 57.1 km	9U 0547078 6011609	+200	I14-209	E14-97	25-Jul-14
4	Unnamed 9.8 km	9U 0521045 5996024	-200	I14-231	E14-114	27-Jul-14
4	Unnamed 9.8 km	9U 0520852 5996112	+200	I14-236	E14-117	27-Jul-14

eDNA collection

At each site, three 2-L water samples were collected in sterile Nalgene bottles from the upper 5 cm of flowing surface water in riffles (Figure 2-42). When sampling, we collected upstream from our standing location to reduce potential contamination by clothing or

equipment. Immediately after collection, 1-L steam-water samples were vacuum filtered through 47-mm diameter, 0.45 µm pore size, mixed-cellulose filters (Cole-Palmer, Montreal, QC, Catalogue No. A045A047A) for a total of two filter papers per 2-L water sample. Each filter was then preserved in 95 % ethanol and stored at -20 °C until extracted for DNA. In total, nine water samples were taken from each sampling location. For NGS analysis, only one of the 2-L water replicates was used from the -200 m and +200 m locations, except for Chist Creek, where all three replicates were used (Table 2-76). To monitor for contamination from field and laboratory equipment, a 1-L Nalgene bottle filled with distilled water was taken to each stream and subjected to the same filtering process using the sterilized equipment.

Benthic invertebrate collection

Promptly after water samples were taken, benthic invertebrates were collected from the three sites. Three replicates were collected at each site, each from a different nearby riffle. Sampling methods were adapted from protocols developed for streams in the Greater Vancouver Regional District (Page et al. 2008). At each replicate location, benthic invertebrate sampling was conducted using a Surber sampler (Dynamic Aqua Supply, Surrey, BC, Catalogue No. SBN250) with a 30 × 30 cm frame (0.09 m^2) and 250-micron mesh size. One replicate Surber sample was a composite of three 3-min placements within one riffle working sequentially upstream for each sampling incident. During a placement, the person sampling would rub the large rocks within the sampling frame, causing invertebrates to float downstream into the mesh, and the rock would be removed from the sampling area. Once large rocks were removed, the person sampling would swirl the gravel and sand to stir up invertebrates into the mesh. At the end of the 3-min interval the person sampling would move upstream to an undisturbed area in the same riffle for another 3-min placement. A third placement in the same riffle, upstream of the previous two samples, completed the composite Surber sample.

After each sample was collected, five elutriation steps were performed to concentrate the organic material and discard the inorganic debris such as rocks and sand. A sterilized 5-L bucket was used to swirl the sample contents and suspend the organic material, which was then poured back into the funnel of the Surber sampler. After the inorganic debris was rinsed

five times, it was discarded. The organic material was then transferred to a 1-L plastic bottle and preserved in 95 % ethanol.

Sorting and identification of benthic invertebrate samples

Benthic invertebrate specimens were identified following standard CABIN protocols (Reynoldson et al. 2001). Sample contents were spread out in a large tray and examined using a dissecting microscope. Large samples estimated to contain more than 600 invertebrate specimens were placed in gridded trays, the cells of which were then subsampled randomly, using a random number generator until at least 300 specimens were sorted from the sample for identification. If the sample contained less than 600 individuals, the entire sample was sorted. All aquatic insect taxa (Insecta) were included in the specimen count, including the Orders dominant in stream habitats Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies), Diptera (true flies), and Coleoptera (beetles), as well as water mites (Hydrachnida: Trombidiformes). Other invertebrates were identified but not included in the count, such as worms, copepods, ostracods, terrestrial fall-in (e.g., spiders and aphids), which can represent a substantial food source for some fish. Invertebrates were identified to the Family level when possible, or to Order level, using taxonomic keys from McCafferty (1981), Merritt et al. (2008), and Clifford (1991).

DNA extraction

DNA was extracted from the filters using bead beating and the DNeasy Blood and Tissue Kit: spin-column protocol (Qiagen, Toronto, ON, Catalogue No. 69506). To avoid contamination, all extractions were performed in a room dedicated to low-quality DNA sources. No DNA samples from other species were handled in this room and separate laboratory coats, pipets, pipette filter tips and laboratory equipment were used to perform the extractions at that location. Additionally, all equipment was first sterilized using a 10 % bleach solution and exposed to UV light for a minimum of 15 min. To monitor for contamination, a negative control was included for each set of water filter DNA extractions.

Filters were removed from the ethanol and cut into approximately 1-mm slices using sterile forceps and tweezers. The filter pieces were placed into 2-mL tubes that contained two 5/32’’ steel grinding beads (Ops Diagnostics, Lebanon, NJ, Catalogue No. GBSS 156-5000-01)

and left to air-dry overnight. Next the filter pieces were shaken in the Genogrinder2000 (BT&C Inc., Burlington, ON) at 1500 oscillations × min^{-1} for 90 s. To prevent contamination, the filter particles were spun for 1 min at 6,000 × g before opening the tube and adding 870 µL Buffer ATL and 30 µL Proteinase K solution. The tubes were incubated at 56 °C for 30 min with 150 rpm agitation, then shaken in the Genogrinder at 1,000 oscillations × min^{-1} for 60 s. Samples were incubated once again at 56 °C with agitation for 1.5 h. After the final incubation step, samples were vortexed for 15 secs and spun for 3 min at 10,000 g. The supernatant (~600 µL) from each tube was transferred into a new, labelled 2 mL tube without disturbing the filter paper pellet. Since multiple filters were required to filter each 2-L water sample, the supernatants from the appropriate samples were combined before proceeding with the wash steps. Hereafter the supernatant was subjected to a classical DNA extraction for the remaining steps with the Qiagen DNeasy Blood and Tissue Kit (manufacturer's protocol) using the following modifications; 600 µL Buffer AL, 600 µL 95 % ethanol, and final elution steps of 2 × 50 µL AE Buffer for each sample. The DNA concentration of each eDNA extract was quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay, Life Technologies, Burlington, ON, Catalogue No. Q32854) and stored at -20 °C until PCR amplification.

Selection of universal invertebrate primers

To target a 157-bp fragment located at the 5' end of the barcoding gene cytochrome c oxidase subunit I (COI), DNA was amplified using a modified version of the general invertebrate COI primers published by Zeale et al. (2011), ZBJ forward and reverse (ZBJ F and ZBJ R). These primers were designed to capture a broad range of arthropod taxa, and successfully amplified single bands at the expected size in preliminary trials. However, the use of DNA extracted from a single specimen of the taxa most abundant in stream habitats — including mayflies (E), stoneflies (P), caddisflies (T), and flies (D) — revealed that the ZBJ primers did not consistently amplify representative specimens selected from various EPTD Families, which was being masked by amplification of other taxa in mixed-DNA samples.

To improve amplification of the target sequence from EPTD taxa obtained in Surber samples, an additional reverse primer was designed using COI sequences of 12 EPTD Families commonly identified from study streams. It was chosen by aligning sequences from 12 common

Families (Baetidae, Ephemerellidae, Heptageniidae, Perlodidae, Chloroperlidae, Nemouridae, Rhyacophilidae, Hydropsychidae, Glossosomatidae, Simuliidae, Tipulidae, and Psychodidae) and manually selecting bases in the ZBJ R-binding region that were more similar to the aligned sequences. Combined with ZBJ F, this primer (EPT reverse, EPT R) targets the same 157-bp amplicon as the ZBJ primers, located within at the 5' end of standard COI barcode region (Hebert et al. 2003).

PCR amplification

We prepared the samples for unidirectional sequencing on a PGM Ion Torrent sequencer in a two-step PCR amplification method that incorporated fusion primers designed by the Heath Lab at the University of Windsor in Ontario (D. Heath, *pers. comm.*). Two tailed-end sequences, UniA and UniB, were added to the end of the primer sequences used in the current study: UniA onto the forward primer (ZBJ F) and UniB onto reverse primer EPT R (Table 2-77). These "universal" tailed-end sequences were used in the second PCR step to incorporate the Ion Torrent adaptor sequences into the final amplicon sequence: an IonA adaptor sequence and a unique 10-bp identifier sequence (used to identify the individual samples sent for sequencing) binds to UniA sequence, and the P1 adaptor sequence used to initiate the sequencing reaction during an Ion Torrent sequencing run, binds to the UniB sequence. The P1 adaptor sequence is required for correct attachment of prepared amplicons to the Ion Spheres, which rest in the wells of the semi-conductor chip and the IonA adaptor the priming site for the sequencing reaction on the Ion Torrent chip (data not shown).

During the first PCR step (PCR1), the target 157-bp COI sequence with ZBJ F, EPT R and UniA and UniB sequences (Figure 2-61) was amplified in 35 µL reaction volumes in triplicate using the Qiagen Multiplex PCR kit (Toronto, ON, Catalogue No. 206143). Each reaction contained 17.8 µL Multiplex MasterMix, 0.75 µL UniA-tailed forward primer (10 µM), 0.75 µL UniB-tailed reverse primer (10 µM), and 15.0 µL of nuclease-free water and extracted DNA which combined for a total of 20 to 60 ng of template DNA per reaction. Also included were a negative extraction control, a negative PCR control, and a positive PCR control, each in 35 µL reaction volumes and in triplicate. PCR positive controls consisted of two mock communities, each prepared using DNA extracted from 20 benthic invertebrate specimens (Table 2-78)

obtained in Surber samples and representing taxa common in study streams. DNA from each specimen was extracted with the Qiagen DNeasy Tissue Kit according to manufacturer protocols, quantified with QUBIT® 2.0 Fluorometer (dsDNA HS Assay), and pooled in equimolar concentrations.

Table 2-77 – List of primers used to amplify extracted DNA for next generation sequencing. The ZBJ forward and reverse primers (ZBJ-F) are from Zeale et al. (2011). We designed a complementary reverse primer for the same region (see Section 2.7); EPT reverse (EPT-R) was specifically designed as a degenerate primer to amplify the COI gene for common aquatic invertebrates in central BC. Base pair Y = CT; R = AG; H = ACT; and N = ACGT. UniA and UniB sequences included within the ZBJ-F and EPT-R primer (shown in bold). Primer P1UniB was attached to the amplified product in the PCR2 step.

Name	Sequence	Sequence length
ZBJ-F: UniA_ZBJ-ArtF1c	**ACCTGCCTGCCG**AGATATTGGAACWTTATATTTTATTTTTGG	157
ZBJ-R-UniB_ZBJ-ArtR2c	**ACGCCACCGAGC**WACTAATCAATTWCCAAATCCTCC	
ZBJ-F: UniA_ZBJ-ArtF1c	**ACCTGCCTGCCG**AGATATTGGAACWTTATATTTTATTTTTGG	157
EPT-R: UniB_EPTD4d	**ACGCCACCGAGC**ACTAAYCARTTNCCRAAHCCHCC	
P1UniB	CCTCTCTATGGGCAGTCGGTGAT**ACGCCACCGAGC**	-

PCR reactions were amplified using the following thermal cycling conditions: 95 °C for 15 min, 30 cycles of 94 °C for 30s, 46 °C for 60s and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR products were visualized on 2% TBE agarose gels stained with ethidium bromide to verify the presence of single bands at the expected size (235 bp) for the COI amplicon in eDNA samples and the absence of bands in extraction and PCR negative controls. PCR1 products were purified using Agencourt AMPure XP purification beads (Beckman and Coulter, Mississauga, ON, Catalogue No. A63881) and quantified using a QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

The second PCR step (PCR2) was performed to attach the IonA plus unique identifier and P1 adaptor sequences to the amplicons produced during PCR1 (Figure 2-61). Each PCR reaction contained 3.6 µL 10 x reaction buffer, 1.5 µL $MgCl_2$ (25 mM), 0.75 µL dNTPs mix (10 mM), 0.75 µL IonX-UniA primer (10 µM), 0.75 µL P1-UniB primer (10 µM), and 0.15 µL Platinum Taq polymerase (5 Units × μL^{-1}) (Invitrogen, Burlington, ON, Catalogue No. 1096634). The IonX-UniA primer contained a single unique identifier sequence for each sample or positive/negative

control that was used for sample identification in downstream data analyses. The amount of purified PCR1 product added to the reaction varied depending on the concentration. As a standard, we added approximately 30 – 50 ng of purified PCR1 template to each reaction and adjusted the amount of nuclease free water accordingly for a total volume of 15 µL. Samples were amplified at 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 5 min.

Table 2-78 – Benthic invertebrates used as a positive control for PCR amplification of eDNA water filter samples. Each mock community was prepared using 20 different specimens using 10 ng of each individual extraction.

	Mock community 2			Mock community 2	
	Order	**Family**		**Order**	**Family**
1	Ephemeroptera	Ephemerellidae	1	Ephemeroptera	Ameletidae
2	Ephemeroptera	Heptageniidae	2	Ephemeroptera	Heptageniidae
3	Ephemeroptera	Baetidae	3	Ephemeroptera	Ephemerellidae
4	Ephemeroptera	Leptophlebiidae	4	Ephemeroptera	Baetidae
5	Plecoptera	Chloroperlidae	5	Plecoptera	Perlidae
6	Plecoptera	Perlidae	6	Plecoptera	Pteronarcyidae
7	Plecoptera	Perlodidae	7	Plecoptera	Sabicosa
8	Plecoptera	Nemouridae	8	Plecoptera	Perlodidae
9	Trichoptera	Hydropsychidae	9	Trichoptera	Limniphilidae
10	Trichoptera	Glossosomatidae	10	Trichoptera	Rhyacophilidae
11	Trichoptera	Rhyacophilidae	11	Trichoptera	Hydropsychidae
12	Trichoptera	Brachycentridae	12	Trichoptera	Glossosomatidae
13	Diptera	Ceratopogonidae	13	Diptera	Tipulidae
14	Diptera	Chironomidae	14	Diptera	Ceratopogonidae
15	Diptera	Tipulidae	15	Diptera	Simuliidae
16	Diptera	Psychodidae	16	Diptera	Athericidae
17	Coleoptera	Elmidae	17	Hymenoptera	-
18	Trombidiformes	Hydrachnidae	18	Coleoptera	Dytiscidae
19	Araneae	Lycosidae	19	Araneae	Lycosidae
20	Hymenoptera	Apidae	20	Trombidiformes	Hydrachnidae

A subset of PCR2 products were analysed using an Experion DNA 1K Analysis kit (Bio-Rad, Mississauga, ON, Catalogue No. 7007107) to verify that unique identifier and PI adaptor sequences were attached correctly to PCR1 amplicons. Successful PCR2 reactions produced three bands per sample: (1) PCR1 product plus both the IonX-unique identifier and P1 adaptor sequences (~299 bp), (2) PCR1 product plus the IonX-unique identifier sequence only (278 bp), and (3) PCR1 product plus the P1 adaptor sequence only (~258 bp). Following confirmation that

PCR2 reactions were successful, triplicates of each sample were then pooled (~60 µL), purified with Agencourt AMPure XP purification beads, and quantified using QUBIT® 2.0 Fluorometer (dsDNA HS Assay).

Preparation of final COI amplicons for ion torrent sequencing

Samples and positive controls were normalized by pooling equimolar ratios of each sample, as well as ~5 µL of each negative control, which had DNA concentrations too low to be read by QUBIT® 2.0 Fluorometer, as expected. Two 40-µL aliquots of the final normalized COI amplicon library (all samples and positive/negatives controls) were then run on 1.5% TAE agarose gels stained with ethidium bromide at ~200 v for 1.5 h to allow sufficient separation of the 295-bp bands (amplicons plus all required adaptor sequences) from those containing amplicons with only one adaptor sequence attached (255 and 275 bp). The ~295-bp fragment was extracted from the gel using a clean, sterile scalpel and the DNA was purified using the Qiagen MinElute Gel Extraction Kit (Toronto, ON, Catalogue No. 28606). To verify that the correct band was excised from the gel and the final concentration of the product, the purified DNA was analysed using the Experion DNA 1K Analysis Kit. The resulting COI amplicon library was submitted to the Great Lakes Institute for Environmental Research (GLIER) Lab at the University of Windsor in Ontario where the library was sequenced using a PGM Ion Torrent Sequencer with an Ion Torrent 318 chip.

Data analysis

Bioinformatics analyses were performed on the Rocks server through the UNBC High Performance Computing Lab. A python script was used to strip non-biologically informative sequences (primer and UniA sequences) from the raw reads (raw sequences generated by Ion Torrent PGM Sequencer), and to relabel reads with the unique identifier label (Sample ID) associated with each read. USEARCH (Edgar 2005) was used to filter the resulting sequences for quality using a maximum error rate of 0.5 (allows one error for every 200 bp) and to truncate sequences to the expected amplicon length (157 bp). Sequences were sorted by size using a minimum size of two sequences to remove singletons, and then clustered into operational taxonomic units (OTUs) using a sequence identity of 97 % (i.e., sequences that are 97% to similar to each are clustered around a central sequence), with the resulting sequences

relabelled with sequential OTU numbers, and a fasta file containing all OTUs generated. OTUs were then mapped back onto the original reads (COI sequences labelled with unique sample identifiers), including singletons, and a taxonomy table generated that included the number of all sequences for each OTUs contained in each eDNA sample and positive control, as well as negative controls if present.

MegaBLAST (www.ncbi.nlm.nih.gov) was used to assign taxonomy to the resulting OTU sequences. The BLASTn file containing all OTUs and their top hits in the GenBank database (i.e., taxonomic identity of the organism with closest sequence identity to the queried OTU) and the USEARCH OTU fasta file were imported into MEGAN (MEtaGenome ANalyzer; www-ab.informatick.uni-tuebingen.de/software/megan) to allow the taxonomic assignments for each OTU to be exported as a tab separated value (.tsv) file for manipulation in Excel. Taxonomic assignments were added to the OTU table containing all OTUs present in each sample/control and the data analysed in Excel to determine which taxa were detected in each of the eight streams analysed. Taxa that had a minimum of 48 sequences per read were included in the analysis and results were then compared to the morphological identifications of the specimens collected from the corresponding Surber sample.

Results

Metabarcoding analysis was performed on DNA extracted from the cellulose filters used to collect eDNA from 2-L water samples obtained from eight streams along the route of the proposed pipeline RoW (Table 2-76). PCR 1 was successful and amplified the desired 230-bp band, which represents the 157-bp length of the COI gene region combined with the F (ZBJ) and R (EPT) primers (Figure 2-86). In addition, no bands were produced for the negative PCR controls, negative extraction controls or for the field control negatives. PCR2 was performed on the purified PCR1 products and produced the desired ~300-bp length amplicons for all samples, indicating amplicons have successfully been bonded with the Ion Torrent specific UniA and UniB sequences.

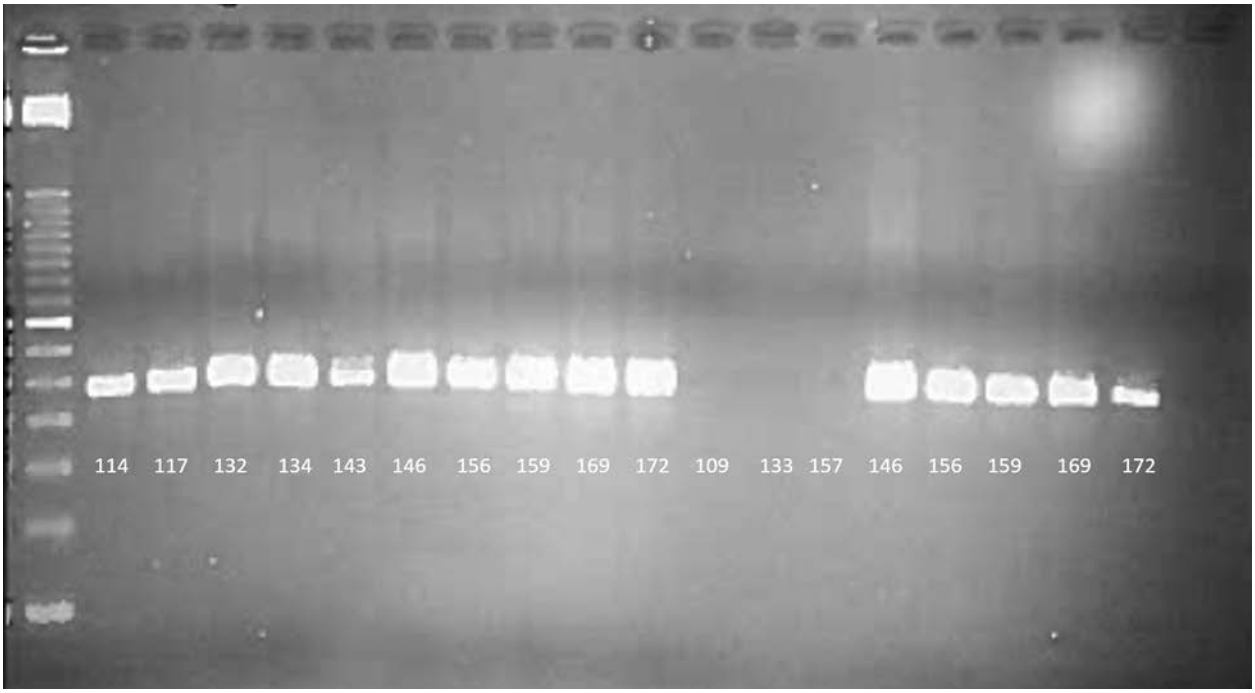
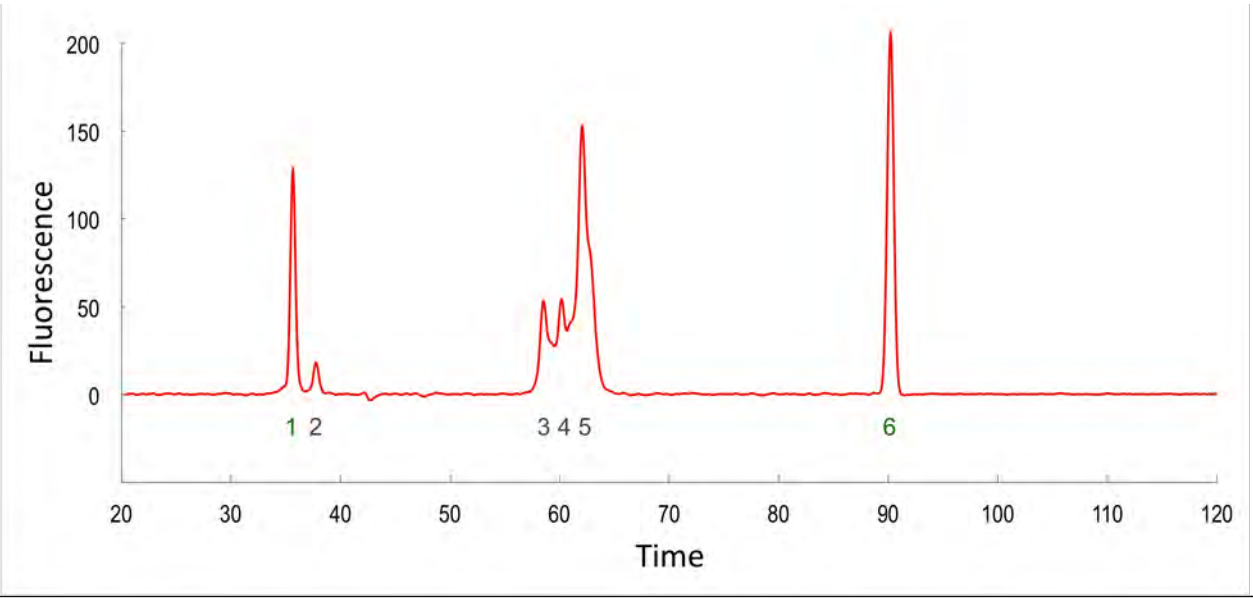


Figure 2-86 – Selected examples of PCR1 amplification products of the COI gene region from water filter samples collected from the eight streams. Sample identification is provided in Table 2-76. Primers amplified a 157-bp fragment. Samples were amplified in triplicate and the 50-bp ladder is shown in the left lane. Negative field and negative extraction controls are samples E14-109, E14-133, E14-157.

An example of an Experion run for PCR2 amplification is shown in Figure 2-87. The Experion results showed three congregated peaks, each representing a different binding outcome of the PCR1 product with the UniA and UniB sequences. The two smaller fragment lengths indicate PCR1 product has combined with only one of these two sequences, while the larger ~300-bp fragment indicates the desired outcome of having both UniA and UniB sequences attached to the PCR1 product. The largest fragment for each sample (300 bp), also had the highest concentration, and was isolated through gel extraction and submitted for sequencing.



Peak Number	Size (bp)	Concentration ($ng \cdot \mu l^{-1}$)	Observation
1	15		Lower Marker
2	25	0.8	
3	255	2.0	
4	274	1.5	
5	**295**	**7.1**	
6	1500		Upper Marker

Figure 2-87 – Experion output indicating the COI fragment sizes and their corresponding concentrations following the PCR2 amplification step for a single Coastrange sculpin stomach content sample. The desired amplicon size of approximately 300 bp is shown in bold.

Mock community (positive controls)

To verify that the primers selected in this study successfully amplified the targeted region of the COI gene for the dominant taxa present in Surber samples obtained in study streams, mock communities were analysed using the same methods as those for eDNA samples. Eighteen of the 20 species comprising the mock community were detected by Ion Torrent sequencing, including all 16 EPTDs, as well as the water mite (Trombidiformes) and ground beetle (Coleoptera: Carabidae) (Table 2-79). Not detected using these primers were samples of a spider (Araneae: Lycosidae) and a bee (Hymenoptera: Apidae); a crane fly sample (Diptera: Tipulidae) was detected in only one of the three replicates, represented by a single

sequence. Except for Tipulidae, all other EPTD taxa were detected in all three replicates; number of sequences for all of the EPTD samples ranged from 78 – 43282 copies.

Table 2-79 – Morphological identifications of specimens comprising the mock community used as a PCR positive control in metabarcoding analyses, and the number of replicates and sequences of each taxa present in mock community positive controls. Data is shown for Mock Community 2.

	Morphological identification		Metabarcoding	
	Order	Family	replicates	sequences
1	Ephemeroptera	Ephemerellidae	3	1729
2	Ephemeroptera	Heptageniidae	3	1611
3	Ephemeroptera	Baetidae	3	1140
4	Ephemeroptera	Leptophlebiidae	3	102
5	Plecoptera	Chloroperlidae	3	1605
6	Plecoptera	Perlidae	3	78
7	Plecoptera	Leutricidae	3	14733
8	Plecoptera	Nemouridae	3	651
9	Trichoptera	Hydropsychidae	3	1552
10	Trichoptera	Glossosomatidae	3	220
11	Trichoptera	Rhyacophilidae	3	149
12	Trichoptera	Brachycentridae	3	43282
13	Diptera	Ceratopogonidae	3	4068
14	Diptera	Chironomidae	3	2310
15	Diptera	Tipulidae	1	1
16	Diptera	Psychodidae	3	375
17	Coleoptera	Carabidae	3	61
18	Trombidiformes		3	42
19	Araneae	Lycosidae	0	0
20	Hymenoptera	Apidae	0	0

Section 4 (coastal streams)

COI sequences were successfully generated by Ion Torrent sequencing for seven eDNA samples collected from four streams located in section 4, one from each of the -200 m and +200 m sites in Unnamed Creeks 9.8 km and 57.1 km, and one from the -200 at Trout Creek. For Chist Creek, however, three samples were analysed for the upstream and downstream sites (Table 2-80). Of the four main aquatic invertebrate Orders — Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies), and Diptera (flies) — all were detected in each stream using water filters samples. The composition of Families and species, however, differed

among streams. Generally, detection of species present in a stream was not dependent on the sampling location (-200 m or +200m); the vast majority of Families were detected at both sampling sites for each stream. The most abundant Families in the four streams were from the Orders Ephemeroptera (Ephemerellidae, Heptageniidae and Leptophebiidae) and Diptera (Chironomidae, Simuliidae), whereas Plecoptera had the fewest Families detected in the streams. Overall, eDNA analysis from water samples detected the presence of five different Families from Ephemeroptera and Plecoptera, ten Families from Trichoptera, and thirteen Families from the Order Diptera.

Table 2-80 – Comparison of taxa identified morphologically (Morpho. ID) from Surber samples or by Ion Torrent sequencing (eDNA) from water filter samples obtained from coastal streams in section 4. Data represents the number of times each Family was detected in the different types of samples — it is not a representation of the number of different taxa. List beside the name of the Order is the total number of samples that contained the different Families for each of the two different methods.

Sample ID	I14-204	E14-93	I14-209	E14-93	I14-231	E14-114	I14-236	E14-117	I14-240	E14-70	I14-249	combined	I14-253	combined		
Stream	km 57.1	km 57.1	km 57.1	km 57.1	km 9.8	km 9.8	km 9.8	km 9.8	Trout	Trout	Chist	Chist	Chist	Chist		
Site	-200	-200	+200	+200	-200	-200	+200	+200	-200	-200	-200	-200	+200	+200		
Date	25-Jul-14	25-Jul-14	25-Jul-14	25-Jul-14	27-Jul-14	27-Jul-14	27-Jul-14	27-Jul-14	28-Jul-14	28-Jul-14	29-Jul-14	29-Jul-14	29-Jul-14	29-Jul-14		
Run	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Total Streams combined (Morphological ID)	Total streams combined (eDNA)
Ephemeroptera															25	29
Ameletidae										✓		✓		✓	0	3
Baetidae	✓	✓	✓	✓		✓	✓		✓	✓	✓	✓	✓	✓	6	6
Ephemerellidae	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	7	7
Heptageniidae	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	7	6
Leptophlebiidae	✓		✓	✓	✓	✓	✓	✓	✓	✓		✓		✓	5	7
Siphlonuridae															0	0
Plecoptera															19	15
Capniidae												✓		✓	0	2

Sample ID	I14-204	E14-93	I14-209	E14-93	I14-231	E14-114	I14-236	E14-117	I14-240	E14-70	I14-249	combined	I14-253	combined		
Chloroperlidae	✓		✓		✓	✓	✓		✓		✓	✓	✓	✓	7	3
Leutricidae		✓				✓		✓		✓		✓		✓	0	6
Nemouridae	✓		✓		✓	✓	✓		✓		✓		✓	✓	7	2
Perlodidae	✓		✓		✓		✓		✓			✓		✓	5	2
Pteronarcyidae															0	0
Taeniopterygidae															0	0
Trichoptera															11	29
Arctopsyche												✓		✓	0	2
Brachycentridae															0	0
Goeridae		✓		✓						✓		✓		✓	0	5
Glossosomatidae		✓	✓				✓		✓			✓		✓	3	3
Helicophidae												✓		✓	0	2
Hydropsychidae			✓				✓		✓						3	0
Hydroptillidae															0	0
Lepidostomatidae		✓		✓								✓		✓	0	4
Leptoceridae												✓			0	1
Limnephilidae								✓				✓		✓	0	3
Parapsyche												✓		✓	0	2
Polycentropodidae															0	0
Rhyacophilidae	✓	✓	✓		✓		✓		✓			✓		✓	5	3
Uenoidae		✓		✓								✓		✓	0	4
Diptera																
Bibionidae														✓	0	1
Blephariceridae															0	0
Cecidomyiidae															0	0
Ceratopogonidae			✓	✓	✓		✓	✓	✓	✓					4	3
Chironomidae	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	7	7
Culicidae				✓											0	0
Empididae														✓	0	1
Hybotidae												✓			0	1
Limoniidae												✓			0	1
Muscidae				✓											0	1
Phoridae															0	0
Pipunculidae	✓														1	0
Psychodidae			✓		✓		✓	✓							3	1
Ptychopteridae								✓							0	1
Simuliidae				✓	✓	✓	✓	✓	✓			✓	✓	✓	4	5
Tachinidae												✓			0	1
Tipulidae		✓							✓	✓	✓	✓	✓	✓	3	4
Coleoptera	✓	✓	✓						✓						3	1
Elmidae	✓		✓						✓						3	0
Chrysomelidae		✓													0	1
Hydrachnida									✓	✓	✓		✓		3	1
Hymenoptera		✓													0	1
Orthoptera															0	0
Acrididae															0	0
Hemenoptera															0	0

Sample ID	I14-204	E14-93	I14-209	E14-93	I14-231	E14-114	I14-236	E14-117	I14-240	E14-70	I14-249	combined	I14-253	combined		
Hemiptera															0	0
Trombidiformes															0	0
Eupodidae															0	0

Comparison of benthic invertebrates in an individual Surber sample to a single eDNA sample showed that the morphological identification of specimens in a Surber sample had a greater number of Families present within the stream. However, eDNA was able to detect a greater number of Families present from multiple samples in multiple streams (Table 2-81). Overall, eDNA analysis was able to identify eight more Families of Trichoptera, seven Families of Diptera, two Families of Plecoptera and one additional Family in Ephemeroptera compared to morphological identification of Surber samples.

Table 2-81 – List of taxa identified morphologically (Morph. ID) or in the environmental DNA from water (eDNA) samples collected from coastal streams in section 4. The list includes only Families that were identified in only one of the sampling methods.

	Morph. ID	eDNA		Morph. ID	eDNA
Ephemeroptera			**Diptera**		
Ameletidae		✓	Bibionidae		✓
Trichoptera			Empididae		✓
Arctopsyche		✓	Hybotidae		✓
Goeridae		✓	Limoniidae		✓
Helicophidae		✓	Muscidae		✓
Hydropsychidae	✓		Pipunculidae	✓	
Lepidostomatidae		✓	Ptychopteridae		✓
Leptoceridae		✓	Tachinidae		✓
Limnephilidae		✓	**Plecoptera**		
Parapsyche		✓	Capniidae		✓
Uenoidae		✓	Leutricidae		✓

Section 2 (interior streams)

COI sequences were successfully generated by Ion Torrent sequencing for eight eDNA samples collected from four streams located in section 2, one from each of the -200 m and +200 m sites in Nine Mile Creek, Ormond Creek, Salmon River and Tatsutnai Creek (Table 2-82).

Detection of species present in a stream was not dependent on the sampling location (-200 m or +200m); the vast majority of Families were detected at both sampling sites for each stream. Unlike section 4, only two of the main aquatic invertebrate Orders — Ephemeroptera (mayflies), and Diptera (flies) — were detected in each stream using water filters samples, the most abundant Families in the four streams being Heptageniidae (Ephemeroptera) and Chironomidae (Diptera). eDNA analysis was unable to detect the presence of species from the Orders Plecoptera (stoneflies) and Trichoptera (caddisflies) in Salmon River and Ormond Creek, and only the Family Goeridae (Trichoptera) was detected in Ormond Creek. Overall eDNA analysis detected the presence of fewer Families in section 2 for Plecoptera (four Families), Trichoptera (six Families), and Diptera (four Families), whereas the presence of six Families of Ephemeroptera were detected.

Table 2-82 – Comparison of taxa identified morphologically (Morph. ID) from Surber samples or by Ion Torrent sequencing (eDNA) from water filter samples obtained from coastal streams in section 2. Data represents the number of times each Family was detected in the different types of samples — it is not a representation of the number of different taxa. List beside the name of the Order is the total number of samples that contained the different Families for each of the two different methods.

Sample ID	I14-293	E14-132	I14-300	E14-134	I14-305	E14-143	I14-308	E14-146	I14-284	E14-156	I14-291	E14-159	I14-276	E14-169	I14-281	E14-172		
Stream	Nine Mile	Nine Mile	Nine Mile	Nine Mile	Ormond	Ormond	Ormond	Ormond	Salmon	Salmon	Salmon	Salmon	Tatsutnai	Tatsutnai	Tatsutnai	Tatsutnai		
Site	-200	-200	+200	+200	-200	-200	+200	+200	-200	-200	+200	+200	-200	-200	+200	+200		
Date	5-Sep-14	5-Sep-14	5-Sep-14	5-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	9-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	2-Sep-14	27-Aug-14	27-Aug-14	27-Aug-14	27-Aug-14		
Run	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Morph. ID	eDNA	Total Streams combined (Morphological ID)	Total streams combined (eDNA)
Ephemeroptera																	29	31
Ameletidae	✓	✓	✓	✓	✓	✓					✓			✓		✓	4	5

Sample ID	I14-293	E14-132	I14-300	E14-134	I14-305	E14-143	I14-308	E14-146	I14-284	E14-156	I14-291	E14-159	I14-276	E14-169	I14-281	E14-172		
Baetidae	✓	✓		✓		✓	✓	✓	✓		✓		✓	✓	✓	✓	6	6
Ephemerellidae	✓	✓	✓	✓			✓				✓		✓	✓		✓	5	5
Heptageniidae	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	8	8
Leptophlebiidae			✓		✓	✓	✓	✓	✓		✓			✓	✓	✓	6	4
Siphlonuridae						✓								✓		✓	0	3
Plecoptera																	24	11
Capniidae			✓	✓													1	1
Chloroperlidae	✓		✓		✓		✓		✓		✓		✓		✓		8	0
Leutricidae		✓		✓										✓		✓	0	4
Nemouridae	✓		✓		✓		✓						✓		✓		6	0
Perlodidae	✓	✓	✓	✓			✓		✓		✓		✓	✓	✓	✓	7	4
Pteronarcyidae									✓		✓			✓		✓	2	2
Taeniopterygidae																	0	0
Trichoptera																	28	15
Arctopsyche																	0	0
Brachycentridae		✓	✓	✓	✓		✓		✓				✓		✓		6	2
Goeridae		✓		✓		✓								✓		✓	0	5
Glossosomatidae							✓		✓				✓	✓		✓	3	2
Helicophidae														✓		✓	0	2
Hydropsychidae	✓		✓				✓		✓		✓		✓	✓	✓	✓	7	2
Hydroptillidae	✓				✓		✓										3	0
Lepidostomatidae			✓		✓		✓		✓		✓						5	0
Leptoceridae																	0	0
Limnephilidae	✓		✓														2	0
Parapsyche																	0	0
Polycentropodidae																	0	0
Rhyacophilidae		✓	✓	✓									✓				2	2
Uenoidae																	0	0
Diptera																	28	16
Bibionidae																	0	0
Blephariceridae																	0	0
Cecidomyiidae																	0	0
Ceratopogonidae	✓		✓		✓				✓				✓				5	0
Chironomidae	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	8	8
Culicidae														✓		✓	0	2
Empididae																	0	0
Hybotidae																	0	0
Limoniidae																	0	0
Muscidae																	0	0
Phoridae																	0	0
Pipunculidae																	0	0
Psychodidae	✓		✓		✓		✓						✓		✓		6	0
Ptychopteridae																	0	0
Simuliidae		✓		✓	✓	✓			✓				✓	✓	✓	✓	4	5
Tachinidae																	0	0
Tipulidae	✓		✓		✓		✓	✓	✓								5	1
Coleoptera	✓				✓		✓		✓		✓		✓				6	0
Elmidae	✓				✓		✓		✓		✓		✓				6	0

Sample ID	I14-293	E14-132	I14-300	E14-134	I14-305	E14-143	I14-308	E14-146	I14-284	E14-156	I14-291	E14-159	I14-276	E14-169	I14-281	E14-172		
Chrysomelidae																	0	0
Hydrachnida	✓		✓		✓		✓		✓		✓		✓		✓		8	0
Hymenoptera																	0	0
Orthoptera																	0	0
Acrididae										✓		✓					0	2
Hemenoptera		✓		✓													0	2
Hemiptera						✓											0	1
Trombidiformes																	0	0
Eupodidae						✓											0	1

When comparing the eDNA samples to their corresponding Surber samples, morphological identification of benthic invertebrates was able to detect a greater number of Families present within the streams for Plecoptera, Trichoptera and Diptera (Table 2-83). Overall, 22 Families were identified based on morphological data. There were seven additional Families identified morphologically but not detected by eDNA analysis: two Families (Chloroperlidae and Nemouridae) from the Order Plecoptera, three Families (Hydroptillidae, Lepidostomatidae and Limnephilidae) from the Order Trichoptera and two Families (Certopognidae and Psychoidiae) from the Order Diptera. Although eDNA analysis identified fewer Families overall in section 2, eDNA was still able to detect five Families not observed through morphological identification. These Families include Siphlonuridae (Ephemeroptera), Leutricidae (Plecoptera), Goeridae and Helicophidae (Trichoptera), and Culcidae (Diptera).

Combined results for coastal and interior streams

Overall, among all samples and streams analysed from section 2 and section 4, 23 Families were identified based on morphological data, 37 Families based on eDNA analysis, a total of 39 for both methods combined. This indicates that NGS sequencing was able to detect the majority of taxa present in the 15 Surber samples examined. Two Families, Hydroptilidae (microcaddisfly or purse-case caddisfly) and Pipunculidae (big-headed fly) were identified morphologically but not detected through NGS sequencing. eDNA analysis detected 16 Families not found based on morphological data including one mayfly Family (Siphlonuridae), one

stonefly Family (Leuctridae), six caddisfly Families (Arctopsyche, Goeridae, Helicophidae, Leptoceridae, Parapsyche, and Uenoidae), and eight fly Families (Bibionidae, Culicidae, Empididae, Hybotidae, Limoniidae, Muscidae, Ptychopteridae and Tachinidae). Of the Families identified by eDNA analysis only, most are known from study streams and have been identified based on morphological data through this study, suggesting that they were likely in the Surber samples in which they were detected but not processed due to subsampling, and are not the result of contamination or incorrect taxonomic assignments using the NCBI database and MEGAN software.

Table 2-83 – Comparisons of taxa identified morphologically (Morph. ID) to eDNA samples collected from interior streams in section 2.

	Morph. ID	eDNA		Morph. ID	eDNA
Ephemeroptera			**Trichoptera**		
Siphlonuridae		✓	Goeridae		✓
Plecoptera			Helicophidae		✓
Chloroperlidae	✓		Hydroptillidae	✓	
Leutricidae		✓	Lepidostomatidae	✓	
Nemouridae	✓		Limnephilidae	✓	
Diptera					
Ceratopogonidae	✓				
Culicidae		✓			

Discussion

Our results demonstrate that using eDNA with NGS techniques is an effective way of identifying the dominant EPTD taxa present in stream habitats. Overall, eDNA analysis detected more benthic invertebrate Families present in streams than the morphological methods. Although our findings were reported only to Family level, eDNA analysis provided a significantly greater taxonomic resolution for benthic invertebrates found within the streams. Majority of the COI sequences generated from NGS were identifiable to Genus or species level (Section 2.7). This lower taxonomic resolution provides further insight into baseline biodiversity that is often difficult to obtain through morphological identification alone due to unidentifiable immature specimens, damaged specimens, or lack of taxonomic keys.

In addition to the dominant stream taxa presented — mayflies (Ephemeroptera), stoneflies (Plecoptera), caddisflies (Trichoptera), and flies (Diptera) — many additional taxa were identified by NGS sequencing. Many unicellular taxa were detected including Bacteria, Amoebozoa (freshwater and marine amoebas), Apusozoa (flagellate protozoans), Cryptophyta (freshwater and marine algae), Haptophyceae (algae), and Malawimonadidae (protists). Multicellular taxa detected include Viridiplantae (green algae and land plants), Stramenopiles (algae and oomycetes, parasites of plants and fish), Rhodophyta (red algae), Nucleariidae (freshwater amoeba), Porifera (sponges), Cnidaria (hydrozoans, pararsites, etc.), Rotifera (rotifers), Mollusca (slugs and snails), Gastrotricha (hairybacks), and Annelida (earthworm-type worms). These findings suggest that eDNA analysis provides greater insight of an aquatic community compared to just morphological identification.

The success of eDNA providing thorough information about an aquatic community depends on several variables, including target species, stream size, and season (Goldberg et al. 2011). Our study found that eDNA detection using only one 2-L filtered water sample in triplicate was less effective as stream size increased. Salmon River and Ormond Creek, both larger systems situated in section 2, detected less EPTD Families present compared to smaller systems such as Tatsutnai Creek and Nine Mile Creek (Table 2-82). The sensitivity of eDNA detection can be increased by combining filtered water replicates together. For example, samples from Chist Creek (the largest system sampled in section 4) comprised of three 2 L replicates instead of only one sample and detected the most Families of EPTD compared to any other stream sampled (Table 2-81). For eDNA analysis to be successful, however, future studies are required to determine what is the minimum number of replicates needed to detect multiple taxa for various aquatic communities. In addition, samples should be tested from other sampling periods (early spring, summer, early fall) to detect if season affects the prevalence of specific taxa within the system.

Another factor that may increase the number of taxa detected within a stream is analysing all the sequences that were produced and not having a minimum number of reads per specimen. Since there were over 9 million reads produced for one run on the Ion Torrent platform, based on efficiency and time restraints, if an individual for each stream did not

produce a minimum of 48 sequences per read, we did not include the taxa in the analysis. By not analysing all the sequence data, it is possible that rare or uncommon species were deemed undetected. Lowering or removing the threshold would decrease the likeliness of missing rare taxa or sequences that were low due to PCR bias or preferential amplification of certain taxa over others present in the sample, but may lead to false positive identification for some species.

Although combining the ZBJ forward primer with our EPT reverse primer was able to detect the dominant EPTD taxa better than the Zeale et al. (2011) primer combination, recent metabarcoding studies have revealed that using multiple primer sets targeting more regions of the genome can improve the accuracy of taxonomic identifications of specimens present in bulk samples (Hajibabaei et al. 2011; Carew et al. 2013). Furthermore, metabarcoding markers targeting other genes such as 12S or 16S rDNA can provide greater taxonomic coverage than using the COI gene alone (Clarke et al. 2014). The addition of multiple primers would improve the overall success of eDNA detection of benthic invertebrates in aquatic communities.

2.12. Mini-COI barcoding primers to target freshwater salmon (Salmonidae) and sculpins (Cottidae) species of central British Columbia

Adam O'Dell, Mark Shrimpton

Introduction

The use of genetics to identify taxonomic groups has greatly enhanced our ability to properly assess biodiversity for animals that are difficult to visually identify. Most notable are insects where structural differences between species and even Genera are often subtle and difficult to distinguish — made even worse by multiple life stages and poor taxonomic description down to the species level for all developmental stages. The literature is now replete with examples of how genetics has provided a means of identifying invertebrate species — even in vertebrate fecal matter (Zeale et al. 2011). In contrast, taxonomic groups that are Familiar and more easily identified commonly rely on visual measures to characterize species groupings; consequently the primary method to identify vertebrates to species is not usually genetics. Determining vertebrate composition, therefore, depends on taxonomic sampling surveys — although they not technically demanding can be difficult and may not capture all organisms in a system, particularly if they are rare or elusive.

For aquatic systems, genetic approaches to sampling for biodiversity offer substantial benefits over traditional taxonomic methods. The development of genetic tools to successfully assess stream biodiversity relies on environmental DNA (eDNA) and amplification of specific loci commonly used for "barcoding" species. To achieve appropriate results, primers are required to amplify a specific region of DNA, but will bind to multiple target species' DNA in a region that has sufficient variability to differentiate among species (Miya et al. 2015). Being described as the optimal region for differentiating all living species, the cytochrome oxidase subunit 1 (COI) loci of the mitochondrial gene contains at least a 2 % sequence divergence within 98 % of species (Hebert et al. 2003). Having well established COI sequence databases available for referencing species identification, GenBank® (http://www.ncbi.nlm.nih.gov) and Biodiversity of

Life Database (BOLD) version 3 (Ratnasingham and Hebert 2007), the COI gene region is therefore the ideal region of focus for barcoding primers.

Barcoding the COI gene region has typically been accomplished with primers which target and sequence DNA of approximately 650 bp in length (Ward et al. 2005). Samples that are degraded through preservation methods or environmental factors, however, may not allow for this sequencing approach as the DNA can become fragmented, often resulting in the inability to target lengths of this size. Mini-barcoding is therefore a method that can be used to sequence degraded DNA which may only be present at a fraction of its entire length, while only losing a small level of species predictive ability (Meusnier et al. 2008).

The objective of this project was to develop primers to amplify species-specific short COI sequences of potentially degraded DNA. Primers were developed for a Family of fish with a wide geographic range and that are highly mobile — the Salmonidae (Bahr and Shrimpton 2004; Flores et al. 2012). Primers were also developed for a Family of fish with wide geographic range that are considered to be resident — the Cottidae (Gray et al. 2004; but see Clarke et al. 2015). Our target was to develop universal primers for each Family that would differentiate species from central and coastal BC. Amplicons of ~150–250 bp in length were targeted to be suitable for next generation sequencing (NGS), with the goal of being able to describe taxa to the species level.

Methods

Primer development

Primers were developed using CLC Main Workbench 7.6. Known COI sequences for species of Salmonidae and Cottidae were downloaded from Genbank and BOLD; two sequences for each species to account for potential variation within species. Sequences for Salmonidae and Cottidae were aligned and then visually inspected for a forward primer region that was conserved across all species. Potential primers for the target region were selected based on size (16 – 22 bp in length), nucleic acid composition (GC content greater than 40%), and with a melting temperature between 43 – 60 °C. Forward primers identified that met all criteria were submitted to Integrated DNA Technologies' OligoAnalyzer 3.1

(https://www.idtdna.com/calc/analyzer) to test for occurrence of hairpins, dimers, and self-dimers. Primers with a high likelihood of secondary structure were excluded. For each forward primer that met all conditions, the sequence alignment was searched for a reverse primer that would produce an amplicon of approximately 150–250 bp and the process repeated to design the reverse primer.

Selected forward and reverse primer sets were tested in CLC Main Workbench 7.6 (Qiagen company, 2014) for effectiveness and to confirm ability to bind to and form a fragment from downloaded sequences for each target species (Table 2-84).

Table 2-84 – Species and accession numbers for sequences used to development primers for the Families Salmonidae and Cottidae.

Family	Genus	Species	Genbank accession number
Salmonidae	*Oncorhynchus*	*clarkii*	FJ998612
Salmonidae	*Oncorhynchus*	*mykiss*	KP013084.1
Salmonidae	*Oncorhynchus*	*gorbushca*	EU524209
Salmonidae	*Oncorhynchus*	*nerka*	JX960923
Salmonidae	*Oncorhynchus*	*tshawytscha*	JX960926
Salmonidae	*Oncorhynchus*	*kisutch*	JX960917
Salmonidae	*Salvelinus*	*malma*	JX960963
Salmonidae	*Salvelinus*	*confluentus*	JX960956
Salmonidae	*Salvelinus*	*namaycush*	EU522425
Salmonidae	*Prosopium*	*williamsoni*	EU524304
Cottidae	*Cottus*	*aleuticus*	EU523993
Cottidae	*Cottus*	*asper*	JQ354065
Cottidae	*Cottus*	*cognatus*	EU524520
Cottidae	*Leptocottus*	*armatus*	KF930040

Sequences for the primers that were successfully created in CLC Main Workbench 7.6 are presented in Table 2-85; one was designed for Salmonidae (SAL_1) and two Cottidae (COT_1 and COT_2). Sequences for each target amplicon were used to create phylogenetic trees and verify visually that each primer set would differentiate among the selected species within each Family.

Laboratory verification

Tissue samples from nine Salmonidae and five Cottidae were used to verify that the designed primers amplified the targeted loci region (Table 2-86). Some of the samples were

from archived tissue in the lab, others from our biodiversity stream sampling in 2013 and 2014, and three *Cottus cognatus* samples were from the UNBC teaching collection. All three *C. cognatus* tissue samples were removed from whole specimens stored in 95 % ethanol, but may have been originally preserved in formalin. These three samples were soaked in 1 mL of TRIS 2 M solutions for one week, replacing the TRIS after two days, in an attempt to remove any residual formalin. *Oncorhynchus gorbuscha* and *Leptocottus armatus* were not included in the laboratory analysis as DNA samples were not available.

Table 2-85 – Sequences for primer sets targeting the cytochrome oxidase subunit 1 (COI) region of the mitochondrial genome. Primers were designed to differentiate freshwater species within Families Salmonidae and Cottidae. Length and amplicon size (fragment length) are given in base pairs.

Primer	Sequence	Length (bp)	GC%	Annealing temperature (°C)	Amplicon size (bp)
SAL_1	F ATTTAGTATTTGGTGCCTGAGC	22	40.9	53.2	258
	R GGAGTCAGAAGCTTAT	16	43.8	44.3	
COT_1	F TAGTATTTGGTGCTTGAGCC	20	45.0	52.5	138
	R TTATTACGAAAGCATGGGC	19	42.1	50.5	
COT_2	F ATATGGCCTTTCCTCGAAT	19	42.1	51.0	189
	R CTGCTAGGTGAAGGGAGAAG	20	55.0	54.5	

DNA extraction and PCR amplification

DNeasy Blood and Tissue® kit (Qiagen) was used for DNA extraction, following company specification. DNA was then quantified using Qubit dsDNA HS Assay Kit (Life Technologies) and the 2.0 Qubit flourometer (Life Technologies) following company specifications.

Primers were ordered from Integrated DNA Technologies Inc. (IDT). Each PCR reaction consisted of 3 µL of DNA template, 5 µL of Multiplex Master Mix (Qiagen), 0.2 µL of each primer (10 µM), and 1.6 µL ddH_2O in a total volume of 10 µL. PCR conditions were an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 52 °C annealing temperature for 90 secs, 72 °C for 90 secs, with a final step at 72 °C for 10 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide and illuminated with UV light.

Table 2-86 – List of species, type of tissue, and capture location for genetic samples used to validate primers designed in this study to amplify the cytochrome oxidase subunit 1 (COI) region. All samples were preserved in 95 % ethanol unless otherwise indicated. The three *Cottus cognatus* samples original preservation method was not known, but the samples may have been fixed initially in formalin.

Species	Tissue	Population
Salmonidae		
Oncorhynchus clarkii clarkii	Fin clip	Trout Creek, Kitimat River catchment
Oncorhynchus mykiss (2)	Fin clip	Ormond Creek, Fraser River catchment and Chist Creek, Kitimat River catchment
Oncorhynchus keta	Fin ray	Gisasa River, Yukon River catchment
Oncorhynchus nerka	Gill	Chilko River, Fraser River catchment
Oncorhynchus tshawytscha	Fin clip	Tchesinkut Creek, Fraser River catchment
Oncorhynchus kisutch	Fin clip	57.1 km Stream, Kitimat River catchment
Salvelinus malma	Fin clip	57.1 km Stream, Kitimat River catchment
Salvelinus confluentus	Opercular punch	Coldwater River, Fraser River catchment
Cottidae		
Cottus aleuticus	Fin clip	Chist Creek, Kitimat River catchment
Cottus asper	Fin clip	Ormond Creek, Fraser River catchment
Cottus cognatus (3)	Fin clip	Teeter Creek, Racing River, and Calendar Creek, Peace River catchment

PCR conditions were further optimized for each Sculpin primer set to maximize efficiency for amplification. Each PCR reaction was as described above, however a gradient of annealing temperatures from 50 to 60 °C were compared and products visualized on 2% agarose gels stained with ethidium bromide and illuminated with UV light. Optimal temperature for both Cottidae primer sets was 50 °C. Volumes for each PCR reaction were also doubled for all subsequent analyses to ensure adequate template for quantification and sequencing.

PCR product was purified using ExoSAP-IT (Affymetrix). Purification mixture per sample contained 10 µL PCR product, 0.01 µL Exo, 0.1 µL SAP and 3.89 µL nuclease free water. Incubation conditions were 37 °C for 30 min, followed by 80 °C for 15 min. Purified PCR product was then quantified using NanoDrop 3300 Fluorospectrometer (Thermo Scientific) following

company specifications and samples were then submitted to the UNBC Genetics Facility for sequencing using an Applied Biosystems sequencer.

Sequence results were compared to online databases (Genbank and BOLD) for species determination. When both forward and reverse sequences produced matches, only the greater similarity was recorded. When percent similarity was relatively low or absent for both sequences, trace data was assessed for "N" base pairs and visual determination of peaks was conducted to edit the corresponding nucleotides.

Results

Primer development

The phylogenetic trees from the published sequences for Salmonidae and Cottidae species indicated good separation among species, but not within species for all three primer sets designed (Figure 2-88). SAL_1 amplified a section of the COI gene that was 258 bp in length. The region of the COI gene for SAL_1 primer did not bind to the *Prosopium williamsoni* sequence and was not included in the phylogenetic tree. The duplicate samples for each species separated together except for *Oncorhynchus clarkii*; the two subspecies *O. clarkii clarkii* and *O. clarkii lewisi* differed, but were on the same branch (Figure 2-88A). The SAL_1 primers, however, did not separate species of char (*Salvelinus sp.*). *Salvelinus malma* and *Salvelinus confluentus* did not differ in the targeted region and were also on the same branch as *Salvelinus namaycush*.

Leptocottus armatus was included in the development of the sculpin primer; however the initial primer (COT_1), which appeared to work for all target sculpin species, amplified only a relatively short fragment, 138 bp. A second sculpin primer was developed, COT_2, which was 189 bp, but the reverse primer had two base pair mismatches with *L. armatus*. Both sculpin primers were used to test their effectiveness. Both COT_1 and COT_2 primers amplified variable regions among the species and showed good separation (Figure 2-88B and C).

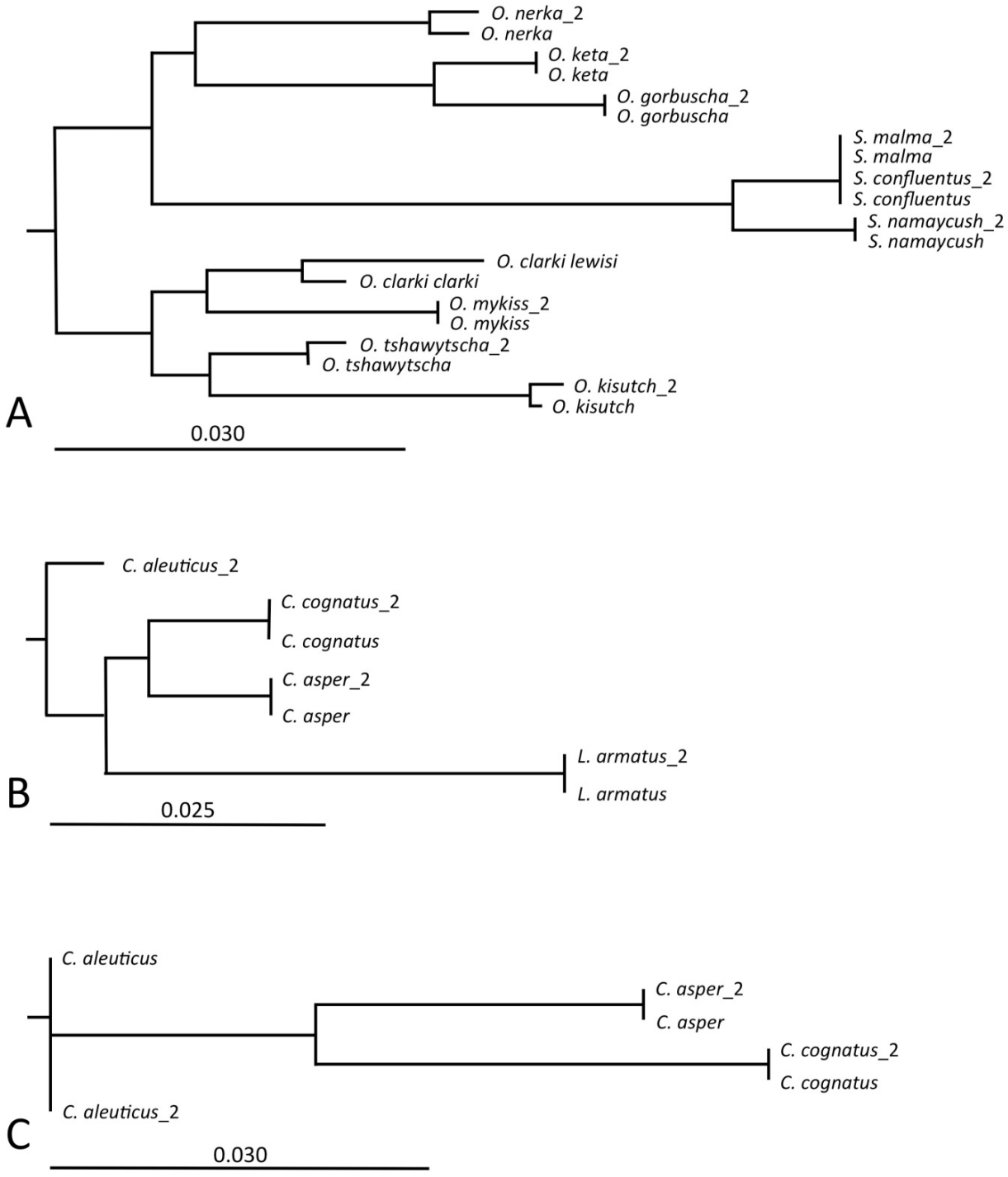


Figure 2-88 – Phylogenetic trees created for (A) SAL_1 primer set, (B) COT_1 primer set, and (C) COT_2 primer set for published sequences for Salmonidae and Cottidae species for cytochrome oxidase subunit 1 (COI).

DNA extraction and PCR amplification

We were able to obtain high quality DNA from all samples with the exception of the three *C. cognatus* samples. DNA concentration was greater than 6 ng × μL^{-1} for all samples except *C. cognatus* which was between 0.9 and 3.6 ng × μL^{-1}. Figure 2-89 also shows the presence of sculpin DNA for both primers at their predicted fragment size, with the exception of the *C. cognatus* samples which show bands that are faint or absent in the desired location. The temperature gradient test for amplification efficiency for the two sculpin primers indicated an optimal annealing temperature of 50.3 °C (data not shown) for both primers. *C. aleuticus* and *C. asper* DNA appeared to amplify consistently across all temperatures, but *C. cognatus* DNA began to fade with an annealing temperature greater than approximately 53 °C. There was no relationship between initial amplicon concentration and post purification PCR products. All purified products were high with a range from 685.5 to 1464.8 ng × μL^{-1} — even the two C*. cognatus* samples for COT_2 primer, which did not appear to have any PCR product (data not shown).

Sequences

DNA sequences amplified with SAL_1 produced 100 % match with the correct species for seven of the nine samples. *S. malma* and *S. confluentus* sequences could not be identified to species, but there was a 100 % correspondence to the Genus *Salvelinus*. All but one sequence for *C. aleuticus* and *C. asper* matched sequences in Genbank or BOLD for the correct species — the last sequence resolved to the Genus *Cottus* (Table 2-87).

The results for *C. cognatus* indicate poor quality DNA. The sequencing of DNA from two of the *C. cognatus* samples failed for each of the sculpin primers. The third *C. cognatus* sample produced sequences with no clear match, although editing the chromatographs resulted in a sequence 98.8% match in BOLD to *C. asper*. One of the top results for this sequence, however, was a 97.5% match for *C. cognatus*. Multiple sequence alignments for the variable regions of the COI gene are shown in Figure 2-90.

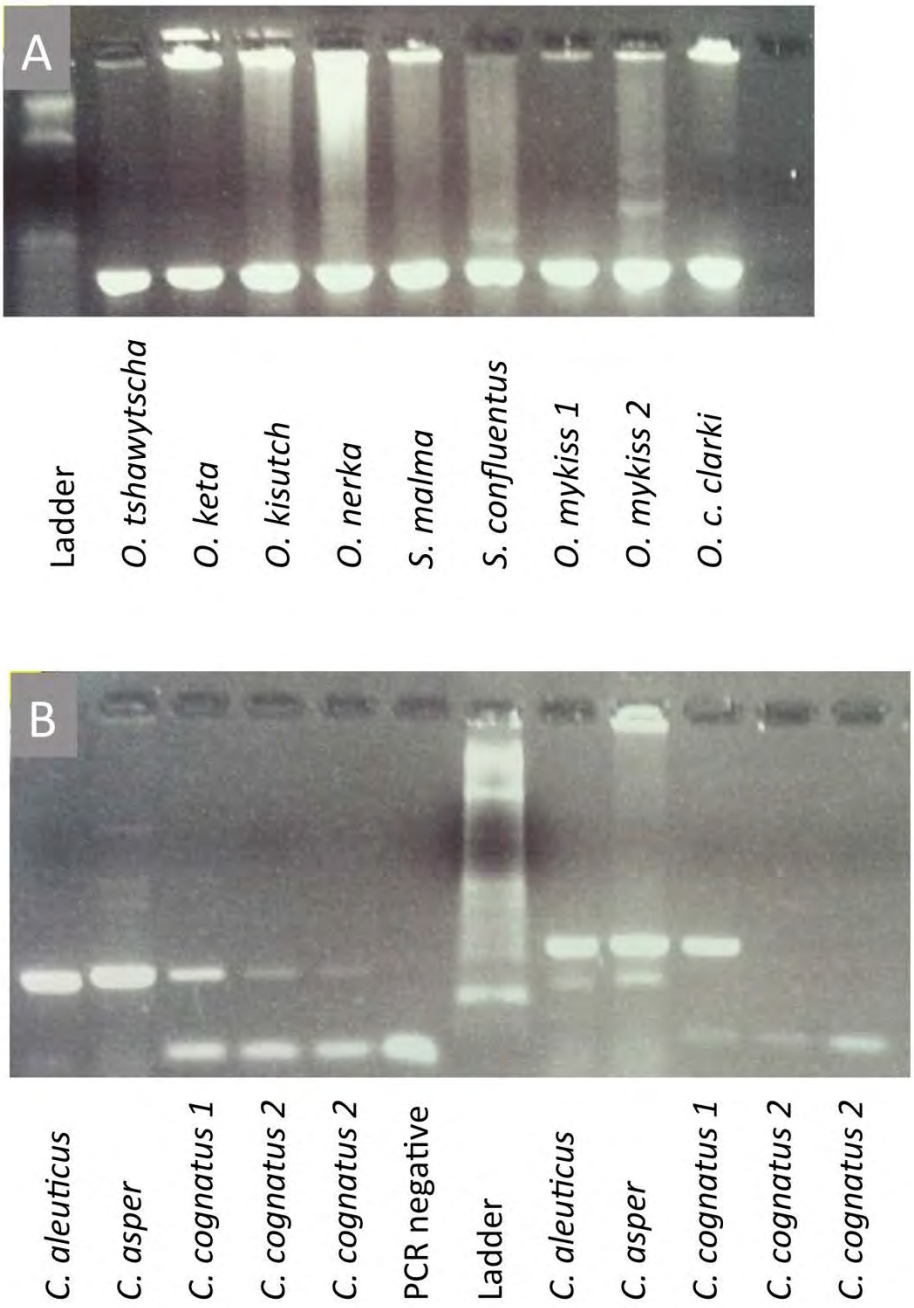


Figure 2-89 – A 2 % agarose gel visualizing the final PCR product for cytochrome oxidase subunit 1 (COI) for (A) Salmonidae tissue samples amplified with SAL_1 primer set and (B) Cottidae tissue samples amplified with COT_1, left lanes, and COT_2, right lanes. Description and location of origin for tissue samples is given in Table 2-86.

Table 2-87 – Sequences produced with COI mini-barcode primers designed for Salmonidae and Cottidae primers compared to BOLD and GenBank databases for similarity with existing sequences. Sample collection information is given in Table 2-86.

ID	primer	Source	Sequence ID	Lowest Level	Similarity
Salmonidae					
O. tshawytscha	SAL_1	BOLD	RDATC016-05	*tshawytscha*	100%
O. keta	SAL_1	BOLD	BCFB945-07	*keta*	100%
O. kisutch	SAL_1	BOLD	TZFPC002-05	*kisutch*	100%
O. nerka	SAL_1	BOLD	BCF451-07	*nerka*	100%
S. malma	SAL_1	BOLD	BCF523-07	*Salvelinus*	100%
S. confluentus	SAL_1	BOLD	BCF506-07	*Salvelinus*	100%
O. mykiss 1	SAL_1	BOLD	RDATC019-05	*mykiss*	100%
O. mykiss 2	SAL_1	BOLD	RDATC019-05	*mykiss*	100%
O. c. clarkii	SAL_1	BOLD	BCF428-07	*clarkii*	100%
Cottidae					
C. aleuticus	COT_1	GenBank	JNO24987.1	*aleuticus*	97%
	COT_2	BOLD	BCF056-07	*aleuticus*	100%
C. asper	COT_1	BOLD	BCF062-07	*Cottus*	100%
	COT_2	BOLD	BCF062-07	*asper*	99.3%
C. cognatus 1	COT_1	BOLD	BCF062-07	Cottidae	98.8%*
	COT_2	BOLD	BCF062-07	*Cottus*	100%
C. cognatus 2	COT_1	Sequencing did not work			
	COT_2	Sequencing did not work			
C. cognatus 3	COT_1	Sequencing did not work			
	COT_2	Sequencing did not work			

*This sequence was 97.53% similar to *C. cognatus* in BOLD.

Discussion

The use of molecular protocols in biodiversity analysis requires methodology and the tools to effectively identify all species present within a system. Whether the DNA is rare or highly degraded, universal primers that target small gene regions can help to effectively identify all DNA and lead to a broad assessment of biodiversity. The use of fragmented and degraded DNA for species distinction is more problematic when genetically similar species coexist. Properly developed primers can lead to species distinction in these situations and allow for more certainty in the outcomes. Further, additional steps can be taken which benefit from the use of these universal mini primers. The analysis of eDNA through quantitative PCR (qPCR) which includes these primers can lead to a more efficient method to identify the presence of target species within aquatic environments.

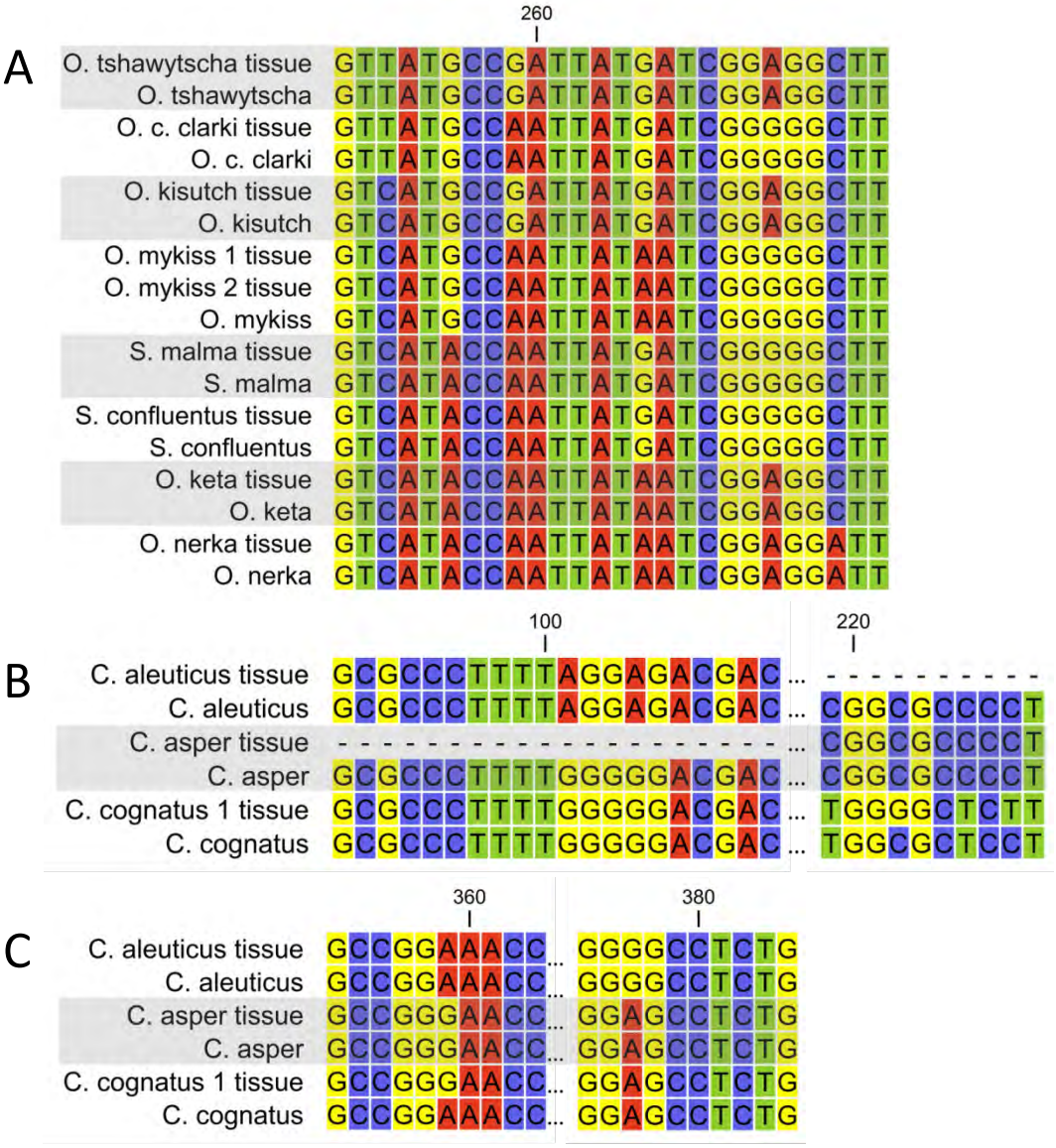


Figure 2-90 – Multiple sequence alignments for the cytochrome oxidase subunit 1 (COI) gene showing regions that vary among species using (A) SAL_1 primer set, (B) COT_1 primer set, and (C) COT_2 primer set. Sequences were generated from amplified DNA extracted from tissue samples and compared to the published sequences (Table 2-86).

The primers developed worked well to differentiate among species within the Families Salmonidae and Cottidae. Both the analysis with published sequences and our sequencing results correctly classified species — except for the samples within the Genus *Salvelinus* and for the *C. cognatus* samples. Our sequencing results for the two Cottidae primers were

unsuccessful for the three *C. cognatus* samples — despite the preliminary analysis with published sequences. DNA extracted from the *C. cognatus* samples, however, was poor quality and the bands were faint or missing on the agarose gel and was likely attributable to the poor preservation of DNA in these particular samples.

Species level identification for the Salmonidae sequences was consistent with the phylogenetic analysis (Figure 2-88) and did not distinguish *S. confluentus* and *S. malma*. Sequences of the COI gene retrieved from Genbank and BOLD for these two species were nearly identical for the target region. The SAL_1 primer set, however, will still be useful for determining species presence in eDNA samples collected from the Fraser River catchment and Kitimat River catchment. There is a good ability to differentiate among all the Salmonidae species that are found in both watersheds of our study. *S. malma*, however, is found throughout the Kitimat River catchment, but not in the central interior region of the Fraser River catchment (McPhail 2007). In contrast, *S. confluentus* is found throughout the interior, but not the Kitimat River catchment (McPhail 2007). Consequently, for the samples that we have collected for the Biodiversity Monitoring and Assessment Program, SAL_1 primers will be effective at detecting the presence of Salmonidae in eDNA of water samples in the Kitimat River and north Fraser River systems.

The SAL_1 primers also worked well to differentiate two closely related species that are known to hybridize, *O. mykiss* and *O. clarkii clarkii* (Bettles et al. 2005). It was also interesting to note that the top matches for our sample of *O. c. clarkii* from the BOLD database were 100% consistent with sequences from fish obtained along the west coast of North America from Washington, BC and Alaska. The second published sequence used in the development of the Salmonidae primers was from eastern BC, within the geographic range for the subspecies *O. c. lewisi*.

Both primer sets developed for Cottidae discriminated among the three freshwater species of sculpin found within the region sampled for our Biodiversity Monitoring and Assessment Project. Both *C. aleuticus* and *C. asper* are widely distributed in the Kitimat River catchment and *C. asper* and *C. cognatus* are widely distributed in the Upper Fraser River and Nechako River catchments (McPhail 2007) — so discrimination of these two species are

important. Both primer sets worked well for resolving sequence differences for the *C. aleuticus* and *C. asper*, but COT_2 amplified regions with greater ability to differentiate between *C. asper* and *C. cognatus*.

Two of the primer sets developed, SAL_1 and COT_2, produced amplicons close to 200 bp in length; a fragment likely to be amplified from degraded DNA as is likely for eDNA samples, but also a length well suited for next generation sequencing. Our ability to discriminate among Salmonidae species with SAL_1 was good, except within the Genus *Salvelinus*. One wide-ranging species of Salmonidae, *P. williamsoni*, differed in the region of the COI gene where our reverse primer was designed; consequently, SAL_1 is not expected to work for this species. We did not, however, capture any *P. williamsoni* in the streams sampled for the BMAP — suggesting that SAL_1 would still be useful for detecting the presence of Salmonidae species from our previously collected eDNA samples. Although COT_2 was not designed to work for *L. armatus*, this is a marine species and unlikely to be widespread in the Kitimat River catchment and not present in the Upper Fraser River. Our ability to discriminate among Cottidae species with COT_2, therefore, is high. The primers developed will be useful for determining presence of species within these two important Families of freshwater fish from water samples collected for eDNA — a useful tool for assessing biodiversity that will complement our fish sampling efforts.

2.13. Determining fish movement patterns and habitat connectivity using otolith microchemistry

Anne-Marie Flores, Adam O'Dell, Brandon Boucher[*], Chris McFarlane[*], Mark Shrimpton

Introduction

Aquatic animals found in temperate streams are intimately associated with bottom substrates. Many benthic invertebrates depend on gravel and cobble of streambeds to provide spaces for attachment, protection, feeding, and the interstitial flow of water for oxygen consumption (Wood and Armitage 1997). There is also a link to vertebrate fauna as many invertebrate species that reside on coarse substrates are preferred prey for fish. Changes to substrate characteristics from instream works, altered sediment-loading, and sedimentation will have implications not only for benthic invertebrates, but for fish as well. Fish depend on substrate for habitat, including cover, spawning substrate and forage, and respond behaviorally and physiologically to altered sediment dynamics. Fish, therefore, are sensitive to both the direct and indirect effects of increased sediment-loading. In response to habitat changes, fish can be displaced from, or avoid, disturbed areas that are no longer suitable. Fish abundance has been found to decrease downstream following pipeline installation (Anderson et al. 1998), but recovery of abundance occurred when higher flows removed disturbed sediments from the stream bed. If displacement of fish populations persists, however, such changes can have effects on long-term population structure as groups of animals become fragmented and isolated. Larval fish and small, stream-resident fish may be affected by disturbances from linear development as dispersal is often restricted to specific development stages in most species.

Movement patterns, however, are quite difficult to study and have traditionally relied on capturing, marking and recapturing individuals. Tracking fish through multiple life-history stages with conventional tagging techniques has contributed useful information to identify the timing and duration of habitat use, but there is an inherent bias in many tagging techniques. Tagging programs may be limited to the re-captured non-mobile portion of the population as migrating fish often leave the study area (Gowan et al. 1994), or to the members that are

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physically large enough to receive tags (Benjamin et al. 2014). Differences can exist in migratory tendencies among and even within species in studies where individual fish were followed (Gowan et al. 1994; Bahr and Shrimpton 2004; Chapman et al. 2012). Small fish species and early-life stages of larger fish are difficult to tag leading to a paucity of information for part or all of the lifecycle for many species.

Life histories of fishes can be revealed with the aid of otoliths — small, aragonite structures that form part of the hearing and balance system in teleosts. Calcium carbonate is accreted daily onto the protein matrix of otoliths (Söllner et al. 2003) resulting in daily and annual growth bands that are possible to resolve microscopically and reveal age of the fish (Pannella 1971; Mugiya et al. 1981). It is also possible using analytical instruments such as laser ablation inductively coupled plasma mass spectrometers (LA-ICP-MS) to characterize the elemental composition of otoliths. The combination of aging structures and chemical analyses provides a powerful set of tools to examine fish life histories (Campana and Thorrold 2001; Elsdon et al. 2008). Otolith microchemistry has shown promise in providing habitat use data for fish. The large difference in the chemical composition between freshwater and seawater is reflected in otolith microchemistry and has been used to assess movement of anadromous fish between these two media (Veinott et al. 1999) and also to identify juvenile progeny of anadromous or resident fish (Zimmerman and Reeves 2002). Potential also exists to assess movement within freshwater systems as elemental stream signatures are based on differences in the underlying bedrock geology (Kennedy et al. 1997). The distinctive chemical signatures that vary among freshwater streams and rivers also correlate with elemental ratios in the bones of a number of freshwater species (Kennedy et al. 1997; Clarke et al. 2007b; Shrimpton et al. 2014). Consequently, changes in elemental signatures in bones of fish reflect habitat shifts and patterns of movement for individual fish within freshwater systems (Shrimpton et al. 2014; Clarke et al. 2015). Such an approach could reveal variation that exists within populations of a species, but also responses of fish to habitat disturbance.

Our objective was to use microchemistry signatures from otoliths to assess patterns of movement in fish prior to construction of the Pacific Trails Pipeline project. The potential for habitat fragmentation of fish populations due to environmental disturbance has been assessed

using a number of different strategies depending on abundance, species composition, and size of individuals — but otolith microchemistry shows promise for assessing not only movement of individual fish, but also connectivity among different locations and habitats, or whether fish are resident to specific locations. For this study, we used putative resident fish from the Family Cottidae and putatively mobile species from the Family Salmonidae. Comparison of movement patterns for species from both Families will reveal connectivity among river systems and whether fish are resident to the study streams. Our initial investigation provides an understanding of fish movement patterns, which is important to assess whether patterns change after the pipeline has been constructed.

Methods

Study locations

This project was conducted within tributaries of the Nechako River and Kitimat River watersheds in central BC. Sample sites were chosen from rivers and creeks that were characterized as fish bearing with good rearing habitat.

In the Nechako River watershed two creeks were sampled; Ormond Creek and Nine Mile Creek (Figure 2-91). Ormond Creek is a small lake headed system that flows into Fraser Lake at 10 U 385484 5994765; distance from Ormond Lake to Fraser Lake is ~10 km. A complex channel with abundant cover and areas of slow moving water provide excellent rearing habitat in Ormond Creek. Wetted width is 5.9 m and ranges from 3.7 – 7.0 m. Nine Mile Creek is a slightly smaller system than Ormond Creek, but not lake headed and flows into the Nechako River at 10 U 406122 5996879. The channel is complex with abundant cover for good rearing habitat. Wetted width is 4.3 m and ranges 3.0 – 5.5 m.

In the Kitimat River watershed, five creeks were sampled; Trout Creek, 9.8 km Stream, Cecil Creek, Chist Creek, and 57.1 km Stream (Figure 2-92). Trout Creek has excellent rearing habitat, created by large woody debris, boulders, and undercut banks. Wetted width is 4.7 m and ranges from 3.0 – 7.0 m. Trout Creek flows into the Little Wedeene River at 9 U 523770 5997825. 9.8 km Stream is a system with abundant cover and good channel complexity for

rearing; flow is good and there are parts with slow moving water for smaller fish. Channel wetted width is 2.1 m and ranges from 1.0 – 4.0 m. 9.8 km Stream flows into Goose Creek at 9 U 521235 5991586; 250 m above the confluence with the Kitimat River. Cecil Creek has abundant gravel with lots of cover and scour pools. Wetted width is 8.5 m and ranges from 7.5 – 10.3 m. Cecil Creek flows into the Kitimat River at 9 U 527700 6005907. Chist Creek has abundant cover provided from cobble, depth, boulders, off-channel, and side-channels. There are also pools up to 2 m deep. Wetted width is 18.5 m and ranges 10.4 – 28.9 m. Chist Creek enters the Kitimat River at 9 U 532797 6011831. 57.1 km Stream has coarse substrate and abundant cover in pools and riffles. Wetted Width is 3.7 m and ranges from 3.1 – 4.7 m. The Stream enters the Kitimat River at 9 U 546437 6011177.

Water sample collection and analysis

To determine the heterogeneity of streams and rivers within the study watersheds, water samples were obtained from geographically distinct locations in the summer of 2014 (Figure 2-91 and Figure 2-92). Methods for obtaining water samples followed the recommendations outlined by Shiller (2003) for sampling dissolved elements in remote locations, with some minor modifications as outlined by Clarke et al. (2007a). High-density polyethylene bottles (50 mL) were cleaned with ultra-pure water and filled with a solution of 2 % high purity nitric acid and left for a minimum of two weeks. Bottles were then rinsed five times in ultra-pure water. Polyethylene 50-mL syringes were cleaned in the same manner as the 50-mL bottles. Nylon filters (25 mm × 0.45 µm) were cleaned by passing 40 mL of a solution of 2 % high purity nitric acid followed by a rinse of 20 mL of ultra-pure water. All filters were blown dry with high-pressure clean air and left under a fume hood until use. All water samples collected in the field were acidified to 2 % high purity nitric acid immediately after collection.

Water analysis was completed at the University of Victoria School of Earth and Ocean Sciences with a Thermo Scientific X-Series 2 Quadrupole inductively coupled plasma mass spectrometer (ICP-MS) (Waltham, MA). A mixture of Rh, In and Re was added on-line as the internal standard. External calibration standards were prepared from single and mixed element stock solutions (NIST traceable). Accuracy was tested by analysis of the certified reference materials SLRS-5 (Ottawa River Water). Precision was determined by repeat analysis of an

"average" solution prepared by combining a small amount of each of the samples. The limit of detection was determined as 3 times the standard deviation of the laboratory acid blank. The elements measured included: Barium (Ba), Calcium (Ca), Strontium (Sr), Magnesium (Mg), Manganese (Mn), Rubidium (Rb), Sodium (Na), Phosphorus (P), Sulfur (S), Potassium (K), Iron (Fe), and Zinc (Zn).

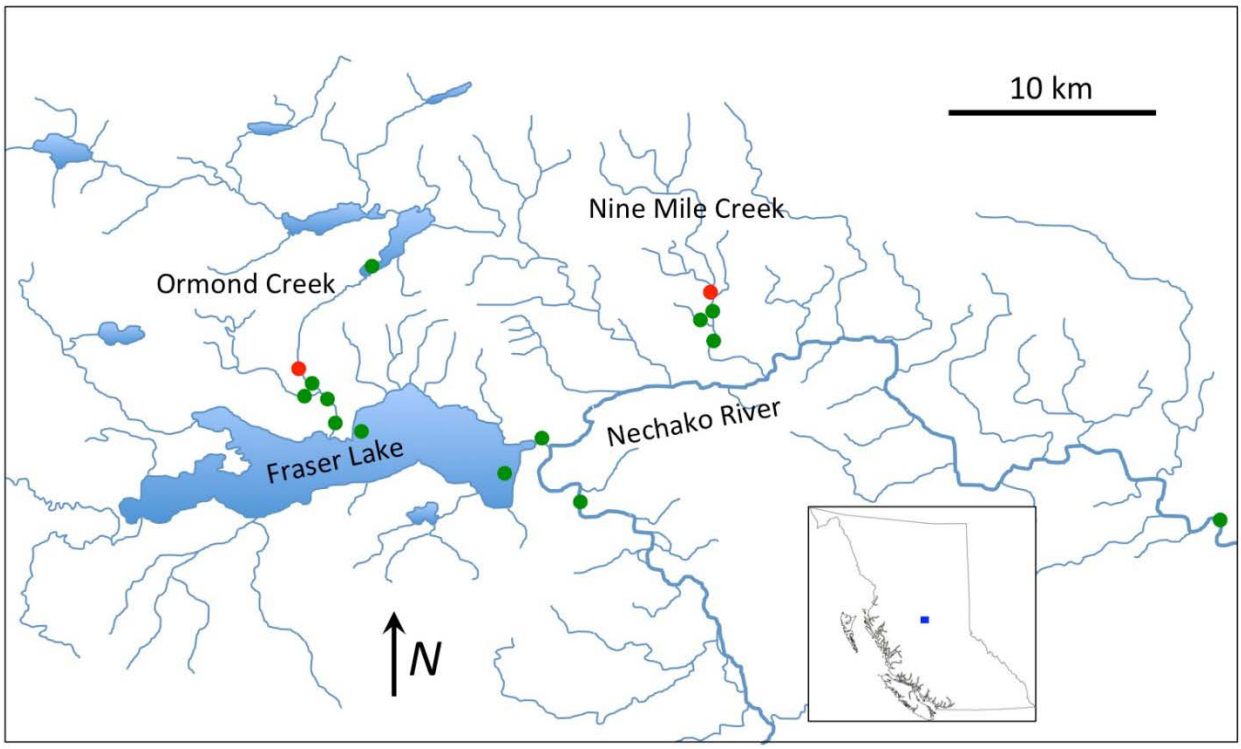

Figure 2-91 – Map of the Nechako River watershed and Fraser Lake showing the locations of rivers where water samples were collected and fish were captured. Approximate locations where fish were captured are shown with red circles (●); electrofishing was conducted from -250 – -150 m below the RoW and from +150 – +250 m above the RoW. Water samples were collected at the RoW for all fish sampling locations and additional water samples were collected at locations shown by the green circles (●). Inset map shows the region of BC where the study was located.

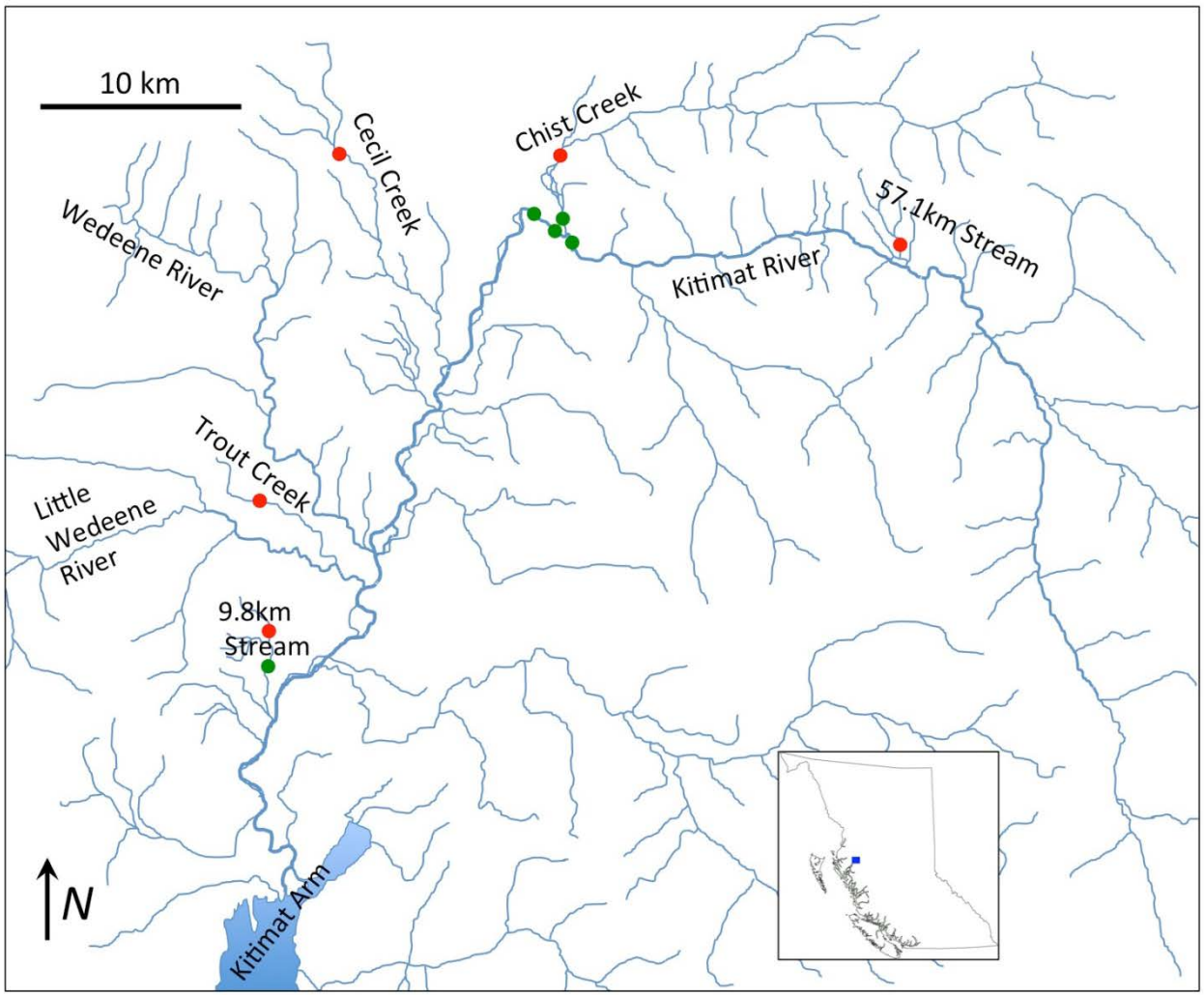

Figure 2-92 – Map of the Kitimat River watershed showing the locations of rivers where water samples were collected and fish were captured. Approximate locations where fish were captured are shown with red circles (●); electrofishing was conducted from -250 – -150 m below the RoW and from +150 – +250 m above the RoW. Water samples were collected at the RoW for all fish sampling locations and additional water samples were collected at locations shown by the green circles (●). Inset map shows the region of BC where the study was located.

Discriminant function analysis (DFA) using jack-knife resampling was used to provide a visualization of geographical separation using water chemistry data collected from the watersheds sampled using a combination of Sr : Ca, Ba : Ca, Mn : Ca, and Rb : Ca (SYSTAT 7.0, San Jose, CA). Water chemistries were obtained from both the tributaries and the mainstem rivers for both the Kitimat River and Nechako River watersheds. Jack-knife re-sampling was

used to validate the robustness of the discriminant functions. The jack-knife approach is appropriate when sample sizes are too small to allow for a split sample procedure (Tabachnick & Fidell 2001). Confidence ellipses were also created for each mainstem river and tributaries. Ellipses were centered on the mean Discriminant Function values with boundaries defined by the unbiased standard deviations. Default probability of 0.683 was used for ellipse creation.

Fish collection

Fish were captured using a Smith Root L24 backpack electrofisher (Vancouver WA) during the summers of 2013, 2014, and 2015. We selected portions of streams below and above the proposed pipeline crossing for electrofishing on each stream. Below the crossing location we fished a representative portion of the reach that was 100 m long that corresponded to approximately -250 – -150 m. Above the crossing location we fished a representative portion of the reach of a similar length that corresponded to approximately +150 – +250 m. After capture fish were transferred to a bucket containing 100 mg × L^{-1} tricaine methanesulfonate buffered with 200 mg × L^{-1} sodium bicarbonate for sampling. Length (to 0.1 cm) and weight (to 0.01 g) were recorded for each fish prior to removal of gastro-intestinal tract to determine stomach contents for food web analysis and then the fish were frozen. In the laboratory, the sagittal otoliths were removed and placed in a small vial with moist cotton wool for storage. Sagittal otoliths were chosen, as they are the largest of the three pairs of otoliths found in teleosts.

Species collected in the Nechako River watershed and tributaries were Prickly Sculpin (*Cottus asper*), Rainbow Trout (*Oncorhynchus mykiss*), Burbot (*Lota lota*), Redside Shiner (*Richardsonius balteatus*), Longnose Dace (*Rhinichthys cataractae*), Leopard Dace (*Rhinichthys falcatus*), Mountain Whitefish (*Prosopium williamsoni*), and Longnose Sucker (*Catostomus catostomus*). Species collected in Kitimat River watershed and tributaries were Prickly Sculpin, Coastrange Sculpin (*C. aleuticus*), Dolly Varden (*Salvelinus malma*), Rainbow Trout, Cutthroat Trout (*O. clarkii*), Coho Salmon (*O. kisutch*) and Chinook Salmon (*O. tshawytscha*). Although 13 species of fish were captured, we limited the analysis to abundant and wide-ranging species so that we could assess variation within streams and among streams for different species. We used putative resident species (*C. asper* and *C. aleuticus*), and potential migratory or mobile

species (*O. mykiss*, *O. clarkii*, and *S. malma*) for the otolith microchemistry analysis. These species were the most commonly found and most abundant species found in streams of the interior plateau and coastal region. A summary of fish used in the study is given in Table 2-88.

Table 2-88 – Summary of species from each study stream of the Nechako River and Kitimat River watersheds used in the otolith chemical signatures study. Data is presented as mean ± standard deviation and the minimum and maximum length of fish caught at each site for each species. n is the number of samples used in the analysis.

	Length of fish below RoW				Length of fish above RoW			
	n	mean	min	max	n	mean	min	max
Nechako River watershed								
Ormond Creek								
Cottus asper	7	8.0 ± 2.2	6.5	12.7				
Oncorhynchus mykiss	5	12.2 ± 2.2	9.7	15.6	3	10.7 ± 1.5	9.0	11.7
Lota lota	2	10.2 ± 0.3	10.0	10.4				
Nine Mile Creek								
Oncorhynchus mykiss	4	11.4 ± 1.0	10.3	12.7	3	11.8 ± 2.4	9.1	13.2
Kitimat River watershed								
Trout Creek								
Oncorhynchus clarkii	10	14.0 ± 2.9	10.9	18.4	8	12.8 ± 3.5	10.1	19.2
9.8 km Stream								
Oncorhynchus clarkii	1	14.1			5	12.8 ± 2.3	10.6	16.3
Salvelinus malma	3	8.4 ± 0.4	8.1	8.3	1	9.8		
Cecil Creek								
Oncorhynchus clarkii					6	10.7 ± 0.8	9.3	11.6
Salvelinus malma	1	7.5			3	9.3 ± 1.9	7.8	11.5
Chist Creek								
Cottus aleuticus					7	9.4 ± 0.5	8.6	10.1
Oncorhynchus mykiss	6	10.2 ± 1.1	9.0	11.8	6	11.7 ± 2.6	8.8	15.1
Salvelinus malma	3	12.6 ± 2.8	9.4	14.6	3	13.6 ± 0.3	13.3	13.9
57.1 km Stream								
Oncorhynchus clarkii	3	9.4 ± 0.2	9.3	9.7	9	14.3 ± 2.0	11.3	17.6
Salvelinus malma	6	13.2 ± 3.7	8.6	17.2				

Qualitative assessment of fish movement

Cathodoluminescence (CL) is light emitted by a substance excited by high-energy electrons and released through fluorescence. Element substitutions in the carbonate matrix of otoliths have been shown to emit CL of different colours (Halden et al. 2004) making this technique useful to assess heterogeneity of chemical composition within fish otoliths. Otoliths were embedded in Struers Epofix epoxy resin (Mississauga, ON) and then sequentially polished

using a combination of 300, 600, and 1200 grit wet/dry sandpaper and 6 µm, 3 µm, and 1 µm diamond paste on a Buehler Minimet polishing machine (Lake Bluff, IL).

The CL imaging was performed using a cold-cathode cathodoluminescence imaging systems (Nuclide Luminoscope cold cathode system, Model PP15-10; State College, PA). A high-energy beam of electrons (12.5 – 17.5 kV; 0.5 – 0.7 mA) was focused on a polished otolith in a vacuum chamber. The incident electrons cause bound electrons to rise to higher energy levels and when the electrons return to their original state, they release the energy through luminescence. The wavelength of the light emitted is specific to each element. Light emitted by the sample is collected with achromate lenses and guided to the entrance slit of the spectrometer. Exposure time was 10 sec with a 10-sec noise reduction correction.

Quantitative assessment of fish movement

Resonetics S-155-LR 193nm Excimer laser ablation system (Nashua, NH) coupled to an Agilent 7700x quadrupole ICP-MS (Santa Clara, CA) was used to determine otolith elemental composition. The instruments are housed in a purpose-built laboratory with tightly controlled air conditioning and ventilation to guarantee minimal instrument drift in the University of New Brunswick, Department of Earth Sciences. We used two different standards, MACS (a synthetic carbonate standard) and NIST610 (a primary concentration glass standard). The MACS standard was used to reduce the data, except for Rb where the NIST610 standard was used. All otolith ablation paths ran from edge to edge through the core. We used a rotating slit to ensure the best spatial resolution possible for the different growth zones, consequently the lines are not perfectly straight but curved to compensate for the complex zoning of each sample and reduce overlap between each zone. We scanned the 60 × 6 µm slit at 6 µm × sec^{-1} and the quadrupole sweep time (i.e., to make a complete measurement of all of the isotopes) was 0.995 sec. The isotopes measured in the otoliths were ^{23}Na, ^{25}Mg, ^{31}P, ^{34}S, ^{39}K, ^{42}Ca, ^{44}Ca, ^{55}Mn, ^{56}Fe, ^{66}Zn, ^{85}Rb, ^{88}Sr, and ^{137}Ba. Calcium was used as the internal standard due to the otoliths aragonite ($CaCO_3$) composition, which has a known and consistent Ca content (40 % molar weight). An internal standard was used to account for variations in aerosol production caused by the variation in the amount of material being extracted from the otolith by the laser.

The relationship between water elemental signature and otolith microchemistry was determined by linear regression of the chemical signature at the outer edge of the otolith as a function of water chemistry from the location of capture. We used the outer 18 µm of the otolith as it represents the most recent time period experienced by the fish before capture. Relationships were examined for Sr, Ba, Mn, and Rb between water and otoliths. Sr and Ba have previously been shown to have a strong relationship between water and otoliths (see Clarke et al. 2007a, 2007b; Shrimpton et al. 2014). There is recent evidence that Rb is a useful element in statoliths of spawning adult sea lamprey (*Petromyzon marinus*) for discriminating populations (Lochet et al. 2014). These four elements also exhibited good variation among watersheds and appeared to show variation in the linescans of otoliths. Linear regression was conducted on mean otolith elemental composition for fish captured in each of the different river systems. Data from two recent publications were also included in the analysis; Shrimpton et al. (2014) examined relationships for juvenile *O. kisutch* and Clarke et al. (2015) examined relationships for *C. cognatus*. Where significant relationships were found between otolith and water elemental composition, putative movement patterns for fish were calculated using the coefficients from the linear regressions. Zinc oscillations were used to estimate age and annuli zonation for each fish. Halden et al. (2000) showed that zinc fluctuations in otoliths provide temporal information consistent with the annual cycle in the biology of the fish. Age of approximate annuli zonation was confirmed by counting annuli of each sectioned otolith using light microscope images.

Results

Water chemistry

Considerable differences exist in water chemistry among the rivers and streams measured in the Nechako and Kitimat River watersheds (Table 2-89). Discriminant function analysis of the elemental signatures in water samples for Mn, Rb, Sr and Ba revealed good separation for each location (Pillai's Trace = 3.713; $F_{48,152} = 40.92$; $p < 0.001$). The percentage of correct classification for each location is provided in Table 2-89. The total number of cases classified correctly using jack-knife validation was 94%. Discriminant function analysis indicated

that some of the samples collected from the Nechako River grouped with the Fraser River samples. Additionally some of the samples collected from 9.8 km Stream grouped with samples from Chist Creek (Table 2-89). For example, in Table 2-89, two water samples from the Fraser River were correctly classified as Fraser River samples (Fr), showing 100% accuracy in the classification of those water samples (cross-validation accuracy). For the Nechako River samples, two were classified incorrectly as Fr, and two were classified correctly as Nechako (Ne), showing only 50% accuracy in the classification. Samples collected from an individual river system reflect changes in water chemistry between the headwaters and mouth of the rivers (Figure 2-93). Rivers and creeks flowing from lakes also grouped with the lakes from which they flowed; these systems were grouped for the analysis.

Table 2-89 – The percentage of correct classification determined by discriminant function analysis for water samples collected from rivers and streams in the Nechako and Kitimat River watersheds using jack-knife re-sampling to validate the robustness of the discriminant functions. Cross-validation accuracy is expressed as percent (%). The elements incorporated into the model were Sr, Ba, Rb, and Mn. Nautley River includes samples collected from Fraser Lake; Ormond Creek includes samples collected from Ormond Lake.

River	Fr	Ne	Na	9m	9t	Or	Ot	Ki	Tr	9k	Ce	Ch	57	%
Fraser River (Fr)	2	0	0	0	0	0	0	0	0	0	0	0	0	100
Nechako River (Ne)	2	2	0	0	0	0	0	0	0	0	0	0	0	50
Nautley River (Na)	0	0	6	0	0	0	0	0	0	0	0	0	0	100
Nine Mile Ck (9m)	0	0	0	6	0	0	0	0	0	0	0	0	0	100
Nine Mile trib (9t)	0	0	0	0	2	0	0	0	0	0	0	0	0	100
Ormond Creek (Or)	0	0	0	0	0	10	0	0	0	0	0	0	0	100
Ormond trib (Ot)	0	0	0	0	0	0	2	0	0	0	0	0	0	100
Kitimat River (Ki)	0	0	0	0	0	0	0	4	0	0	0	0	0	100
Trout Creek (Tr)	0	0	0	0	0	0	0	0	2	0	0	0	0	100
9.8 km Stream (9k)	0	0	0	0	0	0	0	0	0	3	0	1	0	75
Cecil Creek (Ce)	0	0	0	0	0	0	0	0	0	0	2	0	0	100
Chist Creek (Ch)	0	0	0	0	0	0	0	0	0	0	0	6	0	100
57.1 km Stream (57)	0	0	0	0	0	0	0	0	0	0	0	0	2	100
Total	4	2	6	6	2	10	2	4	2	3	2	7	2	94

Otolith microchemistry: cathodoluminescence

Cathodoluminescence (CL) is the emission of photons that produce colour specific to the composition of the materials. Typically, CL images for trout and char showed bands of lighter colouration alternating with darker blue (Figure 2-94). For some fish, the bands appear to

alternate and correspond with the annular rings seen in light microscope images such as *O. clarkii* caught in 57.1 km Stream (Figure 2-94A). For other fish, bands of green were visible — although the pattern did not correspond with annular rings. For *O. clarkii* and *S. malma* caught in Cecil Creek, a brighter green band was prominent near the core and additional bands were also evident towards the edge of the otolith (Figure 2-94B and C, respectively). Otolith colouration also differed between fish caught in the same creek such as the otoliths from two *O. mykiss* from Nine Mile Creek; distinct green bands were visible near the otolith core and edge for one fish with less defined bands in another fish (Figure 2-94D and E, respectively). Further, an otolith from *O. mykiss* caught in Ormond Creek exhibited more green bands than blue (Figure 2-94F).

Otoliths from Cottidae and Gadidae also showed considerable variation in luminescence. *C. asper* from Ormond Creek showed contrasting patterns; green near the centre and edge (Figure 2-95A) compared to blue near the centre and edge (Figure 2-95B). *C. aleuticus* from Chist Creek also showed variation in luminescence, although the lighter and darker blue bands were visible (Figure 2-95C and D). We also analyzed species that did not belong to either the Family Salmonidae or Cottidae. *Lota lota* from the Family Gadidae also showed distinct regions of green luminescence around the outer edge of the otolith and lighter blue near the centre (Figure 2-95E and F).

Otolith microchemistry: elemental composition

To assess whether otolith zonation revealed by the CL images corresponded to elemental composition, we compared the linescan data determined from LA-ICP-MS analysis to the CL images. Changes in elemental signatures for Ba and Sr superimposed on a CL image for a juvenile *O. clarkii* caught in 57.1 km Stream is shown in Figure 2-96A. The CL image shows clear zonation with changes in intensity of blue colouration — the intensity corresponded well with the annuli rings observed from the light microscopy image (inset image on Figure 2-96A). Although the Ba signature shows marked changes across the otolith, there is no correspondence with the intensity of luminescence. Variation in Sr concentration, however, corresponded with the intensity of blue luminescence for the outer rings on the otolith — suggesting a correlation — but not near the core of the otolith. High concentrations of Sr were

observed in a number of otoliths near the core without a corresponding increase in intensity of blue (Figure 2-96B). Variation in blue luminescence did not correspond with changes in Sr signature for *S. malma* (Figure 2-96C). Green luminescence also showed no correspondence with changes in Sr signature. Bands of green luminescence also do not correspond with changes in Sr concentration (Figure 2-96D). Similarly, there was no correspondence between Ba concentrations from the LA-ICP-MS analysis and blue luminescence (Figure 2-96A, B, and C) or green luminescence (Figure 2-96B and D). Consequently, CL images do not appear to be effective at qualitatively detecting changes in Sr or Ba in the boney matrix of otoliths.

In contrast we found good overlap between the peaks in Mn concentration from LA-ICP-MS analysis and green luminescence. Otoliths from *O. clarkii* and *O. mykiss* with striking bands of green luminescence near the core match high Mn content near the core (Figure 2-97A and B). Additionally, an otolith from *O. mykiss* with intense green luminescence exhibited very high Mn content with clear spikes in Mn corresponding with bright green rings in the otolith (Figure 2-97C). Additionally, an otolith from an *O. clarkii* with no green luminescence exhibited very low levels of Mn across the entire linescan (Figure 2-97D).

Another element that corresponded well with morphological changes in the otolith was Zn. Although not seen in the CL images, the annuli observed in the light microscope images corresponded well with oscillations in Zn content. Dark annuli characteristic of annual growth rings overlapped well with low values in Zn oscillations for *O. clarkii* (Figure 2-98A and B) and for *O. mykiss* (Figure 2-98C), but not for otoliths from Cottidae (data not shown).

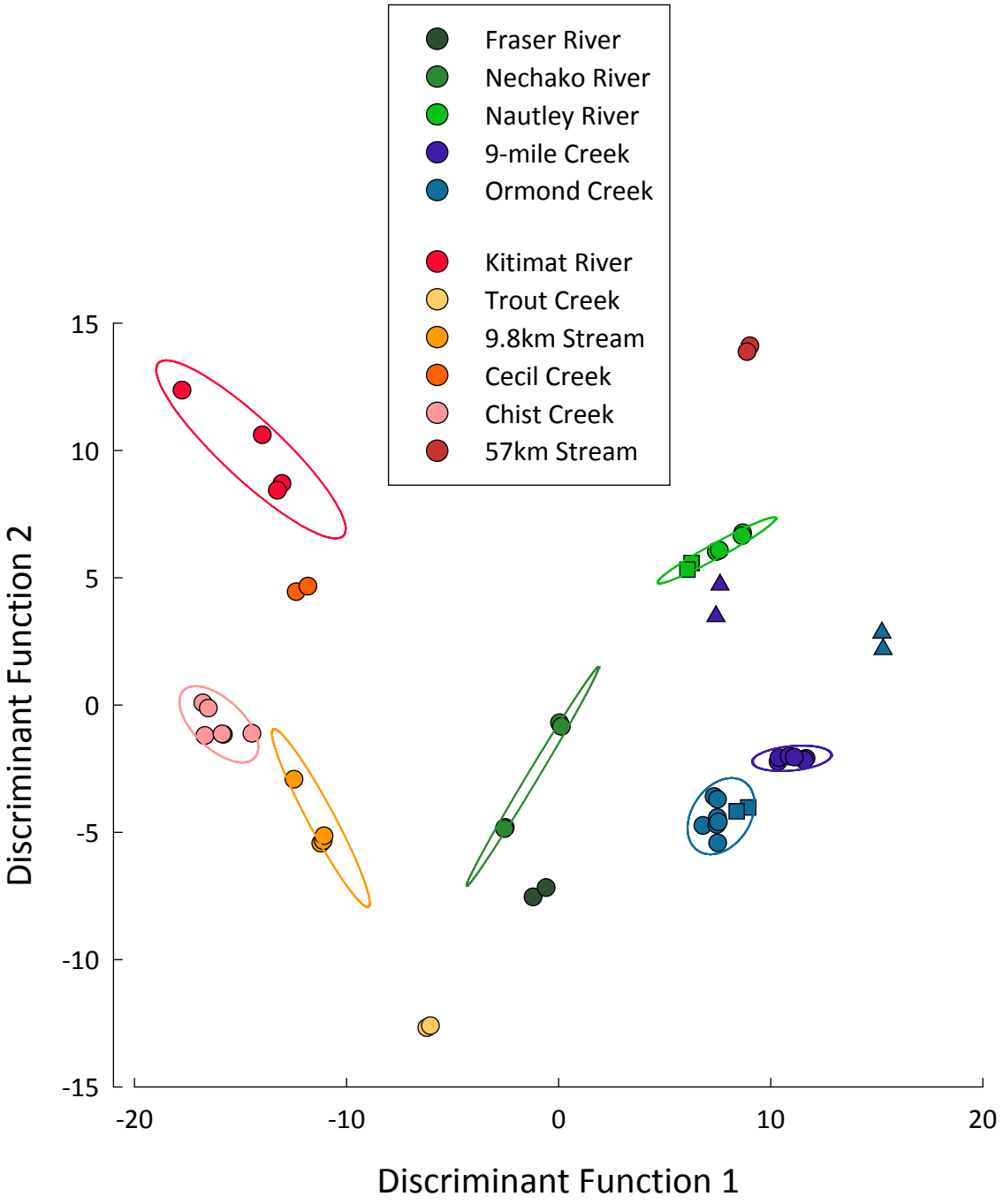

Figure 2-93 – Canonical discriminant function analysis characterizing tributaries in section 2 (Fraser River watershed; blue and green symbols) and section 4 (Kitimat River watershed; red and orange symbols) using multivariate signatures of Sr:Ca, Ba:Ca, Mn:Ca, and Rb:Ca. Samples collected in rivers and streams are defined by circles, lakes by squares, and small tributaries by triangles. Most rivers were sampled at multiple locations; confidence ellipses shown for each system where multiple samples were collected.

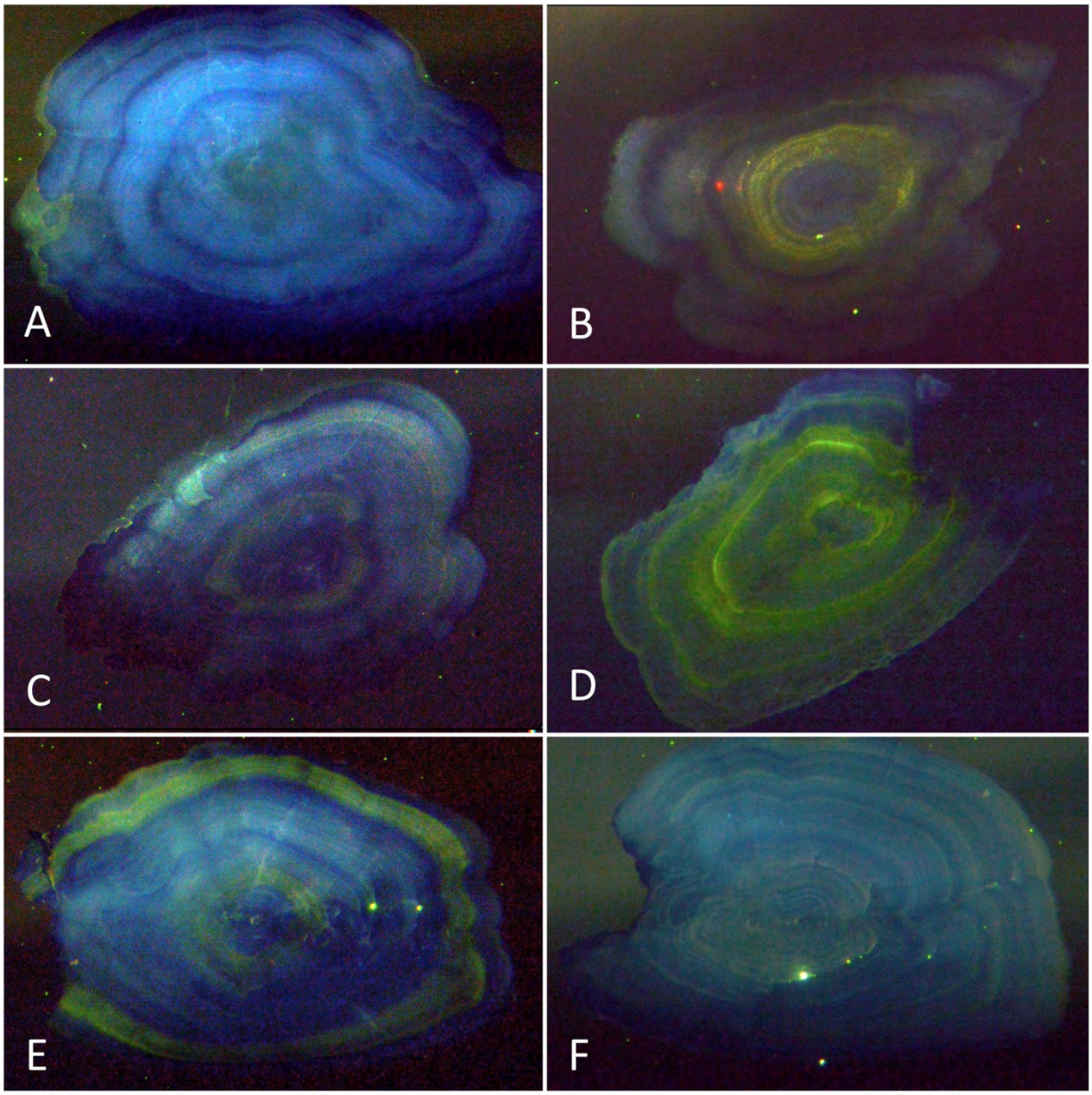

Figure 2-94 – Representative cathodoluminescent images for Salmonidae otoliths. Images are for (A) *O. clarkii* (F15-88) captured on 07-Aug-2015 in Cecil Creek at +200 m above the RoW, 11.6 cm, 18.8 g, (B) *Salvelinus malma* (F14-654) captured on 24-Jul-2014 in Cecil Creek at +200 m above the RoW, 11.5 cm, 14.8 g, (C) *O. clarkii* (F14-858) captured on 09-Aug-2014 in 57.1 km stream at +200 m above the RoW, 16.2 cm, 45.6 g, (D) *Oncorhynchus mykiss* (RB13-1) captured on 20-Sep-2013 in Ormond Creek at a site below the pipeline crossing RoW, 15.6 cm, 38.5 g, (E) *O. mykiss* (F14-1199) captured on 04-Sep-2014 in Nine Mile Creek at +200 m above the RoW, 13.2 cm, 21.1 g, and (F) *O. mykiss* (F14-1200) captured on 04-Sep-2014 in Nine Mile Creek at +200 m above the RoW, 13.2 cm, 24.2 g.

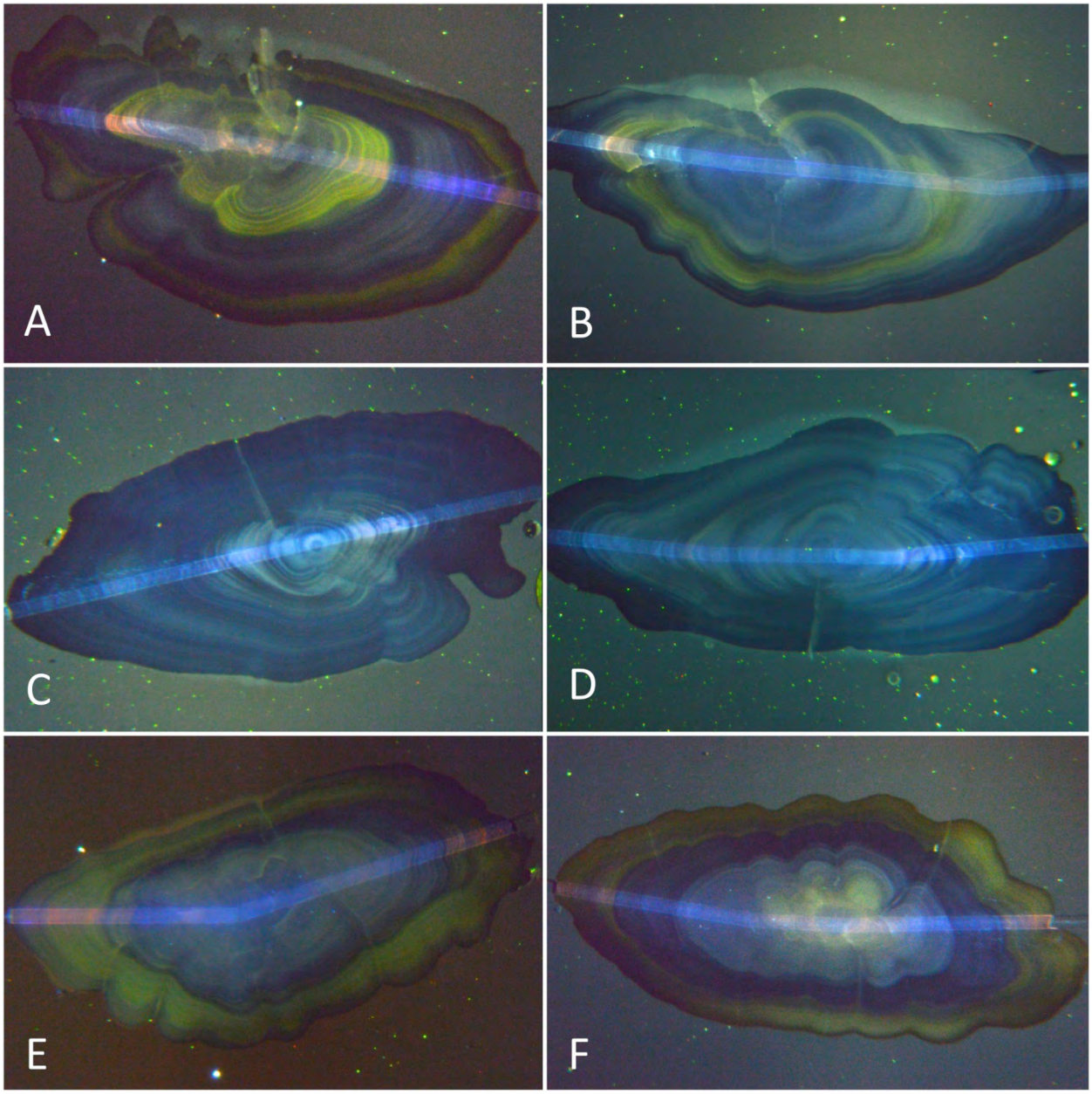

Figure 2-95 – Representative cathodoluminescent images for Cottidae and Gadidae otoliths. Images are for (A) *Cottus asper* (CAS13-2) captured on 20-Sep-2013 in Ormond Creek below the RoW, 6.5 cm, 2.9 g, (B) *C. asper* (CAS13-7) captured on 20-Sep-2013 in Ormond Creek below the RoW, 7.6 cm, 5.4 g, (C) *C. aleuticus* (F14-545) captured on 23-Jul-2014 in Chist Creek at -200 m below the RoW, 9.1 cm, 7.8 g, (D) *C. aleuticus* (F15-67) captured on 6-Aug-201 in Chist Creek at +200 m above the RoW, 9.5 cm, 7.9 g, (E) *Lota lota* (BB13-1) captured on 20-Sep-2013 in Ormond Creek below the RoW, 10.0 cm, 6.1 g, and (F) *L. lota* (BB13-2) captured on 20-Sep-2013 in Ormond Creek below the RoW, 10.4 cm, 7.0 g. Images were taken after laser ablation and bright line transecting each otolith is the laser path.

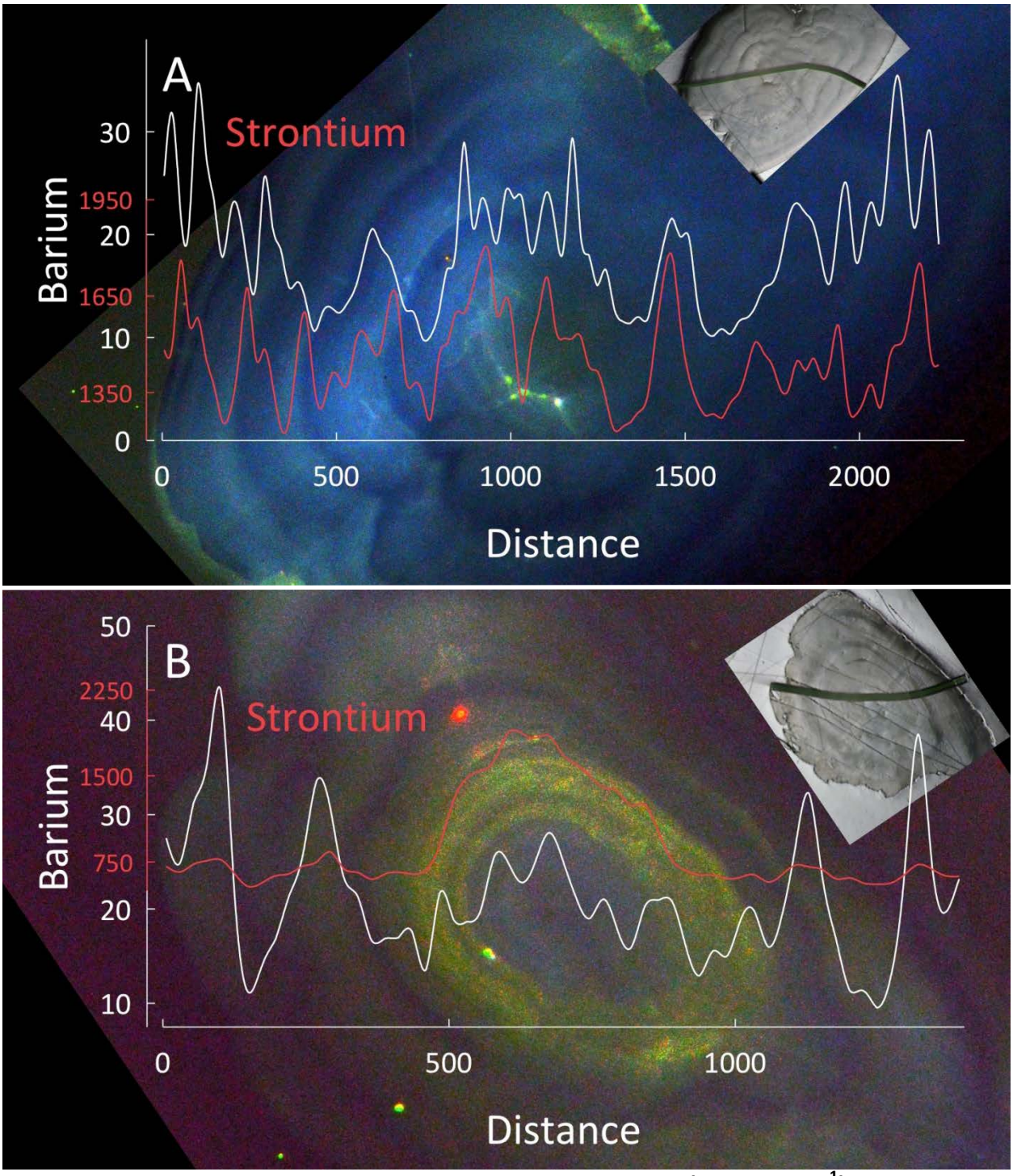

Figure 2-96 – Laser ablation linescans for Barium and Strontium (mmol × mol^{-1}) superimposed on cathodoluminescent images of otoliths from (A) a cutthroat trout (F14-858) captured on 09-Aug-2014 in 57.1 km stream at +200 m above the RoW, 16.2 cm, 45.6 g, and (B) a cutthroat trout (F15-88) captured on 07-Aug-2015 in Cecil Creek at +200 m above the RoW, 11.6 cm, 18.8 g. Insets are low resolution images showing the laser ablation paths.

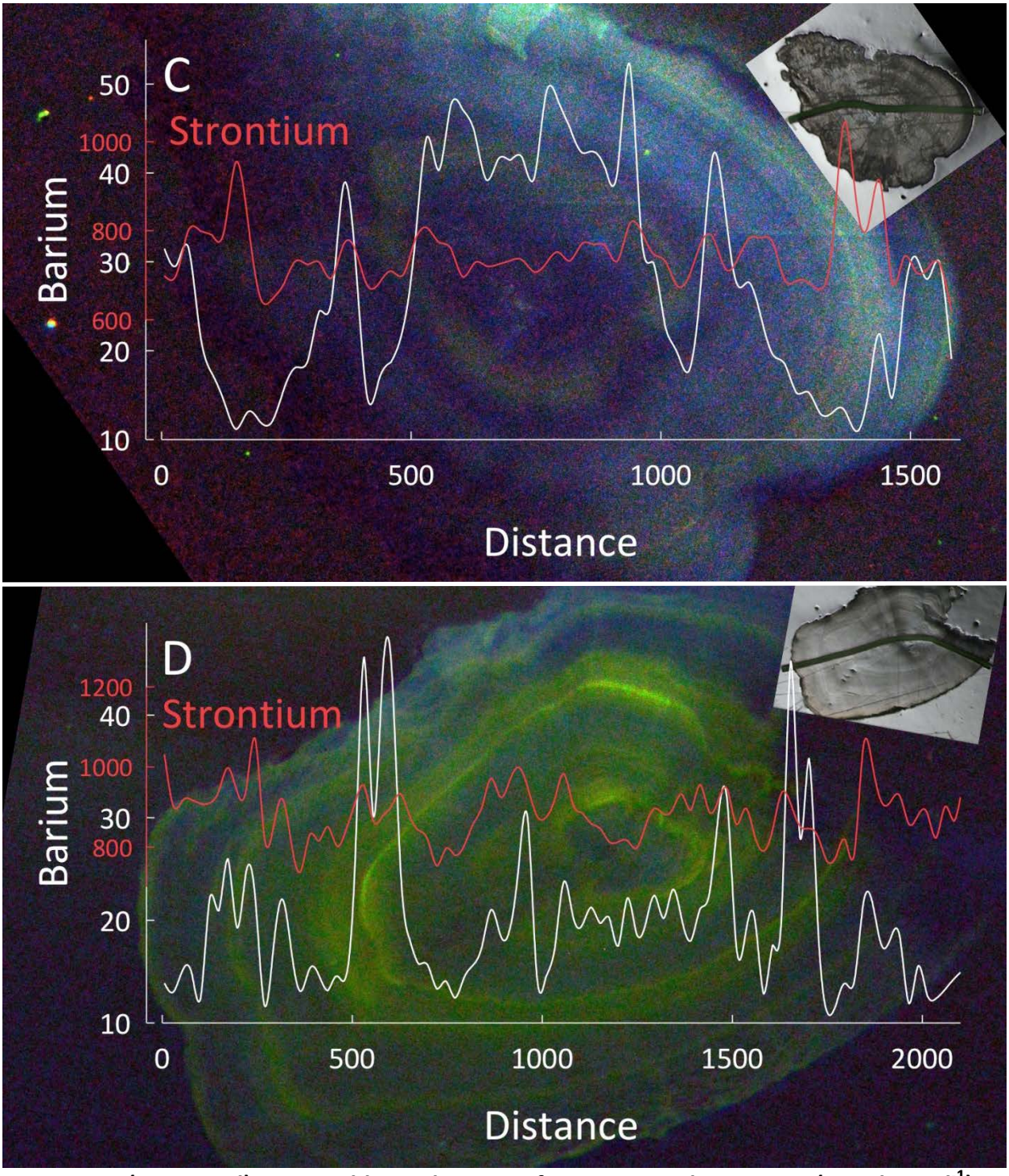

Figure 2-96 (continued) – Laser ablation linescans for Barium and Strontium (mmol × mol^{-1}) superimposed on cathodoluminescent images of otoliths from (C) a Dolly Varden (F14-654) captured on 24-Jul-2014 in Cecil Creek at +200 m above the RoW, 11.5 cm, 14.8 g, and (D) a rainbow trout (RB13-1) captured on 20-Sep-2013 in Ormond Creek below the RoW, 15.6 cm, 38.5 g. Insets are low resolution images showing the laser ablation paths.

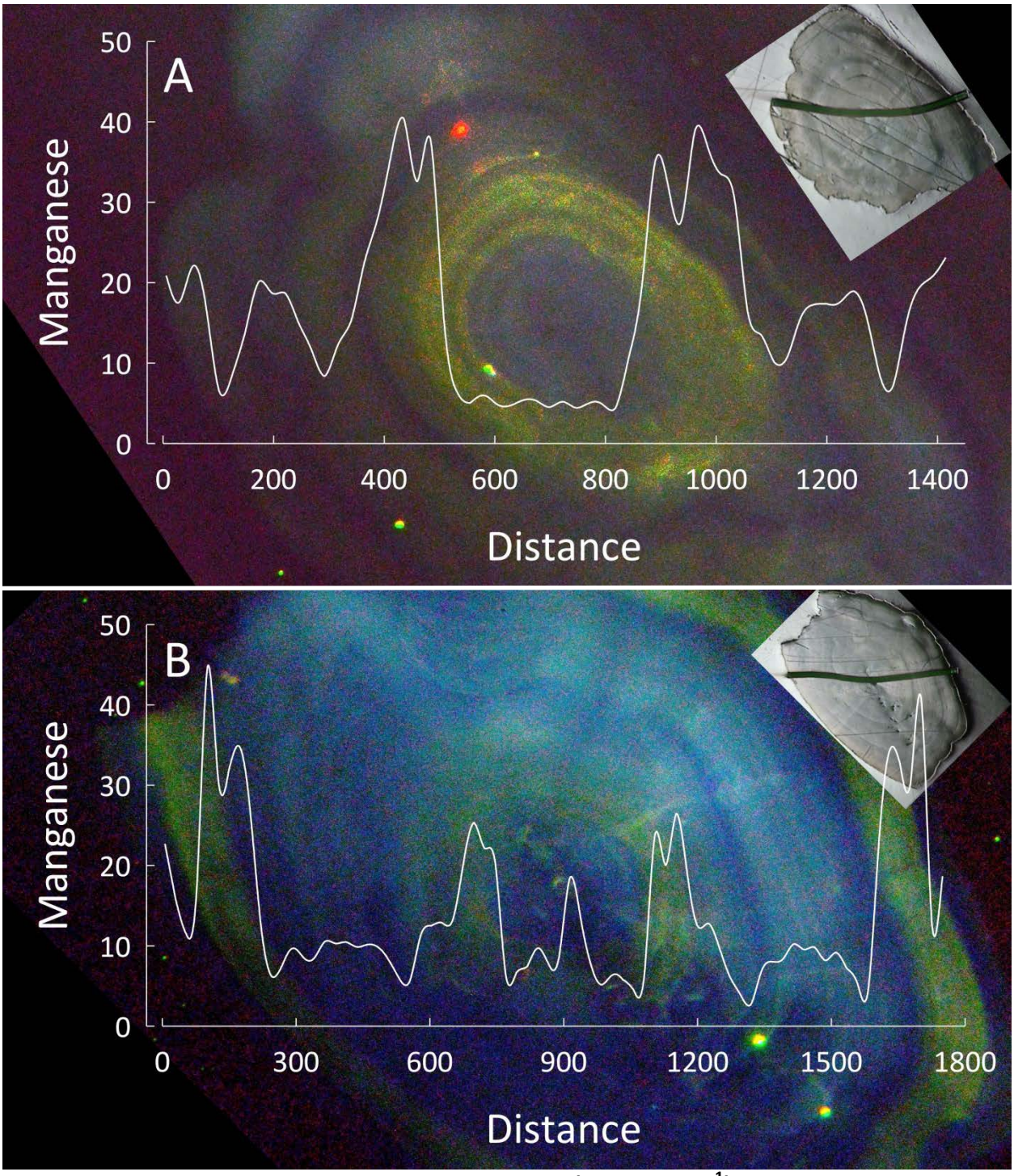

Figure 2-97 – Laser ablation linescans for manganese (mmol × mol^{-1}) superimposed on cathodoluminescent images of otoliths from (A) a cutthroat trout (F15-88) captured on 07-Aug-2015 in Cecil Creek at +200 m above the RoW, 11.6 cm, 18.8 g, and (B) a rainbow trout (F14-1199) captured on 04-Sep-2014 in Nine Mile Creek at +200 m above the RoW, 13.2 cm, 21.1 g. Insets are low resolution images showing the laser ablation paths.

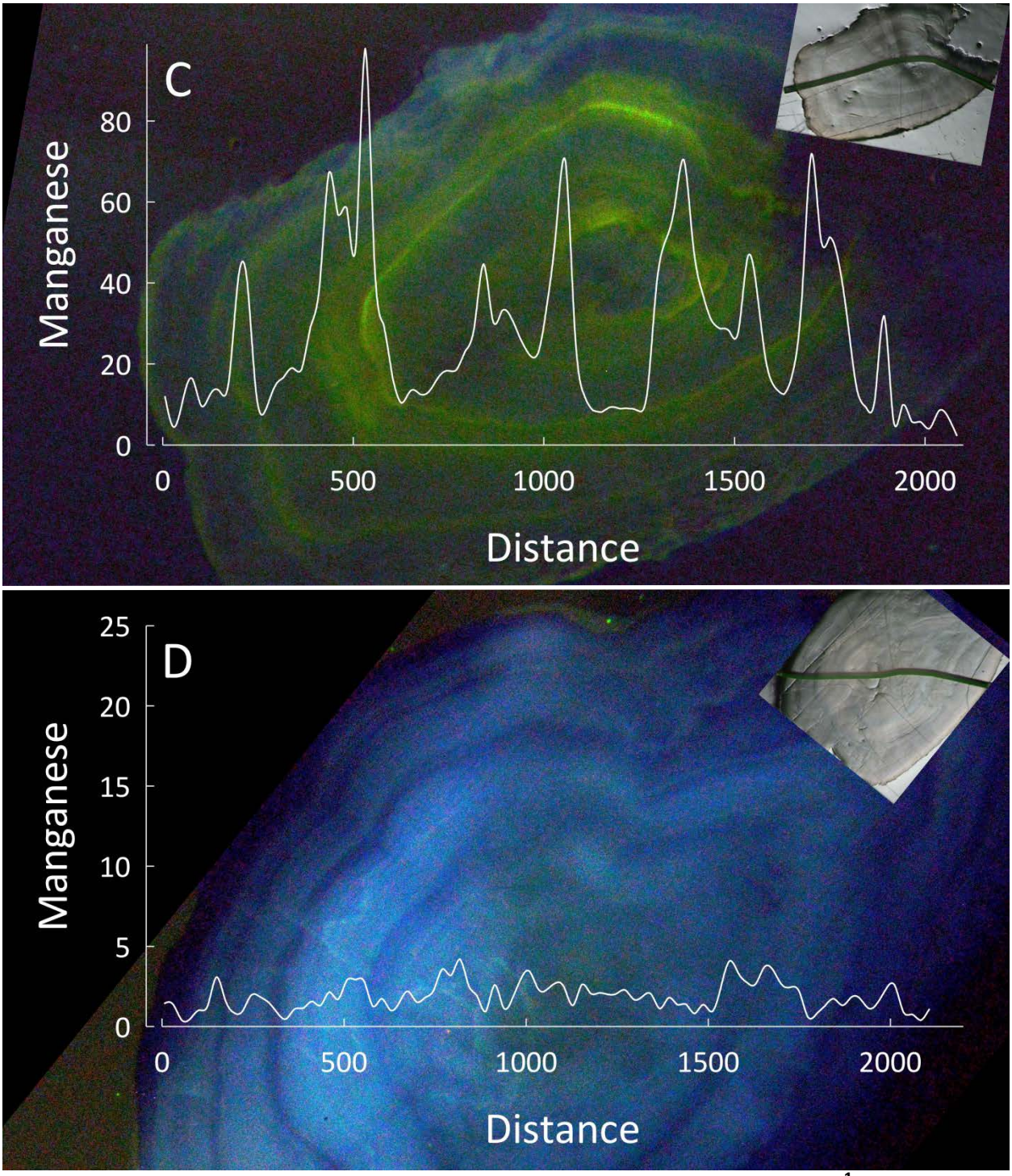

Figure 2-97 (continued) – Laser ablation linescans for manganese (mmol × mol^{-1}) superimposed on cathodoluminescent images of otoliths from (C) a rainbow trout (RB13-1) captured on 20-Sep-2013 in Ormond Creek below the RoW, 15.6 cm, 38.5 g, and (B) a cutthroat trout (F14-857) captured on 09-Aug-2014 in 57.1 km stream at +200 m above the RoW, 14.5 cm, 29.7 g. Insets are low resolution images showing the laser ablation paths.

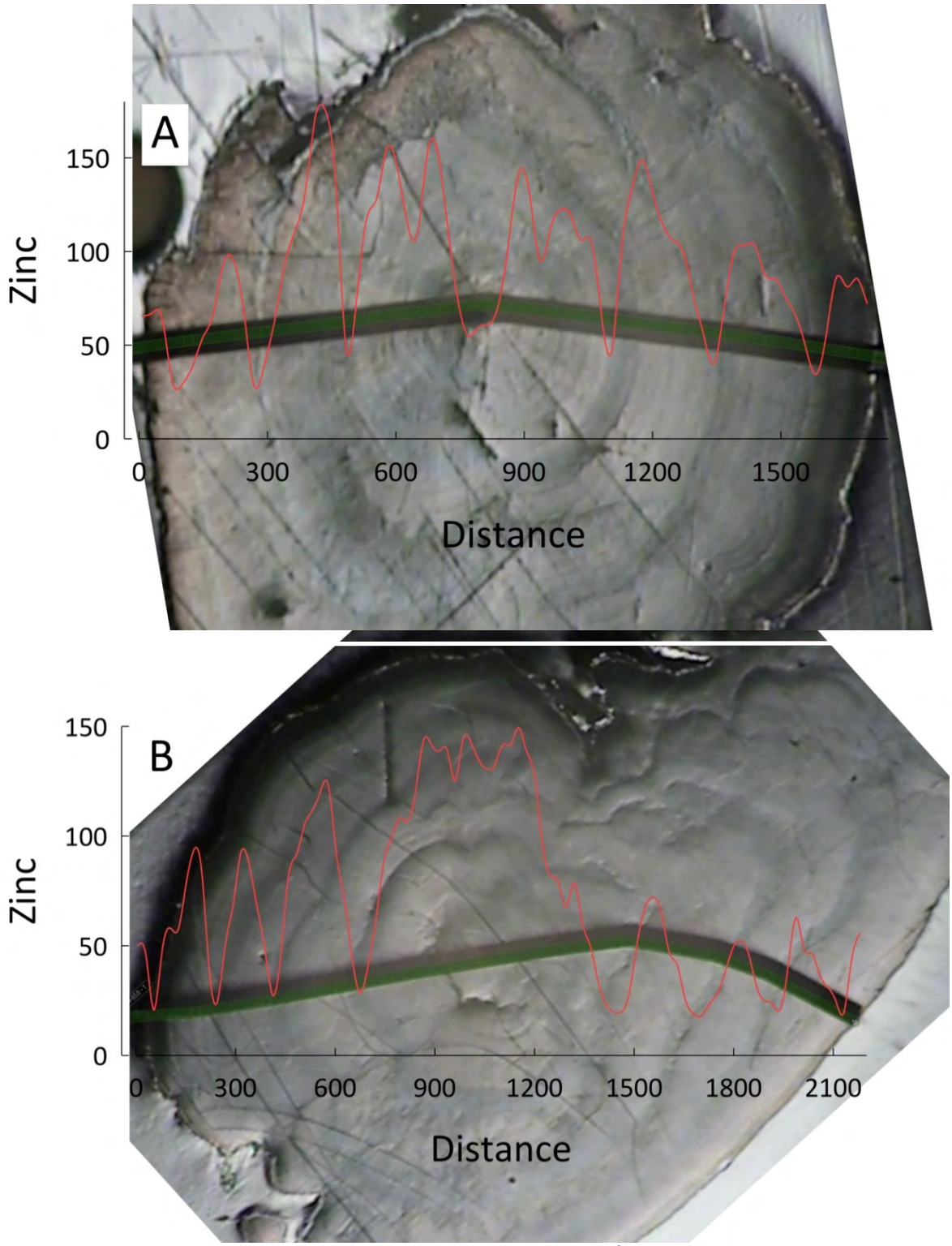

Figure 2-98 – Laser ablation linescans for zinc (mmol × mol^{-1}) superimposed on light microscope images of salmonids otoliths showing annular rings. See next page for description.

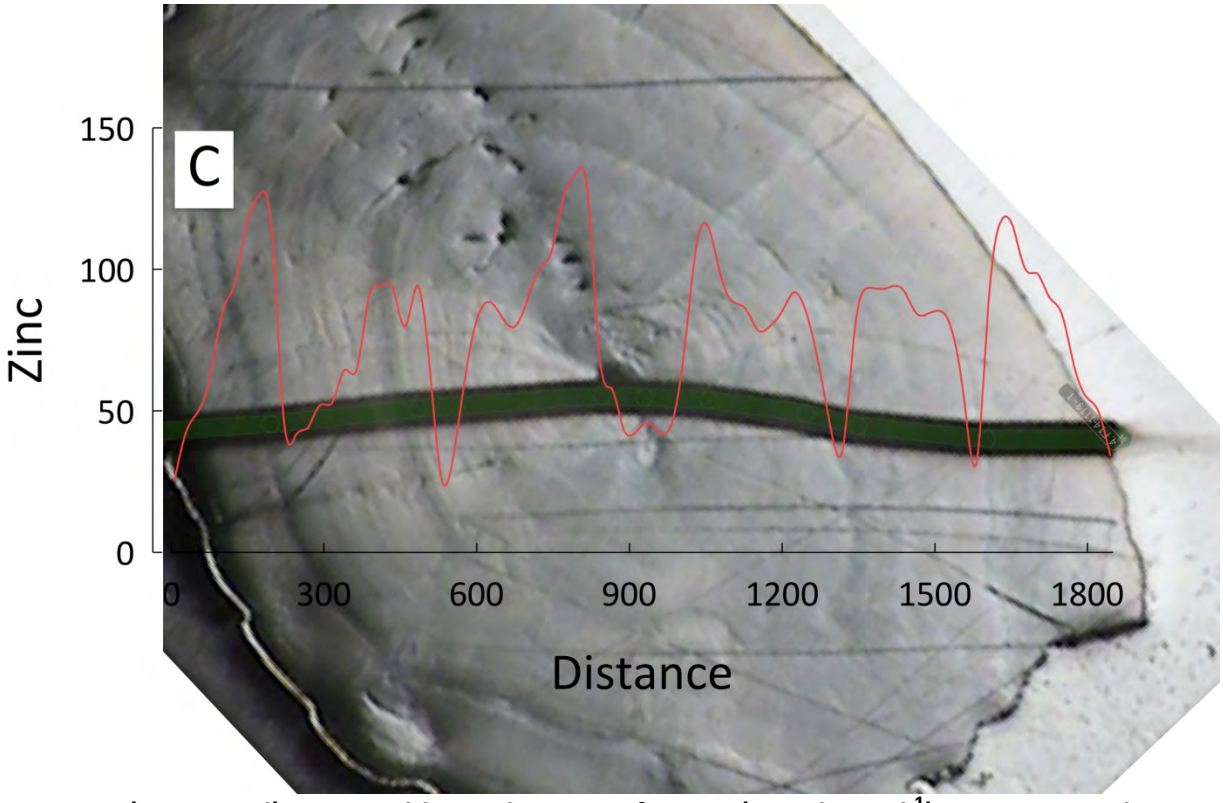

Figure 2-98 (continued) – Laser ablation linescans for zinc (mmol × mol^{-1}) superimposed on light microscope images of salmonids otoliths showing annular rings. Images are for (A) a 3-year old *O. clarkii* (F14-760) captured on 7-Aug-2014 in 9.8 km Stream at a site below the pipeline crossing RoW, 14.1 cm, 27.5 g, (B) a 4-year old *O. clarkii* (F14-858) captured on 9-Aug-2014 in 57.1 km Stream at +200 m above the RoW, 16.2 cm, 45.6 g, and (C) a 2-year old *O. mykiss* (F14-1199) captured on 4-Sep-2014 in Nine Mile Creek at +200 m above the RoW, 13.2 cm.

Otolith microchemistry: relationship to water elemental composition

LA-ICP-MS revealed substantial differences in otolith chemistry among watersheds for Salmonidae and Cottidae. Highly significant relationships between chemical ratios from the outer edge of the otolith and the chemical ratios in the water were found for Sr and Ba for Salmonidae (Figure 2-99) and Cottidae (Figure 2-100). The relationship for Sr in Salmonidae was $Sr_{OTO} = 142.4 \times Sr{:}Ca_{H2O} - 83.75$ ($F_{1,16} = 257$; $p < 0.001$; $r^2 = 0.942$) and for Ba in Salmonidae was $Ba_{OTO} = 10.35 \times Ba{:}Ca_{H2O} + 1.915$ ($F_{1,16} = 336$; $p < 0.001$; $r^2 = 0.955$). The relationship for Sr in Cottidae was $Sr_{OTO} = 178.0 \times Sr{:}Ca_{H2O} - 88.66$ ($F_{1,7} = 465$; $p < 0.001$; $r^2 = 0.985$) and for Ba in Cottidae was $Ba_{OTO} = 15.16 \times Ba{:}Ca_{H2O} + 1.100$ ($F_{1,7} = 598$; $p < 0.001$; $r^2 = 0.988$).

The relationship between otolith elemental concentration and water chemistry was not as strong for the other elements measured (Mn and Rb). The relationship between Mn_{OTO} to $Mn:Ca_{H2O}$ for Salmonidae was not significant ($F_{1,16} = 0.277$; $P = 0.606$; $r^2 = 0.017$; Figure 2-99C). Otolith Mn content increased up to approximately 2 mmol × mol^{-1} for Mn in the water, however, fish caught in two systems with high water Mn content (Cecil Creek and Nine Mile Creek) exhibited lower otolith Mn signatures than values measured for *O. kisutch* in the study by Shrimpton et al. (2014). Consequently, we examined the relationship between Mn_{OTO} to $Mn:Ca_{H2O}$ for *O. mykiss*, *O. clarkii*, and *S. malma* caught in the present study. We found that the relationship was significant for the data; $Mn_{OTO} = 2.923 \times Mn:Ca_{H2O} + 3.490$ ($F_{1,8} = 10.0$; $P = 0.014$; $r^2 = 0.556$) — although not as strong as the relationships determined for Sr or Ba. A significant relationship for Mn was found for Cottidae; $Mn_{OTO} = 1.720 \times Mn:Ca_{H2O} + 1.808$ ($F_{1,7} = 36.2$; $p < 0.01$; $r^2 = 0.838$; Figure 2-100C). The relationship was calculated for mean otolith Mn values for each river system, but tremendous variation in signatures was observed from the otoliths of fish caught in each system. The range in otolith Mn for fish from an individual river was often greater than the range among rivers. We found no relationship between Rb in the otolith to water content (data not shown). Variation in Zn also did not correlate with water chemistry, but increases coincided with the annular rings of the otolith.

Otolith microchemistry: patterns of change and putative movements

Due to the lack of or weak relationship between otolith and water for Mn and Rb, only Sr and Ba were used in subsequent analyses. Elemental signatures for Sr and Ba showed considerable variation among sampling locations (Figure 2-101) and discriminant function analysis for just Sr and Ba indicated the differences for both watersheds were significant (Nechako: Pillai's Trace = 1.936; $F_{12,48} = 121.7$; $p < 0.001$; Kitimat: Pillai's Trace = 1.958; $F_{10,28} = 131.0$; $p < 0.001$). The percentage of correct classification for samples collected in the Nechako River watershed was 100% (Table 2-90) and for samples collected in the Kitimat River watershed was 95 % (Table 2-91).

It is interesting that just modeling Sr and Ba differentiated between the Fraser River and Nechako River samples, whereas the model for Mn and Rb misclassified two of the Nechako River samples. Samples from the Nechako River tributaries were generally higher in Sr. Smaller

tributary streams flowing in to Ormond Creek and Nine Mile Creek were also higher in Ba than the receiving creeks (Figure 2-101A). The DFA model also did not differentiate the samples from Chist Creek and 9.8 km Stream as Ba and Sr elemental ratios to Ca were similar between these two systems (Figure 2-101B; Table 2-91). The differences in elemental signatures among the creeks and streams measured in the Kitimat River watershed, however, generally showed much greater differences among sample sites than the sites sampled in the Nechako River watershed. Comparison of river water chemistry revealed that the mainstem Kitimat River was high in Ba and intermediate in Sr. Trout Creek, 9.8 km Stream, and Chist Creek were also quite low for Sr — Trout Creek also had very low Ba levels relative to Ca. Samples collected from 57.1 km Stream had distinctly higher Sr levels.

Based on the strong relationships for Sr and Ba between otolith and water, changes in otolith elemental signature indicate that the fish are experiencing different water chemistries over the course of their lives. Given the differences in spatial elemental signatures among rivers that we examined, marked changes in otolith elemental signature likely reflect movement of fish among areas of the watershed with different water chemistries. Changes in elemental signatures, therefore, can be used as a proxy for movement of fish among watersheds.

Linescans from the LA-ICP-MS analysis showed considerable variations in elemental signatures of the otoliths for both Sr and Ba — patterns were highly variable within rivers and also among rivers. Figure 2-102 shows changes in Sr and Ba for three fish sampled in the Nechako River watershed; two *O. mykiss* and one *C. asper*. One of the *O. mykiss* caught in Nine Mile Creek showed very large changes in both elements over the entire life history of the fish, whereas the *O. mykiss* caught in Ormond Creek showed less change in elemental signatures over the life history of the fish. Linescans for *C. asper* also showed considerable differences in both Sr and Ba throughout the entire life history of the fish.

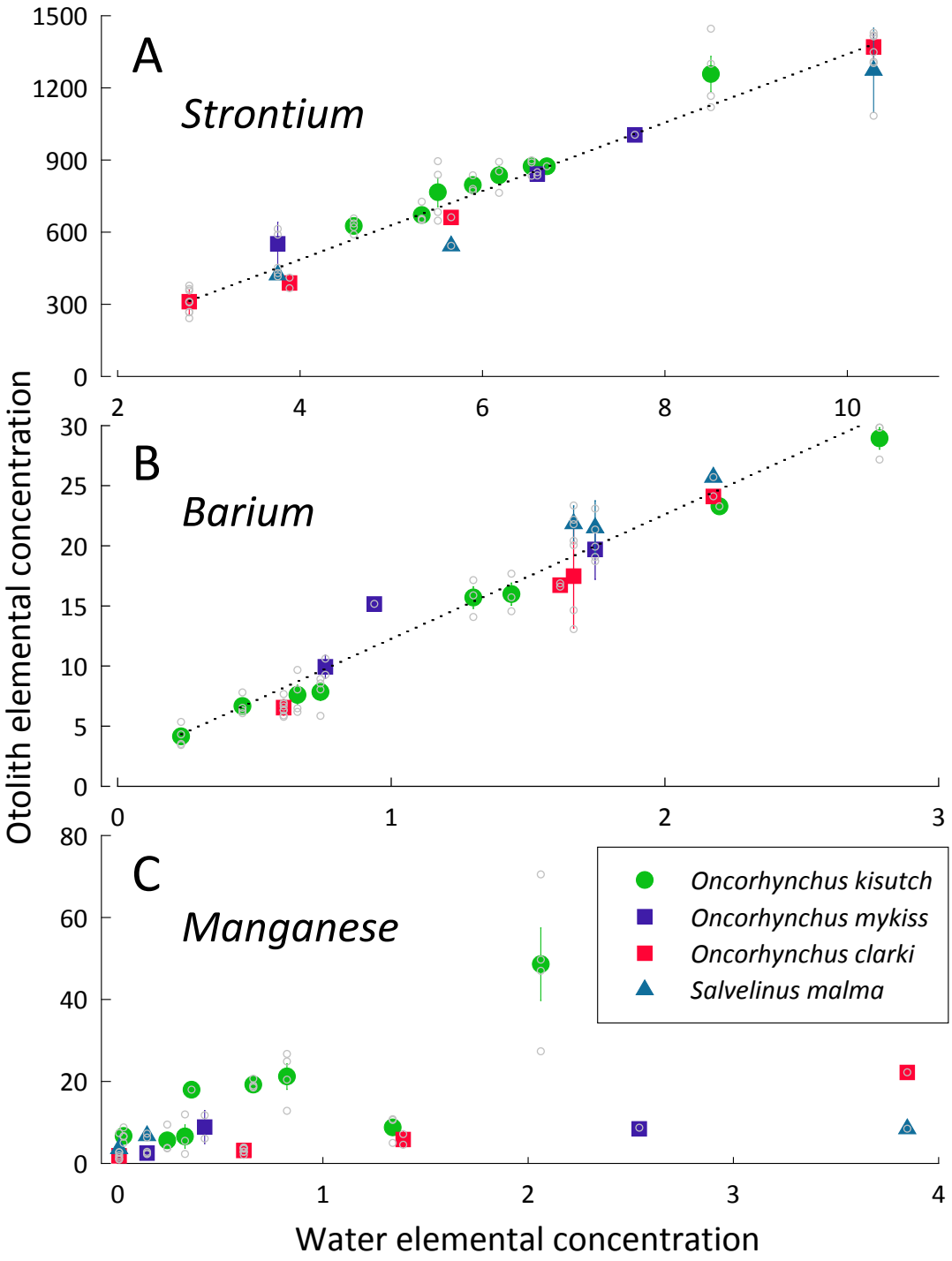

Figure 2-99 – Relationship between elemental concentration in otoliths (mmol × mol^{-1}) and water element to calcium ratios (mmol × mol^{-1}) for (A) strontium, (B) barium, and (C) manganese for Salmonidae. Dotted line determined by linear regression. Large symbols are means ± SD and small symbols are individual fish. *O. kisutch* data is from Shrimpton et al. (2014).

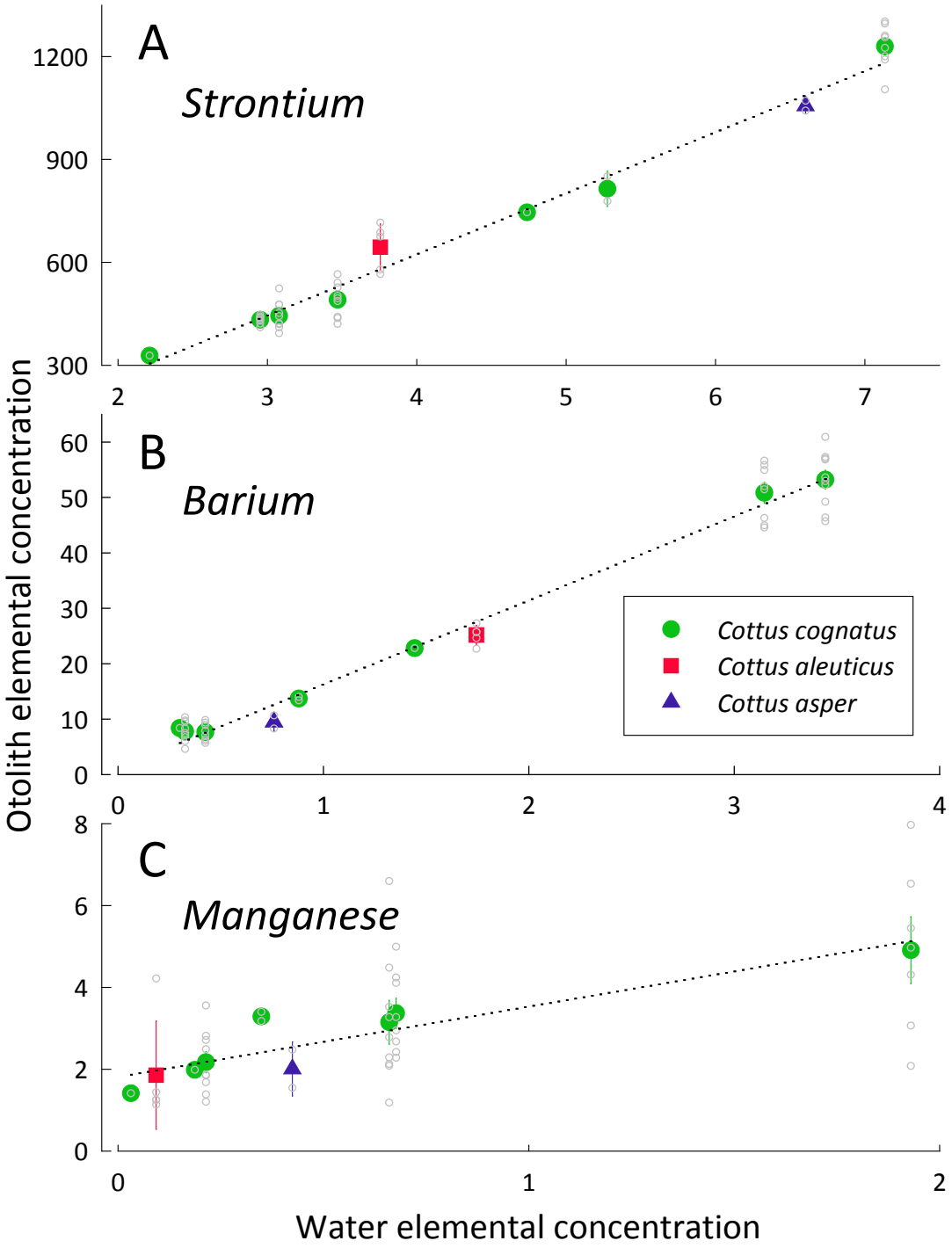

Figure 2-100 – Relationship between elemental concentration in otoliths (mmol × mol^{-1}) and water element to calcium ratios (mmol × mol^{-1}) for (A) strontium, (B) barium, and (C) manganese for Cottidae. Dotted line determined by linear regression. Large symbols are means ± SD and small symbols are individual fish. Data for *C. cognatus* is from Clarke et al. (2015).

Table 2-90 – The percentage of correct classification determined by discriminant function analysis for water samples collected from rivers and streams in the Nechako River watershed using jack-knife re-sampling to validate the robustness of the discriminant functions. Cross-validation accuracy is expressed as a percentage (%). The elements incorporated into the model were Sr and Ba. Nautley River includes samples collected from Fraser Lake; Ormond Creek includes samples collected from Ormond Lake.

River	Fr	Ne	Na	9m	9t	Or	Ot	%
Fraser River (Fr)	2	0	0	0	0	0	0	100
Nechako River (Ne)	0	4	0	0	0	0	0	100
Nautley River (Na)	0	0	6	0	0	0	0	100
Nine Mile Creek (9m)	0	0	0	6	0	0	0	100
Nine Mile trib (9t)	0	0	0	0	2	0	0	100
Ormond Creek (Or)	0	0	0	0	0	10	0	100
Ormond trib (Ot)	0	0	0	0	0	0	2	100
Total	2	4	6	6	2	10	2	100

Table 2-91 – The percentage of correct classification determined by discriminant function analysis for water samples collected from rivers and streams in the Kitimat River watershed using jack-knife re-sampling to validate the robustness of the discriminant functions. Cross-validation accuracy is expressed as a percentage (%). The elements incorporated into the model were Sr and Ba.

River	Ki	Tr	9k	Ce	Ch	57	%
Kitimat River (Ki)	4	0	0	0	0	0	100
Trout Creek (Tr)	0	2	0	0	0	0	100
9.8 km Stream (9k)	0	0	4	0	0	0	100
Cecil Creek (Ce)	0	0	0	2	0	0	100
Chist Creek (Ch)	0	0	1	0	5	0	83
57.1 km Stream (57)	0	0	0	0	0	2	100
Total	4	2	5	2	5	2	95

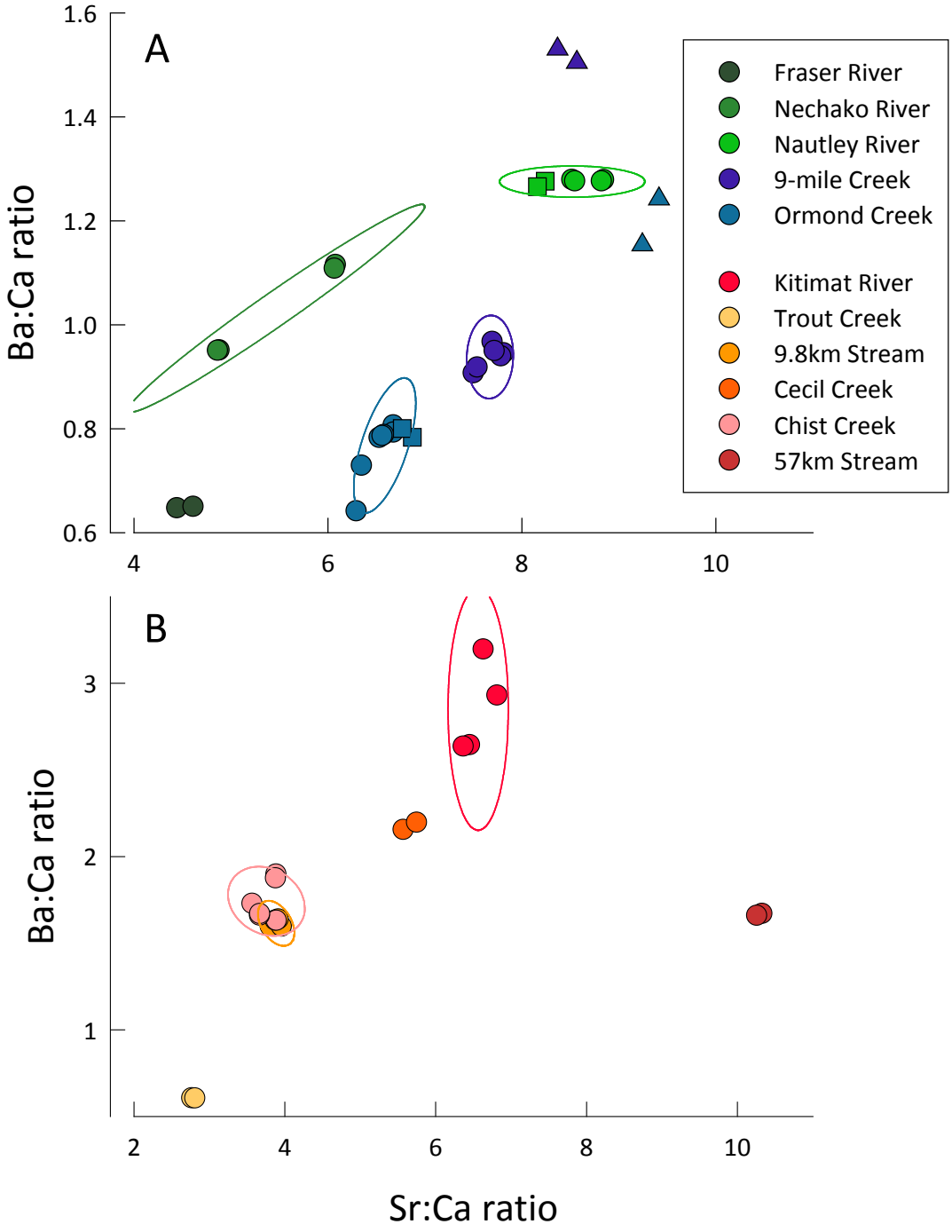

Figure 2-101 – Ratios for strontium and barium relative to calcium (mmol × mol^{-1}) for duplicate samples collected in (A) the Fraser River catchment and (B) the Kitimat River catchment. Samples collected in rivers and streams are defined by circles, lakes by squares, and small tributaries by triangles. Most rivers were sampled at multiple locations; confidence ellipses shown for each system where multiple samples were collected.

The putative water chemistries for these fish suggest movement to locations where water chemistry differed from where they were captured. The Ormond Creek *O. mykiss* appeared to have resided in a system where water chemistry was similar to that of Fraser Lake and the Nautley River when it was younger, but elemental signatures corresponded to Ormond Creek after the fish was older than two years (Figure 2-103A). The Nine Mile Creek *O. mykiss* also showed considerable movement — including a repeated pattern of movement to systems with considerably higher Ba content at variable times over the life history of the fish (Figure 2-103B). Putative water chemistries experienced by *C. asper* captured in Ormond Creek generally overlapped the water samples collected from Ormond Creek — suggesting that movement for this fish was limited to the single creek (Figure 2-103C). Although there is some overlap with the water samples from Nine Mile Creek, it is not likely that the fish moved between these two systems, but likely represents potential seasonal variation in elemental composition of Ormond Creek or more likely movement into the small tributary stream that was sampled — duration of residence in the stream was not long enough to develop a strong enough signature to overlap with the water chemistry values. Elemental signatures that were lower in Sr may represent movement by the fish into a tributary for Ormond Creek that was not sampled.

Fish sampled in the Kitimat River watershed also showed considerable changes in Sr and Ba in otolith microchemistry. Elemental signatures near the core of otoliths for fish sampled in Chist Creek showed two patterns; very high Sr values over 1500 mmol × mol^{-1} or intermediate Sr values of approximately 500 mmol × mol^{-1} (Figure 2-104A). All the otoliths examined from *O. mykiss* samples from Chist Creek exhibited high Sr at the core — elemental composition at the core is characteristic of maternal signatures. Progeny of anadromous females exhibit high Sr values near the otolith core due to the higher Sr to Ca ratios in the marine environment compared to most freshwater systems. In contrast otoliths from *S. malma* caught in Chist Creek had lower Sr values near the core indicating that they were progeny of stream resident fish. Elemental signatures for Ba also varied over the life history of fish sampled from Chist Creek, but showed greater differences as the fish became older (Figure 2-104B).

The overlap between the elemental signatures from otoliths with water chemistries shown in Figure 2-105 suggests that we can use this technique to trace movements of fish over their life history within the Kitimat River watershed. Putative Sr and Ba water chemistries calculated from otolith linescan data for *O. mykiss* from Chist Creek indicate a strong anadromous maternal signature, but that as a young-of-the-year juvenile the fish resided in Chist Creek; at approximately age three years moved into the Kitimat River and a water source of unknown origin before returning to Chist Creek where it was captured (Figure 2-105A). A second *O. mykiss* also shows a strong anadromous maternal signature, but Ba levels were much higher and as a young-of-the-year fish resided somewhere other than Chist Creek where it was captured (Figure 2-105C). Putative movement patterns for a *S. malma* from Chist Creek indicate movement downstream into the Kitimat River as a one-year old and then movement back up into Chist Creek where it remained until capture (Figure 2-105B).

Representative linescans for Sr and Ba for three *O. clarkii* are shown in Figure 2-106 for fish caught in Trout Creek, Cecil Creek and 57.1 km Stream. Trout Creek is characterized by low Sr and Ba; both elements were consistently low in *O. clarkii* captured from this creek. In contrast, 57.1 km Stream is characterized by high Sr levels; *O. clarkii* captured from this stream exhibited much higher levels of Sr in the otoliths. Cecil Creek had intermediate levels of Sr and otolith Sr values at the outer edge reflected the intermediate water chemistry.

The high Sr near the core for many of the *O. clarkii* caught in Cecil Creek indicated that they were progeny of anadromous fish, but as a juvenile likely spent time in the Kitimat River and another river of unknown location (Figure 2-107A). Otolith elemental signatures from an *O. clarkii* captured in Trout Creek directly overlap the water chemistries for the river where it was caught indicating that this fish had been resident within Trout Creek throughout the entire life of the animal (Figure 2-107B). Similarly, otolith microchemistry for a representative *O. clarkii* from 57.1 km Stream indicated that this fish was resident to the stream of capture and did not move into the Kitimat River — although, there was considerably more variation in elemental signature over the life history of the fish.

Three representative linescans from *C. aleuticus* captured in Chist Creek suggest considerable variation not only in patterns of movement for this species, but also life-history

variation (Figure 2-108). Differences in Sr and Ba levels at the core suggest three life-history patterns. The first pattern is low Sr and relatively high Ba at the core indicating that the fish was progeny of a freshwater female sculpin. The second pattern is high Sr at the core that declined just beyond the centre of the otolith with low Ba that increased just beyond the centre of the otolith and indicates that the fish was progeny of a marine female *C. aleuticus* that spawned in freshwater. The third pattern is high Sr at the core that remained elevated throughout the first year and low Ba throughout the first year and indicates that the fish was progeny of a marine female sculpin that may have spawned in the estuary and the juvenile likely reared in the estuary or ocean for a year before moving into freshwater.

Overlap of otolith microchemistries with water elemental signatures further support the variation in life-history patterns among *C. aleuticus* from Chist Creek (Figure 2-109). Marine residence can be seen before movement into Chist Creek and relatively little movement beyond this creek until capture for fish 14-602 (Figure 2-109A). In contrast, little movement was observed in fish 15-49 beyond residence in water with slightly higher Sr and Ba during the first two years — likely to a tributary of Chist Creek that we have not identified (Figure 2-109B). Fish 15-70 likely spent some time during the first years of life in the Kitimat River and an unknown tributary of the Kitimat before spending time until it was captured in Chist Creek (Figure 2-109C).

A summary of the number of fish sampled from each stream, fish with anadromous maternal signatures, and the proportion of fish that showed movement for different age classes is given in Table 2-92. There were no clear patterns as movements varied among streams, but also there were considerable differences within streams.

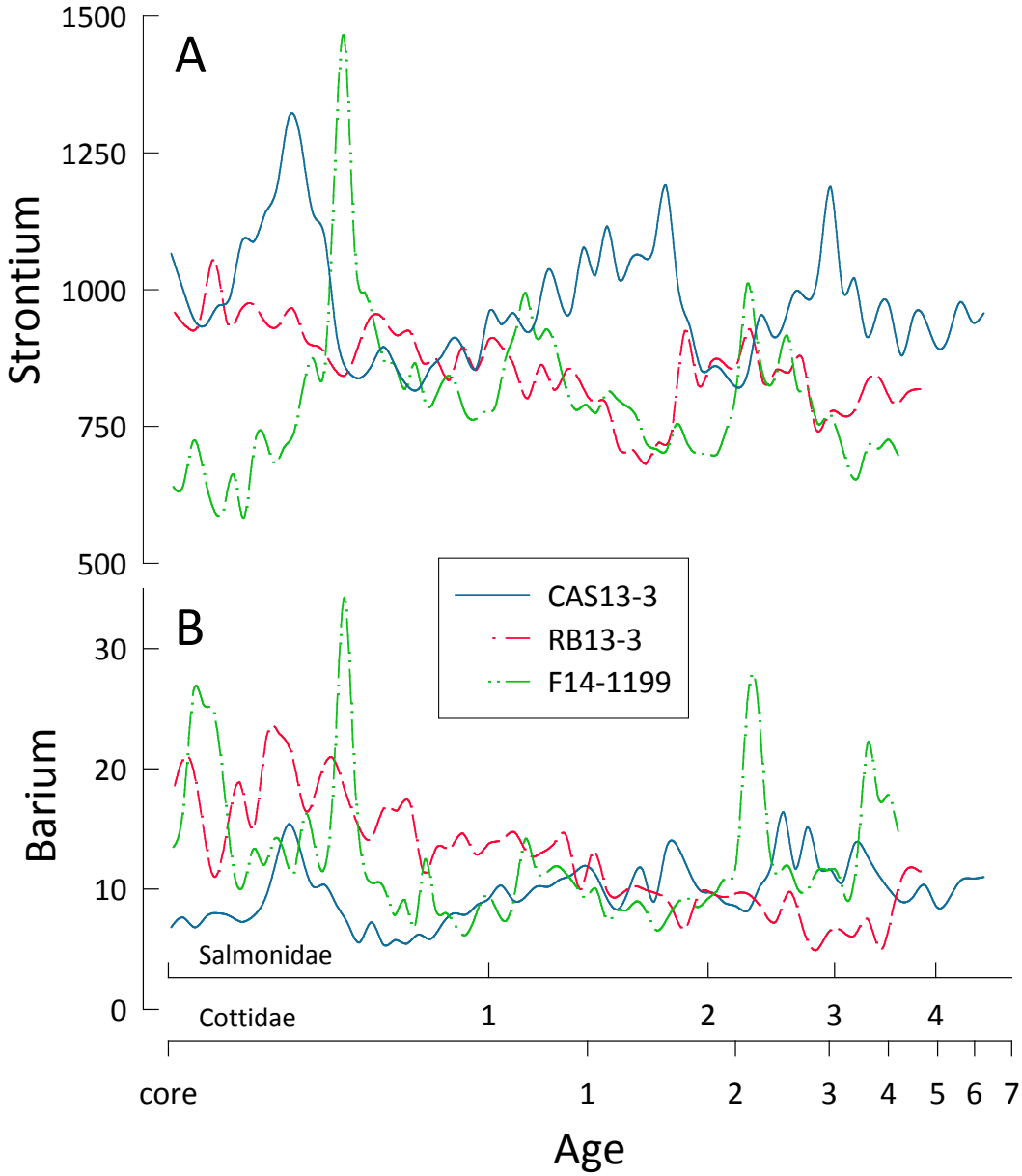

Figure 2-102 – Laser ablation-linescans from otoliths of *Cottus asper* (CAS13-3) and *Oncorhynchus mykiss* (RB13-3 and F14-1199) caught in tributaries of the Fraser River. Changes in (A) strontium to calcium ratios (mmol × mol^{-1}) and (B) barium to calcium ratios (mmol × mol^{-1}) are shown from the core of the otolith to the outer edge. Fish CAS13-3 (8.5 cm, 7.3 g) and fish RB13-3 (12.8 cm, 21.8 g) were caught below the RoW in Ormond Creek. Fish F14-1199 was captured in Nine Mile Creek at +200 m above the RoW (13.2 cm, 21.1 g). Fish age was estimated from otolith annuli and variations in Zn content.

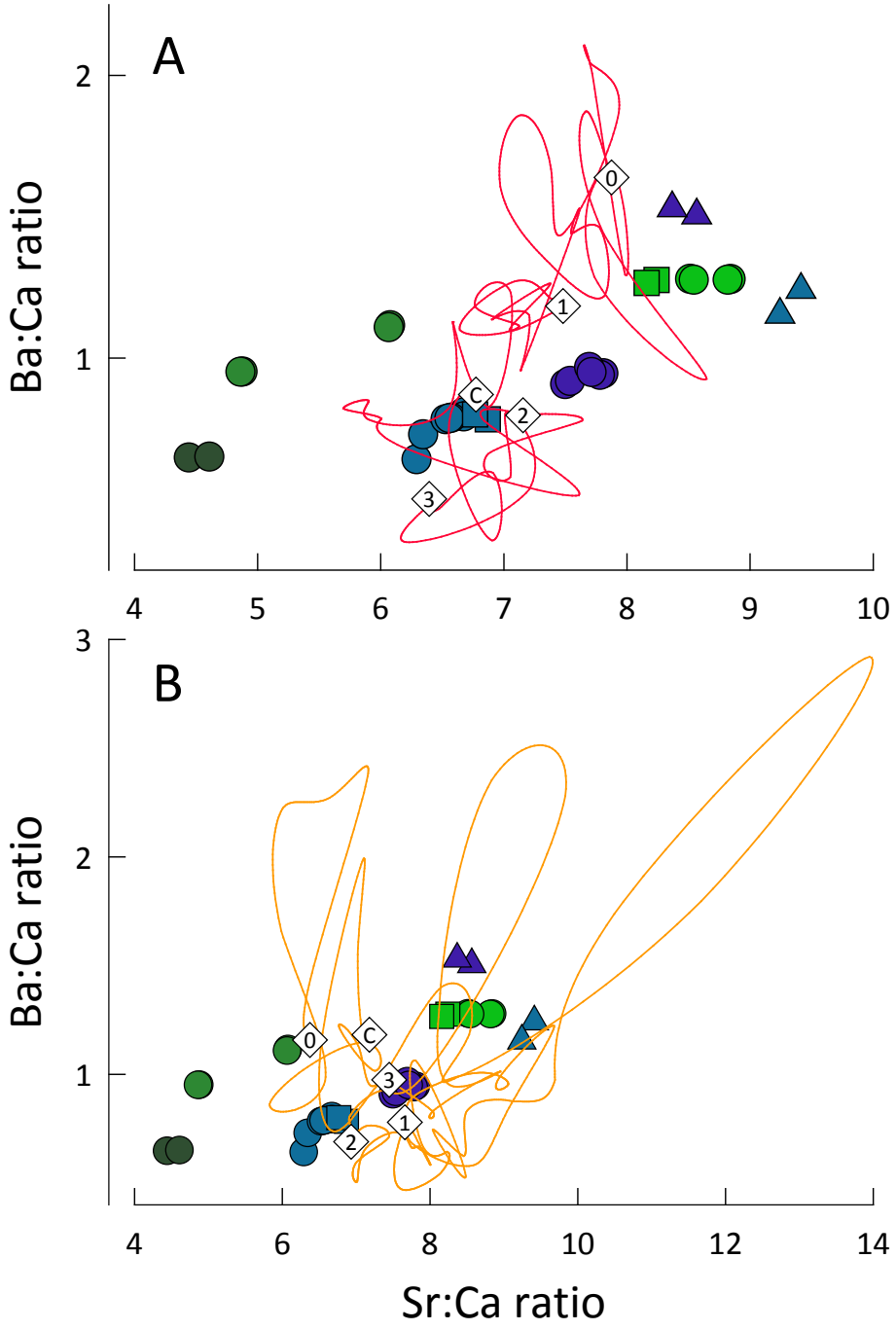

Figure 2-103 – Plots of elemental water signatures for Sr to Ca (mmol × mol^{-1}) and Ba to Ca (mmol × mol^{-1}) calculated from LA-ICP-MS data for *Oncorhynchus mykiss* and *Cottus asper* otoliths based on relationships shown in Figure 2-99 and Figure 2-100, respectively. Plot (A) is for *O. mykiss* RB13-3 caught below the RoW in Ormond Creek. Plot (B) is for *O. mykiss* F14-1199 captured in Nine Mile Creek at +200 m above the RoW. Figure continued on next page with description of symbols.

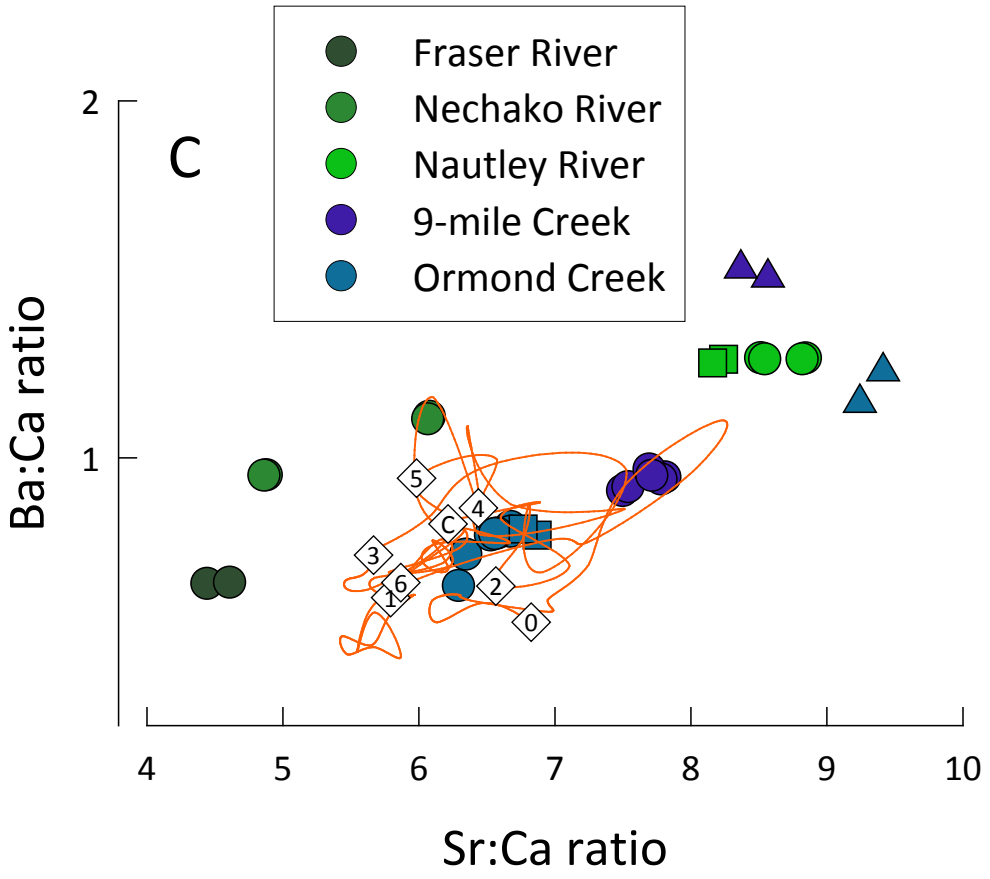

Figure 2-103 (continued) – Plots of elemental water signatures for Sr to Ca (mmol × mol^{-1}) and Ba to Ca (mmol × mol^{-1}) calculated from LA-ICP-MS data for *Oncorhynchus mykiss* and *Cottus asper* otoliths based on relationships shown in Figure 2-99 and Figure 2-100, respectively. Plot (C) is for *C. asper* CAS13-3 caught below the RoW in Ormond Creek. Numbers within open diamonds represent annual age for each fish estimated from annuli visible on light microscope images and variation in Zn to Ca ratios (mmol × mol^{-1}) determined from LA-ICP-MS. The letter "C" within the open diamonds are the Sr and Ba values at the edge of otolith; equivalent to the last elements accreted onto the otoliths before the fish was captured and sampled. Water chemistry from samples collected in rivers and streams are defined by circles, lakes by squares, and small tributaries by triangles. Locations of sites where water samples were collected are shown in Figure 2-91.

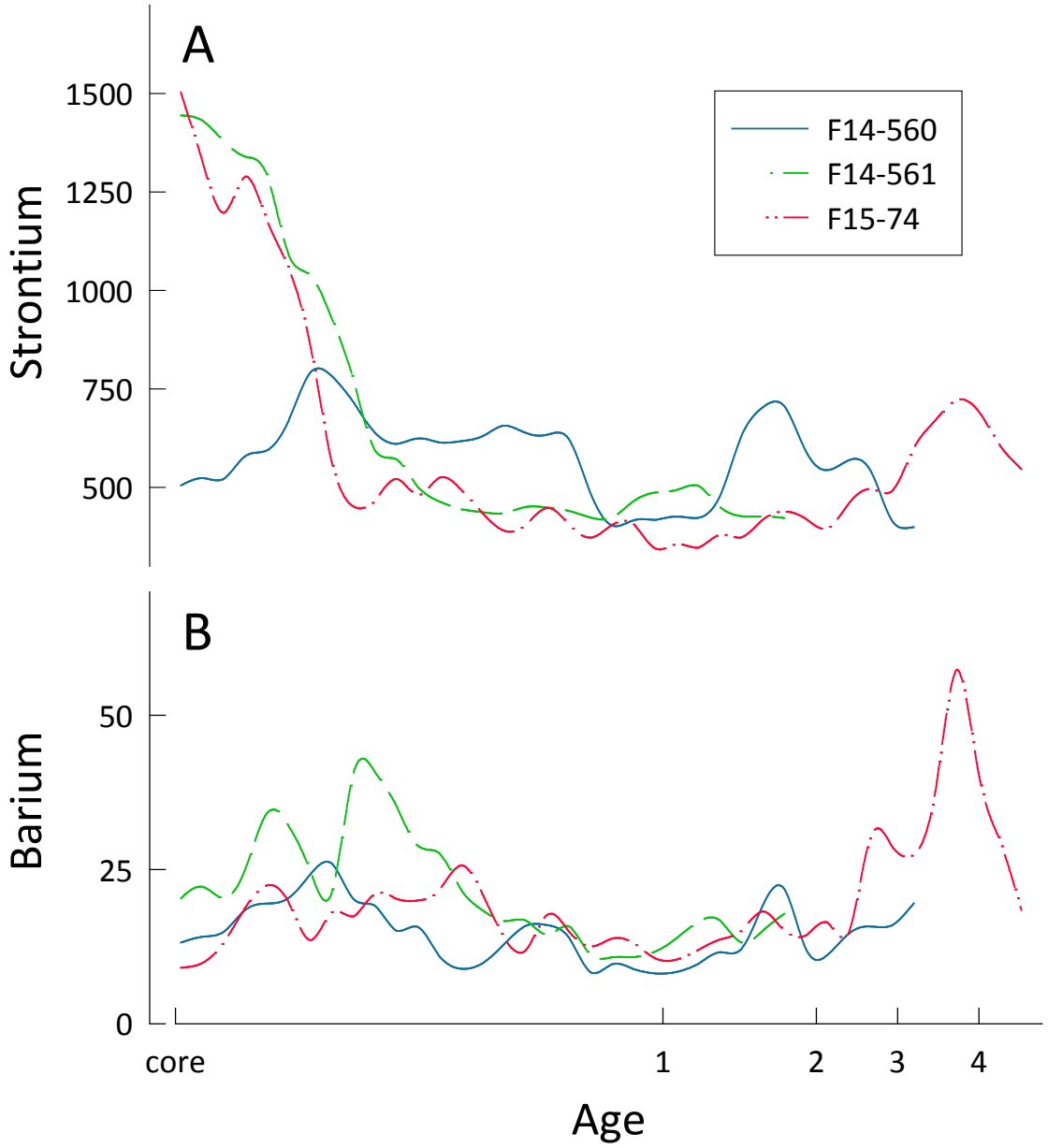

Figure 2-104 – Laser ablation-linescans from otoliths of *Salvelinus malma* (fish F14-560) and *Oncorhynchus mykiss* (fish F14-561 and F15-74) caught in Chist Creek. Changes in (A) strontium to calcium ratios (mmol × mol^{-1}) and (B) barium to calcium ratios (mmol × mol^{-1}) are shown from the core of the otolith to the outer edge. Fish F15-560 (14.6 cm, 26.1 g) and fish F14-561 (11.0 cm, 17.5 g) were caught 200 m below the RoW; F15-74 (14.1 cm, 32.8 g) was caught 200 m above the RoW. Fish age was estimated from otolith annuli and variation in Zn to Ca ratios.

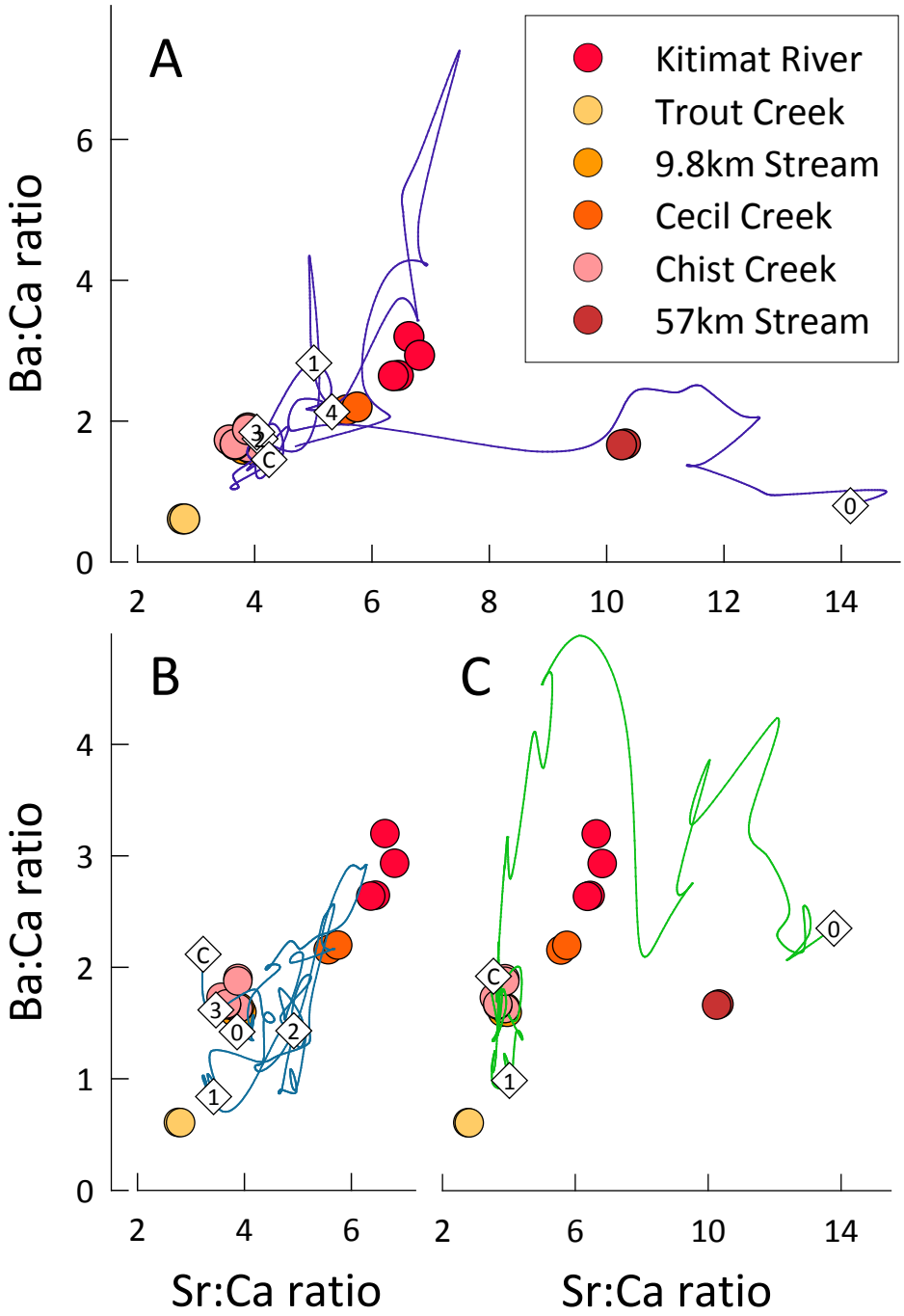

Figure 2-105 – Plots of elemental water signatures for Sr to Ca (mmol × mol^{-1}) and Ba to Ca (mmol × mol^{-1}) calculated from LA-ICP-MS data for *Oncorhynchus mykiss* and *Salvelinus malma* otoliths based on relationships shown in Figure 2-99. All fish were caught in Chist Creek. Plot (A) is for *O. mykiss* 15-74 (purple line), (B) is for *S. malma* 14-560 (blue line) and (C) is *O. mykiss* 14-561 (green line). See Figure 2-103 for symbol description and Figure 2-92 for water sample locations.

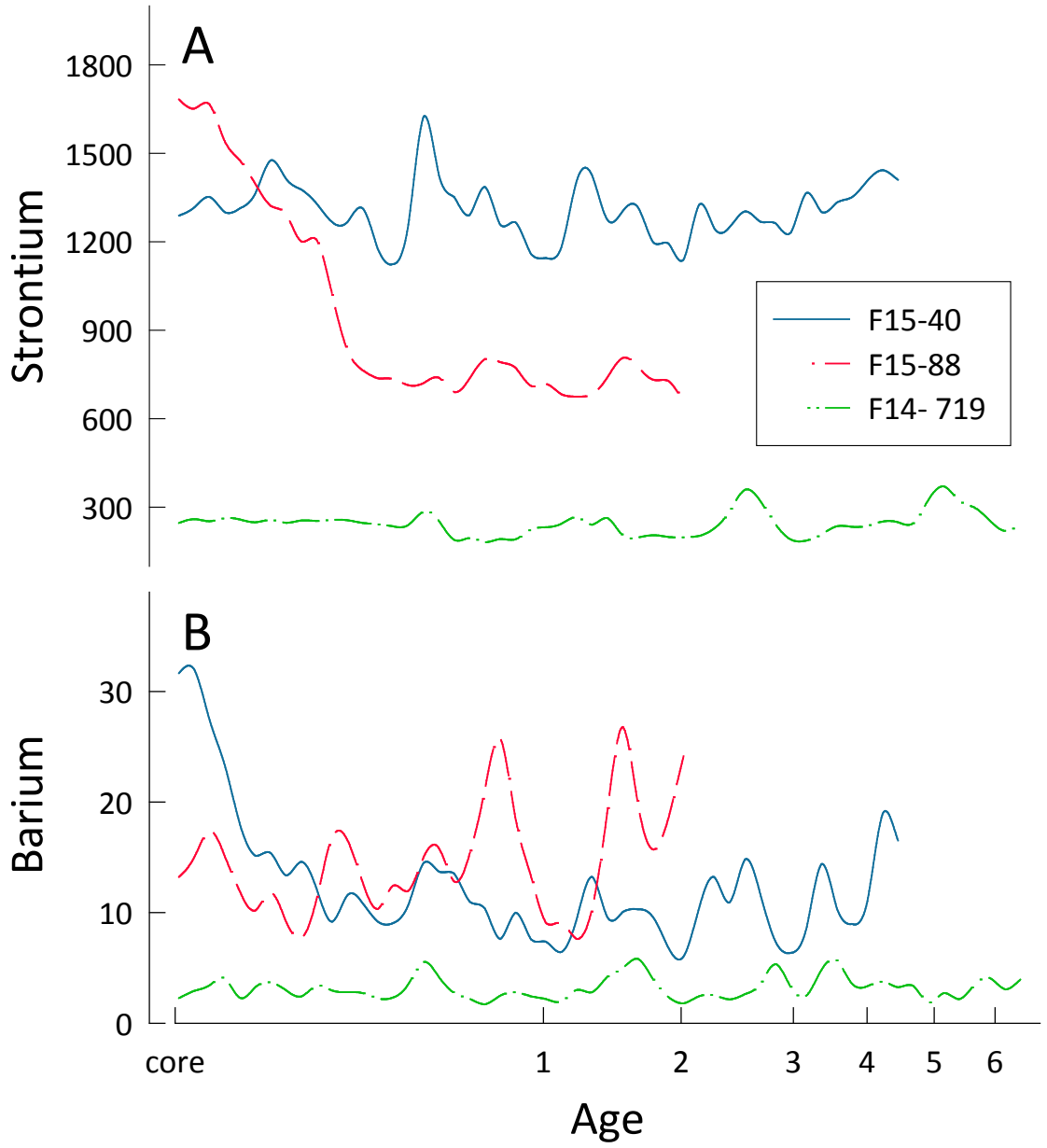

Figure 2-106 – Laser ablation-linescans from otoliths of *Oncorhynchus clarkii* caught in Trout Creek (F14-719), Cecil Creek (F15-88), and 57.1 km Stream (F15-40). Changes in (A) strontium to calcium ratios (mmol × mol^{-1}) and (B) barium to calcium ratios (mmol × mol^{-1}) are shown from the core of the otolith to the outer edge. Fish F14-719 (19.2 cm, 59.8 g), fish F15-88 (11.6 cm, 18.8 g), and fish F15-40 (15.7 cm, 38.2 g) were caught 200 m above the RoW for each stream. Fish age was estimated from otolith annuli and variation in Zn to Ca ratios.

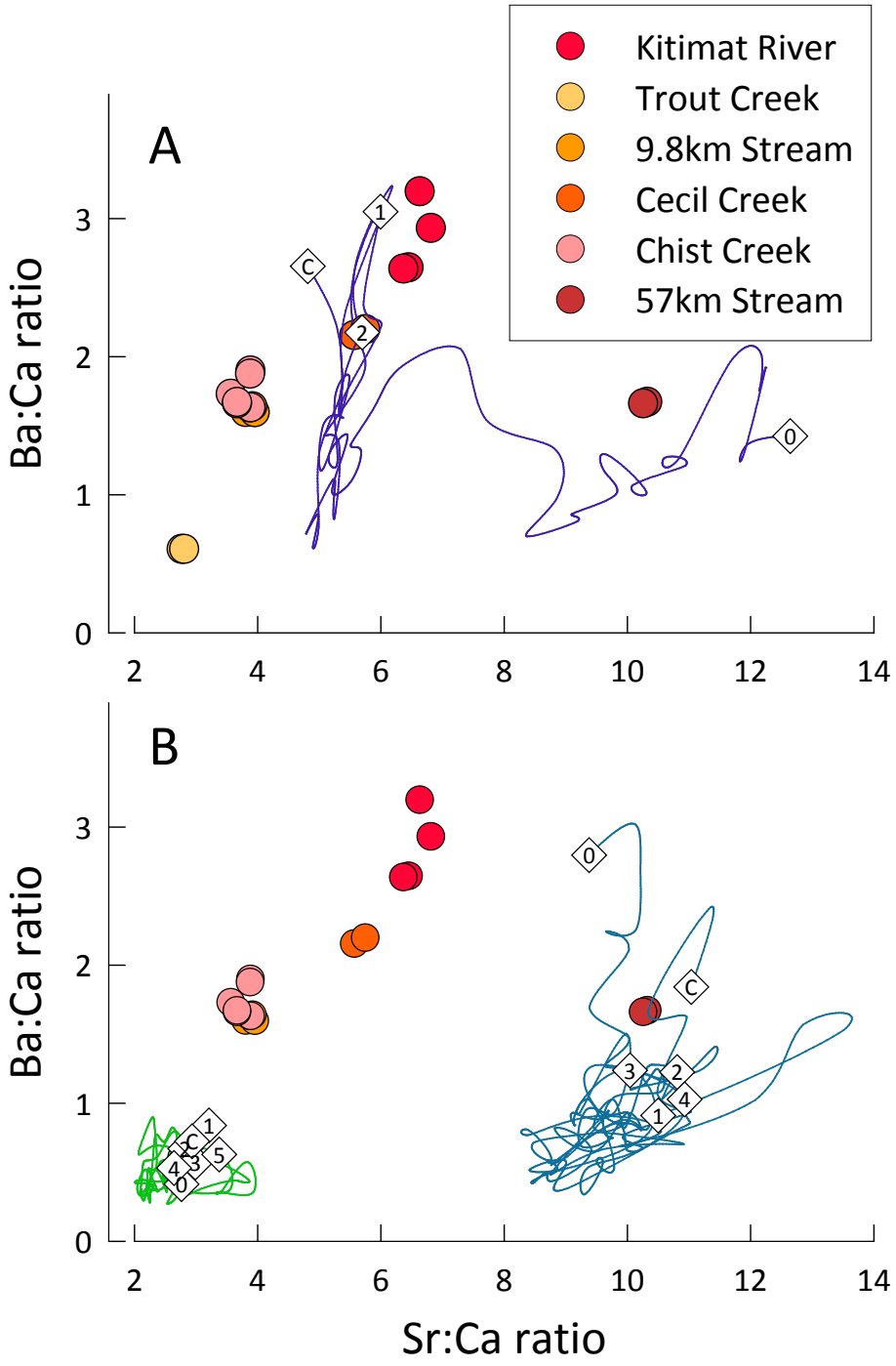

Figure 2-107 – Plots of elemental water signatures for Sr to Ca (mmol × mol^{-1}) and Ba to Ca (mmol × mol^{-1}) calculated from LA-ICP-MS data for *Oncorhynchus clarkii* otoliths based on relationships shown in Figure 2-99. Plot (A) is for *O. clarkii* 15-88 from Cecil Creek (purple line). Plot (B) is for *O. clarkii* 14-719 from Trout Creek (green line) and 15-40 from 57.1 km Stream (blue line). See Figure 2-103 for symbol description and Figure 2-92 for water sample locations.

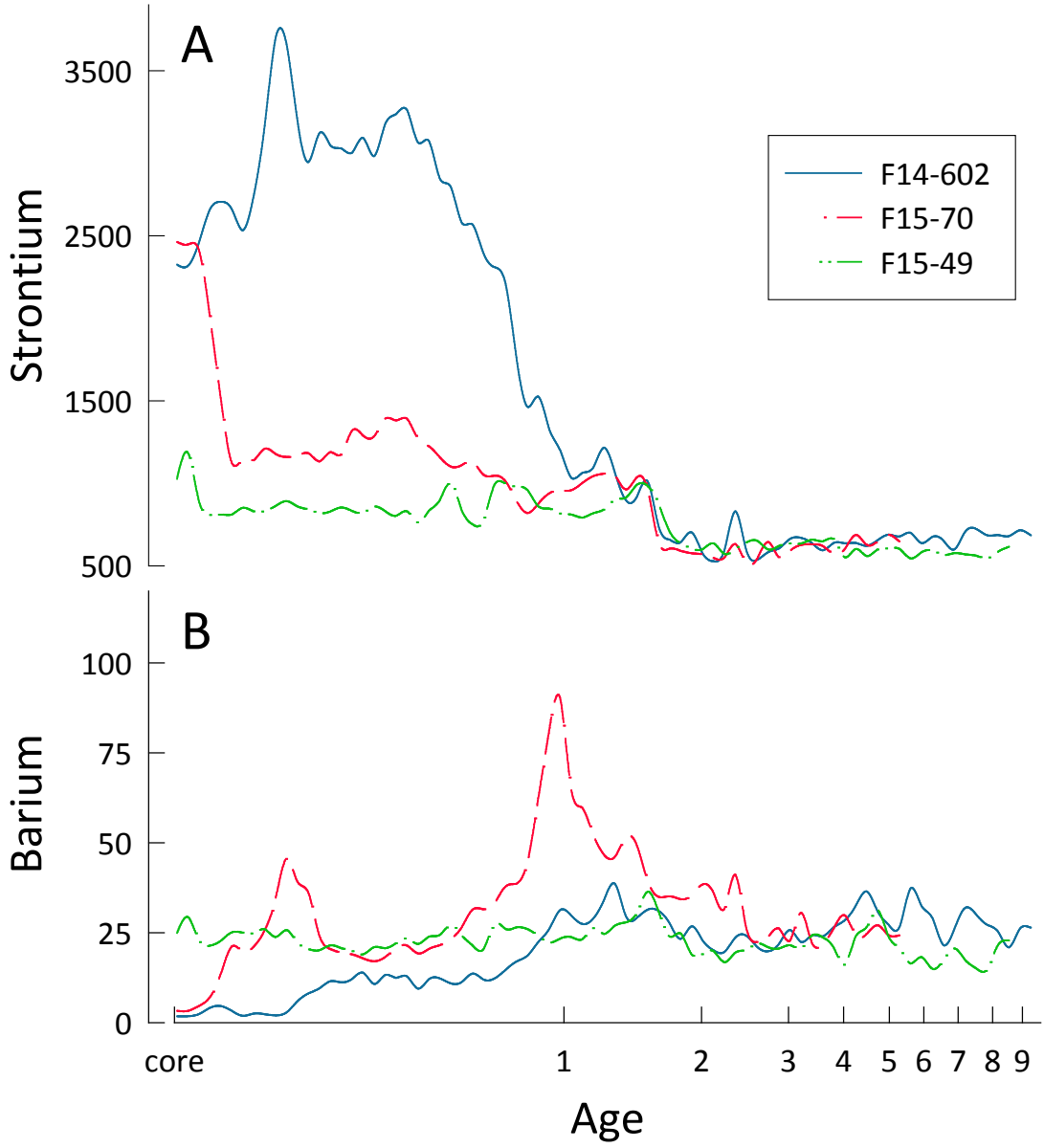

Figure 2-108 – Laser ablation-linescans from otoliths of *Cottus aleuticus* caught in Chist Creek. Changes in (A) strontium to calcium ratios (mmol × mol^{-1}) and (B) barium to calcium ratios (mmol × mol^{-1}) are shown from the core of the otolith to the outer edge. Fish F15-49 (9.5 cm, 8.9 g) was caught 200 m below the RoW; fish F14-602 (9.4 cm, 8.2 g) and F15-70 (10.1 cm, 9.0 g) were caught 200 m above the RoW. Fish age was estimated from otolith annuli.

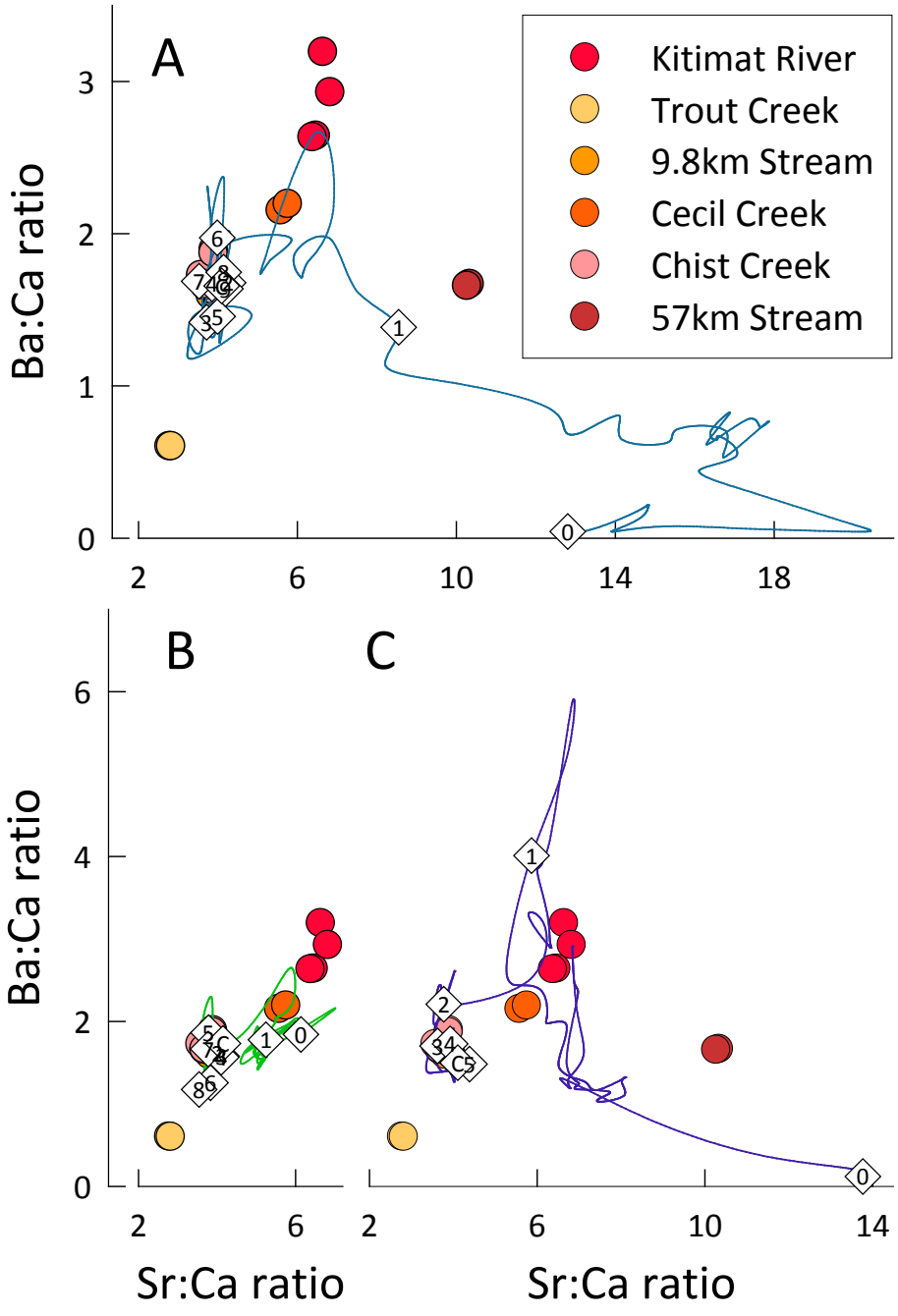

Figure 2-109 – Plots of elemental water signatures for Sr to Ca (mmol × mol^{-1}) and Ba to Ca (mmol × mol^{-1}) calculated from LA-ICP-MS data for *Cottus aleuticus* otoliths based on relationships shown in Figure 2-100. All fish were caught in Chist Creek. Plot (A) is for fish 14-602, (B) is for fish 15-49, and (C) is for fish 15-70. See Figure 2-103 for symbol description and Figure 2-92 for water sample locations.

Table 2-92 – Summary of movement patterns for Salmonidae and Cottidae captured in streams from the Nechako River and Kitimat River watersheds. Samples size is given as n. Mean age (years) with range in parentheses. The proportion of fish with an anadromous maternal signature (AMS) represents a percentage of the total number for that species from each system sampled. The percentage (%) of fish showing movement among habitats with distinct differences in water chemistry is shown for each species by year class. Sample size for the older age classes is less than the value of "n" as some of the fish were not old enough to include in the analysis for older age-classes.

	n	Age	AMS	Proportion moved within year				
			%	0	1	2	3	4+
Nechako River watershed								
Ormond Creek								
Cottus asper	7	4.9 (3–9)	-	86	57	43	71	43
Oncorhynchus mykiss	8	1.8 (1–3)	-	50	75	100	100	
Nine Mile Creek								
Oncorhynchus mykiss	6	2.5 (2–3)	-	83	0	17	100	
Kitimat River watershed								
Trout Creek								
Oncorhynchus clarkii	17	2.6 (2–5)	6	12	47	71	83	100
9.8 km Stream								
Oncorhynchus clarkii	6	2.2 (1–4)	17	17	50	100	100	100
Salvelinus malma	3	1.0	0	0	100			
Cecil Creek								
Oncorhynchus clarkii	6	1.8 (1–2)	100	83	100	100		
Salvelinus malma	3	1.0 (0–2)	33	67	100	100		
Chist Creek								
Cottus aleuticus	12	5.5 (4–8)	92	58	75	83	50	25
Oncorhynchus mykiss	12	1.8 (1–3)	100	33	42	86	100	
Salvelinus malma	6	2.0 (1–3)	0	0	33	100	0	
57.1 km Stream								
Oncorhynchus clarkii	12	2.6 (1–5)	17	58	58	86	100	
Salvelinus malma	6	3.0 (1–5)	0	83	67	100	100	100

Discussion

Our work used changes in elemental composition to track movement patterns for stream fishes. The streams sampled in this study exhibited sufficiently different chemical signatures to allow differentiation based on geographic locations. Additionally, the elemental ratios measured in Salmonidae and Cottidae otoliths were highly correlated with the stream ratios measured where the individuals were captured.

Elemental chemistry signatures within and among watersheds

Streams within the Nechako and Kitimat River watersheds were sufficiently heterogeneous in water chemistry to discriminate their locations. Our data support a number of studies that have also successfully discriminated geographic locations using chemical signatures measured in a freshwater environment (Kennedy et al. 1997, 2000; Wells et al. 2003; Clarke et al. 2007a, 2007b, 2015; Shrimpton et al. 2014). Variation in the freshwater environment, therefore, is common as geology of most regions varies spatially due to differences in composition of bedrock.

Stability of water chemistry was an important component of this study, as large temporal variation in stream chemistry would ultimately confound our ability to trace movements of fish among different river systems. The stability of the stream specific chemical signatures has previously been demonstrated (Taylor and Hamilton 1994). Interannual variability, however, was found in two recent studies, but variation was minor compared to spatial differences (Clarke et al. 2015) and did not limit classification of juvenile Atlantic salmon to natal streams (Martin et al. 2013). Elemental ratios remain consistent during low flows even if element concentrations rise (Taylor and Hamilton 1994), but show the greatest change in elemental ratios during the spring freshet (Clarke et al. 2004, 2015). Variation observed in these studies for each river was due to lower than average values measured during high flow conditions when less interaction with soils and bedrock can occur and values can shift toward those in unaltered precipitation. Additionally, monthly measurements over a two-year period from the Adour Basin in France found that Sr ratios fluctuated seasonally (Martin et al. 2013). Fluctuations in water elemental chemistry were mainly driven by water flow regimes, but interestingly were not reflected in the otoliths of juvenile *Salmo salar*. Otolith Sr ratios during freshwater residency for these fish were stable for each site and were related to water Sr ratios during low flow periods. Elemental ratios, therefore, were likely lower during spring freshet (high flow conditions) for the tributaries examined in the present study, though this is of limited concern, as most growth for otoliths of fish in watersheds with a snow dominated hydrograph has been documented to occur during base flow conditions (Kennedy et al. 2000). Fluctuations in water chemistry were likely for each stream in our study; but the large differences in

elemental ratios among streams and rivers particularly in the Kitimat River watershed should enable us to use otolith microchemistry to discriminate movements of fish.

Elemental signatures in otoliths: maternal signatures reveal connectivity

Our work has shown considerable variation in otolith microchemistry. The strong relationship between elemental chemistry in water and otoliths, particularly for Sr and Ba, indicates that fish incorporated elements from different water sources — reflecting movement among tributaries, rivers and even linkage to the ocean. Such findings are indicative of connectivity among rivers and streams in both the Nechako and Kitimat River watersheds.

Elemental chemistries with high Sr levels at the core of the otoliths reveal anadromous maternal signatures (Zimmerman and Reeves 2002), a pattern seen for *O. mykiss* caught in Chist Creek and *O. clarkii* caught in Cecil Creek indicating that anadromous trout spawn in these two tributaries. Both creeks also have populations of anadromous salmon — so it is not surprising that anadromous trout spawn in these two creeks. The marine signature indicates that there is connectivity between the locations where the fish were caught and these two creeks and the ocean. The presence of a marine signature in *O. clarkii* caught in 9.8 km Stream indicates movements of anadromous spawners into this system — the smallest stream in our study. Substrate size and depth of 9.8 km Stream are not appropriate for anadromous Pacific salmon and to our knowledge no species of salmon spawn in this system, although anadromous salmon were seen in Goose Creek — 9.8 km Stream flows into Goose Creek. The anadromous maternal signatures for these three watersheds indicate that adult spawners make directed movements from the ocean, through the Kitimat River, and into each of the streams.

Marine maternal signatures were also revealed in the otoliths of *C. aleuticus* caught in Chist Creek. Populations of *C. aleuticus* are known to complete their entire life cycle in fresh water (McLarney 1968), although use of estuaries is common as nursery habitat for juveniles (McPhail 2007). Brown et al. (1995) found that *C. aleuticus* populations in upstream areas were maintained by immigration from downstream areas in the Eel River, California). The marine maternal signature at the core of otoliths from sculpins captured in Chist Creek indicates that some of the fish in our study re-invade freshwater to spawn. Connectivity with the marine environment, therefore, also exists between the study streams and the ocean for the Family

Cottidae. The extent of the marine signature at the core for some of the Cottidae otoliths is less pronounced than for Salmonidae otoliths due to the smaller egg size — consequently less available maternal nutrients are available to influence the developing otolith of the embryo.

It is harder to define where fish resided before spawning that do not exhibit a marine elemental signature as elemental ratios within freshwater systems are more similar than the marine signature. Consequently for the study streams in the Nechako River watershed and two of the study streams in the Kitimat River watershed, spawners exhibited a freshwater maternal signature. Such fish are often referred to as resident individuals (Zimmerman and Reeves 2002) — perhaps not the most appropriate term as it implies that fish lacking a marine signature are non-migratory and do not move around. The largest fish caught in our study from any of the streams sampled in the late summer and early fall was an *O. clarkii* caught in Trout Creek weighing 66.8 g and 18.4 cm long. Most of the fish caught were much smaller, less than 10 cm in length, and many were young-of-the-year. Consequently, habitat available in the streams sampled appears well suited for young fish and not adults. Adults, therefore, must migrate into the systems to spawn which further supports connectivity of the streams sampled with larger river systems. It is possible that fish with maternal signatures that do not differ from the water source where the fish were captured are progeny of resident fish or fish from areas where the water signature did not differ appreciably.

Elemental signatures in otoliths: juvenile movement indicates connectivity

There is also evidence of connectivity for the portions of the linescans that were not influenced by the maternal signature. Fish of all species in our study exhibited variation in Sr and Ba consistent with residence in multiple habitats. Based on the large differences in otolith elemental chemistries measured, spatial movement of fish are variable, but substantive for many fish.

For some Salmonidae, the elemental signatures within the first annuli were similar to signatures at the time of capture suggesting residency within the stream of capture. Others showed considerable variation following the loss of maternal signature until time of capture. Overlap in elemental signatures of otoliths with water chemistry for larger mainstem rivers downstream of the site of capture was evident in *O. mykiss* from the Nechako River watershed

and *O. mykiss*, *O. clarki*, and *S. malma* from the Kitimat River watershed. It would appear, therefore, that fish moved downstream at an early age and then returned upstream to their natal location. This pattern has been seen in juvenile Salmonidae in other studies recently published; *Prosopium williamsoni* move downstream into the Columbia River, WA (Benjamin et al. 2014) and *Thymallus arcticus* move downstream into the Parsnip River, BC (Clarke et al. 2015). Movement downstream by juvenile *P. williamsoni* revealed by otolith microchemistry was not found in the same study using passive integrated transponder (PIT) tags, as the fish that migrated were too small for tags (Benjamin et al. 2014) — indicating the utility of otolith microchemistry for resolving movements for fish throughout their entire life history. Consequently, otolith microchemistry enables us to determine patterns of movement for life stages of fish that are too small to physically tag.

We have also presented evidence for movement of juvenile Cottidae in our study. Marine signatures for *C. aleuticus* beyond the maternal signature, but still within the first annuli indicate young-of-the-year Cottidae must also make considerable movements. *C. aleuticus* may adapt a fluvial life history where they spawn in a range of habitats, but juveniles rear in estuaries (Brown et al. 1995). Juvenile *C. aleuticus* that reared in estuaries would have to invade freshwater systems — consistent with otolith microchemistry for fish caught in Chist Creek. A pattern of movement upstream and into smaller tributaries appears to be common in *C. aleuticus*. In fact we caught no small young-of-the-year sculpins in our sampling program. Mean size for *C. aleuticus* caught in Chist Creek was 8.1 ± 0.2 cm in length and 5.8 ± 0.5 g; the smallest fish was 6.2 cm in length — indicating none of the Cottidae captured in Chist Creek were young-of-the-year. Our data indicates considerable movement among habitats for juvenile Salmonidae and Cottidae with a high degree of connectivity of tributary streams sampled with the mainstem rivers. Interestingly, we found no difference in movement patterns between fish sampled from the +200 m site compared to the -200 m site — despite potential differences in species composition for some of the streams sampled (Section 2.11, this volume).

Elemental signatures in otoliths: residency

Although our otolith microchemistry analysis indicates considerable movement of fish among the streams and rivers for both watersheds studied, there were otolith signatures that

remained relatively constant throughout the entire life history of the fish. The lack of variation in elemental signatures of the otoliths suggests the fish remained resident in the stream of capture and did not exploit other habitats. For Salmonidae, little variation in elemental signature was characteristic of the first few years of life and increased movement in older fish. In contrast, variation in elemental signature tended to be greater in the first few years of life and then decline at older ages for Cottidae. Many Cottidae had relatively stable otolith elemental signatures near the otolith edge — suggesting less movement in older fish (or movement of short duration that would not incorporate signatures from a novel habitat). There is a decline in resolution near the edges of otoliths due in part to an averaging effect, because the analytical beam has a constant diameter but traverses increasingly smaller growth bands in the outer parts of otoliths. Yet changes in elemental signatures at the outer edges of otoliths were found — indicating that habitat shifts were still detected in older Cottidae.

Elemental signatures in otoliths: life-history information

Elemental signatures and cathodoluminescence indicate that Mn clearly varies across the otoliths of fish from the present study, but also among fish sampled from different streams. Although, Mn changes can be clearly seen in the LA-ICP-MS linescans for fish in our study, CL images may be better for interpreting changes in abundance for this element. LA-ICP-MS captures an average elemental signature from the otolith that is dependent on the diameter of the laser scan whereas cathodoluminescence is sensitive to trace elements and colour changes in the otolith that are attributable to variations in chemical composition (Halden et al. 2004) — suggesting that resolution of elemental differences should be greater with luminescence than ablation. Additionally for CL images Mn typically causes most of the luminescence associated with carbonates (Marshall 1988).

For otoliths, relationships between Ba and Sr have been shown to strongly reflect water chemistry for *O. mykiss* (Gibson-Reinemer et al. 2009), *O. kisutch* (Shrimpton et al. 2014), *T. arcticus* (Clarke et al. 2007a, 2007b), and *C. cognatus* (Clarke et al. 2015). Consistent findings between water and otolith chemistry have not been demonstrated for Mn. Although, we found a significant relationship for Mn between otolith and water in Cottidae and Salmonidae, no relationship was found for *O. mykiss* (Gibson-Reinemer et al. 2009) or *T. arcticus* (Clarke et al.

2015). Shrimpton et al. (2014) found a weak relationship for *O. kisutch* parr — similar to findings in our study with considerable variation among fish sampled from the same location.

Most trace elements are taken up in a dissolved form via passage through the gills or the gut and are carried through the bloodstream to the endolymph where they are precipitated onto the otolith (Campana 1999). Mn is highly redox sensitive and much more mobile in hypoxic waters than in fully oxygenated waters and varies much more than the divalent cations Ca, Ba, and Mg. Typically groundwater is higher in Mn than surface water; Mn delivered to rivers from groundwater quickly precipitates on rocks and other surfaces (Drever 1997). Dissolved forms of manganese are Mn^{2+}, which is thermodynamically stable in anoxic and low pH waters, and Mn^{3+}, which is an intermediate in the microbially mediated production of Mn oxides (Tebo et al. 2004; Trouwborst et al. 2006). Dissolved Mn may remain in solution for periods of days at a time, even in the presence of oxygen (Thamdrup 2000; Pakhomova et al. 2007). Fish present in water where Mn remains dissolved may incorporate this element into the otolith (Thorrold and Shuttleworth 2000; Clarke et al. 2004, 2015). Recently, the use of Mn in otoliths has been proposed as a method for tracking fish exposure to hypoxia or low oxygen environments (Limburg et al. 2015).

The biological significance of changes in Mn in otoliths from fish in our study is not clear, but Mn in otoliths may provide insight into the type of environment fish are exposed to rather than shifts in habitats used by fish. Given that Mn remains dissolved in water at low oxygen and low pH levels, high Mn in otoliths may indicate exposure to low dissolved oxygen or low environmental pH at some point in the over the life history of the fish. Adult salmonids use specific locations within rivers to spawn and do not use other locations that appear suitable (McRae et al. 2012). Use of spawning locations is due to the interactions between groundwater (phreatic) and surface water (stream) that occur in the hyporheic zone (Valett et al. 1993, Geist et al. 2002). Phreatic water is low in oxygen — but stable temperatures of phreatic water have been suggested to be important for defining development timing (Brannon 1987) and improving survival (Saltvait and Braband 2013). The green luminescence near the core of otoliths, therefore, likely indicates that phreatic water high in Mn is influencing otolith accretion during incubation. Work examining incubation conditions in the Kitimat River

watershed has shown that Cecil Creek is strongly influenced by groundwater (Tuor and Shrimpton, UNBC, unpublished data). For example, the high Sr levels at the core of an *O. clarki* spawned in Cecil Creek indicate an anadromous maternal signature (Figure 2-96B). Beyond the high Sr levels is a dramatic increase in Mn (Figure 2-97A) when chemical composition of the incubation environment began to dominate the incorporation of elements into the otolith. Thus the high Mn near the core of the otolith is likely due to high Mn in the hyporheic water infiltrating the redd.

Not all otoliths from Salmonidae in our study showed a high Mn signature near the core of the otolith — but this does not mean only fish with high Mn near the core of otoliths were exposed to low dissolved oxygen levels during incubation. Surface waters from Cecil Creek were the highest in Mn of the rivers sampled in our study and likely reflect underlying bedrock geology high in Mn. CL images for fish sampled from 57.1 km Stream showed little green luminescence near the core (Figure 2-96A) or no green luminescence and very low Mn values from LA-ICP-MS linescans (Figure 2-97D) — 57.1 km Stream had the lowest concentrations of Mn in the surface waters measured in our study.

Manganese uptake by otoliths not only depends on environmental concentrations, but to some extent is also affected by growth (Limburg et al. 2015). Fish growth slows with age and this is also seen in otoliths; the portions of otoliths that provide the highest resolution for elemental signatures, therefore, are generally near the core — within the first and second annual growth zones. Regions of the otoliths beyond the first two annual growth zones, however, also show variable Mn. There is a relationship between otolith Mn and water chemistry and although the relationship is significant, there is considerable variation among individuals. Consequently, changes in Mn should not be used to define movement patterns; but Mn is clearly varying in otoliths for fish beyond the core region and characterizing water from the incubation environment. The stronger relationships between otolith Mn and water Mn for Cottidae (this study; Clarke et al. 2015) and juvenile Salmonidae (Shrimpton et al. 2014), but lack of a relationship for larger Salmonidae (Clarke et al. 2007a; Gibson-Reinemer et al. 2009) may be related to life-history differences and association with substrate and benthic organisms. Shifts in diet composition from benthic invertebrates to feeding on fish and terrestrial

invertebrates may be reflected in dietary Mn incorporation. Benthic invertebrates dominate the diets of small stream fishes belonging to the Cottidae and also juvenile Salmonidae; but diet shifts away from aquatic invertebrates as Salmonidae grow (McPhail 2007). Consumption of food labeled with ^{54}Mn, a radioisotope, revealed that this element became concentrated in bony and cartilaginous tissues (Baudin et al. 2000). Diet, therefore, may also contribute to the Mn signature in the otoliths and may indicate life-history variation and whether the fish primarily using benthic or pelagic habitats. The green luminescence seen at the outer edge of otoliths from both Cottidae and Gadidae — and some of the Salmonidae otoliths — may indicate an association with the substrate and a diet of benthic invertebrates for these fish.

Elemental signatures in otoliths: other elements

The use of ^{85}Rb in our analysis was expected to increase our ability to differentiate water chemistries and consequently movement patterns of fish among the different sampling locations. The molecular weight of this element is similar to ^{88}Sr, the most abundant stable isotope. Consequently it was expected that Rb might be incorporated into the otolith matrix with a relationship similar to that of Sr. Recent work using otolith microchemistry to understand population specific structure has incorporated the element Rb in the analysis to aid resolution (Anstead et al. 2015). Statoliths of adult *P. marinus* also show variation in Rb content that differs among populations (Lochet et al. 2014). Our water chemistry analysis also revealed good spatial variation in Rb among the locations where we sampled, however, the concentrations of Rb were very low in the streams where we sampled — an order of magnitude lower than Ba and Mn, and two orders of magnitude lower than Sr. Incorporation of Rb into the otoliths of fish in our study were near detection limits of the instruments used and as a result we could not detect any potential variation in otolith Rb signatures.

Consequently, Sr and Ba appear to be of greatest use for tracing movements of fish among freshwater systems. We also quantified otolith Zn concentrations, but found no correlation with water chemistry — a finding consistent with several other reports (Halden et al. 2000; Gibson-Reinemer et al. 2009).

Conclusions

Sampling locations for the present study were selected as they represented sites with good quality fish habitat. Our study has demonstrated that fish exploit these locations where we sampled, but also other locations. Elemental signals indicate connectivity for all streams with the mainstem rivers in both watershed — and also the ocean for the Kitimat River watershed. Such patterns indicate that movement within the watershed is extensive for Salmonidae; a finding that is not surprising. Movement patterns and connectivity among rivers and streams is also extensive for Cottidae; a finding that may be more surprising, but our understanding of movements among habitats for small putatively stream "resident" species is increasing (see Clarke et al. 2015 and references therein). Use of the term "resident" stream fishes may not be appropriate, but dispersal and even partial migration (Chapman et al. 2012) exists among a wide range of fish species.

Chemical composition of otoliths not only depends on environmental concentrations, but also is to some extent affected by growth (Limburg et al. 2015). Fish growth slows with age and this is also seen in otoliths; the portions of otoliths that provide the highest resolution for elemental signatures, therefore, are generally near the core — within the first and second annual growth zones. Consequently, our ability to resolve habitat shifts using otolith microchemistry is the greatest during the early life history of the fish. A gradual decrease in variation of elemental signatures over the course of years as the fish ages is seen in Cottidae otoliths. The decline in variation could be due to an averaging effect, because the analytical beam has a constant diameter but traverses increasingly smaller growth bands in the outer parts of otoliths. Not all fish from our study, however, demonstrated this pattern. In fact, otoliths from Salmonidae tended to show more variation as the fish grew older. The simple explanation would be that Cottidae disperse as juveniles and then become resident to specific habitats, but Salmonidae show the opposite pattern and become more mobile as they become older and larger.

There are weaknesses, however, in our approach. We cannot detect movement of fish to water chemistries that do not differ appreciably. Also, intermediate elemental concentrations do not necessarily mean that fish were in water with that specific elemental

signature. Uptake and incorporation of elements onto the otolith is not instantaneous and fish must remain in water for a period of weeks before the elemental signature of the otolith reflects the water. The size of the laser ablation spot also leads to an average of the elemental signatures.

Further work would benefit our understanding of connectivity among the streams and rivers intercepted by the Pacific Trails Pipeline. More water sampling would further define the extent of movements within streams sampled and whether the unknown signatures represent smaller tributaries. The higher Ba content found for elemental signatures of *O. mykiss* from the Nechako River watershed tributaries to Ormond Creek and Nine Mile Creek (also consistent with Clarke et al. 2015) — suggest that smaller tributary use is highly likely. Sampling these smaller tributaries is needed to define finer scale habitat use for fish in the present study.

Although earlier work has indicated that spatial variations in water elemental signatures differs more than temporal variations, additional water sampling at other times of the year will improve our confidence to define habitat shifts using otolith microchemistry. The relatively stable Sr signatures as *C. aleuticus* aged (elemental composition at the edges of otoliths) is suggestive of stable water chemistry in the Kitimat River watershed. *C. asper*, from the Nechako River watershed, however, did show considerable variation in elemental signature across the otolith. We interpret this to reflect movement patterns, but stability of water chemistry will need to be confirmed by repeated sampling throughout the year.

2.14. Aquatic Communities Protocol: findings and recommendations

We have shown that DNA barcoding and metabarcoding using next-generation sequencing are comprehensive and viable measures for assessing and monitoring aquatic biodiversity. We used DNA sequencing methods to clarify the mis-identified larvae of aquatic moth flies (Genus Pnuemia), to identify individual specimens and life stages that would be impossible to identify using traditional methods, to comprehensively identify bulk samples of insects from preservative ethanol, and to identify invertebrates and vertebrates from filtered stream water. DNA-based biodiversity assessment is a powerful and versatile technology that will only expand in use and scope in the coming decades.

The information that we collected as part of the Aquatic Communities Protocol indicated that the overall health of the study streams was good to excellent in all cases. The streams that we studied along the RoW have a diverse population of benthic invertebrates and unique use by fish. Despite the intensity of our initial field surveys to identify potential study streams, the systems ultimately differed a great deal, offering little chance for replicate streams in our study. Streams sampled in the interior plateau and central coastal region of BC varied in size from small creeks that had limited flow during the dry months to river systems that could only be sampled along the margins. As a potential control for the diverse physical characteristics of each stream, we proposed to sample at the point where the RoW crossed the stream, below the RoW and above the RoW. Such as study design would enable us to use the above site as a potential control within the stream that could be compared to the RoW crossing site and then estimate the persistence of any effects by examining the site below the RoW. Our sampling in 2013 and 2014 was also important to establish baseline – or before treatment – metrics for physical and biological features of each study stream. Such an approach conforms with the Before-After-Control-Impact (BACI) study design. Our results showed that every stream system is unique. Consequently, the upstream sites sampled would have to serve as controls – something that appears to be possible for aquatic invertebrates, but not for fish. Our results show that the measurement of localized and abundant taxa (e.g., aquatic invertebrates) may be an accurate measure of disturbance and of the effectiveness of restoration, but the

larger and more mobile vertebrates would not accurately reflect ecosystem health using the originally proposed BACI study design. Sampling over time, however, is crucial as we showed large variations in invertebrate biodiversity and fish size classes both within and among years.

Sampling for fish indicated that most of the stream contained small species such as sculpins or younger age classes of salmon and trout – although length frequency distribution suggest that most of the salmon and trout do not stay resident in the streams. Elemental signatures in otoliths from juvenile salmonids revealed extensive changes in water signature supporting extensive movement among river systems by the salmon and trout particularly as for larger and older individuals. Sculpin also showed variation in the otolith elemental signatures, but generally when the fish were younger. Our findings, therefore, indicate the importance of movement by stream fishes and ensuring connectivity between tributaries and the mainstem river.

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Chapter 3 – Tailed Frog as a Model for Understanding Connectivity within Aquatic, Riparian and Terrestrial Ecosystems Protocol

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Tailed frog habitat in Trout Creek. Photo credit: Mark Shrimpton; inset: Cherie Mosher

3.1. Introduction

The Tailed Frog Protocol tested a range of techniques for monitoring changes in the distribution and abundance of the coastal tailed frog (*Ascaphus truei*) in order to recommend methods for assessing presence and for maintaining connectivity of montane ecosystems. The coastal tailed frog is distributed from northwestern California to the Nass River north of Prince Rupert, BC (Dupuis et al. 2000). Relatively little is known of the ecology and biogeography of the

species at the northern extent of their range, in the vicinity of the Pacific Trail Pipeline project. Current research, in combination with past studies from southern BC, however, indicate that post-metamorphic tailed frogs use old forest types with a particular affinity to coarse woody debris and associated subterranean structures. Larval frogs and breeding adults are dependent on cool, fast-flowing headwater streams (Dupuis et al. 2000, Matsuda and Richardson 2005, McEwan 2014). Populations of tailed frogs are negatively affected by disturbances that reduce forest and riparian structure and alter stream hydrology (Dupuis and Steventon 1999). In northwestern BC, such effects are related to forest harvesting, hydroelectric developments, and other land developments. Given their biology, limited abundance, and the threats from landscape change, the coastal tailed frog is protected under the federal Species at Risk Act (*Special Concern*) and provincially recognized as a *Species at Risk* under the BC Forest and Range Practices Act. The high sensitivity of tailed frogs to environmental change and their need for a range of aquatic and terrestrial communities suggest that they are a good species for assessing the connectivity of habitats found in wet montane ecosystems that border fast-flowing streams.

The Tailed Frog Protocol included two integrated components designed to monitor and understand changes in the connectivity of aquatic and terrestrial ecosystems by using tailed frog populations as a (qualitative and quantitative) measuring tool. The first component tested mitigation strategies using three pre-existing Experimental Watersheds near Terrace, BC (Todd et al. 2012) in close proximity to the pipeline RoW. These watersheds are representative of habitat conditions found on the RoW. At the Experimental Watersheds, we used systematic pitfall traps and radio-telemetry to reveal multi-scale resource selection and movements of individual frogs in areas that are disturbed by forest harvesting. This component of the study was partially supported by PTP and was reported in McEwan (2014). In the second component, we tested a number of direct (i.e., pitfall sampling and visual encounter surveys) and indirect (i.e., environmental DNA) techniques for measuring and monitoring the distribution and abundance of post-metamorphic tailed frog populations. Also, we conducted two studies to assess the connectivity of tailed frog populations along the pipeline RoW. This included analyses of the genetic structure and by inference the connectivity of populations from across

northwest BC. Second, we studied the environmental parameters that influence the relative abundance of tadpoles in larval streams that may be bisected by the RoW. In combination, these studies investigated the movements and distribution of tailed frogs at both the ecological and evolutionary spatio-temporal scales. When complete, the data will allow for the interpretation of patterns of tailed frog distribution and can be used to guide restoration after pipeline construction.

3.2. Research approach and outline of contributions

We tested a series of monitoring methods for understanding the presence and abundance of tailed frogs in both unaltered and cleared habitats. The Experimental Watersheds were composed of three treatments representing forest harvesting practices adjacent to small headwater streams: unharvested, 50-m harvest buffer, and harvest to immediate vicinity of the stream. These treatments bracket the range of effects associated with the disturbance of tailed frog habitat and have applicability to clearing activities on the pipeline RoW. Because tailed frogs are a visually cryptic species and spend significant amounts of time at great distances from natal streams, often associated with complex woody structures on the forest floor, this species is difficult to census. We investigated the factors that influence the detection probability of coastal tailed frogs using two survey methods: visual encounter surveys (VES) and pitfall trap sampling. Although detection probability via VES and pitfall sampling share similar explanatory variables, the data suggest that VES are more sensitive to environmental conditions. The efficiency of VES can be equal and often greater than that of pitfall sampling, but requires relatively low temperatures and high humidity — environmental conditions that allow frogs to be active during daytime. Pitfall sampling of coastal tailed frogs is more informative about seasonal movements, but VES appear better for an unbiased sample of the entire population. Both methods provide valuable information, but the choice of method should be determined based on study objectives and sampling constraints. The results of these studies are described in Section 3.3.

In Section 3.4, we describe a practical application of stream water environmental DNA sampling used for monitoring the presence of coastal tailed frog in streams along the RoW. We

detected coastal tailed frog in 13 of 15 streams crossing the RoW in the Kitimat River watershed and detected no signal in control streams, sampled outside of coastal tailed frog habitat. The confirmed presence of coastal tailed frog in most of the streams in the coastal RoW presents an opportunity to use this species as an indicator of habitat connectivity and restoration after pipeline construction.

Section 3.5 reports on our studies of the genetic variation within the tailed frog population in the RoW area. Our initial approach was to use an existing microsatellite marker system to conduct a landscape level analysis of genetic connectivity. Our results showed that the RoW-Terrace population of tailed frogs had dramatically reduced genetic diversity when compared with other regions in the Pacific Northwest (BC and Washington). Significant genetic structure is noted among all regions sampled. Consistent with postglacial expansion, the genetic diversity of tailed frog population decreased in a northern direction within the species range. Essentially, the northernmost population found along the RoW is genetically distinct and has the lowest genetic diversity among all of the populations sampled.

The reduction in genetic diversity may contribute to an increase in the sensitivity of this population to environmental change. In order to further study the evidence of reduced genetic diversity and to enable the proposed study of landscape genetics and connectivity, we developed a genotype-by-sequencing marker system, RAD Capture, in Section 3.6. This newest generation of genetic markers genotypes thousands of loci across the genome versus the 10–15 used in traditional microsatellite systems. It enhances the resolution of both individual genotypes and estimates of whole genome diversity. In Section 3.6, we present the research plan and potential outcomes from the development of the RAD Capture approach to assess genetic variation in tailed frog.

Finally, in Section 3.7, we assessed the environmental characteristics of high-quality stream habitat for larval frogs. We used a measure of sampling intensity, tadpoles collected per search hour, to assess the relative abundance and important habitat correlates for this life stage of tailed frog. Data collected at 124 stream reaches suggested that tadpoles had a greater relative abundance in smaller stream reaches with a relatively low embeddedness of substrate in the stream's bedrock.

3.3. The efficacy, applicability and limitations of visual encounter surveys in the detection of *Ascaphus truei*

Ben Millard-Martin, Angela Grob, Brent Murray, Chris Johnson

Introduction

The coastal tailed frog (*Ascaphus truei*) and the recently differentiated Rocky Mountain tailed frog (*A. montanus*) represent the monophyletic Family of Ascaphidae, the only extant representatives of the basal lineage of Anurans (Nielson et al. 2001). As a result of this ancient divergence, they are unique in several respects. Tailed frogs inhabit fast-flowing streams in upland montane habitats across northwestern North America. The streams are used as nurseries for eggs and tadpoles, and as overwintering habitat and refugia for adult frogs during warm and dry periods. The adults spend the majority of their life cycle in terrestrial habitats characterized by mature conifers with horizontal forest-floor structures such as coarse woody debris and root hollows (Metter 1964; McEwan 2014). The frog's ecological niche consists of old-growth forest and fast-moving streams, which is likely a driving force that has resulted adaptation unique to this Family. The "tail" is an external cloaca, found only in males, and functions in internal fertilization. *A. truei* lack tympanum, which is the hearing organ present in other Anurans; tailed frogs are able to sense vibrations through the ground and water (Claussen 1973). The tadpole has a mouth modified into an oral disk that functions as a suction cup for clinging to rocks in swift water and feeding by scraping periphyton from rocks (Metter 1964).

Coastal tailed frogs are distributed throughout the Coast Mountains from northern California to the Skeena Watershed, BC (Corkran and Thoms 1996; Dupuis and Bunnell 1997). The species has highly vascularized skin and is sensitive to desiccation, and therefore is highly dependent on the humidity and mild temperatures provided by the temperate coastal forests (Bury and Corn 1988; Gomez and Anthony 1996; Wahbe et al. 2000; McEwan 2014). When temperatures are high and humidity is low, tailed frogs become primarily nocturnal (Welsh and Reynolds 1986; Wahbe 1996) and seek cool, moist places such as those provided by coarse woody debris and root hollows (McEwan 2014).

The duration of the larval stage for *A. truei* can last as little as one year (Wallace and Diller 1998), but is generally 2–4 years (Metter 1964; Brown 1990). Variation in developmental period among populations is likely the result of differences in temperature and food abundance (Bury and Adams 1999). Sexual maturity is not reached until 8–9 years of age and adults may live as long as 15–20 years (Daugherty and Sheldon 1982).

A. truei is blue-listed by the BC Conservation Data Center and designated as *Special Concern* in Canada (COSEWIC 2011).The species is a habitat specialist during both major life stages, thus, larval and post-metamorphic frogs are sensitive to disturbances of riparian and upland areas. Furthermore, the long larval stage and low reproductive rate of *A. truei*, increases the species' vulnerability to habitat loss and fragmentation (Richardson and Neil 1998). Anthropogenic disturbances such as logging and construction projects can have significant impacts on ambient temperature, humidity and water quality within and around streams directly affecting all stages of the coastal tailed frog's life history (Richardson and Neill 1998; Dupuis and Stevenson 1999; Hawkes and Gregory 2012). Disturbance to areas surrounding streams reduces the understory and forest-floor habitat structure, which in turn, influences the temperatures and humidity of *A. truei*. Dupuis and Stevenson (1999) recorded a significant negative correlation between tadpole densities and fine sediments in the streams. The sedimentation of streams often results from increased mechanical activities from logging, power generation projects, roads, and bridge construction. Sediment infiltrates the stream systems and fills the large pore space in coarse sediments that *A. truei* tadpoles require for refugia (Dupuis and Stevenson 1999).

Due to the elusive nature of *A. truei*, the distribution, movement and habitat associations of post-metamorphic frogs are not well understood. Pitfall traps and time-area constrained visual encounter searches are the two methods used most often to estimate the abundance and population structure of terrestrial amphibians (Bury and Corn 1987; Vonesh et al. 2009). Pitfall traps are an assembly of hard tubes dug into the earth with fencing to direct the frog to the trap. This is a passive detection method in which traps are opened for a predetermined time and checked according to a 12 or 24-h schedule. In comparison, visual encounter surveys (VES) seek to detect frogs by actively surveying habitat. This can be achieved

using an effort or area based study design. Both pitfall traps and VES are often oriented across an environmental gradient (e.g., increasing distance from natal stream) allowing for data that describe habitat associations of observed frogs.

Visual encounter surveys may be a cost-effective rapid assessment technique for establishing amphibian presence, diversity, and relative abundance (Vonesh et al. 2009). Crump and Scott (1994) reviewed this technique and suggested that the precision and accuracy of VES data is potentially limited by a number of factors including searcher bias and site characteristics that affect detection probabilities. Some researchers have suggested that due to low capture rates VES is ineffective for *A. truei* (Matsuda 2001). In comparison, as a passive sampling technique, pitfall traps eliminate observer bias, but are expensive and time consuming to install.

Study objectives

There has not been a systematic comparison of methods for assessing the distribution and abundance of post-metamorphic coastal tailed frogs. Conflicting evidence from pitfall and VES surveys, (Corn and Bury 1989; Dupuis et al. 1995; Dupuis and Stevenson 1999; Wahbe et al. 2004; Kroll et al. 2008), indicate the need to evaluate precision and accuracy as well as the costs and efficacy of each method. Past work by McEwan (2014) fully explored the factors that influenced detection success of coastal tailed frog when using pitfall traps. Therefore, we explored hypotheses focused on habitat, environmental conditions, seasons, as well as potential biases that were inherent to the VES technique. These results were qualitatively compared to pitfall results reported by McEwan (2014). Specifically, we aimed to evaluate the efficacy of VES and pitfall trap sampling as a means of estimating occupancy, distribution and density of coastal tailed frogs across three treatment types: clear-cut, buffer-zone and old-growth forests. Furthermore, we investigated the environmental and sampling factors that influenced the distribution and detectability of coastal tailed frogs.

Methods

Study sites

Visual encounter surveys and pitfall trap sessions were conducted in two watersheds in the Hazelton Range of the Coastal Mountains near Terrace, BC. All sites were located in Coastal Western Hemlock (CWH) (Meidinger and Pojar 1991). McEwan (2014) describes the ecological conditions of the sites in detail.

Within each watershed, three streams were chosen based on forest harvest characteristics: clear cut, old-growth forest and a forest-harvest buffer zone. The streams were typical of coastal montane regions, consisting of steep gradients flowing in a step-pool fashion with riffles in lower gradient ranges (Todd et al. 2013). The clear-cut sites were at various stages of regeneration, being 4–20 years post-harvest. A forest retention zone of 30–50 m adjacent to the stream defined the buffer sites, beyond which was clear-cut. Old-growth forest sites were defined as being undisturbed for more than 140 years.

Survey design

Visual encounter surveys and pitfall sampling were conducted three times per year (June–October, inclusive). Each watershed was surveyed seasonally, once in the spring (May–mid-July), summer (mid-July–August) and fall (September–October). Visual encounter surveys were conducted with fixed time intervals across 20 × 100-m belt transects. At each site during 2012, the VES area was centred on five parallel transects located at distances of 10, 30, 50, 70, and 90 m from the stream channel. The survey area extended 10 m to each side of the transect centerline (Figure 3-1). In the fall of 2012 and through 2013, an additional transect was surveyed along the streambed and adjacent stream banks (0 m from stream). Due to sinuosity of the streams, transect centerlines were altered along their 100-m course to maintain the appropriate distance from the stream and transect width. Transects were assumed to be independent sample units.

At each site, pitfall trap arrays coincided with the VES area. Each trap array consisted of four pieces of PVC piping (15 cm diameter × 37 cm depth) installed flush with the forest floor. Four lengths of drift fencing reaching 5 m from the array centre was installed perpendicular to the trap orientation: toward the stream, away from the stream, upstream and downstream.

Drift fencing was buried at the base and reached 30 cm above the forest floor. Lengths of 3 mm (2012) and 8 mm (2013) twine was attached to trap collar as an escape route for accidental small mammal by-catch (McEwan 2014). Twelve trap arrays were installed at each site at systematic distances from the stream (5, 30, 55 and 80 m; Figure 3-1).

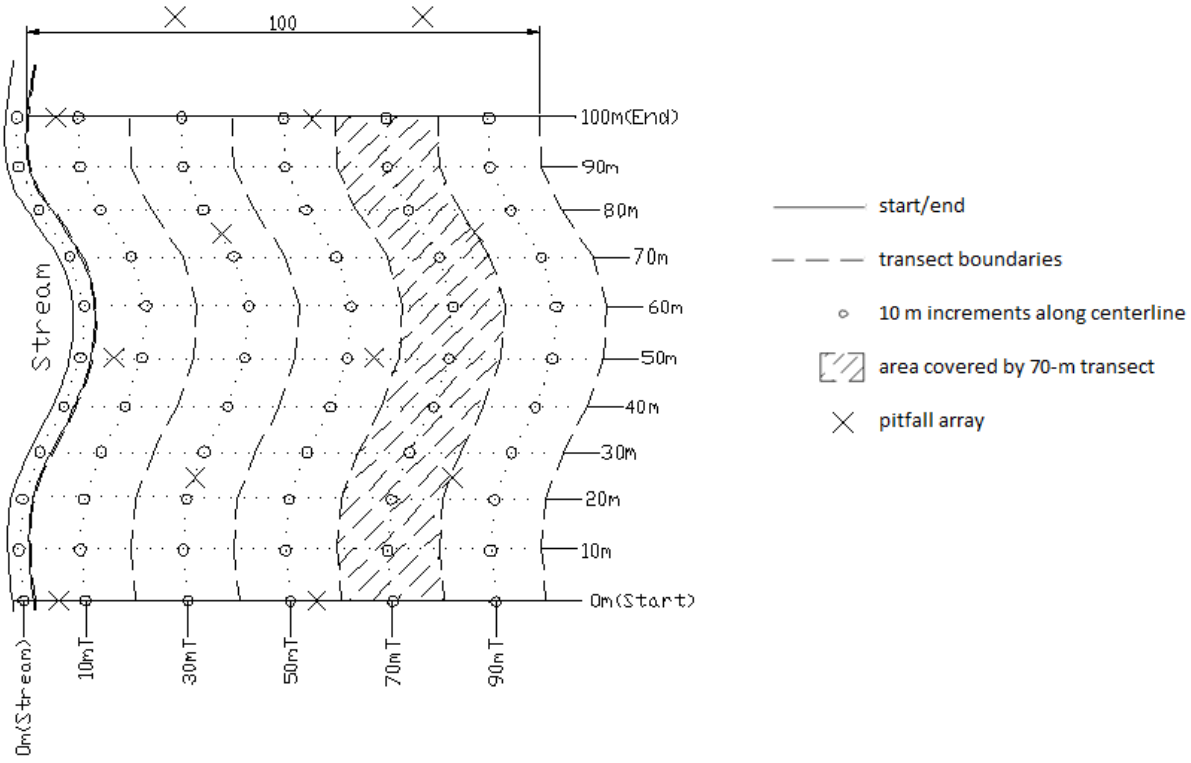

Figure 3-1 – Orientation of hypothetical transects used for visual encounter surveys for tailed frogs. Transect centerlines, indicated by short dashed lines, are parallel to the stream edge at fixed distances (10 mT, 30 mT, etc.). The hatched portion represents the area searched for the 70 m belt transect. Numbers on the right side of the figure (0 m, 10 m, 20 m, etc.) represent linear distances along each transect. Crosses indicate the locations of pitfall trap arrays.

Sampling

Sampling using VES or pitfall traps was offset by a minimum of one week to limit the effect of observer activity on frog distribution. Each seasonal VES session consisted of three replicate searches of each site within a watershed. Typically, a VES session occurred over a 3-day period with one survey per site per day. Depending on weather and staffing, however, the

period within which the three replicates were completed could extend over seven days. To account for differences in the temporal activity of frogs, the order of survey for each site varied across the typical 3-day period (e.g., Day 1: site 1, site 2, site 3; Day 2: site 2, site 3, site 1; Day 3: site 3, site 1, site 2).

VES sampling began following daybreak, thus, start times varied across the year. A handheld digital thermometer was used to measure temperature at the beginning and end of each transect (Table 3-1). The survey crew was careful not to disturb transects until they were to be searched. Each transect was searched for a total of 60 min. The lead technician ensured that the survey crew allocated consistent search time in each segment. A VES required no more than three h from the beginning of the first transect to the end of the sixth transect.

Searchers walked through a transect surveying open areas, taking care to search as much microhabitat and ground cover as time permitted. Although a constant search rate was desired, complex habitat was given more attention than open areas where detection of frogs was easier. During the VES, habitat was not searched using destructive methods: coarse woody debris and rocks were lifted or overturned, but always replaced and flora was pushed aside to improve visibility, but care was taken to cause as little damage as possible.

All frogs captured by VES were held in a plastic container with moist moss, capture locations were flagged, and the containers placed in shaded locations. Following the VES for the site, each frog's morphometrics (i.e., sex, snout-vent length, shank length, weight and age-class) were recorded along with habitat and climate data at the capture site. Frogs were released at the same location that they were captured. Animal care and handling protocols were followed as outlined by Todd et al. (2013).

Pitfall sessions consisted of six consecutive nights of trapping at each site. For each session, the three sites in a watershed were surveyed. Typically, traps were opened from 0700–1000 on day one and closed from 0700–1000 on day seven. The traps were checked every morning of the seven-day trapping period.

For pitfall traps, frogs were processed sequentially following their discovery in a trap. As with VES, morphometrics were recorded along with climate data. Habitat data were only collected once for each trap array. Frogs were released beyond the drift fencing on the

opposite side of the trap array from which they were caught. We assumed that frogs would travel in the previous direction of travel and, therefore, would not be recaptured on the following trap-night.

Data analysis

Capture rates were compared between methods as well as survey year, age class, sex and distance-from-stream. Due to differences in sampling effort, capture rates could not be directly compared, instead a detection efficiency metric based on person-hours of sampling was generated. Efficiency was calculated as the number of daily survey person-hours required for a single detection.

Data gathered from systematic VES sessions was used to generate a series of logistic regression models to identify factors that influenced the distribution and detectability of tailed frogs. This allowed for a comparison of factors identified by McEwan (2014) as influencing the probability of observing a frog using pitfall traps across the same research sites. The primary sampling unit (PSU) of the VES method was the daily survey of individual transects whereas the PSU of the pitfall trap array was an individual trap-night.

Counts of frog detections were used on each VES transect to generate presence-absence data (i.e., ≥1 frog surveyed per survey day). The models were parameterized using a set of variables that were categorized into two groups of hypothesized explanatory factors: ecological factors that affected frog activity and survey factors that influenced the detectability of frogs (Table 3-1). The ecologically plausible variables were further categorized as temporal, site/habitat and climate/weather. Model β-coefficients for explanatory factors were deemed significant if $p < 0.05$.

The Akaike Information Criterion (AIC_c; Burnham and Anderson 2001) for small sample sizes was used to identify the most parsimonious model of the set. The models were ranked according to their difference (ΔAIC_c) from the model with the lowest AIC_c score. Also, we reported the Akaike weights (w) that represent the probability that a model is the best of the set were reported. Models were considered equal in parsimony when $AIC_c < 2$ (Burnham and Anderson 2001).

An information theoretic approach provides a relative, not absolute, measure of model fit and utility. Thus, the receiver-operating characteristic (ROC) was used to quantify the discriminatory capabilities of the most highly ranked logistic regression models. A jackknifing process was used, wherein each individual record was systematically withheld from the model building process and sequentially used to evaluate the predictive accuracy of the model. Several models were created for each category of variables, followed by an integration of these categories.

Model variables were categorized into explanatory groups: temporal, site/habitat, climate/weather, and sampling bias (Table 3-1). Variables were equivalent to those applied by McEwan (2014) to pitfall data with the exception of variables representing sampling parameters specific to VES. Temporal and site variables were categorical delineations of year, month, watershed, and treatment type. At each pitfall array, crown closure, canopy cover, distance to stream, distance to hard edge and elevation were categorized or measured. Once categorized, distance to hard edge can be interpreted as transect treatment (i.e., old-growth forest, edge and clear cut). Crown closure, canopy cover and distance to hard edge were categorized, and elevation and distance to stream were measured as continuous distances (m). The values from each pitfall array were extrapolated to the nearest VES transect. Daily weather variables were collected during site surveys or when traps were cleared. Cloud cover and precipitation were recorded as categorical variables and temperature, humidity and days since last rain as continuous variables. Data-loggers were also installed at each site at standardized distances from the streams and provided hourly measurements of temperature and humidity. From these data, calculations of daily (24 h) maximum temperature and minimum humidity for each pitfall and VES PSU were conducted.

We developed a number of variables to quantify the accuracy and precision of each VES survey. A categorical variable describing ground visibility was created by asking technicians to rank individual transects based on the percent of the forest floor that was visible. A simple categorical variable categorized each technician as experienced (>10 days surveying experience) and inexperienced (<10 days surveying experience).

Table 3-1 – Hypothetical variables used to describe the detection of tailed frogs using visual encounter surveys and pitfall traps at study sites in the Kitimat Range of the Hazelton Mountains during the spring, summer, and fall of 2012 and 2013. Numbers in parentheses represent the coded values used for levels of each categorical variable within the analyses.

Variables	Description
Temporal:	
Year	The year in which search was completed: 2012 (1) and 2013 (2)
Month	The month in which the search was complete: June (1) July-early (2), July-late (3), August (4), September (5) and October (6)
Site/Habitat:	
Elevation, Elevation^2	Elevation of site (meters) and corresponding quadratic term (meters^2)
Treatment type	Forest harvest treatment type: old growth (1), buffer (2), clearcut (3)
Distance from stream (dfs)	Distance of centerline of transect or pitfall array from stream edge
Distance to edge (de_c)	Categorical distance of transect to hard edge of forest stand: >10 m from edge under canopy (mature, 1), >10 m from edge in cut block (edge, 2), <10 from edge (cut, 3)
Crown closure (cancov_c)	Categorical measure of forest overstory: old growth (1), buffer-treed, but with an edge (2), clearcut-no trees (3), regeneration (< 20 years growth - 4)
Climatic/Weather:	
Relative humidity	Percent relative humidity (%): collected at the beginning and end of 0, 10, 50 and 90-m transects
Instantaneous temperature (temp_inst)	Temperature (°C) and corresponding quadratic term collected at the beginning and end of 0, 10, 50 and 90-m transects
Cloud cover (cc)	Percent cloud cover (%) recorded before and after each transect search: 0 (1), <50 (2), >50 (3), 100 (4)
Precipitation (precip_c)	Categorical estimate of rainfall recorded at beginning and end of each transect search: none (1), light (2), medium (3), heavy (4):
Pre-search climate data (temp_max); (hum_min)	Maximum temperature and minimum humidity over the 24 h previous to the search via stationary climate data-loggers
Days since last rain (dslr)	Number of days since rain was recorded at the survey site
Time of day	Time at which transect search was completed: morning (1), mid-day (2), afternoon (3)
Sampling bias (VES only):	
Ground visibility (vis)	Categorical estimate of percent (%) visibility, from standing, of forest floor: 0–25 (1), 26–74 (2), 75–100 (3)
Experience (exper)	Number of days experience of a searcher conducting visual encounter surveys: <9 (1), >9 (2)

Results

Total captures

VES surveys were conducted over a total of 41 and 36 days, totaling 206 and 213 transects in 2012 and 2013, respectively. The increased number of transects in 2013 relative to total days of surveying was due to the additional 0-m transect. In 2012 and 2013 51 and 158 frogs were detected, respectively. When corrected for person-hours of effort, VES had slightly

higher detection efficiency (person-hours × detection^{-1}; Figure 3-2). Pitfall traps were monitored for 36 and 41 days, totaling 1296 and 1476 trap-nights in 2012 and 2013, respectively. In 2012, 129 tailed frogs were detected while 31 individuals were detected in 2013.

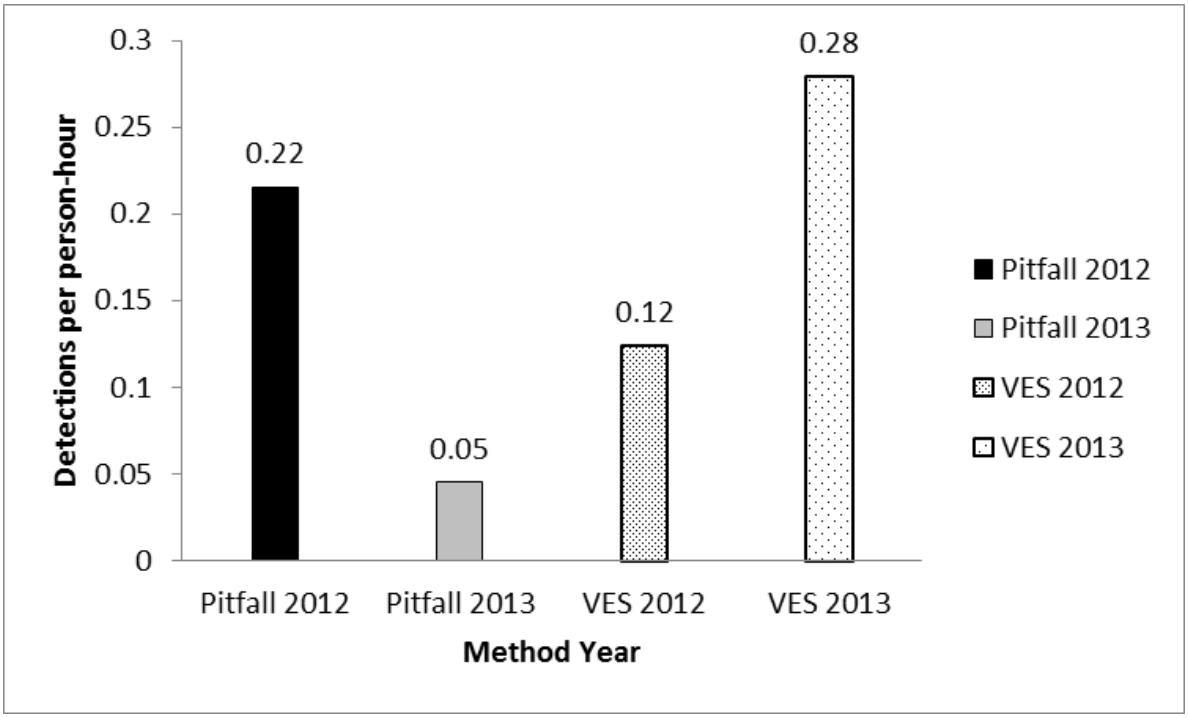

Figure 3-2 – Detections per person-hour of tailed frogs in two watersheds, located east of Terrace, BC for 2012 and 2013.

Sex and age differences in detection

Due to limited resources, each method was not applied in every month, making it difficult to compare data among months. In June, July and August there were no apparent differences in the proportion of adult frogs detected by the two survey methods (Figure 3-3). When using pitfall traps, the greatest proportion of adults was captured during September, whereas in October a higher proportion of adults was detected via VES. Overall, VES sampling resulted in a lower proportion of adult detections when compared to pitfall sampling (Figure 3-4).

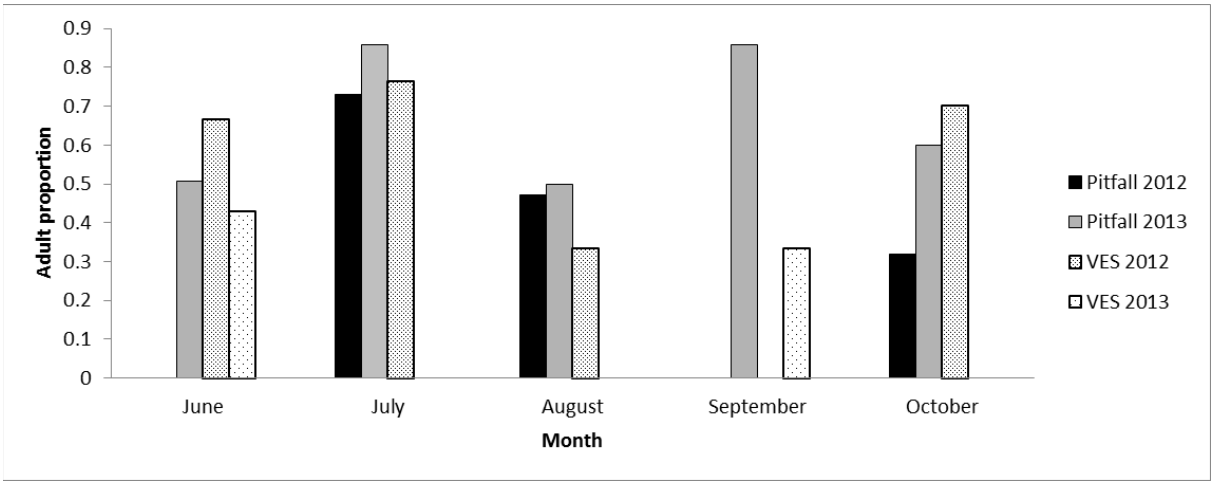

Figure 3-3 – Proportion of detections of tailed frogs that were classified as adults, by method and month in two watersheds, located east of Terrace, BC for 2012 and 2013.

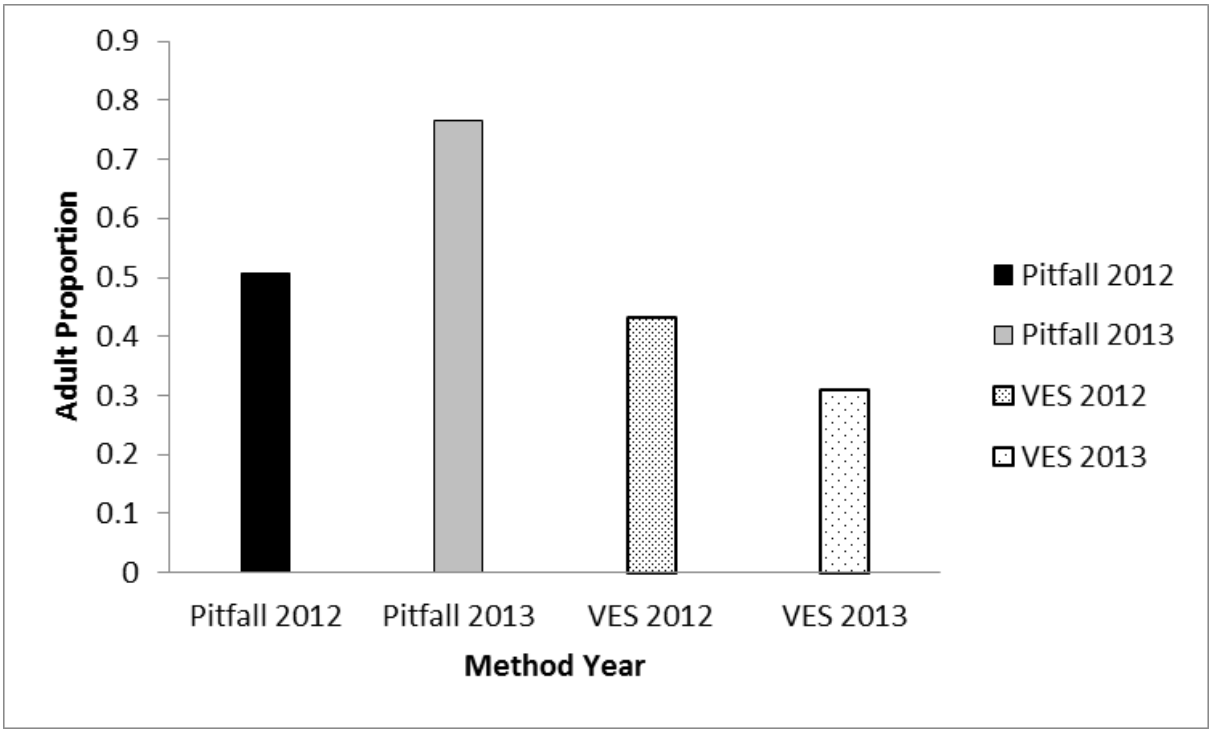

Figure 3-4 – Proportion of detections of tailed frogs that were classified as adults by method and year in two watersheds, located east of Terrace, BC for 2012 and 2013.

The male-female composition of detections varied between methods and across months. In 2013, a lower proportion of females were caught using both pitfall and VES methods (Figure 3-6). In 2012 and 2013, however, the VES method detected proportionally fewer females when compared to the pitfall method. For most months the composition of detections was similar between methods, but in July and August proportionately fewer females were detected via VES (Figure 3-6).

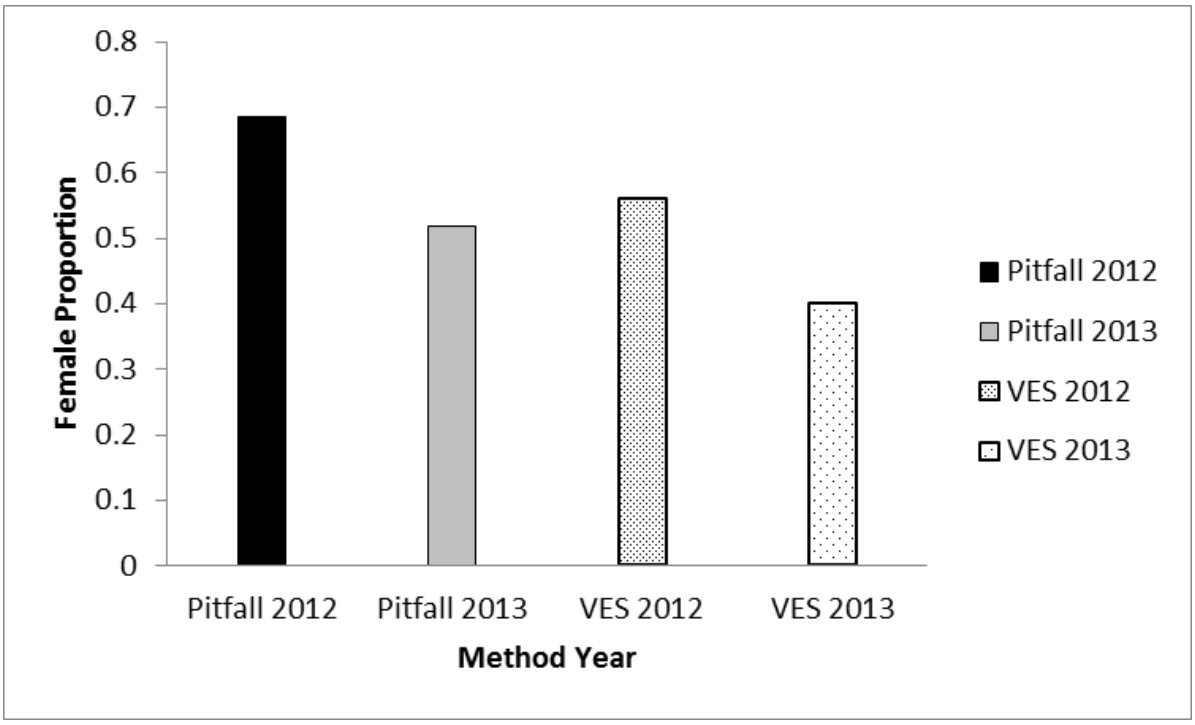

Figure 3-5 – Proportion of detections of female tailed frogs by method and year in the two watersheds.

Distance from stream

The proportion of total detections at systematic distances from the natal stream varied slightly between methods. Pitfall sampling resulted in the highest proportion of total detections nearest to the stream, and fewer as distance increased (Figure 3-7).

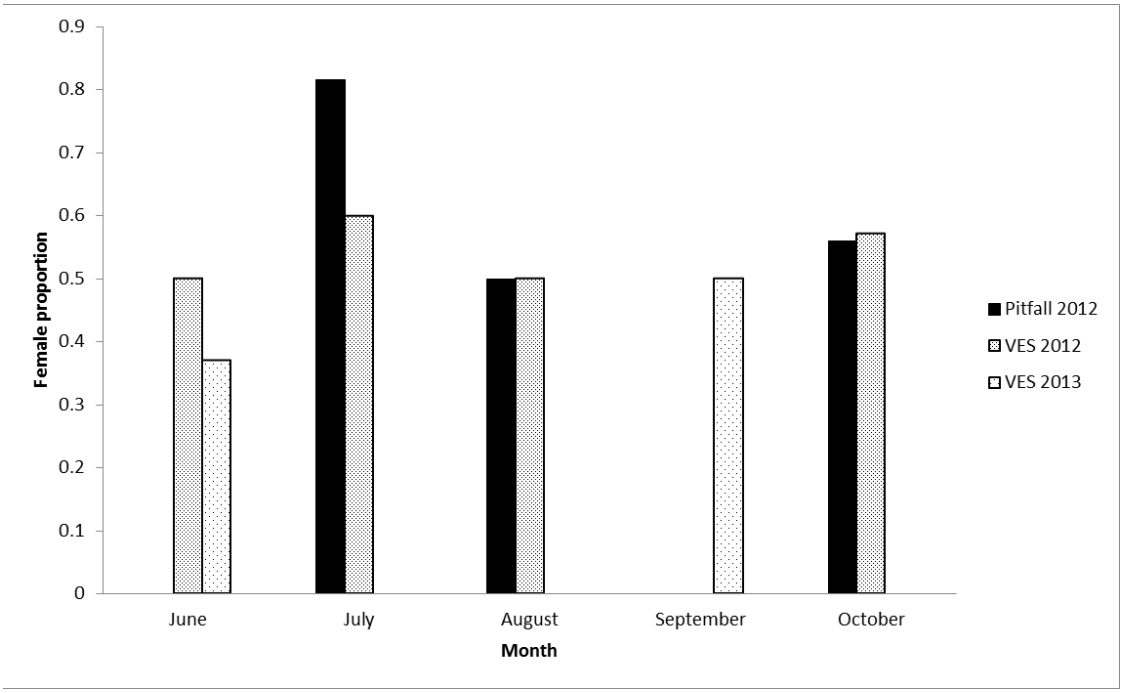

Figure 3-6 – Proportion of detections of female tailed frogs by method, year, and month in two watersheds.

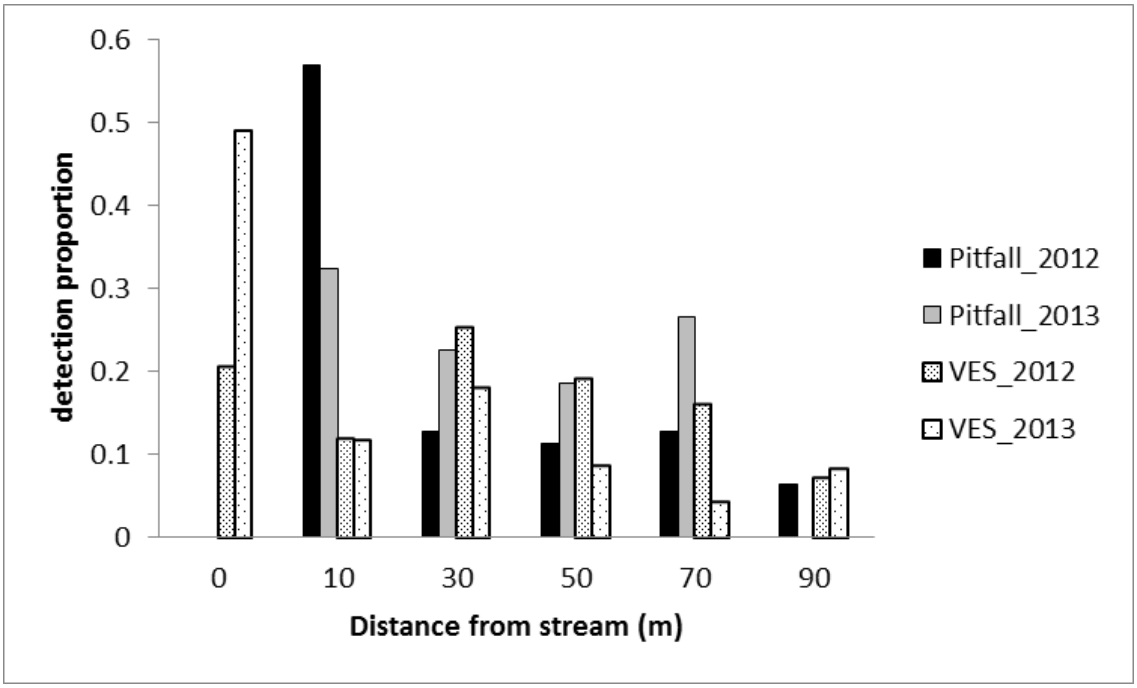

Figure 3-7 – Proportions of total detections by distance from streams. Proportions are corrected for effort at different distances.

Efficiency

After controlling for sampling effort, detection efficiency (detections per person-hour) differed between methods through the sample season (Figure 3-8). Although detections from pitfall sampling were similar across months, VES detections were relatively low in July, August and October.

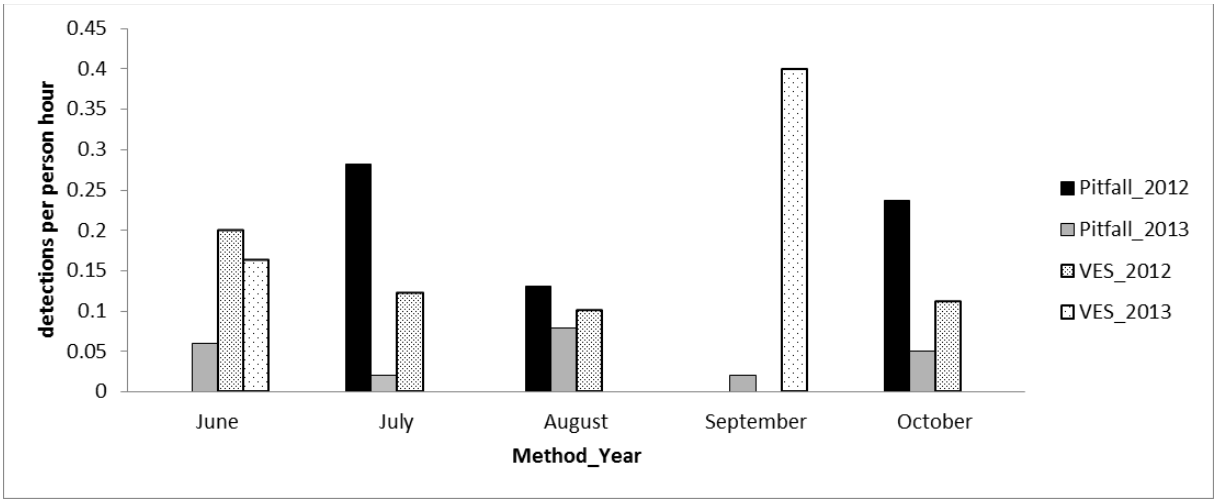

Figure 3-8 – Detections per person-hour of sampling for each month by method and year. Zero values represent no sampling.

Logistic regression: VES model

The top ranked logistic regression model accounted for 57% of the AIC_c weight and the following three models accounted for 43% of the AIC_c weight (Table 3-2). These models included covariates for month (temporal variation), minimum humidity, temperature, days since last rain (climatic variation), distance to stream, canopy cover, elevation (spatial variation) and a covariate representing potential sampling bias associated with vegetation cover along a transect. The first and second ranked models differed only in the presence of a covariate representing days since last rain. Because of its high $AIC_c w$ and fewer variables within the model compared to other competing models, the top ranked model was selected as the most parsimonious. The top ranked model had relatively poor predictive accuracy (AUC = 0.671; SE = 0.025).

Table 3-2 – Top ranked logistic regression models explaining the detectability of tailed frogs across two watersheds in northwest BC using visual encounter surveys (VES). Models were ranked using Akaike's Information Criterion for small sample sizes (AIC_c).

Variables	Rank	K	AIC_c	ΔAIC_c	AIC_w
month + hum_min + temp_inst + temp2 + dfs +de_c + elevation + elevation2 + vis	1	9	701.3	0	0.566
month + hum_min + dslr + temp_inst + temp2 + dfs +de_c + elevation + elevation2 + vis	2	10	702.8	1.501	0.267
month +temp_max + hum_min + dslr + temp_inst + temp2 + dfs +de_c + cancov_c + elevation + elevation2 + vis + exper	3	13	703.9	2.438	0.167
month +temp_max + hum_min + dslr + temp_inst + temp2 + vis + exper	4	8	722.2	20.833	< 0.001

The majority of coefficients in the top ranked model were statistically significant ($p < 0.05$). Significant negative correlations occurred between detectability and distance from stream (dfs). Minimum humidity (hum_min) was positively correlated with the probability of detecting a frog using VES. The positive correlation between detection and temperature and elevation, and the negative correlations with their quadratic terms suggest nonlinear concave relationships. No significant relationship was found between detections and month of survey and distance to edge (de_c).

The top VES model contained similar covariates to those reported by McEwan (2014) for the corresponding pitfall data. The VES model, however, also contained covariates explaining temperature, distance to edge and visibility (only tested in VES analysis; Figure 3-9). Covariates that were consistent among methods were similar in sign and magnitude.

Discussion

The probability of detecting a frog with either VES or pitfall sampling is dependent on variation in temporal, site or habitat, climate and weather characteristics. Yet, the relative strength and inclusion of some of these variables differed between methods, as indicated by the logistic regression models. In addition to differences in explanatory variables, the summary statistics indicated that demographic parameters influenced detection rates.

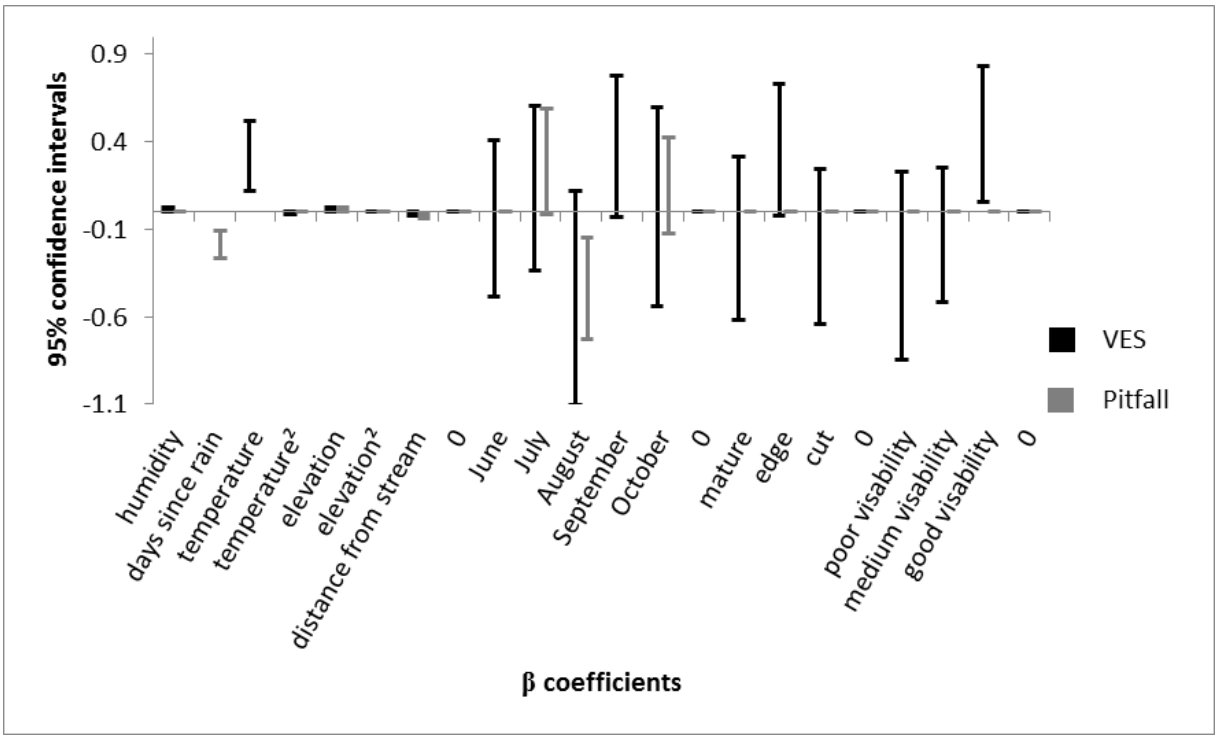

Figure 3-9 – β-coefficients (95 % confidence intervals) for the best VES and pitfall model (McEwan 2014) explaining detectability of tailed frogs in two watersheds in northwest BC.

Pitfall sampling is a passive technique that captures frogs as they move across the landscape, over a 24-h period. Because of this, frogs that are captured can be responding to a suite of conditions that are favourable for activity. For example, during warm periods, activity may be restricted to the nighttime, a period of sampling specific to pitfall traps. Conversely, VES is an active technique, which captures frogs (sedentary or moving) over a period of one h during the day. Conditions over this short period are relatively static and may or may not be favourable to frog activity. For this reason, a successful VES requires conditions that encourage daytime frog activity at the time that the survey is conducted.

A large discrepancy exists in the capture rate for pitfall traps between 2012–2013. In an effort to reduce small mammal mortality, a larger diameter escape twine was installed in traps during the 2013 field season. Although small mammal mortalities were reduced, it is possible that frogs used that larger twine to escape potentially biasing the sample. Alternatively, annual variation in capture rate may be a function of differences in capture schedule that correlated with factors, such as weather or reproductive behavior, that were conducive to frog activity.

Efficiency

Detection efficiency (detection per person-h) varied greatly between method and year. Due to annual differences in the pitfall method (i.e., twine), the diversity of technicians conducting VES, and weather and climatic variation, the capture rates represented the possible range of results that can be expected from each method. We note, however, that the efficiency calculations did not account for the time required to survey and install pitfall traps and VES transects. Installation of transects for VES required relatively little effort (i.e., 10–20 person-h per site), whereas pitfall installation was labor intensive (i.e., 100–250 person-h per site). Although the time required for the installation of pitfall traps varied considerably depending on site conditions (e.g., soils, understory and terrain) and the capacity of the technicians.

Previous work has suggested that because of the cryptic nature of tailed frogs, the VES method is an ineffective survey method. Matsuda (2001), for example, reported extremely poor capture rates. This study, however, resulted in a much greater capture rate with several factors potentially accounting for those differences. First, Matsuda (2001) studied a population of tailed frogs in the Chilliwack Valley, BC. The Terrace region is characteristically wetter and cooler throughout the summer months. Activity of tailed frogs, which is highly dependent on humidity and temperature (Bury and Corn 1988; Gomez and Anthony 1996; Wahbe et al. 2000, McEwan 2014), may have been reduced during the survey periods in the Chilliwack Valley to such a degree as to render them almost undetectable via VES. Secondly, the understory was removed from Matsuda's (2001) transects to improve visibility. It is possible that so much of the understory was removed, which is correlated with habitat use, that frogs selected against the cleared VES transects. Thirdly, although search intensity was not quantified by Matsuda (2001), the effort may have been less than was applied in this study. For example, Matsuda's (2001) survey method may not have required technicians to investigate coarse woody debris and root hollows as we did in this study.

Similarities in VES and pitfall sampling results

The top logistic regression models for pitfall sampling and VES share a number of similarities that are indicative of tailed frog biology. Coefficients for elevation in both models are suggestive of an optimal elevation at which conditions (temperature and snow free period)

are most ideal for tailed frogs. This may influence abundance or the activity of tailed frogs, relative to the survey periods. An optimal elevation and climate window has been observed in a number of other cold adapted amphibians (McCaffery and Maxell 2010). Season is also correlated with frog detections in both VES and pitfall sampling. This corresponds to seasonal movement patterns, associated with reproductive behaviours, and seasonal climate variation that affect frog activity and subsequently detectability.

Differences in VES and pitfall sampling results

Although measures of humidity are significant in the top models, minimum daily humidity and days since last rain capture different aspects of humidity and subsequently are interpreted differently in respect to frog biology. Tailed frogs often modify their behavior in response to humidity and temperature (Welsh and Reynolds 1986; Wahbe 1996). This includes closer associations to climate refugia and reduced daytime activity. Minimum daily humidity generally occurs in the daytime. As pitfall sampling takes place over a 24-h period, it is more likely responsive to long term variation in humidity that may be better represented by days since last rain. Alternatively, VES occurs during daylight hours when minimum daily humidity is more likely to occur. For this reason, VES detections are highly dependent on the conditions at the time of the surveys and not necessarily 24-h or long-term trends.

Temperature was only found to be significant in the top VES model. As with humidity, this is likely because pitfall sampling takes place over a 24-h period, allowing for detections during cooler, more humid, times of the day. Maximum temperatures are realized in the daytime when VES are conducted. The data suggest that behavioral adaptations to weather variation, such as decreased daytime activity, result in a reduced detection probability with VES sampling relative to pitfall sampling. As with elevation, the inclusion of the non-linear temperature term indicates a temperature optimum at which detections are most likely.

The distance to edge, and resulting canopy cover, of individual transects and pitfall arrays were only present in the top VES model. Tailed frogs are known to associate with mature and old growth forests (Metter 1964). But, the only significantly positive coefficient of transect treatment type was edge (<10 m from hard edge). This result is likely due to few sites in the study (resulting in low power) and the coarse categorization of forest type. Kleanza-1, for

example, the highest elevation site and also a mature site, had the least number of frog detections throughout the study. This may be due to watershed characteristics rather than stand type. Kleanza-1 had a very short snow-free period and the natal creek itself was relatively small, filling with fine sediments and organic debris, all of which limit frog abundance (Dupuis and Stevenson 1999; McEwan 2014). In contrast, Kleanza-3 was a low-elevation site in later stages of regeneration that was classified as clear cut; despite the open canopy conditions there were a large number of frog detections at that site. A better metric of forest type and additional watershed characteristics could be used in the future to understand the distribution and abundance of tailed frogs relative to forest harvesting and resulting regeneration.

As a passive technique, pitfall sampling requires a frog to move to the location of detection. This may bias results toward portions of the population that must move to satisfy life-history requirements. For example, females move during early summer to oviposition, metamorphs disperse during the fall, and adult frogs congregating during September and October to breed. As an active technique, VES may better sample the entire population, with respect to population structure.

Although seasonality (month variable) was present in both top models, frog detections were not significantly correlated to the same month in both VES and pitfall models. The significance of the 'July' variable in the top pitfall model and not the top VES model suggests that VES were not responsive to movements associated with ovipositioning, an important reproductive behaviour that occurs during this month (Todd et al. 2013; McEwan 2014). Similar to the model results, a higher proportion of females were captured in July with pitfall sampling, but not VES. VES, however, did locate a relatively high proportion of newly metamorphosed frogs in the months of September and October. This suggests that VES is responsive to the dispersal of metamorphs.

The likely differences in survey results between July and September and October are likely explained by differences in temperature and humidity, which influences the activity of frogs (Welsh and Reynolds 1986; Wahbe 1996). In July, when temperatures are high and humidity low, tailed frogs may become less active during the day and are thus not detected by VES. In September and October, when temperatures are lower and humidity is higher, tailed

frogs may become more active throughout the day. The timing of these movement events differ across study sites and likely differ even further across the tailed frog's range. Prior knowledge of site weather as well as snow-free period would allow for strategic scheduling that may improve results of either pitfall sampling or VES.

VES biases

VES transects varied considerably in the amount of forest floor that could be observed. Some sites had dense understory whereas in others the entire forest floor could be viewed easily from a standing position. The highest visibility categorical variable (i.e., 75–100%) was positively correlated with frog detections. Visibility along a transect was often closely related to the site treatment. Mature stands generally were more open and more easily surveyed whereas clear-cut or regenerating stands were often crowded with understory growth that made surveying more difficult.

In addition to visibility, we tested observer (technician) experience as a potential factor in detecting frogs. The variable was not included in the most parsimonious model, but did appear in the third and fourth ranked models. Observer experience was quantified as the number of days that a technician had spent conducting VES. This metric, however, may be a poor representation of an observer's ability to detect a frog. Observers varied considerably in their fitness, outdoor aptitude and general enthusiasm (B. Millard-Martin, *pers. obs.*). These factors are not expressed in the 'experience' variable, but likely contribute to the effectiveness of the observer in detecting tailed frogs.

Conclusions

Results from this study suggest that VES can detect frogs with a similar or even greater efficiency than pitfall sampling. This is especially the case for short-term studies (e.g., one year of sampling) due to the high cost of installing pitfall arrays. VES may be more applicable to short-term studies, where there is insufficient time and resources to install pitfall traps, or where a large area or many sites are surveyed.

Although detections of frogs are influenced by similar factors, regardless of technique, VES requires a stricter set of conditions to be effective. VES can be informative about

movement patterns, but not to the degree that pitfall sampling achieves. Furthermore, to be effective VES requires cool and wet survey conditions. Thus, the technique may be inefficient in certain regions or periods of the year with a warm and dry climate. This may include the southern and interior portions of the tailed frog's range. When compared to pitfall trapping, VES may provide a better, less biased, sample of the population because it targets both moving and sedentary frogs. However, for VES to be unbiased, it is important to account for visibility within transects and the aptitude of individual technicians. Controlling for these factors is difficult and could be improved upon in future studies.

Acknowledgements

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3.4. Environmental DNA: A detection method for coastal tailed frog in the Kitimat River watershed

Angela Grob, Anne-Marie Flores, Chris Johnson, Brent Murray

Introduction

Ecosystem function is often evaluated using bio-indicator species that reflect current ecosystem states (Hodkinson and Jackson 2005; Horn et al. 2009). Typically these species occupy a highly specialized niche and are reliant on stable environmental conditions; therefore, changes in distribution, abundance, ecology, or behaviour may signify that the ecosystem is experiencing a source of stress (Wahbe et al. 2004; Hodkinson and Jackson 2005; Horn et al. 2009). In BC's northwest coastal rainforest, the coastal tailed frog (*Ascaphus truei*) is an important bio-indicator species that reflects the health of freshwater ecosystems (Dupuis et al. 2000; Wahbe et al. 2004; Horn et al. 2009).

Tailed frogs are the only true stream-breeding anurans that are endemic to the Pacific Northwest (Dupuis et al. 2000; Wahbe et al. 2004). Neilson et al. (2001) describe the tailed frogs in the Pacific Northwest as two geographically distinct species; one inhabiting the Coastal Mountain range (Coastal tailed frog: *Ascaphus truei*) while the other inhabits the Rocky Mountain range (Rocky Mountain tailed frog: *Ascaphus montanus*). Coastal tailed frogs are listed as a species of *Special Concern* on Schedule 1 of the Species at Risk Act (SARA) registry, and are *Blue-listed* on the provincial list of species at risk (COSEWIC 2011).

The distribution of the coastal tailed frog throughout the Coast Mountain range is closely associated with the Coastal Western Hemlock biogeoclimatic zone (Dupuis et al. 2000). The northern extent of their range is in the Terrace area, south of the Nass Valley. They are found between the Pacific Coast and the Rocky Mountains in BC and their range extends southward into northern California (Daugherty and Sheldon 1982; Wahbe et al. 2004). Coastal tailed frogs inhabit perennial streams with clear, fast-flowing water and cold temperatures (Depuis et al. 2000; Neilson et al. 2001). They prefer large cobble to finer substrates that provide interstitial spaces for foraging and shelter (Dupuis et al. 2000; Horn et al. 2009).

Medium-gradient streams provide stable substrate and fast-flowing water that does not allow the accumulation of sediment (Dupuis et al. 2000).

Coastal tailed frogs have a low tolerance for desiccation — their habitats must provide sufficient moisture and appropriate temperatures (Wahbe et al. 2004; Horn et al. 2009). They require foliage cover in summer and adequate snow cover in winter to prevent streams from freezing solid (Wahbe et al. 2004). These physiological constraints make this species sensitive to environmental changes and limit their dispersal and colonization abilities, qualities that are characteristic of bio-indicator species (Hodkinson and Jackson 2005).

Compared to other amphibian species, coastal tailed frogs have a relatively long life span living as long as 15–20 years (Daugherty and Sheldon 1982). Tadpoles are dependent upon streams between 3–5 years before they metamorphose into adults and leave the stream environment (Daugherty and Sheldon 1982; Dupuis et al. 2000). Sexual maturity is reached at 7 years of age in males and not until 8 or 9 years in females (Daugherty and Sheldon 1982; Dupuis et al. 2000). Adult frogs lead a more terrestrial lifestyle, dispersing from their natal streams for terrestrial foraging, but return to the streams to mate in the fall (Daugherty and Sheldon 1982). Male frogs use a tail-like appendage for internal sperm transfer to the female (Neilson et al. 2001). Females store the sperm until the following June when internal fertilization occurs and the eggs are laid in strings on the underside of rocks (Daugherty and Sheldon 1982). After 6 weeks of development, the tadpoles emerge (Wahbe et al. 2004).

Manual detection of coastal tailed frogs is difficult because of their nocturnal lifestyle and cryptic habits. Traditional methods have relied on visual surveys, trapping larvae using electroshocking, or trapping adults with pitfall traps and drift fences (Wahbe et al. 2004; Quinn et al. 2007; Cossel et al. 2012; Pilliod et al. 2013). These methods, however, require expert detection of individuals, they are labour intensive, and they require time-consuming visual surveys and trap checking (Thomsen et al. 2012; Section 3.3). Lastly, these methods are occasionally invasive; handling or trapping can physically harm the frogs and their habitat, or disrupt their natural behaviours (Thomsen et al. 2012).

A new method of species detection, environmental DNA (eDNA) is becoming more widely implemented in species management, supplementing traditional detection techniques

(Ficelola et al. 2008). This method uses DNA collected from the environment, instead of the organism itself, to detect species presence (Ficetola et al. 2008; Thomsen et al. 2012; Pilliod et al. 2013). DNA can be shed from an organism in the form of mucous, skin, feces, gametes, or other sources, and can be collected from the ecosystem using an environmental sample such as water or soil (Goldberg et al. 2011). Environmental DNA sampled from water samples have been widely implemented because many difficult-to-detect species are found in aquatic habitats (Goldberg et al. 2011; Thomsen et al. 2012; Dejean et al. 2011). Unlike traditional methods, eDNA allows for rapid species detection based on non-invasive, standardized techniques that require fewer field resources (Ficetola et al. 2008; Thomsen et al. 2012).

Previously, eDNA has been successfully implemented for the detection of the invasive American bullfrog (*Rana catesbeiana*) in France, to monitor the extent of Asian carp (*Cyprinus carpio*) invasion into the Laurentian Great Lake system, and to determine the distribution of the Idaho giant salamander (*Dicamptodon aterrimus*), and the Rocky Mountain tailed frog, two elusive and hard to detect species (Goldberg et al. 2011; Jerde et al. 2011; Dejean et al. 2012). More recently, eDNA techniques have been used to estimate biodiversity and predict the abundance of species present in specific ecosystems (Lodge et al. 2012; Hajibabaei et al. 2012; Thomsen et al. 2012). Environmental DNA has proven to be an invaluable tool for detecting species present in freshwater ecosystems where other techniques would have been too costly or unfeasible (Goldberg et al. 2011; Lodge et al. 2012; Pilliod et al. 2013).

The location of this study corresponds to the proposed Pacific Trail Pipeline RoW. Industrial development projects disturb natural habitats of tailed frogs, both the larvae by introducing particulate matter into streams and adults by clearing forested areas near streams (Dupuis et al. 2000; Wahbe et al. 2004; Horn et al. 2009). Therefore, it is important to obtain an overview of the population distribution in the affected systems as a standard to compare post project restoration effectiveness. Our goal was to evaluate eDNA as a method for detecting tailed frog and to further delineate the distribution of the species. We tested the effects of water sample volume on total DNA recovered to refine eDNA methods for freshwater streams. Results provided a broad overview of the species' distribution within the Kitimat River watershed and demonstrated the effectiveness of eDNA as a detection method.

Methods

Study sites

Fifteen study sites, located in the Kitimat River watershed, were chosen for sampling. These streams were all within the tailed frog range and chosen based on their location bisecting the Pacific Trail Pipeline route (Chevron Canada; Dupuis et al. 2000). Two negative control streams were located in interior BC, 50 km north of Prince George, BC. These two streams were located 400 km east of the known tailed frog distribution and used as negative controls to test the specificity of tailed frog primers using water containing other amphibian species. An additional stream located in the Kitimat River watershed with known tailed frog occupancy was chosen to sample more intensively. This site was used as a volume control, to determine the effect of different water volumes on eDNA quantity and quality.

Field data collection

At the 15 study sites and the two negative control sites in interior BC, two replicates of 2 L water samples were collected in four 1-L sterile Nalgene bottles. Each 2-L replicate water sample was filtered onto one 47 mm diameter, 0.45 µm pore-size, cellulose nitrate filters (Cole-Palmer, Montreal, QC, Catalog No. A045A047A), using a vacuum-pump system. If the stream water contained high amounts of particulate matter and it took longer than 30 min to filter 250 mL, filters were changed; and, therefore some replicates had more than one associated filter. The volume control site was sampled at three sites each 200 m apart. Water samples with volumes of 1 L, 2 L and 4 L were collected at each of the three sites and 1 L of water filtered onto each filter (Table 3-3). The filters were combined during a subsequent step to ensure appropriate volume per volume control sample.

To control for contamination introduced during filtering, 2 L of distilled water was filtered and included as a blank control after each eDNA filtering day. Each filter paper was folded and stored in 95 % ethanol at -20 °C until extraction, which occurred 3–6 months later.

Table 3-3 – Volume control (VC) samples taken from three sites, located 200m apart from a stream with known tailed frog occupancy. 1 L, 2 L, and 4 L samples were taken at each site; these were quantified for tailed frog eDNA using quantitative RT-PCR.

Sample ID	UTM (09 U)	Mean DNA (pg)	Standard deviation
(VC 1) 1 L	520624 6000175	9.02	± 2.3
(VC 1) 2 L	520624 6000176	5.28	± 1.633
(VC 1) 4 L	520624 6000177	15.72	± 7.254
(VC 2) 1 L	520507 6000181	6.91	± 2.656
(VC 2) 2 L	520507 6000182	9.14	± 4.061
(VC 2) 4 L	520507 6000183	12.96	± 2.492
(VC 3) 1 L	520317 6000197	4.03	± 1.427
(VC 3) 2 L	520317 6000198	3.47	± 2.277
(VC 3) 4 L	520317 6000199	12.42	± 2.663

DNA extraction and amplification

DNA was extracted from filters using spin columns and reagents from the DNeasy Blood and Tissue Kit: spin-column protocol (Qiagen, Toronto, ON, Catalog No. 69506), using a modified spin-column protocol. Each filter was removed from ethanol and cut into 1-mm slices that were placed in a 2-mL microcentrifuge tube containing two 5/32 in steel grinding beads (Ops Diagnostics, Lebanon, NJ, Catalog No. GBSS 156-5000-01) and left to air-dry overnight. The filters were then ground using the Genogrinder 2000 (BT&C Inc., Burlington, ON) at 1500 oscillations × min^{-1} for 90 sec. 870 µL of Buffer ATL was added and the sample was incubated for 30 min at 56 °C. The grinding and incubation steps were then repeated. 30 µL proteinase K was added, and the samples were incubated at 56 °C for 2 h. After the incubation period, the lysis solution was transferred into the Qiagen spin column adding equal amounts of 95 % ethanol and Buffer AL. For the volume control samples, lysis solution from multiple filters was combined to obtain eDNA from 1 L, 2 L, and 4 L sample volumes. The subsequent washing steps using Buffer AW1 and AW2 followed the protocol in the DNeasy Blood and Tissue Kit spin-column procedures. The DNA was eluted twice using two volumes of 50 µL Buffer AE with 5 min incubation times. Negative extraction controls were included for each set of extractions.

Amplification of eDNA was performed using the real-time TaqMan approach on a 7300 real-time PCR system (Applied Biosystems Burlington, ON). Detection primers and probes were previously designed by Flores et al. (2013), specifically targeting a 90-bp region of the coastal

tailed frog mitochondrial *cytochrome b* gene (Table 3-4). The TaqMan probe uses a 5' VIC reporter dye and 3' TAMRA non-fluorescent quencher dye.

Table 3-4 – Primer sequences specific for the cytochrome b gene in the coastal tailed frog (Flores et al. 2013).

Primer name	Primer sequence	Type
ASTR-CYTb-F	CGGAAACGTACTAGTCCAATGGA	Detection
ASTR-CYTb-R	GAAAGCGAAAAATCGTGTTAACG	Detection
ASTR-CYTb	TGCTGGCAAAGGCTGGATTCTATTACAAAA	TAMRA probe

Each 25-µL TaqMan reaction contained 12.5 µL of TaqMan® Environmental Master Mix 2.0 (ThermoFisher Scientific), 1 µL of 10-µM forward and reverse detection primers, 1 µL of 2.5 µM TAMRA probe, 7.5 µL of nuclease free water, and 2 µL of DNA template. PCR cycle conditions included 1 cycle of 5 min at 50 °C, 1 cycle of 10 in at 95 °C, 55 cycles of 30 sec at 95 °C and 1 in at 55 °C. Each sample was conducted in triplicate, including a no-template PCR control.

The volume controls and external primer control samples were quantified using approaches outlined by Ginzinger (2002). A dilution series of 2 pg to 2×10^4 pg of total DNA extracted from the tail of a tailed frog larva were included as standards on the plates containing these samples. The amount (picograms) of DNA present in the standards was used to estimate the relative amount of mitochondrial DNA present in these unknown samples. The DNA quantity was averaged between the triplicates of each sample, with non-detected samples being included as 0.00 pg in the average.

Data Analyses

For each of the streams, a relative eDNA score was given based on the detection of DNA. Where both replicates were positive for tailed frog DNA, the sample was scored of '2', where one replicate failed, the sample was scored '1'. If no eDNA was detected in either replicate, a score of 0 was applied.

Volume control data were analyzed using the Stata statistical software (StataCorp, Texas, USA). Shapiro-Wilk tests were used to test the normality of the dataset. Significant

differences between average eDNA quantity for each volume of water filtered (1L, 2L, and 4L) was tested using analysis of variance (ANOVA). Three technical replicates of DNA amplification were conducted for each filter volume at each of three sites. The technical replicates were averaged to produce the DNA estimate at each site. These estimates were then used to conduct the statistical tests to assess the effect of volume on DNA quatity amplified from filter DNA.

Results

None of the negative field blanks, negative extraction controls, and no-template PCR controls showed any DNA signal indicating the presence of tailed frog. The negative control streams of Interior BC also showed no DNA signal.

Of the 15 study sites, tailed frog DNA was detected at least once in 13 of the streams. Of these 13 streams, six had DNA detected in both 2-L samples, six had DNA detected in only one 2-L sample, and one detected DNA in one 2-L sample but had missing data for the sec 2-L sample. Figure 3-10 details the locations of the study sites as well as the relative strength of DNA signal from environmental DNA.

The volume control study showed that the amount of water filtered affected the amount of eDNA recovered (Figure 3-11). The quantity of eDNA collected from 4 L of water was significantly higher when compared to 1 L and 2 L ($F_{2,6} = 54.99$, $p = 0.015$), however the confidence intervals overlapped showing no specific differences among the volumes filtered.

Discussion

Tailed frogs were found in 13 of the 15 study sites (Figure 3-10). All of the study streams bisect the RoW. Increased sedimentation of streams due to increased traffic, and clearing of forests causing a loss of habitat connectivity and changes in the humidity and temperature of tailed frog habitat pose the greatest impacts to the species (Dupuis et al. 2000; Wahbe et al. 2004; Horn et al. 2009). Therefore, it is important to collect preliminary tailed frog distribution data to allow the effectiveness of post-development restorative efforts to be measured. We have applied eDNA sampling to produce a distribution map of tailed frog presence along the pipeline RoW.

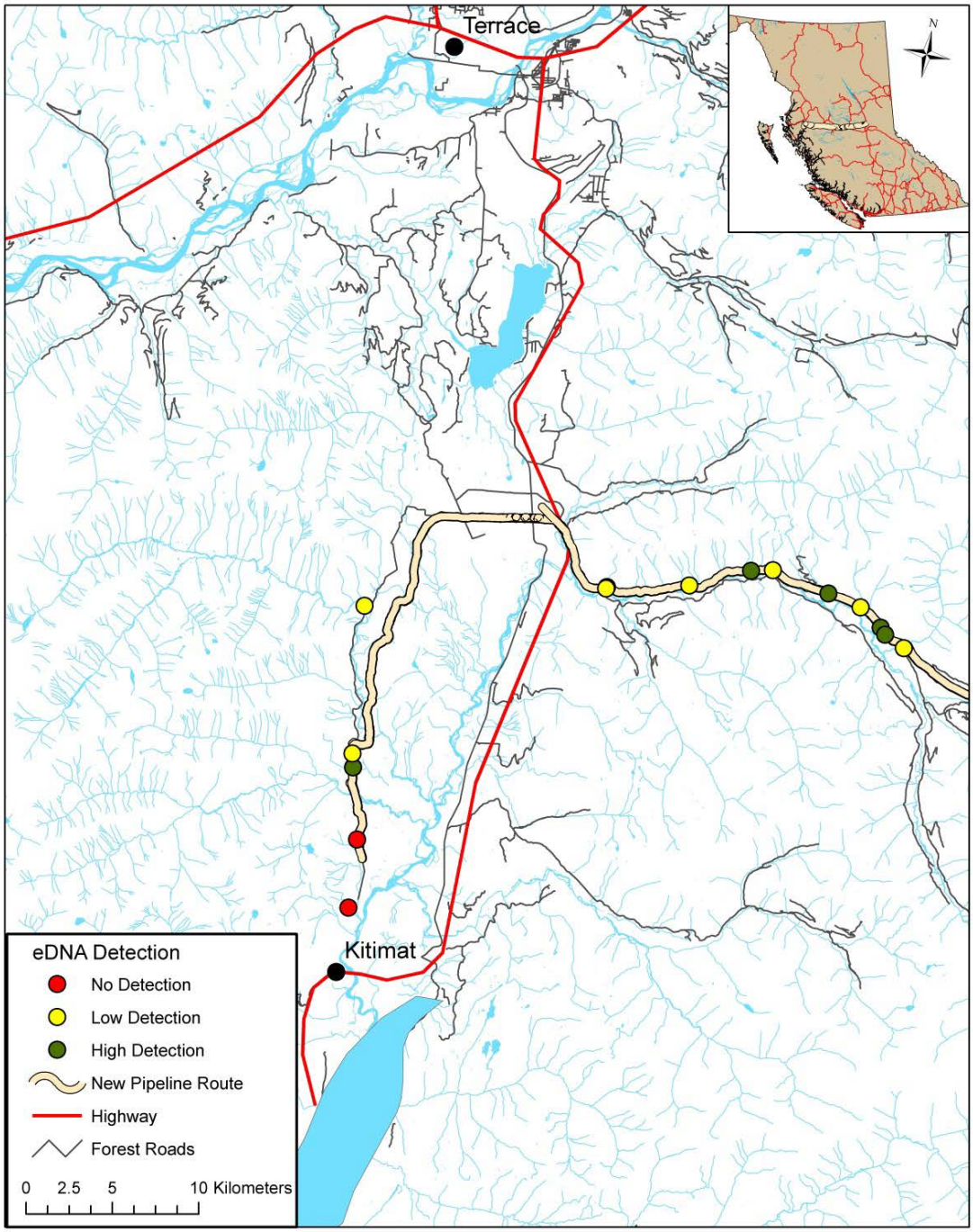

Figure 3-10 – Environmental DNA sampling locations to identify the presence of coastal tailed frog. Red dots indicate no detection of tailed frog DNA in the filtered water sample, yellow dots indicate low detection, and green dots indicated high detection levels.

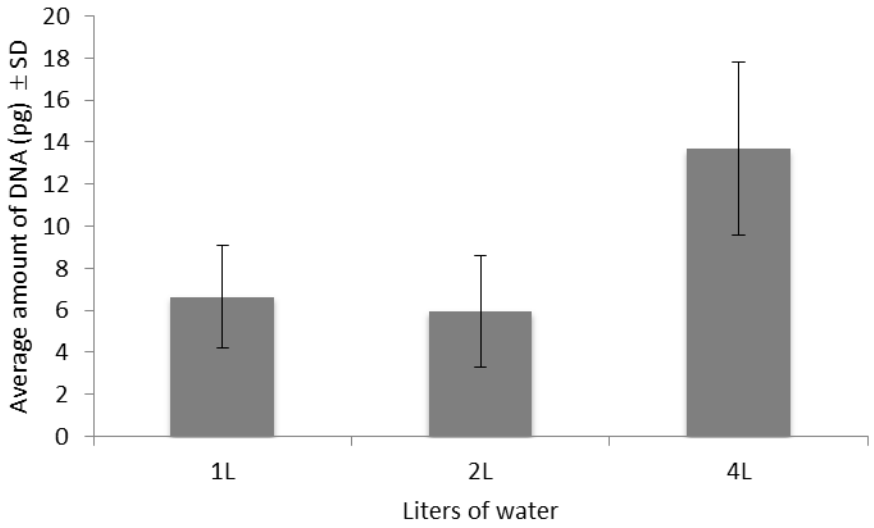

Figure 3-11 – Average eDNA recovered from 1 L, 2 L, and 4 L water samples (± 1SD) at three sites 200 m apart from a stream known to support coastal tailed frog populations.

The method was effective for the detection of tailed frog, but may be improved in future studies. Two negative control streams, located approximately 400 km east of the nearest known tailed frog population, were used to test the specificity of the primers developed by Flores et al. (2012) against other amphibian species that can be found in interior streams. No eDNA was amplified from water samples collected at the control streams. This provides some support for the specificity of these primers for use in studies aimed at detecting the presence of coastal tailed frogs. To unequivocally demonstrate the primer specificity, however, genetic samples from all known amphibian species in the Kitimat River watershed should be assessed for amplification.

Some of the water sample replicates took longer to filter as indicated by the streams with multiple filters required per replicate. These replicates were assumed to contain higher amounts of particulate matter impeding the filtering process. Dupuis et al. (2000) found that particulate matter has adverse effects on tailed frog larvae that could explain the low eDNA scores associated with the streams that required more than one filter per replicate. Moreover, the replicates with multiple filters were found closer to the Kitimat River estuary, which is located at a lower elevation than other streams we sampled. Typically, breeding adults are

found in higher elevation habitats (see Section 3.3) resulting in lower eDNA scores for these sites (Dupuis et al. 2000).

A common eDNA collection protocol in natural environments used by Jerde et al. (2011), Flores et al. (2013), and Takahara et al. (2012) involves extracting eDNA from 2-L water samples collected at stream sites. In our study, there was no significant difference between filtering 1 L or 2 L, however, doubling the volume to 4 L significantly increased the amount of eDNA recovered. This suggests that a standard sampling protocol of 1 L is just as efficient as collecting 2 L of water. The site, from which these data were collected, was located in an area with high tailed frog abundance (B. Murray; unpublished data), which may not necessarily be broadly applicable, although a 2-L sampling volume effectively detected tailed frog in the majority of the streams we tested. If a negative result occurred in a stream with good tailed frog habitat, re-sampling with larger water volumes is recommended to enhance the probability of species detection.

Although eDNA has been widely implemented for species detection, the effects of animal behaviour and species abundance on eDNA results have not been studied extensively. Thomsen et al. (2012) found that aquatic species such as fish and amphibians showed a much higher detection rate compared to semi-aquatic species such as the otters or dragonflies and concluded that animal behaviour affects detectability using water samples. Other effects may be due to differential upstream conditions, patchiness and longevity of DNA in water. Spatial and temporal refinement of sampling methodology may improve detection of species present in low densities.

We have shown a relationship between quantitative RT-PCR values and template eDNA concentration; these methods show promise as a technique for estimating species abundance estimation technique. Previous research in this area has been inconclusive. Thomsen et al. (2012) used experimental manipulation of tadpole densities to show that positive correlations existed between species density and DNA concentration. Other studies conducted in the natural environment found varying results: Pilliod et al. (2013) found a positive correlation between the eDNA and the density of Rocky Mountain tailed frogs and Idaho giant salamander, Takahara et al. (2012) also found a positive correlation between eDNA and carp present in the

natural habitat, while Goldberg et al. (2011) did not find any evidence that eDNA amounts was related to Rocky Mountain tailed frog densities. If eDNA could be reliably applied to species quantification, in addition to detection, it would provide an efficient alternative to traditional species enumeration methods.

In conclusion, eDNA is a valuable species detection tool which can be implemented in future studies investigating coastal tailed frog presence within freshwater ecosystems. Data gathered using eDNA can be applied to the conservation of species, populations and ecosystem functions. In the context of this study, eDNA sampling was successfully implemented as a tool to further define tailed frog distribution in the Kitimat River watershed along the pipeline route and can provide baseline location data to guide post-industrial development restoration efforts in this area.

3.5. Tailed frog distribution and population connectivity

Cherie Mosher, Chris Johnson, Brent Murray

Introduction

Time and space are considered the axes of phylogeography, the field of study relating differences in DNA sequences to the processes governing the geographical distribution of a species or populations (Hedrick 2011). The amount of divergence between populations is a balance between the homogenization of gene flow between populations and the diversification of drift (Allendorf and Luikart 2007). Thus, the assumption is that distant populations have limited gene flow and should accumulate differences due to genetic drift as well as mutation and selection. Migration among populations maintains genetic diversity within a species through gene flow, and there is clear evidence from a variety of species that the quality of the interstitial environment (e.g., cover, humidity, ruggedness, temperature) influences the degree of gene flow by migration (Rothermel and Semlitsch 2002). Recent advances in population genetics (i.e., microsatellite analysis and genotype-by-sequencing) allow the study of regional genetic diversity, genetic differentiations among sub-regions, and the estimation of the rates and patterns of gene flow (Yamene and Nashida 2010). Patterns of gene flow can be compared to landscape features, with enough variation and at the right scale, to determine how they influence migration between populations. These approaches have often been applied in population studies of frogs, as anuran dispersal is historically difficult to study due to their cryptic nature and potentially low dispersal rates (Murphy et al. 2010; Moore et al. 2011; Zancolli et al. 2014; Watts et al. 2015).

Ascaphus truei, belonging to the Family Ascaphidae, is an ancient lineage with *Ascaphus* as the only Genus in the Family. Their unique lineage is reflected in their distinctive morphology and life histories (Brown 1975). They have a slow development, especially for a frog species with a small body length in adults, ranging from 2.2–5.1 cm. Individuals can remain in the larval phase up to four years and may not reach breeding maturity until eight years of age. Longer periods until breeding maturity may occur more often at the northern extent of their range.

Coastal tailed frogs are also one of the longest-lived of all North American frogs (Nussbaum et al. 1983), and are adapted for life in cold, clear mountain streams during the larval phase. They spend the majority of their juvenile and adult lives in terrestrial landscapes. Juveniles and adults feed in riparian zones but are known to disperse more than 100 m from streams when conditions are moist (Wahbe et al. 2004). Adults overwinter on land as well as in water (Bull and Carter 1996).

Many authors have suggested that *A. truei* needs an abundance of cool, moist microhabitats for survival (Hayes and Quinn 2015) and dispersal. Forested riparian areas were positively correlated with both tadpoles and post-metamorphic frogs along the Oregon Coast Range (Stoddard and Hayes 2005). Larval *A. truei* were associated with moderate-sized streams and post-metamorphic *A. truei* occurrence increased with an increase in elevation. Post-metamorphic frogs exhibited a positive relationship to gradient as well as elevation (Hayes and Quinn 2015). Matsuda and Richardson (2005) found that juveniles were more common in clear-cut than in old-growth forests. Wahbe et al. (2004) found juveniles to be 2.9 times more common than adults in clear-cuts, and adults to be 2.3 times more abundant than juveniles in old growth forests. This evidence suggests coastal tailed frogs use a variety of terrestrial landscapes that varies depending on life stage. Dispersal also may vary with life stage. Matsuda and Richardson (2005) also found that pre-reproductive *A. truei* showed the widest dispersal in a study on the south coast of BC, and Bury and Corn (1988) captured recently metamorphosed frogs at greater than 75 meters from their natal stream reaches during the fall in Washington.

During the larval stage, *A. truei* tadpoles have a strong association with coarse substrates and steep gradients within streams (Adams and Bury 2002). Wahbe and Bunnell (2001) found that tadpoles follow a ‘colonization cycle’ described by Muller (1974). According to Muller, stream populations are maintained through the interplay between downstream drift of larval frogs and upstream dispersal of post-metamorphic individuals. Wahbe and Bunnell (2001) identified significant downstream movement by larval life stages. The influence of larval movement downstream on the genetic structure, or even on the definition and boundaries of a particular population, of this species is still unclear.

Spear and Storfer (2008) found that gene flow was extensive through over-land forested habitat in a landscape population genetics study conducted in Olympic National Park (Washington). They found that land cover and topography strongly influence population connectivity in their study region. Genetic clustering was not connected by rivers or streams, and they found little influence of stream connectivity. Spear et al. (2012) used regression analysis with two different paths of gene flow to model landscape influence on tailed frog recolonization after the Mt. St. Helens eruption in 2008. Their landscape genetics models suggest that gene flow through an unmanaged portion of the blast area was influenced by distance between sites and the frost-free period. In contrast, gene flow pathways within the blast area where salvage logging and replanting occurred post-eruption were strongly limited by the physiologically important variables of heat load and precipitation.

We aimed to use microsatellite markers developed by Spear et al. (2008) in a landscape population genetics study at the northern extent of *A. truei's* range (Figure 3-12). Our objective for this study was to compare landscape-level genetic structure to elevation, gradient, wet site type, land cover, distance between streams, and across watersheds. The goal was to determine the landscape features that facilitate tailed frog dispersal and to identify potential landscape barriers to dispersal at the northern extent of *A. truei's* range. Our initial results, using 53 individuals from the streams sampled in 2014, showed a sharp reduction in genetic variability when compared to southern population genetics studies (Table 3-5), making it impossible to conduct a landscape genetics study using these microsatellite markers for *A. truei* at the northern extent of its range. Taking the preliminary genetic data in account, we expanded the scope of the study in order to identify the geographic extent of this reduction in genetic variability. Understanding the patterns and extent of genetic divergence between populations along a range is crucial for protecting species and developing effective management plans (Allendorf and Luikart 2007). As a result, we sampled two additional BC regions with tailed frog populations, and we requested southern population tissue samples from Dr. Stephen Spears that were collected from two regions of Washington (Figure 3-12). Our revised objective was to explore the spatial genetic variation of microsatellite markers among *A. truei* populations collected from five regions throughout the Pacific Northwest.

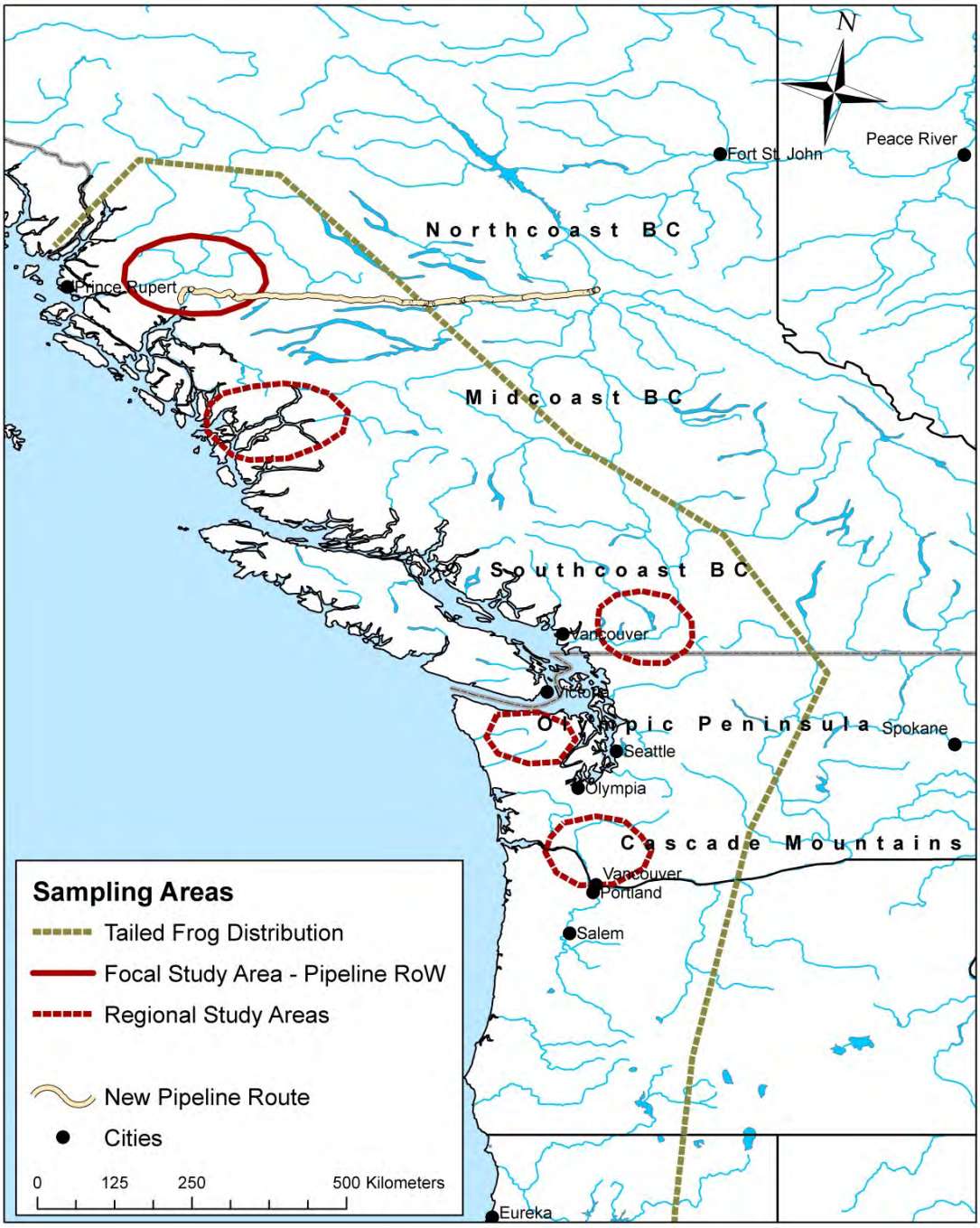

Figure 3-12 – Distribution range of *Ascaphus truei*. The red circles show the regional study areas in British Columbia and Washington.

Table 3-5 – Initial results for microsatellite fragment analysis. Samples were taken from streams at the northern extent of its range and show reduced variability and heterozygosity. Spears et al. (2008) samples are from the Olympic Peninsula and Washington are included for comparison.

Microsatellite locus	Northcoast # alleles	Northcoast observed heterozygosity	Spears et al. (2008) # alleles	Spears et al. (2008) heterozygosity
1	3	0.170	12	0.828
2	3	0.038	13	0.858
4	2	0.216	34	0.875
12	1	0.000	8	0.812
13	2	0.019	10	0.859
15	4	0.377	16	0.906
17	1	0.000	22	0.922
24	3	0.094	17	0.891
26	5	0.774	24	0.828
31	3	0.076	16	0.859

Methods

Data collection

Sampling commenced throughout the area surrounding Terrace and Kitimat, BC, in the summer of 2014, and we defined this region as the 'northcoast' (NC). Streams were sampled based on the number of stream crossings, historical information on tailed frog presence, and their locations in relation to each other. Samples were taken from up to five locations along the stream (Figure 3-13). For each stream crossing (0 m site), a visual survey was conducted to determine the presence of tadpoles. If tadpoles were encountered at a site, we collected tail clips from up to four tadpoles. We then moved to locations ~100 and 200 meters upstream and downstream, sampling up to four tadpoles at each location or a maximum search time of 60 min (Figure 3-14). At each sampled site, we moved upstream from the start point, did visual sweeps and flipped over rocks while swiping nets to capture the tadpoles. The final goal for each stream was to obtain 20 tail clips. We marked the UTM on a handheld GPS and recorded it sequentially (i.e., NC01, NC02, NC03, etc.).

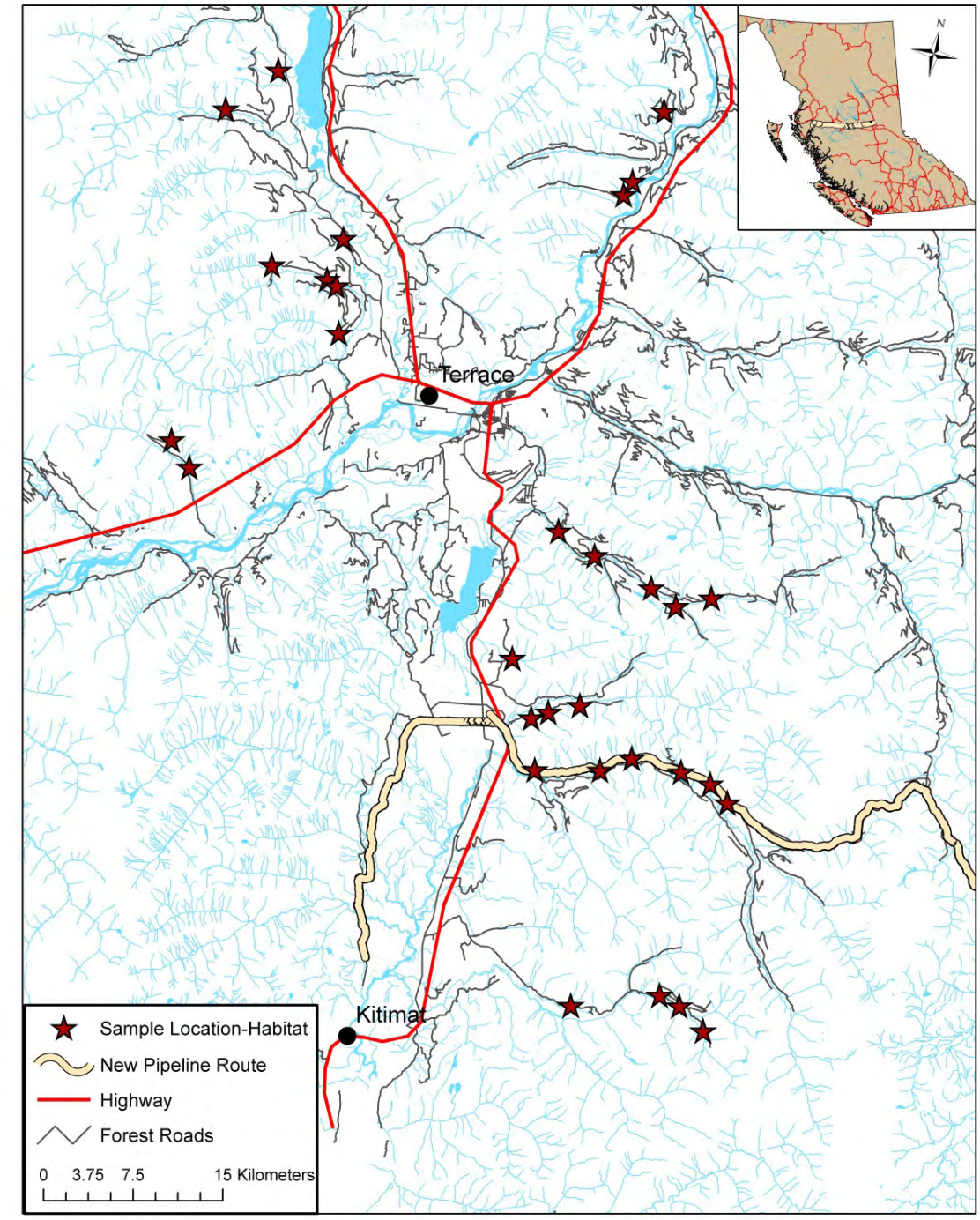

Figure 3-13 – Sampling locations for the north coast region around the proposed pipeline RoW.

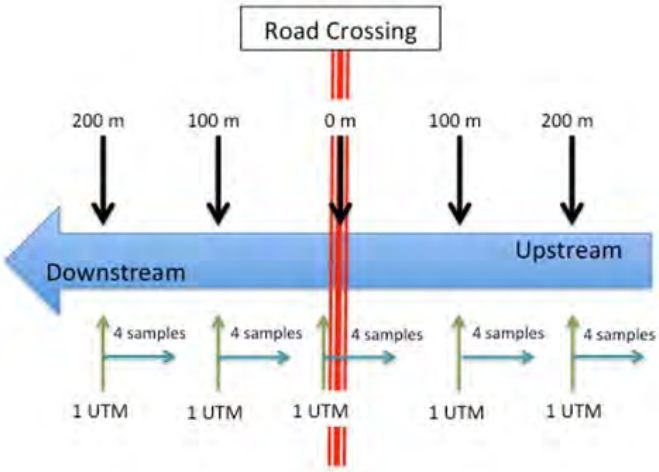

Figure 3-14 – Each stream was sampled first at the road crossing (shown in red) at '0 m', then at 100 and 200 m downstream, and 100 and 200 m upstream. UTM coordinates were taken at each sampled site.

Sampling in the 'midcoast' (MC) and 'southcoast' regions (SC) was less extensive as our phylogeographic analysis was conducted at a coarser scale for comparison. Twelve streams from the NC were randomly selected and four samples per stream were randomly selected. Our goal was to also obtain four samples from 12 streams in the MC and SC regions. Streams were selected based on accessibility and, when possible, streams in close proximity to each other were not sampled. We also received tailclips from five streams for the SC region. To date, we have 11 streams from SC.

Tail clips were lysed and purified using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Toronto, ON), and we amplified ten polymorphic microsatellite DNA markers (Spear et al. 2008). We included negative controls with each PCR run. Multilocus genotypes were generated using fragment analysis with the Applied Biosystems 3130XL and fragments were scored with GeneMapper 4.0 (Applied Biosystems, Burlington, ON).

Observed and expected heterozygosities were calculated using GenAlEx 6.5 in Excel (Peakall and Smouse 2012). Observed heterozygosity is the proportion of n samples that have

two different alleles at any given locus. Expected heterozygosity is the expected proportion based on random mating and the allele frequency at that locus. Wright's F-statistics were calculated using GenAlEx to determine the genetic differentiation within regions versus among regions.

Genetic distance-based analyses were conducted among regions. The genetic data was first converted into a pairwise individual-by-individual genetic distance matrix and an Analysis of Molecular Variance (AMOVA) was performed in GenAlEx. AMOVA is a method of estimating population differentiation directly from molecular data and can be used to test hypotheses. We applied the Discriminant Analysis of Principal Components (DAPC) using the 'adegenet' package for R software to describe genetic clusters in the data (Jombart 2008). Genetic clusters are constructed using linear combinations of alleles that have the largest between-group variance and the smallest within-group variance. DAPC also provides membership probabilities that indicate the 'tightness' of the genetic clusters. DAPC was run on the data in several different ways. Initially, DAPC was run with each region as a separate population (K = 5). We also ran it with each stream as a separate population (K = 58). We adjusted the number of eigenvalues several times to determine if the clusters changed.

F_{ST} refers to the mean reduction in heterozygosity, a form of variability in population genetics, of subpopulations (relative to the total population) due population structure (nonrandom mating) among subpopulations. It is a measure of the extent of genetic differentiation among subpopulations and can range from 0.0 (no differentiation) – 1.0 (complete differentiation — subpopulations).

We also used the Bayesian clustering package STRUCTURE 2.3.4 as an additional test for genetic clustering (Pritchard et al. 2000). We ran models assuming admixture, i.e., each individual draws some fraction of its genome from each of K populations. We ran the simulations with prior sampling location information to assist with clustering. Each of ten iterations was run for 10,000 cycles with a burn-in of 10,000 assuming K from 1–15 (where K is the number of genetic population clusters). STRUCTURE results were imported into the programs CLUMPAK and Structure Harvester to compute statistics, report results, and for plotting (Kopelman et al. 2015; Earl and VonHoldt 2002).

Results

The range of allelic richness varied greatly between regions (Table 3-6), generally decreasing as one moves north along the range (Figure 3-15). The mean number of alleles across all loci ranged from 22.0 (CM) to 2.9 (NC). OP had a mean allelic richness of 18, SC was 11.9, and MC was 5.6. The mean observed heterozygosity also decreased from south to north: 0.882 (OP), 0.877 (CM), 0.678 (SC), 0.363 (MC), and 0.176 (NC).

Table 3-6 – Allelic richness, observed heterozygosity, and expected heterozygosity for all loci separated by region. Included is the mean heterozygosity over loci within region (mean hetero.) and the number of samples per region (samp.)

	Northcoast BC			Midcoast BC			Southcoast BC			Cascade Mtns WA			Olympic Peninsula WA		
Microsatellite Locus	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Number of Alleles	Observed Heterozygosity	Expected Heterozygosity
1	4	0.146	0.138	4	0.354	0.367	7	0.875	0.776	24	0.938	0.944	12	0.833	0.836
2	2	0.042	0.041	4	0.333	0.352	9	0.771	0.743	34	0.896	0.905	21	0.958	0.926
4	4	0.438	0.400	4	0.479	0.576	13	0.833	0.881	22	0.896	0.928	33	0.917	0.962
12	1	0.000	0.000	2	0.021	0.021	2	0.208	0.353	3	0.271	0.448	8	0.646	0.751
13	1	0.000	0.000	3	0.042	0.041	5	0.128	0.279	13	0.667	0.856	8	0.708	0.829
15	3	0.271	0.354	7	0.729	0.709	8	0.813	0.832	21	0.979	0.935	15	0.896	0.889
17	1	0.000	0.000	1	0.000	0.000	9	0.313	0.317	27	0.917	0.945	22	0.917	0.914
24	2	0.042	0.041	4	0.313	0.293	14	0.750	0.839	22	0.917	0.929	16	0.854	0.913
26	8	0.617	0.684	22	0.896	0.872	40	0.875	0.963	38	0.938	0.959	34	0.854	0.948
31	3	0.104	0.100	5	0.396	0.398	12	0.833	0.799	16	0.938	0.916	14	0.729	0.854
Mean heter.	0.176			0.363			0.678			0.877			0.882		
Samp.	48			48			48			48			48		

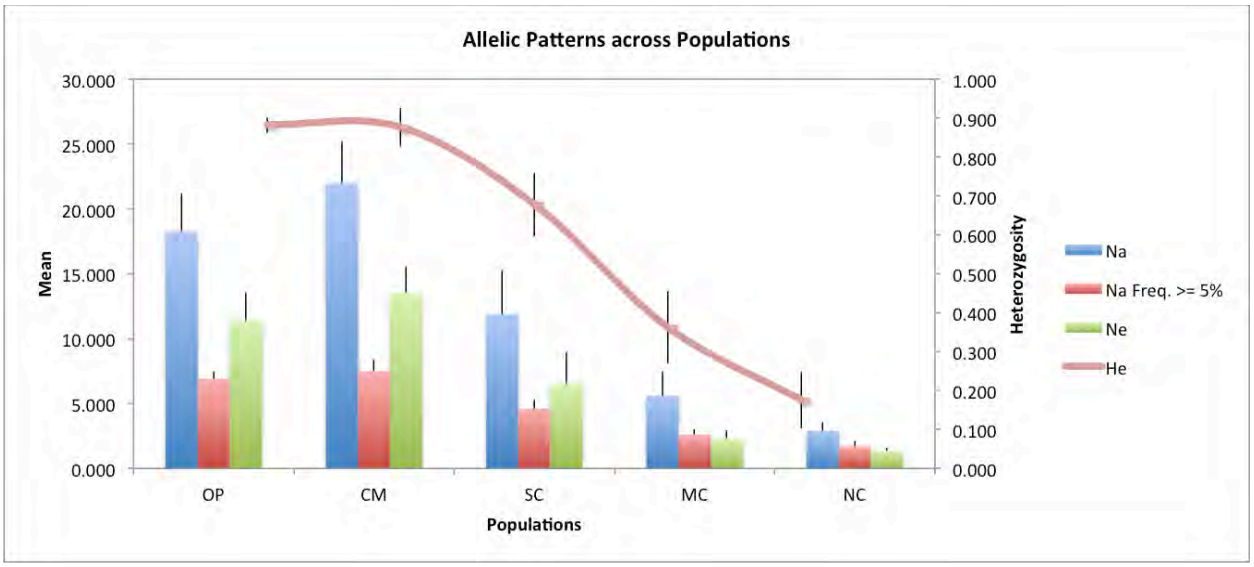

Figure 3-15 – Patterns of allelic richness and heterozygosity in 5 sampled regions of Ascaphus truei genotyped at 10 microsatellite loci. Bars represent mean and SD of number of alleles (blue bars), mean and SD of different alleles with a frequency less or equal to 5% (red bars), mean and SD number of effective alleles (green bars). Mean heterozygosities for each population (across all loci) are represented by the pink line. The two Washington regions are first (OP and CM) and the three BC regions follow (SC, MC, and NC). CM is the southernmost population and NC is the northernmost.

A significant amount of genetic variation is noted among populations. An overall F_{ST} of 0.281 ($p < 0.001$) is found (Table 3-7). A pairwise comparison shows significant genetics structure among all five populations ($p < 0.001$), ranging from 0.069 between OP and CM to 0.446 between OP and NC (Table 3-8).

Table 3-7 – AMOVA Summary Table showing an "among population" F_{ST} of 0.281, $p > 0.001$. Degree of freedom (df), Sum of Squares (SS), Mean Sum of Squares (MS) and Estimated Variance (Est. var.) are shown.

	df	SS	MS	Est. var.	%
Among population	4	463.329	115.832	1.175	28%
Within population	475	1430.260	3.011	3.011	72%
Total	479	1893.590		4.186	100%

Table 3-8 – Pairwise F_{ST} values among each of the 5 populations. F_{ST} values for each pairwise comparison are shown below the diagonal (indicated by ---) and p-values are shown above the diagonal.

Population	OP	CM	SC	MC	NC
OP	---	0.001	0.001	0.001	0.001
CM	0.069	---	0.001	0.001	0.001
SC	0.180	0.138	---	0.001	0.001
MC	0.348	0.320	0.145	---	0.001
NC	0.446	0.417	0.372	0.420	---

The degree of population structure among regions is shown in the DAPC plots (Figure 3-16). The first plot is based on each region as a population and the second is with each individual stream as a population. The Olympic Peninsula cluster was looser when the streams were partitioned as populations; even so, the populations were clustered into the five regions without regional information *a priori*. This reflects genetic structuring between regions, showing a higher level of genetic similarity between NC and MC. The NC cluster is tighter than other regions in the plots, showing a high degree of relatedness within the region compared to the within-cluster relatedness of other regions.

The Bayesian estimates of regional population structure corroborated our discriminant analysis of principle components. The STRUCTURE analysis determined a greater likelihood of 4, 5, or 6 clusters in the data over all others (from 1 to 15) (Figure 3-17). The 'K = 4' plot shows a likelihood of some SC samples falling into a cluster with CM samples, though a new, distinct cluster appears in 'K = 5' and 'K = 6'. 'K = 6' does not deviate from 'K = 5' other than a pink band across all regions. It appears that 'K = 5' accurately represents the relatedness of the data. 'K = 5' shows clustering between MC and SC, and, to a much lesser extent, clustering between MC and NC.

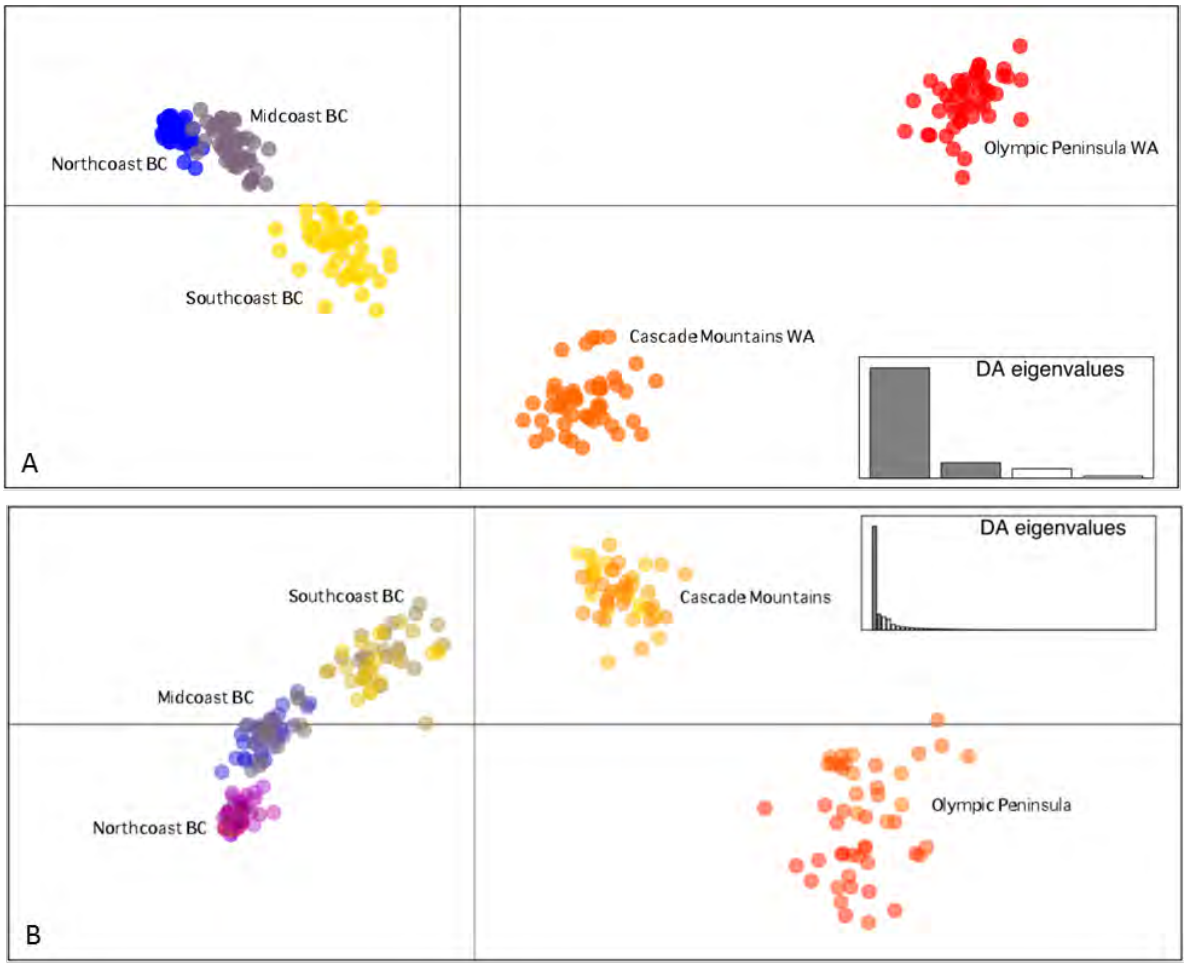

Figure 3-16 – Discriminant Analysis of Principle Components (DAPC) showing five clusters. The top plot (A) represents the clustering based on each region as a population. The bottom plot (B) represents the clustering based on each stream as a population. The tightness of the clusters also reflects the level of variability in that region.

Discussion

We found five statistically significant genetic clusters that relate to five geographic regions. Our phylogeography analysis reflects a sharp reduction in allelic richness and heterozygosity towards the northern range in Terrace, BC (Figure 3-18). The results are consistent with 'bottleneck' or 'founder' effects associated with a northward range expansion. Our results show a south-to-north genetic structure in *A. truei*. The STRUCTURE analysis shows similarities between the genotypes in the southcoast and midcoast regions, suggesting some link between regions. Genotype-by-sequencing analysis (Section 3.6) may shed more light on the links between regions, even though microsatellite analysis reveals a clear regional structure.

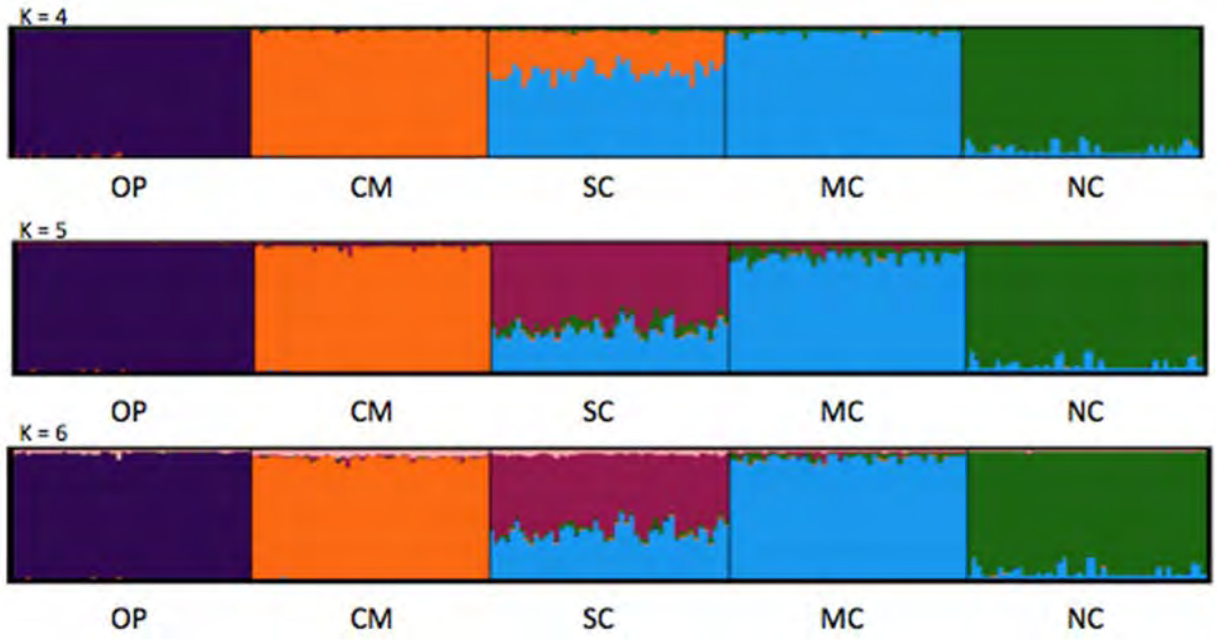

Figure 3-17 – CLUMPAK major clusters for K = 4, 5, and 6 of the STRUCTURE results. '4' is at the top, '5' is in the middle, and '6' is at the bottom. Plots reflect the maximum likelihood of a genotype belonging to a cluster. K = 4 shows a likelihood of some SC samples falling into a cluster with CM samples, though that becomes a distinct cluster in K = 5 and 6. K = 6 does not deviate from K = 5 other than a pink band across all regions. K = 5 likely accurately represents the relatedness of the data.

Ascaphus truei are the only members of Family Ascaphidae and are considered the most ancient frogs in the world, representing sister species to the lineage of the anurans (Nielson et al. 2001). This unique and ancient line is evidenced by a distinctive array of features including tadpoles with adhesive suckers, true ribs, alternate-leg swimming, no functional tongue, inability to hear sound, a cloacal 'penis' in males, and skin peptides called ascaphins with antibacterial and antifungal properties (Hayes and Quinn 2015). *A. truei* is on BC's provincial Blue List and is designated as a *Species of Special Concern* in Canada (COSEWIC 2011). Our findings reflect the importance of dispersal to maintain genetic diversity throughout their range and landscape. Resource management and restoration can help facilitate dispersal, especially at the northern extent of their range where microsatellite marker variability is low. The lack of genetic diversity in this region may make it more sensitive to environmental change as low genetic variation may also result in low phenotypic variation and ultimately less resilience to

environmental changes. For this region in particular, it is important to fully understand the extent of the reduction of diversity at a larger number of genomic markers (i.e., genotype-by-sequencing).

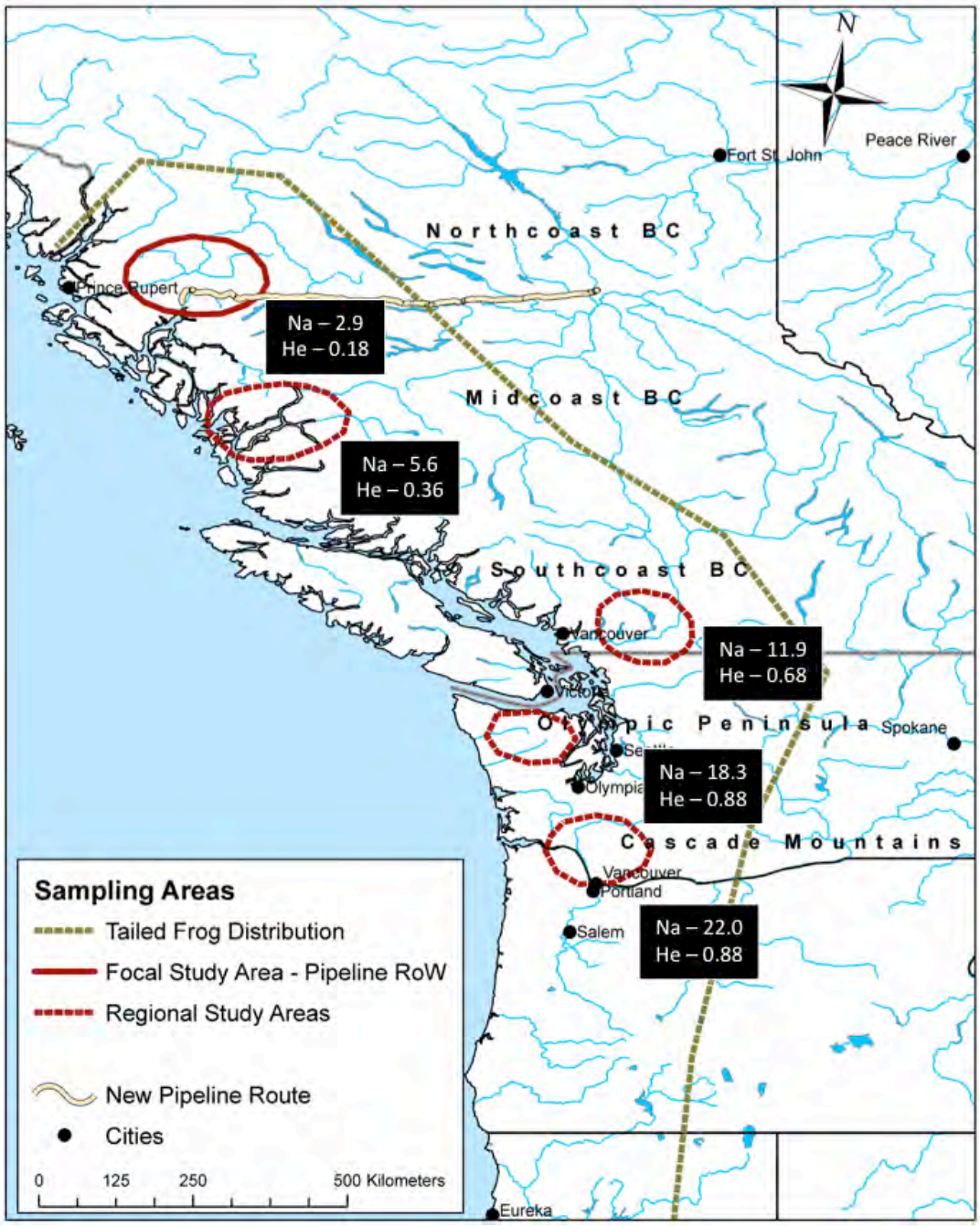

Figure 3-18 – Five sampled regions across British Columbia and Washington with their respective allelic richness (Na) and heterozygosity (He). There is a significant south-to-north reduction of both allelic richness and heterozygosity.

Population genetics allows for a global picture of the spatial processes that are likely to affect genetic structure (Prunier et al. 2013) and can also be scaled down for fine resolution analyses if the genetic structure is amenable to analysis. In our study, the reduced variability in the Terrace region and surrounding the pipeline RoW (i.e., the northcoast population) did not allow for a fine-scale landscape genetic analysis. Consequently, in Section 3.6, we explore a new way to investigate population genetics across all scales. Ideally, the population genetics analyses in the northcoast will provide complementary information about landscape permeability, i.e., gene flow, to other studies on behavior, habitat selection, and their biotic or abiotic interactions (Prunier et al. 2013). Population genetic data is used in landscape ecology for the indirect evaluation of functional connectivity (Pruiner et al. 2013), and can aid in creating more robust ecological management models for *A. truei*.

Our study shows the *A. truei* populations in BC are genetically unique and will require management goals and strategies that account for reduced genetic diversity, especially goals that facilitate natural dispersal among populations. Future work will expand our resolution of genomic variation of *A. truei* across the Pacific Northwest as well as within the northern extent of their range.

3.6. Population genetics of *A. truei* using "RAD capture"

Cherie Mosher, Chris Johnson, and Brent Murray

Introduction

Different molecular genetic markers have dominated landscape population genetics at different phases throughout its history due to rapid technological and theoretical advances. Genetic markers are broadly defined as features of DNA sequence regions that can be used to identify an individual within a population. Marker genotypes are used to quantify genomic variation within and among individuals. Estimates of genetic variation can be used to study demography of a population, revealing geographic structure and distribution or to classify individuals into different subspecies within a species. When population genetics is combined with landscape information, the identification of environmental factors that are barriers or partial-barriers to population connectivity is possible (i.e., dispersal) as is the detection of population declines (Spear and Storfer 2010). Technological advances have allowed the development of genetic markers located in mitochondrial and genomic DNA.

Some of the first population genetics markers used were located in mitochondrial DNA (mtDNA) (Allendorf and Luikart 2006). mtDNA is relatively easy to isolate and is useful for setting up phylogenetic trees. mtDNA on its own, however, is an unsuitable marker for the study of recent genetic divergences. Welsh and McClelland (1990) and Williams et al. (1990) independently described RAPD (randomly amplified polymorphic DNA), nonspecific primers to amplify regions throughout the genome. They are effective for amplifying polymorphic markers, but are difficult to reproduce. Lougheed et al. (2000) determined that RAPD markers and microsatellite markers were comparable at the regional scale, but that microsatellites were superior in discerning fine-scale genetic differentiation. RAPD markers were ultimately dropped due to their lack of reproducibility among studies.

The most commonly used molecular genetic marker today is the microsatellite. PCR primers are developed to amplify regions with consistent tandem repeats of short base sequences (e.g., ATAG ATAG ATAG ATAG). These regions are typically polymorphic because the number of repeats vary; the rate of mutation is estimated at one in every 1,000 or 10,000

meioses (Allendorf and Luikart 2006). Results with microsatellites are reproducible and comparable among studies because the primers are developed for specific regions.

Recently, next-generation sequencing was developed for population genetics analysis. Next-generation sequencing is the broad term for rapid sequencing of small fragments of DNA across entire genomes. Bradbury et al. (2015) used RAD sequencing (a next-generation sequencing technique) and developed 2574 usable single nucleotide polymorphisms (SNPs) across the Atlantic salmon (*Salmo salar*) genome. They used genotypes based on those SNPs to compare to microsatellite genotypes (15 loci) from the same samples. Similarities showed effectiveness in representing common spatial genetic signals for each marker type, while the SNP data set showed signs of introgression that was not clearly present in the microsatellite dataset (Bradbury et al 2015).

The appropriate molecular genetic marker used in a population genetic study is based on its variability and the nature of the information it provides, though variation in one region of a species range may not exist in others. The genetic characteristics of a population do not only determine the results of the genetic analysis, but also the method needed to determine population structure. Coastal tailed frog (*Ascaphus truei*) is an example of this phenomenon. Numerous molecular genetic markers have been used to study this species throughout its range. Much of this analysis was done to examine the taxonomic relationship of the Rocky Mountain and Coastal Mountain populations (currently recognized as *Ascaphus montanus* and *A. truei*, respectively). Ritland et al. (2000) used RAPD markers to examine the range expansion of *A. truei* in BC. Sixteen primers were selected after 84 were screened, and the analysis yielded 169 amplified polymorphic fragments. Each fragment was treated as a dominant locus, genotyped as either present or absent (not amplified). They discovered three major groups at the larger scale (north and mid-coast, south coast, and interior). A note, the north coast refers to King Island, north of Bella Coola, BC, even though the range of *A. truei* extends close to the Nass River, north of Terrace BC. Populations of the south-coastal regions showed the lowest F_{ST} values (0.035), while the interior populations showed the highest (0.119).

Mitochondrial DNA was used by Nielson et al. (2001) to also examine the phylogeography of *Ascaphus*, this time in northwest USA. This data showed that Coastal and

Rocky haplotypes formed monophyletic clades with sufficient genetic divergence to suggest long-term genetic separation and designation as separate species. The lower number of haplotype lineages in the Rocky Mountains represented a more recent divergence.

RAPD's were used by Wahbe et al. (2005) to determine the fine-scale genetic structure of *A. truei* in two streams, one surrounded by clearcut and one by old-growth forest. The streams were located on the south coast of BC. Sixteen primers were screened and six were used for the analysis, yielding 97 band zones. Genetic differentiation among reaches within old growth was higher (F_{ST} = 0.3136) and slightly lower in clearcuts (F_{ST} = 0.2285). Interestingly, they detected the most genetic differentiation within streams and found more genetic relatedness upstream over downstream.

Spear and Storfer (2008, 2010) and Spear et al. (2012) used 13 polymorphic microsatellite DNA markers they developed in various landscape population genetics studies on both *A. truei* and *A. montanus*. Similar to Wahbe et al. (2005), Spear and Storfer (2008) compared the influence of forest age on population genetic structure. Their study incorporated more streams across both forest types, and they found more genetic connectivity in the harvested forests than in the unmanaged forests (though they postulated it may be related to with differences in slope between the two forest types). Spear and Storfer (2010) further compared gene flow in harvested forest versus wildfire-disturbed forest and identified different patterns of genetic connectivity between forest types. The wildfire-disturbed habitat had greater terrestrial connectivity whereas the harvested forest habitat showed connectivity along riparian corridors.

Finally, Spear et al. (2012) described the recolonization of *A. truei* after the eruption of the Mount St. Helens volcano using their microsatellite DNA markers. Their results suggest recolonization from outside source populations within one generation. Post-eruption management practices (i.e., salvage logging and replanting) appeared to limit gene flow compared to natural regeneration. They found, as with the study done in 2008, that increased slope related to increase gene flow.

We set out to use the microsatellite markers developed by Spear et al. (2008) in a landscape population genetics study at the northern extent of its range (Section 3.5). Our initial

results, using 53 samples from streams showed a sharp reduction in genetic variability in the northern range (Table 3-5). Because we cannot therefore associate the microsatellite data with landscape features, we focused on "RAD capture" genotyping. Recently developed genotype-by-sequencing techniques, such as RAD capture, genotype thousands of loci allowing for a more detailed analysis of genetic structure and a better estimate of genomic variation. This is in contrast to microsatellite marker studies that are based on 10–15 genotyped loci. This technique produces loci scattered across the genome, and the variability is based mostly on SNP calls and, to a lesser extent, microsatellite calls.

Because of the range of techniques employed, it is difficult to compare previous population genetics analyses for tailed frog among different locations. At the cusp of a change from microsatellites to next-generation sequencing, we aimed to compare the variability and effectiveness of RAD capture genotyping to microsatellite markers using the same samples from four regions in Washington and BC, including one region close to the northern extent of the species' range (Figure 3-12). Section 3.5 details the results from the microsatellite analysis, and our objective for this study was to determine the effectiveness of RAD capture genotyping among five regions previously genotyped using 10 microsatellite loci.

Methods

Tissue sample collection was done as detailed in Section 3.5. The same samples used in the microsatellite loci analysis were used to develop RAD capture genotypes. Up to 48 individuals were chosen for analysis in the southern populations (OP = 48, CM = 36, SC=44, MC = 48; Figure 3-12). In order to investigate fine scale genetic differences and conduct landscape genetic analyses in the Terrace area, this sample size was increased to 80 for the NC population. To test the reproducibility of genotyping, a number of sample replicates were included in a "blind" sample design. DNA concentration for each sample was measured using the Qubit® Quantitation Platform which uses fluorescent dyes to measure the concentration. At least 50 ng of clean DNA per sample, a concentration of about 5.0–10.0 ng × μL^{-1}, were submitted for RAD Capture genotyping. A "bait" (i.e., capture) library was constructed by producing and sequencing a RAD library from a single individual, similar to creating a map that will guide the

sequencing done in the rest of the individuals (Etter et al 2011). We have completed the data collection to this point.

Within the next two months, SNP saurus will use this bait library and subsets of homologous fragments produced following the NEXTera protocol (Illumina) will be "captured" from all other samples. This procedure allows for the fragments of an individual sample to contain a unique sequence "barcode". Barcoded capture fragments will then be pooled, and fragments will be sequenced using single-end reads on the HiSeq 2000 platform (Illumina, Inc. San Diego, CA.). The sequences of bait-library loci will be used as a reference genome. Self-alignment will be used to identify and remove sequences within the assembly with significant homology and to minimize paralogous DNA regions in the references (Lozier 2014). Each read will be aligned to the 'RAD Bait reference' using BOWTIE (Langmead et al. 2009). SNPsaurus scripts were used to detect SNPs and to call genotypes.

Anticipated results

Analysis of tailed frog genomic variation assessed through RAD-capture sequencing is ongoing. At the point, a bait library has been constructed. This library contains fewer than 10,000 loci (fragments) that will be used capture homologous fragments in all other individuals. One of the main advantages of sequence capture protocols over other genome partitioning techniques is the increased ability of the method to obtain comparable sequence information at all loci for all samples (Jones and Good 2015). The final dataset should therefore contain sequence information at up to 10,000 loci for each individual. This dataset will enable a genome wide, multi-locus genotype that will be unique to each individual. This will drastically improve our resolution of genetic difference within and among populations. It will allow us to confirm the general phylogeographic pattern of reduced variation in northern regions and to further study the levels of gene flow among these regional populations as well as enable a fine scale analysis of landscape genetics. A manuscript with the completed results and analysis will be forwarded to Chevron when complete.

3.7. Habitat features associated with *A. truei* tadpole densities

Cherie Mosher, Brent Murray, and Chris Johnson

Introduction

Ascaphus truei, the coastal tailed frog, is of interest in BC because of its evolutionary uniqueness, habitat specialization, and vulnerability to land development. *A. truei* has brought attention to headwater streams, as non-fish bearing tributaries historically have received little management priority (COSEWIC 2011). The biggest obstacles to maintaining this species are: our lack of understanding about their specific habitat requirements, from the patch to the landscape; and identifying the causes and impacts of habitat loss and fragmentation.

Although *A. truei* is a federal Species at Risk, much of their habitat is located in areas that are logged or are crossed by linear corridors (i.e., roads and pipelines). Near 500,000 streams were crossed by roads as of 2005, with an average increase of 13,369 crossings per year (over a five year period) (COSEWIC 2011). To maintain this species across BC's managed landscapes, we require a better understanding of the stream characteristics that influence the abundance of *A. truei*.

Ascaphus frogs have relatively low fecundity and a lengthy larval period, as tadpoles occupy streams from one (in the warmer climes) to four years (cooler climes). They are among the longest-lived North American frogs, at 15–20 years, and reach sexual maturity around eight years for males and nine years for females (Daugherty and Sheldon 1982). Ascaphidae have highly specialized habitat requirements: headwater creeks that must flow year round because of the long developmental period for larvae (Nussbaum et al. 1983).

Tadpoles possess a flattened oral disc that serves as a suction cup for clinging to the undersides of rocks (Figure 3-19). *A. truei* tadpoles are the top grazers in streams throughout their northern distribution (Section 3.5 Figure 3-12); although, biotic interactions are among the more poorly understood aspects for this species (Hayes and Quinn 2015). Their grazing influences primary production and invertebrate grazers, the biomass of periphyton and

chlorophyll *a* being reduced as great at 98% in tadpole-grazed experimental streams (Lamberit et al. 1992). As a potentially dominant grazer, due to the low diversity of species in headwater streams, protecting their habitats safeguards more complex community interactions in these ecosystems. Removing amphibians from their habitats can affect algae communities, invertebrate populations, predator dynamics, leaf litter decompositions, and nutrient cycling. Also, maintaining functional headwater ecosystems will have benefits for species found at lower elevations in the watershed.

Figure 3-19 – Coastal tailed frog tadpole's oral disc; it is used to cling to the underside of rocks and scrape food.

The Pacific Trails Pipeline project transects four main ecological units: interior, mountainous, coastal, and marine ecosystems. Over 600 watercourses are crossed by the project. Over 30% of the project footprint is in mature or old forests, and several species of conservation value are found within this footprint. Examining the stressors for these species will be crucial in revealing the sometimes subtle and often unpredictable ways in which human disturbance might influence ecosystem dynamics.

In the search for identifying harvest treatment effects, fine-scale influences have been ignored, at least as part of any directly expected result. When Walsh (1990) was posed the question of why patterns of tailed frog abundance were not more strongly associated with forest age, his response was that forest age was an indirect measure of limiting factors, and that microhabitat or microclimate features that varied in parallel with forest age were the real limiting factors (Hayes and Quinn 2015). If this is the case, studies addressing *A. truei* tadpoles intended to identify forest management effects must address site-specific variation to minimize potentially substantial confounds.

Hawkins et al. (1988) studied the distribution of tadpoles in streams disturbed during the 1980 eruption of Mt. St. Helens. They found that older tadpoles (year two) were aggregated, and they linked that pattern to the non-random distribution of microhabitats. Densities increased at intermediate water velocity, relatively low embeddedness of substrate, and relatively coarser substrate sizes (10–30 cm in diameter). Tadpoles remain in natal stream systems from 1–4 years (or 2–5 different summer seasons) and the length of larval cycle in *A. truei* varies based on latitude and elevation. Tadpoles in northern BC appear to have a 3–4 year larval cycle. Hayes and Quinn (2015) suggest a need to understand how different inorganic clast size combinations influence habitat suitability for the various tadpole life stages.

Our objective for this study was to quantify the relationship between tadpole density and factors that were hypothesized to influence the distribution and abundance of larval *A. truei*. We calculated an index of tadpole density and related that measure to a range of environmental parameters that described stream reaches where tailed frogs were likely to occur. The results will increase our understanding of the ecology of this life stage of *A. truei*. Such understanding can be used operationally to predict stream reaches with a relatively high density of larval frogs.

Methods

Streams were sampled throughout the area surrounding Terrace and Kitimat, BC (Figure 3-13) at the northern extent of the *A. truei* range (Figure 3-12) in the summer of 2014. Data were collected between mid-June and mid-November in conjunction with sampling for genetic

material (see Section 3.5). We employed an opportunistic, non-random sampling scheme, accessing only streams that crossed operational roads. Based on our primary objective of obtaining genetic material, we only sampled streams that we assumed served as habitat for tailed frogs. Streams were selected based on the number of stream crossings by operational roads as well as historical information on *A. truei* presence. Streams were selected based on their size. In addition, we did not sample streams that had a high likelihood of drying out during the summer or that were in close proximity to other sampled streams. If a stream emptied into a different watershed (i.e., the Zymoetz versus the Kitimat), it was sampled even if it was directly adjacent to another sampled stream. The goal was to sample as many streams as were accessible while also ensuring there was adequate time to visit streams throughout the broad study area.

Samples were taken from up to five locations along the stream reach as in Section 3.5, Figure 3-14. We sampled from just below the road crossing (0 m site) to determine whether tadpoles were present. If they were present, we sampled at 200 m downstream, at 100 m downstream, 100 m upstream and 200 m upstream. UTM coordinates were taken at the beginning of each sampled site. The goal was to get four samples from each sampled site with a maximum search time of 60 min. We moved upstream from the start point, did visual sweeps and flipped over rocks while swiping nets to capture the tadpoles. Sampling sites were dropped or moved if accessibility was limited or if the mouth of the stream was less than 200 m from the road crossing.

At each sampling site we recorded the time until detection, the distance from site start to capture location, water temperature, the number of tadpoles, the distance in relation to the start point, and the cohort size of each tadpole captured. We also placed a pin with a flag at the exact location the tadpole was found (Figure 3-20).

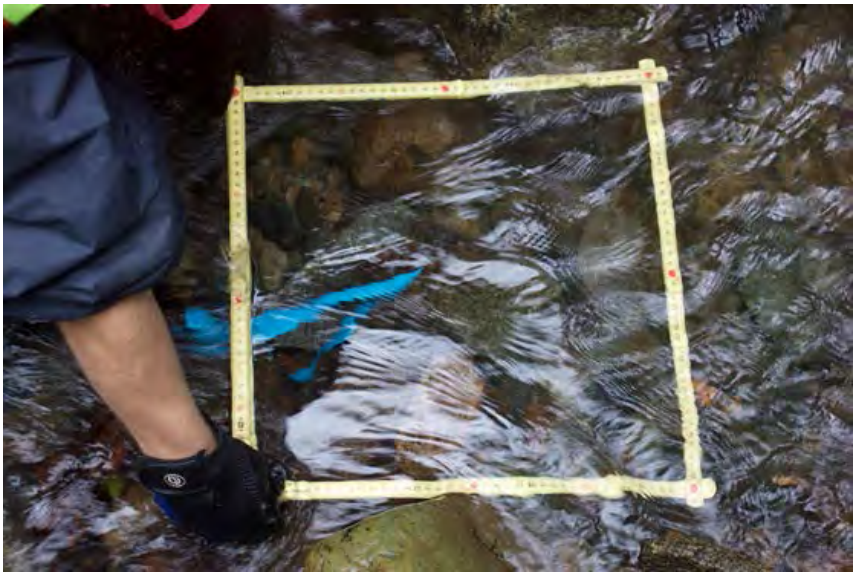

Figure 3-20 – Blue flagging is attached to a pin that is placed under the rock where the tadpole was found. The 0.4 × 0.4-m measuring tape was used to record substrate sizes.

We recorded wetted width, wetted depth, and stream profile over the pin where the tadpole was found. Profile was related to three classes: pool, riffle, and cascade. We used a 40 cm × 40-cm quadrat placed along the stream's flow of water to record benthic substrate classes (Figure 3-20). We used a gravelometer to estimate the percent cover and number of stones of each size class of substrate (i.e., ≤45 mm, 46–64 mm, 65–90 mm, 91–128 mm, 129–180 mm, >180 mm). Stones were placed in size classes based on their total size, but only the portion of the stone that was within the plot was included in the estimate of percent cover. We used a spherical crown densiometer to measure the percent cover of shade due to vegetation overstory. We took densiometer readings downstream, leftbank, and rightbank; the three measures were averaged.

Data analysis

We calculated the rate of tadpoles collected per hour as the number of tadpoles divided by the total sampling time at a site. We recorded environmental data at the flagged location and averaged the data per sampling site. Mode, instead of an average, was calculated for the categorical variables (i.e., profile and embeddedness).

We used statistical count models to relate tadpoles-per-hour to date, temperature, stream width, stream length, profile, embeddedness, substrate percent cover, substrate class numbers, and vegetation percent cover; for this approach, we used a negative binomial distribution. Combinations of variables within each count model served as hypotheses representing ecologically plausible explanations for the influence of microhabitat features on the density of tadpoles (Table 3-9).

Table 3-9 – Independent variables used for negative binomial count models representing the rate of collection of coastal tailed frog tadpoles in stream reaches from northwestern BC.

Variable Name	Coding/ Levels	Description
Date	Categorical:4	Date sampled (2014): 1 - August 26th – 29th, 2 - September, 3 - October, 4 - November 1st thru 10th.
Temp	Continuous	Temperature in Celsius (from 3.5 to 12) for a sampled site.
Width	Continuous	Wetted width in m, taken at the tadpole capture location and averaged over a sampled site.
Depth	Continuous	Wetted depth in centimeters, taken at the tadpole capture location and averaged over a sampled site.
Profile	Categorical:3	Nature of water in a water channel: Pool, Riffle, and Cascade. Mode was calculated for each sampled site.
Substrate percent cover	Continuous in 6 categories	Percent cover of six size classes (45 mm, 64 mm, 90 mm, 128 mm, 180 mm, and >180 mm) in a 0.4 × 0.4 m square with tadpole capture site at its center. Percent cover equals 100 and is averaged over a sampled site.
Substrate size number	Continuous in 5 categories	Number of individual stones of five size classes (64 mm, 90 mm, 128 mm, 180 mm, and >180 mm) in a 0.4 × 0.4 m square with tadpole capture site at its center. Averaged over a sampled site.
Embeddedness	Categorical:4	Degree to which substrates are embedded into bedrock: 0 - none, 1 - up to 25% of substrates are embedded, 2 - 26 to 75% are embedded, 3 - 76 to 100% are embedded (includes sites that are all bedrock). Mode was calculated over a sampled site.
Vegetation percent cover	Continuous	Vegetation cover using a spherical densiometer. The three percent covers were averaged for a total and averaged over a sample site.

We tested a series of hypotheses constructed as statistical models that served as ecologically plausible explanations for the rate of tadpole capture. Models corresponded with three sets of explanatory factors: temporal, environment, and *a priori* based on published literature (Hayes and Quinn 2015). We used variance inflation factors (VIF) to assess multicollinearity among covariates. An individual VIF greater than 10 or a mean VIF greater than 1 suggests that a model has high levels of multicollinearity (Chatterjee et al. 2000).

We used Akaike's Information Criterion (AIC) to rank and select the most parsimonious model from the set of ecologically plausible models. We used ΔAIC to identify the 'best' models of the set and Akaike weights (w_i) to quantify model selection uncertainty. Where models were nearly equivalent (i.e., ΔAIC < 2), we selected the model with the fewest number of parameters (Johnson et al. 2006). We used 95 % confidence intervals to assess the relative strength of the coefficients within the selected model. When intervals do not overlap zero, covariates are considered as a significant factor influencing the density of tadpoles. Multiple sites were sampled within a stream. Thus, we used a clustering algorithm to correct the confidence intervals for an autocorrelated data structure. All statistical analyses were conducted using Stata (ver. 12.1, StataCorp LP, 2012).

Results

We captured 532 tadpoles from 124 sites along 32 stream reaches. Using those data, the most parsimonious count model describing the relative abundance of tadpoles (i.e., tadpoles collected per hour) was also the most complex (Table 3-10). This count model included nonlinear covariates for stream width and depth as well as covariates representing the gravel and stone composition of the streambed (i.e., gravelometer) and the embeddedness of that substrate in bedrock ($AIC_c w$ = 0.639). The next highest ranked model was less parsimonious (AICc Δ = 2.90) and consisted of only nonlinear terms for stream width and depth.

Table 3-10 – Number of model parameters (*k*), differences in Akaike Information Criterion (AICc) scores (Δ) and AICc weights (*w*) for negative binomial count models representing the rate of collection of coastal tailed frog tadpoles in stream reaches from north western BC. The top 95 % of AICc weights are presented.

Model	*k*	$AIC_c\Delta$	$AIC_c w$
$Width^2$ + $Depth^2$ + Substrate Size Class (except 180) + Embeddedness	12	0.00	0.639
$Width^2$ + $Depth^2$	5	2.90	0.150
$Width^2$ + $Depth^2$ + Substrate Size Class (except 180)	9	3.39	0.118
$Width^2$ + $Depth^2$ + Substrate Size Class (except 180) + Embeddedness + Profile	10	6.00	0.032
$Width^2$ + $Depth^2$ + Substrate Percent Class (except 180) + Embeddedness	13	6.43	0.026

The rate of tadpole collection at each reach, an index of abundance, was greatest in narrow and shallow streams, and decreased in a nonlinear fashion with stream dimension (Table 3-10; Figure 3-21 and Figure 3-22). Tadpole collection was negatively correlated with large pieces of rock substrate (>180mm), but increased in substrate with a low level of embeddedness in bedrock (Figure 3-23 and Figure 3-24).

Table 3-11 – Model coefficients, standard errors (SE) and 95 % confidence intervals for the most parsimonious negative binomial count model representing the relationship between the rate of collection of coastal tailed frog tadpoles and environmental parameters measured at stream reaches from north western BC (see Table 3-10).

Covariate	Coefficient	Standard Error	95 % Confidence Interval
Stream width	-0.513	0.139	-0.786 – -0.240
Stream width^2	0.047	0.015	0.018 – 0.076
Stream depth	-0.077	0.037	-0.149 – -0.004
Stream depth^2	0.002	0.001	0.000 – 0.004
Substrate - size class 64	0.112	0.104	-0.092 – 0.317
Substrate - size class 90	-0.037	0.095	-0.223 – 0.149
Substrate - size class 128	0.042	0.102	-0.158 – 0.241
Substrate - >180 size class	-0.202	0.071	-0.341 – -0.063
Embeddedness - low	0.493	0.162	0.174 – 0.811
Embeddedness - medium	0.349	0.187	-0.017 – 0.715
Embeddedness - high	0.581	0.307	-0.020 – 1.182
Constant	3.748	0.427	2.910 – 4.586

Discussion

We designed the sampling and collected data to address the connectivity of tailed frogs among populations and within individual streams. The analysis of landscape-level connectivity was premised on genetic samples taken from tadpoles within each stream. Thus, for efficient sampling we prioritized our sampling to streams that would have some likelihood as habitat for larval frogs. This limited the analysis and inference from the environmental data. Results provide inference to streams that contain tailed frogs, not the full range of streams that are found across the pipeline RoW.

For those streams that we sampled, tadpole abundance decreased as width increased, with an increase at the greatest widths (Figure 3-21). This increase may be an artifact of sampling, as larger streams are more difficult to search for tadpoles. It may also be due to an

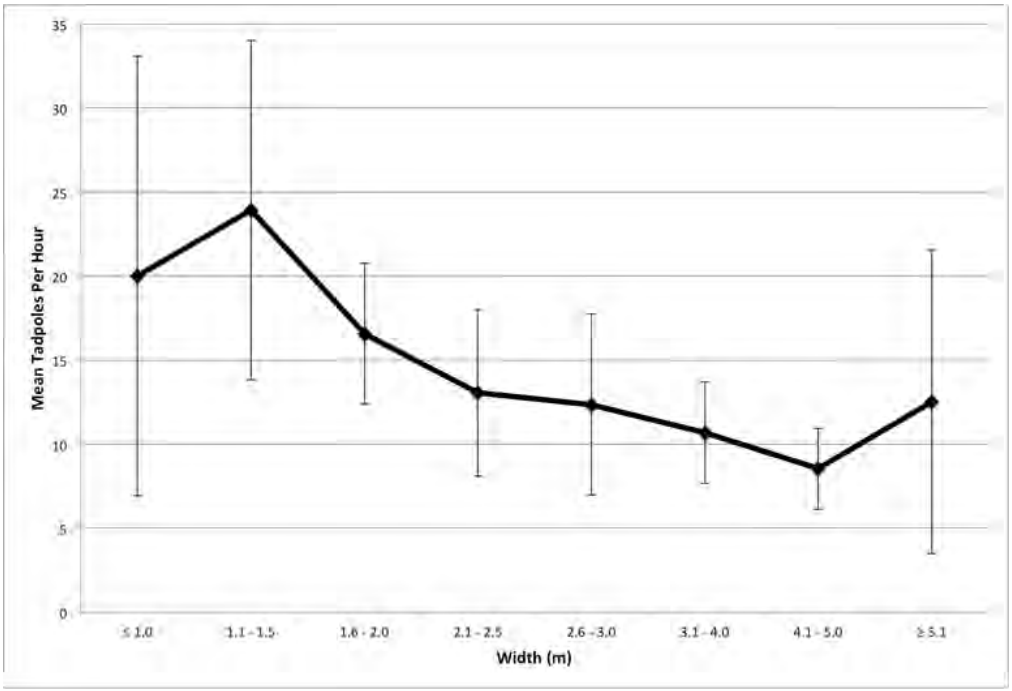

Figure 3-21 – The mean tadpoles per hour (averaged per site) based on the average width (in meters) per sampling site. The averages were divided into eight categories.

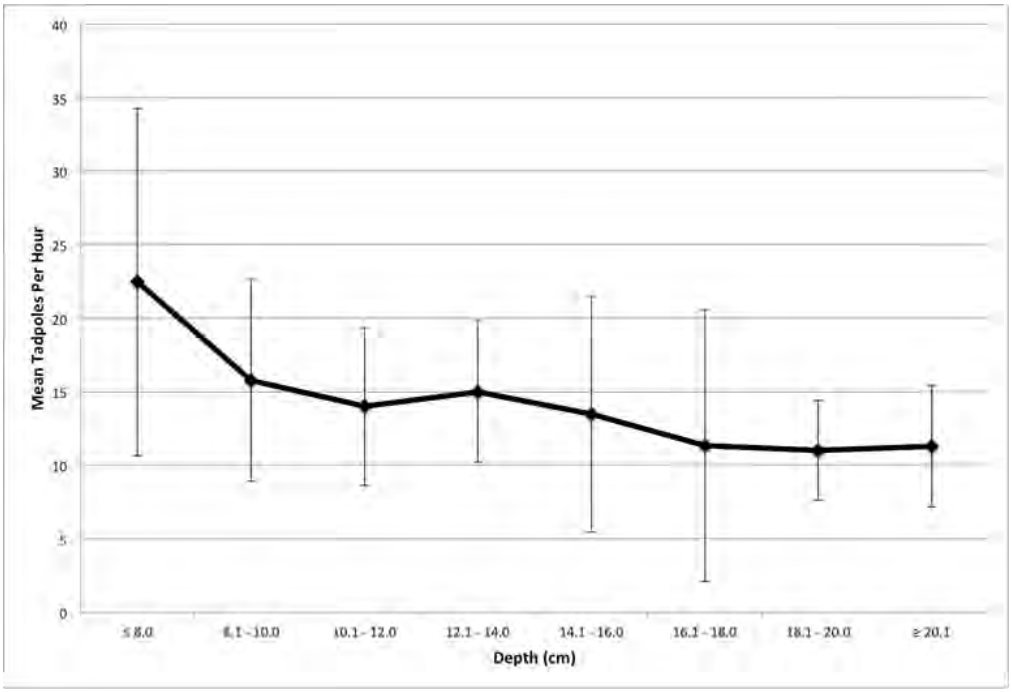

Figure 3-22 – The mean tadpoles per hour (averaged per site) based on the average depth (cm) per sampling site. The averages were divided into eight categories.

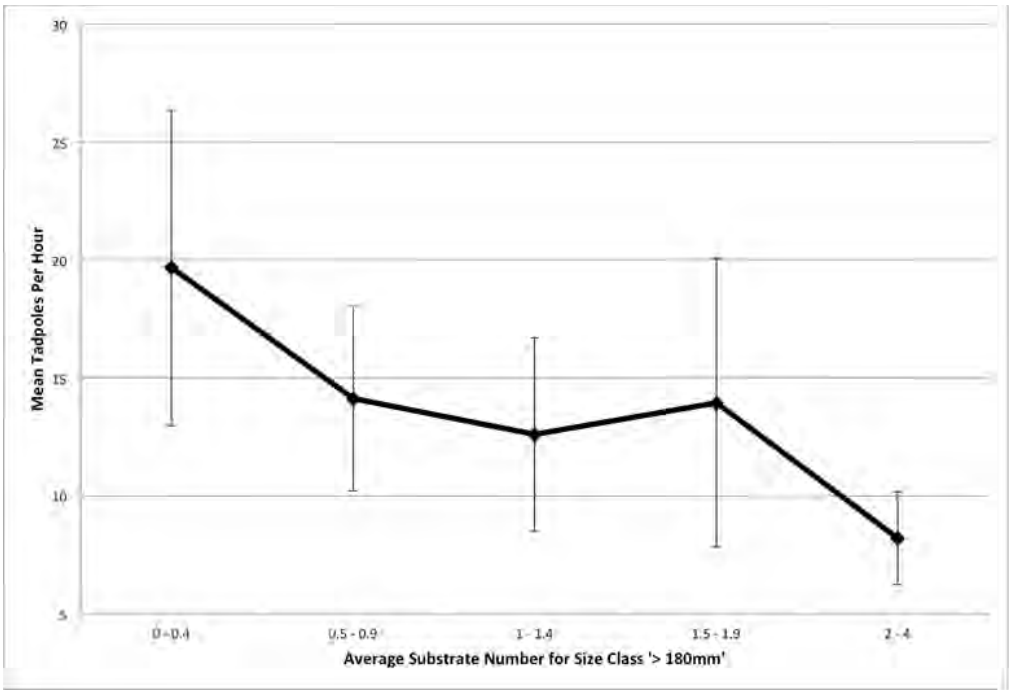

Figure 3-23 – The mean tadpoles per hour (averaged per site) based on the average number of stones (0–4) in the greater than 180mm category per sampling site. The averages were divided into five categories.

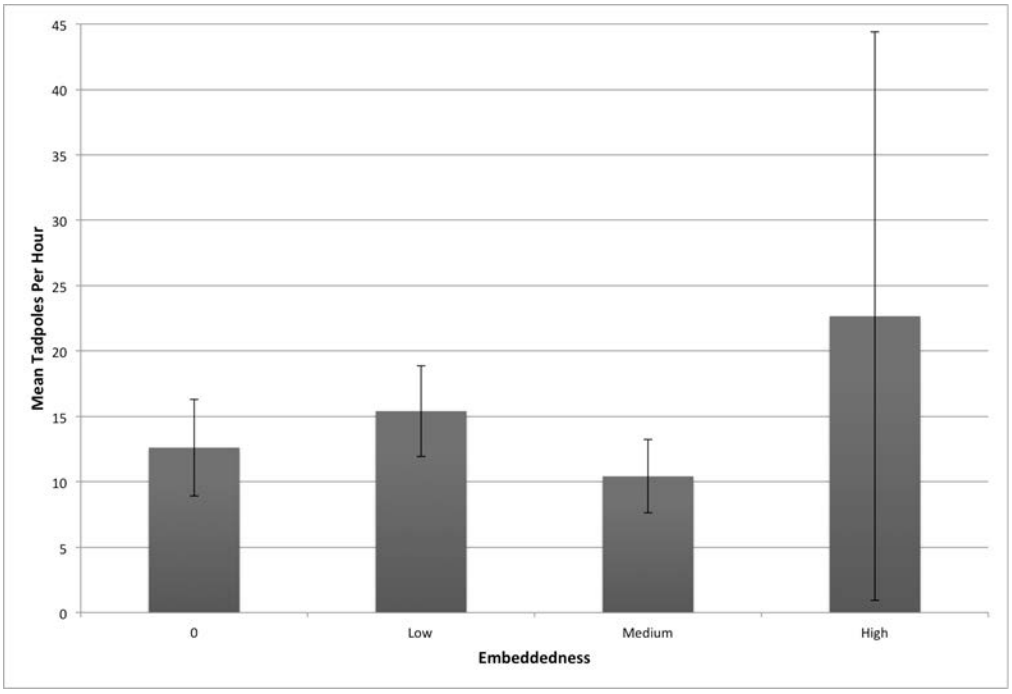

Figure 3-24 – The mean tadpoles per hour (averaged per site) based on the categories of embeddedness in bedrock (zero, low, medium, and high).

increase in the number of older age cohorts in larger streams. Because of age-specific survival, we would expect fewer tadpoles in the older age cohorts.

Elevation, gradient, and aspect had significant relationships to relative abundance of tadpoles (captures × m^{-2}) on the Olympic Peninsula, WA (Adams and Bury 2002). Density, in that study, peaked at both moderate and steep gradients, and differences in habitat selection among cohorts was included as a potential explanation for the two peaks. Our stream width category may relate to their measure of gradient as stream width decreases with an increase in gradient. In future work, we will determine if selection for stream gradient and width differs among cohorts.

Dupuis et al. (1999) related changes in tadpole densities to stream width, substrate composition, and the amount of coarse material. Tadpole density was negatively correlated with fine materials filling interstitial spaces, a classical assessment of 'embeddedness'. We defined 'embeddedness' as levels of substrate rooted in bedrock, as fine materials were uncommon in the streams we sampled. A higher embeddedness score equals either complete bedrock or a large number of stones fixed into the bedrock. Tadpole densities decreased as embeddedness in bedrock increased. Tadpole density also decreased when the number of large stones within a 0.4 × 0.4-m quadrant increased. Though this is a different way of assessing embeddedness, the underlying concept may be the same. *A. truei* tadpoles prefer habitats that have interstitial spaces among rocks and cobbles; potentially for foraging, and predatory or flooding refuges. An increase in fine sediments or in the dominance of bedrock reduces interstitial spaces, lowering the amount of tadpole habitat. Any disturbance that scrapes away substrate, exposing bedrock or increases fine sediment, may negatively impact tadpole abundance.

The observed relationships between sampling intensity and environmental parameters suggest that activities that decrease freestanding stones in small streams will contribute to a reduction in the productivity of *A. truei* populations. Furthermore, our data and preliminary analyses suggest that small streams, likely associated with steeper gradients, are ideal habitat for *A. truei* tadpoles. Thus, we recommend that industrial activities near small streams should minimize the generation of fine sediments and gravels and maintain the natural rock and

cobble substrate. Future work may show differential selection of habitat across the age cohorts of tadpoles (0-4 years). This may reveal that larger streams have fewer tadpoles, but these demographically important age classes are essential for the recruitment of reproductively active post-metamorphic frogs.

3.8. Tailed Frog Protocol: findings and recommendations

Results from this Protocol provide new understanding on the current and long-term distribution of the coastal tailed frog across northwest BC. Also, we provide guidance on the choice of technique for further monitoring of this species in both terrestrial and aquatic habitats. The coastal tailed frog is strongly associated with headwater streams found in montane areas that will serve as the route for the proposed PTP pipeline. Thus, pre- and post-construction monitoring of this species will provide insights on the connectivity of those habitats for a range of aquatic and terrestrial species.

An extensive sample of tadpoles provided not only genetic material for the study of population structure and landscape genetics, but also revealed factors that influence the distribution of larval frogs at ecological time scales. Larval frogs were most abundant in smaller sized streams (i.e., stream width and volume). The data also suggest a strong correlation between larval frogs and the size and embeddedness of stream substrate in bedrock. As is reported for more southern populations, larval frogs select substrate with interstitial spacing that provides refugia from predators and appropriate surface area for feeding. Thus, preliminary results suggest that activities in the RoW that result in siltation or that lays bare bedrock in small streams will likely lead to a reduction in the productivity of populations of tailed frogs. Additional analyses will look to potential differences in the habitat selection of different cohort sizes.

Future work designed to detect the presence or abundance of tailed frogs can be implemented using a range of methods that differ in sampling effort and potential biases. When assessing terrestrial habitats for post-metamorphic frogs, our results suggest that pitfall traps and VES can be effective, but the choice depends on the monitoring objective. VES is less expensive to implement and for some environments can be effective at detecting the presence and abundance of frogs across a range of site conditions. The technique, however, is sensitive to intra-day variation in frog activity that may be dictated by breeding behavior and local weather. Also, the data suggest that the results from VES are biased by dense understory vegetation, restricting the view of the forest floor, as well as the experience of the technician.

Pitfall traps are less sensitive to local weather, as they sample over a 24-h period. This technique is expensive to implement and is passive, depending on the movement of individual frogs. Thus, long periods of dry or warm weather may result in relatively few captures. Likewise, the movement of frogs to or from natal streams could result in a high, but temporally discrete capture rate.

This is one of the first studies to assess the effectiveness of eDNA for detecting tailed frogs in fast-flowing montane streams. Results suggest that the technique can reliably detect the presence of tailed frogs. Relative to VES and pitfall sampling, the field logistics of water sampling are much less expensive; however, there are additional costs associated with laboratory analysis. Although the data appear to be useful for identifying streams occupied by tailed frogs, we are uncertain if the technique is appropriate for monitoring the absolute or relative abundance of frogs. Also, the technique is spatially indeterminate in describing the location and demographic composition of frogs that contribute DNA to the water sample. Depending on the time of year, we suspect that DNA may be more associated with larval or adult frogs. We are uncertain on the dilution rate of DNA within a stream as well as factors that may degrade DNA. Thus, a positive identification within a water sample may be associated with a population that is adjacent or relatively distant from the sampling location.

From an evolutionary perspective, genetic data shows that populations of tailed frog near the northern extent of their distribution are genetically distinct from southern populations and have a significant reduction in microsatellite markers developed using samples from Washington. The reduction in genetic diversity may contribute to an increase in the sensitivity of this region to environmental change. The reduced amount of genetic diversity using microsatellite markers may be correlated with an overall reduction in genomic diversity and hence phenotypic diversity. Although the degree of the effect is unknown, a reduction in phenotypic diversity may reduce individual variation in response to environmental change. Thus, we recommend caution, as the response to environmental change in RoW tailed frogs populations may be limited. Although landscape genetic studies are needed to confirm our findings, we also recommend that measures be taken to ensure population connectivity on the local scale and the provincial scale, to allow for natural gene flow patterns. Maintaining natural

connectivity may enhance both the response to change as well as the evolutionary potential of these unique northern populations of tailed frogs.

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Chapter 4 – Soil Integrity and Revegetation Protocol

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Cleared land exposing the soil on the RoW. Photo credit: Mark Shrimpton

4.1. Introduction

Soils are naturally occurring materials resulting from interactions between parent geological material, biota, climate, topography and time. Geological materials are minerals from rocks and sediments that form the bulk (i.e., 90–99% by mass) of inorganic soil while biota or organic matter comprises both the living and decomposed organic materials (i.e., carbon, hydrogen, oxygen, nitrogen-containing materials). Soil properties continually change with time because matter (e.g., minerals, organic matter and water) and energy are constantly subject to

four basic soil-forming processes of addition, loss, transformation and translocation within and outside of the soil. The intensity of each soil process is regulated by rainfall and temperature (i.e., climate) and the location of soil on the landscape (i.e., slope or topography). Soils are dynamic and any significant additions or losses (e.g., deposition of new minerals by flooding, turn over and exposition of underlying rocks) of geological materials reset soil formation and alter its ecosystem functions. Thus, soils are sensitive to effects in the environment including anthropogenic activities such as pipeline construction.

Healthy soil delivers many ecosystem functions, including medium for plant growth, habitat for organisms, natural filtering of water, recycling of nutrients, sources of pharmaceutical ingredients and, in many places, cultural values as well (Brady and Weil 2008). Any measure of soil integrity should center on the ability of soil to carry out its ecosystem functions, and it is the microbial community that is integral to soil's ability to carry out these functions. Fungi in soil decompose organic matter and form mycorrhizal associations that act as the liaison between plant roots and soil nutrients. Soil bacterial communities cycle nutrients between forms that differ in their availability to the living biomass of the soil. One of the most important functions is nitrogen fixation, which fixes atmospheric nitrogen into a form that can be taken up by plants. Any measure of soil health must include assessment of the taxonomic and functional diversity of the soil microbial community.

Developing microbial diversity and establishment of pioneer native plant species are initial indicators of a properly functioning soil system. Pioneer plant species deposit roots, leaves, twigs and other plant debris and their subsequent decomposition enriches the organic matter content of soils (e.g., Ottenhof et al. 2007). Recruitment of native species will then follow the natural vegetation succession in steady state with the climatic and edaphic factors in the environment. The continued active biological processes — both plant roots and organisms — will effectively mix mineral and organic materials in soils to generate optimal soil granular structure for efficient flow of air and water within the soil (Young and Crawford 2004). The formation of granular structure was observed under pioneer plant species established in new soil parent materials resulting from intensive mining activities in southeast Spain (e.g., Zanuzzi et al. 2009; Arocena et al. 2012).

A healthy soil should have optimal soil structure (e.g., granular) to continuously provide the plants and microorganism with sufficient supply of water and oxygen. Soil structure is assessed through several physical properties including bulk density, water-holding capacity, soil strength, hydraulic conductivity, aggregate stability (e.g., Burt 2004) and fabric analysis (Stoops 2003).

The current practice for pipeline placement is to strip off the upper 20 cm of duff or mineral horizons and place this in a pile, or continuous row, along the Right-of-way (RoW); this is considered “topsoil”. Then, the underlying mineral horizons are removed and placed in another pile; this can be considered “subsoil”. Once the pipe has been placed in the ground, the subsoil is first placed in the trench, followed by the topsoil. Although root debris might serve as an inoculum or seed of soil organisms for reclamation, it is our understanding that it is not acceptable to include debris in the trench directly over the pipe during backfilling operations. That said, fine root fragments will be present in the duff (i.e., LFH) and topsoil horizons; these surface layers are saved and replaced during reclamation activities (i.e., normal practice). These horizons should contain sufficient microbial inoculum for reclamation and restoration activities. The post-construction replacement of coarse-woody debris (e.g., fallen logs; decomposing vegetation) on the soil surface has also shown to serve as an effective biological inoculum in other reclamation studies.

By using environmental DNA (eDNA), a technique that is sensitive enough to detect changes due to disturbance, we had the potential to monitor the reestablishment of soil ecosystem function throughout the restoration process. We first needed to assess the ability of eDNA methods, however, to detect different types of perturbations of the soil (i.e., to see how specific disturbances affect the soil community and its function). Because there is a lot of environmental noise around the eDNA ‘signal’ associated with various aspects of soil function, we conducted a series of experiments — the microcosm experiments — to determine bacterial and fungal community function with soils that have been manipulated to simulate disturbance by pipeline installation. Given the taxonomic richness of soil microbial communities, we used next-generation sequencing methods to broadly profile microbial diversity over 12 months. In addition, we measured enzymes involved in carbon degradation, available soil nutrient (e.g.,

total N, P) and the abundance of genes involved in biochemical cycling (e.g., N mineralization) and soil physical properties (bulk density, aggregate stability, hydraulic conductivity and fabric analysis such as soil structure) as measures of soil ecosystem function.

The Soil Integrity and Revegetation Protocol was designed to monitor the ecosystem functions of soils through the phases of construction to operation of the pipeline. Specifically, the research team investigated microbial community composition, key soil properties and processes (i.e., chemical, physical), both of which are indicative of soil health. The studies described, and data presented, in this report correspond to biological, chemical and physical characterization of soils from interior region near Summit Lake, BC and coastal region near Kitimat. Information from this study can contribute to the scientific literature on reclamation and restoration ecology of linear projects in general, on management policy of those sites, and on the development of the pipeline project long-term restoration plan in the future. Because of the early termination of the BMAP program, we were not able to address any of the restoration aspects of the Protocol, either during or after construction.

4.2. Approach and outline of contributions

In order to understand how the soil metagenome can be maintained or restored, baseline data of current conditions must be collected and analyzed. We addressed this objective in two phases: first, using a series of microcosm experiments as a pilot study and, subsequently, transferring those research findings from the trials into several 'temporary' research in-field plots. We completed two types of experiments, the first one was a soil microcosm experiment was designed to: 1) look at how mechanical disturbance of soil structure (shaking) might change microbial community structure detailed in Section 4.3; and 2) develop work-flows for marker gene-based community profiling and assays for potential hydrolase activity to be applied as methods for post-RoW soil monitoring. The second experiment involved applying several types of physical disturbance treatments in several temporary research plots set-up in a forested area located close to the pipeline RoW and is reported in Section 4.4. These two experiments enabled us to characterize the soil fungal and bacterial diversity, characterize functional diversity (potential litter decomposition enzyme activities, and

surveys of genes related to biogeochemical processes) and acquire some physical measurements of the soil properties (soil moisture and temperature monitoring).

4.3. Soil microcosm experiment

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Introduction

In order to understand how the soil metagenome can be maintained or restored, baseline data of current conditions must be collected and analyzed. The soil microcosm experiment examined changes in microbial (i.e., bacterial and fungal) diversity and community composition accompanying treatments that mimic the disruption of soil layers during the establishment of a pipeline RoW. This experiment was established at the end of November 2013 when soil cores were obtained from the Summit Lake (sub-boreal spruce) and Kitimat (coastal western hemlock) areas. Organic soil cores and mineral-soil cores were used to create combinations of soil layers having different soil disturbance characteristics. Our objectives were to: 1) determine whether short-term disturbance of soil structure and re-establishment of the soil profile affects microbial community composition; 2) use combinations of upper and lower soil profile disturbance to evaluate whether microbial communities are differentially susceptible to soil handling activities; 3) determine whether soil litter decomposition activity levels, specifically hydrolytic enzyme activity levels, change with disturbance of soil structure and with soil organic matter constituents buried in mineral soil layers; and 4) establish “in-house” work-flows for DNA-based community profiling and soil potential enzyme assays for field experiments. Throughout our work, we defined LFH and organic-layer soils by visually identifying the transition zone in soil colour from darker (i.e., predominantly organic soil) to lighter (i.e., predominantly mineral soil).

Methods

Soil cores collected from the Kitimat (Latitude 54.2007; Longitude -123.1600) and Summit Lake Latitude 54.2007;Longitude -128.4942) areas were used to construct microcosms that simulated the order of trench re-filling during construction (i.e., mineral soils first followed by capping with organic soils). We used combinations of physically disturbed (i.e., by shaking contents of a soil core in a mason jar for 30 sec and returning the contents to the core sleeve

used in initial sample collection) and undisturbed (i.e., intact) soil layers in constructing the treatment levels. We established five different soil treatments, each with three replicates, from both the Kitimat and Summit Lake sites. In each treatment a total of 15 cm of core were collected, but the organic and mineral portions were treated differently. We used the following five treatments:

A. intact organic and intact mineral collected together in a single 15-cm long core;
B. intact organic 7.50cm core and intact mineral 7.5-cm core collected sequentially, but then reassembled into a single intact core;
C. the organic 7.5-cm core was collected and disturbed by shaking and then combined with an 7.5-cm intact mineral core;
D. an intact organic 7.5-cm core was combined with a disturbed mineral 7.5-cm core ; and
E. a disturbed organic 7.5-cm core was reassembled with a disturbed mineral 7.5-cm core.

Treatments A–B are referred to as intact treatments while treatments C–E are our disturbed treatments.

The shaking (or homogenization) in one or both components of the disturbed treatments simulated disturbance of soil structure (i.e., aggregate distribution and particle ordering) and disruption of the soil profile (i.e., burial of organic components when soil is bulldozed) that likely occurs when excavation is performed during the creation of a pipeline trench. During pipeline construction, the approximate orders of the soil profile are maintained during site restoration by ensuring that mineral soils are first used to refill the trench followed by organic soils at shallower depths.

In order to assess changes over time, we assessed the treatments at five time periods: 0 months, one month, three months, six months, and 12 months. To account for possible variation among cores, we made our measurements on three replicates for each treatment at each sampling period — 75 initial cores from both the Kitimat and Summit Lake sites. At each sampling period, three cores from each site were destructively sampled, composited (i.e., combined), and characterized. Here we present results for eDNA community profiling (i.e.,. bacterial and fungal) at 0 and six months, while the potential enzyme activity data is described for sampling at three, six, and 12 months.

Laboratory analyses of soil samples

We employed several techniques to monitor changes in biological, and chemical parameters of the soils. We used polymerase chain reaction (PCR) amplification and sequencing of the bacterial 16S ribosomal RNA gene and the fungal ribosomal RNA Internal Transcribed Spacer 2 (ITS2) region from soil DNA extracts to profile bacterial and fungal diversity. In addition, litter decomposition activities, important for the cycling of nutrients, were measured by conducting hydrolytic enzyme assays using fluorogenic substrates to measure potential hydrolase activities associated with chitin degradation, cellulose degradation, and mineralization of phosphorus compounds in soil-buffer suspensions.

Community profiling (eDNA)

Sampling of bacterial and fungal diversity through soil (eDNA) extracts provides a broad survey of cultivable and uncultivable organisms (especially the latter), across a short vertical profile — DNA-based community profiling uses non-invasive sampling and minimal molecular methodology. Changes in fine-scale taxonomy (i.e., relative abundance of bacterial and fungal genera) as a consequence of soil disturbance treatments were monitored through eDNA methods.

Soil DNA was extracted using the Mobio PowerSoil® DNA extraction kit, using 250 mg of frozen (storage at -80 °C) unsieved soil from composited samples. Once the DNA was extracted from composited samples, we used PCR to amplify the bacterial 16S gene and fungal ITS2 regions. The resultant sequences were well suited to marker-gene surveys because those genomic regions are highly conserved in bacteria and fungi, yet contain enough intraspecific diversity to discriminate between taxa at the species or Operational Taxonomic Unit (OTU) level. PCR primers targeting the V4 region of the 16S gene were used for bacterial community profiling (as in Caporaso et al. 2012); PCR primers targeting the ITS2region (as in Vancov and Keen 2009) were used for fungal community profiling. Amplicon pools (visualized by agarose-gel electrophoresis and selection of bands corresponding to expected sizes of the gene fragments) were extracted and purified prior to sequencing. The purified amplicons were pooled and submitted to Genome Quebec Innovation Centre (McGill University, Montreal, Quebec) for sequencing on an Illumina Miseq high-throughput sequencer.

Data from these sequencing results allowed us to describe bacterial and fungal community composition, as well as follow any treatment-specific shifts in community structure. The microbial community structure inferred from those data is described as "total" because template eDNA may originate from metabolically active organisms (at the time of sampling), but includes DNA signatures from dormant or dead microorganisms present as well as free microbial DNA in the soil (Blagodatskaya and Kuzyakov 2013). Bioinformatics analysis of the amplicon sequencing was also performed at Genome Quebec. We used the provided taxonomic summaries to explore patterns in relative abundances of fungal and bacterial taxonomic groups across time and between treatments.

Hydrolytic enzyme assays

Hydrolytic enzyme assays provide an indicator of organic nutrient availability for microbial populations, as well as an indicator of microbial activity related to nutrient cycling (especially for assimilating polymeric carbohydrate and phosphorus reservoirs) — other studies have used hydrolytic enzyme assays as an index of soil health in reclamation studies (Jamro et al., 2014). The substrate analogs that we selected for our panel of enzyme assays were chosen because of their relevance to litter decomposition: 1,4-β-N-acetylglucosaminidase (NAG, involved in chitin degradation), cellobiohydrolase (CBH, involved in cellulose degradation), and acid phosphatase (AP, involved in mineralization of organic phosphorus compounds).

Enzyme assays were prepared as follows. Soil suspensions (using 1.0 g wet weight of soil) were prepared by vortexing soils in 50 mM sodium phosphate buffer (10 mL buffer per gram of moist soil), pH 5.2 for 30 min, to re-solubilize soil-associated stable enzymes and re-suspend soil particles containing immobilized yet functional hydrolases. Aliquots of the soil suspensions were then incubated with substrate analogues containing the 4-methylumbelliferone (MUB) fluorophore, which is detectable by spectrofluorimetry (using 360-nm excitation and 460-nm emission filters) if hydrolysis occurs. Enzyme activity values were interpreted as hydrolytic functional potential per specific substrate as the sample preparation and assay does not discriminate between biotic and abiotic substrate degradation. Hydrolytic enzyme activities provided us with a measure of soil functional activity associated with soil particles or with the accessible surfaces of soil microorganisms. At each sampling interval,

enzyme assays were performed on the three soil cores (for treatment and site) after disassembly (i.e., separation of organic/litter from the mineral layer) and compositing (i.e., combining) of the three replicate samples. Method development did not occur until after the baseline zero months (T = 0) samples were collected and stored for eDNA extraction. Therefore, assays of potential soil hydrolase activities were applied to soil cores destructively sampled in the microcosm experiment at three months, six months and 12 months. Soils were stored at 4 °C after initial processing until the time that the enzyme assays were conducted.

Quantification of extractable nutrients

As an indicator of major limiting nutrients in a forest soil setting, the chemical characterization of test soils was performed by sending samples to the BC Ministry of Environment (BCMOE) laboratory, which uses well-established methods to measure the total and available nutrients in the soil. Major nutrient measurements include total carbon, nitrogen (in various forms of nitrate, ammonium, or mineralized), available phosphorus, soil acidity (pH), gravimetric moisture content (time of sampling), and soil cation exchange capacity (CEC). These measurements were performed on mineral and organic soil samples submitted directly to BCMOE labs. Chemical characterization methods used to inform soil-pit descriptions for sites in Kitimat and Summit Lake where soil cores were collected for the microcosm experiment.

Results and Discussion

Fungal community profiling by ITS2 amplicon sequencing

Phylum-level analysis indicated that all microcosm disturbance treatments, across both forest soil types (Summit Lake- sub-boreal spruce and Kitimat-coastal western hemlock), shifted the fungal community composition to contain abundant Zygomycota at six months (Figure 4-1). This is a marked contrast from phylum-level profiles of all cores at baseline (T = 0 months), where fungi belonging to the phylum Basidiomycota were the dominant community members. The death of mycorrhizal Basidiomycota accompanied by an increase in decomposer Zygomycota fungi accompanying soil “trenching” is a previously reported phenomenon (Fernandez and Kennedy 2015), where fungal necromass (i.e., dead fungal tissue) accumulates in soils when mycorrhizal connections to plant roots are severed. This necromass provides

growth substrates to the Zygomycota fungi that colonize in the presence of detrital materials such as dead mycorrhizae.

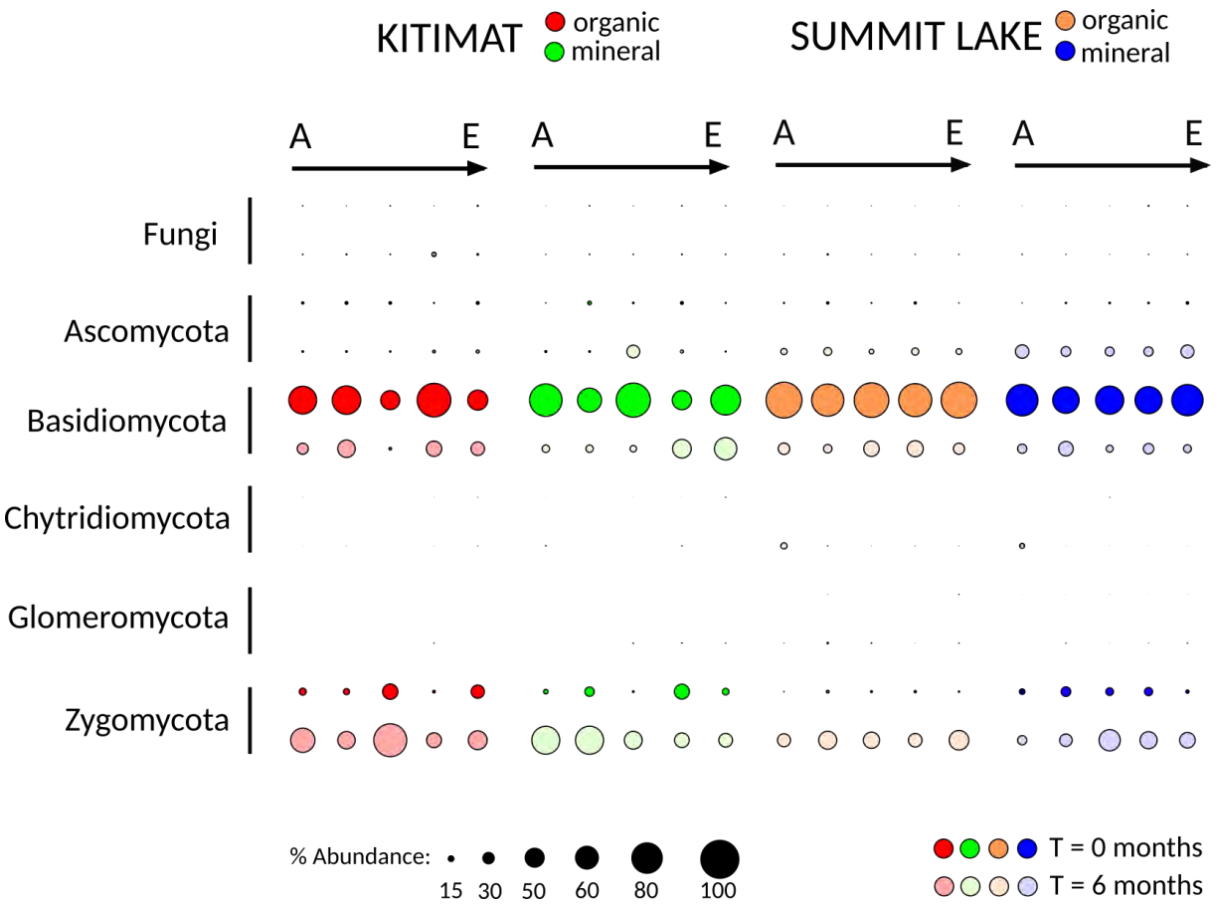

Figure 4-1 – Summary of fungal sequence abundance by phylum level groupings of taxa. The category labeled Fungi contains taxa that could not be classified beyond the kingdom level. % Abundance means that the relative abundance of sequences in each phylum is a percentage of the total sequence count per sample unit. For each phylum upper rows of bubbles represent abundances at T = 0 months and lower rows of bubbles represent abundances at T = 6 months.

The emergence of a highly abundant Zygomycota population in the control (i.e., intact) cores after six months in the microcosm study contrasts with the saprotrophic shifts observed in the Summit Lake field experiment (Section 4.4) where control plots did not exhibit a dominance of Zygomycota until 12 months. The uniform trend towards Zygomycota colonization in all the cores including the undisturbed (Treatment A) core, indicate that the root

severing associated with soil core collection for this experiment was a sufficient disturbance to create a pool of substrates for decomposer fungi. In the field experiment (see Section 4.4), the intact control plots were sampled by digging, which would disturb some but not all mycorrhizal networks. In contrast to the saprotrophic shift in the microcosm experiment, DNA data for control samples in the field experiment indicated that the below-ground integrity of mycorrhizal taxa were structurally preserved.

Because the soil-coring process itself severs connections between mycorrhizal fungi and the plants they symbiotically associate with, it is not surprising that the Kitimat and Summit Lake soil cores were similar at zero months because they exhibit low abundances of Zygomycota and dominance of Basidiomycota fungi. This is notable because the Kitimat soil cores were collected two months prior (August 2013) to the collection of Summit Lake soil cores (October 2013) and all cores were stored at 4 °C prior to the establishment of the microcosm experiment in November 2013. We know that the final gravimetric water contents of soil cores could be quite high, and soil cores were waterlogged in some instances during sampling times in the microcosm experiment. In contrast, the soil cores prior to experimental set up were kept at cool temperatures but were not irrigated on a regular basis. We believe that the combination of root severing (i.e., the initial creation of mycorrhizal necromass) and the regular watering regimen employed during the microcosm experiment contributed to conditions in the soil cores that encouraged the rapid growth of decomposer fungi with subsequent loss of mycorrhizal Basidiomycota. No significant treatment effects, however, were observed between composited soil cores representing the five combinations of soil physical disturbance in this phase of our research. Further, our decision to composite triplicate cores per treatment at every time point, did not allow enough replicates to robustly evaluate treatment effects within and across time points, even though those replicates would have been nested with treatment and sampling period. An important and unexpected insight from the pre-microcosm storage conditions of soils, however, is that protection of excavated, salvaged soils from excessive moisture will minimize populations of decomposer Zygomycota and preserve Basidiomycota abundance. Preserving the abundance of fungi from the latter phylum should

ensure that major representatives of mycorrhizal taxa, important for plant health during re-vegetation, are available to provide ecosystem services during RoW reclamation activities.

A major insight from our investigation of the soil microbiota is that the Summit Lake and Kitimat forest soils had distinct populations of mycorrhizal Basidiomycota (Figure 4-2). Highly abundant mycorrhizal taxa decreased over time in the disturbed treatments, but saprotrophic Basidiomycota abundances were largely unchanged. Our analytical approach taken with the fungal community profiling data for the microcosm experiment was to look at the shifts in Basidiomycota and Zygomycota relative abundance at a higher level of taxonomic detail, beyond phylum level summaries. For those analyses we defined “abundant” Basidiomycota and Zygomycota genera (i.e., looking only at sequence counts that were classified to the Genus level) as genera for which the average relative abundance across all composited soil samples and sampling periods was >1 %.

The decline of mycorrhizal taxa was not more pronounced in disturbed treatments of soils (Treatments C, D and E) compared to intact treatments (A and B), reinforcing that root severing during soil core collection was likely the main driver of this shift. Saprotrophic Basidiomycota were also detected as abundant members of this phylum in Kitimat and Summit Lake soils. Unlike the Zygomycota, however, these saprotrophs did not exhibit a large increase in relative abundance in response to the loss of mycorrhizal communities. Some saprotrophic genera such as *Cryptococcus* emerge in abundance across all samples at six months, and *Hygrocybe* is emergent in the fungal communities of Kitimat soils at six months.

Abundant saprotrophic Zygomycota communities primarily consist of *Mortierella* in Kitimat soils, and *Mortierella* as well as *Umbelopsis* in Summit Lake soils (Figure 4-3). As mentioned previously, the dominant saprotrophic fungal communities observed in composited soil cores across all treatments and soil layers at six months were associated with the phylum Zygomycota. In the Kitimat soils, the genus *Mortierella* dominated within this phylum at both 0 and six months. This suggests that high abundance (i.e., >25% relative abundance compared to other fungal genera) of *Mortierella* in soils from this area might be a robust indicator of disturbance in a monitoring context.

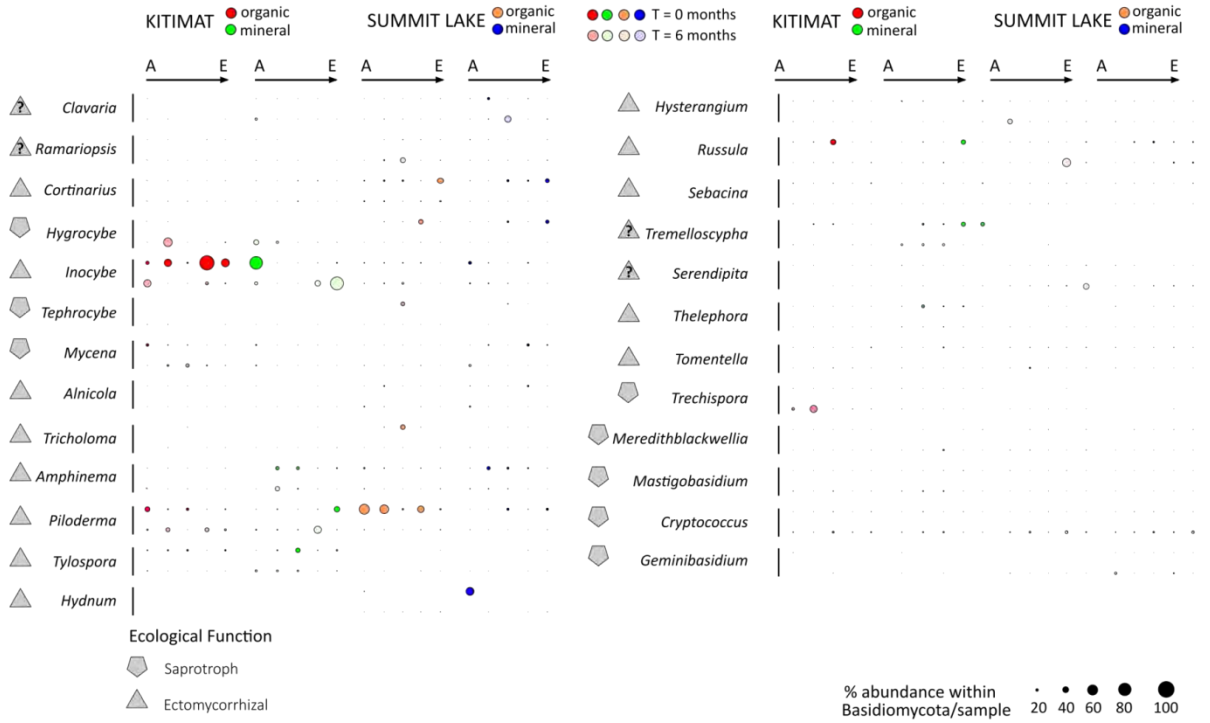

Figure 4-2 – Relative abundance (%) for fungal genera within the phylum Basidiomycota and their ecological functions. Functional classifications were assigned by Dr. Keith Egger. Shading of bubbles in the plot distinguish abundances at T = 0 months from abundances at T = 6 months. For each genus upper rows of bubbles represent abundances at T = 0 months and lower rows of bubbles represent abundances at T = 6 months.

In contrast, the soils from Summit Lake contained *Mortierella* and *Umbelopsis* as dominant genera within the phylum Zygomycota. Interestingly, *Umbelopsis* diminished in abundance in Summit Lake organic soils after six months and remained more abundant compared to *Mortierella* in mineral soils after six months. In the Summit Lake field plot experiment (Section 4.4), a similar response was observed in the disturbed soil treatments, such that disturbed organic soils eventually had a higher ratio of *Mortierella* to *Umbelopsis*, but in disturbed mineral soils both of these genera were comparable to each other for relative abundance. The reasons why *Umbelopsis* seemed to prefer mineral soils is unclear, but it is encouraging that this differential distribution of Zygomycota was observable in the microcosm as well as the larger field experiment.

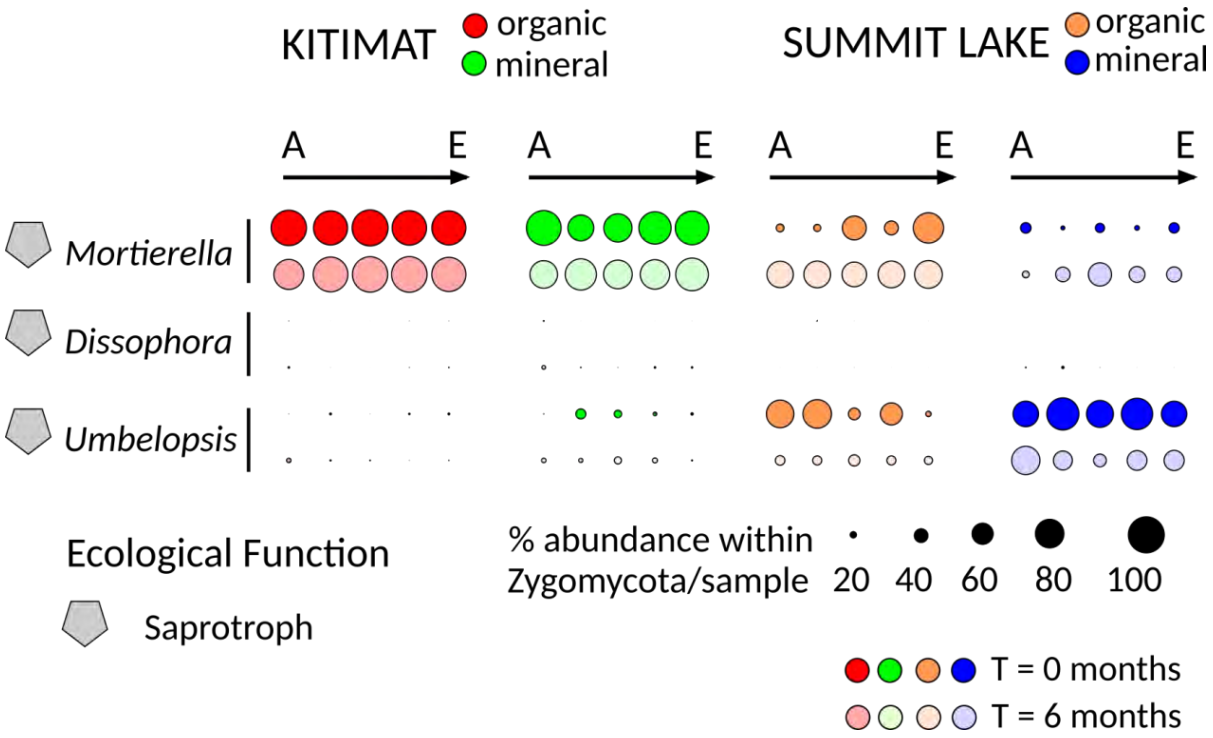

Figure 4-3 – Relative abundance for fungal genera within the phylum Zygomycota. For each genus upper rows of bubbles represent abundances at T = 0 months and lower rows of bubbles represent abundances at T = 6 months.

Bacterial community profiling by 16S amplicon sequencing

In contrast to fungal communities, only subtle changes in bacterial community composition at the phylum level were observed during the experiment. Some of those shifts were specific to forest soil type (Summit Lake-sub-boreal spruce and Kitimat-coastal western hemlock), but there were no differences among treatments.

The Kitimat and Summit Lake soil cores showed minimal changes in phylum-level relative abundances of bacteria between zero and six months, such that the overall order of phylum abundance was stable after six months (Figure 4-4). Notable changes in bacterial abundance at this broad level of taxonomic classification included: decreases in the relative abundance of Acidobacteria in Kitimat organic soils and Summit Lake soils for both layers (organic and mineral); increases in the relative abundance of Proteobacteria in Summit Lake soils for both layers; increases in the relative abundance of Actinobacteria in Kitimat soils for

both layers; increases in the relative abundance of bacterial candidate division AD3 in Kitimat mineral soils; and increases in the relative abundance of Nitrospirae observable for Kitimat mineral soils and Summit Lake mineral soils.

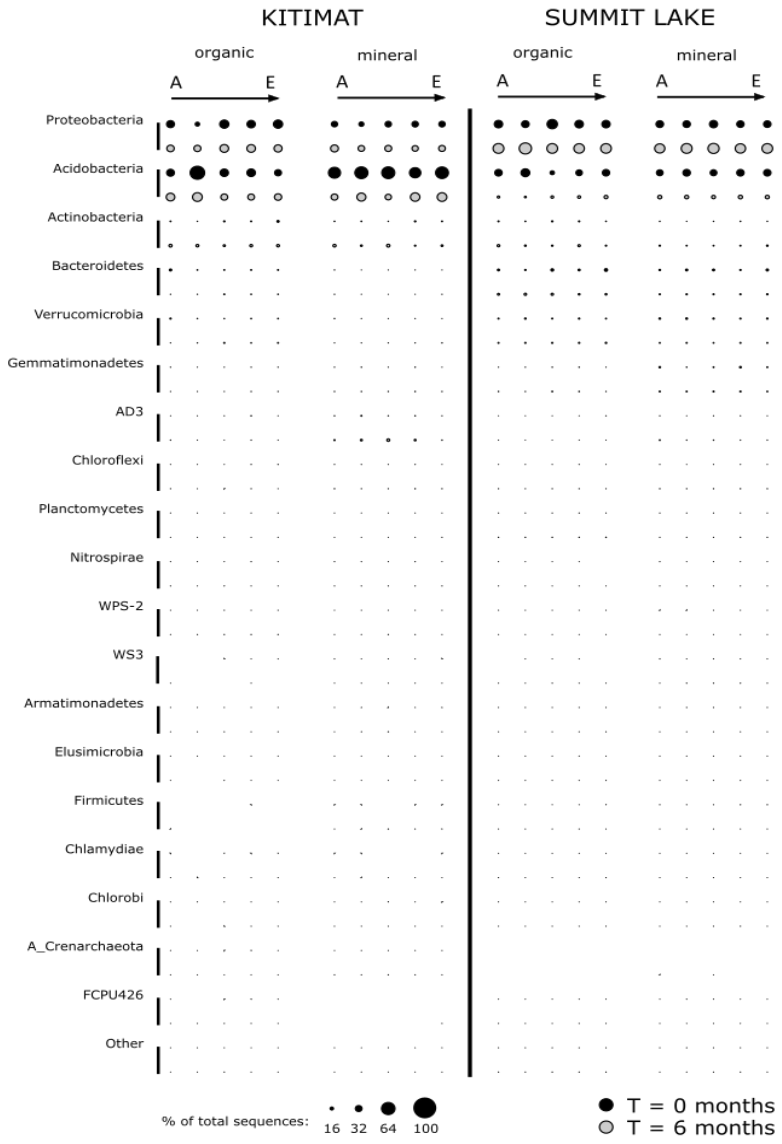

Figure 4-4 – Summary of bacterial community composition at phylum level composition comparing soil cores harvested at T = 0 months (black) and T = 6 months (grey). The category Other includes all bacterial phylum that had an average relative abundance across all samples (N = 40) of < 0.1%.

All of the above changes in bacterial community composition were observed in composited soil core samples from all treatments including the intact control (Treatment A) as of six months. This result implies that these changes in bacterial community composition are

more likely driven by growth chamber conditions (i.e., moisture and temperature) as opposed to the physical disturbance treatments. The ecological roles and responses of highly representative bacterial groups, specifically Acidobacteria, are not well described in literature, so we cannot explain the observed decrease in their abundance at six months. That this phylum decreases in abundance for soils from two BEC zones suggests that some aspect of our soil handling, which has been identical for all soil cores (most likely the storage and incubation conditions), stimulated a similar response of these bacteria despite coming from geographically distant locations.

Non-metric multidimensional scaling (NMDS) of bacteria 16S sequences classified into 766 terminal taxa, indicating that Kitimat soils were relatively unchanged in community composition after six months, while Summit Lake soils showed a temporal shift. In addition, NMDS indicated that Kitimat soil cores exhibited weak clustering by soil layer, and minimal shifts in community composition when comparing zero to six-month cores (Figure 4-5). The ordination showed separation of organic and mineral soil bacterial communities for both sampling sites (along Axis 2) yet clustering by soil layer for Kitimat soils was weakly defined, considering that two mineral soil sample units for six months fall within the oval that defines ordination space primarily occupied by the organic samples for this location. The shift in Kitimat soil bacterial community structure over time is more pronounced for mineral soil sample units, and almost negligible for organic soils because they do not cluster tightly by sampling period. This contrasts with the Summit Lake data points, where sampling time separation occurred for both soil layers along Axis 1. Considering that the watering regimen applied to these soils was frequent and that the growth chamber was set to 50 % relative humidity, these environmental parameters likely reflect precipitation as well as humidity levels to which soils from coastal western hemlock forests are typically exposed.

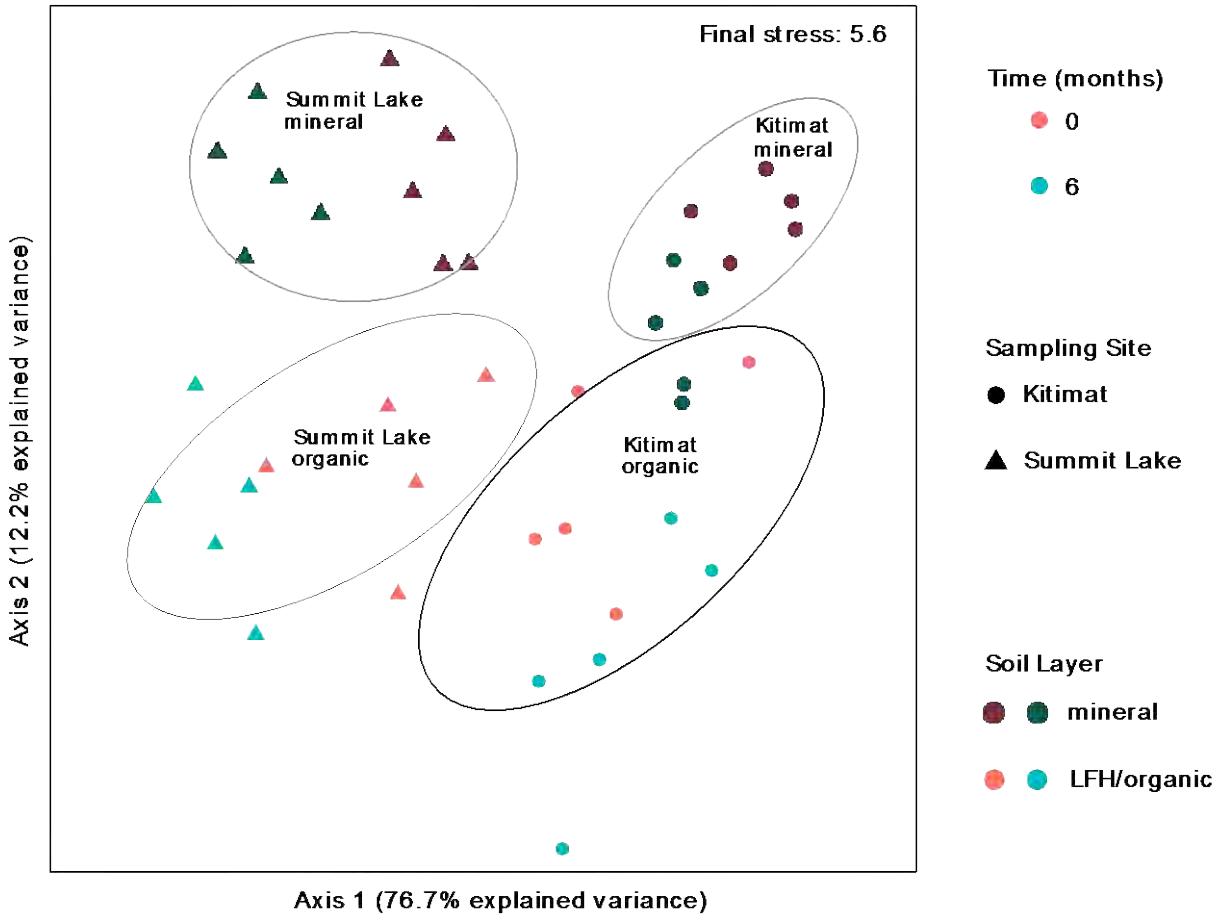

Figure 4-5 – NMDS ordination using a Sorensen distance measure to compare bacterial community composition using 766 bacterial terminal taxa between sample units (n = 40 sample units defined by site (Kitimat or Summit Lake) treatment (A–E), soil layer (organic or mineral), and time (0 or 6 months). The ovals indicate clustering of data points (sample units) corresponding to site and soil layer grouping.

Therefore, the absence of well-defined shifts in overall bacterial community composition in Kitimat soils likely reflects a tolerance for incubation conditions that were probably within their natural range for exposure to high moisture. The practical implications of this finding are that the local moisture and temperature ranges of soil excavation sites can inform the acceptable range of storage conditions that should be used while soils are kept in salvage piles.

Summit Lake treatments exhibited strong retention of bacterial community composition that cluster by soil layer. Temporal shifts in bacterial community composition were observed

across all treatment levels, similar to continuous shifts observed in the temporary field plot experiment (see Section 4.4). The Summit Lake treatments, in spite of the minimal changes in phylum level bacterial community composition, exhibited shifts that are resolved at the terminal taxon level, which included a substantial number of organisms classified to genus level. This result is similar to the field plot bacterial community profiling results (Section 4.4), showing that bacterial community composition shifts from 0 months, while maintaining dissimilarities that define organic soils from mineral soils. Because the microcosm experiment only used one set of growth temperature conditions and one watering schedule, we cannot account for how much of the change in Summit Lake soil core bacteria communities can be attributed to the waterlogging that was frequently observed at the time of soil core destructive sampling. The growth-chamber conditions and watering schedule, however, were regular enough to not resemble seasonal variations present in the field experiment over several months, suggesting that a percentage of the bacterial communities in Summit Lake soils inherently cycle in abundance on an ongoing basis. In contrast to the fungal communities, the stability of bacterial community composition at the phylum level over time might reflect that nutrient availability for these organisms is not severely affected by soil excavation (simulated by removal during core collection in combination with the shaking treatments) or changes in soil structure (simulated by shaking treatments).

Microbial function: hydrolytic enzyme assays

Because the fungal and bacterial community composition of soils in the microcosm experiment did not differ among treatment levels, and because of insufficient replication of treatment due to compositing soil cores, the potential enzyme activity data were summarized as the average activity across treatment levels (n = 5) for samples grouped by site (Kitimat or Summit Lake), layer (organic or mineral) and sampling time.

Higher enzyme activities are present in organic soils compared to mineral soils

Enzyme assays on microcosm soils demonstrated differences in hydrolytic enzyme activities between organic from mineral soil layers. Potential activity levels for all three hydrolases were higher in organic soils from both sites at all time-points compared to mineral soil layers (Figure 4-6). The range of observed values of all three potential enzyme activities

were also more variable in the organic soils for both sites compared to the mineral soils, possibly reflecting a greater heterogeneity of substrate availability or extracellular enzyme distributions in upper horizons. The distributions of chitin, cellulose and organic phosphates (substrates for 1,4-β-N-acetylglucosaminidase, cellobiohydrolase and acid phosphatase, respectively) can be expected to be more heterogeneous in organic soils as these upper horizons contain organic inputs (from dead microbial, plant and animal sources) in varying stages of decomposition.

Temporal shifts in potential hydrolase activities are not uniform when making comparisons between soil layers and sampling sites

One of the expectations during the microcosm experiment was that incubation of all soil cores in a growth chamber (given a regular irrigation regimen) would equalize temperature and humidity effects on soil microbial communities over time. The implementation of homogenous moisture and temperature conditions should have made it easier to identify shifts in soil microbial community and changes in litter decomposition activities attributable to the soil disturbance treatments implemented as part of the experimental design. Again compositing of the three replicate soil cores at each sampling period (and resultant loss of replication from this approach) affected the statistical power for directly evaluating the five disturbance treatments for differences. Saprotrophic fungi, however, are thought to be dominant contributors of extracellular hydrolase activities present in soils environments (Schneider et al., 2012). Based on this assumption, along with the observation that soil fungal communities shifted towards having abundant saprotrophic Zygomycota across all treatment levels and sampling sites, it might be expected that an increase in hydrolase activities would be observed at or several months after the time of disturbance.

Temporal changes in organic layer and mineral layer potential hydrolase activities were not similar when compared between Kitimat and Summit Lake soils. For the Kitimat organic soils potential 1,4-β-N-acetylglucosaminidase and acid phosphatase activities stabilized after six months, while potential cellobiohydrolase activity declined after six months. In contrast, Kitimat mineral soils showed an increase in all three potential hydrolase activities at the 12-month observation period. This increase in Kitimat mineral soil decomposition activities might reflect a

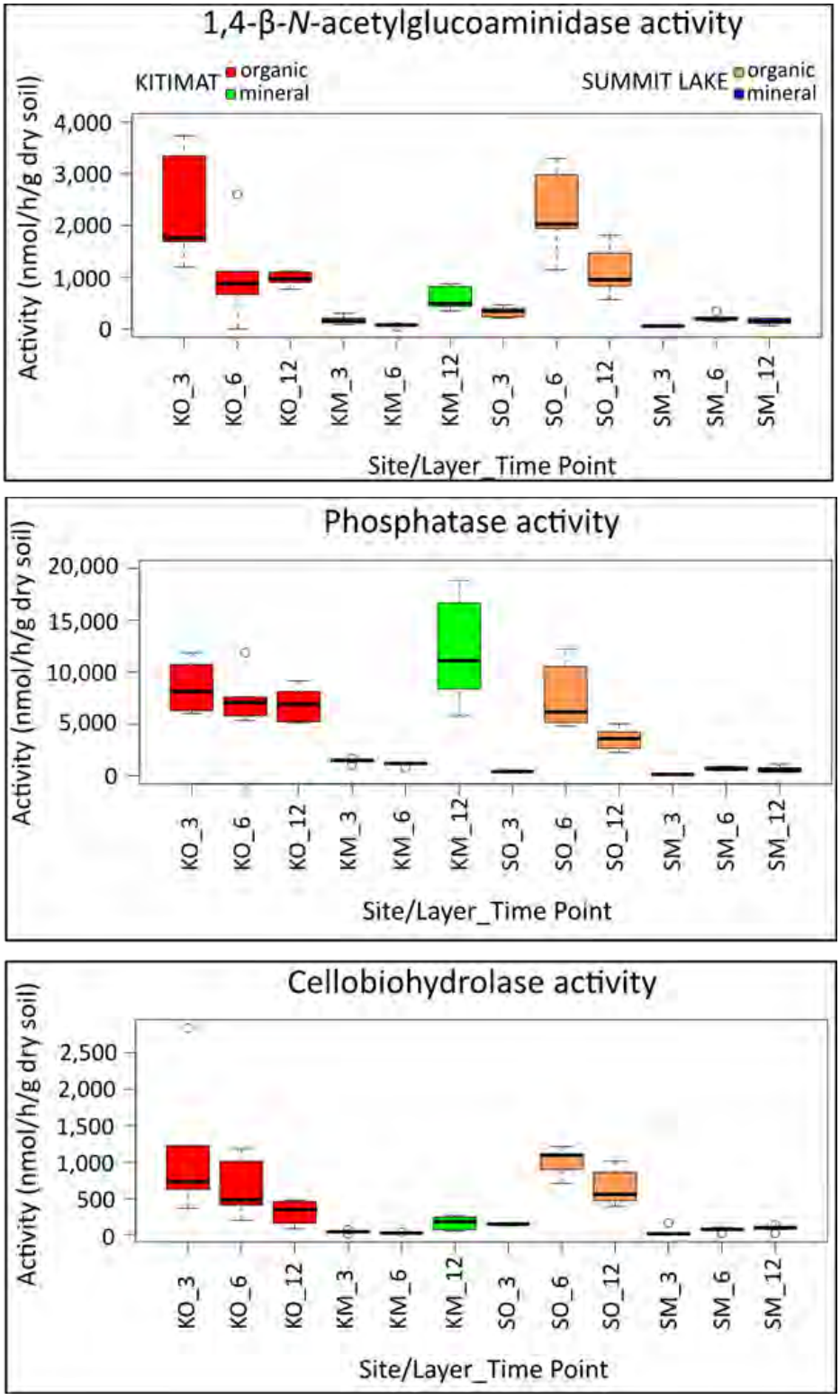

Figure 4-6 – 1,4-β-N-acetylglucosaminidase activities (top), acid phosphatase activities (middle), and cellobiohydrolase activities (bottom) grouped by site and layer at 3, 6 and 12 months.

delayed increase in extracellular enzyme activities in response to organic inputs introduced by the shaking treatments. Alternatively the increase in activity at 12 months may reflect increased substrate availability for the existing pool of stabilized enzymes in the mineral layer, which were re-located from upper layer soil due to shaking or transport during the irrigation regimens. In accordance with this observation, the stabilization of potential 1,4-β-N-acetylglucosaminidase and acid phosphatase activities and slight decrease of potential cellobiohydrolase activities, might reflect lowered substrate availability in the organic soil layer because of substrates that moved lower in the soil profile during shaking treatments or watering. The absence of hydrolase activity increases in the Kitimat organic soils also reflect that rates of secretion of extracellular enzymes or the general abundance of fungal hydrolase enzymes do not correspond to increased saprotroph abundance at the DNA level.

The Summit Lake soil cores exhibited very different temporal trends compared to the Kitimat soil cores. The organic soil cores from this site show a peak in all three potential hydrolase activities at six months, followed by a decline at 12 months although the range of potential activities at this time-point are higher than those observed at baseline (i.e., 0 months). This increase appears to fit with a working model of litter decomposition activities increasing in a soil with a saprotrophic shift in fungal community composition, where either the abundance of enzymes increased (because of more saprotrophic organisms present to secrete hydrolases), or the substrate availability to existing pools of hydrolases increased with the input of mycorrhizal necromass (i.e., dead organic matter from declining Basidiomycota community members). Soil physical mixing as implemented in the three disturbed treatments (i.e., C–E) may also be the agent of increasing substrate availability to extracellular hydrolases by changing distributions or depths of litter components in the re-assembled soil cores. For the Summit Lake mineral soils very slight increases in all three potential hydrolase activities were observed over time, suggesting minimal shifts in substrate availability or enzyme abundance in this soil layer.

Conclusions

Community profiling

Saprotrophic fungal taxa such as *Mortierella* and *Umbelopsis* can be monitored by marker gene (ITS2) profiling as performed in this study, and high abundances of these fungi can be considered indicators of soils that have been subjected to recent disturbance. In our investigation, the high abundances of these taxa were accompanied by lowered abundances of mycorrhizal Basidiomycota, and which reinforces previous observations that physically disrupting symbiotic fungal communities should be accompanied by significant "blooms" of saprotrophic Zygomycota that can use substrates from dying mycorrhizae. Therefore, high abundances of *Mortierella* and *Umbelopsis* detected by ITS2 surveys of soil DNA seem to indicate, not only relatively recent disturbance in soils, but that mycorrhizal abundance might be suboptimal for establishing plant communities. Further research into the success of revegetation regimens on physically disturbed soils would be required to establish whether the presence of saprotrophic Zygomycota are a reliable indicator of this attribute of soil health.

Low abundances of saprotrophic Zygomycota and high abundances of mycorrhizal Basidiomycota in baseline soil cores indicate that symbiotic communities may be preserved by limiting exposure to moisture. The Kitimat and Summit Lake soil cores were collected three months and one-and-a-half months, respectively, before the start of the microcosm experiment. Soil cores from both sites were refrigerated in plastic bags prior to assembling the microcosm experiment. The baseline ITS2 community profiling (i.e., at 0 months) results indicated that mycorrhizal Basidiomycota were still quite abundant in the soil cores and saprotrophic taxa like *Mortierella* were low in abundance, in spite of the initial disturbance caused by root severing during core collection. The placement of cores in a growth chamber with humidity around 50%, combined with a weekly watering regimen may have increased the accessibility of dead mycorrhizal substrates to saprotrophs, resulting in their growth and establishment as dominant community members. The implication for soil stockpiling practices during pipeline construction is that although mycorrhizal populations in soils can be negatively affected by excavation or physical disturbance, the loss of mycorrhizal taxa (and significant gain

of decomposer taxa) might be reduced by limiting the access of a soil salvage pile to large quantities of water.

Bacterial community profiling

Bacterial community composition was less responsive to disturbance of the soil profile. Fungal taxonomic profiles are a better indicator of soil disturbance, whereas physiological characterization of bacterial activities, such as nitrogen fixation rates or respiration rates may provide more meaningful descriptions of whether the bacterial community present in soils is at an optimal configuration.

The stability of community composition observed for Kitimat soils in contrast to Summit Lake suggests that local environmental factors have shaped community tolerance to changes in parameters such as frequent contact with moisture. Microbial biogeography can therefore be useful in establishing guidelines for the preservation of bacterial communities and their associated functions when soils are stockpiled.

We cannot suggest any contributing factor to the strong temporal shifts in Summit Lake soil bacterial communities or why the Kitimat soils did not show a similar trend in the microcosm experiment. The “moisture” priming of coastal western hemlock soils to the growth chamber and watering regimen, due to the characteristics of their BEC zone seem to be a plausible explanation however for the remarkable stability of bacterial community structure in this investigation. The fungal community profiling results summarized earlier strongly suggest that stockpiled soils should have limited exposure to moisture to preserve beneficial fungal communities separated from plant hosts during excavation (and to limit proliferation of Zygomycota saprotrophic organisms). Bacterial communities in contrast reflect a broader range of tolerance to soil handling and storage, but this can be further informed by the typical climate conditions for specific soils.

Potential soil hydrolase activity profiling

Organic and mineral soil potential hydrolase activities from different sites will exhibit different temporal variations although upper layers tend to exhibit higher ranges of activity than lower layers. The results of the microcosm experiment suggest that distributions of enzyme activities and substrate availabilities might reflect “local” dependencies on soil texture

and microsites characteristics, because temporal changes in hydrolase activities for organic and mineral soil layers were not similar between sub-boreal spruce (Summit Lake) and coastal western hemlock (Kitimat) forest soils. From a practical standpoint, manipulations of soils that do not drastically alter microbial community composition will not impair the capacity for hydrolase activities to be present, but soil structure may influence the arrangements of sites of nutrient density that are important for soil ecosystem function. Preserving local soil materials for reclamation and stockpiling these materials in a way that limits the loss of soil structure will likely maintain the nutrient distributions reflected by potential extracellular enzyme activities, when measured.

4.4. Chemical, physical, and biological analysis of soil disturbance and storage treatments in the field

Ewing Teen, Nicole Sukdeo, Keith Egger, Hugues Massicotte, Michael Rutherford

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Introduction

Permanent field plots could not be established until pipeline placement has occurred (2016 or later). Therefore, an experiment off the right-of-way (RoW) was implemented in 2014 to field test what we had learned from the lab microcosm experiment (Section 4.3). Our experimental plots, monitored for approximately 12 months, examined whether disturbance regimes in the microcosm experiment mirrored changes in microbial communities in the field, and examined the effect of stockpiling of topsoil during pipeline construction. These field trials were important because the preliminary findings, using a variety of disturbance regimes, provided for refinement of research methodologies.

In our field experiment, we examined how manually reconstructed soil profiles re-configured microbial communities and litter decomposition. We predicted that symbiotic associations and access to nutrient provisioning from overstory vegetation and local detrital inputs could be maintained or re-established by the microbial community, in spite of mechanical disturbance. Unlike the microcosm experiment, however, we included an additional treatment in which the organic soils were completely mixed into lower soil horizons — simulating the approach to trench refilling during RoW construction. We anticipated that this additional treatment would differ from the control and from disturbed but refilled with approximate ordering of mineral and organic horizons soil profiles in microbial community composition and potential litter decomposition activity profile. Although salvaged organic soils have proven to be effective contributors of plant propagules during soils reclamation (Naeth et al. 2013), the stability of microbial communities in stockpiled soils is not well-described. We anticipated that stockpiled organic soils might undergo changes in microbial community composition, potential litter decomposition, and in the biogeochemical repertoire because of their isolation from the soil profile.

The objectives of our field experiments were to determine whether short-term disturbance of soil structure, with approximate re-establishment of the soil profile, affected microbial (i.e., bacterial and fungal) community structure and the profile of potential hydrolase enzyme activities. Simultaneous monitoring of a control treatment accounted for seasonal shifts in bacterial or fungal relative abundance over the first 12 months of the experiment. In addition, we: 1) compared changes in community structure and in the profile of potential hydrolase activities when soil horizons are indiscriminately mixed; 2) determined if short-term stockpiling of organic soils affected microbial (bacterial and fungal) community structure and the profile of potential hydrolase activities; 3) determined if nutrient levels are affected by all treatments and levels of soil disturbance and in stockpiled organic soils over a 12-month period; and 4) determine whether moisture and temperature attributes (e.g., stability, range of variation) were affected by all treatments and in stockpiled organic soils over a 12-month period.

Methods

The weather in the central interior of BC is predominantly dry and continental. Using Climatic (Wang et al. 2006) and historical data from the last decade (2001–2010), we estimated the mean annual mean annual temperature and mean annual precipitation for the study sites to be ~3.5 °C and 696 mm. The field experiment was conducted near Summit Lake, BC (Site #1: 10U, 0489554E, 6005881N, Site #2: 10U, 0489558E, 6005700N).

Before establishing the field plots, we conducted a plant diversity assessment at each of the two sites: there were minimal differences between the two sites in terms of total number of tree, shrub and herb species, varying from 32–34 species. Lodgepole pine (*Pinus contorta*) was present surrounded both sites, albeit at a low level, given the recent mortality due to mountain pine beetle. The soils of the field plot sites were predominantly Eluviated Dystric Brunisol (E.DYB) and minorly Orthic Gray Luvisol (O.GL); four of the five soil pits we dug at the sites were classified as E.DYB, with the other classified as O.GL. The parent material for the area was glacial till. The soil texture ranges between silt loam and loam for the upper horizons (Ahe/Ae) to transition to sandy loam with depth (mineral B-horizon), except for O.GL that

showed an increased in illuviated silicate clay (compared to the higher sandy soils in E.DYB). The Soil Associations were a complex of Crystal (sandy E.DYB) and Barrett (silty O.GL). The soil type on both sites was Eluviated Dystric Brunisol, most often associated with the Crystal-Barrett complex.

Experimental Design and regimes of soil disturbances (treatments)

The experiment was set up in the summer of 2014 using a randomized block design on two sites (with three replicates per site) — we used a block design to capture small-scale differences among replicate treatments within a site. Each block contained four plots (each 1 m × 2 m) and each plot received one of four disturbance regimes. Therefore 24 plots (12 at each of the two sites) were established in a random orientation. Distance between the two sites was ~150 m; within each site, blocks were >10 m apart (Figure 4-7).

Individual treatments, each assigned to a plot (Figure 4-8) were: A) undisturbed control in which there was no mixing between the organic and mineral horizons; B) complete mixing of the organic and mineral horizons; C) initial removal of the organic horizon, mixing of the mineral layer with the intact organic horizon immediately replaced back on top of the disturbed mineral layer; and D) removal and storage of the organic horizon (D_o in Figure 4-8) under a cool, shaded-area for five months (May–November) when it was then replaced on top of the mineral layer that had been mixed when the treatment was first applied. In Treatment D, the mixed mineral soil, therefore, was exposed from May–November until replacement of the organic soil. The complete mixing (Treatment B) of organic and lower horizon soils simulated a non-precise method of refilling a trench, where no attempt is made to reconstruct the approximate order of soil horizons. Treatments C and D more accurately simulated trench refilling approaches endorsed in contemporary soil reclamation exercises. All of the disturbance treatments were established in the summer (mid-May) of 2014, right after the snowmelt.

Within each plot, soil samples for biological and chemical analysis were collected three times: shortly after the plots were established, at the end of autumn (five months after treatment), and approximately one year after treatment. Soil samples collected for fungal and bacterial community profiling at baseline (T = 0 months) were collected two weeks (May 26, 2014) after the application of soil mechanical disturbance treatments during May 12–14, 2014.

This delay in sampling time was meant to allow soils to re-equilibrate after the initial manipulations. For each treatment plot, each soil horizon (organic or mineral) was sub-sampled three to five times; subsamples were then pooled to represent a single composite sample per plot. Because Treatment B combined both the organic and mineral layers, only one composite sample was obtained at each sampling interval. During sampling of plots with separate horizons, the organic horizon was sampled at a depth of 0–9 cm, and the mineral horizon was sampled from the upper surface of the mineral soil to a depth of 15 cm. Consequently, in each block at each sampling interval, we collected two composite samples from Treatments A, C, and D, and only one composite sample from Treatment B (because the treatment entailed complete mixing of the organic and mineral layers).

Plant Root Simulator (PRS) probes were installed to measure diffusible ionic nutrients in control and manipulated soils. Physical properties were measured using soil moisture-temperature (SMT) probes (see below).

Laboratory analyses

We used several techniques to follow changes in the microbial biodiversity along with some fungal-biomass measurements. We employed sequencing of amplified 16S gene and ITS2 regions from soil DNA extracts to profile bacterial and fungal diversity, respectively. The fungal and bacterial community profiling methods were conducted as described in Section 4.3. Fungal biomass changes were measured by quantifying ergosterol extracted from soil samples collected at zero (for mineral soils only), five months, and 12 months (methods detailed under "Fungal biomass (ergosterol)" below). Assays of potential soil hydrolase activities (methods detailed under "Hydrolytic enzyme assays" in Section 4.3) were applied to samples collected at zero (for mineral soils only), five months, and 12 months. The enzyme activities targeted for assay were 1,4-β-N-acetylglucosaminidase (involved in chitin degradation), cellobiohydrolase (involved in cellulose degradation) and acid phosphatase (involved in mineralization of organic phosphorus compounds). Two additional hydrolases, β-glucosidase (which liberate glucose from cellobiose units originating from cellulose decomposition) and β-xylosidase (involved in hemicellulose decomposition) were assayed for at five and 12 months.

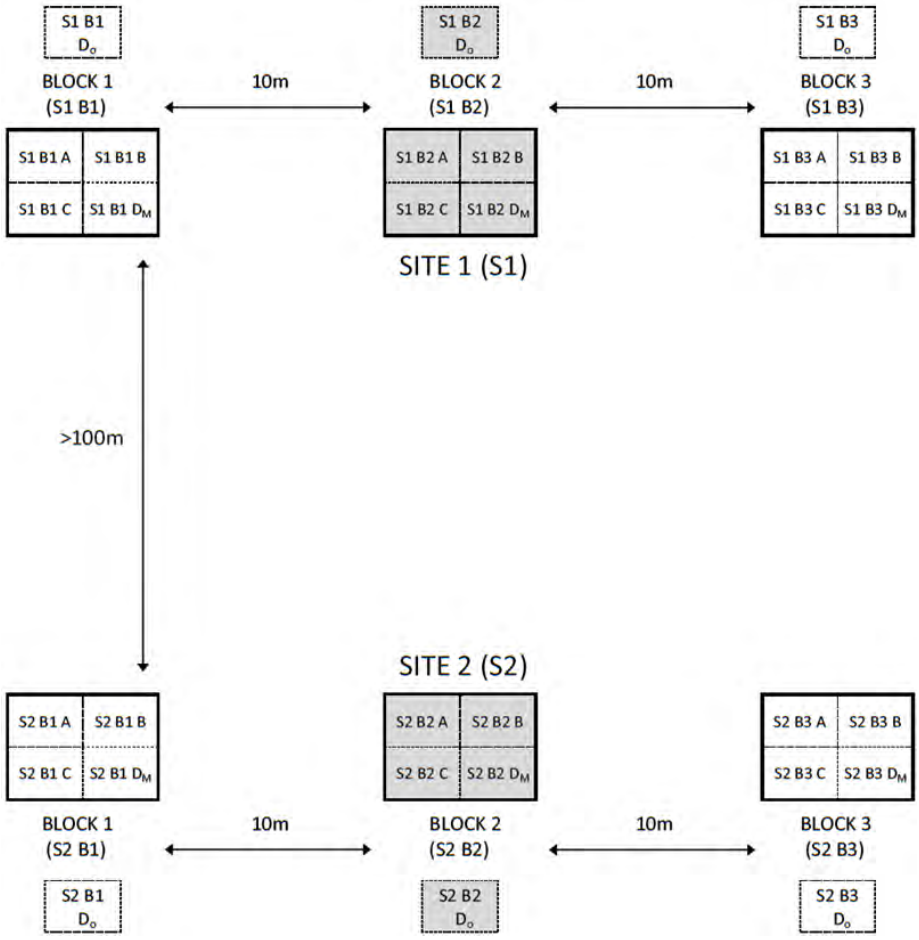

Figure 4-7 – Schematic diagram showing field plot design, treatments (i.e., D_o, A, B, C, D_M blocks (R), and sites (S). Replicates where soli-moisture-temperature probes were installed are indicated in grey.

Fungal biomass (ergosterol)

The main limitation of describing the microbial community by DNA-based methods is that nucleic acids can be isolated from dead or dormant organisms. Although 16S and ITS2 community profiling provide great taxonomic detail about soil microbial community composition, we cannot say with certainty how much of the viable, metabolically active microbial community these datasets represent. Ergosterol is considered a measure of fungal biomass (i.e., the sterol derives from fungal cell membranes and is thought to rapidly degrade in the environment when organisms die). We chose to analyze soil ergosterol to see if "viable fungal biomass" shifted in response to soil disturbance (i.e., are live or active fungal populations increasing or decreasing in response to soil excavation and horizon mixing).

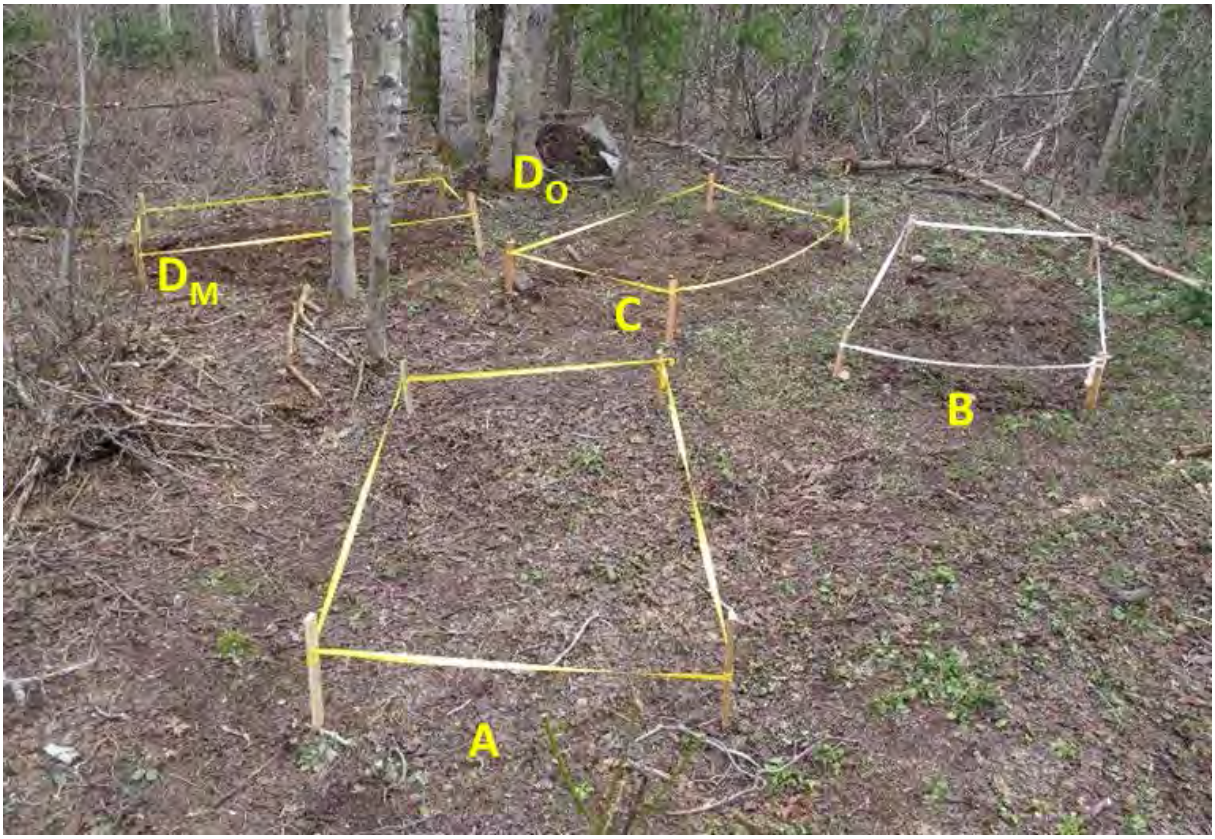

Figure 4-8 – Example of one replicate set up in Summit Lake containing the four treatments (A, B, C, D_O [stockpiled organic] and D_M [bare minerals]) in the summer of 2014.

Microbial membranous components such as fungal ergosterol are considered labile and short-lived in the environment if the organism dies. The recovery of ergosterol from soils is, therefore, considered a good estimator of viable fungal biomass. Soil samples submitted to BC Ministry of Environment (BCMOE) laboratory in Victoria, BC were extracted the sterol from milled soils with a KOH and methanol mixture, transferred the extract to an organic solvent, and then performed quantitative high performance liquid chromatography against standards.

DNA microarray of genes related to biogeochemical cycling (Geochip 5.0 S microarray)

The abundance of functional genes involved in microbially-mediated biogeochemical processes using DNA-hybridization microarray (Geochip 5.0S) was used to monitor for the presence and abundance of genes coding for nutrient cycling in soil DNA extracts from Treatments A and D only, at zero, five, and 12 months. Fluorescence intensities ("hits") to gene

targets in the hybridization array are interpreted as “presence” of genes in functional categories such as carbon degradation, sulfur metabolism, and phosphorus metabolism. As part of the biodiversity monitoring aspect of this Protocol, we chose this method as a means of estimating the stability of nutrient cycling functions in forest soils, in response to disturbance, that does not require detailed bioinformatics expertise for interpretation. This microarray analysis is offered by Glomics Inc. and contains 60,000 DNA probes relevant to “core biogeochemical cycles (C, N, P, and S) as well as common pollutant degradation and metal and antibiotic resistance based on oxidation, reduction, or degradation of those compounds” (Glomics 2014).

The BCMOE performed chemical analyses of the soils from the Summit Lake experiment as in Section 4.3, including total carbon, nitrogen, available phosphorus, soil acidity (pH), gravimetric moisture content, and soil cation exchange capacity. This analysis was performed on samples collected during the Summit Lake field plot experiment at zero, five, and 12 months.

Quantification of extractable nutrients

The BC Ministry of Environment (BCMOE) laboratory in Victoria, BC performed chemical analyses of the soils from the Summit Lake experiment as in Section 4.3 including total carbon, nitrogen, available phosphorus, soil acidity (pH), gravimetric moisture content, and soil cation exchange capacity. This analysis was performed on samples collected during the Summit Lake field plot experiment at 0, 5 and 12 months.

Quantification of diffusible ionic nutrients using Plant Root Simulator (PRS) probes

Unlike soil nutrient quantification methods that rely on harsh chemical methods, the Plant Root Simulator (PRS) probes use ion exchange resins to capture soluble, diffusible ions in soil pore spaces. The use of this device was chosen to provide a better profile of bioavailable ionic soil nutrients (i.e., nutrients that are not immobilized due to organo-mineral complex formation) that are accessible to the plant and microbial communities inhabiting our field sites (Skogley and Doberman, 1996). PRS has a broad range of practical applications, ranging from its utility in management of agricultural nutrients that affect crops to environmental soil research amendments for reclamation (Qian and Schoenau, 2002). PRS probes were installed in the organic surface horizon and the upper mineral horizon to capture the nutrient availability of

nitrogen (nitrate, ammonium), phosphate, calcium, potassium, magnesium, zinc, aluminum, iron, manganese, and sulfate. PRS probes were sampled from late May to early November 2014, at intervals between 3 to 5 weeks for each time point.

Soil moisture-temperature (SMT) probes

The seasonal and temporal warming and water retention in the Summit Lake field plot soils were monitored using a DECAGON EM50 Data Logger and five 5TM Soil Moisture-Temperature (SMT) sensor probes. The data logger was set up to record individual measurements at 30-min intervals (i.e., 336 points per week). The SMT probes measured the physical properties of soil moisture and temperature for three months in 2014 (13 weeks, August 9th – November 6th), and for five months in 2015 (20 weeks, May 12th – September 29) (Figure 4-9). The probes measure volumetric water content (%VWC) and soil temperature (°C). Ten SMT-probes were installed in in one block at each site (Site1-Block#2, and Site2-Block#2). Each block had its own SMT probes installed in each of the plot; four of the probes were inserted into the mineral horizons, at a depth of 8 cm or more. The fifth probe was used to monitor the organic of treatment D that was separated from the subsoil. In 2014, the probes measured the storage pile of the organic horizon; in 2015, the measurement was on the same, previously displaced topsoil from May 2014 that had now been replaced to its original location on top of the mineral horizon of Treatment D in November 2014.

For this report, we have summarized the nutrient, ergosterol and potential hydrolase data as averages (with standard errors) for each treatment level (A–D) across sites and blocks (n = 6). Those data are described with emphasis to overall trends, which can be considered as site descriptions for soil nutrient status. Inferential statistical analysis is in progress and will be reported in peer-reviewed publications to indicate whether significant treatment effects have occurred. For those data derived from DNA-based community profiling (bacterial 16S and fungal ITS2 amplicon sequencing) and functional profiling (GeoChip), we present various multivariate analyses —analysis methods preface the presentation of data products in the relevant sections below.

Figure 4-9 – Soil Moisture-Temperature Sensor Probes (SMT-probes) were inserted at a depth of 8 cm into the mineral horizons of the field plots, in Summit Lake to monitor the volumetric water content (%VWC) and temperature (°C) between different treatment blocks for the period of August-November of 2014 and May-September of 2015.

Results and Discussion

Plant recolonization of the field plots

None of the treatments discouraged plant recolonization of treatment plots, with species of mosses, grasses, *Epilobium*, and members of Asteraceae (i.e.,. *Aster, Hieracium*), some with invasive attributes, being early colonizers. Several indicator plants, often used in the context of biogeoclimatic classification (i.e., *Chimaphila*, *Cornus*, *Linnaea*, *Orthilia*, *Goodyera*), slowly recolonized the plots, possibly from root cuttings or adjacent rhizome recolonization. Germinants from seeds were unlikely during the short time period, but we did not explicitly test or control for this. The shrub stratum, however, represented mainly by *Alnus*, *Vaccinia* species, *Lonicera*, *Rosa* and *Rubus*, repopulated slowly, probably from buried cuttings.

Among treatments Treatment D was the harshest treatment and showed minimal regrowth during the experiment. In the herb stratum, the results varied, but again Treatment D was almost always the lowest in plant species richness. Of relevance to the microbial response during the experiment, the differences noted in plant communities (and their accompanying mycorrhizal guilds) are overall minimal, and would likely contribute minimally to changes in microbial communities.

Fungal biomass (ergosterol)

Soil fungi make up the bulk of the microbial biomass in most soils (Joergensen and Wichern 2008). Their key roles include decomposition, nutrient cycling and in the structural formation of soils. Mycorrhizal associations aid plants in the acquisition of water and nutrients (Taylor and Sinsabaugh 2015). Numerous methods are used to determine the size of the fungal biomass in soils, including the extraction and quantification of ergosterol (Kandeler 2015). Ergosterol is the dominant sterol of many soil fungi and does not occur in plants or bacteria, making it an ideal biomarker for soil fungi (both free living and mycorrhizal; Joergensen and Wichern 2008; Wallander et al. 2013). In our experiment, we compared ergosterol concentrations as influenced by treatment and time since disturbance.

Ergosterol concentrations were much greater (typically 10-fold or more) in the organic layer compared to mineral horizons (Figure 4-10 and Figure 4-11) The main findings from the ergosterol quantification results were that organic horizon soils generally contain higher fungal biomass compared to the mineral soils, and the mixing treatment B resulted in a higher ergosterol content within this treatment compared to A, C and D mineral soils over time. Soil fungi tend to exhibit greater biomass within surface organic horizons as compared to underlying mineral horizons (Prescott and Grayston 2013). In general, soil microbial biomass is greater in surface horizons than in subsurface ones (Voroney and Heck 2015).

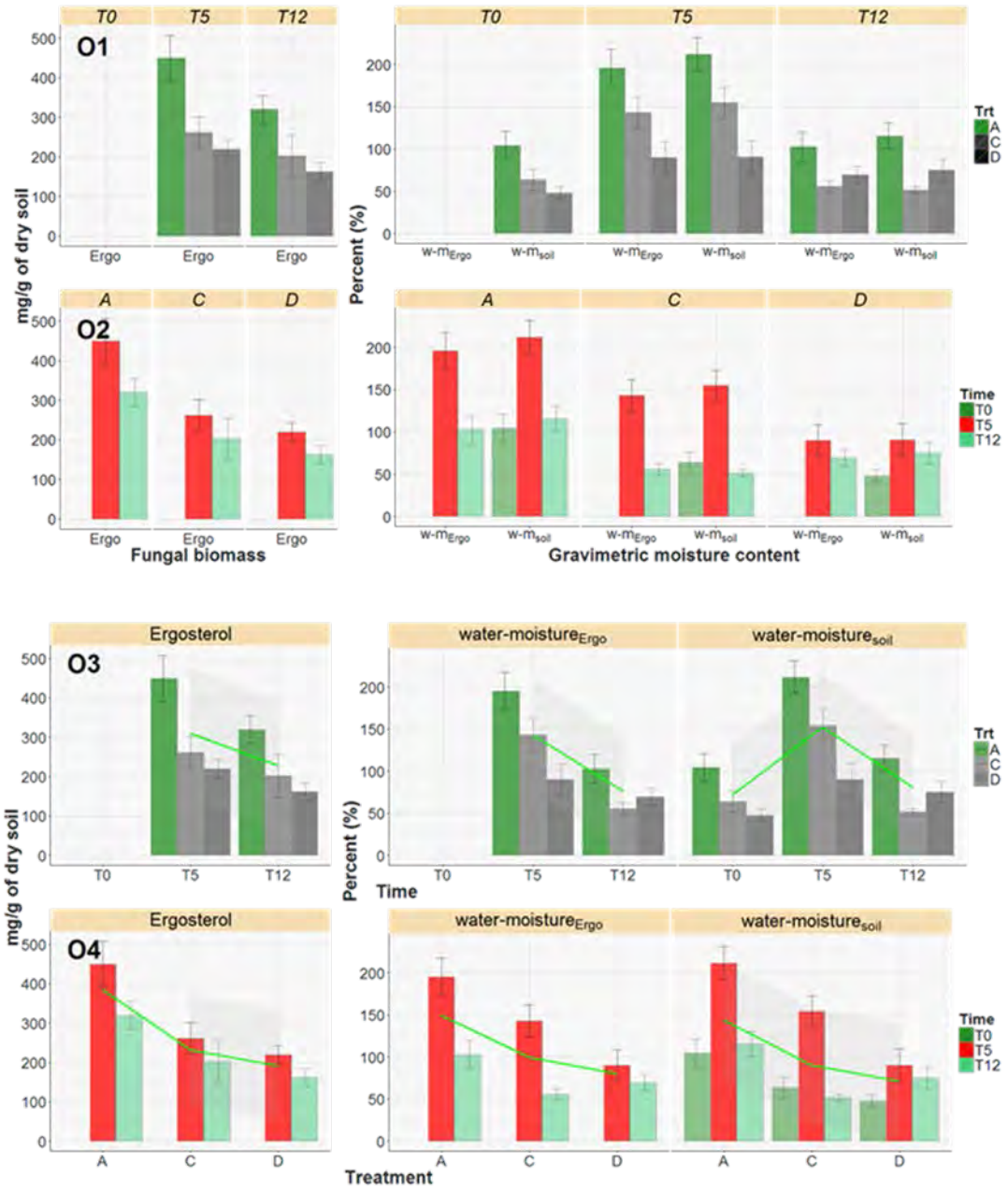

Figure 4-10 – Summary of organic soil ergosterol measurements, soil water content determined during ergosterol quantification (water-moisture$_{Ergo}$) and comparison to gravimetric water content determined at the time of sampling (water-moisture$_{soil}$) for the Summit Lake field experiment. Data is summarized as the mean (±SE) fungal biomass (ergosterol) and soil moisture of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015, across the four treatments for the organic horizon, relative to each sampling time (O1: comparison between treatments at each time-point, O2: comparison within treatments between time-points, O3: comparison between treatments at each time-point with trend line, O4: comparison within treatments between time-points with trend line).

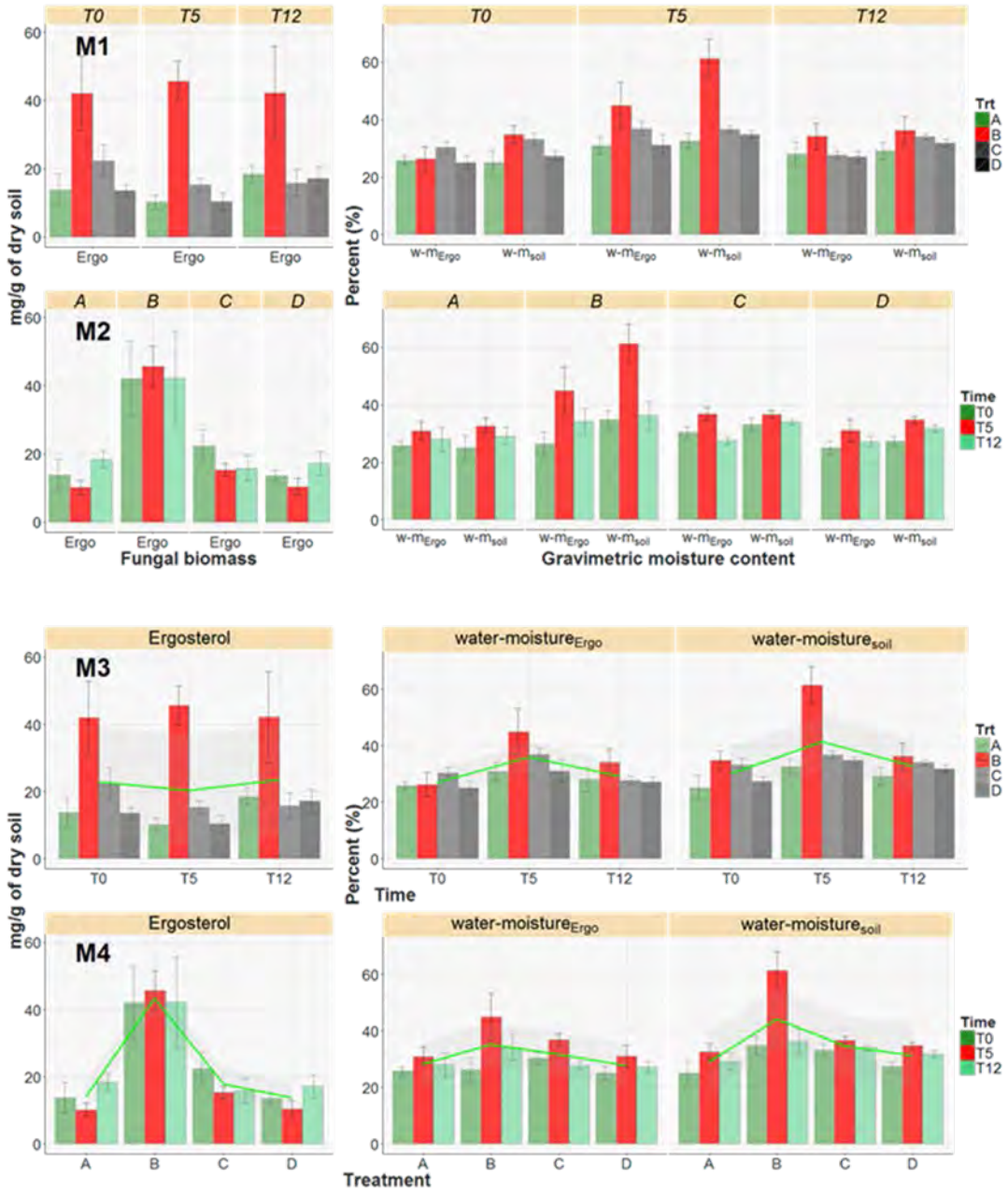

Figure 4-11 – Summary of mineral soil ergosterol measurements, soil water content determined during ergosterol quantification (water-moisture$_{Ergo}$) and comparison to gravimetric water content determined at the time of sampling (water-moisture$_{soil}$) for the Summit Lake field experiment. Data is summarized as the mean (±SE) fungal biomass (ergosterol) and soil moisture of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015, across the four treatments for the mineral horizon, relative to each sampling time (M1: comparison between treatments at each time-point, M2: comparison within treatments between time-points, M3: comparison between treatments at each time-point with trend line, M4: comparison within treatments between time-points with trend line).

The concentration of ergosterol was highest in the undisturbed organic layer of Treatment A, as compared to the organic layers of treatments C and D (Figure 4-11). This makes sense considering that Treatment A plots had greater cover of actively growing plants, which would have provided the microbial community with organic substrates through root turnover and release of organic materials into the rhizosphere. Treatment A would have had a relatively intact mycorrhizal community with intact connections to plant roots to derive photosynthetically-sourced carbon. In contrast, the removal of the organic horizon, followed by either replacement (immediate with treatment C), or by storage and then replacement (treatment D) would have disrupted access to root and rhizosphere carbon inputs. Removal of organic horizons would have also compromised the mycorrhizal community. It is noteworthy that the concentration of ergosterol in the organic layer seemed to vary with water content; that is, greater ergosterol concentrations were associated with greater gravimetric water contents. Water availability may affect the size and activity of the microbial biomass (Voroney and Heck 2015), and will also influence the growth and activity of plant roots, an important source of organic substrates and nutrients for the soil microbial biomass (Jones et al. 2009). There was a decrease in ergosterol in most treatments between five and 12 months. Although the decrease in soil moisture content may be associated with a decrease in soil fungal biomass, it is also possible that microbial biomass and activity in soil collected in May 2015 (i.e., T = 12 months) had not fully recovered from the winter.

Considering mineral layers, the concentration of ergosterol was much greater in soil collected from Treatment B as compared to the other three treatments (Figure 4-11, M1 panel). This is logical as the soil in treatment B was a mix of organic surface soil with underlying mineral soil. The mixing of organic substrates and nutrients into the mineral horizon increased total carbon, nitrogen and sulfur concentrations (Section 4.3). This would have stimulated microbial activity and biomass. Unlike the organic layers, the concentration of ergosterol did not seem to vary greatly with time since treatment.

In order to examine whether “abundant” fungal genera exhibited distributional patterns associated with treatment or time since treatment (Figure 4-12), the aggregated genus-level table of sequence counts (not shown) was sub-sampled to retain genera that: 1) had taxonomic

labeling all the way down to genus level; and 2) had an minimum average relative abundance of 1 % of sequence reads across all sampling replicates, treatments and time-points (n = 126). This data sub-set consisted of 27 genera, with 22 belonging to the phylum Basidiomycota, 3 belonging to the phylum Ascomycota and two belonging to the phylum Zygomycota. The average relative abundance of these 27 genera was calculated for samples groupings of Treatment, layer, and time so that relative abundances could be compared among disturbance regimens (Treatments A, B, C, and D) and sampling events (T = 0 months, T = 5 months and T = 12 months). Functional guilds (i.e., mycorrhizal, pathogenic or saprotrophic) of 27 abundant genera ("abundant" was defined as minimum 1 % relative abundance across all samples and time-points) were derived from assigned designations to these genera when the operational taxonomic units (OTU) table was analyzed using FUNGuild (Nguyen et al. 2015). FUNGuild is a database that takes a fungal OTU table, containing taxonomic classifications, and assigns ecological functions to fungal taxa from an open source database that contains appropriate references to scientific literature that support the guild classifications.

Mycorrhizal genera *Cortinarius, Inocybe, Piloderma* and *Russula* were dominant taxa in intact soil profiles that persist in abundance over time and represent a significant fraction of the soil fungal community in the control (Treatment A) plots, with average relative abundances ranging from 1.1 % (*Inocybe* in Treatment A, organic horizon, T = 0 months) to 15.5 % (*Piloderma* in Treatment A, organic horizon, T = 12 months) in the organic soil layers, and average relative abundances ranging from 8.7 % (*Cortinarius* in Treatment A, mineral horizon, T = 12 months) to 15.5 % (*Russula* in Treatment A, mineral horizon, T = 5 months) in the mineral soil layers (Figure 4-12). With the exception of *Inocybe*, these genera exhibited strong relative abundance across all three sampling periods, indicating that these mycorrhizal taxa are temporally persistent members of the soil microbial community, with appreciable levels of colonization in the upper 20 cm of soils. *Amphinema, Clavulina, Rhizoctonia, Sebacina, Tomentella* and *Tricholoma* were minor mycorrhizal genera in intact soil profiles that persist in abundance over time.

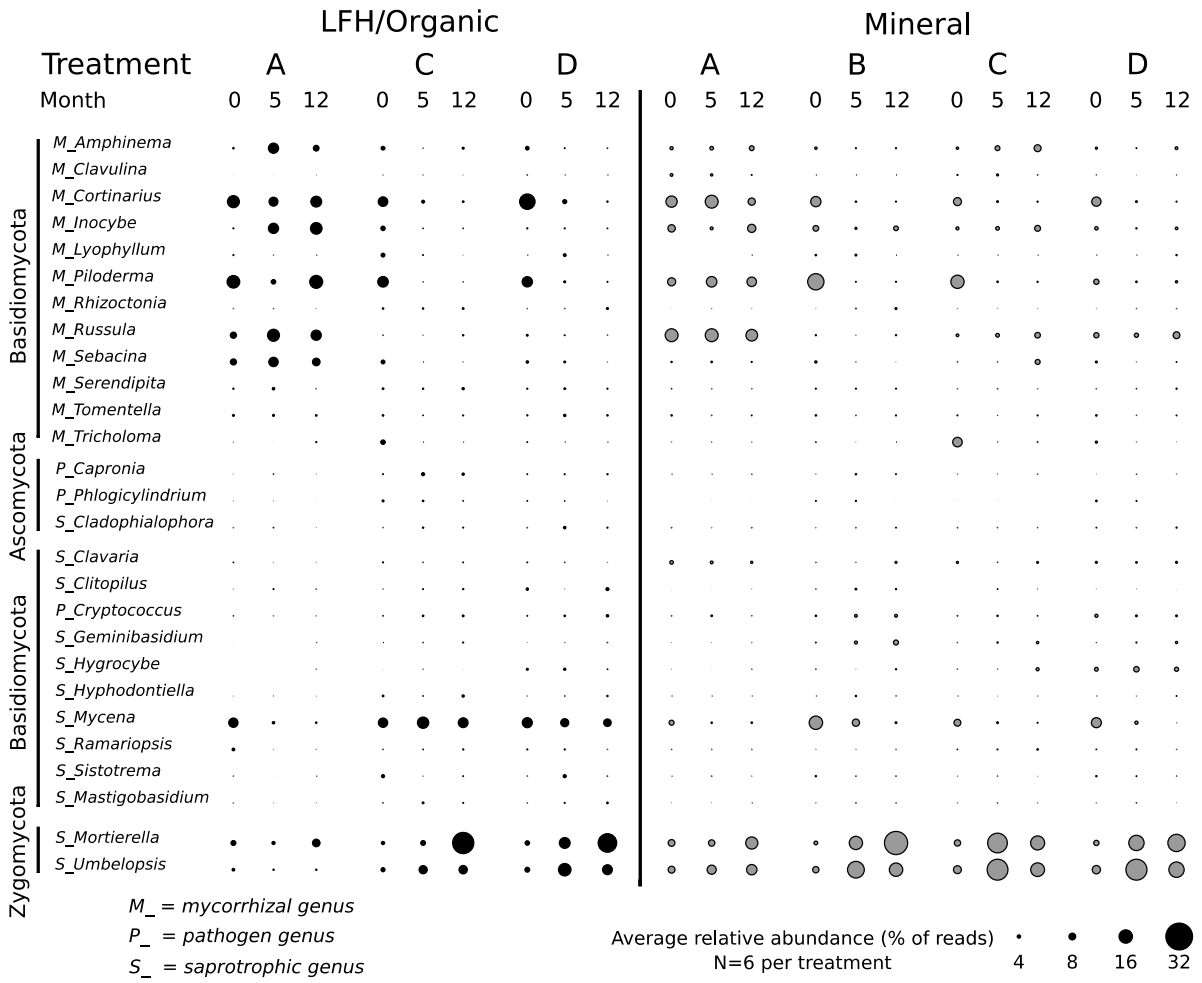

Figure 4-12 – Bubble chart showing average relative abundance of "dominant" fungal genera over 0, 5 and 12 months of the Summit Lake field-plot experiment. The one-letter prefixes preceding the names of each genus designates ecological guild (M_ mycorrhizal, P_ pathogen, S_ saprotroph). Abundance values are proportional to the radius of bubbles in the diagram. Black bubbles represent data for the organic soil samples and grey represents data for the mineral soil samples.

The above mentioned genera are known to engage in symbiotic associations with plants and, with the exception of *Sebacina,* appear to have comparable distributions between control (Treatment A) organic soil samples and control mineral soil samples in this investigation. Non-zero average relative abundances are observed for these genera across all sampling periods for Treatment A organic and Treatment A mineral samples. Together with the dominant mycorrhizal sequence counts for *Cortinarius, Inocybe, Plioderma* and *Russula,* these data

indicate that mechanically undisturbed soils in the Summit Lake area can maintain a stable pool of mycorrhizal fungal communities.

Saprotrophic and pathogenic fungal communities exhibited mostly low and temporally stable abundance in intact soil profiles. Most of the genera with saprotrophic or pathogenic functions did not exceed the average relative abundances observed for the dominant mycorrhizal taxa (i.e., *Cortinarius, Inocybe, Plioderma* and *Russula*) in control (Treatment A) organic and mineral soil samples across all three sampling periods. *Mortierella* and *Umbelopsis*, however, exhibited higher abundances in control mineral soil samples compared to organic samples across time. These two decomposer fungal genera exhibited relative abundances that fall within the range of abundance values observed for *Cortinarius, Inocybe, Piloderma* and *Russula* in Treatment A mineral soils. This suggests that, for intact soils in the Summit Lake field experiment region, saprotrophic communities are stable throughout the year in mineral horizons with the dominant taxa being *Mortierella* and *Umbelopsis.* Further, the relative abundance of these two saprotrophs is comparable to that of the four most dominant mycorrhizal taxa across all sampling periods, implying that strong saprotrophic presence can be observed in soils not subjected to mechanical disturbance. The saprotroph *Clavaria* also exhibited higher average relative abundance in control mineral soils compared to Treatment A organic layers across time. Also notable is the abundance of *Mycena* in Treatment A, which had the highest relative abundance in organic and mineral soils samples at T = 0 months, but lower average relative abundances at later times.

All mechanical disturbance treatments (i.e., B, C and D) reduced the abundance of mycorrhizal community members after T = 0 months. For the mycorrhizal genus *Russula,* abundance appeared to be reduced at T = 0 months (right after treatment application) for all disturbance treatments for both levels of soil sampling (organic and mineral) compared to Treatment A. A similar trend was apparent for *Sebacina*, another mycorrhizal genus. These results suggest that certain mycorrhizal fungi show early sensitivities to mechanical disruption of soils compared to other co-occurring fungi (e.g., *Piloderma* or *Cortinarius*) with similar functional guild membership.

A stronger recurring pattern that distinguished the fungal communities of Treatments B, C and D from the intact soil profiles (Treatment A) was the persistent reduction in average relative abundance of several mycorrhizal genera, apparent at five and 12 months post-disturbance. These persistent reductions in relative abundance were seen in both organic and mineral soil layers for *Amphinema*, *Cortinarius*, *Inocybe*, *Piloderma*, *Russula* and *Sebacina*. Other mycorrhizal taxa listed in Figure 4-12 did not exhibit a disturbance-associated reduction in relative abundance, possibly owing to their comparatively low presence in relation to the aforementioned six genera. The relative abundance of these minor mycorrhizal community members appeared comparable between organic and mineral soil samples for all treatments, in addition to appearing stable in relative abundance over all time-points.

Disturbance increased the average relative abundance of the saprotrophic genera *Mycena*, *Mortierella*, and *Umbelopsis* (T = 0 months compared to five and 12 months). As observed in the microcosm experiment (Section 4.3), the mechanical disturbance of soils (Treatments B, C and D) resulted in noticeable increases of the decomposer fungi *Mortierella* and *Umbelopsis*. The increases in relative abundance for these two genera were more pronounced for the mineral soils compared to organic soils, although the average relative abundance in Treatments C and D organic samples is comparable to values observed for mineral soil at 12 months. Responses of these two saprotrophic genera appeared to differ post-disturbance (Treatments B, C and D) depending on their location in the soil profile, such that *Umbelopsis* relative abundance remained lower compared to *Mortierella* in organic samples, but was nearly equivalent in relative abundance to *Mortierella* in mineral soil samples. Treatment B, which involved mixing of the organic layer into lower mineral soils with accompanying mechanical severing of roots and mycelia via mechanical disturbance, did not show a distinct profile for the relative abundance of *Mortierella* and *Umbelopsis* compared to Treatments C and D, mineral at T = 5 months or T = 12 months. This result suggests that the addition of litter to mineral soils does not enhance colonization of saprotrophic taxa, at least for *Mortierella* and *Umbelopsis*. The genus *Mycena* exhibits an increase in average relative abundance for disturbance Treatments C and D, specifically in the organic samples for these treatments when compared to control (Treatment A) organic layers. The increased relative

abundance for *Mycena* persists in the C and D treatments when compared to the control at 12 months.

Low "recovery" of relative abundance by mycorrhizal taxa was observed for disturbance treatments (B, C and D) in mineral soil layers, but responses were variable among disturbance treatments. For several mycorrhizal genera, increases in average relative abundance were observed in mineral soil samples for disturbance treatments (B, C and D) albeit at lower levels than the observed relative abundances across time for Treatment A. Examples of this included the increased average relative abundance of *Amphinema* in Treatment C, mineral soils (T = 5 months and T = 12 months), as well as *Russula* in Treatments C and D mineral soils (T = 5 months and T = 12 months). With *Russula,* average relative abundance in Treatment B remains low and relatively constant at the 5-month and 12-months compared to C and D mineral soils. Treatment B was distinct from C and D because the organic layers of soils were mixed into lower mineral soil. Because it was also apparent that *Russula* did not show increases in relative abundance at T = 5 months and T = 12 months in Treatments C and D organic samples, the presence of organic soil in the mineral horizon of the B treatment may have inhibited colonization by this mycorrhizal genus, although the reasons for this are unclear. The genus *Inocybe* exhibited a similar pattern for shifts in relative abundance across all treatments in the mineral soil samples, in that average relative abundance decreased at 5 months and increased again at 12 months. Based on the similar response of this genus across all treatments, this shift may simply reflect seasonal variation in *Inocybe* abundance and also indicate that this seasonal variation is insensitive to the disturbance treatments applied during this field study. Saprotrophic basidiomycetes exhibited increased relative abundance in disturbed mineral soil layers in Treatments B and D. With the exception of *Mycena, Mortierella* and *Umbelopsis,* the majority of saprotrophic taxa exhibited relative average abundances less than 10 % in organic and mineral soils across all treatments and sampling periods. Several saprotrophic basidiomycetes, however, appeared to have elevated relative abundances in Treatments B and D, mineral soil samples. For instance the genera *Cryptococcus* and *Geminibasidium* increased in average relative abundance at T = 5 months and T = 12 months in Treatment B compared to all other.

Storage of organic soils from zero to five months (Treatment D) did not affect the distribution pattern of abundant fungal taxa, while replacement of organic layer on plots after five months reversed the trends towards post-disturbance loss of *Cortinarius*, *Inocybe*, *Piloderma*, *Russula* and *Sebacina* in the mineral soil layer. Treatment D was meant to simulate a brief period of topsoil segregation away from the original soil profile, similar to a salvage pile that would be created during RoW construction. Dominant soil fungal genera were similar between Treatments A and D indicating that distributions of abundant fungal taxa were not sensitive to the stockpiling of organic soil. Also the exposure of mineral soil with no organic cover in Treatment D plots did not produce any marked differences in the mineral soils when compared to the control (Treatment A) or the other disturbance plots (Treatment C).

Comparison of fungal communities using sequence counts for 234 “genus-classified ITS2” groupings showed that fungal community structure remained relatively constant for control (Treatment A) samples, while non-control samples (Treatments B, C and D) exhibit significant dissimilarity compared to control plots at later time points (Figure 4-13).

Based on the NMDS analysis, there was higher similarity between the T = 0 months community composition for Treatments B, C and D to controls at T = 0 months, implying that holistic shifts in community composition were not immediately induced by the disturbance treatments. The control (Treatment A) sample data points for five and 12 months shifted away from the cluster of T = 0 sample data points (for all treatments) but occupy ordination space opposite to the cluster formed by disturbed (Treatments B-D) data points collected at later time-points (Figure 4-13). The clustering of disturbed soil sample unit data points (Treatment B, C, and D) for later time points shifting away from baseline (T = 0 months) data points for the same treatments reflects that community dissimilarity is being driven by the disruption of dominant mycorrhizal genera (Figure 4-12), which primes the soil environment (likely providing available substrates from mycorrhizal necromass as well as increased plant carbon leaking from disrupted mycorrhizal networks) for the observed increases in primarily two proficient saprotrophic colonizers.

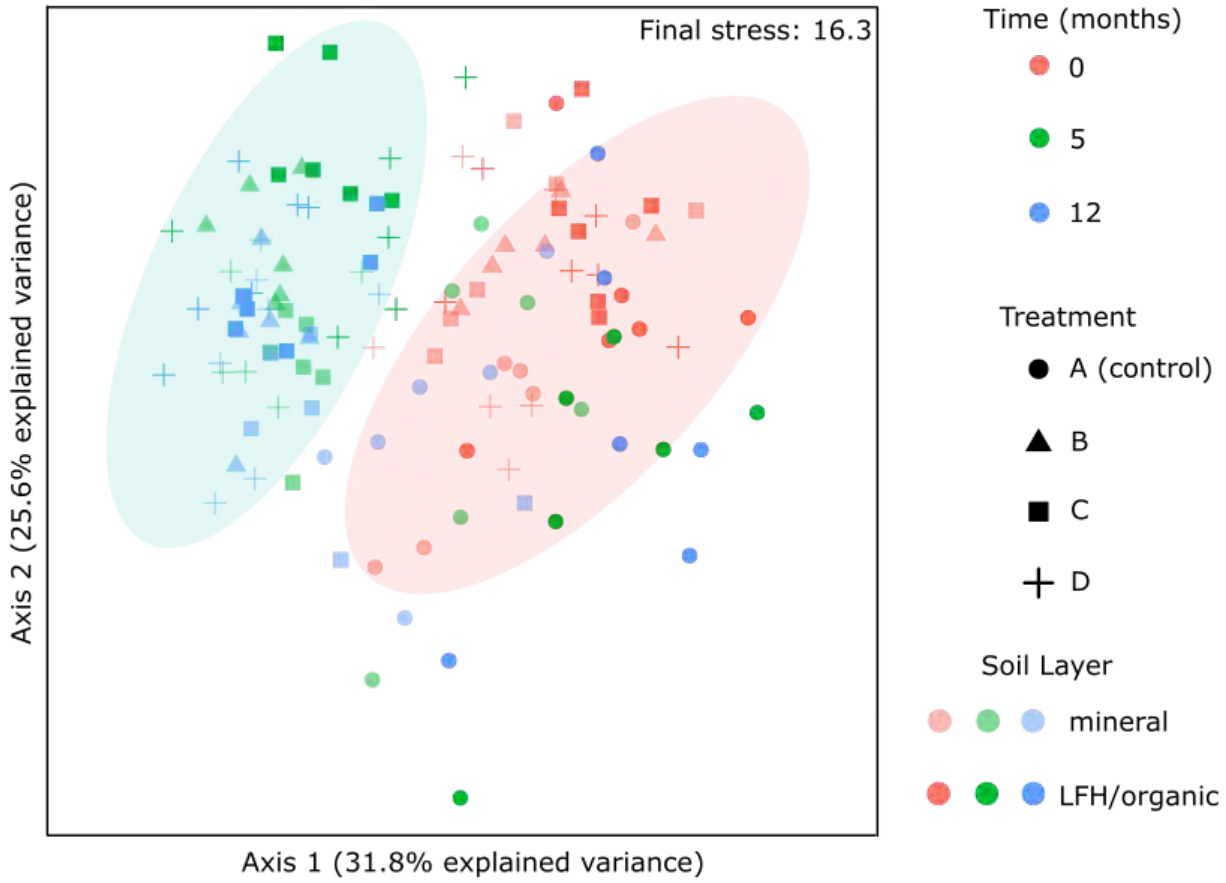

Figure 4-13 – Non-metric multidimensional scaling (NMDS) ordination using a Sorensen distance measure of all individual samples (n = 126) at 0, 5 and 12 months from the Summit Lake Field Plot Experiment, comparing fungal community structure derived from ITS2 amplicon sequencing data categorized at the genus level (comparing relativized abundance of 234 genera). The pink oval indicates ordination space predominantly occupied by samples corresponding to T = 0 months and "A" control plots, while the green oval indicates ordination space dominated by non-control samples collected at T = 5 months and T = 12 months.

The practical implications of this observation is that "health" of the fungal community in Summit Lake forest soils might be inferred from the abundances of only a few dominant taxa representing the mycorrhizal guild. The limitation in moving forward with this inference as a practical index of soil microbial community health is our lack of data establishing a clear link between levels of mycorrhizal fungi abundance to soil nutrient acquisition processes and re-vegetation performance, both of which are highly relevant outcomes in soils reclamation.

Bacterial community profiling (eDNA)

Bacterial 16S amplicons were generated for all samples. At 12 months, we selected only the organic layers (Treatments A, C and D) and the organic-with-mineral soil mixing treatment (Treatment B) for all plots such that only 24 samples were characterized for the following analysis. The reduced set of samples for T = 12 months was selected to inform the comparison of stockpiled, versus immediately replaced versus undisturbed organic soils, and to gain insight into the effects of organic-mineral soil mixing on bacterial communities over time. We performed multivariate and group-specific analyses of bacterial 16S sequences grouped by taxonomic classification to the "terminal taxon" (i.e., using a taxonomic label inventory of sequence counts where groups of organisms where resolved to the genus level where possible, but also had groups where high-confidence label designations were assigned to only order or family level) instead of finer scale 97 % OTU bins because the data processing associated with the zero, five, and 12-month samples were performed separately. The merging of sequence data across time was accomplished by matching of taxonomic labels generated a list of 526 taxonomic bins with terminal taxonomy labels. The 526 taxonomic bins used for analysis did not include taxa found only in single time-points because the sequence counts were sparsely distributed and extremely low in abundance.

Non-metric multidimensional scaling (NMDS) analysis using a Sorensen distance measure was used to compare the overall similarity of patterns of relative abundance of the 526 taxonomic bins between individual sample units (n = 108) between treatments, soil layers (organic versus mineral) and time-points in the field experiment. For these ordination-based analyses, sample units were mapped onto a 2- or 3-dimensional space where the closeness of data points (sample units) in the ordination space reflects the overall similarities of bacterial communities (specifically the relativized abundances of specific taxa).

To look at whether "abundant" bacterial terminal taxa were exhibiting distributional patterns associated with time-point or treatment, the "aggregated terminal taxa level table of sequence counts" were subseted to only retain taxa that had a minimum average relative abundance of 1 % of sequence reads across all sampling replicates, treatments, horizons, and sampling periods. The results consisted of 25 terminal taxa, with four belonging to the phylum

Acidobacteria, three belonging to the phylum Actinobacteria, three belonging to the phylum Bacteroidetes, seven belonging to the Alpha- division of phylum Proteobacteria, three belonging to the Beta- division of the phylum Proteobacteria, one belonging to the Delta-division of the phylum Proteobacteria, three belonging to the Gamma-division of the phylum Proteobacteria and one belonging to the phylum Verrucomicrobia (Figure 4-14). The average relative abundance of these 25 taxa were calculated for samples groupings of Treatment-Layer-Time (n = 6) so that relative abundances could be compared between disturbance regimens (Treatments A, B, C, and D) and sampling periods (i.e., 0, 5 and 12 months).

Abundant bacterial community members in LFH/organic soils do not undergo significant changes in relative abundance during stockpiling or immediate replacement, compared to control LFH/organic soils. The stability of abundant bacterial terminal taxa (based on the abundance of the DNA signature) of the Summit Lake LFH/organic soils sampled in this investigation mostly persisted at all time-points regardless of whether soils were immediately stockpiled and replaced over mixed mineral soils (Treatment C) or stockpiled for five months prior to replacement over mixed mineral soils (Treatment D). The relative abundance of sequences assigned to the Genus *Afipia* does not remain stable, and a decrease from approximately 8% relative abundance to values less than 0.01% relative abundance can be observed in Treatments A, C and D between zero and five months (Figure 4-14).

This response may reflect the sensitivity of this bacterial organism to sampling as it is only strongly detected at T = 0 months, but difficult to interpret in the absence of functional data that can be strongly linked to this taxonomic affiliation. The initial (T = 0 months) average relative abundance of Genus *Nitrobacter* is lower in the disturbance treatments (C and D) compared to the control, but later (T = 5 months and T = 12 months) increases in relative abundance for this Genus are similar between all LFH/organic soil treatment levels (Figure 4-14). Overall the most abundant representatives of the bacterial community in LFH/organic layer community composition appear to be unaffected by stockpiling of this soil layer, implying that bacterial ecosystem services should be preserved by the soil salvage measure.

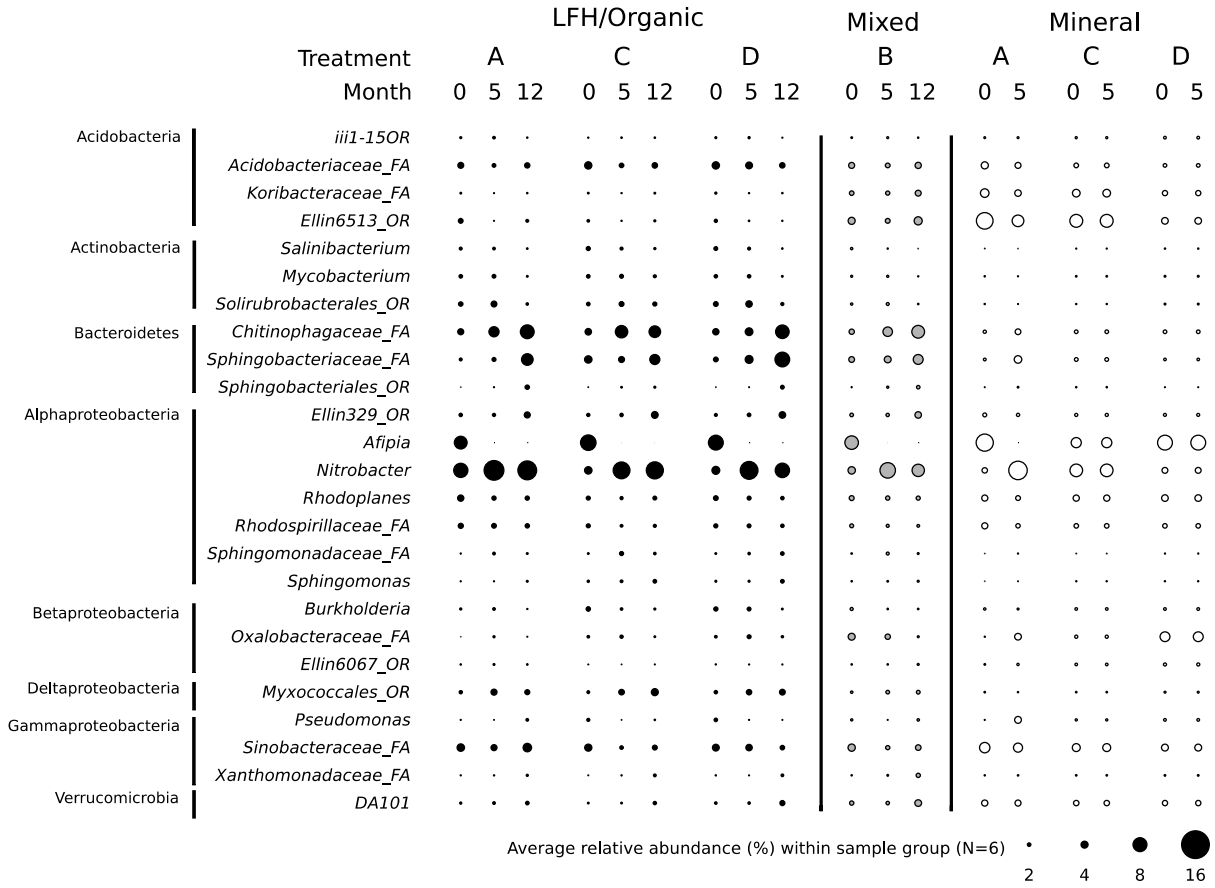

Figure 4-14 – Bubble chart showing average relative abundance of "dominant" bacterial terminal taxa over zero, five, and 12 months (Treatments A,- C,- D- organic soils and Treatment B only for this time-point) months of the Summit Lake field plot experiment. The underscore followed by two capitalized letters in some taxon labels indicates that assignment was only possible to: _OR, order level; _FA, family level. Abundance values are proportional to the radius of bubbles in the diagram. Black bubbles represent data for the organic soil samples, grey represents data for the mixed organic with mineral soil treatment (B) and white represents data for the mineral soil samples.

Mixing LFH/organic soils into the mineral layer (Treatment B) creates a soil bacterial community profile that has group abundances common to both layers but the overall community composition shifts back towards mineral soil. From the highly abundant bacterial taxa, Treatment B closely resembles the LFH/organic soil (Treatments A, C and D) for relative abundance and seasonal shifts in relative abundance for Chitinophagaceae, Sphingobacteriaceae, and *Nitrobacter*. However, the average relative abundances of Acidobacterial taxa Koribacteraceae and Ellin6513 are more similar between Treatment B and

the relative abundances in mineral soil for Treatments C and D. This observation suggests that mechanical disturbance or mixing of upper soil horizons creates conditions in the short term that favor the abundance of these Acidobacterial taxa.

Short-term removal of LFH/organic layers (Treatment D, mineral soil) results in observable increases of *Afipia* and Oxalobacteraceae and observable decreases of Ellin6513 (Figure 4-14). Mineral soil communities for Treatment D (stockpiling of LFH organic soil for five months prior to replacement over mineral soil) had differences in the abundance of the above-mentioned taxa that distinguish it from Treatments A and C (undisturbed and disturbed with immediate replacement of LFH/organic soils, respectively). This result indicates that some changes in the bacterial community might distinguish "uncapped" (mineral soils without LFH/organic cover) from "capped" soils. Further, the differences observed for relative abundance of *Afipia*, Oxalobacteraceae and *Ellin6513* in Treatment D are apparent at zero months, which raises the question as to whether these taxonomic abundances reflect only initial differences in these bacterial populations relative to Treatments A and C or whether they reflect fairly immediate effects of disturbance or absence of LFH cover (recall that T = 0 months sampling of soils occurred two weeks after the installation of plots and disturbance treatments (Figure 4-14).

An interesting comparison can be made between Treatment B (organic soils mixed into lower mineral layer) and Treatment D, mineral soils, because both treatments lack an organic cover. Treatment B relative abundances of *Afipia,* Oxalobacteraceae and *Nitrobacter* are more similar to Treatments A and C mineral layers but less similar to Treatment D (Figure 4-12), suggesting that the presence (or absence) of partially decomposed organic matter is a major determinant for colonization or stability of these taxa in soils. Furthermore, mineral soils of Treatments C and D were the only sample groups, where *Afipia* did not decrease from initial relative abundance. These results indicate that re-construction of excavated soils with defined lower mineral and upper organic regions are likely important habitat attributes for this bacterial genus.

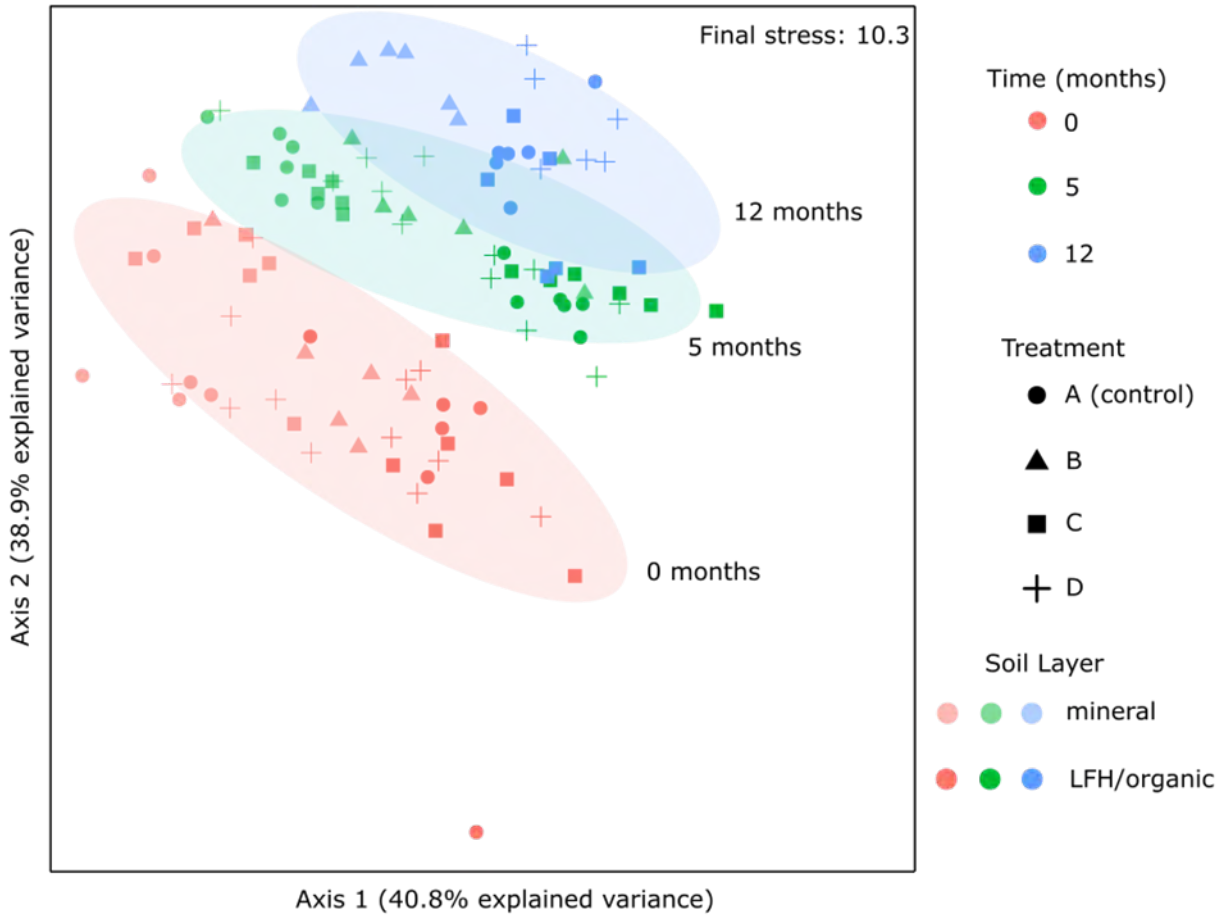

Figure 4-15 – NMDS ordination (using a Sorensen distance measure) comparing bacterial community composition using 526 bacterial terminal taxa between sample units (n = 108 sample units defined by treatment (A-D), soil layer (organic or mineral), site (1 or 2), block (1-3), and time (0, 5, or 12 months). The ovals indicate clustering of data points (sample units) corresponding to time-points in the field experiment.

Dominant clustering patterns from NMDS analysis of 526 bacterial terminal taxa indicated that bacterial communities in all treatments shifted over time, and organic versus mineral soil bacterial communities were weakly distinguishable from each other (Figure 4-15).The optimal NMDS ordination (using PC-ORD with a Sorensen distance matrix) was 3-dimensional, with 79.7 % of variance in the data explained by Axis 1 and Axis 2 (Figure 4-15). Consequently we only present these axes, which sufficiently reveal the main clustering patterns in the bacterial community data. Three main clusters that separate along Axis 2 indicate that

sample units within each time-point are more similar to each other than among sampling periods.

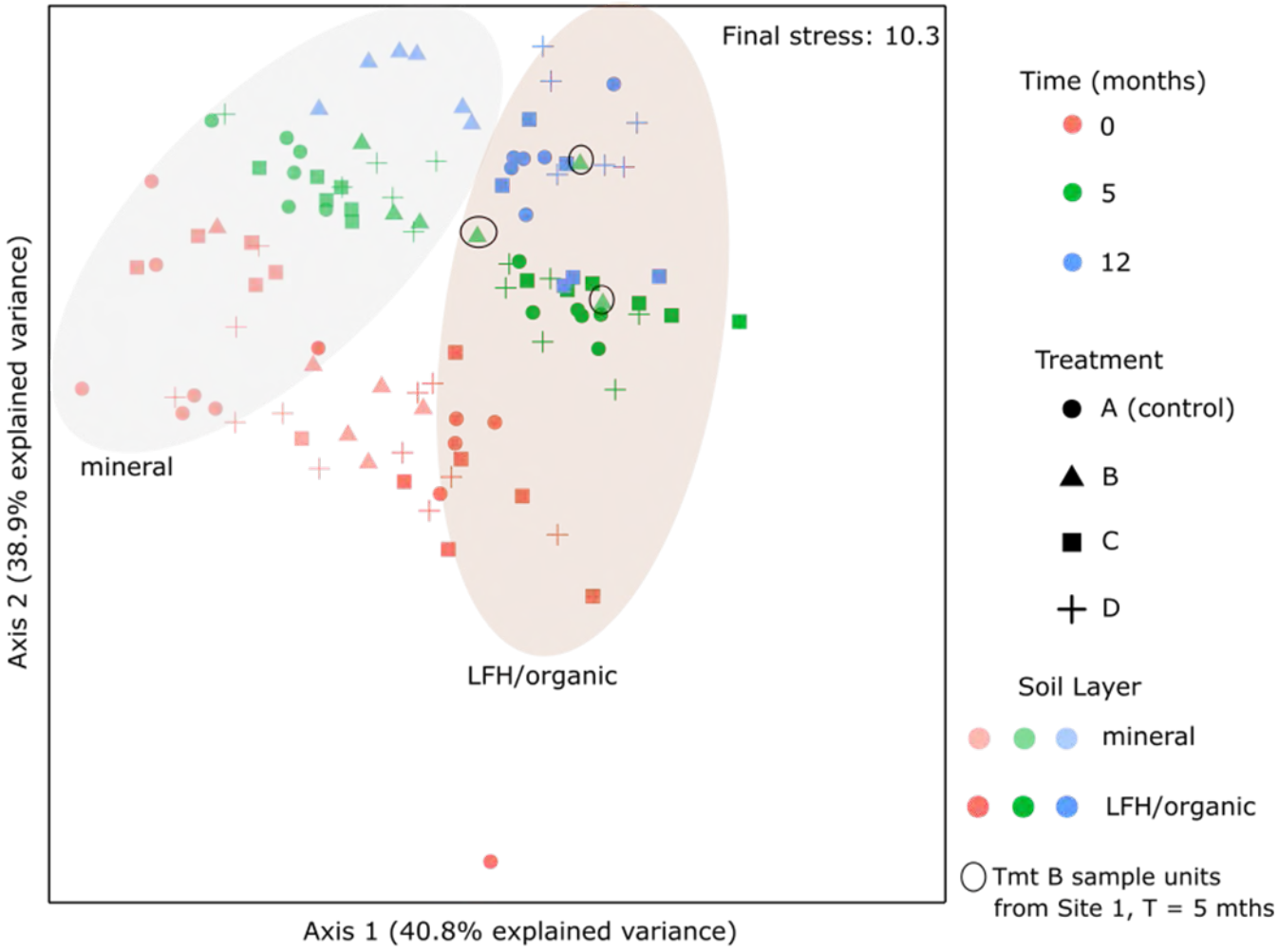

Figure 4-16 – NMDS ordination (using a Sorensen distance measure) to compare bacterial community composition using 526 bacterial terminal taxa sample units (n = 108 sample units defined by treatment (A-D), soil layer (organic or mineral), Site (1 or 2), block (1-3), and time (0, 5, or 12 months)). The brown oval indicates clustering of data points corresponding to organic soils and the grey oval indicates clustering of data points corresponding to mineral soils. Black ovals highlight the Treatment B sample units from Site 1, that have bacterial community compositions which resemble organic soils more closely at T = 5 months. The location of all Treatment B sample units for t = 12 months (blue triangles) indicates that all samples from this treatment level eventually shift in bacterial composition to resemble communities from mineral soils.

The clusters corresponding to sampling periods in Figure 4-16 suggests that independent of physical disturbance, forest soil bacterial communities continuously vary in their distributions of organisms over time, and the NMDS clusters likely represent seasonal fluctuations in abundances of different soil bacteria. Samples from all periods separated weakly along axis 2 to form clusters defined by organic soil bacterial communities and mineral soil bacterial communities. The Treatment B sample units (pink triangles in Figure 4-16) at T = 0 months are located in ordination space between the organic- and mineral-clusters (Figure 4-16), which indicates that the mixing disturbance applied to these plots effectively removed the vertical partitioning of the soil bacterial community in the upper 20 cm. Interestingly the Site 1, Treatment B sample units at five months occupy ordination space within the organic sample cluster, indicating that the bacterial community members originating from the surface organic layers of the soil become dominant in this mixed profile, possibly as a consequence of seasonal conditions after several months. At T = 12 months, however, all six Treatment B samples units (replicates from Site 1 and Site 2) were again observed to occupy ordination space between the organic and mineral clusters (Figure 4-16). Organic layers in the sites for our field experiment were quite thin, not exceeding 5 cm in depth, so the initial abundance of the mineral soil bacterial community may drive this observed “return” of the Treatment B soils (predominantly consisting of soil material from the mineral horizon) to a community composition that resembles lower horizon soil in spite of initial LFH inputs.

The implication of this finding is that organic bacterial communities can be diluted or shifted in composition by the addition of mineral soils, which underlines the need for topsoil salvage to occur during RoW construction, in the interest of preserving bacterial communities of ecological importance within the organic section of a soil profile.

Physically disturbed soils (Treatments B, C and D, organic and mineral) and stockpiled LFH (Treatment D, organic) do not significantly differ in bacterial community composition from control plots across all time-points. No defined clusters consisting of sample units from the same treatment level are observable in the NMDS ordination. Based on our observations, soil handling practices including mixing, disruption of original soil structure and organic soil

stockpiling are minimally consequential to bacterial community composition in Summit Lake area soils immediately after and within a year from the time of original disturbance.

Microbial function

Extracellular enzymes may be associated with living (e.g., membrane-bound) or dead microorganisms, or with the abiotic soil environment (e.g., associated with clay, or soil organic matter; German et al. 2011; Nannipieri et al. 2012; Burns et al. 2013). Enzyme assays are conducted in the laboratory and may be used as a relative index of potential enzymatic activity; they are not used to directly measure enzymatic activity in the field. Our premise was that a diverse and metabolically active microbial community will result in a relatively greater hydrolytic enzymatic activity, as influenced by treatment, soil biotic and soil abiotic factors (Caldwell 2005; Błońska and Januszek 2013; Kivlin and Treseder 2014), keeping in mind that this approach has limitations in that potential activity is not a direct measure of *in situ* enzymatic activity (Nannipieri et al. 2012). The hydrolytic enzymes used in our work were: β 1, 4-N-acetylglucosaminidase: catalyzes hydrolysis of chitin-derived oligomers β 1,4-cellobiohydrolase: catalyzes hydrolysis of glucosidic linkages in cellulose and cellotetraose, releasing cellobiose β 1,4 glucosidase: a starch-degrading enzyme that catalyzes terminal, non-reducing glucose residues β 1,4-xylosidase: degrades short xylan chains to xylose Acid phosphatase: mineralizes organic phosphorus to phosphate under acidic conditions.

In general, potential enzymatic activities were greatest for the organic layers in Treatments A, C and D, and least in the mineral layers and in the mixed soil from Treatment B (the exception being cellobiohydrolase at T = 5 months). This trend was expected as nutrient concentrations and ergosterol concentrations were generally greater in the surface organic layers. We expected that potential enzyme activity would be greatest when fungal biomass (i.e., ergosterol) was large, and when soil moisture conditions were optimal for microbial activity (Table 4-1; Figure 4-17).

Table 4-1 – Comparison of mean ± SE soil potential hydrolase activities, total fungal biomass (ergosterol) and gravimetric moisture content values for organic (O) and mineral (M) soils, grouped by treatment level and sampling period. Data were collected from samples taken at the experimental field plots at Summit Lake in 2014 and 2015, for each treatment, horizons and sampling period as indicated in the table.

				Methylumbelliferone (MUB) enzyme-assays				*Ergosterol, Gravimetric moisture content*		
	Hor	Trt	n	*Time 0*	*Time 5*	*Time 12*		*Time 0*	*Time 5*	*Time 12*
				mean ± SE	mean ± SE	mean ± SE		mean ± SE	mean ± SE	mean ± SE
4-N-Acetyl-Glucosaminidase	O	A	6	642.5 ± (115.2)	2562.5 ± (954.1)	1178.9 ± (329.2)	Gravimetric-water (%)	104.4 ± (16.5)	211.9 ± (19.4)	115.5 ± (15.2)
	O	C	6	400.5 ± (46.6)	2195.4 ± (898.7)	720.2 ± (92.3)		63.9 ± (12.5)	154.6 ± (18.1)	51.6 ± (4.1)
	O	D	6	498.9 ± (82.3)	1955.1 ± (872.3)	793.7 ± (263.7)		47.7 ± (7.4)	90.4 ± (19.5)	75.2 ± (12.9)
	M	A	6	127.8 ± (5.0)	303.1 ± (44.9)	209.4 ± (15.4)		25.1 ± (4.2)	32.6 ± (2.7)	29.1 ± (3.0)
	M	B	6	250.9 ± (26.9)	395.0 ± (110.7)	417.0 ± (29.6)		34.8 ± (3.1)	61.3 ± (6.7)	36.3 ± (4.7)
	M	C	6	166.4 ± (22.9)	418.4 ± (76.1)	286.7 ± (35.1)		33.2 ± (2.2)	36.6 ± (1.3)	34.1 ± (1.0)
	M	D	6	147.4 ± (18.7)	391.5 ± (107.9)	268.2 ± (42.4)		27.4 ± (1.8)	34.7 ± (1.2)	31.8 ± (1.3)
Cellobiohydrolase	O	A	6	131.7 ± (44.7)	516.4 ± (204.5)	394.6 ± (123.9)	Ergosterol (mg/g)	NA	449.3 ± (57.8)	319.8 ± (35.1)
	O	C	6	99.6 ± (9.1)	1352.2 ± (686.6)	363.5 ± (57.2)		NA	261.9 ± (40.2)	202.3 ± (53.6)
	O	D	6	92.2 ± (39.6)	768.1 ± (379.9)	366.5 ± (110.7)		NA	219.6 ± (23.5)	162.0 ± (22.6)
	M	A	6	22.8 ± (7.4)	51.6 ± (22.0)	49.1 ± (12.9)		13.8 ± (4.6)	10.2 ± (2.0)	18.5 ± (2.6)
	M	B	6	37.4 ± (12.3)	413.6 ± (128.7)	1113.7 ± (437.9)		42.0 ± (10.8)	45.7 ± (5.9)	42.2 ± (13.6)
	M	C	6	24.8 ± (8.1)	143.8 ± (35.2)	103.7 ± (19.4)		22.3 ± (4.7)	15.3 ± (1.9)	15.8 ± (3.8)
	M	D	6	21.5 ± (6.8)	114.2 ± (31.5)	109.4 ± (15.0)		13.6 ± (1.7)	10.4 ± (2.5)	17.1 ± (3.4)
β-glucosidase	O	A	6	NA	2492.1 ± (536.3)	2016.8 ± (384.3)	Grav. water (*Ergo*) (%)	NA	195.5 ± (22.5)	102.8 ± (16.9)
	O	C	6	NA	4128.1 ± (1638.6)	1487.2 ± (174.6)		NA	143.0 ± (18.6)	55.7 ± (7.0)
	O	D	6	NA	2446.5 ± (985.3)	2205.6 ± (783.3)		NA	90.1 ± (18.2)	69.7 ± (9.8)
	M	A	6	NA	387.9 ± (106.9)	311.3 ± (59.8)		25.8 ± (1.5)	31.0 ± (3.2)	28.0 ± (4.2)
	M	B	6	NA	609.1 ± (87.1)	1626.4 ± (426.1)		26.3 ± (4.2)	44.9 ± (8.3)	34.2 ± (4.5)
	M	C	6	NA	603.4 ± (82.3)	471.2 ± (59.7)		30.3 ± (2.1)	36.9 ± (2.3)	27.7 ± (1.5)
	M	D	6	NA	314.2 ± (37.8)	515.7 ± (72.3)		25.1 ± (2.4)	31.1 ± (3.9)	27.1 ± (2.0)
β-xylosidase	O	A	6	NA	358.7 ± (89.8)	243.6 ± (41.7)				
	O	C	6	NA	524.4 ± (242.6)	173.4 ± (42.8)				
	O	D	6	NA	322.3 ± (142.0)	232.9 ± (89.1)				
	M	A	6	NA	86.0 ± (39.7)	45.0 ± (12.4)				
	M	B	6	NA	114.3 ± (18.2)	213.4 ± (51.8)				
	M	C	6	NA	127.0 ± (30.3)	76.5 ± (7.3)				
	M	D	6	NA	109.0 ± (32.0)	60.8 ± (9.0)				
Acid Phosphatase	O	A	6	2219.4 ± (306.3)	5941.8 ± (1717.9)	6293.7 ± (1006.5)				
	O	C	6	1532.0 ± (166.1)	5409.0 ± (1706.0)	3480.1 ± (423.3)				
	O	D	6	1308.1 ± (130.7)	3003.2 ± (965.6)	3190.2 ± (743.9)				
	M	A	6	564.8 ± (49.9)	1542.6 ± (339.1)	1136.4 ± (52.2)				
	M	B	6	984.2 ± (72.0)	1151.2 ± (220.8)	2022.5 ± (144.5)				
	M	C	6	617.5 ± (50.5)	1527.0 ± (317.5)	1267.1 ± (92.0)				
	M	D	6	534.8 ± (62.9)	1806.9 ± (481.1)	1101.1 ± (124.6)				

*MUB measurements in nmol per hour/gram of dry soil

For Ergosterol/WaterErgo at Time 0:
Trt.A-M, n=2
Trt.B-M, n=5
Trt.D-M, n=4

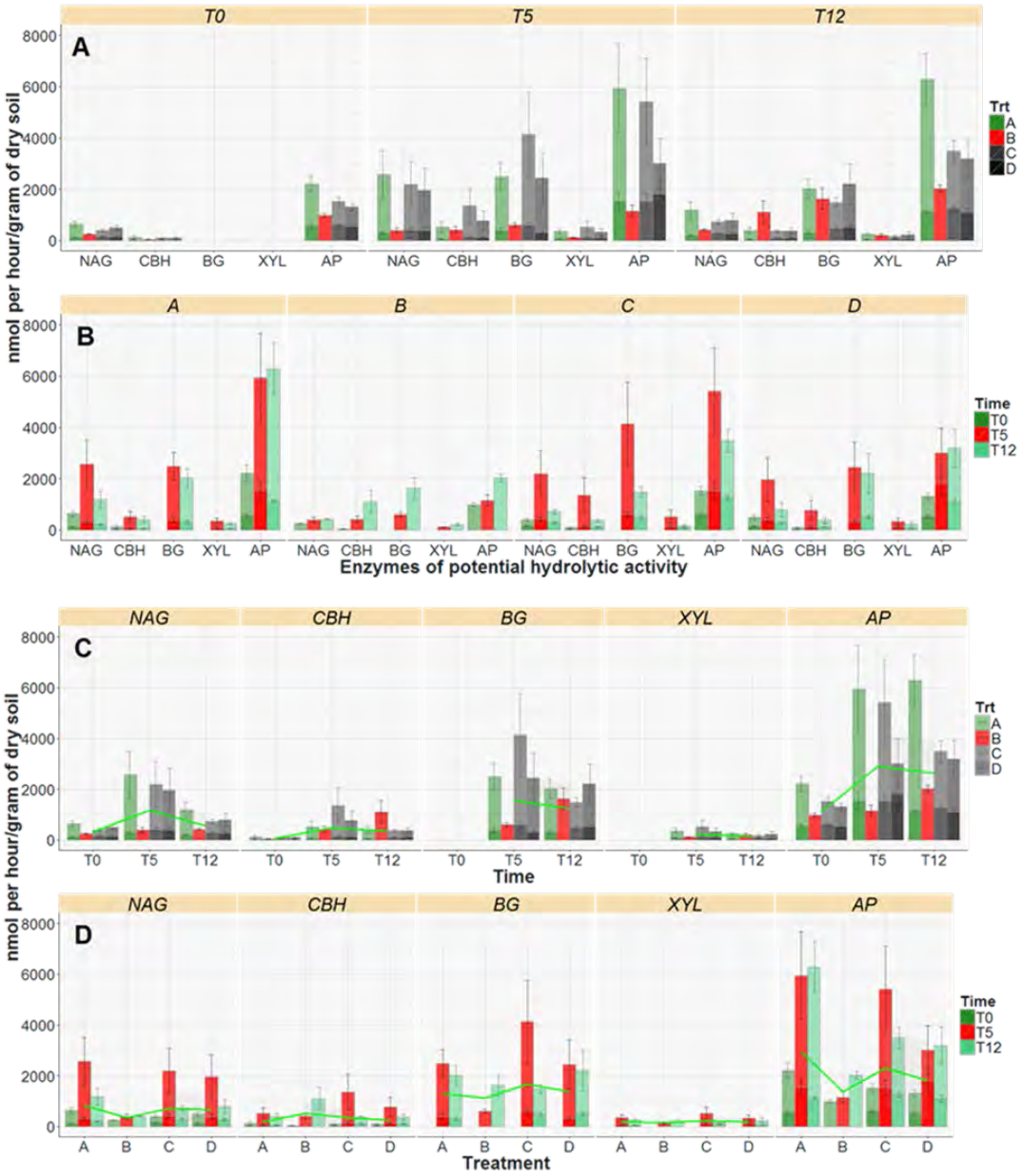

Figure 4-17 – Mean (±SE) potential hydrolase activities of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015. Panel-A: comparison between treatment levels at each sampling period. Lighter shade bars indicate measurements for organic soils and darker shaded bars indicate measurements for mineral soils, Panel-B: comparison within treatment levels across sampling periods. Lighter shade bars indicate measurements for organic soils and darker shaded bars indicate measurements for mineral soils. Panel-C: comparisons from Panel-A split by hydrolase activity with trend line for activity changes over time. Panel-D: comparisons from Panel-B split by hydrolase activity with trend line for comparison of activity between treatment levels. NAG = 1,4-β-N-acetylglucosaminidase, CBH = Cellobiohydrolase, BG = β-glucosidase, XYL = β-xylosidase, AP = Acid phosphatase.

Storage of the organic horizon followed by subsequent replacement (Treatment D), did not appear to diminish potential activity of most enzymes (relative to control treatment A), with the possible exception of acid phosphatase (Table 4-1; Figure 4-17). Peak enzyme activities for most treatments tended to be at the five months (most common) or 12 months (second most common). The five months sampling was also associated with high moisture contents, high ergosterol concentrations and peak concentrations of ammonium. It is likely that substrate availability and microbial activity was greater in the fall of 2014, than in the spring of 2015. Potential activity of acid phosphatase did not correlate with Bray available phosphorus.

DNA microarray of genes related to biogeochemical cycling (Geochip)

The specific question we sought to answer with microarray analysis was whether storage of the organic layer for five months (Treatment D) changed the genomic potential for litter decomposition activities or other microbial soil ecosystem services (including nitrogen cycle components, phosphorus metabolism and sulfur metabolism). To test this effect, we used DNA extracted from Treatment A (serving as the control) and Treatment D organic soils collected at 0 months (organic soil was "stockpiled"), five months (when the organic soil was returned), and at 12 months. Twelve, 1-ug samples were submitted to Glomics Inc. at the University of Oklahoma for each of the three sampling periods. As mentioned previously the GeoChip 5.0 S array contains 60,000 target sequences from genes with known and/or predicted functions in biogeochemical cycling.

The number of targets (a target refers to the DNA oligonucleotide of known sequence and function used as a hybridization probe for genes in an eDNA sample) detected across 12 samples for each time point was as follows: 27,077 hybridization targets detected for T = 0 months. 40,940 hybridization targets detected for T = 5 months. 38,851 hybridization targets detected for T = 12 months.

The DNA samples for T = 0 months were handled differently prior to submission because they were exchanged from Tris buffer in to water prior to analysis. The DNA samples for later samples were purified and eluted directly into water before analysis. The difference in sample handling may account for the lower number of detected targets for the T = 0 months samples. Furthermore, the total number of hybridization "hits" as listed above includes infrequently

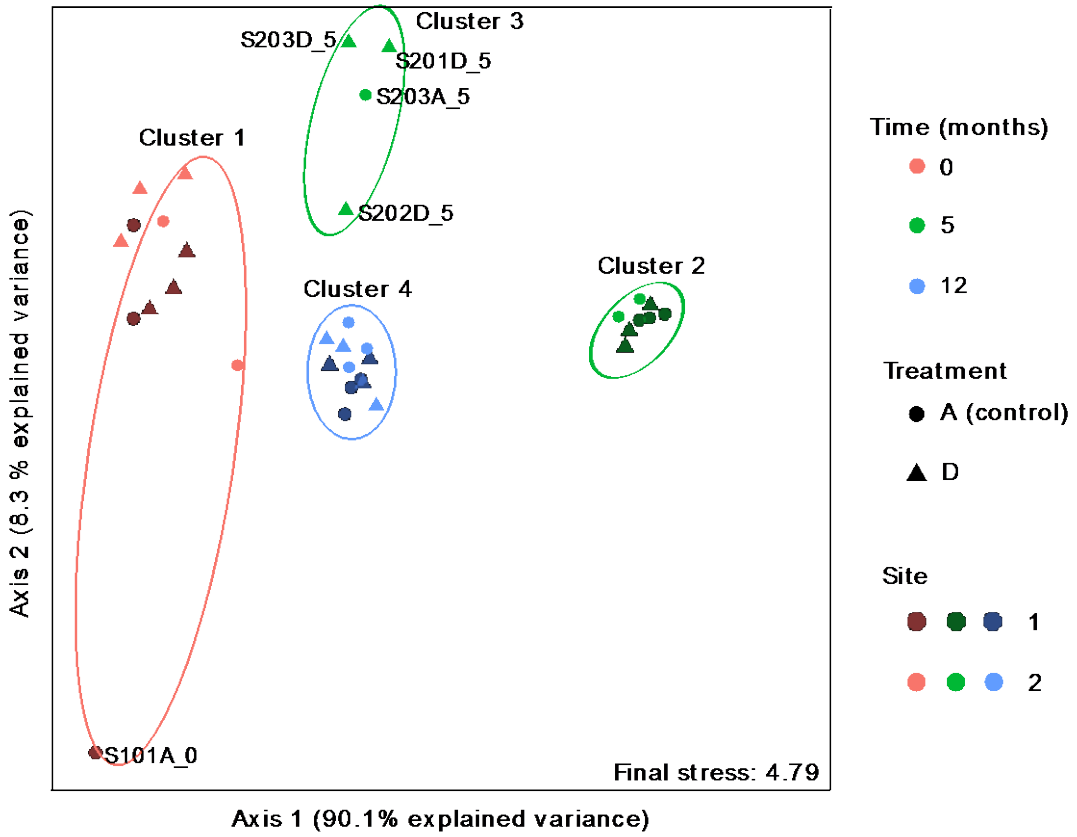

Figure 4-18 – 2-dimensional NMDS ordination using a Euclidean distance measure, for 15,110 Geochip gene targets, detected in a minimum of 75 % of sample units). The final solution for this analysis was 2-dimensional, exhibiting primarily seasonal (time) effects for clustering along Axis 1, and no strong treatment effects within time-points. The division of 5-month sample units (green) into two clusters is difficult to interpret as to whether it indicates a site or treatment difference. Clusters are labeled in accordance with the sample grouping patterns in the hierarchical clustering diagram (Figure 4-19).

detected genes (i.e., detected in less than 50 % of samples for that time-point). To minimize the inclusion of data that might represent genes that are erroneously detected (i.e., not being detected repeatedly in replicates of the same treatment we merged the lists of detected hybridization targets for all three periods to retain gene targets found at least once per sampling period, which resulted in a sub-set of 26,401 detected hybridization targets. Initial multivariate analysis (Figure 4-18) of detected genes between samples used a dataset reduced

from 26,401 targets to 15,110 hybridization targets detected in a minimum of 27 samples out of 36 (i.e., minimum presence in 75 % of 36 samples).

We used an unconstrained ordination approach to determine whether the patterns of relativized functional gene abundances within organic soil samples differed by treatment or by time. Non-metric multidimensional scaling suggested that sample data points cluster by sampling period, and interestingly the five months samples split into two clusters with one cluster (Cluster 3 in Figure 4-19) almost completely composed of Site 2 samples. Although the units of the axes (not shown) in the ordination have no numerical significance, the closeness of sample unit coordinates along Axis 1 (i.e., sample units appear "vertically stacked" in the ordination space) suggest minimal shifts in the functional gene composition for organic soils between five months (specifically for the cluster composed of samples S203_A, S201_D, S202D and S203_D) and 12 months. Because the percent of variation explained by Axis 2 is quite low (i.e., only contributes 8.3 % information to explain sample unit distributions) the coordinate differences of sample units along this axis are very minimal, which indicates that all samples in the ordination exhibited similar patterns for functional gene detection during Geochip analysis. Further, the clustering of sample units when represented as a dendrogram suggests that Clusters 1, 3 and 4 are sub-groups within a larger configuration of functional gene abundances that are more similar to each other than to the Geochip profile of functional genes in Cluster 2.

The ecological significance of the clusters of sample units was tested using the multiple response permutation procedure (MRPP). This test evaluates whether the average within group differences, or distance between sample units in a user-defined cluster of samples, is greater than the differences between pre-defined clusters of sample units (McCune and Grace 2002). The A statistic is interpreted as "ecologically significant" when its value is greater than 0.3 (McCune and Grace 2002). The T statistic describes the separation between clusters where more negative values indicate stronger separation (McCune and Grace 2002).

Although ecologically significant, A-statistic values were generated when comparing Clusters 1 versus 3, Clusters 1 versus 4 and Clusters 3 versus 4, the observed range of T statistics for these pairwise comparisons (12.83–14.69) was narrow, suggesting that these

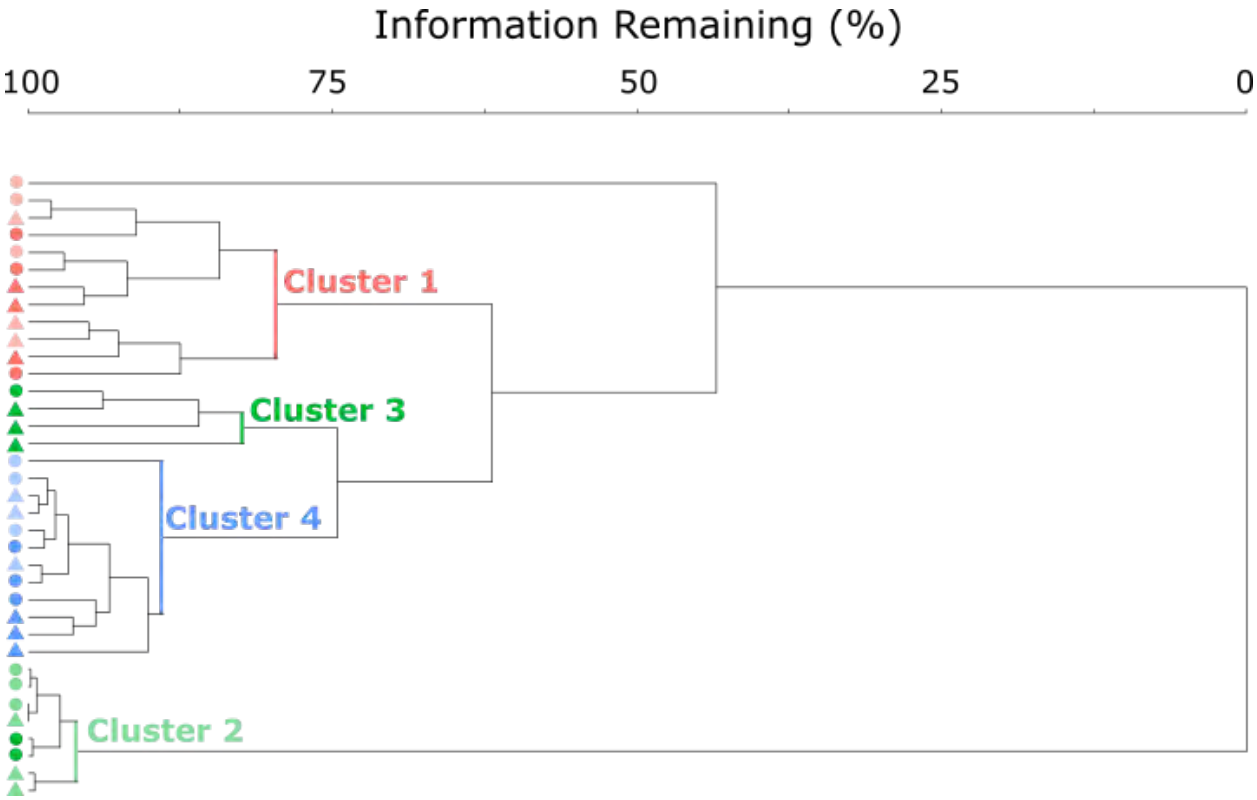

Figure 4-19 – Hierarchical clustering of samples units using a Euclidean distance measure, based on a matrix of 15,110 Geochip hybridization targets. Colours represent time (0, 5 and 12 months), shading represents sites (1 and 2) and shapes represent treatments (A and D) in accordance with the legend in the ordination.

clusters do not exhibit distinct patterns of functional gene distributions from each other (Table 4-2, top). The Cluster 2 versus 3 comparison had the lowest observed T-statistic such that the observed division of T = 5 months samples in the NMDS ordination did not reflect two distinct profiles of genes relating to soil function within this time-point. The low, non-significant A statistics observed for the Clusters 1 versus 2 and Clusters 2 versus 4 comparisons reinforce that shifts in relative functional gene abundance between time-points is probably subtle. Further, pairwise MRPP comparisons of sample groups defined by time-point only (Table 4-2, bottom) revealed highly similar A statistics (0.32–0.4) alongside a narrow range of T statistics (10.39–14.70), which can be interpreted as minimal seasonal variation in soil biogeochemical functional potential.

Table 4-2 – Clusters of sample units defined in Figure 4-19 and groupings by time-point are tested for ecological significance of groupings using the multiple response permutation procedure (MRPP). The A statistic is "ecologically significant" when it is greater than 0.3. The T statistic describes the separation between clusters where more negative values indicate stronger separation.

Comparison	T statistic	A statistic (* if > 0.3)	P value (* if < 0.01)
Group Comparison			
All clusters	-18.33	.56*	0*
Cluster 1 vs 3	-12.83	0.61*	0.000006*
Cluster 1 vs 2	-8.75	0.22	0.000022*
Cluster 1 vs 4	-14.69	0.35*	0.000001*
Cluster 2 vs 3	-.7.30	0.53*	0.000253*
Cluster 2 vs 4	-9.55	0.22	0.000019*
Cluster 3 vs 4	-12.96	0.61*	0.000005*
Time-Point Comparison (0,5,12 months)			
All time-points	-17.60	.43*	0*
0 vs 5	-12.17	0.40*	0.000009*
0 vs 12	-14.70	0.35*	0.000001*
5 vs 12	-10.39	0.32*	0.000034*

Analysis of a "core" set of functional genes detected by Geochip for control (Treatment A) and stockpiled (Treatment D) organic soils

For this analysis, the list of detected Geochip hybridization targets for each treatment, at each time point, were ranked separately in order of highest (i.e., most abundant) to lowest average raw fluorescence intensity across six samples (1 treatment × 2 sites × 3 replicate blocks). The 3,000 Geochip targets with the highest average fluorescence intensities were compared between treatment levels, across sampling periods to determine whether treatment-specific shifts in dominant biogeochemical functional gene composition were occurring, and within treatment levels across sampling period to determine whether the most abundant functional genes remain relatively constant in presence in the environment. The 3,000 gene targets with highest fluorescence intensity, contribute 50–66 % of total fluorescence signal (compared against the total fluorescence intensity for the initial sub-set of 26,401 genes) for each treatment and time grouping (Table 4-3). These results imply that the majority of soil biochemical processes in organic soils were coded for by a small yet abundant set of genes, whose presence in the soil remains fairly constant across seasons.

Table 4-3 – Proportion of hybridization signals represented in sample unit groupings by the "3,000 gene" core set ± 1 standard error. Average % signal was calculated as (Total Fluorescence Intensity from "core 3,000 genes" per Total Fluorescence of 26,401 gene sub-set) x 100 for each sample unit.

Time	Treatment	Average % signal contributed by "core 3,000 genes" ± std. err.	Number of sample units (2 sites × 3 replicate blocks)
0 months	A	65.1 ± 1.4	6
0 months	D	66.2 ± 0.9	6
5 months	A	50.0 ± 0.3	6
5 months	D	51.3 ± 0.6	6
12 months	A	59.7 ± 0.4	6
12 months	D	59.9 ± 0.4	6

Stability of the "3,000 gene core" set between treatments at each time-point between and across time-points

The A versus D Venn diagrams (Figure 4-20) indicate that at 0 months, 5 months and 12 months 2, 862 (95.4 % of the pool of the 3,000 most abundant functional genes for each treatment) functional genes on average, are shared between Treatments A and D. This retention of the abundant genetic repertoire for nutrient cycling indicates that stockpiling organic soils for a 5-month period does not alter the functional gene content. Within treatments, the "core" functional gene repository appears stable to seasonal variation (Figure 4-20).

NMDS analysis with a Euclidean distance measure was used to examine the similarities of the "3,000 gene core" sets between sample units (n = 36). The data matrix used for this analysis contained 3,796 hybridization targets because it included targets that were unique to sample + time-point groupings. All of the genes in this matrix were detected in all of the 36 sample units. Ordination with Euclidean distance measures tends to be more robust when the data matrix used has few zero values (Peck 2010). Therefore, analysis with this "core" gene sub-set should provide a comparison of treatment and temporal effects that can be interpreted with more confidence compared to the larger matrix used for the previous ordinations. The resulting 2-dimensional NMDS solution (Figure 4-21), when compared to the ordinations generated with the matrix of 15,110 Geochip hits, exhibited similar proportions of explained

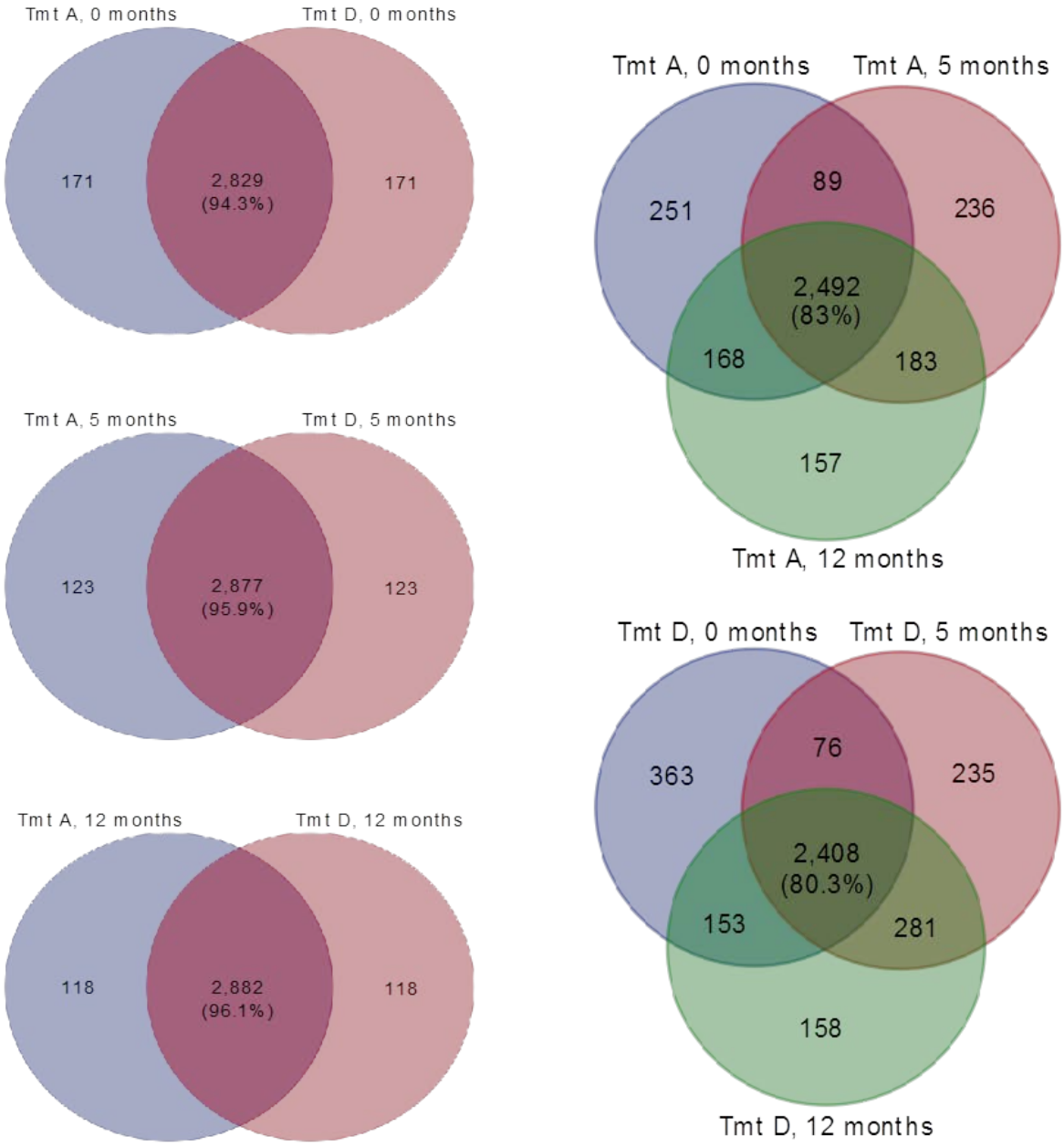

Figure 4-20 – Venn diagrams showing the proportion of the 3,000 most abundant functional genes (as detected by Geochip 5.0 S microarray) that are shared between Treatment A (undisturbed organic soil) and Treatment D (stockpiled organic soil) at each time-point in the Summit Lake field plot experiment (left column). Venn diagrams showing the proportion of the 3,000 most abundant functional genes (as detected by Geochip 5.0 S microarray) that are shared within Treatment A and within Treatment D across time-points in the Summit Lake field plot experiment (right column).

Non-metric multidimensional scaling (NMDS) analysis of "3,000-gene core" set

variance on both axes. Similar patterns of clustering of sample units, including the absence of clusters formed by samples of similar treatment levels (i.e., no differences in functional gene content between undisturbed and stockpiled organic soils) were observed when comparing the 3000-gene NMDS ordination to the 15,110-gene ordination.

The retention of clustering patterns from the smaller matrix indicates that distributions of highly abundant functional genes explain most of the variation (or lack of variation) between sample units. The most noticeable difference between the NMDS ordination of the "3,000-core gene" sets compared to the 15,110-target matrix is the narrower range of ordination space along Axis 1 occupied by Clusters 1, 3, and 4. With the very low percentage of explained variation on Axis 2, the data points representing sample units within these clusters are almost super-imposable in the ordination space, implying that core functional gene composition is stable between treatments and across time-points.

Interestingly, the sample units from T = 5 months (green clusters in Figure 4-21)cluster away from each other with Cluster 3 containing all Site 2 replicates of Treatment D and one Site 2 control (Treatment A) sample unit. Indicator analysis was used to look at which hybridization targets had signal intensities that differentiated Cluster 2 from Cluster 3. This analysis assigns an indicator value (i.e., higher values means they are more strongly associated with a specific cluster) based on the frequency and consistency of detection signal occurring for each specific target in the 3,796-target data matrix. From these indicator lists however, we observed the Cluster 3 samples appear to have a more diverse representation of potential litter decomposition activities (starch, pectin, lignin, hemicellulose, phospholipids, chitin) compared to Cluster 2 samples (starch, pectin, cellulose, chitin). This may be related to differences in litter chemistry between Site 1 and Site 2 or perhaps site differences in microbial community composition.

The clustering patterns in the NMDS analysis indicate real differences in functional gene composition in soils sampled during the Summit Lake field plot experiment, with differences reflecting fluctuations that are time-dependent and site-dependent (i.e., the Cluster 2 versus Cluster 3 differences in Figure 4-21).The majority of hybridization targets, however, were

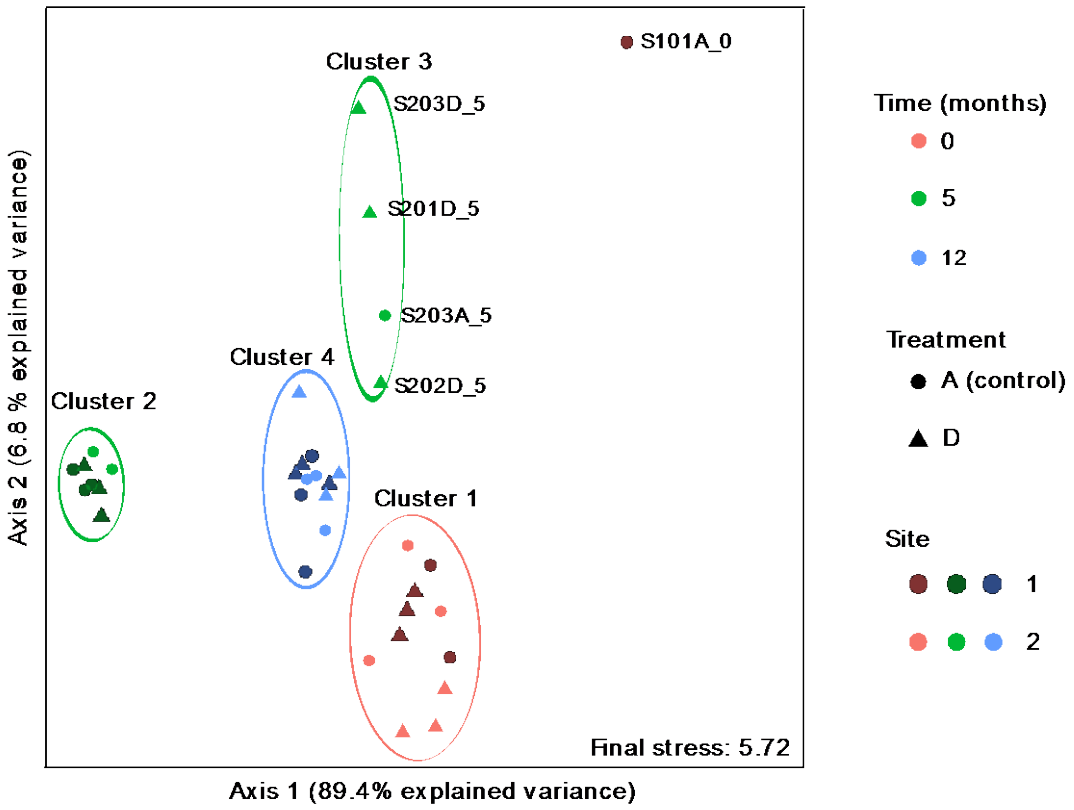

Figure 4-21 – The relative abundance of broad functional genes categories in the "3,000 gene core" set remains stable in undisturbed and stockpiled organic soils.

repeatedly detected among treatments and across time when comparing 3,000 -gene core sets between samples. The list of detected genes from the Geochip 5.0S microarray analysis contains a hierarchical categorization of the genes by function. The average relative abundance of contributed broad gene categories in the within the 3,000-gene core set for each treatment and sampling time grouping (n = 6) was calculated to compare broad biogeochemical function representation across treatments and time. The results of this comparison showed a robust preservation of genomic potential for nutrient cycling functions in the Summit Lake area organic soils, with no overt differences in relative abundance of functional categories by treatment or across the three monitoring points (Figure 4-22; Figure 4-23; Figure 4-24; Figure 4-25; Figure 4-26). This provides more evidence that short-term (5-months or less) stockpiling of organic soils is not detrimental to the potential reservoir of metabolic activities *in sit*.

Major nutrients in soil

Nitrogen: available (ammonium, nitrate) and potentially mineralizable N (anaerobic incubation)

The mineral-nitrogen pools (i.e., inorganic forms) of ammonium and nitrate tend to be the most available forms of nitrogen for many plants and soil organisms (Geisseler et al. 2010; Robertson and Grossman 2015), although it is recognized that ectomycorrhizal fungi play a role in providing some organic nitrogen to plants in some forest soils (Read et al. 2004). The concentrations of ammonium and nitrate are quite dynamic due to the numerous additions and losses to these pools in the plant-soil environment (note that nitrite concentrations tend to be very low in most soils, and are not usually reported). Ammonium and nitrate often make up a small portion of total nitrogen in both organic and mineral soils, with the bulk of nitrogen being in present in organic forms (Brady and Weil 2002; Robertson and Grossman 2015). Concentrations of ammonium-N tend to exceed nitrate-N in many forest soils. The much higher total nitrogen concentrations in organic soil horizons usually results in greater concentrations of mineral N in organic horizons as compared to mineral horizons. A lab-based incubation test can be used to provide a relative index of the potentially mineralizable nitrogen (frequently referred to as mineralizable N) in a soil; this test assesses the net conversion (i.e., net N mineralization) of organic N to mineral N during incubation. Even though this laboratory test has no bearing to a field situation, it is still useful to examine potential treatment effects on the ability of a soil to produce mineral N (Bundy and Meisinger 1994).

Treatment and seasonal effects

In general, ammonium-N concentrations exceeded those of nitrate-N in most treatments (Figure 4-27). Concentrations of mineral N were much greater in organic layers than in mineral soil layers. It is interesting to note that the stored topsoil (Treatment D) exhibited a large increase in ammonium-N between zero and five months; ammonium-N concentrations for this treatment greatly exceeded those for any other treatment. It is likely that ideal moisture and temperature conditions during storage promoted net N mineralization; and, lack of plant growth allowed the ammonium-N concentrations to build over the spring and summer (without any plant removal). The lower ammonium-N concentrations in the following spring (T = 12

months) were likely due to plant uptake, nitrification, net N immobilization and early 2015 spring losses (e.g., leaching) following the replacement of the topsoil to the plots in late 2014. In general, concentration of total mineral N in the mixed treatment (treatment B) was lower than that observed in the organic layers of the other treatments (T = 5 and T = 12 months). This was expected as the mixing in treatment B resulted in much lower concentrations of total carbon and nitrogen.

Potentially mineralizable nitrogen is a laboratory test that can be used to examine potential treatment effects on the ability of a soil to provide mineral N. In general, there was greater mineralizable N in organic layers, with the lowest values produced for the mixed soil in treatment B. The intact organic layer (treatment A) had the greatest potentially mineralizable N. These trends correspond to the concentrations of total N for these soils. In general, soils with the greatest total N tended to exhibit the greatest potentially mineralizable nitrogen. Soil C:N ratios can play a role in net N mineralization, but the C:N ratios (presented later in this Section) were very similar in these soil samples (Table 4-4). In general, potentially mineralizable N decreased from five to 12 months. The potentially mineralizable pool is thought to be composed of organic N components originating from fresh biotic inputs, microbial biomass and their residues (Robertson and Grossman 2015). Concentrations of these components would be expected to be greater at the end of the growing season (five months) than early in the spring (12 months) following a cold winter.

Phosphorus (available, Bray-method)

Total soil phosphorus is made up of both inorganic and organic pools; the proportions can vary greatly in soils (Brady and Weil 2002; Kertesz and Frossard 2015). The Bray 1 test is a simple, widely used test to examine relative treatment effects: here we used it to determine the easily extractable phosphorus in acid soils (Kalra and Maynard 1991; Pierzynski et al. 2005). One limitation of the Bray test, however, is that it cannot predict the fraction of organic phosphorus that might be mineralized through phosphatase activity.

In general, soils collected from disturbed treatments had equal to or greater available phosphorus concentrations than the undisturbed control organic layer (Treatment A). Available

phosphorus concentration was quite low in the mineral layer of Treatment A. There were no strong temporal trends observed for available phosphorus concentrations.

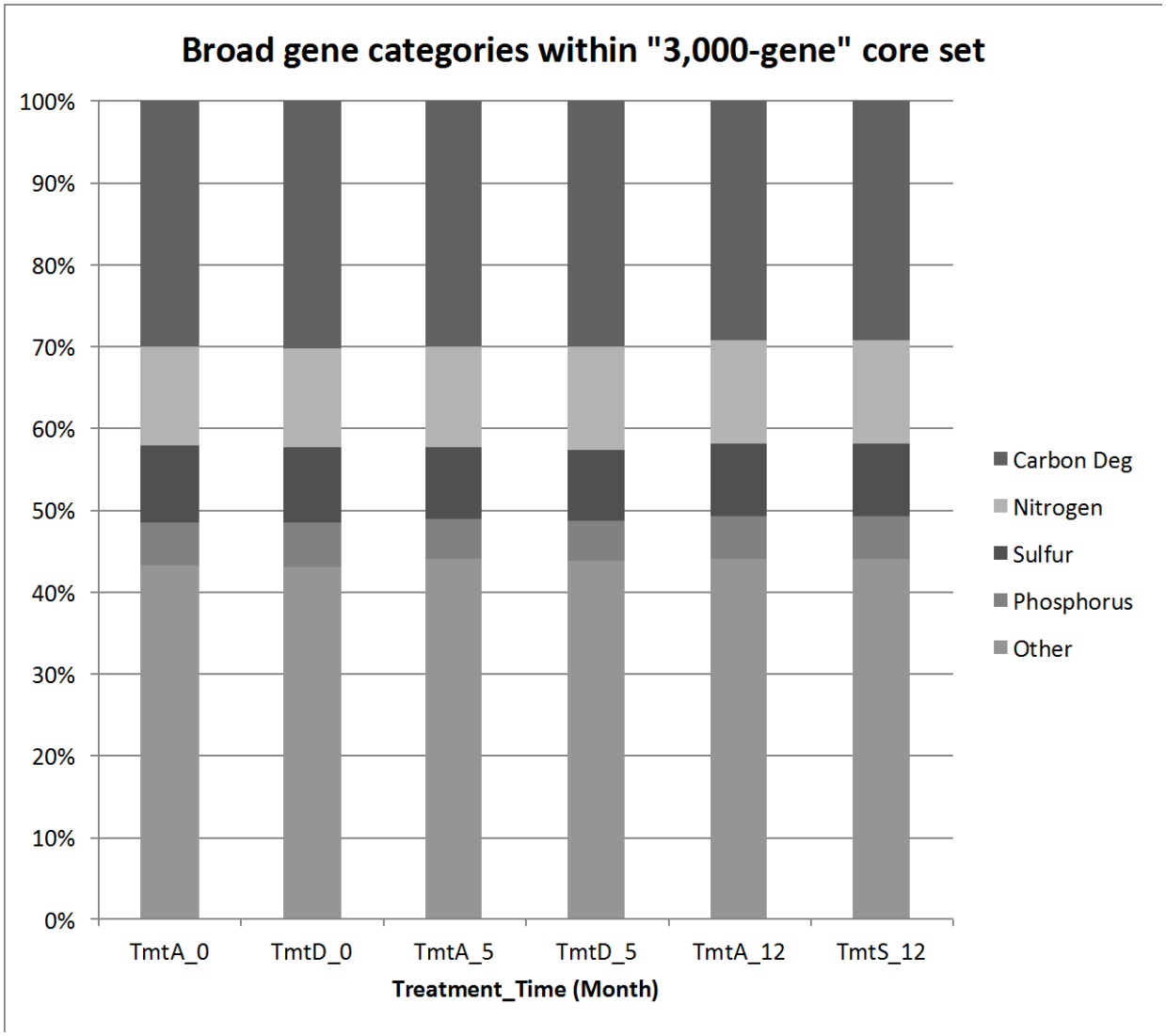

Figure 4-22 – Percentage composition of 3,000-gene core set for sample-time-point groupings (n = 6) by broad functional gene categories. "Other" includes genes coding for antibiotic resistance, bioremediation and carbon fixation functions.

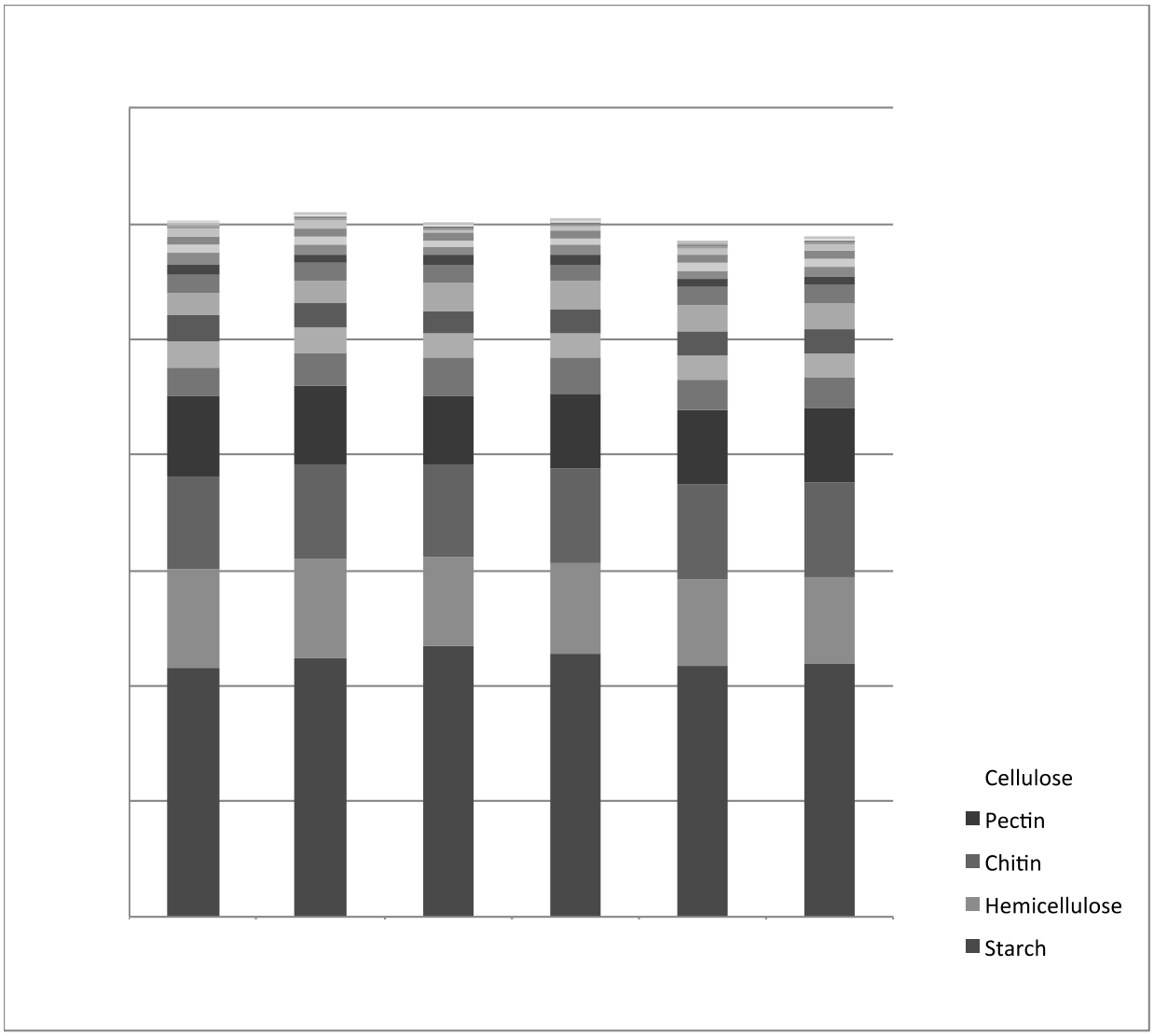

Figure 4-23 – Percentage of total 3,000-gene core set for sample-time-point groupings (n = 6) by sub-categories of carbon degradation genes.

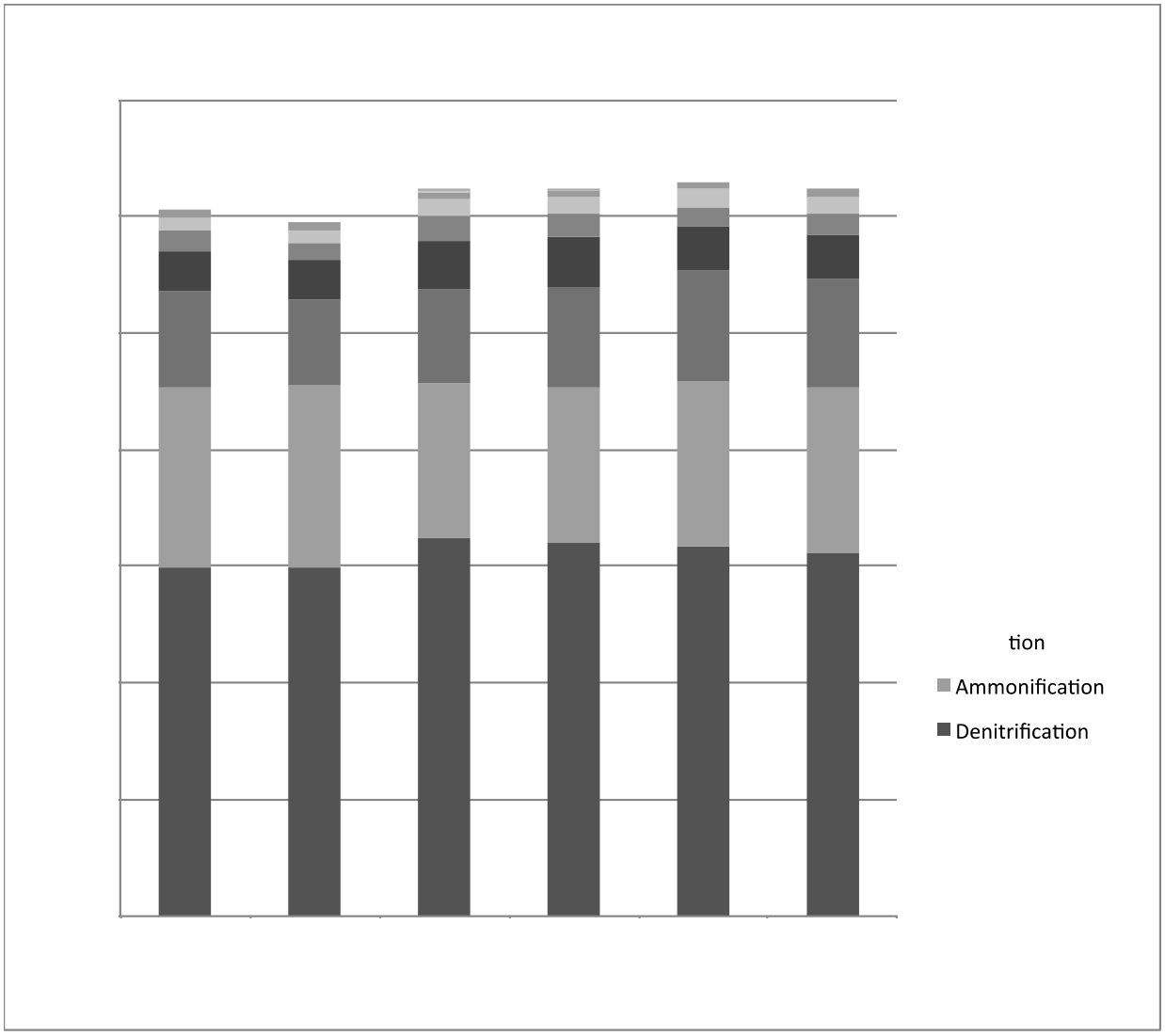

Figure 4-24 – Percentage of total 3,000-gene core set for sample-time-point groupings (n = 6) by sub-categories of nitrogen metabolism genes.

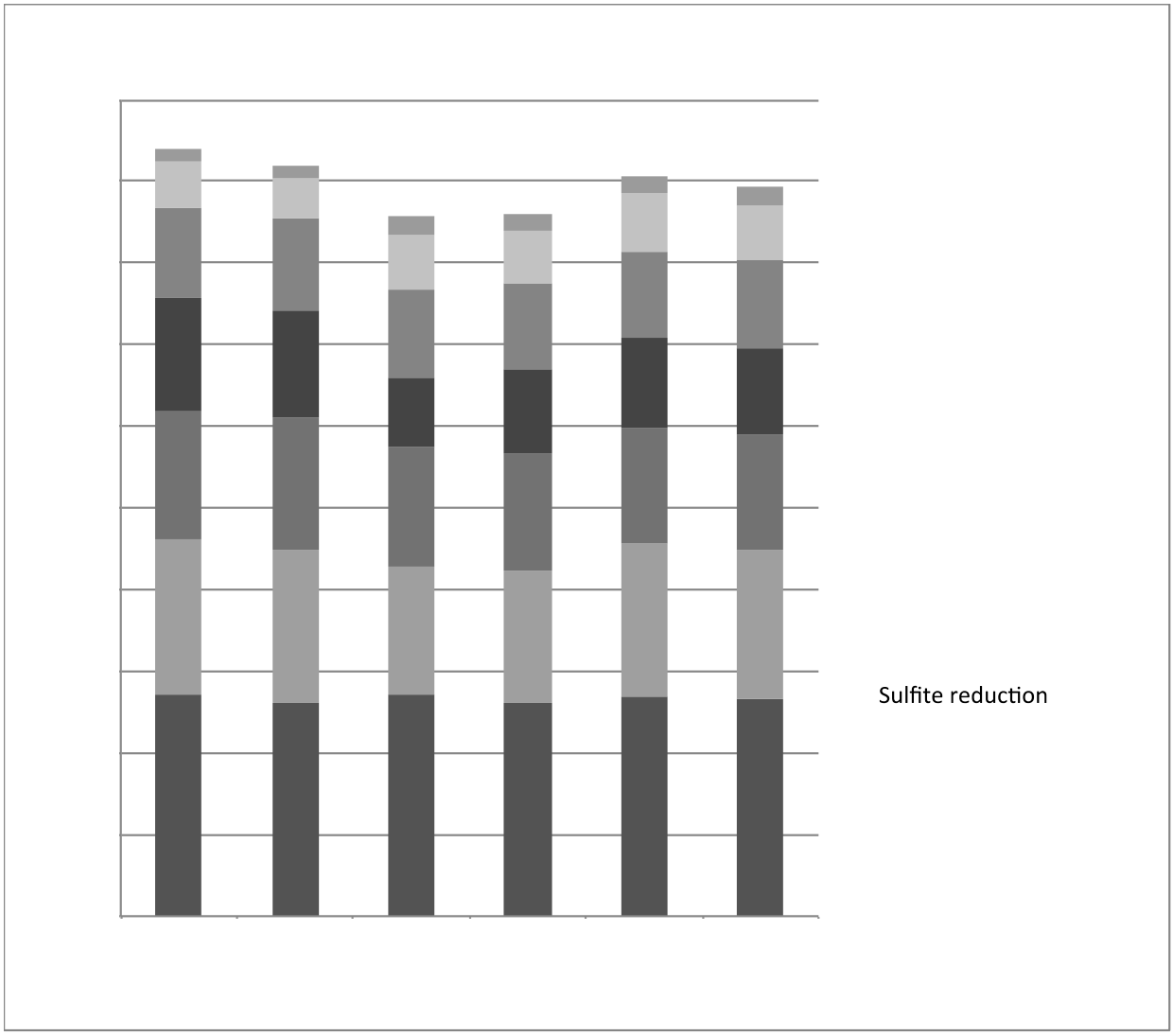

Figure 4-25 – Percentage of total 3,000-gene core set for sample-time-point groupings (n = 6) by sub-categories of sulfur metabolism genes.

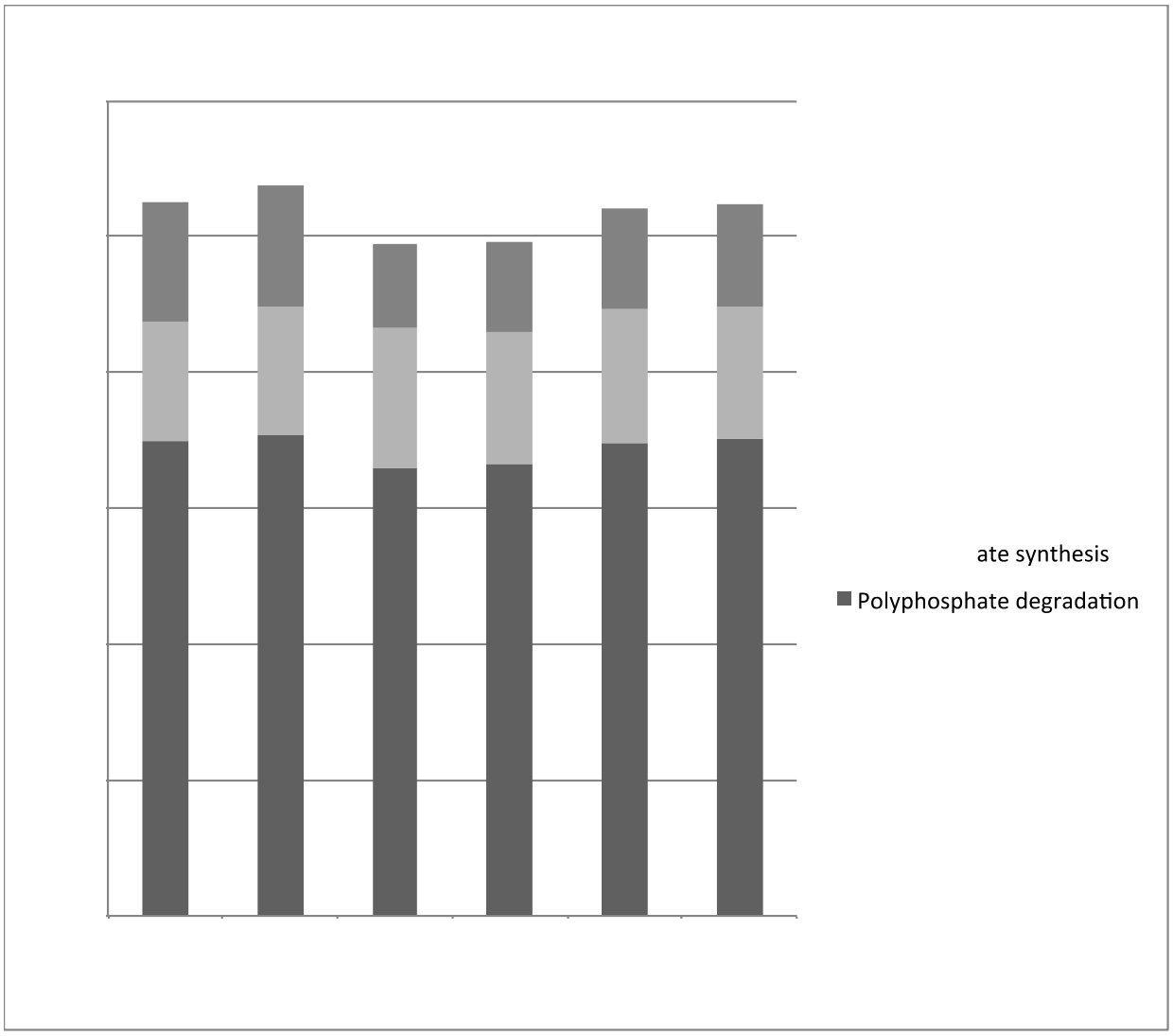

Figure 4-26 – Percentage of total 3,000-gene core set for sample-time-point groupings (n = 6) by sub-categories of phosphorus metabolism genes.

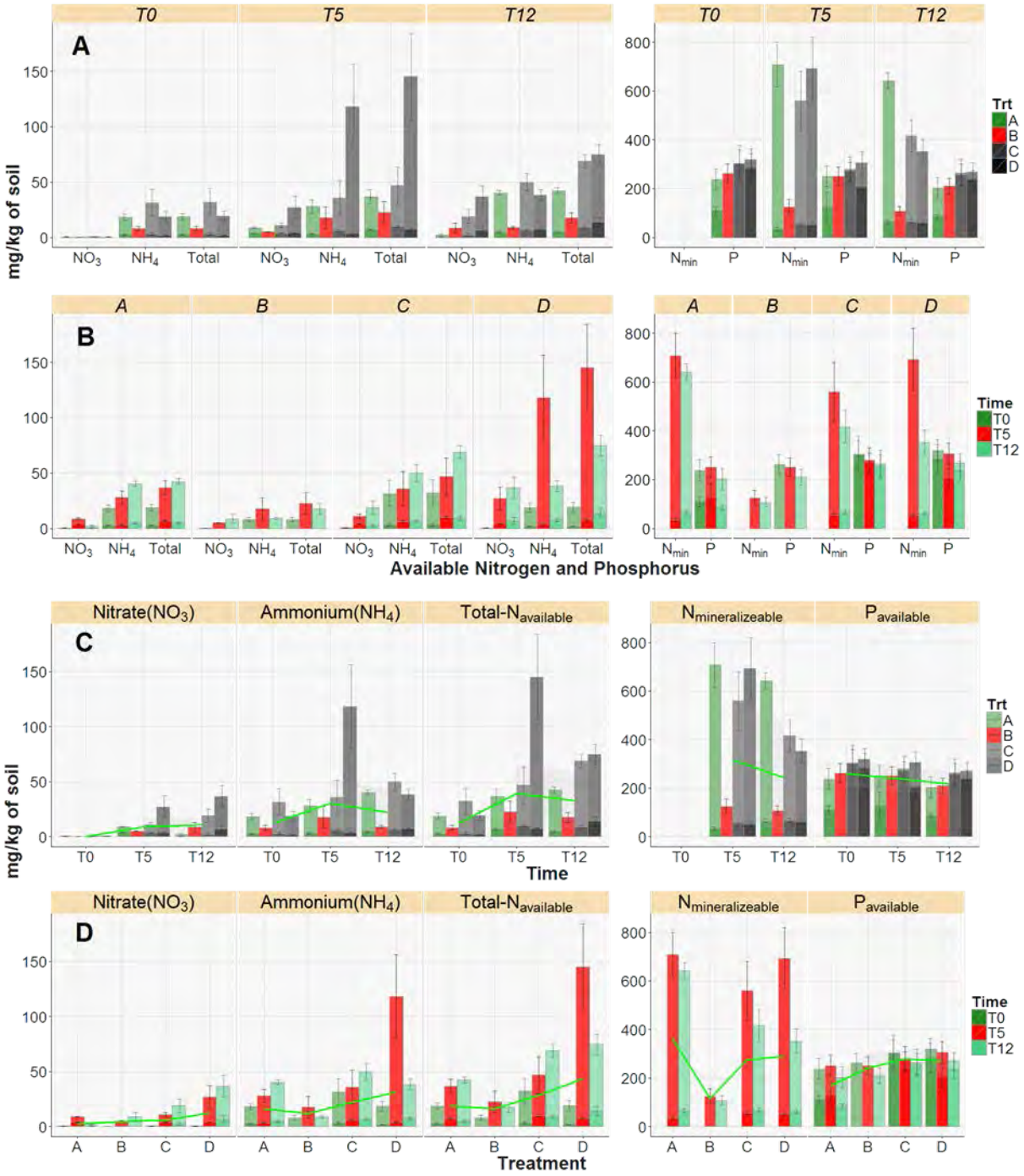

Figure 4-27 – Mean (±SE) available nitrogen and phosphorus of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015 across the four treatments and between horizons, relative to each time points (A: treatment effect), and relative to each treatment (B: seasonal effect). Mean (±SE) available nitrogen and phosphorus of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015 across the four treatments and between horizons for each time point (C: treatment effect), or for each treatment (D: seasonal effect).

Table 4-4 – Summary of total mineral N, total N, total C and total S at the experimental field plots at Summit Lake in 2014 and 2015, across treatment-between-horizons (vertical), over time differences (horizontal) for all sites.

	Hor-Trt	n	Total Nitrogen (Nitrate / Ammonium) Time 0 mean ± SE	Time 5 mean ± SE	Time 12 mean ± SE		Total Nitrogen, Carbon, Sulfur (%) Time 0 mean ± SE	Time 5 mean ± SE	Time 12 mean ± SE
Nitrate (mg/kg)	O A	6	0.43 ± (0.16)	8.72 ± (0.54)	2.05 ± (0.90)	Carbon (%)	30.2 ± (4.4)	33.5 ± (4.2)	31.3 ± (3.1)
	O C	6	0.53 ± (0.06)	10.84 ± (1.92)	18.91 ± (6.08)		17.1 ± (2.6)	24.8 ± (3.5)	21.8 ± (2.4)
	O D	6	0.45 ± (0.16)	27.02 ± (10.36)	36.53 ± (9.72)		15.5 ± (2.0)	26.6 ± (3.9)	18.6 ± (1.8)
	M A	6	0.06 ± (0.03)	3.81 ± (0.08)	0.15 ± (0.07)		2.7 ± (0.3)	2.3 ± (0.1)	2.7 ± (0.3)
	M B	6	0.07 ± (0.02)	4.95 ± (0.36)	8.62 ± (4.18)		5.3 ± (0.7)	6.4 ± (1.2)	6.9 ± (2.2)
	M C	6	0.06 ± (0.02)	3.82 ± (0.17)	2.26 ± (1.22)		2.9 ± (0.2)	2.8 ± (0.2)	3.0 ± (0.3)
	M D	6	0.05 ± (0.02)	3.92 ± (0.11)	6.68 ± (3.17)		2.4 ± (0.2)	2.8 ± (0.3)	2.9 ± (0.4)
Ammonium (mg/kg)	O A	6	18.2 ± (2.9)	27.9 ± (6.1)	40.2 ± (2.2)	Nitrogen (%)	1.20 ± (0.13)	1.54 ± (0.23)	1.37 ± (0.09)
	O C	6	31.5 ± (11.9)	35.9 ± (15.3)	49.8 ± (7.9)		0.70 ± (0.09)	1.02 ± (0.15)	0.90 ± (0.09)
	O D	6	18.6 ± (4.4)	118.1 ± (38.1)	38.2 ± (4.9)		0.66 ± (0.07)	1.16 ± (0.19)	0.81 ± (0.09)
	M A	6	2.7 ± (0.5)	3.0 ± (0.4)	4.7 ± (0.7)		0.13 ± (0.01)	0.11 ± (0.01)	0.13 ± (0.01)
	M B	6	8.0 ± (2.1)	17.7 ± (9.7)	9.0 ± (1.2)		0.22 ± (0.03)	0.27 ± (0.04)	0.29 ± (0.08)
	M C	6	3.2 ± (0.9)	6.1 ± (0.7)	6.6 ± (0.8)		0.14 ± (0.01)	0.14 ± (0.01)	0.14 ± (0.01)
	M D	6	1.8 ± (0.3)	3.7 ± (0.3)	7.4 ± (1.1)		0.12 ± (0.01)	0.13 ± (0.01)	0.14 ± (0.02)
Total N = NO_3 + NH_4	O A	6	18.6 ± (2.8)	36.6 ± (6.2)	42.3 ± (2.5)	Carbon : Nitrogen	24.9 ± (1.9)	22.2 ± (0.8)	22.6 ± (0.9)
	O C	6	32.1 ± (11.9)	46.8 ± (16.7)	68.7 ± (5.7)		24.2 ± (1.6)	24.8 ± (1.4)	24.1 ± (0.6)
	O D	6	19.0 ± (4.4)	145.1 ± (38.8)	74.7 ± (9.2)		23.1 ± (1.2)	23.4 ± (1.1)	23.1 ± (0.6)
	M A	6	2.7 ± (0.5)	6.8 ± (0.5)	4.9 ± (0.7)		20.6 ± (1.7)	20.2 ± (1.0)	20.4 ± (1.1)
	M B	6	8.1 ± (2.1)	22.6 ± (9.7)	17.6 ± (4.6)		23.2 ± (2.0)	23.2 ± (1.7)	22.7 ± (1.4)
	M C	6	3.2 ± (0.9)	9.9 ± (0.8)	8.8 ± (1.5)		21.4 ± (1.1)	20.5 ± (0.9)	20.8 ± (1.0)
	M D	6	1.8 ± (0.3)	7.6 ± (0.4)	14.0 ± (4.0)		20.4 ± (1.0)	22.2 ± (1.0)	21.0 ± (0.9)
N*mineralized (mg/kg)	O A	6	NA	707.8 ± (91.9)	642.2 ± (32.8)	Sulfur (%)	0.095 ± (0.011)	0.132 ± (0.017)	0.120 ± (0.008)
	O C	6	NA	559.0 ± (120.8)	415.8 ± (65.6)		0.055 ± (0.007)	0.090 ± (0.012)	0.085 ± (0.008)
	O D	6	NA	692.5 ± (126.7)	352.8 ± (50.4)		0.052 ± (0.007)	0.104 ± (0.014)	0.076 ± (0.008)
	M A	6	NA	34.2 ± (6.2)	64.6 ± (7.7)		0.011 ± (0.001)	0.010 ± (0.000)	0.013 ± (0.001)
	M B	6	NA	123.9 ± (30.6)	107.5 ± (18.4)		0.018 ± (0.002)	0.020 ± (0.003)	0.026 ± (0.007)
	M C	6	NA	54.8 ± (5.4)	66.1 ± (8.4)		0.012 ± (0.001)	0.011 ± (0.001)	0.026 ± (0.013)
	M D	6	NA	51.3 ± (5.2)	61.2 ± (6.8)		0.010 ± (0.001)	0.011 ± (0.001)	0.012 ± (0.002)

*Nitrogen-mineralized, or anaerobic incubation

Phosphorus** (mg/kg)	O C	6	108.8 ± (18.5)	125.6 ± (59.0)	84.5 ± (9.3)
	O D	6	303.4 ± (72.7)	269.8 ± (37.2)	264.1 ± (54.8)
	M A	6	283.6 ± (59.1)	203.7 ± (43.1)	236.5 ± (37.9)
	M B	6	237.7 ± (43.0)	250.0 ± (42.6)	202.1 ± (43.2)
	M C	6	261.4 ± (39.6)	250.5 ± (36.6)	210.1 ± (31.8)
	M D	6	302.0 ± (56.9)	278.3 ± (52.0)	257.0 ± (43.4)
	O A	6	318.9 ± (45.0)	304.8 ± (44.5)	269.2 ± (36.2)

** available Phosphorus using Bray-P1

Total Carbon, Nitrogen and Sulfur (%)

Total concentrations of carbon, nitrogen and sulfur were much greater in organic layers than mineral layers. Typically, organic horizons of upland forest soils have much greater concentrations of these elements than mineral horizons (Fisher and Binkley 2000; Prescott and Grayston 2013). Generally, our results indicated that total concentrations (%) of carbon, nitrogen and sulfur did not vary greatly over time, as might be expected for a study with such a short duration. The major pools of these elements are expected to reside in organic fractions (i.e., both organic and mineral horizons) and are not subject to as rapid changes as inorganic forms (e.g., ammonium-N, nitrate-N, sulfate-S) (Brady and Weil 2002; Kertesz and Frossard 2015; Robertson and Grossman 2015). Minor fluctuations over time were likely due to sample variability. Both carbon and nitrogen concentrations followed similar trends, as is indicated by the relatively constant C:N ratios measured over the study period (Figure 4-28).

The undisturbed organic layers in the Treatment A had the greatest concentrations of total carbon, nitrogen and sulfur, while Treatment B (organic and mineral horizons mixed) had much lower concentrations (Figure 4-28). These trends were expected as mixing (Treatment B) diluted high concentrations of these elements in organic layers with much lower concentrations in the mineral layers. It is interesting to note that the organic layers in Treatments C and D had lower concentrations of carbon, nitrogen and sulfur than the intact organic layer in Treatment A. Even though care was taken to remove and later replace these organic layers (Treatments C and D), there was still some mixing of mineral matter into the organic layers thereby lowering total nutrient concentrations.

Conclusions

Significance of fungal community profiling

The use of ITS2 amplicon sequencing in this experiment revealed that soils of sub-boreal spruce ecosystems contain several hundred fungal genera but these soil fungal communities tend to be dominated by a small (4-6) number of genera with mycorrhizal ecological functions in “undisturbed” conditions.

Figure 4-28 – Mean (±SE) total carbon, nitrogen and sulfur of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015 across the four treatments and between horizons for the major nutrients, relative to each time points (A: treatment effect), and relative to each treatment (B: seasonal effect). Mean (±SE) total carbon, nitrogen and sulfur of the experimental field plots (n = 6) at Summit Lake in 2014 and 2015 across the four treatments and between horizons for each time point (C: treatment effect), or for each treatment (D: seasonal effect).

Mechanical disturbance in our experiment, consistent with previous investigations of trenching and soil physical disturbance (Fernandez and Kennedy 2015) led to the flourishing of saprotrophic fungal communities (particularly of the genera *Mortierella* and *Umbelopsis*) in organic and mineral soils that persist five to 12 months post-disturbance.

All configurations of physical disturbance in soils during this investigation led to a reduction in mycorrhizal taxa (specifically *Cortinarius*, *Inocybe*, *Piloderma*, *Russula* and *Sebacina*) observable in organic soils as well as mineral soils (with the exception of *Sebacina*, which was mostly found in control organic soils). This reduction persisted for the aforementioned taxa over the duration of this experiment. Our results indicate that short-term topsoil salvage and segregation does not exacerbate the loss of mycorrhizal taxa because the disturbance Treatments C (immediate replacement of organic soil) and D (stockpiled and eventually replaced organic soils) exhibited similar fungal community structure over the course of the experiment.

Mycorrhizal fungi that have reduced relative abundance in disturbance treatments are still detectable five and 12 months post-disturbance. We did not observe any absolute loss of any abundant mycorrhizal taxa through the course of this experiment as a consequence of mechanically disturbing soils or mixing horizons. This is a particularly important observation when considering the short-term storage of soils in Treatment D, because it indicates that symbiotic associates relevant to plant health and nutrient acquisition are being maintained, albeit at reduced levels. If this field experiment were to continue, an important issue to address, which would directly link observations of fungal community composition to their ecosystem service effects, is how re-vegetation (likely with appropriate seed mixtures) regimens would perform in these disturbed soils and how live root systems would contribute to re-establishment of mutualistic fungal communities. The other limitation of our investigation is the length of the observation periods. Extended characterization of the Summit Lake Field Plot Experiment after 12 months would enable us to monitor the dynamics of mycorrhizal and saprotrophic fungal taxa that appeared to undergo major changes in abundance during this initial investigation.

Significance of bacterial community profiling

The use of bacterial 16S amplicon sequencing in this experiment revealed that soils of sub-boreal spruce ecosystems contain diverse (>500 taxonomic bins, where most can be defined to the genus level) bacterial communities with compositions that are resilient to change in the presence of physical disturbance.

In contrast to the fungal community profiles of Summit Lake forest soils, the distribution and abundance of DNA signatures for bacterial communities reflect differences between mineral and organic soil. This characteristic of bacterial community profiles could be useful in a monitoring context for evaluating the extent of soil mixing that happens during re-construction of a soil profile along a RoW. The observation that organic soils mixed with mineral soils (Treatment B) eventually shifts in bacterial community structure to resemble mineral soils reinforces that best practice for preserving microbial communities associated with decomposing organic matter is salvaging organic soil for reclamation.

Highly abundant bacterial community members (such as the 25 abundant bacterial taxa that were compared across treatments in the earlier part of this Section) do not change much in relative abundance in the presence of soil physical disturbance. The seasonal shifts in bacterial community composition and the shifts in mineral soil mixes (Treatment B) over time suggest that minor bacterial taxa (<1 % relative abundance compared to the total detectable bacterial community) are responding to environmental changes, and maintaining soil ecosystem services. Although we have not directly interrogated ecological functions of bacteria in this field study, the dynamic composition of soil bacterial communities, particularly by lower abundance taxa, underscores the need to preserve local soils (and hopefully their associated biogeochemical functions) for reclamation of pipeline RoW areas post-construction.

Significance of soil enzyme activity profiling

Organic soil layers generally exhibit higher potential hydrolase activity values compared to mineral soil layers. Practical implications of enzyme activity profiles (from the microcosm and field plot experiments) are that organic and mineral soil layers can be functionally distinguished from each other, such that suboptimal ordering of the soil profile might be detectable through such assays. The Summit Lake soils in particular have very thin and uneven LFH and Ae horizons,

which could be sensitive to losses of organic material during soil handling. Therefore it is important to reconstruct organic horizons, and ideally have a method for evaluating the reconstruction status, so enzyme activity profiles could inform this aspect of restoration. The monitoring of these types of activities in a vertical soil-sampling scheme might be useful in assessing the quality of reconstructed soil profiles, since a gradient of higher to lower activity should be observable. Deviations from this gradient might reflect excessive mixing of upper and lower soil horizons. The hydrolase assays require minimal processing of soil other than suspension in an appropriate buffer prior to assaying with synthetic substrates, so this method of interrogating i) stability of litter decomposition activities or ii) organic substrate availability is relatively easy to implement as a soil biological monitoring protocol.

Significance of functional gene survey (Geochip)

The use of Geochip 5.0S in this experiment revealed that soils of sub-boreal spruce ecosystems exhibit detectable seasonal fluctuations in the composition and abundance of genes that code for important biogeochemical functions. However highly abundant gene sequences relevant to nutrient cycling persist in organic soils even though microbial communities (bacterial and fungal) can change drastically in composition due to physical disturbance. The two soil treatments compared with the functional gene microarray, specifically undisturbed (Treatment A) and temporarily stockpiled (Treatment D) organic soils did not differ in their content of highly abundant functional genes. Further, seasonal variation in functional gene content appears to minimally affect the “3,000 gene” core set.

The implications of the microarray data are that short-term LFH stockpiling during soil excavation is not subtractive to the potential biogeochemical functions endowed by microbial communities *in situ*. Although the microbial community composition can change as a consequence of disturbance, there appears to be enough functional redundancy in this forest soil bacterial and fungal community to maintain the abundance of dominant genes that code for nutrient cycling functions.

Significance of total and available nutrients in soil within field plots

The high concentrations of total carbon, nitrogen and sulfur (%), and high cation exchange capacity (CEC) of organic horizons, provide a number of benefits to the soil-plant

environment. Carbon (mainly organic carbon in these soils) serves as an important source of energy, fueling the soil microbial community. The large pools of total nitrogen and sulfur in organic horizons also serve as sources of nutrients to the microbial community, and to plants. High cation exchange capacity (a property of most organic horizons) retains many ionic forms of nutrients in plant-available forms, which can also be utilized by many soil organisms. In addition, organic matter provides soil physical benefits such as increased water holding capacity, resistance to soil erosion and (sometimes) moderation of soil temperature changes. Organic horizons also serve as seed banks of native vegetation and as an inoculum source for soil microbes. The function of the soil ecosystem therefore depends on maintaining the order of the soil profile and spatial availability of nutrients as a function of depth. The greater concentrations of most available nutrients measured in this study (e.g., ammonium-N, nitrate-N and sulfate-S) in organic layers supports the premise that maintenance of organic matter concentrations is critical to the reclamation process.

4.5. Soil Integrity and Restoration Protocol: findings and recommendations

The observation period of 12 months used in the Summit Lake field experiment is a relatively short time frame for drawing conclusions about long-term soil responses to disturbance. We believe, however, that our efforts at characterizing soil fungal and bacterial community structure, potential hydrolase activities, chemical composition as well as temperature and moisture dynamics provides insight into short-term soil handling practice during RoW construction.

Within this time frame, we can summarize several key findings. First, bacterial community composition is not a good indicator of disturbance, and topsoil storage for a period of five months has a negligible effect on bacterial community composition. In future studies, we would shift the focus of DNA-based characterization from taxonomic surveys using 16S and ITS2 amplicon sequencing to sequencing of amplified gene targets coding for biogeochemical functions such as nitrogen fixation or glycosyl hydrolases (involved in litter decomposition). This would allow us to track shifts in the abundance of genes coding for microbially-mediated soil ecosystem services, and their taxonomic distribution for comparison between different soil handling protocols.

Second, mycorrhizal fungi are not eliminated during mechanical disturbance, but they decrease in relative abundance (compared to other ecological guilds of fungi present) as short-term consequence of soil excavation. Topsoil storage for a period of five months did not eliminate the pool of available mycorrhizal fungi, although relative abundances were reduced. Mycorrhizal communities appear to be the most vulnerable following disturbance; and therefore, longer monitoring of field plots would allow a better assessment of community recovery (i.e., we would measure the increased relative abundance of dominant mycorrhizal taxa) and to understand the effect of how soil mixing (e.g., our Treatment B) inhibits the re-establishment of mycorrhizal dominance in soil fungal communities.

Third, hydrolytic litter decomposition activities are maintained in stockpiled topsoils, and the presence of genes coding for oxidative litter decomposition activities also appear to be stable during short-term topsoil storage. In addition, the data indicate that short-term LFH stockpiling during soil excavation does not affect the relative abundance of genes coding for

microbial biogeochemical *in situ*. Although the microbial community composition changes over time (bacterial specifically) and as a short-term response to disturbance (i.e., the observed shifts in abundant mycorrhizal and saprotrophic fungal taxa), there appears to be enough redundancy to maintain the abundance of dominant genes that code for nutrient cycling functions. The difference in hydrolytic litter decomposition activities between mineral and organic horizons can be used as an assessment of soil mixing and of correct reassembly of the soil profile during reclamation along a RoW.

Finally, soil chemical composition reflects the vertical distribution of nutrients in the soil profile as well as the nutrient holding capacity (e.g., CEC); it is therefore important to carefully separate the organic horizons prior to pipeline construction and to replace it back intact following pipeline placement. The nutrient distribution (profile) is critical to maintaining soil microbial community structure, necessitating careful replacement of organic layers prior to restoration. Soil physiological measurements (e.g., respiration; net N mineralization) need to be introduced in monitoring work-flows to assess the functional aspects of soil bacterial communities and to establish correlations between taxonomic composition in soils and observed biogeochemical function in the context of physical disturbance or reconstruction. In addition, measures of soil respiration could potentially identify differences between stockpiled topsoil and undisturbed soils and evaluate the stability of soil microbial metabolism *in situ*. Coupling diffusible nutrient availability measures to soil functional measures would allow us to better evaluate relationships between nutrient availability and changes in the level of measurable microbial ecosystem activities.

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Chapter 5 – Anadromous Movement and Estuarine Habitat Use of Coastal Cutthroat Trout

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Tagged cutthroat trout from Bish Cove. Photo credit: Allan Costello

5.1. Introduction

The Cutthroat Trout Protocol was designed to assess the potential effects of the Kitimat LNG facility on the use of Bish Creek estuary, near-shore, and marine environments by coastal cutthroat trout (*Oncorhynchus clarkii clarkii*). The coastal cutthroat trout is an anadromous fish with an extended nearshore-marine residency period, and one known to be sensitive to anthropogenic activities affecting freshwater habitat quality. Unlike pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon, which utilize the estuarine environment around Bish Creek and Cove for a relatively limited time prior to migrating offshore, anadromous coastal cutthroat trout remain in the nearshore marine environment throughout their life, often in the immediate vicinity of their natal stream. They repeatedly move between freshwater and nearshore-marine habitats to access productive habitats and locally abundant food resources like salmon fry (Giger 1972; Behnke 1992; Pearcy 1997; Trotter 1997). Furthermore, the species is listed as *of special concern* in BC and are known to be particularly

sensitive to anthropogenic effects to their freshwater habitats (Johnson et al. 1999; Costello and Rubidge 2005; Slaney 2005).

Throughout its range, the number and distribution of coastal cutthroat trout populations have been steadily declining in response to the cumulative impacts of habitat loss and degradation, overexploitation, and detrimental interactions (i.e., competition, predation, hybridization) with introduced species (Allendorf and Leary 1988; Liknes and Graham 1988; Nehlsen et al. 1991; Johnson et al. 1999; Costello and Rubidge 2005; Shepard et al. 2005). The declines over the last century clearly indicate that the greatest threats to cutthroat trout are the anthropogenic manipulation and degradation of the environment in which it lives. All available information for BC suggests that many populations are depressed relative to historic levels, particularly in the Georgia Basin where numerous local extirpations have been documented (Costello and Rubidge 2005). Although believed to be less impacted than populations further south, the status of most anadromous salmonids along the north coast of BC is largely unknown outside of human-populated areas where declines in Coho (*O. kisutch*), steelhead (*O. mykiss*) and coastal cutthroat trout were first noted in the early 1980s. At that time, it was apparent that anadromous, fluvial, and resident forms of coastal cutthroat trout in some parts of the region were being overharvested to the point where populations were no longer capable of sustaining even modest fishing pressures. Severe harvest restrictions, reduced catch limits and closures were recommended for the Terrace-Kitimat area to prevent further declines and extensive habitat restoration, inventory and assessment of remaining stocks was deemed ‘mandatory’(Whatley 1984).

Anadromous cutthroat appear to rely heavily on marine-derived protein (e.g., meiobenthic fauna, salmon fry and smolts, juvenile smelt and perch, etc.) that promotes overwintering survival and reproductive success. Rocky beaches, eelgrass (*Zostera marina*), or kelp beds near creek mouths provide excellent estuarine habitat for coastal cutthroat, especially if structures such as old pilings and docks are located nearby. Construction of marine terminals and jetties at Bish Cove could, therefore, potentially benefit anadromous cutthroat, as they often use the cover provided by such structures to evade marine predators.

5.2. Approach and outline of contributions

This Protocol focused on the marine environment and incorporated multiple ecosystem indicators to identify physical, biological, and community-level changes associated with the plant site for use in future operational planning and decision making. Further, this Protocol was designed to evaluate the success or the utility of using anadromous fishes as an indicator of long-term ecosystem function. This approach had the added advantage of providing some basic biological information for coastal cutthroat trout, an understudied species for which the relationships between life-history types, their habitat requirements, and the scope and variation typical of cutthroat movements remain poorly resolved, particularly during the marine portion of the lifecycle.

Two potential types of plant–related effects have been previously identified for Bish Cove (Archipelago Marine Research Ltd. 2012): 1) habitat alteration resulting from placement of over-water and in-water structures, sediment runoff, and propeller scour; and 2) biotic community changes (including behavioural disturbance) resulting from noise, increased vessel traffic, changes to seabed substrates, barriers to nearshore fish passage, night lighting, or the introduction of non-native species. In this Chapter, we use two independent and complementary data sets to assess any potential effects of habitat alteration or community change on Bish Creek cutthroat trout — one gathered through fish tagging and acoustic telemetry (Section 5.3) and one gathered through genetic analyses of DNA markers (Section 5.4).

5.3. Acoustic telemetry reveals diverse nearshore marine movements and the importance of intertidal feeding areas for Bish Creek coastal cutthroat trout.

Allan Costello

Introduction

The Bish Cutthroat Trout Protocol was designed to assess the potential effects of the Kitimat LNG facility on the use of Bish Creek estuary, near-shore and marine environments by coastal cutthroat trout (*Oncorhynchus clarkii clarkii*; hereafter CCT), an anadromous fish with an extended nearshore-marine residency period. Determining how animals move within their environment is fundamental to effective management and conservation (Fryxell et al. 2014). Although considerable progress has been made in recent years in developing indices to gauge the ecological health and resilience of estuarine environments (e.g., Borja and Dauer 2008; Beliaeff and Pelletier 2011), no consensus has emerged as to the most sensitive or efficient elements for long-term monitoring. Indeed, the challenge is to integrate indicators for a wide range of individual ecosystem elements, as well as substantive measures of ecosystem structure, function, and process (Borja and Dauer 2008). Fishes, in particular, have often been used as economically valuable and publicly relevant indicators of environmental change (Whitfield and Elliott 2002) and there exists numerous community- or assemblage-based measures of estuarine integrity and ecosystem function that focus exclusively on fish fauna (e.g., Whitfield and Elliott 2002; Pérez-Domínguez et al. 2012; Ramos et al. 2012).

Unlike salmon, which typically utilize the estuarine environment around Bish Creek and Cove for a relatively limited time prior to migrating offshore, anadromous CCT remain in the nearshore marine environment throughout their life, often in the immediate vicinity of their natal stream. They repeatedly move between freshwater and nearshore-marine habitats to access productive habitats and appear to rely heavily on marine-derived protein (e.g., meiobenthic fauna, salmon fry/smolts, bait fish, etc.) to promote overwintering survival and reproductive success (Giger 1972; Behnke 1992; Pearcy 1997; Trotter 1997). Furthermore, the species is listed as *of special concern* in BC and are known to be particularly sensitive to

anthropogenic effects to their freshwater habitats (Johnson et al. 1999; Costello and Rubidge 2005; Slaney 2005).

The primary variables that we investigated were behavioural, and relate to the anadromous movements of CCT. In particular, we were interested in determining the timing and duration of estuarine residency at Bish Creek as well as diel activity patterns for this species. Salmonids often show complex and diverse patterns of diel activity among populations, life-history stages, habitats and even seasons (Groot and Margolis 1991; Quinn 2005). As Goetz et al. (2013) indicate, however, the diel activity period for anadromous CCT is almost entirely unknown. As visual predators, CCT may be most active during daylight hours, but a solid understanding of those movements is required to identify and assess times when CCT may be most sensitive to plant construction and operational effects

We examined the patterns of habitat use in the Bish and Emsley systems (e.g., are trout primarily feeding in the eelgrass habitats, rocky headlands, or river mouths?). Rocky beaches, eelgrass, and kelp beds near creek mouths, for example, provide excellent estuarine habitat for coastal cutthroat, especially when structures such as pilings and docks are located nearby. Construction of marine terminals and jetties at Bish Cove could, therefore, potentially benefit anadromous cutthroat as they often use the cover provided by such structures to evade marine predators. Foraging ecology is thought to be sensitive to changes in the abundance of potential prey and predator species (Werner et al. 1983; Lima 1998); changes in dietary composition may, therefore, offer a window into larger events happening in the environment (species turnover, displacement, perhaps even the arrival of invasive species). As a complementary source of information, we examined the stomach contents from tagged fish to get a better sense of the prey items and habitats they may rely on in salt water. These variables are known to be sensitive to environmental perturbation and thus, useful for monitoring purposes. Specifically, our objectives for this study were to determine estuarine and near-shore environment residency, timing and usage by CCT prior to, during, and following marine construction in Bish Cove and a control site (Emsley Creek).

Methods

Study design

Acoustic telemetry has become a powerful tool for studying animal behaviour and patterns of movement in coastal ecosystems (reviewed by Heupel et al. 2006; Adams et al. 2012; How and de Lestang 2012). Realistically, however, it can be difficult to specifically identify and separate out "plant-specific" effects from any number of environmental covariates. To investigate the possible effects of the LNG facility on estuarine habitat use and residency by CCT, we therefore proposed following a standard Before-After-Control-Impact (BACI) approach. Initially we planned to capture and acoustically tag 20 CCT in the vicinity of Bish Creek (i.e., potentially impacted) and the nearby and similarly sized Emsley Creek estuary (i.e., control site) so that we could observe patterns of estuarine/near shore marine habitat use prior to, during, and following construction of the LNG facility. Emsley Cove is approximately three km west of Bish Cove and appears to be comparable to the Bish site in terms of size, species composition, substrate and bathymetry, as well as the degree of exposure to wind and wave. Logistical constraints and an early end to the BMAP project, however, prevented us implementing the BACI design. Although we did not tag as many fish as we had hoped, we were able to install acoustic telemetry arrays at both the Bish and Emsley sites. Our report focuses primarily on the May 2014 – August 2015 period, as that represents the time when most fish were tagged and when both acoustic arrays were fully operational.

Acoustic tags and hydrophone equipment

To monitor the movements of anadromous CCT at Bish Creek, we selected Vemco 69kHz coded acoustic transmitters, commonly referred to as "tags". These tags emit a coded acoustic signal (specific to a particular animal), which could be detected and recorded with a date and time stamp by any acoustic receivers within range. Two different sizes of acoustic tags were used: a larger one for anadromous adults (Vemco V13-1X tags: 13 x 36 mm; 11 g air, 6 g water; 147 dB re 1uPa @ 1 m); and a second smaller tag for subadults and smolts (Vemco V9-2X tags: 9 x 29 mm; 4.7 g air, 2.9 g water; 145 dB re 1uPa @ 1 m). The different tag sizes allowed us to consider a wider range of fish sizes for tagging and to ensure that tags accounted for no more than 2% of the fish's body mass (as per Mulcahy 2003), although we did not end up tagging any

smolts during sampling. Tags were programmed with a nominal ping rate of 45 sec (varied from 25–65 sec) during the spring and summer (when CCT are most likely to be in nearshore marine environments) and only once every 450 sec (varied from 350–650 sec) during the fall and winter (when fish would be expected to be in freshwater; see Table 5-1). This programming helped reduce the likelihood of tag collision (i.e., when two or more tags transmit simultaneously and can't be distinguished by a receiver) and was expected to greatly extend tag battery life (here estimated to be ~286 days for V9 and 533 days for V13 tags).

Table 5-1 – Programming parameters for the Vemco acoustic tags used in this study. L = 145 dB re 1uPa @ 1 m for V9 tags; 147 dB re 1uPa @ 1 m for V13 tags.

	Tag family	
Parameter	**V9-2x**	**V13-1x**
Est tag life (days)	286	533
Step 1 Status	ON	ON
Step 1 Time (days)	150	150
Step 1 Power (L/H)	L	L
Step 1 Min Delay (sec)	25	25
Step 1 Max Delay (sec)	65	65
Step 2 Status	ON	ON
Step 2 Time (days)	90	65
Step 2 Power (L/H)	L	L
Step 2 Min Delay (sec)	350	350
Step 2 Max Delay (sec)	650	650
Step 3 Status	ON	ON
Step 3 Time (days)	46	150
Step 3 Power (L/H)	L	L
Step 3 Min Delay (sec)	25	25
Step 3 Max Delay (sec)	65	65
Step 4 Status	OFF	OFF

Vemco VR2W 69kHz acoustic receivers (http://vemco.com/products/vr2w-69khz/) were used to detect acoustic tags and monitor the movement of tagged fishes. These low-cost single-channel acoustic receivers are fully submersible and autonomous once deployed. With a battery life of 12–15 months, the receivers are able to record 1–2 million unique tag detections and can be downloaded *in situ* with diver assistance or by pulling them to the surface. We employed a simple mooring system whereby VR2W receivers were attached to sealed concrete moorings and held upright about 1 m off the substrate by a large subsurface crab float.

Moorings were deployed in the narrow subtidal zone so that the float remained a minimum of 1–2 m below the surface during low tide. This was to ensure that hydrophones would remain vertical during all tide stages and to deter tampering or fouling by passing boats.

Range testing and installation of the acoustic telemetry arrays

VEMCO has an online range calculator utility (http://vemco.com/range-calculator/) that we used to predict the likely detection range for a particular tag under typical ocean conditions. Based on our tag specifications, the tool suggested a detection range of 250–490 m for V9 tags and 280–540 m for V13 tags. This information served as a starting point for array design, but did not replace the need for *in situ* range testing as local conditions (i.e., substrate type and bathymetry, salinity, boat activity, etc.) may significantly influence detection ability in particular habitats.

Prior to installing the hydrophone arrays, we performed *in situ* range testing to determine the effective detection range for our smaller V9 smolt tags. In July 2013, we deployed six hydrophones along a linear transect near the intertidal shelf separating Bish Creek and Cove. Hydrophones were placed at 0, 150, 250, 350, 450, 550-m distances from a V9 range testing tag that had a fixed 5 sec ping rate. The linear array was left in place for approximately nine days to evaluate detection ability under a range of tide and weather conditions. By determining the number and percentage of transmissions successfully recorded by hydrophones at each distance, we were able to determine the effective detection range of our tags at the study site. Successful detections were made at ranges up to 550 m, but the reliability of detection began to drop off significantly at ~250 m (Figure 5-1), suggesting that a hydrophone spacing of approximately 500 m would ensure near continuous coverage within the array.

We used 500 m as a guideline when designing the telemetry arrays, although the exact placement of hydrophones was often dictated by local bathymetry. As a U-shaped trench, the Kitimat Arm generally contains a narrow subtidal zone that drops off quickly to depths in excess of 200 m. The final Bish hydrophone array was installed in August 2013. It included six listening stations covering the W and NE sides of Bish Cove, Bish Cove Point, the broad intertidal shelf between Bish Cove and Bish Creek, and the mouth of Bish Creek (Figure 5-2; Table 5-2). The

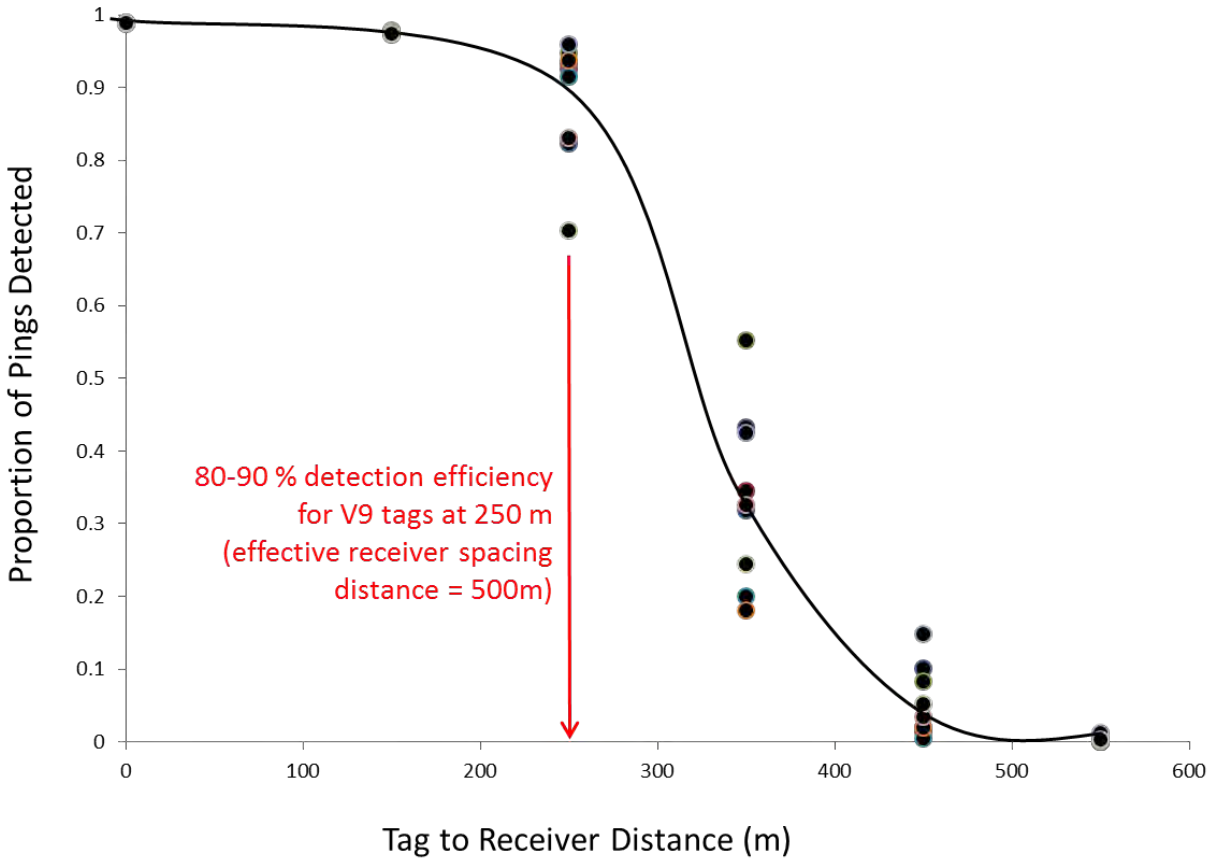

Figure 5-1 – Range testing results prior to installation of the Bish Creek array. Shown are the daily proportion of tag pings received by hydrophones placed 0, 150, 250, 350, 450, and 550 m from the range testing tag over the 9 day trial.

array was designed to allow for some overlap between the detection ranges of the hydrophones (i.e., fish detected at station B6 are often detected by station B5) so as to provide a measure of redundancy in case a receiver went missing. A seventh receiver was added at the Bish Creek tidal boundary marker in June 2014 to better delineate the shift between freshwater and near-shore marine habitats. Due to logistical constraints, the Emsley Creek "control" array was not installed until June 2014, but consisted of a similar deployment pattern covering the majority of available shoreline in that area (Figure 5-2; Table 5-2). The total monitoring period for these stations ranged from 384 – 697 days, but here we focus primarily on the May 2014 – August 2015 period, which represents the time when most fish were tagged and when both arrays were fully operational.

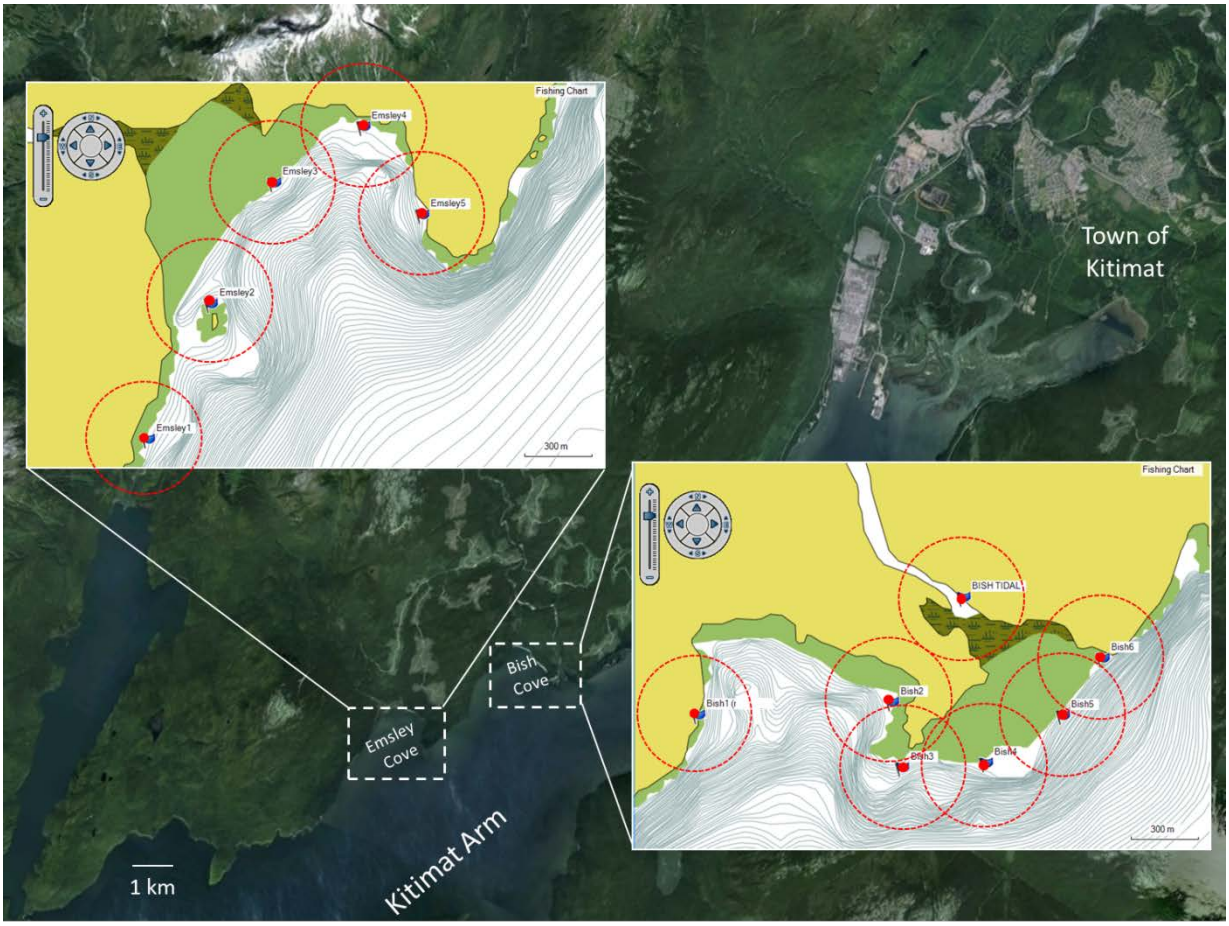

Figure 5-2 – Overview of the study area showing the location of moored hydrophones in the Bish and Emsley telemetry arrays. Red circles represent the approximate detection radius for each hydrophone based on in situ range testing.

Fish capture and biological sampling

Our goal was to tag 20 CCT per year (for two successive years) in both Bish and Emsley Creeks. This was an arbitrary number, but one chosen to balance the logistics of capturing enough suitably sized and apparently anadromous fish against sufficient sample replication that would allow for a robust analysis of the key behavioral parameters. Coastal cutthroat trout populations may sometimes be characterized by single dedicated life-history types, but are more likely to show a range of strategies (i.e., a certain proportion will be stream resident, a certain proportion anadromous, etc.). Accordingly, individual variation for life-history

Table 5-2 – Location data and monitoring period for hydrophones in the Bish and Emsley acoustic telemetry arrays.

Array	Name	Serial #	Location	UTM	Installation Date	Removal Date	Monitoring Period (d)
Emsley	E1	123012	W side of Emsley Cove	9U 513881 5971307	6-Jun-14	3-Jul-15	384
	E2	123010	Big Rock at Emsley Ck mouth	9U 514135 5971903	6-Jun-14	3-Jul-15	384
	E3	123009	mid Cove flats	9U 514391 5972437	6-Jun-14	3-Jul-15	384
	E4	123015	NE corner Emsley Cove	9U 514753 5972686	6-Jun-14	3-Jul-15	384
	E5	123021	E side of Emsley Cove	9U 514993 5972298	6-Jun-14	3-Jul-15	384
Bish	B1	123014	W side of Bish Cove	9U 515988 5974620	5-Aug-13	3-Jul-15	697
	B2	123008	NE side of Bish Cove	9U 516775 5974680	5-Aug-13	3-Jul-15	697
	B3	123011	Bish Cove Pt. (SE)	9U 516817 5974391	5-Aug-13	3-Jul-15	697
	B4	123013	W side of intertidal shelf	9U 517158 5974413	5-Aug-13	3-Jul-15	697
	B5	123017	middle of intertidal shelf	9U 517469 5974623	5-Aug-13	3-Jul-15	697
	B6	123020*, 123022	Big Rock at Bish Ck mouth	9U 517630 5974877	5-Aug-13	3-Jul-15	697
	B7	123018	Bish Ck tidal boundary	9U 517064 5975143	19-Jun-14	26-Aug-15	433

* - receiver went missing from April – August 2014 and was replaced

characteristics is expected to be considerable and individual strategies may change over time (e.g., Goetz et al. 2013). For example, an individual might spend several years in a nonmigratory or freshwater migratory phase before migrating to saltwater. Upon returning to freshwater, the individual may spawn or simply overwinter, repeating this cycle (or a variation of it) numerous times. Importantly, much of this diversity appears to be adaptive in nature and has evolved in response to local environmental conditions (*sensu* Taylor 1991).

Tagging operations were scheduled from late spring to early fall (May – September) which roughly coincides with the period during which anadromous cutthroat trout would be most likely to be found in or moving towards saltwater (Johnson et al. 1999; Saiget et al. 2007; Goetz et al. 2013). Although we did attempt to sample widely in the two sampling areas (i.e., in both coves and creeks, as well as the intertidal shelf and headlands separating areas), our

access to the some of the more productive areas was limited. As such, catch-per-unit effort (CPUE) was generally low in the Bish system with only two fish per 14 rod-days in 2013 and 17 fish per 35 rod-days in 2014. Despite repeated effort, no taggable fish were captured in the Emsley system.

All captured fish were anaesthetized by placing them in an anesthetic bath containing 0.5 ppm tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. Once sedated, we collected standard biological information fork length, weight, sex, condition factor (K) where $K = 105 \times (\text{weight in g})/(\text{fork length in mm})^3$, and photo documentation. All captured fish were marked with a uniquely numbered external Floy tag to provide a visible means of identifying tagged fishes upon subsequent recapture. Adipose fin tissue samples (for DNA analyses), scales or fin rays were also collected; tissue samples were stored in 95 % ethanol while scales and fin rays were stored in dry, labeled scale envelopes. Fish collection and tagging were performed in 2013 and 2014 under the following permits: federal permits XR-115-2013, XHAB-36-2014; provincial permits SM13-86621, SM14-150662; and UNBC ACUC permits 2013-05, 2013-05(2014).

Tagging procedure

As above, fish selected for tagging (based on capture location, size, condition, sex, etc.) were held in the anesthetic bath until the opercular rate slowed and equilibrium was lost. They were then transferred to a padded V-shaped surgery trough containing fresh water. The ventral surface was swabbed with Ovadine solution and a 1.5–2 cm long incision will be made slightly lateral of the ventral midline and anterior to the pelvic girdle. Acoustic transmitters were inserted into the peritoneal cavity and the incision closed with a two sterile Monocryl sutures. The sutured incision was again swabbed with Ovadine solution before placing the fish in a covered holding tank with circulating oxygenated river water for recovery. Actual time spent in anesthetic bath, surgery and recovery time was recorded for each fish as was its condition upon release (qualitative scale from 1 – 5, with 1 being a fish that swims away most vigorously).

Telemetry data downloads and processing

Although the VR2W receivers can store a large amount of detection data, we chose to download data whenever hydrophones were accessed for maintenance (i.e., when replacing

the batteries or defouling). Three main data downloads were performed: April 2014 (data from August 2013 – April 2014), August 2014 (data from April – August 2014), and July/August 2015 (data from August 2014 – August 2015). Data download and processing was facilitated on site by Vemco's VUE Software (v2.2.3 beta), which allowed us to compile time stamp and tag id data from multiple receivers into a central database. The program also offered a set of filtering and processing tools. For example, we used the time correction utility to collect for clock drift between receivers. Due to manufacturing variation in the crystal oscillators used to track time in the VR2W receivers, up to 4 sec of clock drift per day may occur between receivers, making it difficult to accurately distinguish multiple detections from the same signaling event. The time correction utility applies a linear correction based on known initiation and download times (i.e., accurate computer time) to correct for clock drift at each receiver. We also employed the false detection analysis tool (FDA) with default settings to screen the database for poor or questionable detections. These can sometimes occur when numerous transmitters are within range of a detector and multiple tag collisions prevent tags from being properly identified. Only seven of 61,230 total detections were identified as questionable or of low quality by the analysis tool. Each was investigated further to determine if it was a true or "phantom" detection.

Finally, in an effort to simplify our interpretation of the telemetry data and reduce the number of redundant data points in the database, we used the VUE residency search tool. The tool uses an algorithm to reduce the volume of data by combining a series of detections into a single, user-defined residency period. We used a minimum detection threshold of five detections (roughly equating to a fish spending 3–4 min within range of a particular receiver) and an absence threshold of 30 min (maximum time permitted between detections during a single residency period) to determine second "residency" point around a particular hydrophone station. We queried both the complete and reduced datasets to identify the total number of tag detections at each station and for each fish. From those data, we reconstructed the individual patterns of movement and habitat use by tagged fish (both spatially and temporally during the day), estimated the timing of movements in and out of the near-shore marine zone, as well as the residency period for CCT in the area (estimated total number of days spent in the marine

and estuarine zone). To get a sense of where individual fish are spending the majority of their time, we summarized individual detection data by four main "listening" areas, including Emsley Cove (stations E1-E5), Bish Cove (B1-B2), the intertidal shelf between Bish Cove and Bish Creek (B3-B5) and Bish Creek (B5 and B6).

There were, however, some gaps in our temporal coverage. Sometime between April and August 2014, the hydrophone at Station B6 (mouth of Bish Creek) went missing. We believe it may have been tampered with as we found the intact mooring with the hydrophone removed. This is a popular area with fisherman and boaters during the salmon run and although the entire mooring assembly remained sub-surface during low tides, it does incorporate a sub-surface crab float which might have attracted some attention. Because this is an important monitoring area, we deployed a second receiver at the site. Although the physical loss of receiver is unfortunate, we believe that little to no important data were lost. The array was designed to allow for overlap between the detection ranges of the hydrophones so that fish detected at B6 are often detected by B5 and potentially the BT station (Bish Creek tidal boundary), depending on which way they are moving. During that same period, the B2 receiver (NE corner of Bish Cove) was mistakenly pulled up by AMR during their work in Bish Cove. We were able to recover that hydrophone from AMR, download the data and redeploy in the NE corner of Bish Cove. Finally, the E2 hydrophone deployed at the mouth of Emsley Creek malfunctioned (it contained no data when downloaded in August 2015). Again, while unfortunate, the overlap in coverage with the neighboring receivers (E1 and E3) helped ensure that movement data through that area was recorded.

Diet analysis

We were also interested in better understanding the foraging ecology of CCT as it has been relatively understudied relative to other salmonids, and is, more importantly, expected to be highly sensitive to environmental change (and thus useful for monitoring purposes). Although CCT are known to be aggressive and opportunistic feeders (Jauquet 2002), their dietary preferences in the nearshore-marine environment and the degree to which they depend on those habitats are poorly characterized.

Two different approaches were used to obtain stomach contents from coastal cutthroat throat. For all fish caught during tagging operations were sampled for their stomach contents, we employed gastric lavage, a technique that has been found to be highly effective and minimally invasive in collecting stomach contents from live fish (Light et al. 1983; Kamler and Pope 2001; Jones et al. 2008). It involved carefully inserting a clear acrylic tube of an appropriate diameter into the fore-stomach of an anaesthetized fish and injecting a pulsed stream of water to flush stomach contents onto a 400 micron nitex screen for collection. For three fish (i.e., those injured during capture or those responding poorly to surgical implantation of the acoustic tags), we lethally sampled the entire digestive tract for analysis. This allowed for a complete enumeration of prey items in both the stomach and the intestine and as a means of double-checking the efficiency of our gastric lavage procedure.

All prey items were stored in 95 % ethanol prior to analysis. We used a blended approach to stomach content analysis, identifying prey items primarily using traditional taxonomic characters and diagnostic keys (e.g., Merritt and Cummins 1996; Light 2007), supplementing with DNA barcoding of the cytochrome oxidase I (COI) gene as needed. We describe here the dietary composition (in terms of prey species diversity, size and abundance) for sampled all fish; this data is complementary to the telemetry data; particularly where identified prey items have well-defined habitat associations.

Results and Discussion

Fish capture and biological information

As noted, our catch-per-unit effort (CPUE) was generally low in the Bish system with only two fish per 14 rod-days in 2013 (Table 5-3). This sampling occurred primarily in Bish Cove and the estuary and produced only one fish that was suitable for tagging (BishCCT#02). Catch-per-unit effort improved greatly in 2014, with 17 fish per 30 rod-days. Three of those days were spent sampling the lower river where two sites (“the 90”, which is adjacent to the mouth of Skoda and Renegade creeks, and “Stairway to Paradise”, a pool 100 m upstream) were found to be excellent trout water. We captured eight taggable fish at those sites (two males and six females) with six being captured within 30 min at the mouth of Skoda/ Renegade creeks. This is

in keeping with the propensity of cutthroat trout to travel in schools or "packs" (e.g., Giger 1972). We tagged all eight of those fish; one female (BishCCT#06), however, did not recover from the anesthetic and was euthanized. We also had better success in the Bish Creek estuary in 2014. A particularly good spot to find cutthroat trout is near the "Big Rock" at the mouth of Bish Creek. There is a channel that funnels prey items past the rock on ebb tide. Over several days of fishing from the zodiac in June, we caught nine trout in this area (two males and seven females), six of which we ended up tagging. In total, there were 14 coastal cutthroat trout tagged and released in the Bish Creek system. Despite repeated effort, however, no taggable fish were captured in the Emsley system.

The high female-to-male ratio in our sample (~3:1 in both Bish Creek and estuary) is not entirely unexpected for anadromous coastal cutthroat trout and previous studies in coastal cutthroat trout populations have suggested that sex ratios can often favor females, particularly among anadromous populations (Wenburg 1998; Costello 2006). The relative benefits and risks associated with anadromous movement have been well documented for salmonids (Northcote 1997; McDowall 2001; Fleming and Reynolds 2004; Hendry et al. 2004); females, which optimize their reproductive fitness by attaining large size and greater fecundities, would likely benefit to a greater extent from the benefits of rich saltwater feeding areas than would males, even if such movements made them susceptible to increased mortality. Interestingly, baseline genetic data collected from throughout the wider Kitimat Arm suggest that sex ratios in juvenile CCT samples may be highly biased as well, though the direction seems to vary on a site by site basis from nearly entirely female (39:1 FM ratio among Cecil Creek samples) to predominately male (1:13 at Lone Wolf Creek; A. Costello, unpublished data).

Though similar in terms of fork length (average FL = 378 mm vs 381 mm), females tended to be heavier than males, (averaging 605 g vs 560 g, respectively) and were of slightly higher condition factor (K = 1.08 vs 1.01, respectively; Table 5-3; Figure 5-3) . Most tagged fishes were estimated to be 4–5 years old, with one 3-year old: BishCCT#11 (267 mm FL, 220 g) and one or two possible 6-year old fish: BishCCT#16 (455 mm FL, 1000 g) and BishCCT#10 (448 mm FL, 1080 g).

Table 5-3 – Sex, age, and other standard biological measurements for sampled Bish Creek coastal cutthroat trout, including eight (Wt.) and condition factor (K). Tag information and telemetry detection data for the May 2014 - August 2015 period are provided. Acoustic tag (Acous. Tag), total detections (Tot. Det.), the number of hydrophones that detected each tagged fish (No. Hydr.), the first date detected (First Det.), the last date detected (Last Det.), and the estimated marine residency period (in days) is provided for fish with well-defined entrance and exit dates (Res.).

Fish	Sex*	Age	Length (mm)	Wt. (g)	K	Floy Tag	Acoustic Tag	Tot. Det.	No. Hydro.	First Det.	Last Det.	Res. (d)
01	F	3	285	---	---	---	---	---	---	---	---	---
02	M	4+	388	580	0.99	051	11164	0	0	---	---	---
03	F	4	207	80	0.90	---	---	---	---	---	---	---
04	F	4	390	630	1.06	053	11162	6715	11	6-Jun-14	7-Oct-14	125
05	M	4	368	500	1.00	052	11159	1017	6	22-Jun-14	18-Jul-14	---
06	F	5	409	760	1.11	054	11170**	---	---	---	---	---
07	F	4	384	590	1.04	055	11145	15281	7	8-Jun-14	25-Sep-14	29
08	F	5	383	580	1.03	056	11144	9466	6	21-Jun-14	4-Aug-14	46
09	M	4	338	380	0.98	057	11146	3300	11	7-Jun-14	15-Mar-15	124
10	F	5-6	448	1080	1.20	058	11150	2662	4	19-Jun-14	17-Dec-14	---
11	F	3	267	220	1.16	059	11169	7232	11	19-Jun-14	9-Jun-15	---
12	F	5	388	600	1.03	060	11163	9143	7	31-Aug-14	1-Apr-15	---
13	F	5	373	560	1.08	061	11171	0	0	---	---	---
14	M	5	439	720	0.85	062	11158	779	9	22-Jun-14	1-Jul-15	---
15	M	4	372	620	1.20	063	11167	65	6	22-Jun-14	22-Jun-14	---
16	F	5	455	1000	1.06	064	11152	4064	8	23-Jun-14	24-May-15	---
17	F	5	385	540	0.95	065	---	---	---	---	---	---
18	F	4	385	640	1.12	066	11160	1499	6	23-Jun-14	6-Jun-15	---
19	F	4	358	580	1.26	067	---	---	---	---	---	---

* - As determined by the OmyY1 sex-specific marker
** - Fish did not survive surgery

Two main color phases (Figure 5-4) were apparent in our sample: heavily spotted fish with operculum “blush” and a green-brown back typical of freshwater fish, and bright silvery fish with smaller, less conspicuous spotting typical of anadromous fish. BishCCT#05 was a particularly colorful male fish and may have been displaying some spawning coloration (spawning in CCT generally occurs from April – May in this area).

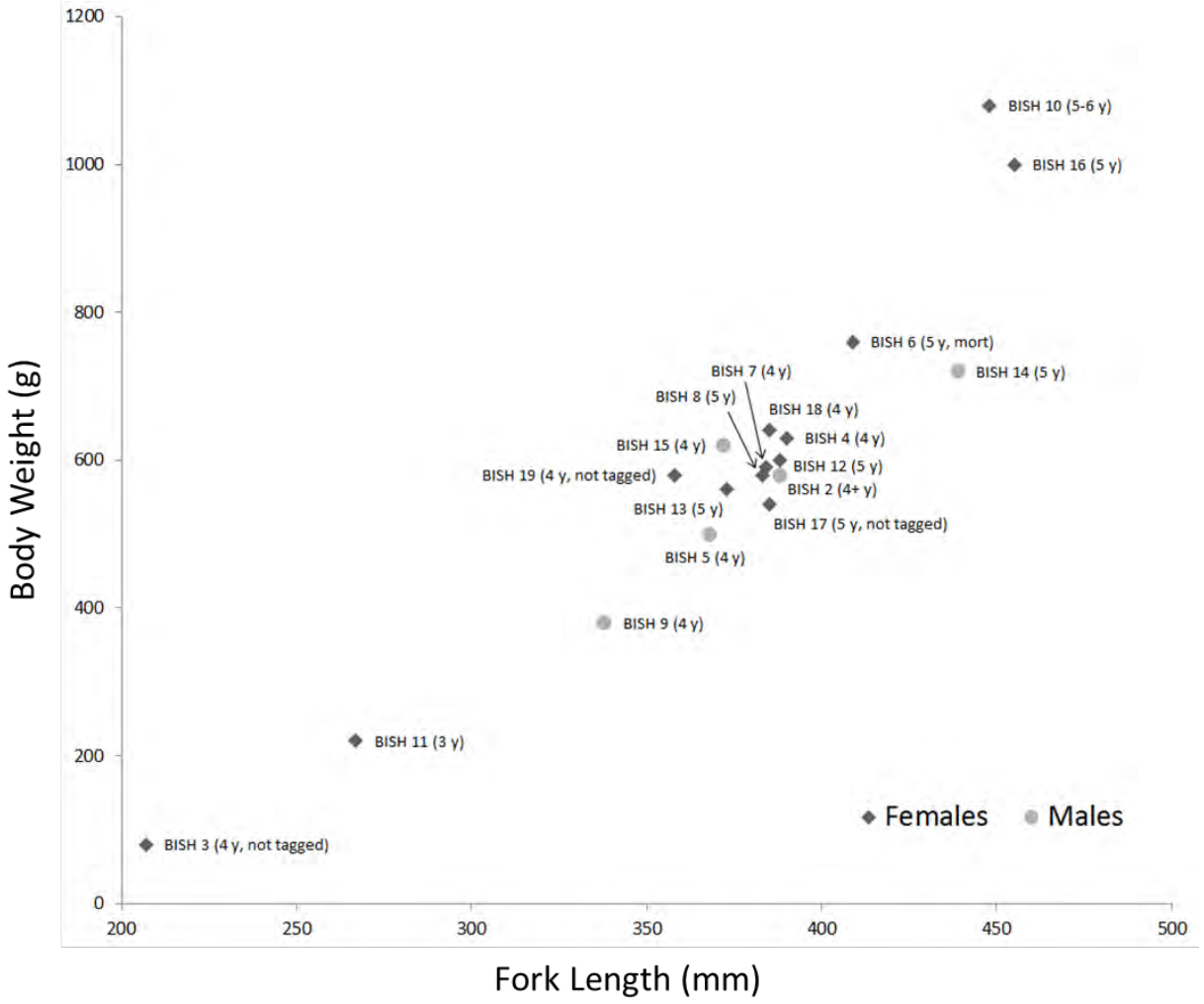

Figure 5-3 – Length-weight relationships for Bish Creek coastal cutthroat trout. Individual fish identification numbers and estimated ages are indicated.

Of interest, a large number of anadromous fish had open wounds and scarring associated with predator attack (e.g., BishCCT#12, 17, Figure 5-5). There are a large number of seals and river otters that inhabit the lower end of Bish Creek and predation is expected to be a significant threat for anadromous cutthroat trout.

Spatial and Temporal Distribution of Tag Detections

A total of 61,223 confirmed tag detections were obtained across the two listening arrays from May 2014 – August 2015. All hydrophones (with the exception of E2, which seems to have malfunctioned) recorded fish presence, with the greatest proportion of detections being made at stations B4 (32 %), B3 (22 %), B5 (13 %), and B2 (12 %; Figure 5-6).

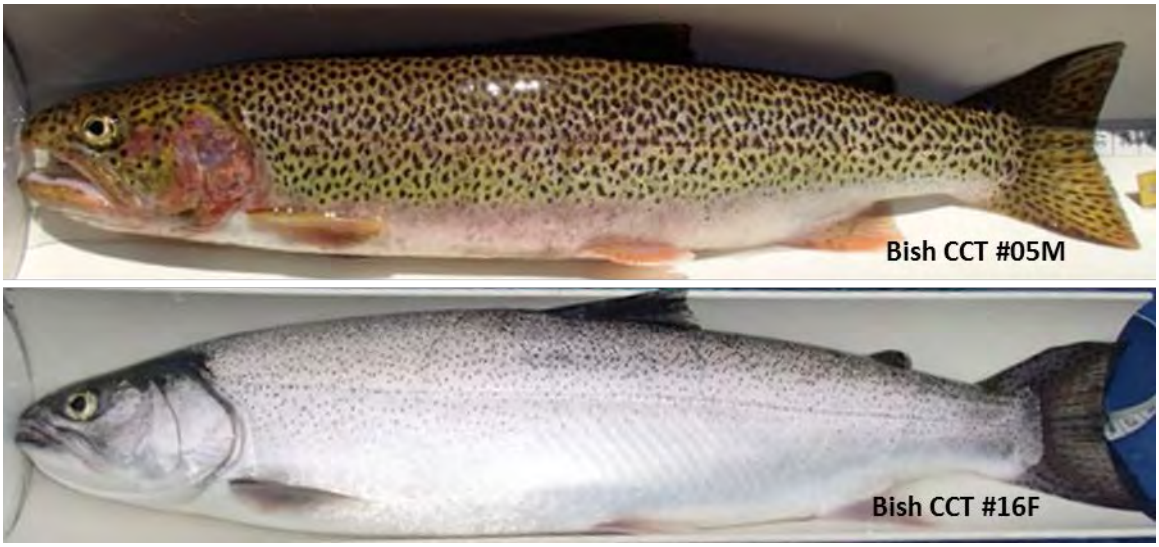

Figure 5-4 – Tagged fish displaying the range of colouration from green-brown typical of freshwater fish to the silvery colour typical of anadromous fish.

Figure 5-5 – Examples of fish with wounds and scarring associated with predator attack.

Spatial and Temporal Distribution of Tag Detections

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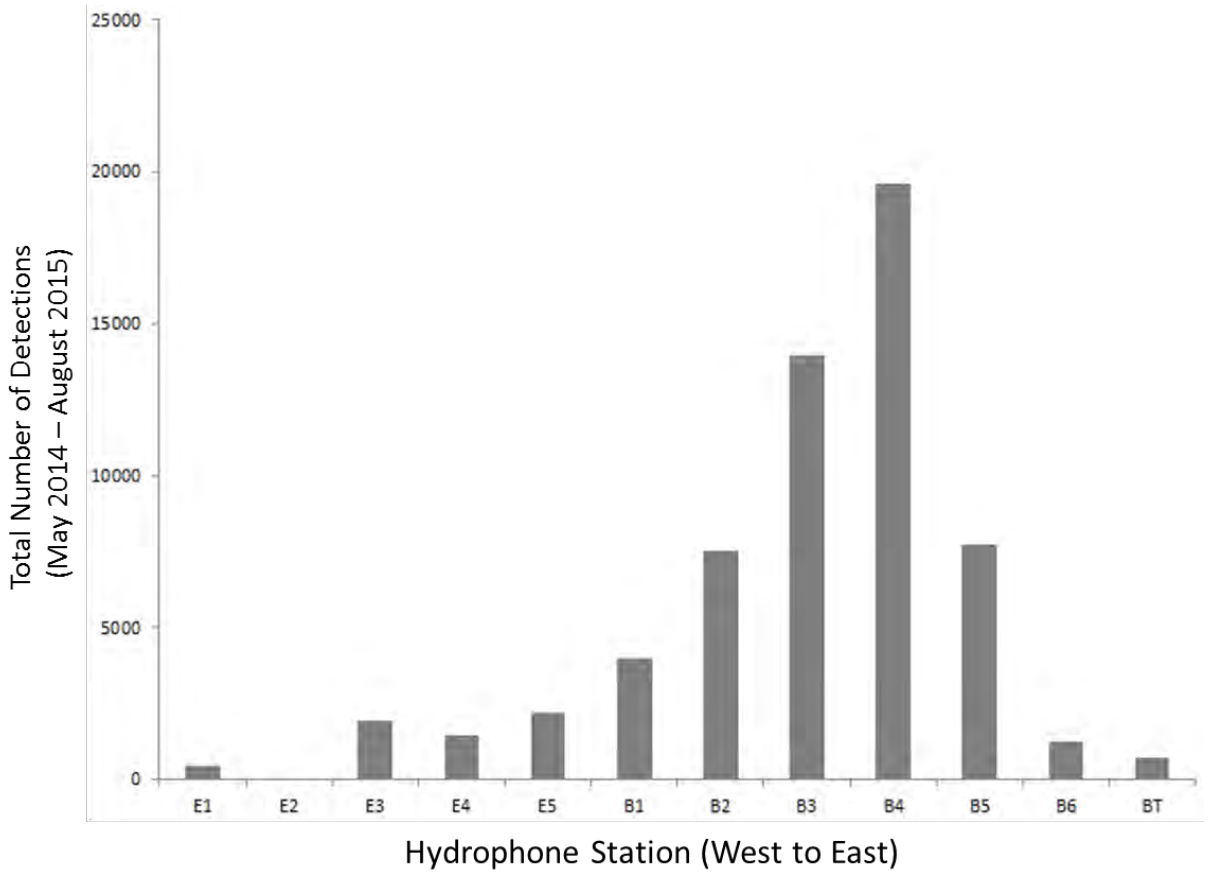


Figure 5-6 – Total number of tag detections by hydrophone station for the May 2014 – August 2015 listening period. Station codes are described in Table 5-2.

These stations cover the area along the rocky intertidal shelf between Bish Creek and Bish Cove, as well as the NE corner of Bish Cove. Relatively few detections were made at in the area of the Bish Creek mouth and tidal boundary (three % in total at B6 and BT), though admittedly there was a period of ~four months where detection data at B6 was lost. More detections were, in fact, made in Emsley Cove (10 % of total) than at Bish Creek. That is not to say that the estuarine portion of Bish Creek is unimportant to CCT; we obviously expect CCT to heavily use that area and indeed, we ended up tagging approximately half of our fish there. It may be, however, that fish are rapidly migrating through that area when leaving or returning to Bish Creek and/or are feeding in the area primarily during a particular time of day or flood stage. We observed this behavior, and over several days, were able to predict when the feeding CCT would arrive (and leave) the mouth of the creek. Detections were made in every month of

the year, but the majority occurred between May and July (the primary period during which tagged fish are feeding in nearshore marine environments), with a secondary peak through September and October (which we interpret as movements associated with the return of trout to Bish Creek to overwinter or feed on the salmon run; Figure 5-7).

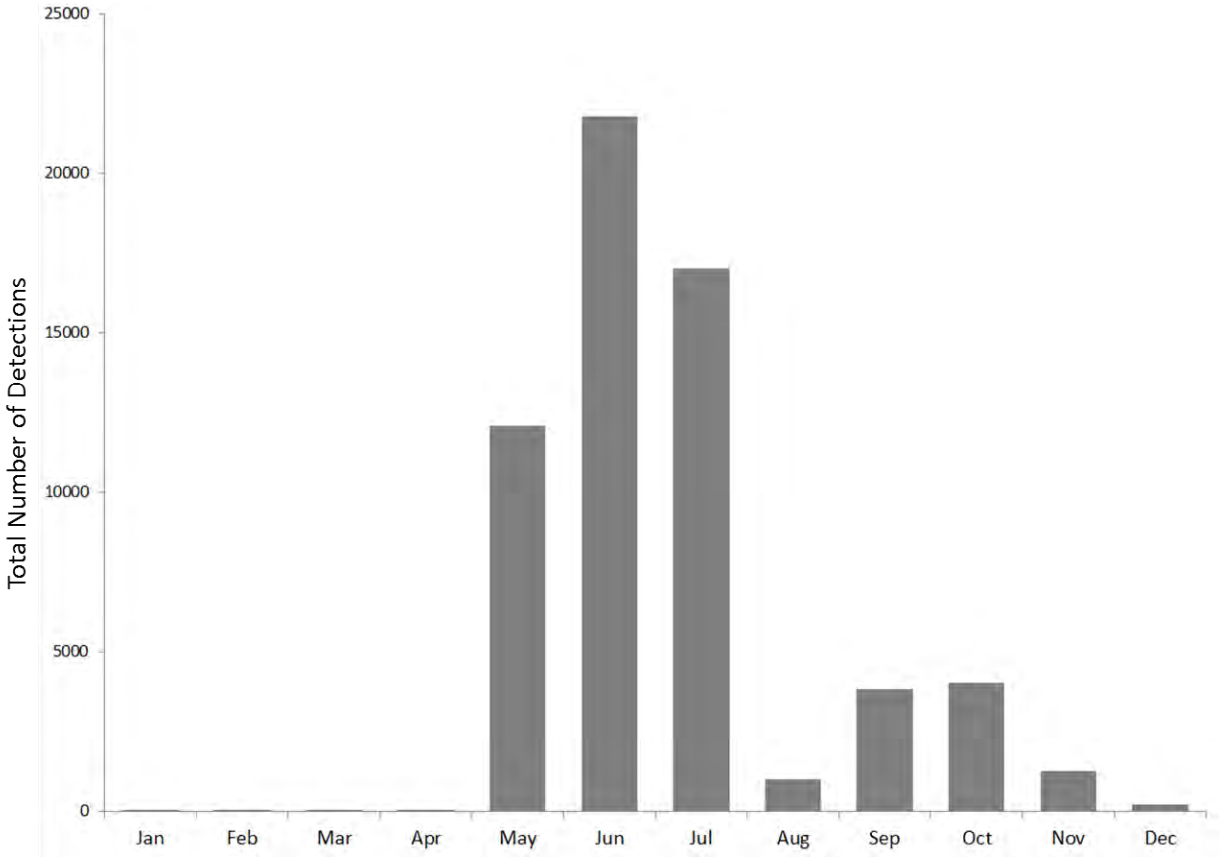


Figure 5-7 – Monthly distribution of tag detections across all hydrophones for the May 2014 – August 2015 listening period.

While tagged cutthroat trout were active throughout the day, daily detection levels were clearly non-random, with 80 % of all detections occurring during daylight hours (approximately 0500 – 2200 h during summer months at this latitude). Relative to the hourly average, peak activity (as much as 55 % above average) occurred between 0400 – 1100 h, declined in the afternoon, and showed a second minor peak at dusk (~2000 h; Figure 5-8). As a visually-oriented predator, CCT are expected to be primarily active during the day (e.g., Goetz

et al. 2013) and many species of piscivorous fishes do show similar bumps in activity during crepuscular periods (e.g., Young et al. 1997; Baldwin et al. 2002). We did, however, observe a distinct exception to this trend whereby CCT were choosing to travel under the cover of darkness during their initial movements to the estuary. Goetz et al. (2013) found a similar trend in the Hood Canal, Washington; the behavior presumably helps to lower predation risk while fish are acclimating to estuarine conditions.

Detection data for individual fish

Twelve of the 14 tagged fish were detected between May 2014 and August 2015 with the total number of detections ranging from 65 – 15,281 for individual fish (Table 5-3). Only two fish went undetected during the period (Bish CCT#02 and #13). As mentioned, Bish CCT#02 was tagged in 2013 and although detected a total of 990 times at five different receivers between June 6th – July 7th of that year, it was not detected again. Bish CCT#13 was tagged in Bish Creek on June 20th, 2014 and does not appear to have descended into the estuary following tagging. It may still be holding in the river upstream of the receivers or may have subsequently died. All other fish tagged in Bish Creek (with the exception of BishCCT#006, the tagging mort) ended up going to the estuary and were picked up by the listening arrays.

Tagged fish began leaving Bish Creek in small groups between June 5th and June 22nd. Those movements appear to be non-random and occurred over relatively short time frames; BishCCT# 04, 07, and 09 left the river between June 6 – 8 and BishCCT# 05 and 08 left between June 21 – 22; both of these dates correspond with the smallest tide changes for the month of June. These fish returned to Bish Creek over a somewhat wider window from July 7 – mid-October, although the peak return appears to have occurred in the first half of August. Actual marine residency time for these fish (where there are well-defined dates of outmigration and return) ranged from 29 – 125 d (Table 5-3). The wide range in estimated residency time may be indicative of different movement patterns/foraging strategies among anadromous CCT (see Section 5.4), but are generally in keeping with data from other areas. Unlike populations to the south where anadromous cutthroat trout may spend four to six months at sea (Sumner 1962; Wenburg 1998), northern populations typically spend only a few months in marine waters (Armstrong 1971; Saiget et al. 2007). In the Kitimat River, for example, CCT appear to

outmigrate between May 22 – June 7 with most fish moving between June 1 – 3; these same fish were detected returning to the Kitimat River between June 17th to July 18th, after spending an average of 34 days in marine environments (Costello et al., unpublished data).

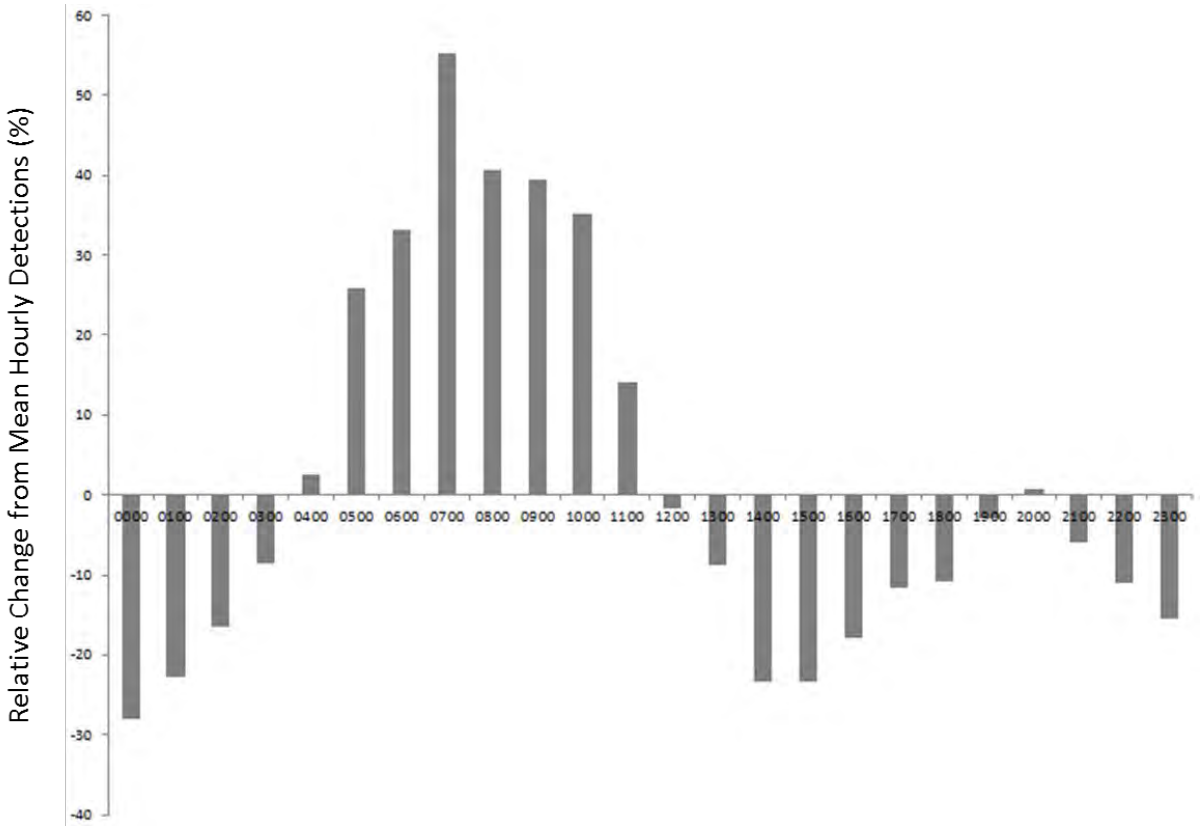


Figure 5-8 – Diel patterns of activity in Bish Creek coastal cutthroat trout for the May 2014 – August 2015 listening period. Bars represent percent change from mean hourly detection rates across all hydrophones. The approximate duration of daylight at this latitude is indicated.

Interestingly, the average number of detections appears to differ significantly between fish tagged in the creek versus the estuary (averaging 7,487 vs. 2,717 detections respectively, $t_8 = 2.05$, $p = 0.04$). Although it is difficult to assess, it may be that fish tagged in the estuary have a lower post-operative survival rate. This would not be entirely surprising; environmental conditions in the estuary (i.e., pH, salinity, temperature, etc.) are highly variable during the day

and may predispose fish to additional physiological stress not encountered in freshwater. Furthermore, the estuary certainly does contain a healthy population of seals and river otters, which do actively target fish. Based on continued tag movement, however, we don't believe tagging-related mortality to be the primary driver of these findings. Alternatively, it may be that a higher proportion of the fish tagged in the Bish Creek estuary are migratory fish that are simply passing through the area, possibly from other systems. Coastal cutthroat trout are known to routinely travel to non-natal streams to feed on locally abundant food sources (Giger 1972; Behnke 1992; Pearcy 1997; Trotter 1997). Given the high-relief topology of Douglas Channel and limited estuarine habitat, we may expect productive estuaries to be utilized by fish from other streams lacking such resources (see Section 5.4).

Near-shore marine movement patterns and habitat use by tagged fishes

As expected for a species with mixed migration strategies and abundant life-history variation, there was considerable deviation between individual fish in terms of the timing, number, and magnitude of their movements in the near-shore marine environment. Some fished moved very little, others moved a lot; fish that moved a lot often did so in different ways (i.e., by making fewer, but longer distance movements vs many shorter movements). Some fish remained in the Bish array for the entirety of the monitoring period, while others travelled to Emsley Cove and beyond, leaving the monitoring area to return at a later date (Figure 5-9).

At least two main strategies seem to be apparent. First, a resident strategy whereby movements were generally confined within the listening area and exhibit daily ranging or commuting behavior while feeding. A total of seven out of 12 fish were identified as undertaking resident strategies with some commuting solely within the Bish array (Bish CCT#07,#08, #10, and #12) and others showing regular movements to and from Emsley Cove (Bish CCT#04,#09, and #14). The majority of these fish were tagged in Bish Creek while two were tagged in the estuary. Second, a migratory strategy whereby fish displayed making more extensive and directed movements out of the listening area. A total of 5 of 12 fish were identified as using this particular strategy (Bish CCT#05, #11, #15, #16, and #18); all but one were tagged in the estuary. Given the geographic scale of our listening area (approximately 5–6 km in total length), we do not have data on where exactly these individuals went when they left

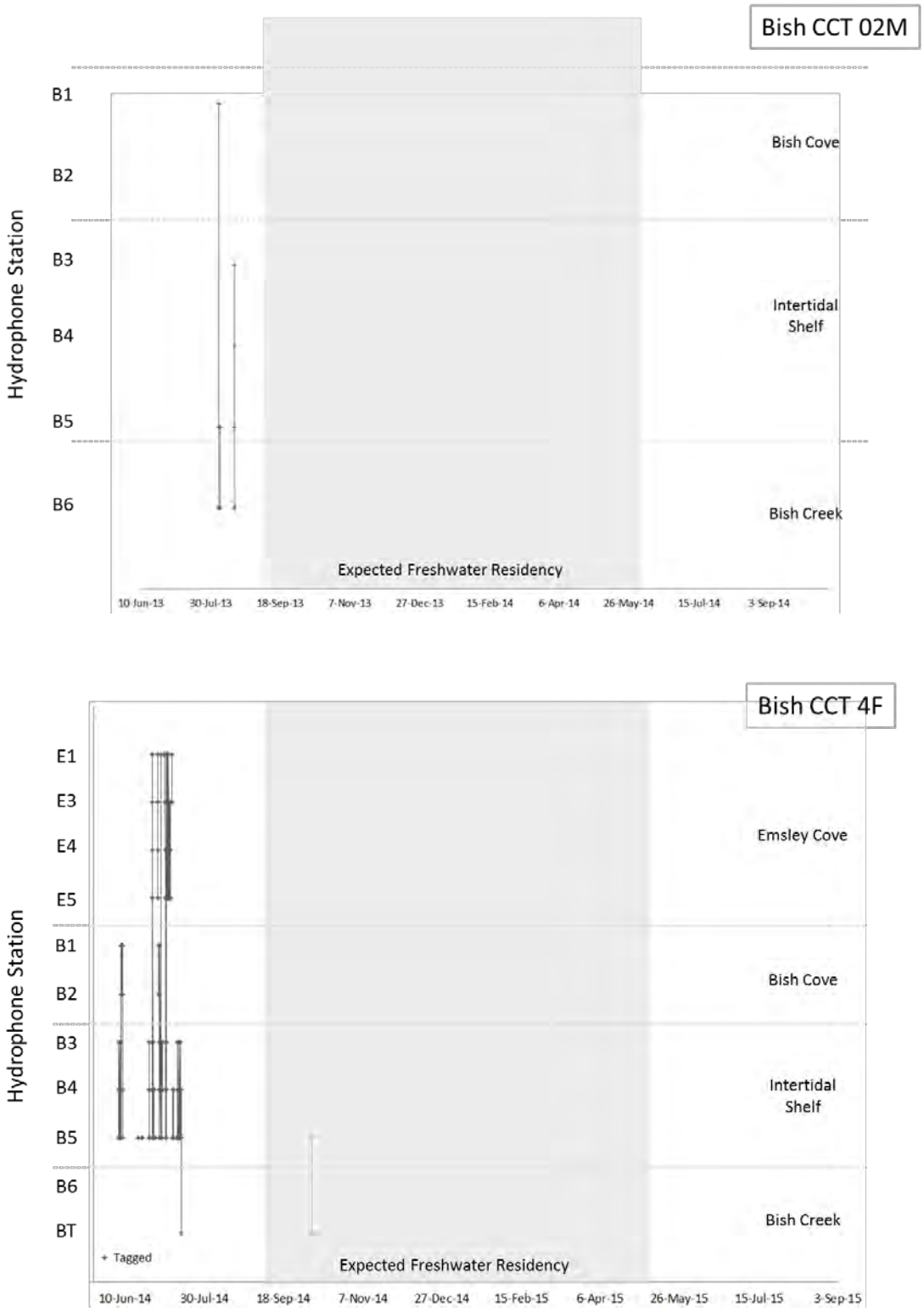


Figure 5-9 – Movement profiles for tagged Bish Creek coastal cutthroat trout for the May 2014 – August 2015 monitoring period (except Bish 02M, 2013-2014). Hydrophone stations are described in Table 2 and arranged roughly west (top) to east (bottom).

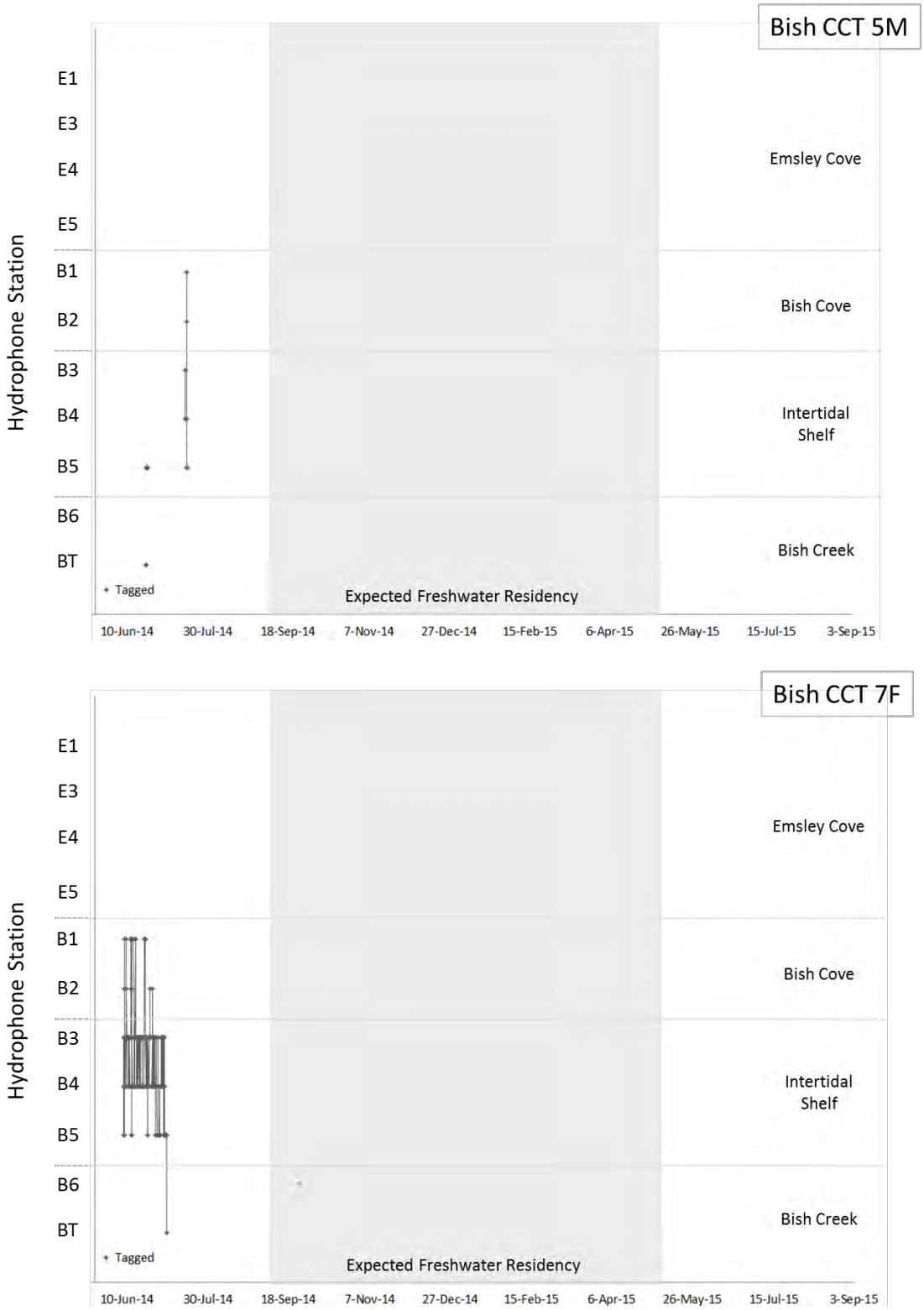


Figure 5-9 continued.

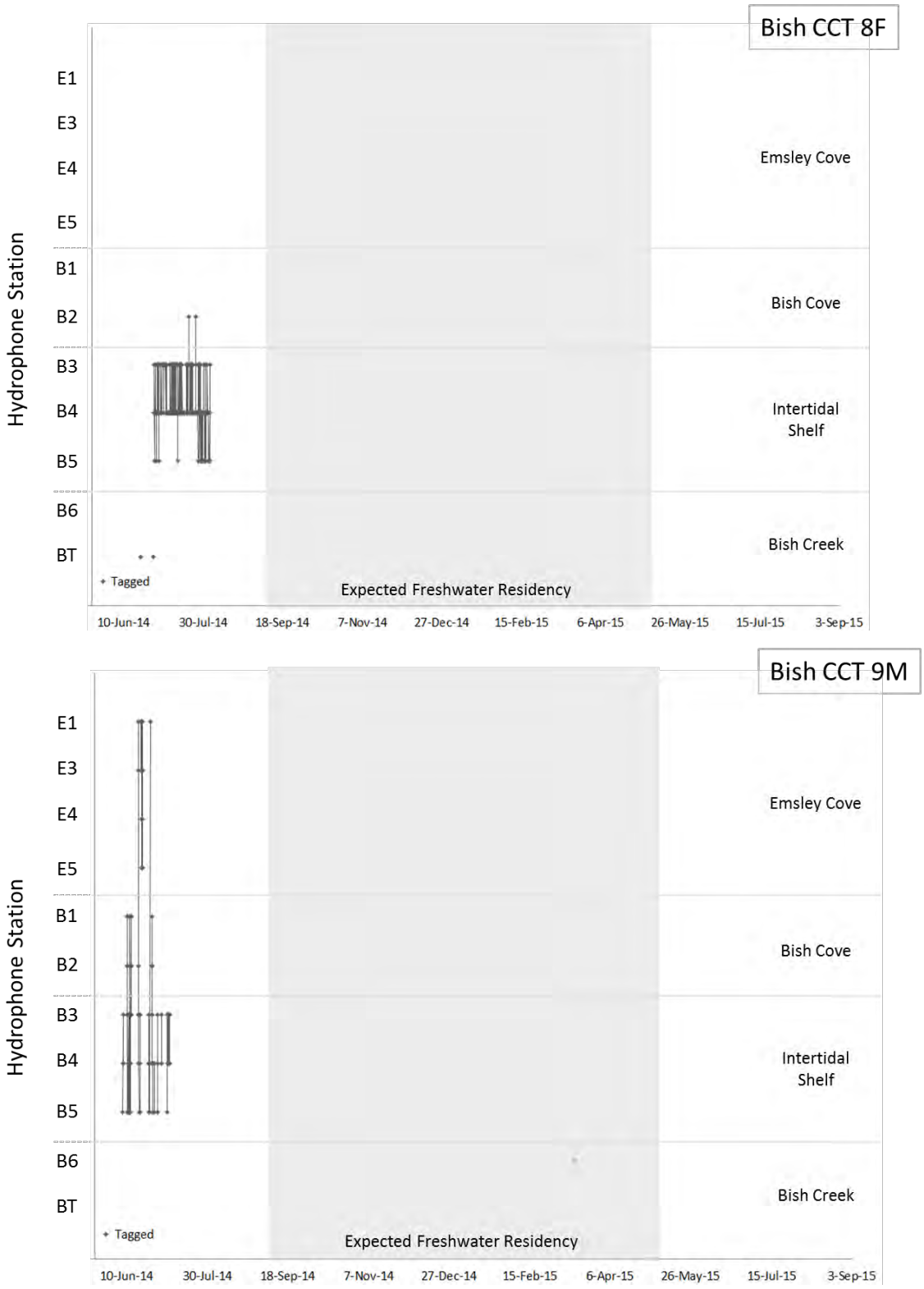
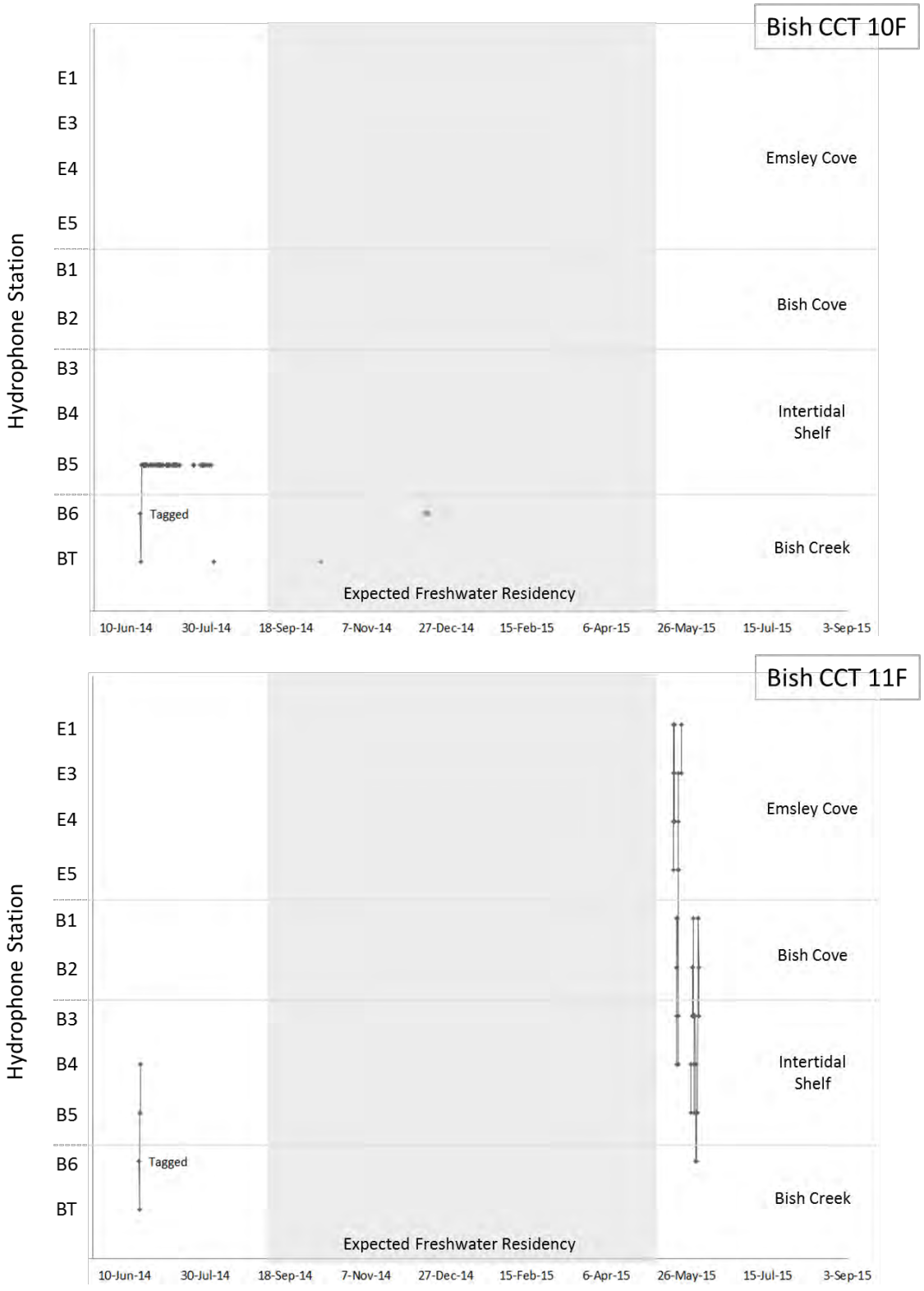


Figure 5-9 continued.



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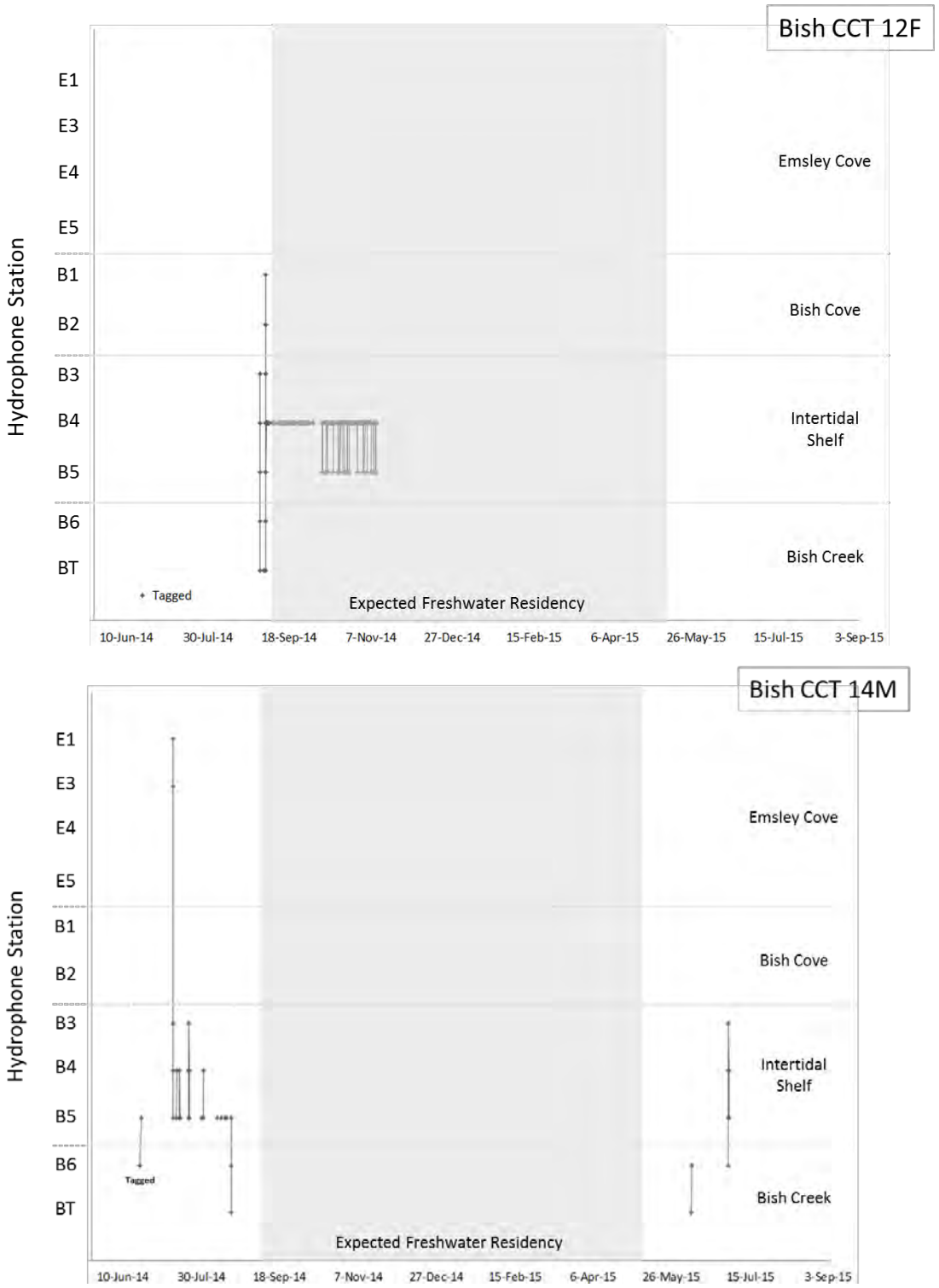

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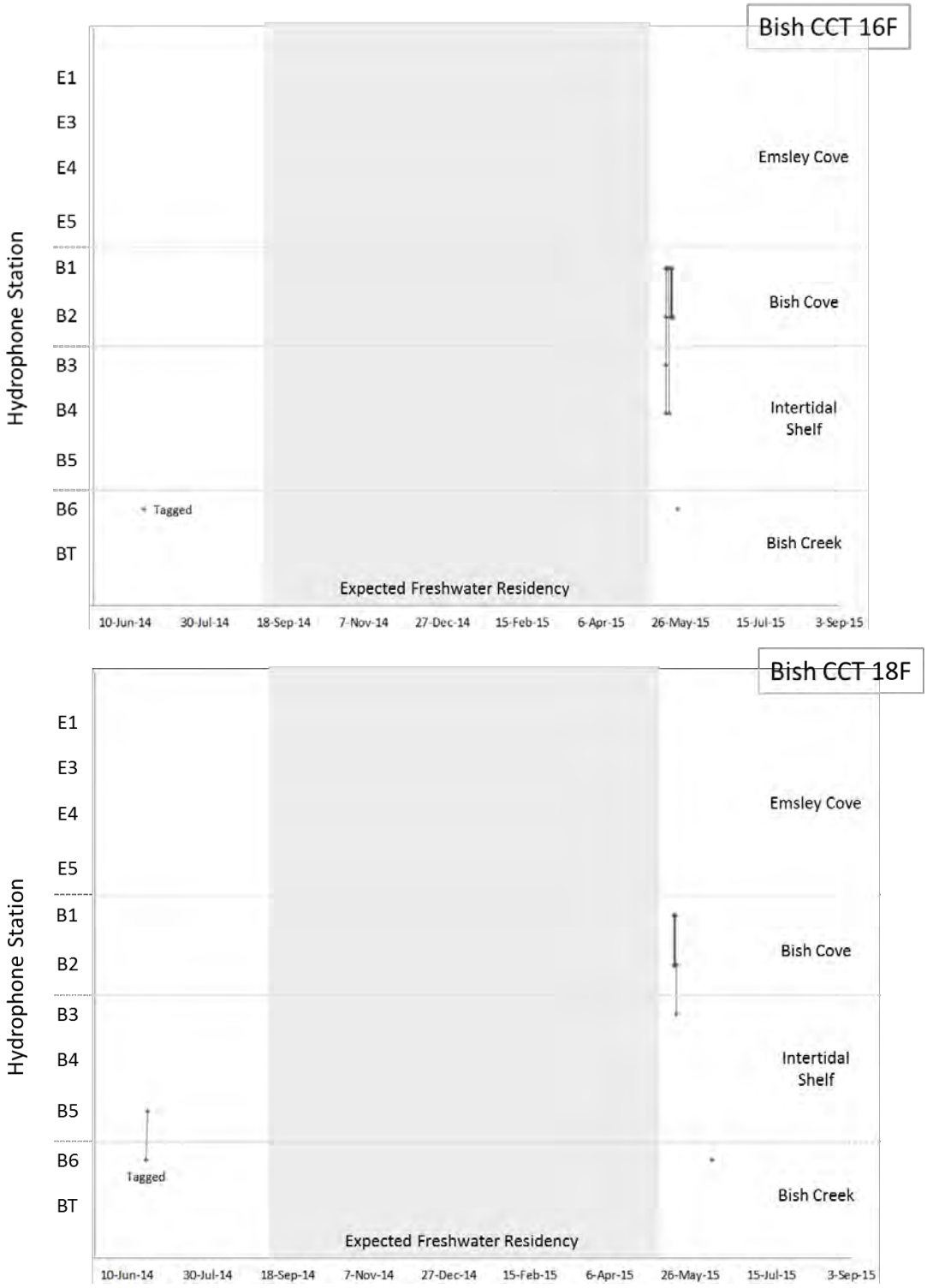

Figure 5-9 continued.

the area, but we do know that at least half of them returned to it in 2015 after presumably overwintering elsewhere (e.g., BishCCT #11, #16, #18).

Anglers of CCT, particularly those who frequent saltwater beaches and estuaries, have long suggested that there might well be two types of anadromous cutthroat, referring to them as “beach trout” (i.e., smaller fish with yellow bellies; mostly males that stay near local bay or estuary); and true “sea-run” trout (i.e., larger silvery fish that travel greater distances and are, mostly female; Massey 1984). With such a limited sample size, however, we are not able to discern the mechanisms involved in the selection of a particular migration strategy; here both males and females undertook both strategies, though a high percentage of what we are calling migratory fish (4 of 5 = 80%) were indeed female.

Goetz et al. (2013) describe similar findings in a study that monitored the movements of 59 coastal cutthroat trout over a three-year period in Big Beef Creek estuary and the Hood Canal, Washington. The Hood Canal is a tributary to Admiralty Inlet and the Strait of Juan de Fuca and is similar to the Kitimat Arm in that it is a narrow (2–4 km wide) and deep (up to 175 m) fjord-like channel. Conducted over a much larger spatial scale (their listening area was over 100 km in length with receivers spaced approximately 3–15 km apart), the authors identified the same general migration strategies we have described. Fish identified as “residents” spent more time in the Big Beef Creek estuary, displayed smaller home ranges (~5 km), covered less total distance, and were less likely to cross open water to the other side of the canal. “Migrants” on the other hand, spent considerably less time in the estuary, had home ranges that extended up to 49 km (averaging 25 km), moved greater total distances (up to 428 km), and crossed the canal more often. The authors did not report sex ratios among the two strategies, but did find the choice of strategy not to correspond with the age or size of tagged fishes.

To get a sense of where Bish Creek cutthroat trout are spending the majority of their time and the types of near-shore marine habitats that might be important for them, we summarized individual detection data by four main “listening” areas: Emsley Cove, Bish Cove, the intertidal shelf between Bish Cove and Bish Creek, and Bish Creek itself. Considerable

individual variation exists in terms of the proportion of individual detection events occurring in each of the four areas (Figure 5-10).

Tagged CCT seemed to have spent most of their time along the intertidal shelf separating Bish Cove and Bish Creek. It was the only area utilized by all tagged fish (with the exception of BishCCT#13 which did not appear to leave Bish Creek), accounting for 1 – 98 % of individual detections and an average 61 % across all fish. Several of the resident fish were observed to make daily ranging or commuting movements back and forth along the shelf, presumably foraging as they went (e.g., Bish CCT#07, #08, #09 in Figure 5-9). The area is primarily a rocky intertidal delta where rockweed (*Fucus gardneri*) and a species of green alga (*Enteromorpha intestinalis*) are the dominant flora and barnacles, mussels, periwinkles and limpets the dominant fauna (Jacques Whitford Limited 2006). This is likely a rich feeding area and would be expected to hold abundant isopods, amphipods, marine worms, and perhaps migrating salmon. Most detections occurred on the western end of the shelf in the area of stations B4 and B3 (see Figure 5-3 and Figure 5-6) in an area that may act as a back eddy to concentrate food on ebb tides. It is also near where a small eelgrass bed was recently found on the intertidal shelf (Horwood 2004).

The next most important habitat (based on number of detections) appears to be Bish Cove itself. It was used by nine of 12 tagged fish and accounted for about 20–40 % of detections for BishCCT #04, #09, and # 11, and in excess of 90 % of total detections for BishCCT #16 and #18. We classified these latter two fish as migrants that left the study area shortly after tagging and overwintered outside the study area. These fish returned to Bish Cove in May 2015, however, spending several days and nights in the cove before visiting the mouth of Bish Creek and again leaving the study area. While exposed bedrock dominates both the western shore and the eastern shores of Bish Cove, the shoreline along the beach contains extensive woody debris over sand and gravel in the west and a mixture of sand, boulder and cobble in the east. The central intertidal region appears to be underlain by coarser substrate (bedrock, boulders and cobbles) than the western region (smaller cobbles, gravel and sand) and the eastern region (mostly sand and silt). Shallow subtidal areas are characterized by sandy substrates with an invertebrate community of bivalves, shelled gastropods, nudibranchs, crabs and lugworms

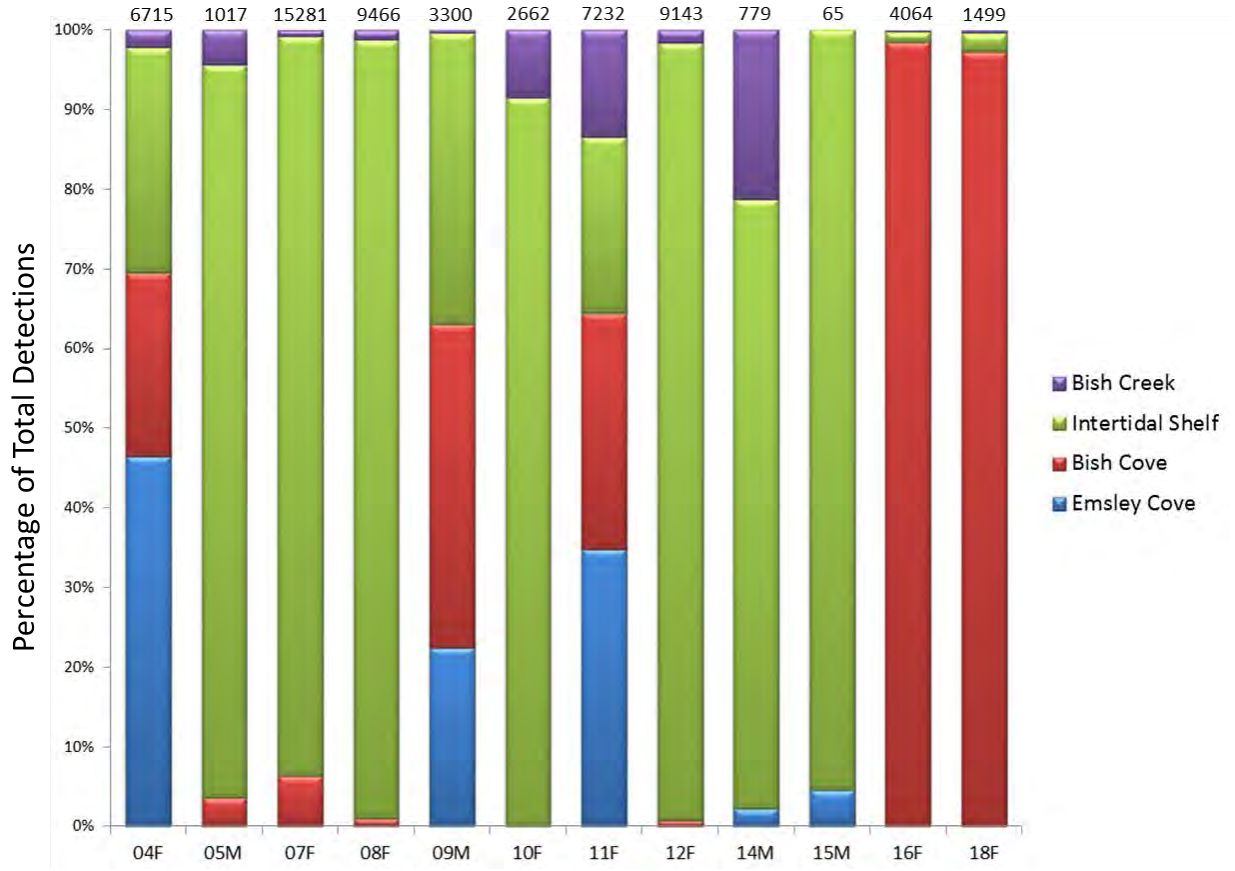

Figure 5-10 – Percentage of tag detections for individual cutthroat trout occurring in each of four main "listening" areas. The total number of detections recorded for each fish appears at the top of each bar.

(Jacques Whitford Limited 2006). At least two eelgrass meadows have been described in the cove: a relatively large, but patchy bed on the NW side of the cove and a slightly smaller, but denser bed located in the easternmost section (Horwood 2004). It was in this area that most detections were made (station B2; see Figure 5-2and Figure 5-6).

We expected that Bish Cove is an important foraging area for CCT. Previous BMAP work by Archipelago Marine Research (AMR), for example, identified the importance of Bish Cove to outmigrating salmon fry, particularly chum and pink salmon. All areas of Bish Cove appear to be utilized with the greatest concentrations near the shoreline in the central part of the cove, followed by the west and east nearshore areas (Archipelago Marine Research Ltd 2014). It is likely that cutthroat trout are seasonally targeting this prey resource as they are travelling

through the area. Though our main monitoring period began at the end of May 2014, data for several fish observed returning to Bish Cove in 2015 (e.g., BishCCT #11, #16, #18) seem to fit within the expected peak period for juvenile pink and chum outmigration of April to May (Archipelago Marine Research Ltd 2014). Cutthroat trout are, however, clearly using the estuary well into June, July and August after salmon fry have largely passed through (Figure 5-9). At those times, the cutthroat trout are presumably targeting invertebrate fauna or shifting to Coho salmon smolts (*O. kisutch*) or juvenile shiner perch (*Cymatogaster aggregata*), which are available year round; these were found to be the dominant fish species in Bish Cove behind chum and pink salmon, comprising 36 % and 28 % of the fish fauna sampled in inshore areas (Archipelago Marine Research Ltd 2014). There was some evidence that many cutthroat trout (e.g., BishCCT #04, #05, #07, #08, #09, #11, #16, and #18) were using Bish Cove as an area of refuge during non-foraging periods. A common pattern among tagged residents was active commuting or presumed foraging along the intertidal shelf by day and relatively inactive periods in the cove at night. It may be that these areas offer a lower energy environment or area of refuge from marine predators during these periods of inactivity.

About half of our tagged fish (six of 12) visited Emsley Cove, located approximately 3 km to the west. Three of those fish (BishCCT #04, #09, and #11) spent a significant amount of time within this area (>20 % detections). With the exception of estuary location (within the cove for Emsley, adjacent to the cove for Bish), both coves are similar semi-protected environments with shorelines characterized by exposed bedrock outcroppings, boulder fields, cobbles and coarse sand. They contain similar flora (including eel grass beds) and fauna, though Coho and surf perch appear absent from Emsley Cove. Instead, staghorn sculpin (*Leptocottus armatus*) and starry flounder (*Platichthys stellatus*) are the dominant species once salmon fry have migrated through, making up 41% and 33% of the inshore fish community, respectively (Archipelago Marine Research Ltd 2014). The majority of tag detections in Emsley Cove occurred between stations E3 and E5, an area that includes the eelgrass flats and cobble-boulder fields on the east side of the cove. BishCCT #04, #09 were observed to make daily ranging or commuting movements back and forth in Emsley Cove, presumably foraging along these areas as they went (Figure 5-9).

As noted earlier, relatively few detections were made at in the area of the Bish Creek mouth and tidal boundary (three % in total at B6 and BT). That is not to say that the estuarine portion of Bish Creek is unimportant to CCT; we obviously expect CCT to heavily use that area and indeed, we ended up tagging approximately half of our fish there. It may be, however, that fish are rapidly migrating through that area when leaving or returning to Bish Creek or are feeding in the area primarily during a particular time of day or flood stage. We have actually observed this behavior and over several days, were able to predict when the feeding trout would arrive (and leave) the mouth of the creek.

Diet analysis

We analyzed stomach contents from 15 Bish cutthroat trout captured between the end of May and beginning of August 2014 (n = 8 from Bish Creek and n = 7 from the estuary). Across all 15 samples, stoneflies (Order Plecoptera) were by far the most common prey item at 66 % of all those identified; this was followed by Dipterans (12 %), fishes (9 %), Trichoptera and plant material (each ~5 %), Ephemeroptera (2 %), Isopoda and Coleoptera (both <1; Figure 5-11). Interestingly, samples from the river had a higher number and greater diversity of prey items than those sampled in the estuary. Freshwater fish stomachs averaged 29.1 prey items (range 6–139) across an average of 3.2 prey groups. Prominent taxa in those stomachs included members of the Ephemeroptera (mayflies, such as *Epeorus*, *Ameletus*/*Parmaletus*, and *Heptagenia* spp.), Plecoptera (stoneflies, including *Suwalia* spp. and other members of the Chloroperlidae), Trichoptera (caddisflies in the Phryganeinae sub-family), and Dipteran orders (true flies, including *Pteromicra* and *Calliphora vomitoria*, the bluebottle fly). Bluebottle fly maggots were, in fact, found in several of the fish sampled at the mouth of Renegade/Skoda creeks. Female bluebottle flies typically lay their eggs in carrion and the maggots then feed on the decomposing matter where they hatch (Greenberg and Tantawi 1993). This suggests that (unless carrion is particularly widespread at that location) several of the CCT captured at this site had been feeding together up one of those tributaries near instream or riparian carrion. We did, in fact, find a small amount of fine black fur in one of our trout stomachs, but were not able to identify it. Finally, the stomach of BishCCT#12, a male caught at the mouth of Skoda and Renegade creek, contained at least 13 juvenile Coho fry (O. kisutch).

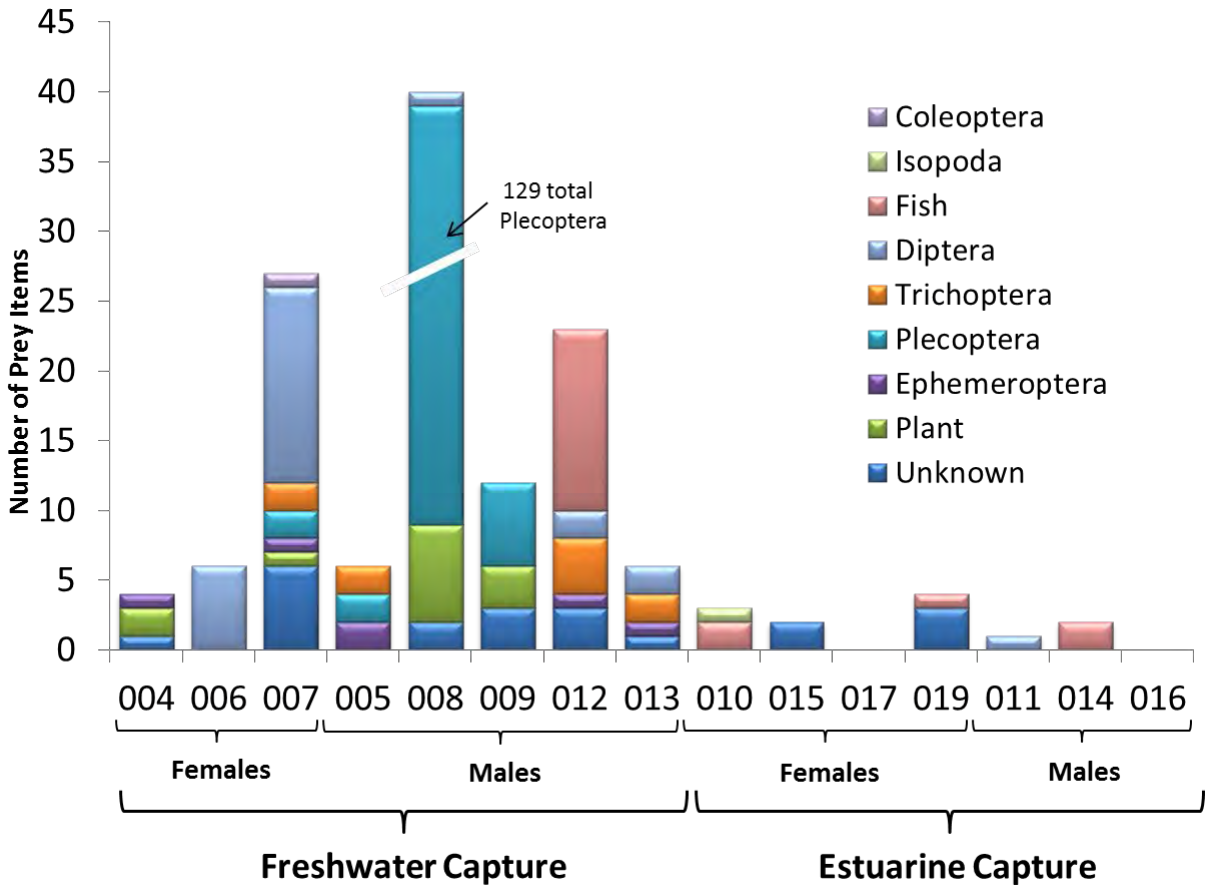

Figure 5-11 – Results of dietary analyses performed on Bish Creek cutthroat trout. The numbers of prey items identified in each of the 15 stomach samples is given by taxonomic order.

Prey number and diversity among our estuarine samples tended to be much lower; stomach samples collected there contained an average of only one prey item (range 0–3; two of the stomachs were empty) across an average of a single prey group, most commonly fish. We are somewhat surprised by this result. One might expect there to be more numerous and diverse prey items available in estuaries which, relative to freshwater systems in coastal BC, should be of much higher productivity. Coastal cutthroat trout are known to be highly predaceous and opportunistic feeders that target many different nearshore food items including amphipods, isopods, shrimp, and small fish including stickleback (*Gasterosteus aculeatus*) , sand lance (*Ammodytes hexapterus*) and various sculpin species, and in the spring,

outmigrating juvenile salmon (Jauquet 2002; Jones et al. 2008). The relative dearth of prey items in the estuarine samples may have something to do with the rapid decomposition rates of soft-bodied marine prey (such as worms and other fish) relative to the freshwater aquatic inverts which are protected by a resistant exoskeleton. Alternatively, it may have to do with the relative reproductive/energetic state of fishes sampled in the two areas. Some of the trout sampled in freshwater, for example, appeared to have been post-spawning fish (e.g., BishCCT#05), which will often feed heavily in early summer to replenish energy reserves expended during the mating season.

Given the timing of our sampling (end of May – early August 2014), we were not expecting to find many chum or pink fry in our estuarine stomach samples (peak outmigration would most likely have passed at that point). We would, however, have expected to find Coho smolts or shiner perch in those stomach samples as these prey items are abundant at the mouth of Bish Creek and Cove. Instead, Pacific herring (*Clupea pallasii*) was the most common fish prey, being found in 3 of the 7 estuarine stomach samples (all were juveniles of ~8-10 cm in length). The Kitimat Arm is known to support a resident Pacific herring population that spawn from February – April in foreshore areas of coastal inlets and bays. Department of Fisheries and Oceans (DFO) records, however, suggest that their primary spawning sites are in Minette Bay and along the foreshore between Kitimat Village and Minette Bay. Minor sites have been recorded in Clio Bay, Kildala Arm and on Coste Island (DFO 2015), but herring have not been previously been identified as rearing in the immediate area of Bish Cove (e.g., Archipelago Marine Research Ltd 2014). The finding of herring in nearly half of our estuarine stomach samples may suggest, therefore, that: 1) herring are indeed using the immediate area around Bish Cove for rearing; or 2) these trout have travelled to foraging areas where herring are more typically found. Unfortunately, given the limited spatial coverage of our listening arrays, we cannot determine where exactly that might have been (e.g., Minette Bay, Clio Bay, etc.).

Other prey items of note in the estuary samples include *Eogammarus confervicolus*, perhaps the most common gammaroidean amphipod on the Pacific coast of North America (Hiebart et al. 2016). This amphipod appears to prefer muddy substrates and is often associated with eelgrass (*Zostera marina*), long green algae (*Carex sp.*) and blatterwrack (*Fucus sp.*)

species. It tolerates full salt to brackish water and is a common inhabitant of the intertidal zone (Carlton 2007; Hiebart et al. 2016). It was found in two of seven estuarine samples (BishCCT #01, #09). We found the Oregon pill bug (*Gnorimosphaeroma oregonensis*), in one tagged fish (BishCCT #10) and several anadromous Dolly Varden (*Salvelinus malma*) we sampled on the intertidal shelf (data not shown). The Oregon pill bug is a small intertidal isopod crustacean. Although a marine species, it is able to withstand long periods of exposure to brackish and/or freshwater and is usually found in the higher intertidal under stones or woody debris (Carlton 2007). Finally, though not shown in Figure 5-11, one fish sampled in 2013 (BishCCT #01) contained a large bolus of 80 – 100 ribbon worms or Nemerteans. For the most part, ribbon worms are bottom dwellers that either burrow in mud or sand or creep among rocks and seaweeds in the intertidal zone (Carlton 2007).

Although the prey species identified above do lend support to our telemetry data and the extensive use of nearshore and intertidal habitats by tagged cutthroat trout, the relatively paucity of prey items in our anadromous samples is surprising. The importance of estuarine and nearshore habitats to coastal cutthroat trout populations likely does vary across geographic areas in accordance with local conditions and predation risk, however, we believe that these numbers underrepresent the diversity of prey items taken by feeding cutthroat trout in the Bish Creek estuary and nearshore marine environments and should, therefore, be viewed with caution. Furthermore, because our sampling period largely falls outside of two major feeding opportunities for coastal cutthroat trout (one occurring earlier in the spring for outmigrating chum and pink salmon fry and a second in the fall when trout will feed almost exclusively on salmon eggs and later, decomposing adults), we cannot fully document the foraging ecology of Bish Creek cutthroat trout during this initial pre-construction phase nor fully assess its value to monitoring efforts going forward. It is clear, however, that the salmon resource makes a significant contribution to overwintering and reproductive success in other coastal cutthroat trout populations (Bilby et al. 1996; Jauquet 2002; Wipfli et al. 2003; Duffy and Beauchamp 2008; Jones et al. 2008) and is deserving of further study

5.4. Determining the origins of Bish Creek coastal cutthroat trout: Genetic assignment tests reveal unusual population structure in the Kitimat Arm

Allan Costello

Introduction

The proposed KM LNG facility is being constructed adjacent to Bish Creek, a productive stream emptying into the Kitimat Arm of Douglas Channel. Bish Creek is known to be an important salmon stream with mean annual escapements of 15,000 pink (*Oncorhynchus gorbuscha*) and 2,500 chum (*O. keta*) salmon (KM_LNG 2005). To evaluate and understand potential effects of the KM LNG plant and pipeline on the surrounding environment, this project focused on ecosystem indicators to help identify physical-, biological-, and community-level changes associated with the plant site for use in future operational planning and policy decision-making. This study was designed to determine the potential effects of the LNG facility and associated marine terminal on the use of the Bish Creek estuary and near-shore environments by salmonids. Estuarine residency is an important life-history stage for most anadromous salmonids and this Protocol addressed potential effects of the Kitimat LNG facility on the use of the Bish Creek estuary, near-shore and marine environments by coastal cutthroat trout (*Oncorhynchus clarkii clarkii*; hereafter CCT).

The CCT is an obvious focal species for this type of study. Unlike salmon, which typically utilize the estuarine environment around Bish Creek and Cove for a relatively limited time prior to migrating offshore, anadromous CCT remain in the nearshore marine environment throughout their life, often in the immediate vicinity of their natal stream. They repeatedly move between freshwater and nearshore marine habitats to access productive habitats and locally abundant food resources like salmon fry (Giger 1972; Behnke 1992; Pearcy 1997; Trotter 1997). Furthermore, the species is listed as *of special concern* in BC and are known to be particularly sensitive to anthropogenic effects to their freshwater habitats (Johnson et al. 1999; Costello and Rubidge 2005; Slaney 2005).

Through ecological analysis of an indicator species subject to potential effects in both their freshwater and marine environments, we believe this work can contribute to the evaluation of the environmental health of the Bish Creek system. Although considerable progress has been made in recent years in the development of indices to gauge the ecological health and resilience of estuarine environments (e.g., Borja and Dauer 2008; Beliaeff and Pelletier 2011), no consensus has emerged as to the most sensitive or efficient elements for long-term monitoring. Indeed, the challenge is to integrate indicators for a wide range of individual ecosystem elements, as well as substantive measures of ecosystem structure, function and process (Borja and Dauer 2008). Fishes, in particular, have often been used as economically valuable and publicly relevant indicators of environmental change (Whitfield and Elliott 2002) and there exist numerous community or assemblage based measures of estuarine integrity and ecosystem function which focus exclusively on fish fauna (e.g., Whitfield and Elliott 2002; Pérez-Domínguez et al. 2012; Ramos et al. 2012). In Section 5.3 we examined patterns of estuarine and near-shore marine habitat use by acoustically tagged coastal cutthroat trout in the area around Bish Cove. In this Section, we introduce and address the question of whether the fish caught and tagged at Bish Creek were actually fish born in Bish Creek, or whether they represent immigrants from elsewhere.

Were the coastal cutthroat trout caught in Bish Creek actually from Bish Creek?

Although it might seem reasonable to assume that CCT using the estuary and lower reaches of Bish Creek are primarily fish born in Bish Creek tributaries, there is evidence to suggest that this may not necessarily be the case. Coastal cutthroat trout exhibit one of the broadest and most variable spectra of migratory behaviors of all the salmonids (Northcote 1997; Johnson et al. 1999) and will undergo a series of different types of movement during their lifetimes: seasonal movements (e.g., feeding, overwintering), spawning runs, and those associated with ontogenic life-history shifts. Fluvial (river-migratory), adfluvial (migrating between lakes and rivers), and resident forms (non-migratory) are present throughout their range (often within the same population), while anadromous forms (migrating to and from saltwater) exist along the coast wherever access to the sea is available (Johnson et al. 1999; Costello and Rubidge 2005).

Like many other salmonids, CCT demonstrate a well-developed ability to "home" or return to their natal stream to breed (Quinn 1993; Dittman and Quinn 1996). Unlike salmon and other anadromous trout, CCT do not make extensive use of rich offshore feeding areas. Instead, they are known to routinely travel to non-natal systems to feed on locally abundant food sources (Bulkley 1966; Pearcy 1997), migrating along beaches in nearshore intertidal areas less than 3 m deep (Pearcy 1997; Trotter 1997). These marine migrations appear limited to two to three months during the summer when feeding forays may be largely confined to bays and estuaries in the vicinity of their natal stream, especially for males (Giger 1972; Behnke 2002). There is considerable evidence to suggest that females are the more migrant sex (Giger 1972; Massey 1984; Wenburg and Bentzen 2001). Female salmonids, which often maximize their reproductive fitness by attaining large size and greater fecundities, would likely benefit to a greater extent from extended saltwater feeding than would males (Gross 1987; Northcote 1997; McDowall 2001; Hendry et al. 2004).

Given the high-relief topology of Douglas Channel and limited intertidal and estuarine habitat, one might expect productive estuaries, like the one at Bish Creek, to be utilized by cutthroat from other streams lacking similar resources. This is an important question to address; if it turns out that local fish use the cove and estuary sparingly, any effects associated with the LNG facility may have limited effect on local trout populations. Alternatively, if the area represents an important feeding, migratory, or reproductive habitat for several distinct populations in the Kitimat Arm, potential effects of the facility may be more widespread. A clear understanding of the Bish Creek coastal cutthroat population structure will, therefore, be essential if we are to estimate the potential effects of construction and plant operation (as well as the success of actions directed towards minimizing adverse effects) on CCT.

Defining population structure in coastal cutthroat trout

As Waples and Gaggiotti (2006) point out, "populations" are the natural focal units for conservation and management. Unfortunately, while the delineation of population structure may easily be made when populations are obviously disjunct and isolated (or in cases where populations may be differentiated by distinguishing morphological or ecological characteristics), the delineation of distinct groups in continuously distributed species often proves more difficult

(e.g., Pearse and Crandall 2004; Hasselman et al. 2010). Perhaps the primary factor determining the nature of population structure in a species is the level of connectivity among adjacent populations (Hastings and Harrison 1994; Hanski 1999). Connectivity between populations may itself be difficult to quantify, but could involve the use of shared feeding or overwintering habitats, co-migration along dispersal corridors, or interbreeding and gene flow among adjacent, but otherwise isolated groups.

In some cases, it may be possible to estimate the connectivity of distinct groups by directly observing the movement of individuals between habitats (as in Section 5.3). For many species, however, direct observation of movement may be difficult and cost-prohibitive, giving at best only a snapshot of movement over typically small temporal and spatial scales. Furthermore, these methods have little ability to distinguish between migration (predictable feeding, breeding or life-history movements, and dispersal which involves interbreeding and successful reproduction in non-natal locales (i.e., it is possible to have physical movement without gene flow, but not vice versa). A full understanding of population structure can, therefore, only be described over a series of spatial and temporal scales that are appropriate for the species under consideration.

To address such questions, a number of complementary indirect approaches have been developed which typically rely on genetic allele frequency data (reviewed by Neigel 1997; Bohonak 1999; Pearse and Crandall 2004; Waples and Gaggiotti 2006). Various individual-based inference methods (e.g., Pritchard et al. 2000; Paetkau et al. 2004; Manel et al. 2005), for example, have been developed to assign individuals to their most likely population of origin for conservation purposes or to determine the composition of mixed stock fisheries (reviewed by Carvalho and Hauser 1994; Begg et al. 1999; Dudgeon et al. 2012; Ovenden et al. 2015). Unlike summary methods based on statistics, individual-based assignment tests are able to capture detailed information about individual patterns of dispersal and gene flow among populations while requiring relatively few assumptions about underlying dispersal models (e.g., Hansen et al. 2001; Fraser et al. 2005; Taylor and Costello 2006; Dudgeon et al. 2012).

Objectives

Using genetic data collected for 13 microsatellite loci, our objective was to identify the likely stream of origin for anadromous migrants tagged in Bish Creek and its estuary. To do this, we performed the following activities: 1) we collected 30–50 DNA samples from multiple source populations in the vicinity of Bish and Emsley creeks to act as a baseline against which tagged fish were compared; 2) we generated multilocus DNA profiles for putative source populations and acoustically tagged cutthroat trout using 10–12 polymorphic microsatellite DNA loci; and 3) we performed genetic assignment analyses to identify the most likely stream of origin for anadromous migrants based on DNA profiling.

As discussed above, CCT are known to routinely travel to non-natal systems to feed on locally abundant food sources. It is necessary, therefore, that we genetically compared fish captured at Bish Creek with other CCT systems in the vicinity. A clear understanding of population structure at Bish Creek is essential to estimate the potential effects of construction and plant operation (as well as the success of actions directed towards minimizing those effects) on CCT. Our results have the added advantage of providing some basic biological information for an understudied species. The relationships between life-history types, their particular habitat requirements, as well as the scope and variation typical of cutthroat movements remain poorly resolved, particularly during the marine portion of the lifecycle. This study could potentially address these information gaps in a unique and deep-water northern setting.

Methods

Sampling of Bish Creek CCT

Our goal was to assign fish collected at Bish Creek during tagging operations to their most likely stream of origin. Those fish, therefore, represented our “unknown” population and were primarily adult and sub-adult fish captured through angling in the estuary and lower reaches of Bish Creek (n = 2 in 2013, n = 17 in 2014; see Table 5-4). Adipose fin clips (for DNA samples) and scale samples (for aging) were collected during tagging operations and stored in 95 % ethanol and dry scale envelopes (respectively) until processing at UNBC in the fall of 2015.

Table 5-4 – Date and location of capture, sex, age, length, weight (Wt.), and condition factor (K) for the 19 "unknown" Bish Creek samples. Floy tag (external) and acoustic (Ac.) tag (internal) numbers are included for reference. Sex was determined by the OmyY1 sex-specific marker.

Fish	Sampling Date	UTM	Site Description	Sex	Age	Length (mm)	Wt. (g)	K	Floy Tag	Ac. Tag
BISH 001	16-Jul-13	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	3	285	---	---	---	---
BISH 002	5-Aug-13	9U 517643 5974893	Big Rock @ Bish Creek mouth	M	4+	388	580	0.99	051	11164
BISH 003	26-May-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	4	207	80	0.90	---	---
BISH 004	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	F	4	390	630	1.06	053	11162
BISH 005	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	M	4	368	500	1.00	052	11159
BISH 006	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	F	5	409	760	1.11	054	11170
BISH 007	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	F	4	384	590	1.04	055	11145
BISH 008	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	F	5	383	580	1.03	056	11144
BISH 009	28-May-14	9U 516418 5976177	Mouth of Skoda/ Renegade	M	4	338	380	0.98	057	11146
BISH 010	19-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	5-6	448	1080	1.20	058	11150
BISH 011	19-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	3	267	220	1.16	059	11169
BISH 012	20-Jun-14	9U 516121 5975940	Stairway Pool	F	5	388	600	1.03	060	11163
BISH 013	20-Jun-14	9U 516121 5975940	Stairway Pool	F	5	373	560	1.08	061	11171
BISH 014	21-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	M	5	439	720	0.85	062	11158
BISH 015	21-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	M	4	372	620	1.20	063	11167
BISH 016	22-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	5	455	1000	1.06	064	11152
BISH 017	22-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	5	385	540	0.95	065	---
BISH 018	22-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	4	385	640	1.12	066	11160
BISH 019	22-Jun-14	9U 517643 5974893	Big Rock @ Bish Creek mouth	F	4	358	580	1.26	067	---

All captured fish were measured for standard biological information including, fork length, and weight, as well as sex (see Section 5.3). Despite numerous attempts, we were unable to tag any

adult or sub-adult fish in Emsley Creek (our intended control site as per the original BACI design).

Collection of genetic reference populations

The collection of potential "source" populations to which we could assign the Bish Creek CCT focused on three main sampling areas: 1) Douglas Channel — these represent populations in the immediate vicinity of Bish Creek (i.e., within 15 km of Bish Creek in the Kitimat and Kildala arms of Douglas Channel; this area is generally characterized by high gradient topology and narrow intertidal ledges which drop off quickly to depths of up to 250 m); 2) Minette Bay — this is a very shallow and semi-isolated estuarine bay at the mouth of the Kitimat River; and 3) Kitimat River — as the largest and most productive system in the area, the Kitimat River may be expected to produce a significant number of anadromous migrants, which might contribute to our tagged fish sample in Bish Creek. Samples were collected at several putative spawning sites throughout the watershed.

Sample sites within each of these three areas were chosen on the basis of accessibility and because they were either known to contain CCT (e.g., based on: BC Government's Fisheries Information Summary System [FISS data]: available at http://www.env.gov.bc.ca/fish/fiss/) or were likely to be trout systems based on stream size and gradient. Coastal cutthroat trout generally prefer smaller systems, reaching their highest densities in drainages with first to third order streams and low gradient anadromous sections (Costello and Rubidge 2005).

Juveniles were the primary focus of this sampling as they are more likely to be in natal stream and thus, be representative of distinct spawning populations. Using pole seining and minnow trapping, our goal was to collect 30–50 juvenile samples per site. Between 2014 and 2015, we sampled approximately 40 different sites for DNA, obtaining sufficient sample sizes (minimum of 15 individuals) from six of 24 sites in the Douglas Channel, two of three sites in Minette Bay, and 11 of 13 sites in the Kitimat River watershed (Figure 5-12, Table 5-5). Over both years, we collected DNA samples from 822 putative CCT juveniles (i.e., putatively identified at CCT until species can be confirmed using genetic testing) in 21 potential source streams. Unfortunately, due to logistical constraints, we were not able to collect juvenile CCT samples from Bish Creek or its tributaries.

Figure 5-12 – Overview of DNA sampling associated with the Bish Creek coastal cutthroat trout project. Red circles represent sampling sites not included in the final analysis due to insufficient sample sizes. Small green circles represent juvenile reference populations (n = 13) and the large green circle represents Bish Creek. Population codes are described in Table 5-5.

CCT: RBT diagnostic species testing

Although many of the sampled streams were smaller than those typically inhabited by steelhead or rainbow trout (*O. mykiss*, hereafter referred to as RBT), RBT were present at several of our sample sites. The two species are similar morphologically, particularly as fry, which makes field identification difficult (McPhail and Carveth 1993; Pollard et al. 1997; Kennedy et al. 2012). Furthermore, both species have been shown to hybridize under compromised environmental conditions (Campton and Utter 1985; Young et al. 2001; Docker et al. 2003). To ensure that only CCT were included in our reference collections, it was necessary that all DNA samples be first screened using diagnostic species testing. Polymerase chain reaction (PCR)-based genetic assays have been used extensively to genetically differentiate CCT,

RBT, and their hybrids. Diagnostic tests are often designed to take advantage of nucleotide polymorphisms or large indels (inserted or deleted DNA sequences) that show fixed differences between species (McKay et al. 1997; Greig et al. 2002; Ostberg and Rodriguez 2004; Pritchard and Garza 2013). Typically, several different species-specific markers are required for the analysis as backcrossed or later generation hybrids individuals are difficult to identify as they have CCT alleles at certain loci and RBT at others.

Table 5-5 – Summary collection data for juvenile reference populations by sampling area, including the number of coastal cutthroat trout (CCT), rainbow trout or steelhead (RBT), and their hybrids (HYB) identified through diagnostic species testing.

Sampling Region	Stream Name	Pop Code	Year	UTM	CCT	HYB	RBT	Site Totals
Douglas Channel	Clio Bay creek	CLIO	2015	9 520852 5972728	47			47
	Eagle Bay creek		2015	9 519617 5962572		1	17	18
	Emsley Creek		2015	9 513254 5972724	2	16	19	37
	Gobeil Bay creek	GOBL	2015	9 521965 5970108	15		19	34
	Jesse Lake		2015	9 508585 5965142	2			2
	Kildala Arm creek	KILD	2015	9 523217 5968775	45			45
	Markland Point creek	MARK	2015	9 510197 5968071	26	8	1	35
	Whatlsto Creek		2015	9 522584 5977689			2	2
Minette Bay	Cordella Creek	CORD	2014	9 525648 5988010	67			67
		CORD	2015	9 525648 5988010	30			30
	Minette Bay Creek		2014	9 525705 5986770	3		1	4
	Pine Creek	PINE	2014	9 525046 5988219	21			21
		PINE	2015	9 525046 5988219	16			16
Kitimat River	Anderson Creek		2014	9 519134 5984488		1	22	23
	Cable Car Creek		2015	9 526819 5997107	2			2
	Cecil Creek	CECL	2015	9 522902 6014757	40			40
	Chicken Creek	CHKN	2015	9 526733 5992732	28			28
	Duck Creek	DUCK	2015	9 519575 5992170	26	15		41
	Goose Creek	GOOS	2015	9 520975 5995724	42			42
	Humphreys Creek		2015	9 528813 6004035	4		33	37
			2015	9 528288 6004324			33	33
	Lone Wolf Creek	LONE	2015	9 521434 6010443	28	2	64	94
	McNeil Creek	MCNL	2015	9 530207 6007856	25	5		30
	Nahlbeelah Creek	NAHL	2015	9 530132 5997047	53			53
		NAHL	2015	9 527343 6000435			41	41
				Species Totals	**522**	**48**	**252**	**822**

DNA was extracted from reference tissue samples using Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For this project, we developed an octaplex PCR reaction using eight fluorescently labelled primers sets based on those developed by Ostberg and Rodriguez (2004). Each primer set produced a single species-specific band. Multiplex PCRs were carried out in 14-µL volumes containing 1 µL of template DNA, 7 µL of 2× Qiagen multiplex PCR master mix (final concentration of 3 mM $MgCl_2$), with primer concentrations given in Table 5-6. Thermal-cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 29 cycles of denaturation at 95 °C for 30 sec, annealing at 61 °C for 90 sec, and primer extension at 72 °C for 60 sec, and a final extension of 60 °C for 20 min. Following PCR, capillary electrophoresis was conducted using an Applied Biosystems 3730S 48-capillary DNA Analyzer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocols. Allele binning and base calling was performed using either GeneMapper5 (Applied Biosystems Inc.) or GeneMarker v2.6.4 (SoftGenetics, LLC).

Microsatellite genotyping of genetically pure CCT samples

All individuals identified as pure CCT (n = 522) were genotyped at a suite of 13 microsatellite loci (Angers et al. 1995; Morris et al. 1996; O'Reilly et al. 1996; Scribner et al. 1996; Condrey and Bentzen 1998; Olsen et al. 1998; Robinson et al. 2009; Brunelli et al. 2013). Microsatellite DNA markers are particularly useful for these types of analyses given their hyper-variability within and between populations. They are one of the few molecular markers that allow researchers insight into recent and fine-scale ecological questions (Hansen et al. 2001; Balloux and Lugon-Moulin 2002; Chistiakov et al. 2006; Selkoe and Toonen 2006). Genotyping was carried out in three multiplex PCR reactions, each in 10-µL volumes containing 1 µL of template DNA, 5 µL of 2× Qiagen multiplex PCR master mix (final concentration of 3 mM $MgCl_2$), with primer concentrations given and annealing temperatures given in Table 5-6. Thermal cycling conditions, capillary electrophoresis, and data analysis were carried out as in the CCT:RBT diagnostic species testing method, described above. To provide some regional context for the genetic relationships observed in the Kitimat area, we also performed these analyses on a sample of 33 pure CCT from streams in Bute Inlet as an outgroup.

Table 5-6 – Microsatellite DNA marker panels used for this study with annealing temperatures, primer concentrations, and expected fragment size ranges. For panel MP-ID, diagnostic fragment size for CCT and RBT are indicated, with minor or alternate fragments given in parentheses. OmyY1 (FAM) is the marker used to determine genetic sex.

Panel	Marker	Primer (µM)	Size range CCT	Size range RBT	Reference
MP-ID	Occ34 (FAM)	0.14	224 (218)	218	Ostberg and Rodriguez 2004
(60 °C)	Occ35 (PET)	0.29	238	205 (238)	Ostberg and Rodriguez 2004
	Occ36 (PET)	0.14	303 (274,288,305)	274 (303)	Ostberg and Rodriguez 2004
	Occ37 (FAM)	0.07	264, 266	254	Ostberg and Rodriguez 2004
	Occ38 (FAM)	0.07	167	143	Ostberg and Rodriguez 2004
	Occ42 (NED)	0.29	193	171 (193)	Ostberg and Rodriguez 2004
	Om47 (VIC)	0.14	247	276	Ostberg and Rodriguez 2004
	Om55 (VIC)	0.14	173	192	Ostberg and Rodriguez 2004
Panel	**Marker**	**Primer(µM)**	**Number alleles**	**Size Range (bp)**	**Reference**
MP-A	Oneu11 (FAM)	0.08	2	139-143	Scribner et al. 1996
(57 °C)	Omy77 (VIC)	0.2	13	108-138	Morris et al. 1996
	Sfo8 (FAM)	0.2	8	191-211	Angers and Bernatchez 1996
	Ssa85 (NED)	0.2	24	104-190	O'Reilly et al. 1996
	Ssa197 (PET)	0.08	3	79-117	O'Reilly et al. 1996
MP-B	Och24 (PET)	0.1	6	171-187	Robinson et al. 2009
(61 °C)	Och29 (FAM)	0.1	9	239-293	Robinson et al. 2009
	Ocl2 (VIC)	0.15	11	115-158	Condrey and Bentzen 1998
	Ogo4 (FAM)	0.1	11	133-151	Olsen et al. 1998
	Ogo8 (NED)	0.2	6	91-98	Olsen et al. 1998
MP-C	Ocl1 (FAM)	0.08	4	136-150	Condrey and Bentzen 1998
(61 °C)	Ocl4 (PET)	0.4	13	73-112	Condrey and Bentzen 1998
	Ocl9 (NED)	0.08	3	135-145	Condrey and Bentzen 1998
	OmyY1 (FAM)	0.2	1	468	Brunelli et al. 2013

Descriptive genetic analyses

Descriptive statistics of the genetic variation present at individual microsatellite loci and within populations were compiled using the GENEPOP v4.5.1 (Rousset 2008) or the GenAlEx v6.5.2 add-in for Excel (Peakall and Smouse 2012). Metrics included observed and expected heterozygosities, and the number of alleles (An). Tests for deviations from Hardy-Weinberg equilibrium were performed for each locus-population combination in GENEPOP using an exact test, in which p-values were estimated using a Markov chain method. Tests for genotypic

linkage disequilibrium (i.e., non-random association among alleles) for all combinations of locus pairs within a population were also made using a Markov chain method with GENEPOP default values. Sequential Bonferroni corrections (Rice 1989) were applied to these calculations to maintain an experiment-wide $\alpha = 0.05$.

Delineation of population structure among reference samples

Pairwise tests for population differentiation (i.e., allele heterogeneity) were performed for each locus and over all loci combined using the contingency methods of Raymond & Rousset (1995) as implemented in GENEPOP. For each locus, the probability that the observed allele frequencies were drawn from the same population was estimated by using Markov-chain Monte Carlo methods. To provide an unbiased estimate of the probability for each randomization test, 10 batches of 10,000 replicates each were run, with 1,000 dememorization steps. For each comparison, Fisher's combined probability test was applied over all loci. We also ran this analysis to examine the temporal stability of allele frequencies for those populations sampled in multiple years (Pine and Cordella creeks).

To further examine the hierarchical partitioning of genetic variation in our samples at variously nested levels, we used the analysis of molecular variance (AMOVA) approach of Excoffier et al. (1992). This analysis was performed using ARLEQUIN v3.5 (Excoffier and Lischer 2010) and allowed us to estimate the percentage of the total observed genetic variation explained by genetic differences within populations (i.e., among individuals in a population in terms of allelic diversity and heterozygosity, VIP), among populations within groups (as defined by the user, VIG), and by differences between groups (VBG) under different grouping scenarios. In practice, it is desirable to identify groupings which maximize the VBG component (i.e., explanatory power of the grouping scenario tested) and to minimize VIG (the within group variation) so as to define homogenous groups.

To estimate long term connectivity or gene flow between populations, we computed pairwise estimates of F_{ST} (Wright's fixation index) and Nm (the effective number of migrants per generation). F_{ST} values measure the reduction in population heterozygosity due to population subdivision and range from 0 – 1, with values near zero indicating a lack of population structuring or subdivision, and values >0.3 indicate moderate to strong genetic subdivision

(Weir and Cockerham 1984). At migration-drift equilibrium, Nm ~ 1 - F_{ST} / 4 in the equilibrium island model (Wright 1931, 1965). Here, estimates of pairwise F_{ST} and Nm were calculated in GENEPOP. Pairwise estimates of F_{ST} are often compared against the geographic distances separating populations to test for isolation by distance, or the tendency towards increasing genetic differentiation among populations with increasing geographic distance (Wright 1943; Slatkin 1993). Indeed, many studies in salmonids have demonstrated that positive correlations do exist between genetic distance and geographic distance across several spatial scales (Campton and Utter 1987; Wenburg et al. 1998; Johnson et al. 1999; Costello 2006). To test for isolation by distance in our populations, we used the Isolation By Distance Web Service (Jensen et al. 2005). The programs compared pairwise F_{ST} values and the shortest pairwise water distance between populations (in km) using a Mantel test, giving a correlation coefficient (r) and its statistical significance based on a permutation process.

A visual representation of the multilocus genetic variation existing among populations was provided by the Factorial Correspondence Analysis (FCA) plotting algorithm in GENETIX v4 (Belkhir et al. 2001). FCA is an exploratory technique suitable for categorical data that has been adapted for use with diploid genetic data. It enables visualization of individuals (or populations) in multidimensional space with no *a priori* assumptions about grouping. Axes were generated from combinations of alleles that explained portions of the total observed ‘‘inertia’’ of the table. Individuals that are genetically similar plot near each other and individuals that are genetically different plot distantly from each other. We used the “sur populations” FCA option in GENETIX, whereby the program first calculates the genetic center of each sample population and then recalculates the position of individual fish in relation to its genetic center to better delineate groupings within overlapping plots of individuals.

To serve as a contrast to FCA ordination, we constructed a neighbor-joining tree based on Cavalli-Sforza-and Edwards distance (CSE; Cavalli-Sforza and Edwards 1967). The CSE distance estimates genetic differences between populations under a model in which all divergence among populations is due to genetic drift. Like the FCA described above, the topology of the tree reflects patterns of genetic similarity between reference populations and was constructed using the GENDIST, NEIGHBOR, and CONSENSE modules of the PHYLIP genetics

package (Felsenstein 1993).The consensus tree is based on 10,000 bootstrapped datasets generated in the PHYLIP SEQBOOT module. Support for particular nodes on the tree is estimated via bootstrapping and values greater than 50 % are indicated on the tree.

Finally, we also used the program BAPS6 (Corander et al. 2008) to further examine the number of distinct genetic clusters (K) in the reference dataset using a Bayesian clustering methodology. The program treats both population allele frequencies and the number of distinct groups as random variables which can be modeled in a Bayesian framework. Like similar methods (e.g., Pritchard et al. 2000), the program assumes population clusters will exhibit Hardy-Weinberg and linkage equilibria as well as low migration rates, but differs from other such programs in that it can consider populations as the basic sampling unit (rather than individuals). It estimates which populations have differentiated allele frequencies, rather than simply partitioning individuals into Hardy-Weinberg populations (reviewed by Pearse and Crandall 2004). Using a stochastic optimization algorithm, BAPS examines the posterior mode of the sampling solutions to determine the partition (i.e., the number of distinct population clusters, K) with the greatest likelihood given the data. We performed five replicate runs for values of population clusters, K = 1 – 15, and based on those results, a second round of 20 runs for population clusters, K = 6 – 9.

Genetic assignment

To determine the most likely stream of origin for our Bish “unknown” sample, we used the maximum-likelihood method implemented in ONCOR (Kalinowski et al. 2007). ONCOR estimates the probability that an individual of unknown origin belongs to each possible source population in the database based on multi-locus genotypic frequencies and anticipated admixture proportions. Assignment analyses depend on the quality of the reference collection (i.e., that it include all contributing source populations). We are reasonably confident that we have identified all significant CCT source populations within a 15-km radius of the plant site and from many representative areas of the Kitimat River, with the following notable exceptions: 1) Emsley Creek — though we sampled it thoroughly in both 2014 and 2015, we were unable to capture enough reference CCT samples (see Genetic species identification results); and 2) Bish Creek — logistical constraints prevented us from accessing the creek and its tributaries.

Assignment was therefore conducted without representation from at least some of the expected primary source populations.

Given a particular reference database, these types of assignment procedures are most accurate when there is a high degree of genetic differentiation between populations, when the number of reference samples for each source population are large, and when large numbers of loci are genotyped. There is no simple formula to calculate how accurate genetic assignment is expected to be; instead computer simulation is often used to test the discriminatory power present in a particular reference database.

To examine the robustness of our baseline reference set and our ability to accurately assign Bish CCT to their most likely population of origin, ONCOR was used to conduct "leave-one-out" (i.e., jack-knifing) assignment tests on reference collections. For this analysis, each juvenile sample in a population is removed from the baseline dataset and treated as an individual of unknown origin. The population allele frequencies are then recalculated without that individual, and the individual is assigned to its most likely population of origin within the baseline dataset (as per Anderson et al. 2008). The number (or proportion) of individuals from the baseline dataset assigned to the population from which they were collected provides a measure of the accuracy of the baseline dataset for population assignment. Having a limited number of reference populations, we also chose to assign these jack-knifed individuals to the most likely geographic area of origin (i.e., Douglas Channel, Minette Bay, Kitimat River, or the Bute Inlet outgroup).

Following leave-one-out tests, we then used ONCOR to then assign our "unknown" sample (i.e., those captured during Bish tagging operations) to their most likely population and geographic area of origin. For comparison, we conducted the same analysis using the Bayesian assignment method of Rannala and Mountain (1997) using the program GeneClass2 (Piry et al. 2004). GeneClass2 calculates the likelihood of drawing an individual's genotype from the population where it was sampled (with the individual in question removed, Lhome) and divides this by the highest assignment likelihood among all reference populations (Lmax). Resampling and probability estimation is performed using the method of Paetkau et al. (2004), whereby the genotypes of 10,000 individuals from each reference population is simulated to generate a

distribution of assignment likelihoods against which the true "unknown's" test statistic is compared. We present the top two assignments for each method (Max Likelihood and Bayesian).

Results and Discussion

Genetic species identification

As expected, species diagnostic testing on the 19 fish captured at Bish Creek during tagging operations suggest that all were pure coastal cutthroat trout (i.e., they possessed zero of 16 RBT alleles). Of the 822 putative CCT collected from reference populations, however, a total of 252 were identified as RBT, 48 as CCT-RBT hybrids, and 522 as genetically pure CCT (Table 5-6).

The RBT were detected in 11 of 23 sites and made up the majority of samples collected at Eagle Bay, Emsley, Gobeil Bay, Anderson, Humphreys, Lone Wolf and Nahlbeelah (site 2, near Hwy 37) creeks. The finding of misidentified RBT among these populations was not unexpected; CCT and RBT coexist in many of the sample locations and, as noted, are difficult to distinguish from CCT as fry (McPhail and Carveth 1993; Pollard et al. 1997). If it had been an option, we could have reduced the likelihood of capturing RBT by concentrating our sampling higher up in the watersheds. CCT generally spawn in the headwaters and uppermost reaches of streams to avoid competition with larger salmonids (Behnke 1992; Trotter 2008).

Hybrids were detected at seven of 23 sites with particularly high proportions in Emsley (16 of 37 samples), Duck (15 of 41 samples), and the Markland Pt. creeks (eight of 35 samples). The majority of hybrids in Duck and Markland Point creeks appear to be F1s (recent hybrids produced by matings between pure RBT and CCT parents). At Emsley Creek, however, only 5 of 16 individuals were F1 hybrids and the remainder were backcrossed RBT hybrids (produced by matings between hybrids and RBT parents). The high proportion and composition of the hybrids in Emsley Creek suggest that hybridization in that creek is well established and ongoing. The reasons for this are not known at this time.

Hybridization between CCT and RBT has been previously identified along much of the west coast (Campton and Utter 1985; Johnson et al. 1999; Young et al. 2001; Ostberg et al.

2004); in excess of one-third of all CCT populations in Washington and Oregon are now expected to contain hybrids (Johnson et al. 1999). Here in BC, Bettles et al. (2005) provided evidence of hybridization in 29 of 30 sympatric trout populations on Vancouver Island with the frequency of hybridization in those populations ranging from 3– 88 %. Generally, however, hybridization between these species is episodic in nature and associated with the stocking of non-native species or habitat degradation that reduces the quality and abundance of spawning habitats (e.g., Heath et al. 2010). There has been previous logging in the Emsley watershed, including some recent logging adjacent to the uppermost sections we sampled in 2014, though a link between the two is not necessarily obvious.

After removing misidentified RBT and hybrids from the reference data base, we were left with 522 pure CCT at 20 of 23 sample sites. Small sample sizes (i.e., n < 15) for Emsley Creek, Jesse Lake, Minette Bay, Cable Car, and Humphreys creeks, however, meant that these were excluded from further analyses. This, leaving 509 CCT samples from 13 Kitimat area streams (as well as 33 samples from the Bute Inlet outgroup) was our reference database.

Levels of genetic diversity

Moderate levels of genetic diversity were observed at the 13 microsatellite loci (Table 5-7). Across all populations, the number of alleles (An) at individual loci ranged from 2 (Oneu11) to 24 (Ssa85), averaging 8.7 alleles per marker. Ocl9 was nearly fixed in the Kitimat area for Ocl9*135; only CECL and NAHL displayed an alternate allele (Ocl9*141); both of these and a third allele (Ocl9*145) were present in the BUTE sample. Observed heterozygosity (Ho) ranged from nearly zero (Ocl9) to 0.73 (Ssa85), averaging 0.52 across all markers.

Tests for conformity to Hardy-Weinberg equilibrium (HWE) indicated significant deviations in 14 of 195 tests (7%) following sequential Bonferroni adjustment (nominal α = 0.05). Nine of these cases were observed at Och29, 3 at Omy77, and one case each at Ogo4 and Ssa85. The deviations at marker Och29 were all caused by an excess of homozygotes relative to HWE, suggesting the presence of a null (non-amplifying) alleles at that marker. As a conservative measure, we dropped this marker from further analysis. Tests for genotypic linkage disequilibrium rejected the null hypothesis of independence in 26 of 1170 tests (2.2%)

following sequential Bonferroni adjustment (nominal $\alpha = 0.05$), but significant results were not restricted to any single locus pair.

Table 5-7 – Summary genetic data for coastal cutthroat trout reference populations and the Bish creek unknowns. 'An' is the number of alleles detected, 'Ho' and 'He' are the observed and expected heterozygosity. Significant deviations between Ho and He following sequential Bonferroni corrections are indicated by an asterisk (*).

	Oneu11	Sfo8	Ssa197	Omy77	Ssa85	Ogo8	Ocl2	Ogo4	Och24	Och29	Ocl4	Ocl1	Ocl9	Population Average
Bish Creek "unknowns":														
Bish Creek (BISH) n = 19														
An	2	5	3	6	9	4	4	3	4	5	6	4	1	4.3
Ho	0.30	0.80	0.60	0.80	0.90	0.50	0.75	0.75	0.40	0.25*	0.85	0.60	0.00	0.60
He	0.32	0.75	0.58	0.72	0.82	0.62	0.72	0.57	0.52	0.66	0.77	0.70	0.00	0.60
Douglas Channel:														
Clio Bay creek (CLIO) n = 47														
An	2	3	2	4	7	4	4	2	3	2	3	3	1	3.1
Ho	0.04	0.68	0.06	0.49	0.64	0.68	0.68	0.26	0.15	0.15*	0.28	0.64	0.00	0.38
He	0.04	0.65	0.06	0.44	0.60	0.56	0.55	0.22	0.14	0.49	0.31	0.52	0.00	0.35
Gobeil Bay creek (GOBL) n = 15														
An	2	4	3	4	11	3	4	5	4	4	5	4	1	4.2
Ho	0.07	0.60	0.47	0.67	0.73	0.60	0.47	0.60	0.67	0.20*	0.93	0.67	0.00	0.54
He	0.06	0.65	0.37	0.56	0.83	0.53	0.72	0.63	0.68	0.65	0.76	0.60	0.00	0.54
Kildala Arm creek (KILD) n = 45														
An	2	4	2	5	9	3	4	4	2	5	5	4	1	3.8
Ho	0.47	0.69	0.53	0.71	0.93	0.42	0.71	0.44	0.44	0.36*	0.64	0.56	0.00	0.55
He	0.44	0.67	0.47	0.64	0.84	0.37	0.65	0.43	0.46	0.68	0.66	0.58	0.00	0.53
Markland Point creek (MARK) n = 26														
An	2	4	2	5	7	4	4	2	3	4	3	4	1	3.5
Ho	0.46	0.42	0.27	0.65	0.46	0.23	0.58	0.54	0.38	0.12*	0.50	0.62	0.00	0.43
He	0.43	0.58	0.33	0.64	0.59	0.24	0.56	0.45	0.33	0.41	0.54	0.67	0.00	0.44
Douglas Channel averages n = 133														
An	2.0	3.8	2.3	4.5	8.5	3.5	4.0	3.3	3.0	3.8	4.0	3.8	1.0	3.6
Ho	0.26	0.60	0.33	0.63	0.69	0.48	0.61	0.46	0.41	0.20	0.59	0.62	0.00	0.45
He	0.24	0.64	0.31	0.57	0.72	0.43	0.62	0.43	0.40	0.56	0.57	0.60	0.00	0.47
Minette Bay:														
Cordella Creek (CORD) n = 97														
An	2	4	3	10	9	4	6	5	5	4	5	4	1	4.8
Ho	0.11	0.75	0.39	0.82	0.73	0.68	0.71	0.66	0.47	0.42	0.66	0.52	0.00	0.53
He	0.11	0.70	0.36	0.82	0.64	0.63	0.69	0.60	0.47	0.54	0.78	0.59	0.00	0.53
Pine Creek (PINE) n = 37														
An	2	4	3	9	6	4	6	4	5	5	8	4	1	4.7
Ho	0.08	0.65	0.16	0.76	0.49	0.65	0.73	0.46	0.19	0.38	0.70	0.68	0.00	0.46
He	0.08	0.70	0.15	0.73	0.52	0.65	0.76	0.55	0.25	0.51	0.75	0.64	0.00	0.48
Minette Bay averages n = 134														

	Oneu11	Sfo8	Ssa197	Omy77	Ssa85	Ogo8	Ocl2	Ogo4	Och24	Och29	Ocl4	Ocl1	Ocl9	Population Average
An	2.0	4.0	3.0	9.5	7.5	4.0	6.0	4.5	5.0	4.5	6.5	4.0	1.0	4.7
Ho	0.10	0.70	0.28	0.79	0.61	0.66	0.72	0.56	0.33	0.40	0.68	0.60	0.00	0.49
He	0.09	0.70	0.26	0.78	0.58	0.64	0.72	0.57	0.36	0.52	0.76	0.62	0.00	0.51
Kitimat River:														
Cecil Creek (CECL) n = 40														
An	2	4	3	7	10	4	7	7	4	5	6	4	2	5.0
Ho	0.33	0.70	0.55	0.55	0.68	0.83	0.90	0.73	0.58	0.15*	0.78	0.60	0.03	0.60
He	0.27	0.67	0.54	0.59	0.78	0.67	0.83	0.75	0.64	0.55	0.79	0.57	0.02	0.59
Chicken Creek (CHKN) n = 28														
An	2	5	3	6	10	4	6	5	5	4	7	4	1	4.8
Ho	0.14	0.86	0.61	0.75	0.93	0.71	0.68	0.75	0.54	0.14*	0.79	0.64	0.00	0.62
He	0.13	0.69	0.61	0.72	0.84	0.66	0.66	0.72	0.62	0.54	0.79	0.70	0.00	0.59
Duck Creek (DUCK) n = 26														
An	2	4	2	7	9	4	5	5	5	5	7	4	1	4.6
Ho	0.19	0.62	0.46	0.88	0.81	0.81	0.85	0.65	0.65	0.31*	0.69	0.58	0.00	0.60
He	0.23	0.61	0.36	0.76	0.73	0.73	0.71	0.66	0.59	0.66	0.75	0.65	0.00	0.57
Goose Creek (GOOS) n = 42														
An	2	3	3	5	7	3	6	5	5	5	5	4	1	4.2
Ho	0.33	0.62	0.50	0.62*	0.76	0.64	0.69	0.60	0.57	0.31*	0.79	0.64	0.00	0.56
He	0.31	0.66	0.57	0.64	0.78	0.47	0.68	0.63	0.52	0.60	0.76	0.59	0.00	0.56
Lone Wolf Creek (LONE) n = 28														
An	2	5	3	10	9	4	8	6	4	3	7	4	1	5.1
Ho	0.04	0.79	0.36	0.75	0.82	0.54	0.68	0.75	0.82	0.29	0.79	0.61	0.00	0.55
He	0.04	0.72	0.55	0.77	0.85	0.55	0.71	0.65	0.63	0.49	0.81	0.48	0.00	0.56
McNeil Creek (MCNL) n = 25														
An	2	4	3	7	10	4	5	5	5	4	5	4	1	4.5
Ho	0.32	0.80	0.64	0.56*	0.72	0.52	0.80	0.60	0.36	0.52	0.96	0.84	0.00	0.59
He	0.32	0.73	0.56	0.69	0.76	0.65	0.74	0.68	0.40	0.64	0.78	0.72	0.00	0.59
Nahlbeelah Creek (NAHL) n = 53														
An	2	5	3	5	11	4	6	5	5	5	6	4	2	4.8
Ho	0.21	0.75	0.40	0.87*	0.81*	0.62	0.68	0.79*	0.70	0.43	0.75	0.62	0.02	0.52
He	0.22	0.72	0.44	0.69	0.85	0.65	0.65	0.72	0.67	0.59	0.76	0.52	0.02	0.58
Kitimat River averages n = 242														
An	2.0	4.3	2.9	6.7	9.4	3.9	6.1	5.4	4.7	4.4	6.1	4.0	1.3	4.7
Ho	0.22	0.73	0.50	0.73	0.79	0.67	0.75	0.68	0.60	0.41	0.79	0.65	0.01	0.58
He	0.22	0.69	0.52	0.69	0.80	0.63	0.71	0.69	0.58	0.58	0.78	0.60	0.01	0.58
Bute Inlet: outgroup (BUTE) n = 33														
An	1	5	3	5	5	5	8	8	6	3	5	2	3	4.5
Ho	0.00	0.42	0.27	0.70	0.58	0.79	0.85	0.79	0.76	0.33	0.30	0.21	0.03	0.46
He	0.00	0.69	0.24	0.74	0.54	0.66	0.81	0.73	0.76	0.49	0.58	0.28	0.17	0.51
Marker Averages across all populations														
An	2	8	3	13	24	6	11	11	6	9	13	4	3	8.7
Ho	0.21	0.68	0.42	0.71	0.73	0.61	0.72	0.62	0.51	0.29	0.69	0.60	0.00	0.52
He	0.20	0.68	0.41	0.68	0.73	0.58	0.70	0.60	0.51	0.57	0.71	0.59	0.01	0.53

Levels of variation within individual populations were also moderate, but varied considerably among sites. Mean number of alleles and heterozygosities were lowest among the Douglas Channel populations (An = 3.6, Ho = 0.45). Diversity levels were substantially higher in the Minette Bay populations (An = 4.7, Ho = 0.49) and Kitimat River populations (An = 4.7, Ho = 0.57). The three regions were statistically differentiated in terms of mean An ($F = 10.058$, $p = 0.003$) and Ho ($F = 4.086$, $p = 0.047$) with differences driven primarily by the Douglas Channel samples; Minette Bay and Kitimat River populations were not significantly different following Tukey's test for multiple comparisons (data not shown).

We extend this analysis by examining the hierarchical distribution of genetic variation among our sample populations under various grouping scenarios (Table 5-8). AMOVA analyses suggested that the majority of the genetic variation we identified (80–91 %) was distributed at the individual population level (i.e., represents the allelic diversity and heterozygosity found among individuals). This is a very typical result for these types of analyses (e.g., Wenburg and Bentzen 2001; Johnson et al. 2010; Heggenes et al. 2011). What we are most interested in are the between (VBG) and within (VIG) group components, as they tell us something about how the distribution of the variation at other spatial scales.

Table 5-8 – Hierarchical partitioning of multilocus microsatellite DNA variation based on AMOVA analyses under various grouping scenarios.

Comparison	Number of groups compared	Between groups (V_{BG})	Among populations within groups (V_{IG})	Within populations (V_{IP})
Kitimat vs Bute	2	13.5%	7.5%	80.0%
Three Kitimat regions	3	2.4 %*	6.9%	90.7%
BAPS clusters	7	9.2%	2.7%	88.1%
BAPS clusters without BUTE	6	7.4%	2.7%	89.9%

The regional groupings we tested general explain a statistically significant, but relatively minor component of the total variation among populations (2.4–13.5 %, depending on the particular scenario). The most basic comparison we looked at was the distribution of genetic differences between the Kitimat and Bute Inlet sampling regions. Approximately 13.5 % of the total variation present in these two groups (68 % of the variation remaining once VIP had been

accounted for) was due to differences between the two regions (VBG); considerable variation was also partitioned among populations within the regions (VIG = 7.5%;). Focusing only on the three sampling areas in the Kitimat region (i.e., Douglas Channel, Minette Bay, and the Kitimat River), relatively little variation was explained by these groups (2.4 %); nearly three times as much variability (6.9 %) was found within these regions as exist between them. The most plausible explanation seems to be the BAPS clustering scenario, where the most likely population clusters identified through Bayesian analyses (k = 7; see below) act as the grouping variables for AMOVA analyses. Under this scenario, the seven clusters explain 7.4 % of the total variation (or 73 % of non-VIP level variation) and provide the least amount of within group variation at VIG = 2.7 % of the total (or 27 % of the non-population level variation). Excluding the BUTE population and testing only the Kitimat region BAPS clusters (k = 6) reduced VBG slightly (7.4 %), but did not affect VIG.

As an aside, the levels of genetic diversity exhibited by Kitimat CCT were quite a bit lower than CCT populations to the south, particularly in terms of allelic diversity. A previous survey of microsatellite diversity in populations around Vancouver Island found an average of 16.7 alleles per marker and a mean heterozygosity of 0.62 (Costello 2006). A substantial reduction in allelic diversity with increasing latitude is not entirely unexpected; similar reductions have been observed in many plant and animal species inhabiting northern latitudes (Soltis et al. 1997; Bernatchez and Wilson 1998; McCusker et al. 2000; Brunsfeld et al. 2001; Costello et al. 2003). This trend results from the repeated population bottlenecking and founder-flush cycles experienced during post-glacial recolonization which tends to reduce allelic diversity to a greater extent than heterozygosity levels (Wade and McCauley 1988; Bernatchez and Wilson 1998). The lower levels of genetic variation in the Kitimat area, however, will likely make genetic assignment of the Bish samples more challenging. Interestingly, the 33 samples from Bute Inlet contained nearly as much genetic diversity as the 509 CCT samples from the Kitimat area.

Population structure among reference samples

We began by examining the temporal stability of allele frequencies for those populations sampled in both 2014 and 2015 (Pine and Cordella creeks). Within sites, neither of the sampling years was significantly differentiated from the other (Fisher's exact $p > 0.05$) and so replicates were combined for all further analyses. All other pairwise combinations of populations (with the exception of the BISH-GOBL and BISH-MCNL comparisons) were similarly differentiated in terms of genotypic frequencies (Fisher's exact $p < 0.0001$ over all 12 loci). Within sampling areas, average pairwise F_{ST} values (a measure of genetic subdivision) ranged from 0.14 (moderate subdivision) in the Douglas Channel to 0.03 (weak subdivision) in both the Minette Bay and Kitimat River areas (Table 5-9). These values correspond with average Nm values of ~2.9 effective migrants per generation between populations in the Douglas Channel group and ~9.4 in both the Minette Bay and Kitimat River areas.

Table 5-9 – Pairwise estimates of F_{ST} (below diagonal) and the effective number of migrants per generation (Nm, above diagonal). Comparisons with no shading occur between populations in the same sampling area, while shaded comparisons are between sampling areas.

	BISH	CLIO	GOBL	KILD	MARK	CORD	PINE	CECL	CHKN	DUCK	GOOS	LONE	MCNL	NAHL	BUTE
BISH	---	1.3	12.2	3.2	2.0	6.5	3.4	11.8	12.7	7.4	12.0	7.2	2499.8	18.7	1.2
CLIO	0.17	---	1.2	0.8	0.7	1.1	0.9	1.1	0.9	1.0	1.2	1.2	1.2	1.3	0.6
GOBL	0.02	0.18	---	5.3	1.4	3.3	2.0	7.1	6.6	6.8	4.8	8.5	8.9	8.8	1.2
KILD	0.07	0.23	0.05	---	1.3	1.7	1.1	2.2	2.5	2.0	2.5	2.4	2.3	2.6	0.8
MARK	0.11	0.28	0.15	0.16	---	1.9	1.4	1.4	1.6	1.4	1.9	1.3	1.8	1.4	0.6
CORD	0.04	0.19	0.07	0.13	0.12	---	9.4	4.3	5.5	5.6	4.9	3.5	6.0	6.2	1.0
PINE	0.07	0.21	0.11	0.18	0.15	0.03	---	2.8	3.0	3.8	3.1	2.0	4.0	3.4	0.8
CECL	0.02	0.18	0.03	0.10	0.15	0.06	0.08	---	7.7	9.3	5.6	7.9	10.1	8.7	1.2
CHKN	0.02	0.21	0.04	0.09	0.14	0.04	0.08	0.03	---	8.5	11.2	8.8	27.5	10.3	1.1
DUCK	0.03	0.20	0.04	0.11	0.15	0.04	0.06	0.03	0.03	---	5.6	6.7	9.9	8.3	1.0
GOOS	0.02	0.17	0.05	0.09	0.12	0.05	0.07	0.04	0.02	0.04	---	5.8	15.0	7.5	0.9
LONE	0.03	0.18	0.03	0.10	0.16	0.07	0.11	0.03	0.03	0.04	0.04	---	5.9	7.9	1.1
MCNL	0.00	0.17	0.03	0.10	0.12	0.04	0.06	0.02	0.01	0.02	0.02	0.04	---	10.8	1.1
NAHL	0.01	0.16	0.03	0.09	0.15	0.04	0.07	0.03	0.02	0.03	0.03	0.03	0.02	---	1.2
BUTE	0.17	0.29	0.17	0.24	0.30	0.20	0.24	0.17	0.19	0.19	0.22	0.19	0.19	0.17	---

The inferred level of subdivision in our samples (average pairwise F_{ST} ranging from 0.03 – 0.14) were comparable with estimates from previous studies. Wenburg and Bentzen (2001), for example, calculated the average F_{ST} value among nine anadromous CCT populations in Hood

Canal, Washington to be 0.03 (range 0.013– 0.115). Johnson et al. (2010) found an F_{ST} value of 0.04 between two tributaries of the lower Columbia River separated by ~80 km. Interestingly, our data suggest that the Bish Creek sample has experienced high levels of gene flow with both Douglas Channel (GOBL) and Kitimat River populations (CECL, CHKN, GOOS, MCNL, NAHL) in the past; all pairwise Nm estimates are greater than 10 effective migrants per generation. The particularly high value between BISH and MCNL (Nm = 2500) should not be taken literally and is partly a consequence of the relationship between F_{ST} and Nm; as F_{ST} approaches zero (i.e., panmixia), Nm approaches infinity (Whitlock and McCauley 1999). Regardless of the exact number, this data suggest that the two populations are essentially undifferentiated (confirmed by contingency testing above). Not surprisingly, populations in the Kitimat area do not seem to display a pattern of isolation by distance (Figure 5-13). Mantel testing of the relationships between pairwise F_{ST} and shortest water distance separating the populations (km) suggest that these distances are poorly correlated (r = 0.056, one-sided p = 0.35).

The factorial correspondence analysis (FCA) displayed in in Figure 5-14A describes the genetic relationships between individual coastal cutthroat trout in the Kitimat and Bute Inlet samples. Displayed are the first three factorial axes which together account for 66% of the variation among populations. The most striking aspect of this Figure is the relatively slight variation among Kitimat area populations relative to the Bute Inlet samples which are widely scattered along Axis 1 (the Kitimat populations scatter mostly along axes 2 and 3). By removing the Bute Inlet outgroup sample and rescaling the axes, we can zoom in on the relationships between the Kitimat samples. In Figure 5-14B, we can see some distinct clustering of populations (e.g., individuals from CLIO, KILD, PINE, and CORD), but for the majority of populations, including BISH, there is considerable overlap in the individual sample genotypes.

This inconsistent level of population structuring is supported by the consensus neighbor joining tree based on Cavalli-Sforza and Edwards distances (Figure 5-15). Populations such as the Minette Bay sites (PINE and CORD), and to a lesser extent, MARK and KILD, form well supported clusters or “branches” on the tree. Branching patterns for the majority of populations, however, remain poorly resolved. In the tree, BISH appears to cluster with the

MCNL and GOBL reference populations, though bootstrap values for most nodes in the consensus tree are quite low.

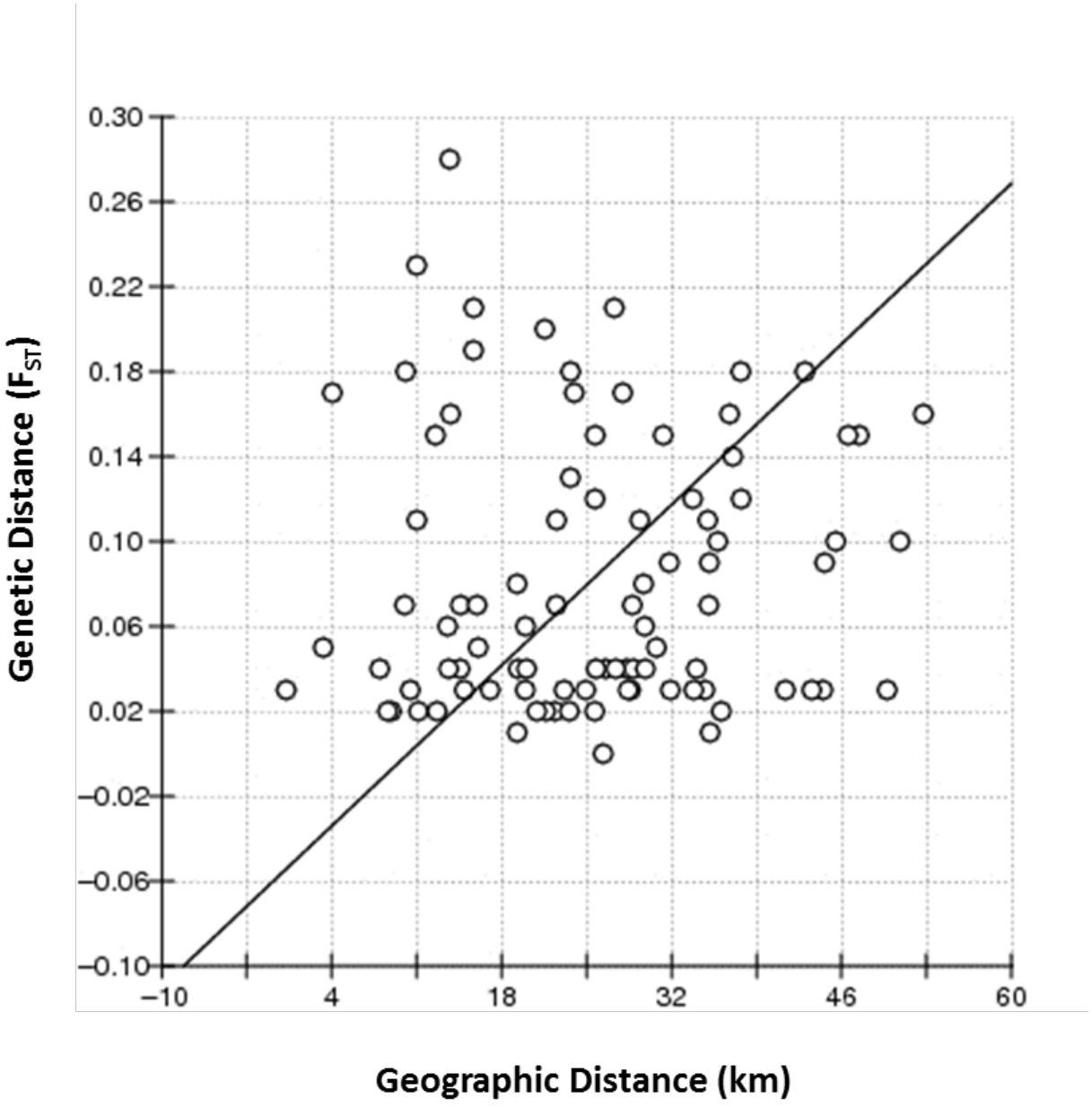

Figure 5-13 – Pairwise isolation-by-distance correlation between genetic distance (F_{ST}) and the shortest water distance (km) separating Kitimat area populations. The Mantel correlation coefficient (r) is indicated and its statistical significance is based on a permutation process.

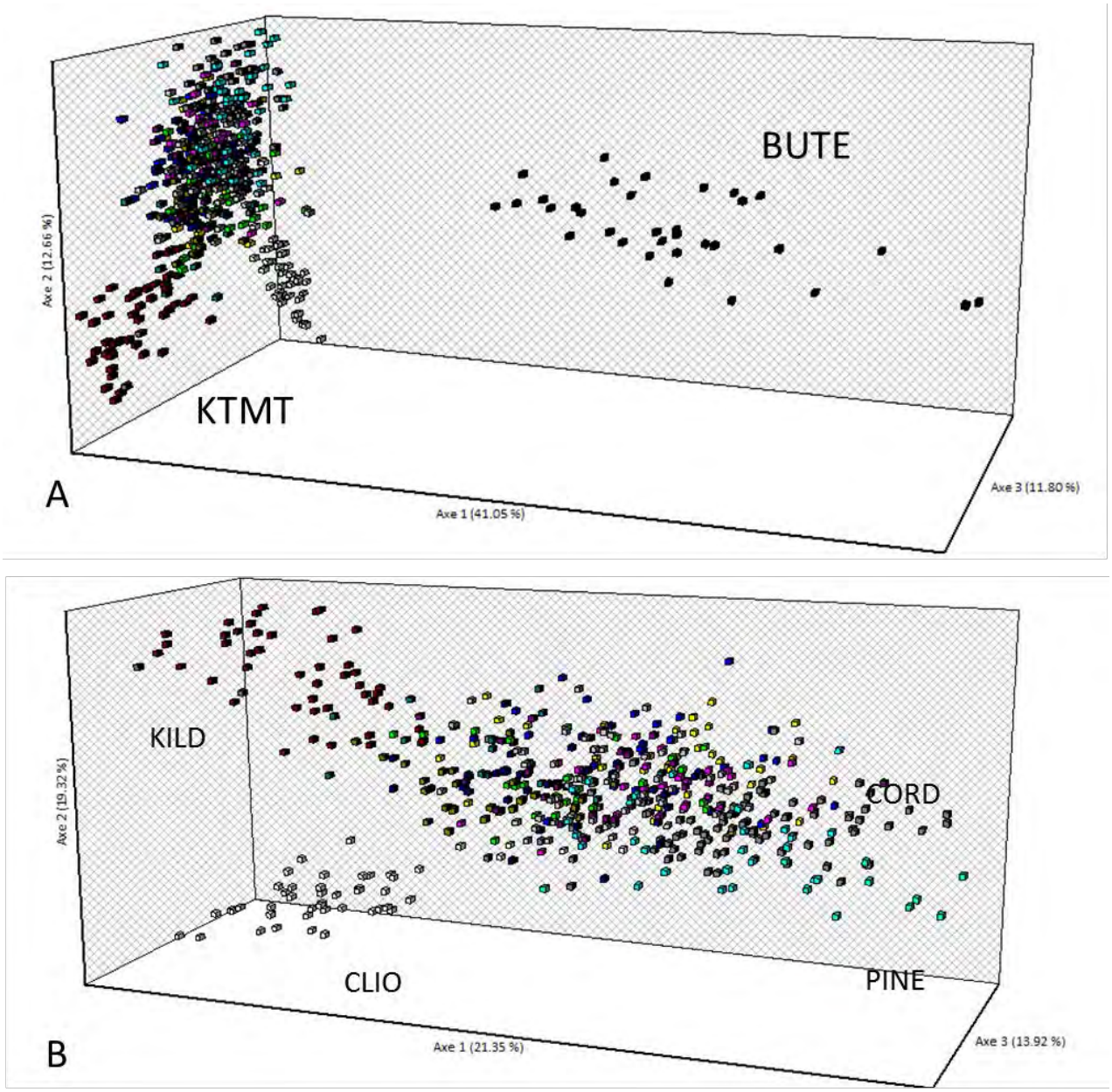

Figure 5-14 – Factorial correspondence analysis ordination of the multi-locus genetic relationships between individual coastal cutthroat trout along the first three factorial axes with the BUTE outgroup included (A) and excluded (B).

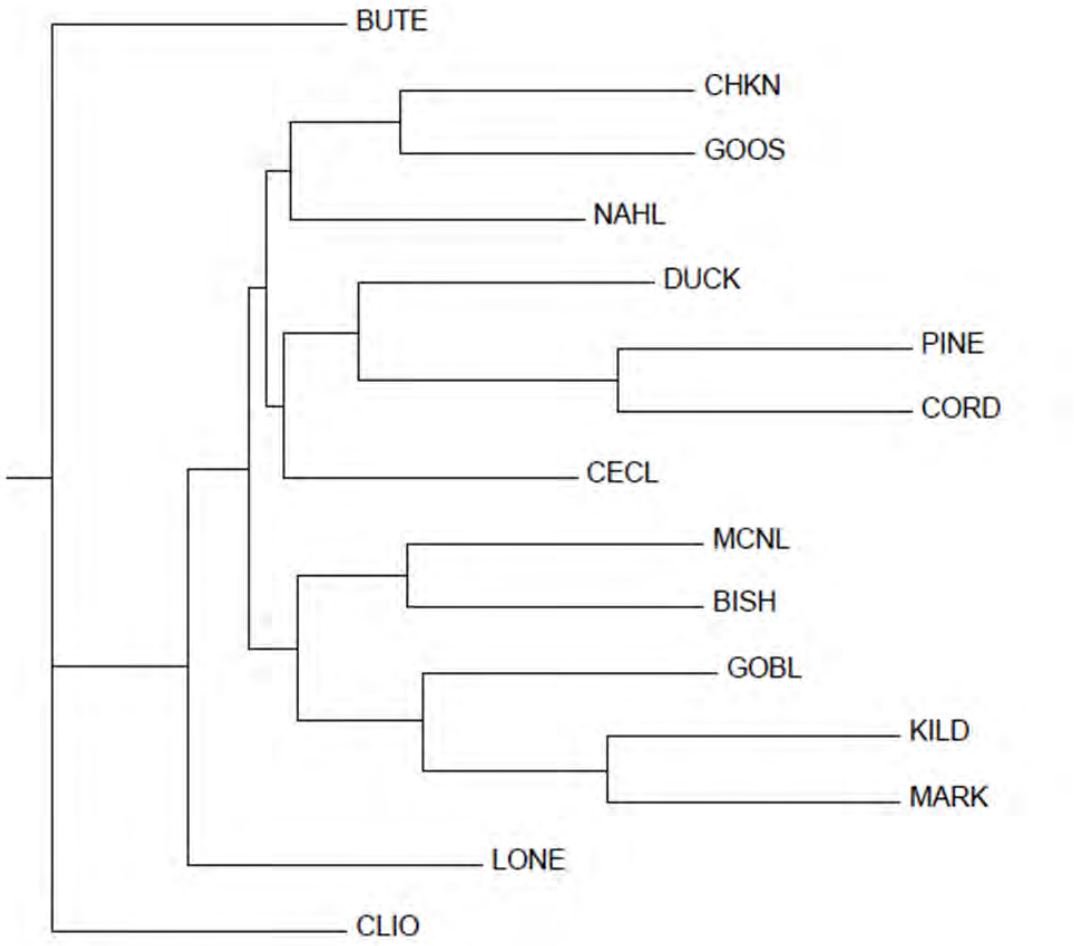

Figure 5-15 – Consensus neighbor-joining tree based on Cavalli-Sforza and Edwards (CSE) chord distances. Bootstrap values greater than 50 percent are indicated (10,000 replicate data sets). Population codes are described in Table 5-5.

This varied level of population structure was further supported by Bayesian clustering analysis. Testing a range of population clusters, K values from 2–15, BAPS consistently identified population clusters, K = 7, as the most likely number of distinct groups (typical ln (L) = -18375). To help visualize the distribution of these clusters in multivariate space, we have projected the population clusters K = 7 partition onto the population centers determined by FCA ordination (Figure 5-16). About one third of the populations (CLIO, KILD, MARK, GOOS, and the BUTE outgroup) cluster individually (i.e., each forms a distinct group). Clustering was observed, however, among the Minette Bay populations (PINE and CORD) and among a large number of

Kitimat River populations (CECL, CHKN, DUCK, LONE, MCNL, and NAHL). Interestingly, the Bayesian analysis suggests that the BISH and GOBL samples cluster within this Kitimat group.

The fact that some of these populations cluster singly is not surprising; previous studies of population subdivision in CCT suggest that most populations are structured at the individual stream level, with adjacent streams separated by as little as a few km supporting reproductively isolated groups (Campton and Utter 1987; Wenburg et al. 1998; Wenburg and Bentzen 2001; Costello 2006). As an outgroup, the Bute Inlet sample (located ~650 km south of Kitimat) would not have been expected to cluster with Kitimat area populations. Similarly, the lack of greater dispersal among the Clio Bay, Kildala Arm, and Markland Pt. creeks is perhaps not overly surprising. Although only 5–10 km distant from each other, these are small, high gradient creeks not likely to be overly productive in terms of generating anadromous migrants or in attracting migrating fish from other systems. That is not to say that some clustering of populations would be expected.

The clustering of the Minette Bay populations (CORD and PINE), for example, was well supported by multiple analyses, each employing different methodologies and assumptions (FCA, NJ tree, Bayesian clustering). As noted, Minette Bay is a shallow estuarine bay, largely isolated from the Kitimat River and the rest of Douglas Channel by a muddy sill through much of the tidal cycle. It is entirely possible that migrants from these creeks use Minette Bay to feed and as a possible dispersal route between streams, but do not generally disperse to the wider area. Similarly, while particular groupings of populations weren't very well resolved in the Kitimat River, the clustering of CECL, CHKN, DUCK, LONE, MCNL, and NAHL in both the FCA ordination and Bayesian analyses is not unexpected. The Kitimat River mainstem undoubtedly serves as a migration corridor for several distinct Kitimat River tributary subpopulations, facilitating gene flow and allowing fluvial and anadromous migrants to access shared feeding and overwintering habitats along the mainstem (Costello et al., unpublished data).

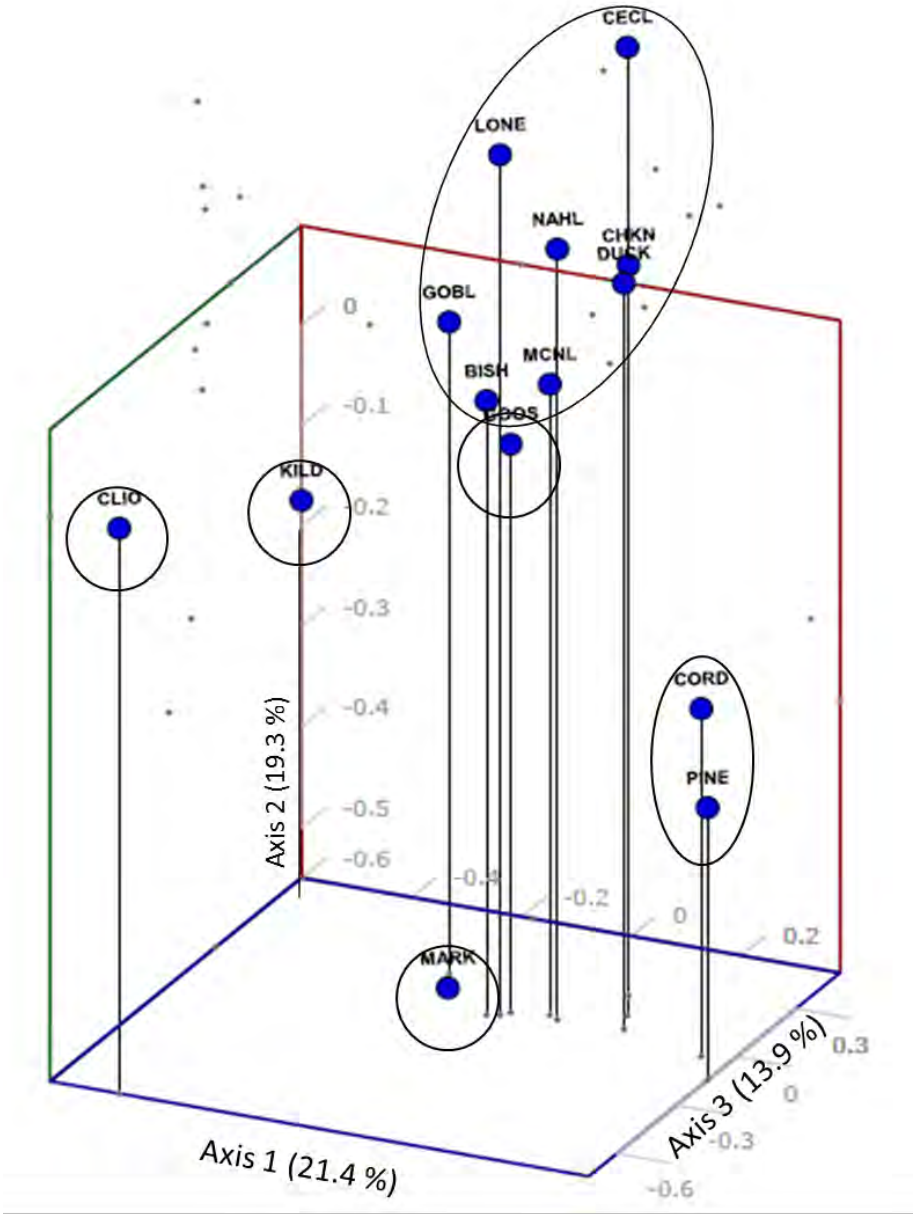

Figure 5-16 – Factorial correspondence analysis ordination of the multi-locus genetic relationships between Kitimat area CCT populations. The blue dots represent population centers as determined by FCA. Population codes are described in Table 5-5. Open circles represent distinct genetic clusters identified by Bayesian analyses (i.e., BAPS).

A recent study of microsatellite variation in steelhead trout from the lower Kitimat River found similarly low levels of genetic subdivision. Examining 333 steelhead trout samples collected from 1976– 2003 in the lower Kitimat River mainstem, Heggenes et al. (2006) found little allele frequency heterogeneity and very low levels of genetic subdivision between

collection years or among age classes (F_{ST} = 0.004). Their samples were likely composed a mixture of several distinct spawning populations which would confound the determination of population structure. Subsequent work by Heggenes et al. (2011), however, focused on the collection of juvenile reference populations which were more likely to be in their natal streams. They found population structure in the upper Kitimat River (i.e., above the 17 Mile bridge) to be better somewhat more developed with F_{ST} = 0.031 (or 0.013 if one suspect marker was excluded), but not well defined spatially. To quote the authors, there were "no clearly separate genetic populations; rather, the genetic structure [within their steelhead samples] appeared to be an overlapping mosaic of modestly genetic divergent localities" (Heggenes et al. 2011: p. 123).

We found a very similar result in our data; while most populations were significantly differentiated in terms of allele frequencies, there was poor or inconsistent spatial resolution of the relationships among populations. There was, for example, no pattern of isolation by distance among these populations and although the clustering of many Kitimat populations (CECL, CHKN, DUCK, LONE, MCNL, and NAHL) is not unexpected, the inclusion of BISH and GOBL within this group is a little less intuitive. Granted, relative to other Douglas Channel streams sampled, BISH and GOBL are larger, more productive systems with extensive anadromous reaches and well developed estuaries. It may be that those creeks have produced (or attracted) significant numbers of anadromous CCT (either historically or more recently) that have facilitated higher levels of gene flow with the Kitimat River populations. Perhaps future work in the area could address this question by extending the telemetry results presented in our previous chapter to look wider movement patterns of CCT in the Kitimat Arm; particularly with regard to determining the historical and contemporary connections between the BISH, GOBL, and MCNL populations which appear to be most similar to each other despite their disjunct spatial locations.

Genetic assignment to population of origin

Leave-one-out tests on the 13 reference populations (and Bute Inlet outgroup) suggest that the discriminatory power of the microsatellite dataset was mixed, with low to high levels of self-assignment success across populations (43%–96 % correctly self-assigned; Figure 5-17).

Not surprisingly, self-assignment success was highest for those populations previously identified as being genetically distinct (CLIO, KILD, MARK, GOOS; average ~88 %). Self-assignment success was somewhat lower in Minette Bay (averaging 64 %), though misassignments were generally to the other Minette Bay population. Self-assignment success to population of origin was lowest for the large Kitimat/GOBL cluster (average 56.4 %, ranging from 43–70%). This would be expected given the limited subdivision we have already documented for those populations (e.g., Figure 5-16). Larger sample sizes or additional DNA markers would be required to improve our ability to distinguish between these populations given their low level of genetic divergence.

Self-assignment success to geographic sampling area of origin (i.e., DC, MB, KTMT, or BUTE) was much better, averaging 89 % over all populations (data not shown). Again, rates were highest among the genetically distinct Douglas Channel populations (average = 96%), and similar for both Minette Bay and Kitimat River samples (~85 %). For comparison, we also calculated self-assignment rates to the Bayesian clusters determined by BAPS. Though circular (the genetic data used to define the clusters is here used to assign them back to clusters, but using different algorithms), self-assignment success of the GOBL reference samples increases dramatically, as it does for several of the other populations in the cluster, further supporting the inclusion of GOBL within this group. Only the Bute Inlet outgroup had 100 % self-assignment success to population, sampling area, and BAPS cluster of origin.

Assignment of our 19 “unknown” samples from Bish Creek and estuary provided some interesting results. ONCOR assigned a high proportion of the Bish Creek samples to the MCNL population (14/19 = 74%) with probabilities ranging from 0.52 - 1. McNeil Creek is a small Kitimat River tributary approximately 30 km from its mouth. The watershed does not appear to be exceptionally productive, but does contain extensive wetlands in its lower reaches which likely provide excellent rearing habitat for juvenile trout and Coho. Two other BISH samples were assigned to NAHL and three individuals were assigned to GOBL. Bayesian assignment by GeneClass2 assigned roughly the same proportions of the Bish Creek sample to NAHL and GOBL, though fewer individuals to the MCNL population (7 of 19). Instead those samples were assigned to other Kitimat populations (CHKN, CECL, LONE;

Table 5-10). In terms of agreement between the two methods, ONCOR and GeneClass2 assigned individuals to the same source population 47% of the time; 63% of the time, both methods resolved the same two top choices, but may have had them reversed. Most disagreement between methods revolved around which population was selected within the Kitimat/GOBL cluster. In only one case out of the 76 total assignments (19 BISH individuals × 2 methods × Top 2 choices per method) was a source population from outside this cluster selected (i.e., GeneClass2 chose CORD as 2nd most likely source pop for Bish 002, p = 0.46).

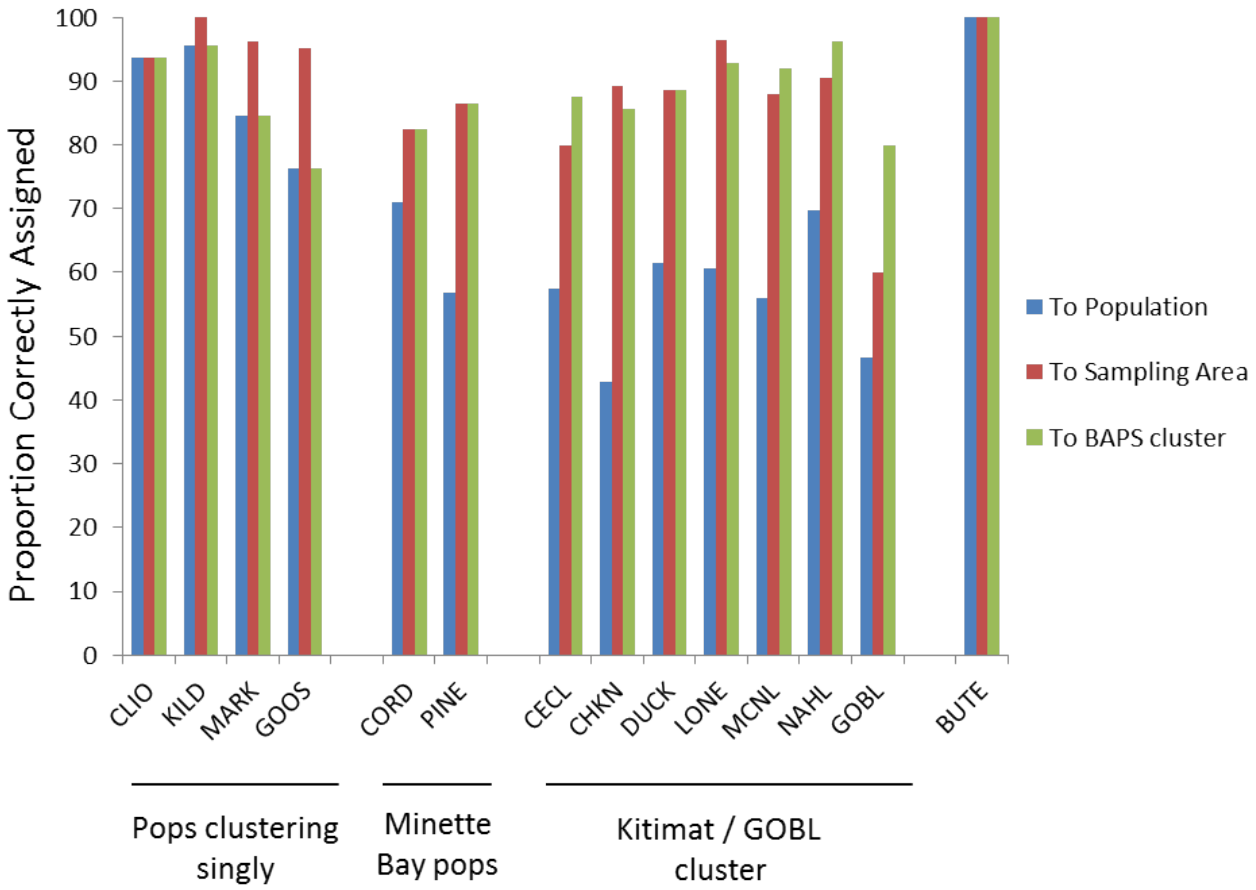

Figure 5-17 – ONCOR self-assignment rates for the 13 Kitimat area reference populations and Bute Inlet outgroup to population of origin (blue bars), sampling area of origin (red bars), and BAPS cluster (green bars). For clarity, samples have been arranged by BAPS cluster. Population codes are described in Table 5-5.

Table 5-10 – Multilocus genetic assignments for the 19 "unknown" samples tagged at Bish Creek. The top two choices for each method (ONCOR, maximum likelihood; GeneClass2, Bayesian) are provided with probabilities based on permutation processes. Source population codes are described in Table 5-5.

				Population origin							
				First choice				Second choice			
				ONCOR		GeneClass2		ONCOR		GeneClass2	
ID	Date	Sample Location	Sex	Source	Prob	Source	Prob	Source	Prob	Source	Prob
001	16-Jul-13	Bish Ck mouth	F	MCNL	0.65	CHKN	0.51	CHKN	0.35	MCNL	0.17
002	5-Aug-13	Bish Ck mouth	M	MCNL	0.94	MCNL	0.56	NAHL	0.06	CORD	0.46
003	26-May-14	Bish Ck mouth	F	MCNL	0.98	LONE	0.07	NAHL	0.01	MCNL	0.06
004	28-May-14	Skoda Renegade	F	NAHL	0.52	CECL	0.86	GOBL	0.21	NAHL	0.69
005	28-May-14	Skoda Renegade	M	MCNL	1.00	MCNL	0.14			LONE	0.13
006	28-May-14	Skoda Renegade	F	MCNL	0.83	MCNL	0.82	NAHL	0.14	NAHL	0.81
007	28-May-14	Skoda Renegade	F	GOBL	0.49	CHKN	0.72	MCNL	0.48	MCNL	0.70
008	28-May-14	Skoda Renegade	F	MCNL	0.55	NAHL	0.49	GOBL	0.26	CECL	0.46
009	28-May-14	Skoda Renegade	M	MCNL	0.98	MCNL	0.50	GOBL	0.01	CECL	0.47
010	19-Jun-14	Bish Ck mouth	F	GOBL	0.47	CHKN	0.57	MCNL	0.41	CECL	0.55
011	19-Jun-14	Bish Ck mouth	F	MCNL	0.59	MCNL	0.64	GOBL	0.36	CECL	0.63
012	20-Jun-14	Stairway Pool	F	MCNL	0.88	MCNL	0.30	GOBL	0.09	CHKN	0.18
013	20-Jun-14	Stairway Pool	F	MCNL	0.87	CECL	0.48	NAHL	0.09	MCNL	0.47
014	21-Jun-14	Bish Ck mouth	M	MCNL	0.63	NAHL	0.45	NAHL	0.35	MCNL	0.37
015	21-Jun-14	Bish Ck mouth	M	MCNL	0.98	LONE	0.06	GOBL	0.02	MCNL	0.03
016	22-Jun-14	Bish Ck mouth	F	MCNL	0.99	MCNL	0.92	NAHL	0.01	LONE	0.65
017	22-Jun-14	Bish Ck mouth	F	MCNL	0.50	GOBL	0.12	GOBL	0.49	MCNL	0.10
018	22-Jun-14	Bish Ck mouth	F	GOBL	0.52	GOBL	0.23	MCNL	0.47	MCNL	0.21
019	22-Jun-14	Bish Ck mouth	F	NAHL	0.68	NAHL	0.28	MCNL	0.31	LONE	0.27

The assignment of the Bish Creek samples to Kitimat River populations of origin (rather than the more proximate CLIO or MARK) is somewhat surprising. As noted, the tendency towards natal philopatry in salmonids suggests that dispersal and gene flow are most often restricted to adjacent populations and leads over time to a pattern of isolation by distance, a pattern characterized by increasing genetic differentiation among populations with increasing geographic distance. That does not appear to be case here; like other studies from the area (e.g., Heggenes et al. 2011), we found no evidence for isolation by distance; rather genetic and geographic distances among these populations appear very poorly correlated (Figure 5-14).

It is important to remember that the assignment of a large proportion of these BISH samples to the MCNL population or other Kitimat River tributaries does not necessarily imply that those fish immigrated to Bish Creek from the Kitimat River (although we cannot completely

discount that as a possibility either). Rather, it suggests that based on our genetic data, the most likely population of origin among the references populations available for comparison, was a population in the Kitimat River/GOBL cluster. Without samples from Bish Creek and its tributaries (and in the absence of larger scale telemetry data), we cannot easily determine the nature of the "connectivity" between Bish Creek and the Kitimat River. The simplest explanation may be that the fish we tagged were born in Bish Creek to parents that are genetically similar to those in the Kitimat/GOBL cluster. The question to ask, however, is why that is? Why are the fish from Bish Creek and GOBL more similar to those in the Kitimat River than they are to other populations in the Douglas Channel?

What is the connection between Bish Creek and Kitimat River CCT?

It is possible that this similarity is historical in nature and is the consequence of the way populations recolonized the Kitimat area during deglaciation. During the height of Quaternary glaciations, ice sheets covered most of the area encompassing present-day BC as well as parts of Alaska, northern Washington, Idaho and Montana (e.g., Clague and James 2002; Hetherington et al. 2004). The bulk of the current flora and fauna of the area are, therefore, the descendants of post-glacial immigrants, which survived glaciation elsewhere (McPhail and Lindsey 1986). In the Kitimat area, deglaciation occurred much later than on the outer coast, was complex and non-uniform; there were rapid periods of glacier retreat and times when glaciers were relatively stable (Clague 1985). Sea levels varied substantially and were up to 200 m higher than they currently are ~10,500 years ago (due to the weight of Cordilleran glaciers), but rebounded quickly to present sea level at about ~8,000 years ago, with about half of this drop occurring in as little as 500 years (Clague 1985; Shugar et al. 2014).

It is possible that different parts of the Kitimat valley were colonized at different times by different "waves" of fish as the glaciers retreated. The similarity of Bish and the Kitimat/GOBL cluster would then be a by-product of their common ancestral origins. We have documented exactly this case in Bute Inlet char, including bull trout (*Salvelinus confluentus*) and Dolly Varden (*S. malma*). Our analyses from that area suggest a marked geographic distribution of divergent lineages with isolated populations of "northern type" Dolly Varden (those that survived in northern refugia, Beringia, etc.) located above migration barriers, and "southern

type" Dolly Varden and bull trout (from southern refugia such as the lower Columbia, Chehalis, etc.) distributed below migration barriers. We believe that this pattern reflects a "double invasion" of Bute Inlet by two distinct waves of fish coming from different source populations. (Costello et al. unpublished data). The same processes are likely to have occurred in the Kitimat River valley, an area where many rivers do indeed have impassable migration barriers above which isolated populations of Dolly Varden and coastal cutthroat trout exist. Future phylogeographic work with more slowly evolving markers (such as mtDNA, reviewed by Avise 1998; Bernatchez and Wilson 1998) could examine the deeper ancestral relationships between these populations to determine whether historical associations could account for the inconsistent geographic structuring of populations we have described.

Alternatively, the similarity between Bish Creek CCT and those from the Kitimat River may have more to do with contemporary levels of gene flow among populations. Relative to other Douglas Channel streams sampled, BISH and GOBL are larger, more productive systems with extensive anadromous reaches and well developed estuaries. It may be that those creeks have produced (or attracted) significant numbers of anadromous CCT which have facilitated higher levels of gene flow with the Kitimat River populations. Coastal cutthroat trout are known to enter other stream systems and have been shown to routinely feed and even overwinter in non-natal systems where presumably there is ample opportunity for interbreeding. In the majority of cases, however, it appears that these individuals are predominately immature during this time and return to their natal stream upon maturation (Bulkley 1966; Jones 1976; Johnston 1982). Indeed, that converse (poor habitat not attracting anadromous migrants) is possibly implicated for the Clio Bay population (CLIO). The sea floor in Clio Bay (including its benthic fauna) is thought to be heavily impacted by organic overburden associated with numerous log sorts that have operated in that bay. We believe that we may actually see an effect of that habitat degradation on our Clio Bay CCT sample. Despite the fact that there are no migration barriers between this population and others in the area, the CLIO sample was the most genetically distinct of all populations (e.g., Figure 5-14B). It displayed the lowest level of genetic diversity we observed, suggesting it experiences low levels of gene flow with other populations in the area.

Although homing precision (or selection against straying) may be particularly well developed in this species (Campton and Utter 1987; Wenburg et al. 1998; Wenburg and Bentzen 2001; Costello 2006), near-perfect homing among CCT may be maladaptive given the small and often unproductive nature of their typical spawning streams. Smaller stream systems are often more severely affected by environmental processes than are larger ones; some coastal cutthroat spawning and rearing habitats may be ephemeral, drying up or going subsurface for large portions of the year. Consequently, some level of dispersal must be maintained (at least periodically) within populations to buffer environmental stochasticity. This may be particularly relevant in the lower Kitimat River which flows through poorly consolidated glacial substrates. Exacerbated by decades of heavy logging (e.g., MacDonald and Shepherd 1983), the loose porous substrate makes the Kitimat River a flashy, dynamic system where the locations of stream habitats and even main channels may migrate annually. This dynamism may predispose CCT to higher levels of dispersal within the Kitimat River and possibly to the wider area. Indeed, previous telemetry work we have done in the Kitimat River does suggest that a small proportion of the population is anadromous and does not return to the river once having left (Costello et al., unpublished results).

It is also entirely possible that recent levels of gene flow between Bish Creek and the Kitimat River have been facilitated by the straying of hatchery reared CCT. From 1985 – 2010, a hatchery supplementation program for CCT operated in the Kitimat River whereby wild cutthroat broodstock were collected and crossed in the DFO's Kitimat hatchery facility (Lough 1990). Over that period, approximately 154,000 CCT smolts were released throughout the watershed after hatchery rearing on a mix of well water and water diverted from the Kitimat mainstem (DFO 2016). A similar program exists for steelhead and was the impetus behind the studies of Heggenes et al. (2006, 2011) for Kitimat River steelhead. Those authors were similarly interested in determining whether hatchery supplementation was reducing levels of genetic diversity or contributing to a breakdown in native population structure in the lower river. While those studies did not find strong evidence to specifically support this, the effects of hatchery supplementation, are known to be complex and varied; from displacement and increased competition, through to hybridization, genetic swamping and a breakdown of local population

structure (reviewed by Hindar et al. 1991; Fraser 2008). If straying and dispersal are promoted in the lower Kitimat (by habitat dynamism or residual hatchery effects), it would certainly help explain the lack of more developed subdivision in the lower Kitimat River and perhaps the similarity between Bish and Kitimat River samples, particularly if anadromous migrants make use of the Kitimat River plume to locate other suitable habitats. Future work in the area could examine this possibility by tagging additional anadromous CCT and expanding the acoustic telemetry array from the immediate area of Bish and Emsley creeks to monitor movements across the wider Kitimat Arm.

5.5. Cutthroat Trout Protocol: findings and recommendations

The Bish Cutthroat Trout Protocol was designed to assess the potential effects of the Kitimat LNG facility on the use of Bish Creek estuary, near-shore and marine environments by CCT, an anadromous fish with an extended nearshore marine residency period. We were particularly interested in documenting the timing and duration of estuarine and nearshore marine residency by Bish CCT, as well as their patterns of habitat use and diel activity near the proposed plant site so that we could identify the times and locations when that species may be most susceptible to disturbance. This information is necessary to evaluate the use of CCT as an indicator of nearshore marine ecosystem health during all phases of plant construction and operation.

We established two listening arrays in the study area (one in Bish Cove and one on Emsley Cove). Though catch-per-unit effort was fairly low initially, we captured a total of 19 cutthroat trout between 2013 and 2014, 15 of which were acoustically tagged. Twelve of those fish provided movement data during our main monitoring period of May 2014 – August 2015.

There was considerable variation between individual fish in terms of the timing, number, and magnitude of their movements in the near-shore marine environment. Two main movement/foraging strategies, however, were identified: a resident strategy, whereby movements were generally confined within the listening area and comprised daily ranging or commuting behaviors, some entirely within the Bish array, others moving back and forth between Bish and Emsley coves; and a migratory strategy, whereby fish made more extensive and directed movements out of the listening area, overwintered elsewhere, and then returned to the study area. We found that the primary residency period was between May and July, though exact dates were highly variable with some tagged trout remaining in the nearshore marine environment well into October. The areas of highest use (based on tag detections within our arrays) appear to be the intertidal shelf between Bish Creek and Bish Cove, Bish Cove itself, Emsley Cove, and Bish Creek, roughly in that order. Trout were active throughout the day, with peak activity occurring from dawn to midday and again at dusk. Many fish displayed a pattern of daily foraging along the intertidal shelf with relatively sedentary nights spent in Bish Cove.

We believe that this baseline behavioral information could be very useful for monitoring purposes at the plant site. By identifying the timing and general pattern of habitat use by this species prior to the construction of marine structures in Bish Cove, we have provided a benchmark against which construction and/or operational activities can be measured. For example, the construction of in and over water structures and its associated lighting is expected to have an effect on migrating pink and chum salmon fry (Archipelago Marine Research Ltd 2014). This may benefit CCT feeding in Bish Cove if these structures attract migrating fry and other baitfish. In that case, we might expect to see a shift towards trout spending more time in Bish Cove as opposed to feeding along the intertidal shelf. There would be a tradeoff, however, if these structures also attracted marine mammal predators (particularly at night when trout are relatively sedentary in the Cove). In that case, we might expect to see fewer trout using Bish Cove as an area of refuge. More generally, we believe that plant-related activities in the area might lead to changes in the relative frequency of resident vs migratory strategies among Bish Creek cutthroat trout (and by extension, that those changes could potentially be identified through ongoing plant site telemetry), depending on whether those activities were benefitting or disadvantaging trout foraging in the area.

Finally, the majority of what we are calling “migratory” CCT, were fish sampled in the Bish Creek estuary and the question remains as to whether these fish were actually born in Bish Creek (and overwintered in non-natal systems as the species is sometimes known to do), or if they were, in fact, fish born in other systems that only happened to be captured in the Bish Creek estuary while foraging. Given the high-relief topology of Douglas Channel and limited intertidal/ estuarine habitat in the Kitimat Arm, one might expect productive estuaries, like the one at Bish Creek, to be utilized by cutthroat from other streams lacking similar resources. This is an important question to address; if the proposed plant site represents an important feeding, migratory, and/or reproductive habitat for several distinct populations in the Kitimat Arm, potential effects of the LNG facility (be they positive and/or negative) could potentially be more widespread. Genetic data presented in the following paper suggest a high degree of genetic similarity between cutthroat trout sampled at Bish Creek, the Kitimat River, and Gobeil Bay. At this point, we are unable to determine whether this similarity is a consequence of the way fish

colonized the Kitimat Arm post-glacially, or whether this similarity is maintained by high levels of contemporary gene glow and dispersal between Kitimat Arm CCT populations (though our telemetry data for migratory cutthroat suggest that the latter scenario is certainly possible). A clearer understanding of the Bish Creek CCT population structure will, therefore, be essential if we are to estimate the potential effects of construction and plant operation (as well as the success of actions directed towards minimizing adverse effects) on coastal cutthroat trout populations in the area.

While we have documented the timing and duration of estuarine and nearshore marine residency by Bish Creek cutthroat trout as well as their patterns of habitat use and diel activity near the proposed plant site, our sample size was relatively small (n = 12) and the study primarily limited to a single monitoring season. Additional tagging and monitoring would provide greater statistical/temporal coverage through other phases of plant construction and operation. Tagging could also be extended to nearby bays and estuaries, as it is possible that several of the fish we tagged in the Bish estuary may have travelled to there from elsewhere. If additional fish are tagged an monitored, it will be important to incorporate detailed and temporally specific construction and operational activities with the movements and behavior of tagged fish to both improve monitoring and to develop best practices at the plant site.

If further monitoring work is undertaken, we suggest maintaining a similar array design in the immediate area of the plant (so as to maintain near continuous monitoring coverage), but that the listening area be extended through strategic placement of hydrophones in the wider Kitimat Arm. Given the limited spatial scale of the current hydrophone deployment (5–6 km total) it is difficult to characterize what cutthroat trout were doing once they left the immediate area and thus, difficult to assess the demographic connectedness of Kitimat Arm populations, their typical home ranges and habitats used, particularly for those adopting migratory strategies. This would mean placing hydrophones on a spatial scale more similar to the Goetz et al. (2013) study, with a hydrophone spacing of 3–10 km covering both sides of the Kitimat Arm in in as many bays and estuaries as possible, and other points of possible importance to CCT (e.g., known spawning/ rearing for herring).

Future work should also extend the juvenile reference collection in terms of sample sizes and geographic scope. It would be very helpful to include other potential source populations in the Douglas Channel (Whatlsto, etc.), but particularly those from Bish Creek and its tributaries (Skoda, Reliant, Renegade creeks). It is a major weakness of this study, that we do not have juvenile reference samples from those creeks in our baseline. Without them, we cannot definitively explain the nature of the apparently close genetic relationship between fish tagged at Bish Creek and populations in the Kitimat River. Given the limited divergence of many reference populations, further genotyping at additional microsatellite may be required to provide additional resolution between Bish and Gobeil creeks and the rest of the Kitimat cluster. To investigate whether postglacial recolonization dynamics and historical associations are responsible for the mosaic-like population structure that we and others have described in the area, future work could investigate patterns of variation at more slowly evolving genetic loci such as mtDNA, which is a more appropriate marker for the time scale of interest (several thousand years before present). Finally, future work could pursue alternate methods of assigning Bish Creek cutthroat trout to their likely stream of origin. Numerous studies, for example have illustrated the utility of using calcified structures in fish (otoliths, fin rays, spines, etc.) as natural records of environmental conditions encountered by a fish throughout its life (Campana 1999; Zimmerman et al. 2013; Pracheil et al. 2014). Indeed, the technique has been used extensively to map anadromous movements, and more recently, wholly freshwater movements with considerable accuracy (e.g., Clarke et al. 2007).

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Chapter 6 – Animal Movement Restoration Protocol

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An ermine (*Mustela erminea*) in the understory. Photo credit: Cherie Mosher

6.1. Introduction

The Restoration Protocol was designed to identify ways to improve the restoration of the pipeline RoW and work areas to increase animal movement along and across the RoW and to decrease the movement of invasive or edge-dependent vertebrate species (e.g., the brown-headed cowbird; *Molothrus ater*) along the RoW and construction working area. Much of the originally proposed protocol focused on post-construction restoration and monitoring — aspects that could not be implemented given the early suspension of the BMAP. Instead, this

Chapter focuses on the analysis of novel methods for baseline mammal biodiversity assessment, the development of DNA-based sampling methods for moose (and other large herbivores) fecal pellets, and the planning for the development of large animal exclosures designed to test restoration effectiveness within the pipeline RoW after construction — all of which could be completed prior to FID.

The edge habitat created by the construction of a RoW can attract large herbivores such as moose (*Alces alces*) (Child 1998), which select the juxtaposition of forest cover and early seral forage in the RoW, as well as birds or other mammals specialized on nesting or foraging in edge or open habitat (e.g., sparrows or microtine voles; Steventon et al. 1998). At the same time, a pipeline RoW across a variety of EUs has the potential to increase the distribution of invasive plant and animal species (both native and alien) during the reclamation period and simultaneously reduce the connectivity of habitats bisected by the corridor (Laurance et al. 2009). The movement of the species in response to habitat disturbance can influence the implementation and success of vegetation restoration.

One means of decreasing movement of invasive species and increasing connectivity among habitats bisected by the RoW is to effectively narrow the width of the corridor, or to create periodic narrowings consisting of vegetation with vertical structure that could act as structural '"bridges" (Jones et al. 2011). Ground cover and other horizontal ground structure within these bridges can be critical for use by small mammals (e.g., Yahner 1986) and meso-carnivores (e.g., Payer and Harrison 2003). Restoring structural components consistent with natural forest conditions — through post-construction revegetation with tree and shrub cover in addition to grasses — increases the gap-crossing ability of many bird and mammal species sensitive to disturbance or reductions in habitat connectivity (reviewed in Otter et al. 2007). Additionally, creating a transitional habitat, rather than leaving an abrupt edge, facilitates can even create ancillary nesting and foraging habitat. This added structure would also decrease the amount of edge habitat that is attractive to nest parasites, invasive weedy plant species or large herbivores that may impede restoration activities. Such mitigation strategies might make the habitat amenable to retaining species present prior to construction, facilitate movement across

the pipeline RoW, and simultaneously make the RoW less suitable for the establishment of invasive species.

Linear RoWs have a range of effects on the mobility of animals. Previous work has shown that in the absence of structural components such as coarse woody debris or shrubs and trees, linear features such as roads (McGregor et al. 2008) and ski-runs (Negro et al. 2013) often act as semi-permeable barriers to the dispersal of small forest-dwelling mammal species. The initial corridor Protocol was designed to assess animal movement and to determine how different forms of mitigation could increase the degree of connectivity across the linear corridor. One proposed method was to use noninvasive sampling of hair for DNA extraction using sticky tape hair snares, followed by species and individual identification. Such data would allow us to determine if the proposed treatments on the RoW enhance perpendicular movement and serve as habitat for forest dependent species.

For other species, RoWs represent increased opportunities for movement and foraging. Increased use of the RoW can occur with some predators (e.g., wolves [*Canis lupus*] and coyotes [*Canis latrans*]) either in search of prey or simply moving across the landscape (Latham et al. 2011). Also, many ungulate species (e.g., deer [*Odocoileus spp.*] and moose) may use the RoW — taking advantage of early seral plants associated with the forest ecotone resulting from land clearing and vegetation management (Rea 2003). An increased presence of large herbivores can also have significant negative impacts on the success of vegetation restoration. In the original Protocol, we proposed to use motion-sensing cameras, standard fixed-plot pellet transects and browse surveys, and DNA from fecal pellets (ungulates only) to assess movement and density of large mammals along the RoW. In addition, fenced exclosures were to be built to monitor the impacts of herbivorous vertebrate species (from hares to moose) on forest regeneration and vegetation complexes — similar exclosures have been used successfully in places such as Isle Royale, Michigan, U.S.A. (Risenhoover and Maass 1987) and Terra Nova Park, Newfoundland (McLaren et al. 2004). Such exclosures can be designed to restrict small, medium-sized and larger herbivores, thus enabling researchers to evaluate the impact of each species, or groups of species, on forest composition and regeneration. Interactions between

vegetation removal due to herbivores and vegetation maintenance activities could likewise be monitored using exclosures.

6.2. Approach and outline of contributions

Because the pipeline construction was delayed beyond the scope of the UNBC BMAP, we report on only pre-FID activities. Specifically, we explored passive, non-invasive approaches for detecting vertebrate presence, tested when fecal-based DNA sampling for ungulates might be most efficient, and conducted detailed literature reviews that could inform exclosure design and DNA fecal pellet sampling for mammals. We briefly outline each of those contributions below.

Section 6.3 outlines a study that combined non-invasive sampling of animal hair with high-throughput DNA sequencing methods to identify the small mammal species present at specific sampling sites along the RoW in both the interior and coastal ecosystem unit. We assessed the efficacy of non-invasive sticky snares for collecting mixed hair samples that could be identified to species with next-generation DNA sequencing methods.

Section 6.4 describes a method development for collecting DNA from moose fecal material. This is a non-invasive method for identifying the distribution of large mammals that deposit easily identified pellet groups. The quality of fecal DNA, however, can be degraded by wet or warm environmental conditions. We tested the effect of pellet-collection time (mid-March–mid-June) and temperature, on the quality of fecal DNA extracted from moose pellets collected from north-central BC. Using the number of loci genotyped as a measure of DNA quality clearly indicated that fecal pellets collected earlier in the year contained higher quality DNA. Fecal pellets collected in March and April from snow, and in cooler conditions, were more likely to remain frozen between moose depositing the pellets and the time of our collections, while those collected in May and June were exposed to warmer temperatures, rain and increased solar radiation, all of which likely contributed to degradation of fecal DNA. Our findings indicate that if high-quality DNA is to be obtained from moose pellets, pellets should be collected in late winter or early spring, rather than in late spring or early summer and are likely best collected from the snow.

In Section 6.5 and 6.6 we provide annotated bibliographies for methods of extracting and using DNA from fecal pellets as well as methods and applications of exclosures for quantifying herbivory. Those reviews of the literature were done in preparation for post-construction studies on the effects of herbivores on RoW restoration.

6.3. Field testing the assessment of baseline small mammal biodiversity using hair-snare techniques in combination with metabarcoding using next-generation sequencing

Jeanne Robert, Philippe Henry, Michael Gillingham

Introduction

The forested ecosystems of central BC have been subject to numerous ecological and industrial pressures over the past few decades. Cumulative impacts of road construction for salvage logging of dead and dying trees from the recent Mountain Pine Beetle (*Dendroctonus ponderosae*) outbreak, in combination with industrial activities such as oil and gas exploration and development has resulted in an increasingly fractured landscape in this area. A number of new linear features in the form of roads and natural gas pipelines are proposed to bisect the landscape through the increasingly fragmented central interior plateau as well as through the coastal ecosystems. Ecosystem responses to fragmentation and industrial activity can vary with time and can depend on the behavior and life history of the species that are present (Debinski and Holt 2000). In addition, linear corridors may function as barriers to the movement of animals that play a role in ecosystem functions such as seed dispersal and forest succession (e.g., Davidson 1993), distribution of mychorizal fungi (Ure and Maser 1982), as predators of insects and eggs (Maxson and Oring 1978; Walters and Miller 2001), and as prey for larger birds and mammals (e.g., Boutin 1995). Ground cover and other horizontal ground structure in openings and gaps can be critical habitat elements for small mammals (e.g., Yahner 1986) and meso carnivores (e.g., Payer and Harrison 2003). Cost-effective, and comprehensive methods for assessing small mammal biodiversity are required to understand rapid changes in landscape fragmentation and patch connectivity.

We identified small mammal presence using non-invasive hair snares in three as yet undisturbed locations along the proposed natural gas pipeline RoW. We used a non-invasive sticky hair snare technique developed by Henry et al. (2011) in combination with DNA sequencing to assess the feasibility of low-tech hair sample collection in combination with

commercially available next-generation DNA sequencing methods for quantifying the presence and distribution of mammals.

Methods

Sampling locations and methods

Packing-tape hair snares (as in Henry et al. 2011; Henry and Rusello 2011) were deployed between June–August 2014 in grids of 45 snares (either 3 × 15 or 5 × 9) at each of three sampling locations (Figure 6-1) at three study sites along the proposed pipeline RoW. Each sampling location contained 4–5 clusters of snare grids within a few km: sample location 1 had 5 grids were in the coastal EU, sampling location 2 had five grids located immediately north of Fraser Lake, and sampling location 3 had had 4 grids north of Prince George. Hair snare traps were set up in grids of 45 (either 3 × 15 or 5 × 9) at each sampling location (Table 6-1).

Table 6-1 – Location and UTM for each hair snare sampling grid.

Ecosystem Unit	Sampling location	Grid	UTM
Coastal	Location #1	1-1	9 U 548176E 6011271
		1-2	9 U 543443E 6012522N
		1-3	9 U 537426E 6011138N
		1-4	9 U 532114E 6014497N
		1-5	9 U 524171E 6014140N
Interior Plateau	Location #2	2-1	10 U 403900E 6000937N
		2-2	10 U 398974E 5999612N
		2-3	10 U 393790E 5997237N
		2-4	10 U 389555E 5998704N
		2-5	10 U 384570E 5997675N
Interior Plateau	Location #3	3-1	10 U 498098E 6007236N
		3-2	10 U 489642E 6005391N
		3-3	10 U 482935E 6004154N
		3-4	10 U 473195E 6003153N

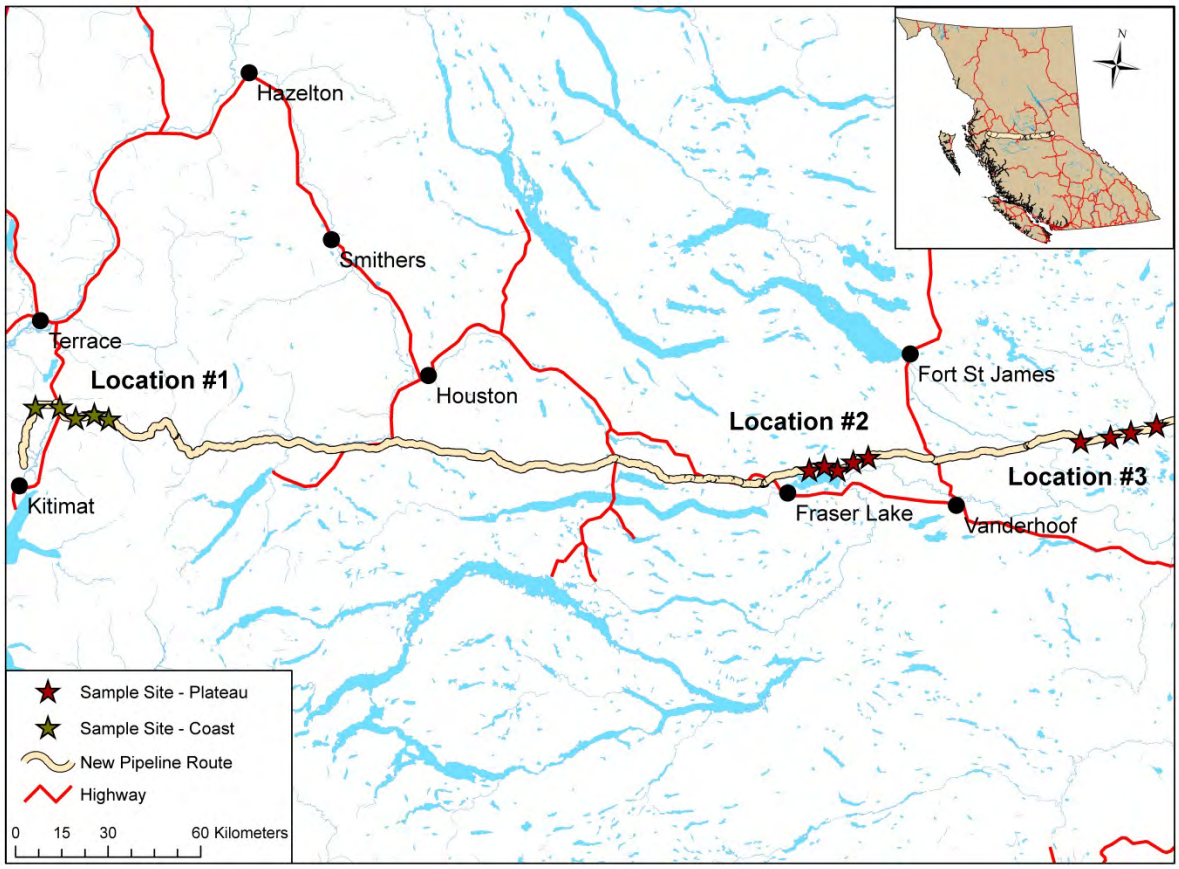

Figure 6-1 – Location of 2014 hair-snare sampling locations along the proposed pipeline route. Location #1 had had 5 replicated grid sites on the west side of the Coast Mountain Range, Location #2 had 5 replicated grid sites north of Fraser Lake, and Location #3 had 4 replicated grid sites west of Prince George, BC.

Grids were designed to measure small mammal presence and species composition by retaining a small number of hairs from each animal that contacted the sticky hair snares within the grid. Snares were placed within the grid along habitat features (such as coarse woody debris or shrub cover) such that the largest possible number and diversity of small mammal species would contact the tape. Traps were disassembled, checked for hair samples, and re-set every three weeks. Between June and August 2014, over 1500 hair samples were collected from the three sampling locations. Hair samples were removed manually or washed from the sticky tape using 70% ethanol and then stored at -80 °C in the laboratory for further analysis.

Taxonomic identification

For each grid collection date, up to five hair samples were pooled from each unknown species — pooling of samples was based on similar morphological hair characteristics. Hair samples were sent to the Canadian Centre for DNA Barcoding (CCDB; Biodiversity Institute of Ontario, University of Guelph, Ontario) for DNA extraction and sequencing using an ion torrent platform. At CCDB, hair samples were immersed in 400 µL of VLB buffer with proteinase K and incubated overnight at 56 °C. One hundred µL of the lysate were retained for genomic DNA extraction using a CCDB glass fibre plate protocol (Ivanova et al. 2007a).

The Canadian Centre for DNA Barcoding used an in-house primer cocktail (Ivanova et al. 2007b) targeted at the standard DNA barcoding region, the cytochrome oxidase I (COI) region (Ivanova and Grainger 2007), of mammal mitochondrial DNA using primers AquaF2 and VR1d_t1 (Ivanova et al. 2012). All 48 samples were successfully amplified and CCDB reported no visible PCR product in negative extraction and amplification control reactions. Sequencing was performed at CCDB using an Ion PGM platform (ThermoFisher Scientific) for next-generation sequencing of the amplicons according to the manufacturer's protocols using Ion PGM template OT2 400 kit and Ion PGM sequencing 400 kit with 314 and 316 chips. Operational taxonomic units (OTUs) were assigned by CCDB using Cutadapt version 1.8.1 (Martin 2011) to trim primer sequences, Sickle version 1.33 (Joshi and Fass 2011) for filtering the data (removing sequences less than 100 bp) and Uclust version 1.2.22q (Edgar 2010) to cluster OTUs with 98% identity and minimum read depth of 2 and to filter out singletons. Mothur (Schloss et al. 2009) was used to assign posterior probability of match to the Barcode of Life Data system (BOLD: http://www.barcodeoflife.org/) reference library of Canadian mammals. Common reagent contaminants (human and chicken) were filtered out of the resulting identifications as well as identified nuclear mitochondrial pseudogenes (defined as having an average posterior probability of less than 0.5 in Mothur).

An animal care and handling permit was obtained from UNBC (UNBC ACUC #2013-04). Permits to collect animal hair in the coastal ecosystem unit and the interior ecosystem unit were also approved from the BC Ministry of Forests, Lands and Natural Resource Operations (Permit PG14-95048 and Permit PG13-87411). These permits included the Wildlife Act of British

Columbia General Permit and the Fish, Wildlife and Habitat Management Branch Animal Care Permit.

Results

Across all 48 samples, we identified 29 species within 12 Families (Table 6-2). Red squirrel (*Tamiasciurus hudsonicus*) was detected in nearly every sample with the exception of three samples collected at the end of June in location 3 (grids K39, K46, and K56). There were two hair samples that could be identified with certainty to Genus only: a *Tamias* species (i.e., chipmunk) and a *Canis lupus* species (i.e., gray wolf or domestic dog).

The hair snares set in the interior EU unit predominantly yielded red squirrel and snowshoe hare (although squirrel and hare were also detected in the coastal ecosystem unit), but other species that were only detected in the interior ecosystem unit included cattle, elk, lynx, wood rat, two species of vole, two species of mouse, and two species of chipmunk (Table 6-3). Species that were detected in the coastal plots only included porcupine, long-tailed vole, northwestern deermouse, Trowbridge's shrew, and Northern bog lemming. The remaining species occurred in both ecosystem units and show a wide range of Genus and species including moose, black bear, flying squirrel, groundhog, marten, ermine, voles, rat, shrews, and mice.

Discussion

We successfully identified an array of small and large mammals using our sticky tape technique combined with metabarcoding using next-generation sequencing. A total of 29 species within 12 were identified from 48 sampling locations and dates in this study. There were only two sequences that could not be conclusively identified to the 'species' taxonomic unit. The first was a *Tamias* species or chipmunk; a possible candidate species is *Tamias townsendii* (Townsend's chipmunk) because the collection area is north of this species known range (Nagorsen 2002), and because this species is not yet vouchered in the ever-expanding BOLD reference library. The second was a *Canis* species. Barcoding using the COI sequence marker does not distinguish between wolf (*Canis lupus*) and domestic dog (*Canis lupus familiaris*). Although the database contains a separate sequence cluster identification BIN for

coyote (*Canis latrans*), hybridization between wolf, dog, and coyote makes identification using DNA markers less reliable than for species that do not hybridize (Bozarth et al. 2011; Monzón et al. 2014).

Table 6-2 – Identified taxonomic units (Family, Genus, and species) in the 48 grid samples submitted for next-generation sequencing.

Family	Genus	Latin binomial	Common name
Bovidae	*Bos*	*Bos taurus*	Cattle
Canidae	*Canis*	*Canis lupus (familiaris?)*	Gray wolf / domestic dog
Cervidae	*Alces*	*Alces americanus*	Moose
	Cervus	*Cervus canadensis*	Elk
Cricetidae	*Microtus*	*Microtus longicaudus*	Long-tailed vole
		Microtus pennsylvanicus	Meadow vole
	Myodes	*Myodes gapperi*	Southern red-backed vole
	Neotoma	*Neotoma cinerea*	Bushy-tailed wood rat
	Peromyscus	*Peromyscus keeni*	Northwestern deermouse
		Peromyscus maniculatus	North American deermouse
	Phenacomys	*Phenacomys intermedius*	Western heather vole
	Synaptomys	*Synaptomys borealis*	Northern bog lemming
Dipodidae	*Zapus*	*Zapus hudsonius*	Meadow jumping mouse
		Zapus princeps	Western jumping mouse
Erethizontidae	*Erethizon*	*Erethizon dorsata*	North American Porcupine
Felidae	*Lynx*	*Lynx canadensis*	Canada lynx
Leporidae	*Lepus*	*Lepus americanus*	Snowshoe hare
Mustelidae	*Martes*	*Martes americana*	American marten
	Mustela	*Mustela erminea*	Ermine
Sciuridae	*Glaucomys*	*Glaucomys sabrinus*	Northern flying squirrel
	Marmota	*Marmota monax*	Groundhog
	Tamias	*Tamias amoenus*	Yellow-pine chipmunk
		Tamias sp.	Chipmunk sp.
	Tamiasciurus	*Tamiasciurus hudsonicus*	Red squirrel
Soricidae	*Sorex*	*Sorex cinereus*	Cinereus shrew
		Sorex preblei	Preble's shrew
		Sorex trowbridgii	Trowbridge's shrew
		Sorex vagrans	Vagrant shrew
Ursidae	*Ursus*	*Ursus americanus*	American black bear

Table 6-3 – The percentage of grids showing the presence of each of the species identified in the DNA pool for Location #1 (coastal ecosystem unit near Terrace, BC), for Location #2 (interior ecosystem unit north of Fraser Lake), and Location #3 (interior ecosystem unit north of Prince George, BC). The dash indicates that the species was not detected in any of the grids for that location. Int. = interior ecosystem unit.

Ecosystem Unit	Latin binomial	Common name	Loc. 1 Coast	Loc. 2 Int.	Loc. 3 Int.
Interior EU	*Bos taurus*	Cattle	-	13%	13%
	Cervus canadensis	Elk	-	-	13%
	Lynx canadensis	Canada lynx	-	-	19%
	Microtus pennsylvanicus	Meadow vole	-	13%	6%
	Neotoma cinerea	Bushy-tailed wood rat	-	7%	-
	Peromyscus maniculatus	North American deermouse	-	27%	38%
	Phenacomys intermedius	Western heather vole	-	13%	13%
	Tamias amoenus	Yellow-pine chipmunk	-	20%	6%
	Tamias sp.	Chipmunk sp.	-	-	19%
	Zapus hudsonius	Meadow jumping mouse	-	7%	6%
Interior and Coastal	*Alces americanus*	Moose	-	20%	-
	Canis lupus	Gray wolf / domestic dog	13%	-	6%
	Glaucomys sabrinus	Northern flying squirrel	47%	60%	44%
	Lepus americanus	Snowshoe hare	7%	53%	100%
	Marmota monax	Groundhog	7%	27%	6%
	Martes americana	American marten	20%	27%	6%
	Mustela erminea	Ermine	7%	-	13%
	Myodes gapperi	Southern red-backed vole	53%	80%	38%
	Sorex cinereus	Cinereus shrew	27%	27%	25%
	Sorex preblei	Preble's shrew	13%	7%	6%
	Sorex vagrans	Vagrant shrew	-	7%	-
	Tamiasciurus hudsonicus	Red squirrel	100%	100%	81%
	Ursus americanus	American black bear	13%	27%	69%
	Zapus princeps	Western jumping mouse	7%	13%	25%
Coastal EU	*Microtus longicaudus*	Long-tailed vole	27%	-	-
	Erethizon dorsata	North American Porcupine	7%	-	-
	Peromyscus keeni	Northwestern deermouse	80%	-	-
	Sorex trowbridgii	Trowbridge's shrew	33%	-	-
	Synaptomys borealis	Northern bog lemming	20%	-	-

Our method is effective for detection the presence of small animals that are difficult to observe using wildlife cameras or to live trap. For example, Fitzgerald (1994) notes that the population size of western heather vole is not well known because of the high mortality rate during live trapping. We identified western heather voles in two locations (Location #2 and Location #3), and were able to obtain samples from at least three individuals across a large

geographic area (at least two individuals at grid K141 and K151 in Location #2, and at least one individual at grid K39 in Location #3) with no evidence of mortality in the sampling area.

Small mammal diversity did differ between ecosystem units as expected, and none of the taxonomic identifications were unreasonable given the known range of the 29 species identified (Nagorsen 2002). Biodiversity differences between coastal and interior ecosystem units are reflected in the ecosystem-specific species lists that we identified. The presence of small mammals such as voles, mice, lemmings, and shrews may be an indication of sufficient cover in undisturbed ecosystems (Pearce and Venier 2008), especially in the coastal ecosystem unit. Restoration of vegetation and coarse woody debris after harvesting for industrial operations would likely aid in the maintenance of the small mammal populations that we identified as important components of ecosystem function and resilience (Hull Sieg 1987; Carey and Harrington 2001).

In addition, the combination of hair snare sample collection with high throughput DNA sequencing could potentially be used to obtain a much wider range of information on the small mammal diversity and condition in a given study area. One of the drawbacks of our current approach is that there is only a presence or absence measure of each species for a particular grid, on a particular collection date. Standardization of sampling effort (e.g., species encounters per trap and day effort) could provide some estimate of relative presence/absence across sampling sites, but there is no control over the number of hairs 'captured' per trap visit nor any way of correcting for multiple 'captures' at the same trap site during a sampling period. Because we show that sufficient DNA can be extracted from as little as five hairs, it is likely that individual identification could also be included with species identifications. Similar methods to those employed for individual identification of large mammal fecal pellets, for example, could possibly be applied to the DNA extracted from hair samples (see Brinkman et al. 2010). Finally, this technique could be combined with other hair analysis techniques such as cortisol analysis (Bryan et al. 2013) in order to add life-history and health information of targeted species or individuals.

Conclusions

We describe a method for easily and comprehensively assessing the presence of small mammal. DNA extractions from pooled hair samples can be used to distinguish a wide array of mammals, without taxonomic expertise. Further experiments can determine the optimal density and timing of hair snare traps to thoroughly sample all mammals in a defined area of interest. We have shown that DNA-based methods are non-invasive and effective with cryptic, or hard to identify small mammals (i.e., shrews, mice, voles, lemmings, weasels). With further development, the technique could ultimately be used to gather an increased amount of information including counts of the number of species, movement patterns of individual animals, and life-history data.

Acknowledgements

We sincerely wish to thank Daniel Rissling, David Graham, Alexander LePage, Matt Scheideman, and Courtney Berdan for assistance with data collection and processing. In addition, we thank Evgeny Zakharov, Natalia Ivanova, and Janet Topan at the Canadian Centre for DNA Barcoding (CCBD) for the DNA extraction, next-generation sequencing and taxonomic identification services. We would also like to thank KNLG, PTP, and Chevron Canada for funding this research through the BMAP.

6.4. Timing the collection of moose fecal pellets to obtain high quality DNA

Roy Rae, Dexter Hodder*, Shannon Crowley*, Chris Johnson, Brent Murray

Introduction

The collection of fecal DNA is an increasingly popular noninvasive technique being used by wildlife biologists to identify individual animals, establish the presence of rare or elusive species, determine gender (Fernando et al. 2003; Waits and Paetkau 2005) and to examine, among other things, population structure, mating systems, genetic diversity and dispersal patterns (Ball et al. 2006; Brinkman et al. 2011). These techniques allow the sampling of a large number of animals quickly and at lower cost (Wehausen et al. 2004; Waits and Paetkau 2005). For populations or species that are rare, elusive or difficult to capture, fecal sampling may be the only technique for collecting DNA (Zhang et al. 2006).

Fecal DNA is DNA extracted and isolated from sloughed cells of the intestinal mucosa, which coat the outer surface of the feces. When compared to blood and tissue, some investigators consider fecal DNA to be of inferior quality because fecal DNA can be subject to degradation (Piggot 2004; Ball et al. 2006; Brinkman et al. 2011), while others simply caution against contamination and laboratory error (Fernando et al. 2003). Degradation is known to increase with time after deposition events, rainfall events, and a myriad of other environmental conditions that can occur (Brinkman et al. 2009 and 2010). In some cases, those processes are not well understood (Mowry et al. 2011). For those reasons, Maudet et al. (2004) and Wehausen et al. (2004) suggest that more research be conducted to clarify the effect of season and environmental moisture on the quality of fecal DNA collected from ungulates.

Our objective was to determine the optimal timing of moose (*Alces alces*) pellet collections in order to obtain samples with DNA of sufficient quality to determine animal identity and sex through analysis of microsatellite markers. To this end, we collected fecal pellets of moose during the middle of March (6–12), April (17–22), May (13), and June (19). Our null hypothesis was that fecal DNA quality would be unaltered by collection date.

* John Prince Research Forest, Fort St. James, British Columbia

Methods

Study site

Moose fecal pellets were collected in the John Prince Research Forest (JPRF; 54°40'10.1"N 124°24'52.1"W; Figure 6-2). The JPRF is a 16,500-ha portion of forested land 45 km northwest of Fort St. James, BC and is co-managed by UNBC and Tl'azt'en Nation. The JPRF is characterized by rolling terrain with low mountains (700 m–1267 m a.s.l) and is within the Sub-Boreal Interior ecoprovince with representation of the Babine Uplands, Manson Plateau and Nechako Lowlands ecosections. The JPRF is situated between two large lakes, Tezzeron (8079 ha) and Pinchi (5586 ha), and has a relatively high density of streams. It represents the northern extent of contiguous Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) forests in the interior of BC and is dominated by the Sub-Boreal Spruce biogeoclimatic (BGC) zone. The area has experienced a wide variety of logging activities over the past 50 years and contains a mosaic of old and young forest with interspersed deciduous stands. The stands have a relatively rich understory of deciduous shrubs and regenerating conifers (for a more detailed description see Crowley et al. 2012).

Pellet collection and analysis

Complete groups of moose fecal pellets were collected opportunistically and repeatedly along the same gravel resource roads (unsalted and unplowed with no traffic) and trails in the JPRF (see transect lines on Figure 6-2). Moose fecal pellets were easily distinguished from the pellets of elk (*Cervus canadensis*) and mule deer (*Odocoilus hemionus*), which are also found in the research forest, by experienced collectors using pellet morphology (Hodder et al. 2013). We conducted sampling sessions during the middle of each month from mid-March–mid-June, 2013. Pellets were collected throughout the JPRF (Figure 6-2). Ten samples were collected during each collection period and placed in Ziploc® bags. Within 6 h., sample bags were moved from field containers to a -20ºC freezer.

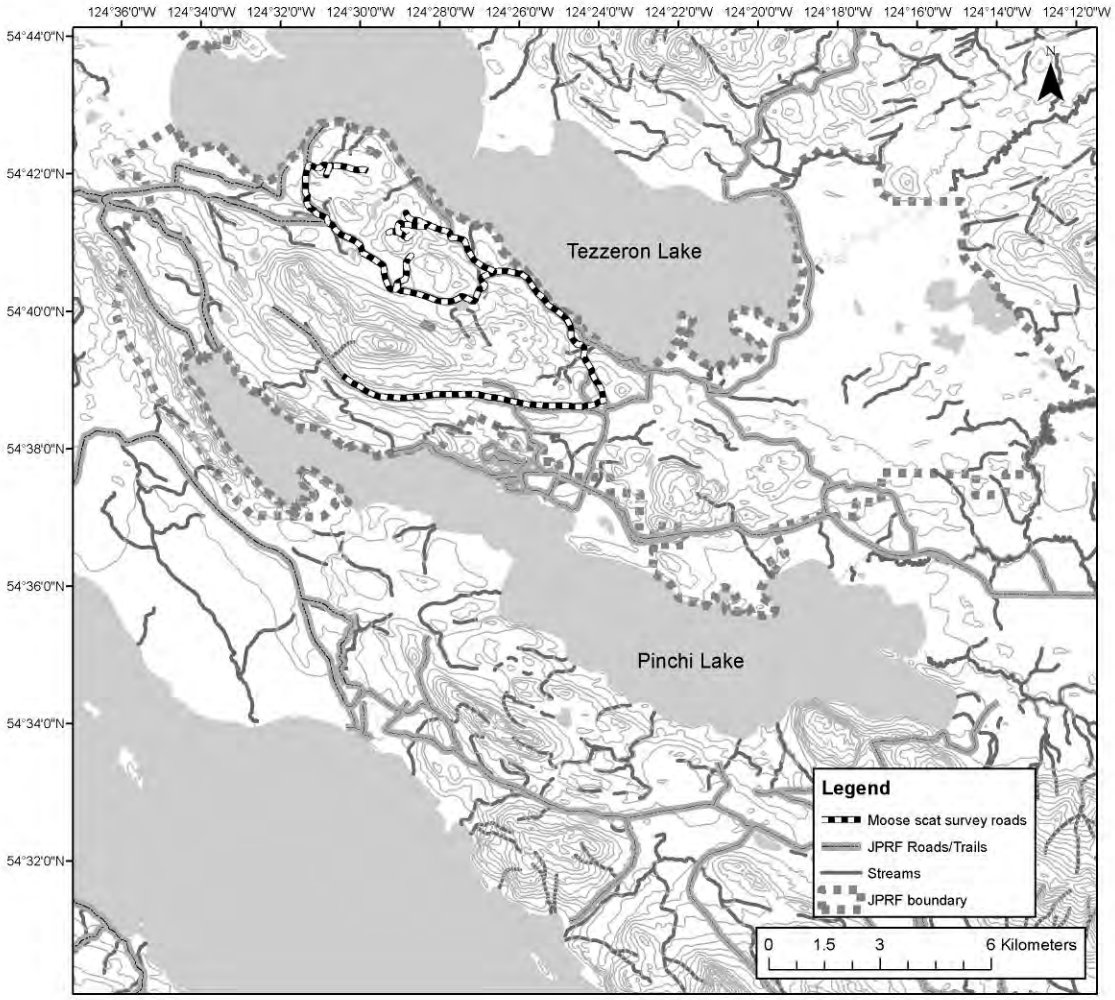

Figure 6-2 – Map of the study area located in the heart of the John Prince Research Forest (boundaries are the grey and white squares), north-central BC. Moose scat survey lines are delineated with black and white squares.

Sample bags were removed from the freezer and individual pellets were separated prior to DNA extraction. As recommended by Wehausen et al. (2004), two porous wooden toothpicks were gently rubbed on the exterior of three individual pellets from each sample to collect epithelial cells; we were careful to ensure that no fecal matter was collected. The toothpicks were placed in individual paper envelopes to dry, after which they were stored for shipping.

Toothpicks containing DNA were sent to Wildlife Genetics International in Nelson, BC for analysis. There, DNA was extracted by clipping a ~3 mm piece of each toothpick and processing the clippings using QIAGEN (Toronto, Ontario) blood and tissue (full kits) DNeasy spin columns, as per QIAGEN's tissue protocol. All samples were genotyped (ultimately for identification of individuals) using eight markers (seven microsatellites plus gender) that had been previously used by Wildlife Genetics for individual ID in other moose pellet studies in northern BC. In order to compare genotyping results among all samples only a single round of genotyping was conducted for most samples. Following the standard operating procedure of Wildlife Genetics International, results for each sample were reported as number of loci with any genotype information and the number of loci with high-confidence genotype information. Low-confidence genotypes were determined through a combination of objective (peak height) and subjective (appearance) criteria. For this project, we used primers Bl42, BM1225, BM 4513, BM848, OarFCB193, Rt24, Rt30 and a ZFX/ZFY gender marker (Buchannan and Crawford 1993, Bishop et al. 1994). We obtained weather records from Environment Canada's National Climate Data and Information Archive (2015).

Statistical analysis

We used Poisson count models to test the relationship between the number of successfully genotyped loci and indices of environmental conditions that may have influenced the degradation of DNA. We conducted two analyses, relating the total number of loci and the number of high-quality loci to average temperature and month that the fecal samples were collected. For month, we used deviation coding to contrast March, April, May, and June. Two samples collected on February 21 were coded as March. The occurrence of snow was highly correlated with temperature and month and the effects of daily precipitation were too ephemeral to consider as a factor in this analysis.

We used Akaike's information criterion (AIC_c) for small samples to identify the most parsimonious model (Anderson et al. 2000). To assess the predictive accuracy of the final models, we used a Wilcoxon Signed-Rank test to determine if the distribution of observed loci differed from the number predicted by the count models. We used a boot-strapping procedure to sequentially withhold each record during model-fitting and then predicted the count for that

withheld record. This allowed for an out-of-sample test, but sufficient data to fit the models (i.e., n = 39).

Results

In an initial round of genotyping, 32 samples yielded high-confidence data for ≤ 1 of the 8 markers, including all 30 samples with collection dates later than mid-March (Figure 6-3). In contrast, samples collected in March yielded more complete genotype information with an average of 7 loci with genotype information (four with high-confidence data).

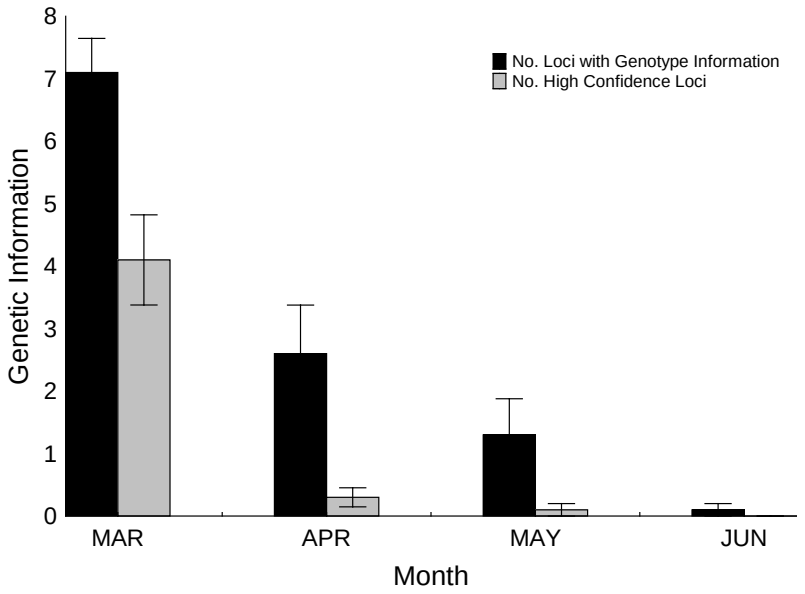

Figure 6-3 – Mean(±1 SE) number of (8 possible) loci obtained from fecal samples containing genotype information and of the number of loci containing high confidence genotype information (i.e., high confidence loci) obtained from moose fecal samples collected during mid- March, April, May and June of 2013 (n = 10 each month).

To ensure that failures to extract DNA of sufficient quality were not caused by a problem in the laboratory, five samples with ≤1 locus with high-confidence data were re-extracted. The re-extractions also failed, suggesting that the low success rate reflected poor sample quality. Positive control samples (from another moose fecal pellet study) analyzed alongside fecal

samples produced clean, strong data, providing further evidence that the low success was not caused by problems with laboratory protocols or reagents.

Weather records indicated that temperature and precipitation changed considerably over time; samples collected from March–June were exposed to an increasingly warmer and wetter environment (Table 6-4). Likewise, the greatest number of samples with either some genotype information or high-confidence loci was collected during March with very few successful samples having been collected in June.

Table 6-4 – Average daily temperatures and precipitation for mid (10th to the 20th of each month) March–June, 2013 from Fort St. James, BC (Environment Canada 2015).

Collection Period	Ave. Daily Temp (°C)	Ave. Max. Daily Temp (°C)	Ave. Min. Daily Temp (°C)	Ave. Daily Rainfall (mm)	Ave. Daily Snow (cm)
March	-4.9	0.0	-6.3	0.0	2.5
April	3.1	7.8	-1.6	2.4	0.0
May	9.8	16	3.5	0.0	0.0
June	14.3	19.8	9.2	2.4	0.0

The most parsimonious Poisson models representing the count of informative loci for each sample consisted of the categorical variable month, not average temperature, for the day that the sample was collected (Table 6-5). The number of loci (both those with some genotype information and those with high-confidence information) was positively correlated with sample collection during the month of March and negatively correlated with collection in June.

Table 6-5 – Number of model parameters (k), differences in Akaike Information Criterion (AICc) scores (Δ) and AICc weights (w) for Poisson count models representing the high-quality and total number of successfully genotyped loci from moose fecal pellets collected in north central BC from March–June, 2013. Predictive ability of each model was tested through a comparison of observed and predicted number of loci using the Wilcoxon Signed-Rank test.

Model	*k*	$AIC_c\Delta$	AIC_cw	Wilcoxon *P* (z)
High quality loci:				
Average Temperature	2	8.9	0.012	0.010 (-2.590)
Month	4	0	0.988	0.312 (-1.012)
Total loci:				
Average Temperature	2	15.2	0.001	< 0.001 (-4.887)
Month	4	0	0.999	< 0.001 (-5.514)

Likewise, as average daily temperature warmed, the number of useful loci decreased (Table 6-6). The Wilcoxson Signed-Rank test revealed that the most predictive models were those constructed from the data representing the number of high-quality loci. The month of sampling was a good predictor for that count model (p = 0.312, z = -1.012; Table 6-5).

Table 6-6 – Model coefficients, standard errors (SE) and 95 % confidence intervals for Poisson count models representing the high-quality and total number of successfully genotyped loci from moose fecal pellets collected in central BC from March–June, 2013 (see Table 6-5).

Covariate	Coefficient	Standard error	95 % confidence interval
High quality loci:			
Temperature Model			
Constant	0.085	0.248	-0.400–0.571
Average Daily Temperature	-0.164	0.018	-0.198–-0.129
Month Model			
Constant	-4.705	0.281	-5.256–-4.155
March	6.116	0.305	5.518–6.714
April	3.502	0.445	2.629–4.375
May	2.403	0.735	0.962–3.843
June	-12.020	0.271	-12.550–-11.490
Total loci:			
Temperature Model			
Constant	1.227	0.131	0.971–1.483
Average Daily Temperature	-0.100	0.013	-0.126–-0.074
Month Model			
Constant	0.219	0.273	-0.317–0.754
March	1.741	0.278	1.196–2.286
April	0.737	0.340	0.070–1.404
May	0.044	0.407	-0.755–0.842
June	-2.521	0.732	-3.957–-1.086

Discussion

Genetic analyses of intestinal mucous from moose fecal pellets indicated that pellets collected in March contained the most samples with DNA of sufficient quality to genetically identify individual moose (n = 8). Samples collected during April were also viable, but the failure rate to successfully genotype samples was much higher. Pellets collected after April contained poor quality, if any, DNA. Collection season and average monthly weather data suggest a strong

correlation between sample viability and average temperature at or below 0 °C and snow cover, as recorded for March 2013.

Because DNA degrades with time (Fernando et al. 2003; Piggot 2004), knowing when pellets were deposited and the amount of time that passed between deposition and collection would have helped separate the confounding effects of pellet age from collection season (Piggot 2004). Given that pellet age has a significant impact on genotyping success (*pers. comm*. Tessa Tjepkes, Strasburg Molecular Ecology Laboratory, University of Minnesota, Duluth, November 2015), determining a way to age pellets would have allowed for a more detailed analysis. Unfortunately, we cannot be sure that pellets deposited in May and June (on the ground) were as fresh as those deposited in March and April (mostly on melting snow pack where some pellets may have been frozen since deposition). We are confident, however, that pellets were relatively recent (being deposited during the winter of our collections) based on procedures used during other pellet surveys conducted in north-central BC (Rea et al. 2010). Also, our objective was not to age pellets *per se*, but to determine DNA quality as a factor of warmer and wetter environmental conditions. Ultimately, whether changes in DNA quality were due to seasonal effects or age of scats or a combination of the two is irrelevant — DNA recovered from pellets collected earlier was better.

Although Piggot (2004) reported that DNA collected from summer-deposited scats of red fox (*Vulpes vulpes*) and brush-tailed rock wallaby (*Petrogale penicillata*) had better quality DNA than winter deposited scats, Harris et al. (2010) suggests that Afghan argali sheep (*Ovis ammon*) fecal materials yielded better DNA when collected in winter. Maudet et al. (2004) also obtained better DNA from Alpine Ibex (*Capra ibex*) and Corsican mouflon (*Ovis musimon*) pellets collected in Italy and France in winter (November–February) when compared to collection periods during spring (April–May), suggesting that diet may significantly affect the quality of DNA obtained from feces. They postulated that the low-quality, high-fiber diets in winter are more likely to increase the amount of time digesta spend in contact with the gastrointestinal tract and that increased fiber will scour and accumulate intestinal mucosal cells.

Wehausen et al. (2004) suggests that pellets collected from herbivores foraging on plants high in secondary compounds may be hard to assess for fecal DNA because of inhibitory effects of plant secondary compounds on PCR reactions — the success of which may depend on maximizing the concentration of intact DNA. Admittedly, plant secondary compounds eaten by moose may have potentially influenced the recovery of useful loci from pellets we collected in June. Secondary compounds, however, are unlikely to explain the drop in loci recovered in April and May because these months precede the flush of forages containing such compounds. Additionally, mucosal DNA collected from wolf and otter scat was of higher quality when collected in winter compared to summer (Lucchini et al. 2002; Mowry et al. 2011), suggesting something other than diet (such as environmental conditions at the time of collection) may be influencing DNA recoverability.

In summary, our findings suggest that if fecal pellets are to be used in identifying individual moose, that collections of fecal pellets should be conducted earlier, rather than later in the spring or early summer. Our data suggest that pellets collected in the snow or shortly after snow melt are more likely to have intact DNA than those collected once the snowpack has melted, temperatures warm, snow turns to rain, and leaf flush begins. Although finding fecal pellets of moose in mid-winter can be challenging given pellets are increasingly covered by snow, late winter and early spring collections provide a unique opportunity to obtain pellets exposed by melting snows that, we contend, contain relatively high quality DNA, at least when compared to pellets exposed to the warmer, wetter temperatures of late spring and early summer.

Acknowledgements

We would like to thank the staff at the John Prince Research Forest for fecal collections and in-kind contributions and Matt Scheideman for assistance in the laboratory. We would like to thank KNLG, PTP, and Chevron Canada for providing funding for the DNA analysis through the BMAP. We would also like to thank Michael P. Gillingham, Leanne Harris, and Tessa Tjepkes for comments on an earlier draft of this manuscript.

6.5. Annotated bibliography: moose fecal pellet analysis

Matt Scheideman, Roy Rea

Introduction

In accordance with our original research goals to determine whether restoration treatments (RoW and WAT) influence the assemblage and relative abundance of mammal species (herbivores, in particular) on the pipeline RoW, we investigated several methods of determining a relative abundance measure. One method was to use non-invasive sampling of fecal pellets to collect DNA (Figure 6-4). Towards this end, we used a literature review to identify potential sampling and analytical methods. We focused our review on ungulates with particular attention on moose, the largest herbivore and the species with the broadest distribution across the RoW. The Section lists a number of papers, followed by a brief description of their relevant contributions to our research objectives for this Protocol.

Figure 6-4 – Removal of DNA-containing tissue from moose fecal pellets. Photo credit: Matt Scheideman

Ball MC, Pither R, Manseau M, Clark J, Petersen SD, Kingston S, Morrill N, and Wilson P. 2007. Characterization of target nuclear DNA from feces reduces technical issues associated with the assumptions of low-quality and quantity template. Conservation Genetics. 8:577–586. DOI: 10.1007/s10592-006-9193-y

Since DNA from feces can have high error rates, many researchers increase PCR cycles to counteract this problem, but this has been shown to increase the probability of amplification errors such as allelic dropout and false alleles. The authors collected 20 woodland caribou samples of 15 pellets in February. The pellets were shipped frozen from the field and stored at -20 °C. In the laboratory, each sample was divided into three subsamples, of three pellets each (60 subsamples). A control DNA sample was taken from muscle tissue and quantified using Picogreen^{TM} fluorescence assay. Samples were thawed in 2 mL of 0.1 M phosphate buffered saline (PBS) which re-hydrated and thickened the mucous. After wiping the pellet, the applicator was immersed in 300µl of lysis buffer. Samples were digested using 20 units of

Proteinase K and incubated at 65 °C for 2 h, followed by additional 20 units of proteinase K and incubation at 37 °C for 12 h. DNA then extracted using the Qiagen DNeasy kit. Where the mucous layer was thin, the authors noted that wiping the pellet may remove more solid fecal material into the extraction process. They used a standard PCR protocol of 30 cycles and experienced no amplification errors the target DNA from caribou pellets.

Brinkman TJ and Hundertmark KJ. 2008. Sex identification of northern ungulates using low quality and quantity DNA. Conservation Genetics. 10:1189-1193. DOI: 10.1007/s10592-008-9747-2

Shaw et al. (2003) amplified sex of deer and moose using length differences in Z*fx* and Z*fy* alleles, but the fragment lengths were greater than 800 basepairs, which is often too long to be consistently amplified from degraded DNA extracted from pellets. While amplification of a 236-bp PCR product was a reasonable task when using low-quality and quantity DNA from feces, amplification of longer (greater than 300 bp) fragments was problematic because of high amplification failure and allelic dropout. There was a large methods section where the authors used blood and tissue samples to determine a procedure using amelogenin-based PCR assays to determine the sex of ungulates from low quantity and quality samples, such as feces. This technique was applicable for determining sex ratios from feces samples. Females consistently showed a single band and males a double band. Moose showed a 210 bp X band a 174 bp Y chromosome-linked allele. The shorter length bands better fit the objective of determining sex from low-yield DNA. The pellets used in this study were collected from the ground under natural conditions and were known to be between 1 and 14 days old.

Brinkman TJ, Person DK, Chapin S, Smith W and Hundertmark KJ. 2011. Estimating abundance of Sitka black-tailed deer using DNA from Fecal Pellets. The Journal of Wildlife Management. 75:232–242. DOI: 10.1002/jwmg.22

In this study, the authors sampled of deer pellets between the beginning of snow melt (March 15) until appearance of leaves (May 15) by using belt transects. 67% of pellets were found on buffered deer trail networks that encompassed 30% of the total belt transects in

earlier sampling. As a result, the authors used predetermined locations and a set compass bearing to follow deer trails. From each fecal pellet group encountered on transects, they sampled 4-6 pellets, then resampled each transect a mean of 6.2 times with 10 days between sampling to ensure that most pellet groups would yield useable DNA. Remaining pellets were removed from transects during each sampling occasion to ensure that all pellet groups encountered on the next sampling expedition were deposited within that 10 day period. The authors collected each sample with new latex gloves, placed samples in plastic conical tubes filled with 90% ethanol, and stored the samples at room temperature for 1–6 months until DNA extraction. They followed a procedure to extract genomic DNA (Brinkman 2010). Since the sampling was done between late winter and early spring, we assumed that forage intake of deer may increase with greening and growth of vegetation and that "capture" opportunities would also increase because pellet deposition would increase. 51% of the samples were successfully genotyped out of 2,248 samples and 737 deer were identified. The deer trail transects were advantageous over straight line transects because they could apply it to all habitat types (ex. unmanaged forests vs. clear-cuts or slash piles), had better pellet detection rates, had easier travel through dense vegetation, and had greater repeatability. The authors suggested that experimenting with a grid-like array of transects may better fit likelihood-based estimators of density.

Brinkman TJ, Person DK, Schwartz MK, Pilgrim KL, Colson KE and Hundertmark KJ. 2010. Individual identification of Sitka black-tailed deer ( ) using DNA from fecal pellets. Conservation Genetics Resources. 2:115–118. DOI: 10.1007/s12686-010-9176-7

Sitka black-tailed deer pellets were collected from transects that were cleared of existing pellets 10 days prior to sampling period. Pellets were preserved in 95 % ethanol and stored at room temperature until DNA was extracted. During collection, pellets were classified based on physical appearance: Good (freshly deposited in a clumped distribution with pellets intact, surface a glossy sheen), Average (slightly older or more weathered pellet group that still had intact pellets with smooth surfaces, but lacked a tightly clumped distribution, glossy sheen and mucus) and Poor (spread-out groups with rough surfaced pellets which were often showing signs of decomposition). Early experiments revealed that "poor" samples consistently failed to

amplify DNA, and therefore they were excluded from further analyses. Genomic DNA was extracted using DNeasy Tissue kit and a protocol described by Maudet et al. (2004) with minor modifications. Cell lysis was performed on fecal pellets in 25 mL scintillation vials on a rocker at room temperature for 20 min using 900 µL of lysis washing buffer to harvest intestinal mucosal cells from exterior of pellet without breaking apart the pellet. The PCR process is listed in the methods section where they performed the multi-tube approach. Good pellets had a genotyping success of 66% and Average had a genotyping success of 33%, showing DNA quality can be assessed in the field by physical appearance.

Brinkman TJ, Schwartz MK, Person DK, Pilgrim KL and Hundertmark KJ. 2010. Effects of time and rainfall on PCR success using DNA extracted from deer fecal pellets. Conservation Genetics. 11:1547–1552. DOI: 10.1007/s10592-009-9928-7

The authors outlined problems associated with fecal DNA including contamination by microorganisms or digested food items, sensitivity to seasonal weather, high PCR inhibitor to DNA ratios, and relatively high amplification and genotyping errors. Small errors genotype identification can lead to large overestimates of population size. During August, the authors collected muscle tissue and pellets from the intestines of five hunter-killed deer. Some pellets were immediately placed in 90% ethanol and stored at room temperature until DNA extraction. Of remaining pellets, half were placed in a wax-coated box sheltered from rainfall, and the other half were placed outdoors on soil exposed to natural processes. Pellets were sampled at intervals of 2, 7, 14, 21 and 28 days after deer harvest. All samples were then sent to Rocky Mountain Research station's genetics laboratory in Missoula, Montana for genotyping. The authors extracted genomic DNA using the DNeasy Tissue Kit and a protocol described by Maudet et al. 2004. Descriptive statistics of the genetic variability were calculated using GENALEX. When evaluating all samples, 1.3% of loci had amplification failures in controlled environment and 39.5% of loci had failures in a wet environment. 1.4% of alleles had errors in the controlled environment versus 15.6% of alleles in the wet environment. In pooled time periods, amplification success was similar among all individuals in dry and wet environments (Dry: $\chi 2 = 3.133$, $p = 0.536$; Wet: $\chi 2 = 2.818$, $p = 0.589$). In addition, amplification success was

similar after 2 days between environments, but was different at each following time period (7, 14, 21 and 28 days). 80% of dry and 22% of wet environment samples were successfully genotyped during the experiment. This study shows that rainfall significantly increases degradation rates of DNA from ungulate pellets. To reduce failure during PCR, the authors recommend clearing collection areas of pellets before the initial sampling and collecting pellets within 14 days, particularly in wet environments.

Forsyth DM, Barker RJ, Morriss G and Scroggie MP. 2007. Modeling the relationship between fecal pellet indices and deer density. The Journal of Wildlife Management. 71:964–970. DOI: 10.2193/2005-695

To count pellets in enclosures (n = 20), the authors used random locations and bearings for 30 to 150m. Pellets were counted along transects in circular 3.14 m^2 plots, spaced 5m apart (30 plots per transect). They obtained the number of deer from estimates by enclosure managers. An increase of 1 animal per ha corresponded with an increase of 140 pellets and 8 pellet groups. The results of this study showed that total pellets found, pellet groups, and pellet frequency changed positively and approximately linearly with increasing deer density. Because the enclosures are artificial and do not show how animals may actually move across the landscape, results may vary with much different environmental conditions or other geographic areas (fecal decomposition and decay rates).

Harris RB, Winnie J, Amish SJ, Beja-Pereira A, Godinho R, Costa V and Luikart G. 2010. Argali abundance in the Afgan Pamir using capture-recapture modeling from fecal DNA. The Journal of Wildlife Management. 74:668–667. DOI: 10.2193/2009-292

Because little was known about the argali population size in this area, the authors tried to determine whether the population was isolated by using genetic testing and aerial surveys. Since argali are sporadic in their movements, the authors did not systematically sample the area. They instead used vantage points to determine animal locations, and then concentrated sampling in those areas. They used three fecal pellets from each pellet group that were "reasonably" fresh. Pellets were not collected if they were deposited in an area greater than 0.1m^2 or if the animal was moving while depositing pellets. Low quality samples (broken or

malformed pellets) or those produced by lambs were not sampled (abundance estimate was therefore for animals greater than 1 year old). Pellets were stored in 30 cm centrifuge tubes with tight-fitting screw-top caps and sporks attached to inside of cap (to handle pellets individually without risk of contamination). Four parts of 95 % ethanol for each pellet was added. They used a Qiagen test kit and lysis buffers for DNA extraction and genotyping. The authors found that genotyping success was higher in winter (although handling and storage differences may have influenced genotyping success). Amplification success was relatively low (85%) but false allele (1%) and allelic drop out (5%) rates were also relatively low in this study.

Hettinga PN, Arnason AN, Manseau M, Cross D, Whaley K and Wilson PJ. 2012. Estimating size and trend of the North Interlake Woodland Cariboo population using fecal-DNA and capture-recapture methods. The Journal of Wildlife Management. 76:1153–1164. DOI: 10.1002/jwmg.380

Using a fixed wing aircraft, the authors flew transects 3 km apart after a snowfall to locate tracks and cratering sites. A helicopter crew then went to those locations and collected fecal pellets within two days. Samples were collected using wooden stick and were placed in sterile bags in a cooler on the aircraft. Ten pellets were collected from each sample. 1.4 times more samples were collected then the number of caribou thought to have been present. Pellets were frozen at -20^{o}C. For DNA extraction, the pellets were thawed in the laboratory, and then the mucosal coat was removed and sent to Trent University forensic laboratory for analysis.

Maudet C, Luikart G, Dubray D, Von Hardenberg A and Taberlet P. 2004. Low genotyping error rates in wild ungulate feces sampled in winter. Molecular Ecology Notes. 4:772–775. DOI: 10.1111/j.1471-8286.2004.00787.x

This paper addresses the efficacy of fecal sampling for Alpine ibex and Corsican mouflon in winter (Nov–Feb) versus spring (April–May). Fresh feces are considered to be less than 12 h from defecation and were collected as a baseline. One dropping from each pellet group was collected and stored in a 25 mL vial with 15 mL of 95 % ethanol. Mucous was removed using a lysis washing buffer for 15 min on a rotary agitator. The supernatant was used for DNA extraction using the DNeasy Blood Kit. Extraction and amplification failures occurred

significantly more frequently (Wilcoxon signed ranked test p = 0. 018 for ibex and p = 0.008 for mouflon) in spring feces than from winter feces. Successful extractions were 71 % and 95 % for ibex, 83% and 99% for mouflon for spring and winter, respectively. All winter sample extractions yielded visible amplification products, where 27% (ibex) and 29% (mouflon) of the spring samples failed to provide an amplifiable DNA. The total success rates for spring feces were only 52% (mouflon) and 59% (ibex). The authors conclude that the higher quality spring diet reduces digestion time and thus decreases contact time with intestinal membranes.

Piggott M. 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of fecal DNA. Wildlife Research. 31:485–493. DOI: 10.1071/WR03096

The purpose of this study was to determine the impacts of age and season on red fox and brush-tailed rock-wallaby in Australia. Feces were used from captive animals to measure amplification rates and genotyping error rates from one day to six months. 1, 2, 3, 4, 5 days, 1, 2, 3 weeks, 1, 3 and 6 months were used as sampling days to determine how age of sample affected genotyping efficacy. The collected samples were placed in paper bags and allowed to dry prior to DNA extraction. Amplification rates decreased significantly over time from one day to three months for both species ($p < 0.0001$), while rates for both genotyping errors error increased significantly over time for both species. The effect of season was highly significant for red fox as evidenced by lower amplification rates and higher genotyping error rates in winter (July, 6.8-14.8^{o}C, 66.6mm rainfall) when compared to summer (December, 13.5-25^{o}C, 50.3 mm rainfall). According to this study, collecting only fresh (less than 1 week old) feces in warm and dry periods is the optimal condition to ensure the fewest genotyping errors and the highest amplification rates. Seasonal differences are most likely due to increased rain fall and moisture which may facilitate in degradation of DNA on the surface of feces than if they remain dry. In contrast, Lucchini et al. (2002) showed that wolf feces collected in winter performed better than those collected in spring and summer because the cold temperatures better preserves the feces and reduced DNA degradation.

Poole KG, Reynolds DM, Mowat G and Paetkau D. 2011. Estimating mountain goat abundance using DNA from fecal pellets. The Journal of Wildlife Management. 75: 1527–1534. DOI: 10.1002/jwmg.184

In this study, the authors completed an aerial survey to determine mountain goat counts, and then collected fecal pellet and hair samples from areas that were safe to access by helicopter. Pellets were collected fresh (<1–2 weeks old) with a mucous sheen, but if recent pellets were not available, old pellets were collected as well. The old pellets were distinguished by dull brown colour, dry texture, mold or vegetation growth, and cracks. Four pellets were collected from each pellet pile, and they were placed in paper coin envelopes. Pellets were dried at 65–70 °C for 6–8 h and sent to Wildlife Genetics International. For DNA extraction, pellets were first covered with Qiagen's ATL digest buffer and agitated for an hour to remove mucous. Out of 490 samples, 16% were not suitable for analysis (e.g., the pellets were crushed or broken apart), 19% were not analyzed (due to subsampling), 31% failed during genetic analysis, but the remaining 35% produced high confidence scores. Out of the pellets collected, fresh (91%), recent (67%), and old (27%) pellets were successfully genotyped. Successful genotyping of samples was correlated with collections during heavy rain periods. Only 1 out of 12 pellet samples collected on snow fields produced a viable genotype. The authors suggest that this is probably due to the high moisture levels encountered on snowfields during summer melting conditions that degraded the DNA or dissolved the mucous coating. The authors note that this DNA-based abundance estimate cost nearly six times more than the aerial survey method, even with volunteers for pellet collection. The DNA-derived estimate was 28% higher than the raw aerial survey estimate and 10% lower than the corrected estimate based on 0.70 sightability. Combining the two methods gives an estimate equivalent to using 0.78 sightability factor with an aerial survey. The authors conclude that this is a reasonable estimate using DNA. In addition, the authors conclude that 14–21 days between sampling sessions was a reasonable period to maximize pellet deposition and genotyping success, while minimizing the risk of repeatedly sampling the same pellet groups.

Rönnegård L, Sand H, Andrén H, Månsson J and Pehrson Å. 2008. Evaluation of four methods used to estimate population density of moose *Alces alces*. Wildlife Biology. 14:358–371. DOI: 10.2981/0909-6396(2008)14[358:EOFMUT]2.0.CO;2

This paper did not include information on DNA methods from moose pellets, but discusses the benefits drawbacks for other types of population estimation methods.

Rutledge LY, Holloway JJ, Patterson BR and White BN. 2009. An improved field method to obtain DNA for individual identification from wolf scat. Journal of Wildlife Management. 73:1430–1435. DOI: 10.2193/2008-492

The aim of this paper was to compare fresh scat swabbed in the field during the summer months to frozen scat collected during the winter and later thawed for DNA analysis. Scat was sampled from gravel roads travelled almost once a day and were categorized as: <12 h, 12–24 h, 24–36 h, and >36 h. Scat estimated to be greater than five days old were not sampled. Sterile cotton-tipped applicators were used to swap the scat sample, they were then transferred into 1.5 mL of DET in 2 mL screw can vials and rotated vigorously to release the mucous from the swab. Samples were then stored at room temperature for approximately 1-2 weeks until analysis — these samples are defined as "field swabbed" (FS). Frozen scat samples collected during winter were stored at -20^{o}C until thawed, swabbed, and placed in a lysis buffer — these samples were defined as "laboratory swabbed" (LS). A significant difference ($p = 0.02$) was observed between FS feces (10 amplified out of 20 samples) and LS samples (3 amplified out of 20 samples) showing that amplification was more likely when fresh scat was swabbed in the field than when frozen samples were swabbed after being thawed. In addition, the results of this study showed that scat less than 36 h old were more likely to yield DNA template that amplifies microsatellites in multiplex reactions (i.e., large fragment nuclear DNA). The author's recommendations include the following: use lysis buffer instead of DET salt solution; don't store samples at room temperature for more than 1 month as the absorption of lysis buffer into swab reduced DNA yields; and scat swabbed in the field has benefits as there is no need to immediately freeze the sample and many samples can be safely stored in a small container and can be handled without protective equipment.

Waits LP and Paetkau D. 2010. Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. The Journal of Wildlife Management. 69:1419–1433. DOI: 10.2193/0022-541X(2005)69[1419:NGSTFW]2.0.CO;2

In this study, the analysis of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) target regions was achieved PCR. The most common method for extracting DNA from herbivore fecal samples was silica-binding extraction kits (Qiagen). The authors suggest silica desiccation, emersion in ethanol, or salt buffers may all be good alternatives for sample collection, but because of species-specific effects, environmental effects, or interactions between storage methods and extraction methods, more study is needed to determine optimal collection methods for each species of interest. The authors state that when testing DNA for individual identification, a researcher must: (1) analyze enough loci to have adequate resolution to distinguish individuals, and (2) minimize genotyping errors. If insufficient loci are used, underestimation of the population abundance will result, but on the other hand, genotyping errors (allelic dropout, false alleles) tend to overestimate population abundance.

Wehausen JD, Ramey RR and Epps CW. 2004. Experiments in DNA extraction and PCR amplification from bighorn sheep feces: The importance of DNA extraction method. Journal of Heredity. 95:503–509. DOI: 10.1093/jhered/esh068

Herbivores pose a challenge for DNA extraction because their diet is rich with plant secondary compounds that interfere with efficient PCR reactions conducted on their fecal material. This paper describes a method using 60 mg of outer mucous layer that was scraped off fecal pellets with a razor blade. The mucous was placed in a 2 mL vial, 1 mm zirconia/silica beads were added as well as 1.6 mL of the initial extraction buffer. The authors used a bead-beater8 to homogenize the material at medium speed for 45 sec followed by full speed for 15 sec (the use of high speed was limited in order to avoid degrading DNA). They then heated the samples for 10 min at 56^{o}C. Subsequently, a Qiagen kit procedure was followed with two alterations: (1) after addition of ethanol, the samples are left for 1 h to maximize DNA precipitation; and (2) the elution buffer was heated to 70^{o}C, allowed to incubate in the spin columns for three min, and the eluate was centrifuged through the spin columns a second time

to maximize DNA collection. The authors found that decreasing the amount of outer mucous scraped from 60 mg to 15 mg showed a slight decrease in PCR success ($p = 0.022$ for 60 vs 30 mg, $p = 0.061$ for 60 vs 15 mg). Where they used 30–60 mg of outer pellet material in combination with bead-beating in the DNA extraction, 99.7% of samples yielded the correct genotypes. The outermost layer of feces are the first to dry, and therefore should experience the least microbial growth, it is therefore important to dry fresh samples as quickly as possible. Fecal pellets from herbivores that live in wetter environments may undergo more DNA degradation if they are not collected fresh and dried immediately. Because scraping the outermost pellet is a time consuming and tedious process, other methods should be implemented (i.e., possibly a wiping or toothpick method).

Zhang BW, Li M, Ma LC and Wei FW. 2006. A widely applicable protocol for DNA isolation from fecal samples. Biochemical Genetics. 44:494–503. DOI: 10.1007/s10528-006-9050-1

This paper introduced a new technique — the QIAamp DNA stool mini kit. This kit was efficient and convenient, but was comparatively expensive and did occasionally fail in samples from herbivorous animals in their study. For the study, the authors collected 585 samples from 23 species. 20g of each sample were stored in 95 % ethanol at room temperature for one day to 23 months. A very complex DNA extraction was described in this paper where different lysis conditions and starches are used in combination with heat, centrifuging, and incubating times. When looking at successful PCR, fresh samples from a zoo (giant panda = 97.1%, Asian elephant = 94.3%) fared much better than old samples collected from the wild (giant panda 60.2%, Asian elephant = 77.5%). For larger samples it was better to store in the feces in ethanol and vortex strongly to remove small debris. This method was advantageous because it uses fecal pellets that have been stored in ethanol (up to 2 years at room temperature), so field sampling is more convenient than using silica beads and DMSO solution. The method also uses whole and crude feces with no special pretreatment. This method did take about 20 min longer than with the Qiagen test kit per sample, but the cost is lower and has a higher PCR success rate for herbivores.

6.6. Annotated bibliography: large animal exclosure fencing

Matt Scheideman, Roy Rae

Introduction

In preparation for our objective to determine whether restoration treatments (RoW and WAT) influence the assemblage and relative abundance of mammal species (herbivores) on the pipeline RoW, we proposed to construct exclosures that would help us to understand the influence of mammals on the vegetation community (including exotic invasive species) within and outside of the corridor. To this end, we conducted a review of both the scientific and grey literature sources to identify potential construction designs as well as the types of data that are typical of mammal exclosures. We could not identify studies that used exclosures within a pipeline RoW, however, the broader literature on the subject provided some guidance on potential methods. Should pipeline construction proceed in the future, this information can be used as a basis for studying the effectiveness vegetation restoration on the RoW.

Gage E and Cooper D. 2009. A study of the effectiveness of a fence design in excluding elk and moose but allowing the movement of other wildlife. Final Research Report, Department of Forest, Rangeland, and Watershed Stewardship, Colorado State University, Fort Collins, CO.

In this study, the fence is approximately 30 acres; it crosses a valley and excludes both moose and elk out of riparian habitat. This study was designed to allow smaller mammals and deer access, but not moose or elk. The fence is 76 inches high, with a 16-inch gap below made of woven wire, designed to allow for additional wire to the top or bottom of the exclosure if too many elk are entering. Gaps of greater than 15 cm are sufficient for deer to enter under fences, supported by this paper and Vercauteren et al. (2006).

Greenwald KR, Petit LJ, and Waite TA. 2008. Indirect effects of a keystone herbivore elevate local animal diversity. The Journal of Wildlife Management. 72:1318–1321. DOI: 10.2193/2007-491

This study for deer done in Cuyahoga Valley National Park. The authors erected twelve 10 × 10 m deer exclosures (height was not given). They concluded that the presence of deer had a positive, indirect, effect on invertebrate species.

Howe HF and Lane D. 2004. Vole-driven succession in experimental wet-prairie restorations. Ecological Applications. 14:1295–1305. DOI: 10.1890/03-5182

In this study, plots were designed to measure succession driven by voles. Plots were 4 × 4 m. 0.6 m trenches were dug around each plot and 1.2 m hardware cloth fences with 1 cm mesh were erected. Each hardware cloth fence was topped with a 40 cm sheet of aluminum flashing bent over the top to exclude climbing rodents. A 2 m poultry netting barrier was erected inside each exclosure to further exclude deer. Finally, the vegetation next to fences was clipped to 0.5 m to prevent access by climbing rodents.

John E and Turkington R. 1995. Herbaceous vegetation in the understory of the boreal forest: Does nutrient supply or snowshoe hare herbivory regulate species composition and abundance? The Journal of Ecology. 83:581–590. DOI: 10.2307/2261626

In this study, plots were 5 × 5 m by 1 m tall galvanized chicken wire with 2.5 cm mesh supported by 2 m long steel T-bars which were firmly stapled to the ground to prevent animals intruding under the fence. The exclosures were successful in excluding snowshoe hares and ground squirrels, but probably not voles. The perimeter around the exclosure was cut to the depth of a spade length to ensure colonial plants were not gaining nutrients from outside of study area. Maintenance work on the exclosure was completed annually in early June.

Kay CE. 2001. Long-term aspen exclosure in the Yellowstone Ecosystem. In: Shepperd WD, Binkley D, Bartos DL, Stohlgren TJ, and Eskew LG. Sustaining aspen in western landscapes: symposium proceedings. Grand Junction, CO. USDA Forest Service, Rocky Mountain Research Station, Fort Collins, CO. pp. 225–240, Proceedings RMRS-P-18

The authors did not set up new experiments, but observed pre-established exclosures in aspen stands in Yellowstone National Park. The stands were highly overgrazed, and so he contrasted the grazing with the exclosure vegetation in order to identify the remedial work necessary to restore the aspen stands. Exclosures were located on ungulate winter ranges and ranged in size from 0.004 ha to approximately 240 ha. The publication does not include information on the height or dimensions of the exclosures.

Kay CE and Bartos DL. 2000. Ungulate herbivory on Utah aspen: Assessment of long-term exclosures. Journal of Range Management. 53:145–153. DOI: 10.2307/4003274

This study used two different exclosure methods. In the first method, an open area was accessible by all ungulates and an inside fence area excluded free range cattle (i.e., it was accessible to moose, deer, and elk that could jump the barbed wire farm fencing). The second method is a three part design: part was open to all ungulates, part was fenced for cattle, and a third part was fenced for all ungulates. There was no information on type of fence used, but one picture showed cattle fencing. The authors use the term “highlining” to describe vegetation browsing by shorter ungulates such as mule deer on lower stems of aspen. This results in a distinct horizontal line of vegetation that they can reach from the ground.

Kielland K and Bryant JP. 1998. Moose herbivory in Taiga: Effects on biogeochemistry and vegetation dynamics in primary succession. Oikos. 82:377–383. DOI: 10.2307/3546979

The authors used the same dimensions as in previous studies published by this group: 50 × 20 × 3 m exclosure with 2.5 cm fence and held up with 5 m posts buried 2 m into the ground. The authors note that the average mass of a single winter fecal drop represents almost the equivalent of an annual 10 m^2 are of litter fall, or the equivalent of 12 m^2 of litter nitrogen. The presence of moose directly modifies the nitrogen input to the soil.

Kielland K, Bryant JP, and Ruess RW. 1997. Moose herbivory and carbon turnover of early successional stands in interior Alaska. Oikos. 80:25–30. DOI: 10.2307/3546512

Nine exclosures measuring 50 × 20 m held up by treated 5 m wooden (10 cm diameter) posts planted 2 m into ground were used in this study. High tensile strength steel wire and 2.5 cm mesh fence wire were used as fencing. The exclosures prevented browsing by moose and hares (but there may have been some grazing by juvenile hares). Control plots were separated by 10 m buffer strip that limited the edge effects of fencing and access trails. Again, the authors note that moose urine and fecal input into the soil represent about 30% of the total nitrogen input in the willow stage of succession. They suggest that herbivory speeds up soil organic matter turnover, and facilitates vegetation change (from willow to alder).

Maron JL, Pearson DE, and Fletcher RJ. 2010. Counterintuitive effects of large-scale predator removal on a midlatitude rodent community. Ecology. 91:3719–3728.

The study objective was to determine the effects of predation on Columbia ground squirrel, deer mice, and meadow vole by excluding ungulates (food competition), weasels, and avian predators. There were 4 study areas, each containing three 100 × 100-m plots separated by at least 100 m. Predator exclosure plots were surrounded by game fencing (Bekaert Industries, Kortrijk, Belgium) topped with two strands of high tension wire (2.6 m). Avian predators were excluded by using overhead parallel strands of 0.025 mm diameter stainless steel wire strung tight and spaced 20 cm apart. The ungulate control fence consisted of 2.4 m tall fence comprised of 10 strands of barbed wire. This allowed for the free movement of predators, with the exception of bears. The control plots had 2 m fence posts to account for raptor perch sites, but no fencing.

The online appendix A2 of this publication further details the exclosure design. Predator exclusion fences were constructed of 1.88 m “Solid Lock” high-tensile game fencing with 7.5 × 20 cm mesh at the bottom graduating to 20 × 20 cm mesh on top, which excludes all mammalian predators, except weasels. In September 2005, the authors retrofitted fences with 180 cm high, 12.7 mm hex-mesh chicken wire, attached to the outside of the game fence. The bottom of this hex-mesh fence had a horizontal flange that extended 20 cm out from the base and was buried and staked 5–10 cm into the ground. The top of the fence was fitted with 20 cm of metal flashing on the outer face that prevented entry but not exit for animals that entered by climbing the fence. Their experiments showed that *P. maniculatus* and *M. montanus* (smaller

than 22 grams in size) readily pass through the mesh. *S. columbianus* can freely disperse by climbing over or digging under the fence from the inside. When *S. columbianus* were active (April–July), the authors checked the perimeter of the predator exclusion plots weekly and filled in the *S. columbianus* tunnels beneath the fences to prevent weasel entry. The snow tracking data and game cameras indicated that predators readily access and hunt on ungulate exclosure plots.

McLaren BE, Taylor S, and Luke H. 2009. How moose select forested habitat in Gros Morne National Park, Newfoundland. Alces. 45:125–135.

This study used 25 × 25 m plots with two stacked spans of cattle fencing for a height of 2.5 m. The fence restricted moose, but not any other smaller herbivores such as snowshoe hare, red squirrel, and meadow voles.

Persson I-L, Danell K, and Bergström R. 2005. Different moose densities and accompanied changes in tree morphology and browse production. Ecological Applications. 15:1296–1305. DOI: 10.1890/04-0499

This experiment was designed to determine plant growth under different "concentrations" of moose activity within each test area. Eight replicates of four treatments were measured. Eight 70 × 70 m, three m high fences excluded moose. Inside each exclosure, they set up four 25 × 25 m treatments where they simulated feeding, defecation by moose. For their study, the 25 × 25 m plots were too small to measure the full effect of their predetermined density at high levels.

Risenhoover KL and Maass SA. 1987. The influence of moose on the composition and structure of Isle Royale forests. Canadian Journal of Forest Research. 17:357–364. DOI: 10.1139/x87-062

The moose exclosure in this study measured 232.3 m^2 and used cedar posts with 3 m high woven wire fencing that permitted free movement of hare. Within each exclosure, a block of 25 plots were established, each plot was 4.05 m^2.

Suominen O, Danell K, and Bergström R. 1999. Moose, trees, and ground-living invertebrates: Indirect interactions in Swedish pine forests. Oikos. 84:215–226. DOI: 10.2307/3546716

In this study, exclosure plots measured 40 × 40 m and were 3 m tall to exclude moose and roe deer, but not hare. The distance between exclosure and reference site was 10–20 m. In another study area, they also used 25 × 25 m plots with 2.8 m high fencing.

Thilenius JF. 1990. Dimensional weights and forage of Barclay willow and sweetgale on moose ranges in the wetlands of the Copper River Delta, Alaska. Forest Ecology and Management. 33-34:463–483. DOI: 10.1016/0378-1127(90)90210-3

Sample sites in this study were 60 × 60 m with a 3.5 m high hog wire fence to exclude moose. To minimize environmental influence of the fence, the sampling done within the exclosure was confined to the inner 40 × 40 m area. There was no evidence to show whether the fence attracted or repelled moose during the study.

6.7. Restoration Protocol: findings and recommendations

There are a number of finding and recommendations for pipeline construction and restoration that arose out of the Restoration Protocol work on small mammals, fecal sampling for large mammals, and for building exclosures.

The diversity of small mammal species that we identified along the proposed RoW (including voles, mice, shrews, rats, and lemmings) suggests that restoration techniques to maintain habitat connectively for small mammals during and after construction of linear landscape features are important. Additionally, RoW construction increases early-seral habitat that may attract herbivores and ecotone species (Child 1998) such as moose, elk, and hares that are clearly present in our baseline sampling. Advancing the restoration of forest structure along the RoW may simultaneously facilitate the movement of native species across the forest gap and decrease the area of disturbed habitat that allows the establishment of invasive species.

In addition, our studies showed that fecal sampling is a feasible method for obtaining information on the distribution and abundance of populations of large mammals. Specifically for moose, pellet samples collected during the late winter or early spring are likely to yield the best information for studies of large herbivores. Furthermore, a review of the current literature suggests that winter will ultimately be the best time of the year to collect moose pellets for several reasons. First, the pellets deposited on the snow should be visible and easily collectible. Second, during winter, moose browse on low-quality food. As a result, their digestion process is longer, the digesting food has more contact time with epithelial cells within the digestive tract, and therefore deposits more DNA on the surface of the pellets when they are excreted. If collected in winter, sampling should be done during cold, stable weather conditions as repeated freeze-thaw cycles degrade DNA. In addition, moose tracks and direction of travel are easier to determine in fresh fallen snow making the collection of fresh pellets much simpler. Should summer sampling be required, different collection techniques should be used. Feces sampled during non-freezing periods should be relatively recent (e.g., using fixed transects) and swabbed in the field. This technique also allows field crews to carry much less equipment during collections. In addition, other studies have shown that pellets collected after a rainfall

had much lower PCR success than the identical pellet sheltered from the rain. For this reason, we recommend that pellets are collected before rainfall events whenever possible. Pellets groups should still be ranked for quality before collection and all uncollected pellets should be removed from the study site to ensure that future collections consider only recently deposited fecal material.

In addition to season, the condition of the pellet and the sampling method employed are also important for optimal results from DNA-based analyses. The condition of the pellet is very important — a visible layer of mucous on the surface of the pellet should be present. Time since defecation is correlated with DNA amplification success; the longer the pellet has been left outside in the elements, the higher the DNA sequencing error rates. Based on the literature review, however, age may be subordinate to pellet condition. For this reason, we recommend the development of standardized field collection procedures to assess fecal sample quality based on the physical appearance (e.g., dried out, little-to-no mucous, crushed, spread out pellet group, mold, etc.). Finally, for optimal DNA yield and analysis, we recommend a sample size of 6 pellets per individual. If collected in winter, pellets should be placed in a sterile plastic bag and kept frozen at -20^{o}C. Once in the laboratory, pellets should be thawed and the mucous containing the epithelial cells wiped or removed using a wooden stick. Care should be taken during this process not to remove any of the internal pellet material because of the high concentration of plant secondary compounds and their inhibitory effect on DNA extraction and amplification.

Our review of methods used by others to test differences between the response of plants inside and outside of herbivore exclosures suggests that larger exclosures are better. Large exclosures allow researchers to sample plants in the interior of the exclosed area that are less influenced by edge effects and disturbances outside the exclosure. All exclosure designs, however, are constrained by costs, and for this project, were also importantly constrained by RoW design, particularly RoW width when considering access for pipeline maintenance.

If construction and restoration of the pipeline RoW had gone ahead, we had planned to build a prototype exclosure (15 m × 25 m of hurricane fencing with mesh sizes for both small and large herbivores, concrete set poles and a 0.5 meter buried apron for approximately

$15,000.00 for supplies and installation). The prototype exclosure would have allowed us to assess its effectiveness for installation on the RoW. We recommend that future researchers start with a similar design and where possible, test and compare plant response inside and outside the exclosure for 2–3 years before fully implementing the Protocol (i.e., replicate exclosures across multiple ecosystems). This will ensure that the design meets their particular research objectives and is conducive to maintenance access within the RoW.

6.8. Literature cited

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Chapter 7 – High Elevation Terrestrial Invertebrates and Lichen Restoration Protocol

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Nimbus Mountain in bloom. Photo credit: Dezene Huber

7.1. Introduction

The flora and fauna of high-elevation habitats are often particularly sensitive to disturbance. The Coast Mountains of BC receive very high levels of snow fall and, with the combination of high elevation and northern latitude, typically have very short growing seasons. Plant species that grow and animals that live at high elevations often are narrowly adapted to this specific habitat and thus potentially very sensitive to disturbance. Due to the magnitude of

disturbance expected to result from the proposed pipeline work, these communities are likely to be locally heavily impacted by blasting and construction. The slow-growing plants and lichen mats, a very short growing and reproductive season for vegetation and animals, and a generally harsh and resource-limited environment that characterize high-elevation communities create unique challenges for restoration. Compounding the challenges for restoration in the high elevation is the small number of studies that have examined high-elevation biodiversity and ecosystem function (i.e., Hooper et al. 2005; Lecerf and Richardson 2009; Dangles et al. 2011), the inherent difficulty in accessing the sites, and the likely changes in substrate material (i.e., to cobble from blasting residuals) along much of the high-elevation portions of the route.

Much of the high-elevation route of the pipeline is covered with lichens and slow-growing alpine plants and, therefore, restoring the vegetation cover on the RoW will be challenging; particular care will be needed not to introduce invasive species. Revegetating the RoW and associated work areas will not necessarily ensure that ecological restoration has been accomplished (e.g., Majer 1983, 1989; Jansen 1997; Bowler 2000; Longcore 2003). Arthropods have been recognized as efficient indicators of ecosystem function and have been recommended for use in restoration assessment (Rosenberg et al. 1986; Kremen et al. 1993; Finnamore 1996). Similarly, the restoration of soil crusts and lichens not only promotes soil conservation, but also supports many biological processes that promote restoration of ecosystem functions (Bowker 2007) including nitrogen fixation.

Arthropods comprise a large proportion of the biodiversity present in any environment and can be valuable indicators of ecosystem health (Hodkinson and Jackson 2005), particularly in alpine ecosystems where the harsh environmental conditions exclude many other groups of animals. Different invertebrate species, within groups such as hexapods (i.e., insects, collembolans) and arachnids (i.e., spiders, mites), each have specific biological, physiological and ecological requirements, making them ideal taxa with which to assess environmental changes resulting from factors such as climatic events or anthropogenic disturbances (Gerlach et al. 2013). Due to the essential ecological services they provide, such as wildlife nutrition and pollination (Losey and Vaughan 2006), and their intermediary position in food web dynamics, they are intricately involved in the overall functioning of ecosystems.

Biological soil crusts are complex communities containing bacteria, cyanobacteria, algae, mosses, liverworts, fungi and lichens (Figure 7-1), which are important contributors to N_2-fixation in alpine and polar environments (Nakatsu and Ohtani 1991, Wojciechowski and Heimbrook 1984; Stewart et al. 2011). Diazotrophic (i.e., nitrogen fixing) bacteria are ubiquitous soil surface micro-organisms in high-elevation environments and they are typically the primary source of newly fixed nitrogen in what are otherwise often nutrient-limited environments (Alexander et al. 1978, Holzman and Haselwandter 1988, Turk and Gartner 2003). Although high-elevation soils contain diverse diazotrophic communities, cyanobacterial species are the major component (Duc et al. 2009), often growing profusely within biological soil crust communities. Cyanobacterial symbioses with lichens are also important in alpine habitats, especially in xeric microsites, providing a major source of newly fixed nitrogen (Alexander et al. 1978, Crittenden and Kershaw 1979, Gunther 1989). As a functional group, soil surface diazotrophs typically colonize early successional and newly disturbed alpine habitats, where they play an important role in facilitating the development of soil properties (Belnap et al. 2001, Schmidt et al. 2008). Not only do soil crusts contribute newly fixed nitrogen to disturbed soils, but complex carbohydrates exuded by biological soil crusts increase soil organic matter content, binding soil particles together, and promote moisture retention in surface horizons (Belnap 2001). Soil crusts may act as keystone communities in establishing primary successional processes and returning disturbed ecosystems to a desirable trajectory (Bowker 2007).

Unlike commercial stabilization products, initial restoration efforts that incorporate the use of soil crusts may offer soil protection, as well as initiate a number of biological processes promoting restoration of ecosystem functions (Bowker 2007; Doudle and Williams 2010). Nitrogen fixing cyanobacteria are common pioneering species during the amelioration and revegetation of degraded ecosystems and have been frequently regarded as biofertilizers and soil conditioners (Rao and Burns 1990; Zimmerman 1993; Acea et al. 2001). Inoculation of soils with cyanobacterial species leads to the formation of organo-mineral aggregates composed of cyanobacterial filaments and extracellular polysaccharides (EPS), where coating, enmeshment, binding and gluing of aggregates and isolated mineral particles significantly improves soil stability (Zimmerman 1993; Neuman et al. 1996; Zulpa de Caire et al. 1997; Acea et al. 2001;

Malam Issa et al. 2001; Malam Issa et al. 2007). In addition, EPS increase soil organic matter content and can be an important source of carbon, helping to ensure microbial growth and survival in soils by their capacity to buffer nutrient supply to microorganisms closely associated with their surfaces (Zulpa de Caire et al. 1997). Biological soil crusts can also change the spatiotemporal pattern of soil moisture and influence re-allocation of moisture by decreasing rainfall infiltration, increasing topsoil water-holding capacity and altering evaporation (Li et al. 2010; Spröte et al. 2010). These changes in hydrologic conditions within the soil are important in controlling floristic and structural changes in vegetation.

Figure 7-1 – A biological crust sampled on Nimbus Mountain. Photo credit: Annie-Claude Letendre.

7.2. Approach and outline of contributions

In this Protocol, we identified terrestrial invertebrate species associated with, and important to, the ecological function of high elevation soil crusts and vegetation, and developed techniques to assist with the restoration of lichen communities and biological soil crusts in the high-elevation ecosystem. Section 7.3 details the baseline biodiversity of alpine invertebrates along the high elevation portions of the proposed pipeline RoW on Nimbus Mountain. A variety of invertebrate sampling methods were tested during the 2014 summer field season to determine the most suitable methods for a field study in a remote region with limited access. Sampling methods (i.e., soil cores, pitfall traps, malaise trap, arachnid aspirations, and sweep netting) were selected to allow collection of a broad a range of taxa in order to determine to most abundant and ecologically important groups present in the study area. Invertebrates were typically identified to the Order taxonomic level, but certain taxonomic groups of interest were identified further to the Family level. DNA barcoding was used to provide greater taxonomic resolution of select representative taxa present in the study area, and revealed relatively high levels of diversity in targeted groups such as spiders, flies and beetles. Because most specimens were identifiable to species using this method, future studies incorporating the use of next-generation sequencing methods would allow very detailed assessments of taxa richness and community composition throughout construction and remediation work.

In addition to the baseline invertebrate biodiversity assessment, we studied restoration techniques for a key component of the high elevation ecosystem, lichen and biocrust communities. Although several studies have examined biocrust development and function in arctic and alpine environments, the potential to use biocrusts in the restoration of disturbed soils in alpine environments requires further study. In a greenhouse trial outlined in Section 7.4, we evaluated the active restoration of biocrust communities through artificial inoculation of alpine soils with mature biocrust fragments. We examined the influence of site preparation (flat compact, microrills, pit and mound, without topsoil) on biocrust recovery. Each treatment received biocrust fragments harvested from alpine sites. This experiment provides key

information regarding the efficacy of using biocrusts for RoW restoration and suggests that creating microtopography may further aid in creating successful restoration conditions.

In a field trial described in Section 7.5, we evaluated the active restoration of biocrust through artificial inoculation of alpine soils on Nimbus Mountain with locally harvested mature biocrust. We examined the influence of fertilizer addition on biocrust establishment and function. This study demonstrates that inoculation and fertilizer addition can successfully accelerate the recovery of biocrusts communities.

7.3. Survey of alpine invertebrates and assessment of their suitability for use as bioindicators of ecosystem function

Ansley Thielman and Dezene Huber

Introduction

Invertebrates are an important component of high-elevation food webs and ecosystems, in which they are common, abundant, and intricately associated with vegetation. They comprise a large proportion of the biodiversity present in any environment and can be valuable indicators of ecosystem health (Hodkinson and Jackson 2005), particularly in alpine ecosystems where harsh environmental conditions exclude many other groups of animals. Different invertebrate species, within groups — such as hexapods (i.e., insects, collembolans) and arachnids (i.e., spiders, mites) — each have specific biological, physiological, and ecological requirements, making them ideal taxa with which to assess environmental changes resulting from factors such as climatic events or anthropogenic disturbances (Gerlach et al. 2013). Due to the essential ecosystem services they provide — such as nutrition for wildlife and pollination (Losey and Vaughan 2006), and their intermediary position in food-web dynamics — they are intricately involved in the overall functioning of ecosystems.

Although the use of aquatic invertebrates as indicators of stream and wetland quality is well known, investigations of the use of terrestrial invertebrates for similar purposes are increasing and recent studies have shown their potential as bioindicators of biodiversity and environmental and ecological change (reviewed in Gerlach et al. 2013; for examples, see Williams 1993; Frouz 1999; Kevan 1999; Kimberling et al. 2001; Pearce et al. 2005; Mgobozi et al. 2008). Due to their pervasive ecological importance to vegetation, and consequential effects on wildlife and other vertebrates, the abundance and diversity of different insect assemblages can be used to assess disturbance and to detect potentially inconspicuous, but important, changes to ecosystem function (Danks 1996). In high-elevation ecosystems, the invertebrate taxa with significant roles in ecosystem function and that are likely to be sensitive to disturbance include: (1) soil-dwellers (e.g., mites, collembolans, dipteran larvae), which are

involved in litter decomposition, soil formation and nutrient cycling; (2) pollinators (e.g., bees, flies), which are required for reproduction of many native vascular plants; and (3) predators (e.g., spiders and ground beetles), the diversity and abundance of which are dependent upon habitat and prey availability and thus intricately linked to plant community composition.

Monitoring changes in invertebrate communities has been an effective tool in assessing and managing disturbance to natural systems (Samways 1993; McGeoch et al. 2011; Guareschi et al. 2012). High-elevation invertebrate assemblages are currently underrepresented in the literature, causing knowledge gaps that prevent management action in these areas. The purpose of this research was to identify and develop a baseline list of key terrestrial macroinvertebrate groups, such as spiders, beetles, flies, and bees, as well as soil microinvertebrates (e.g., mites and collembolans) in a high-elevation, northern Pacific coastal habitat. These groups are associated with the vegetation types targeted for restoration and play important ecological roles such as pollination and maintenance of soil structure through engineering, decomposition, and nutrient cycling. Success of a restoration project often is evaluated on the basis of plant cover only, but recovery of a native invertebrate fauna is also important to achieve conservation goals (Longcore 2003).

The objectives of this project were to establish a baseline measure of invertebrate biodiversity of invertebrate fauna in the high-elevation regions of Nimbus Mountain, assess their potential for use as bioindicators of ecosystem function, and determine the most effective sampling methods to target these taxa in order to develop protocol for monitoring key bioindicator invertebrate taxa throughout the construction of the proposed pipeline and subsequent vegetation remediation.

Methods

Study area

Sampling sites were located on the high-elevation plateau of Nimbus Mountain between km 390 and 394 of the proposed pipeline route. Sampling in this area is restricted to the relatively short snow-free season (June–September) with snow remaining in some areas all year round. Dominant vegetation included *Micranthes tolmiei - Luzula piperi* (Tolmie's Saxifrage -

Piper's wood-rush), a late snow-bed community characterized by few species, and *Cassiope mertensiana - Luetkea pectinata* (White Mountain-heather - Partridge-foot), a community dominated by ericaceous shrubs (BC Conservation Framework 2014; available: http://a100.gov.bc.ca/pub/eswp/). Sampling was conducted primarily in the alpine tundra areas (above the tree line) of the RoW from four broad habitat types including alpine meadow, alpine dwarf shrub (heather), lichen and soil crusts, and talus slopes (Figure 7-2).

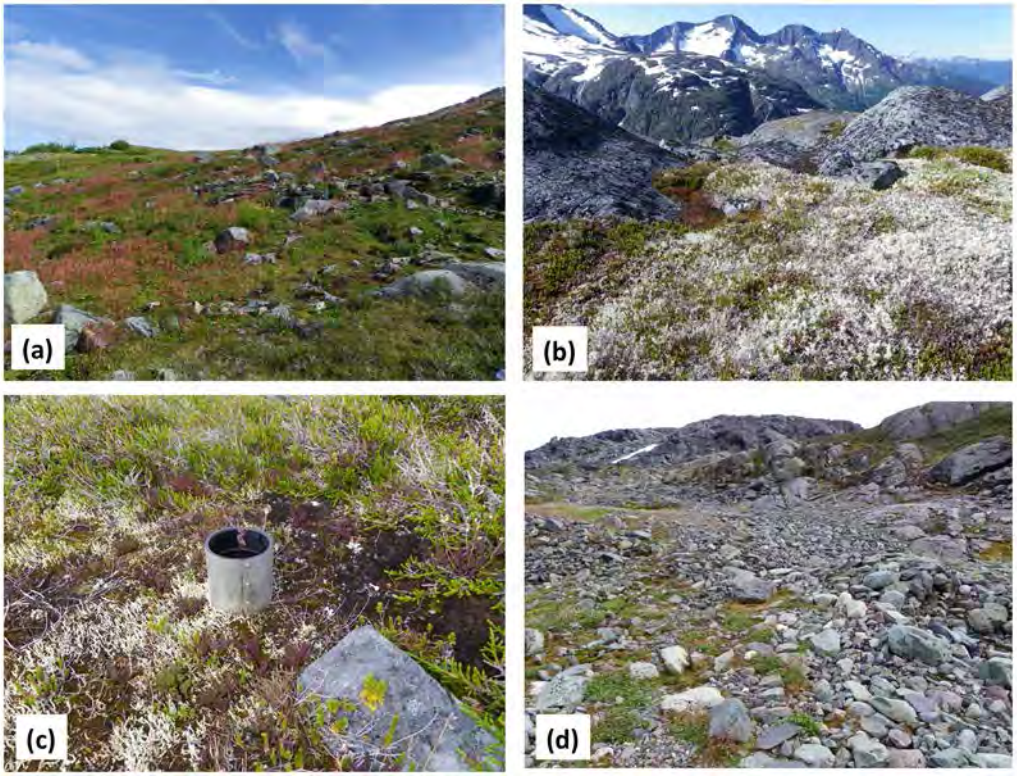

Figure 7-2 – Habitat types sampled for invertebrates at Nimbus in 2014 including (a) alpine meadow, (b) alpine dwarf shrub (heather), (c) lichen and biological soil crusts, and (d) talus slopes.

Sampling sites

Reconnaissance and sampling was conducted at 14 sites on Nimbus during eight days of sampling in 2014 (Figure 7-3). Due to the high degree of patchiness of habitats throughout the study area, all four main habitat types (i.e., alpine meadow, alpine dwarf shrub (heather), lichen and biological soil crusts, and talus slopes) were present in most of the 14 sites sampled.

Sampling information, including dates and GPS coordinates, are provided in Table 7-1. Sites were selected as close to the RoW and planned work areas as possible, but sampling was conducted at lower elevations when cloud cover prevented helicopter access to higher elevations (e.g., NIM1, NIM3 and NIM12).

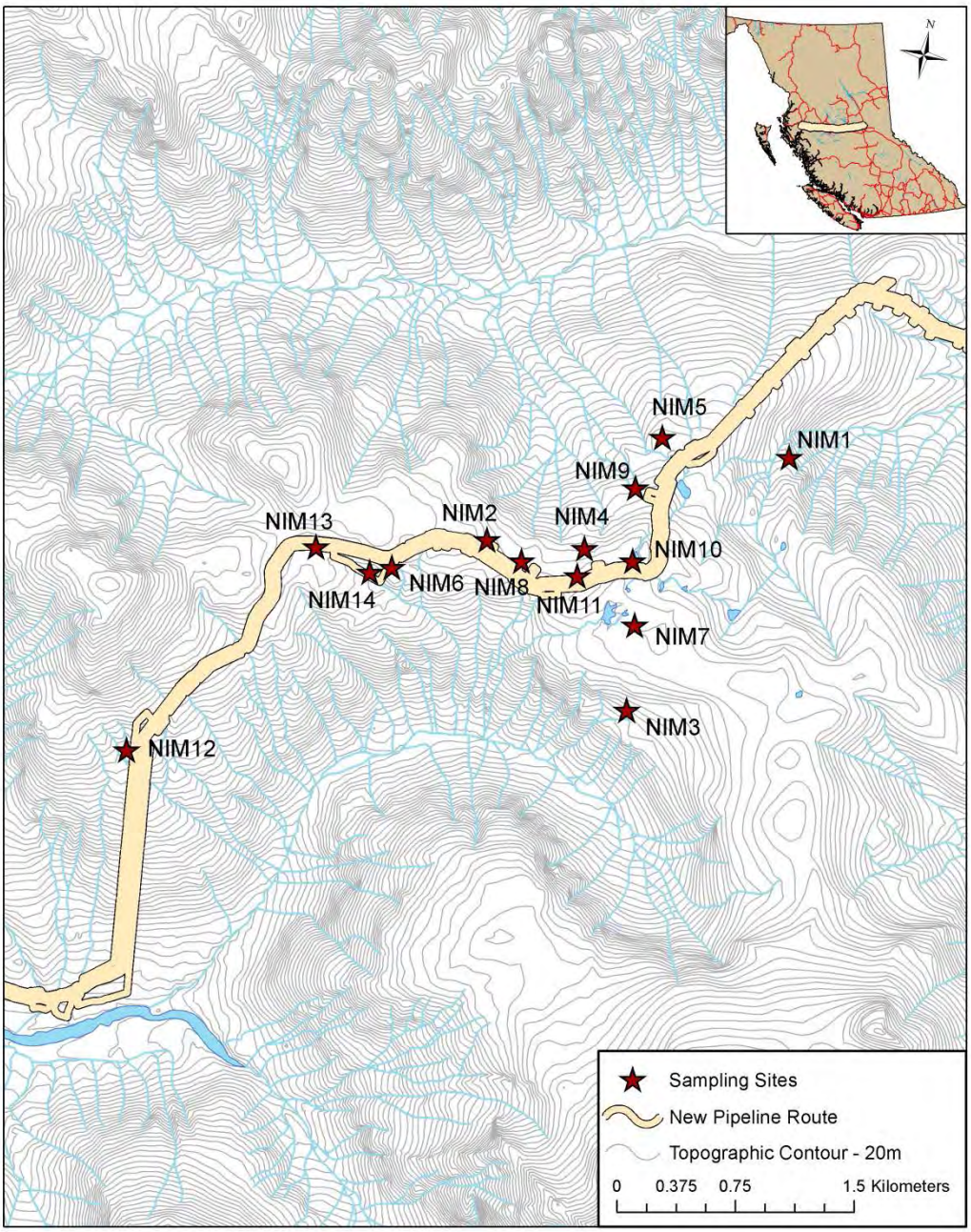

Figure 7-3 – Invertebrate sampling sites on Nimbus in 2014 (NIM1 – NIM14). The pipeline route is designated by the pale yellow line.

Table 7-1 – Site codes, sampling dates, and locations (UTMs all in zone 9U with Easting presented before Northings) of invertebrate sampling sites, including the dominant habitat present at each site. Sites were located at elevations between 1331 m and 1569 m.

Site	UTMs (9U)	Sampling date	Habitat types
NIM1	566191 6010196	22-Jul-14	Dwarf shrub
NIM2	564297 6009595	22-Jul-14	Dwarf shrub
NIM3	565230 6008557	22-Jul-14	Dwarf shrub
NIM4	564918 6009566	23-Jul-14	Lichen and biological soil crust
NIM5	565381 6010287	23-Jul-14	Lichen and biological soil crust
NIM6	563702 6009395	9-Aug-14	Alpine meadow
		19-Aug-14	
NIM7	565258 6009097	8-Sep-14	Dwarf shrub
NIM8	564522 6009471	17-Aug-14	Dwarf shrub
		08-Sep-14	
NIM9	565225 6009961	17-Aug-14	Alpine meadow
		08-Sep-14	
NIM10	565226 6009501	17-Aug-14	Lichen and biological soil crust
		09-Sep-14	
NIM11	564879 6009392	11-Aug-14	Dwarf shrub
		09-Sep-14	
NIM12	562069 6008174	9-Sep-14	Alpine meadow
NIM13	563217 6009505	9-Sep-14	Talus
NIM14	563563 6009360	9-Aug-14	Lichen and biological soil crust

Invertebrate sampling methods

Multiple sampling methods were employed at each main sampling site whenever possible in order to collect as broad a range of invertebrate taxa in order to establish baseline biodiversity in the study area and determine their potential for use as bioindicators(Table 7-2).

Table 7-2 – Invertebrate sampling methods employed on Nimbus, primary taxa collected, associated ecological functions, and habitat types in which they were used.

Method	Dominant Taxa Collected	Ecological Functions	Habitat Types
Soil cores:	Collembola (springtails)	Soil Formation	Lichen and moss mats
	Acari (mites)	Nutrient Cycling	Biological soil crusts
	Diptera (flies) - larvae	Prey Source	Alpine meadow
Pitfall traps:	Araneae (spiders)	Predators	Lichen and moss mats
	Coleoptera (beetles)	Decomposition	Biological soil crusts
	Collembola (springtails)	Nutrient Cycling	Alpine meadow
Sweep netting:	Hymenoptera (bees)	Pollination	Alpine meadow
	Diptera (flies) - adults		Lichen and moss mats
Arachnid aspiration:	Araneae (spiders)	Predators	Talus slopes
	Coleoptera (beetles)		Alpine dwarf shrub

Figure 7-4 – (a) Soil-core sampling in biological soil crust and lichen habitat, and dominant taxa collected including (b) Protura (proturans), (c) Collembola (springtails) (d) Diptera (fly larvae), and (e) Acari (mites).

Soil-core samples were obtained by pushing a sharp metal cylinder (15 cm × 10 cm) lined with a PVC tube as deep into the soil as possible (Figure 7-4a), using a knife to cut through roots and remove rocks if necessary for removal of the core, and stored in plastic containers at 4°C until processing. Soil-cores samples were taken from a variety of microenvironments including alpine meadow, biological soil crusts, and moss and lichen mats to collect soil- and lichen-dwelling invertebrates such as spiders and mites (Arachnida), springtails and proturans (Entognatha), and fly larvae (Diptera) (Figure 7-4b to Figure 7-4e). After completion of the 2014 field season, soil-core samples were removed from the refrigerator (up to 10 at a time), and placed in Berlese funnel traps, which uses heat generated by light bulbs to drive invertebrates present in soil-core samples into a collection jar filled with 95 % ethanol for preservation of the specimens for morphological and DNA barcoding analyses.

Pitfall traps were used in similar microenvironments as those for soil-core sampling, set up in the same holes left after soil-core sampling. Plastic containers containing preservatives (i.e., mineral oil or vegetable oil) were lowered into the ground and covered with metal grid to exclude small mammals, and the pitfall container covered with a white plastic plate to exclude rain-water (Figure 7-5a). Pitfall-trap contents were collected on the next possible sampling date, approximately every 14 days. After returning from the field, preservative fluid was

drained from pitfall samples, the invertebrates rinsed with soapy water, and specimens preserved in 95 % ethanol for long-term storage and subsequent morphological and DNA barcoding analyses. Dominant taxa collected using pitfall traps included primarily predatory taxa such as spiders and beetles, as well as those involved in soil formation and nutrient cycling, as well as a prey source for larger animals, including springtails and mites (Figure 7-5b to Figure 7-5e).

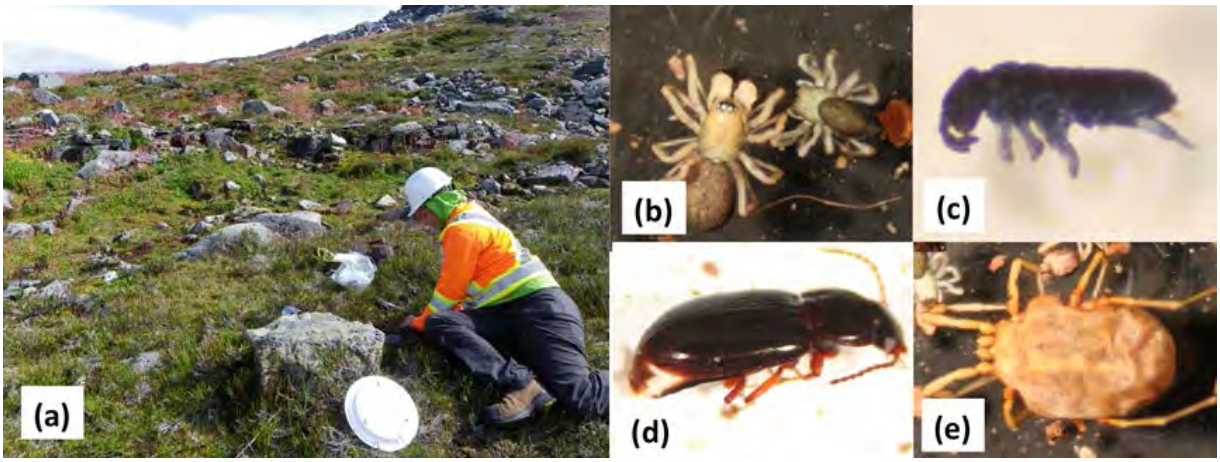

Figure 7-5 (a) Pitfall sampling in alpine meadow habitat, and dominant taxa collected including (b) Arachnida (spiders), (c) Collembola (springtails) (d) Coleoptera (beetles), and (e) Acari (mites).

Arachnids and other predatory groups were collected from talus slopes using an aspirator (Figure 7-6a). Aspirations of arachnids were conducted at each site by lifting rocks and aspirating the invertebrates present below into a temporary storage vials, with two field workers each aspirating for 30-min intervals at each site when possible. Specimens were kept in coolers with chill packs and, upon return from the field, the invertebrates were transferred into vials with labels and 95 % ethanol for preservation and long-term storage. Dominant taxa obtained using the aspirator method included sheet-weaver spiders (Linyphiidae) and wolf spiders (Lycosidae), as well as other spiders, centipedes and beetles (Figure 7-6b to Figure 7-6e).

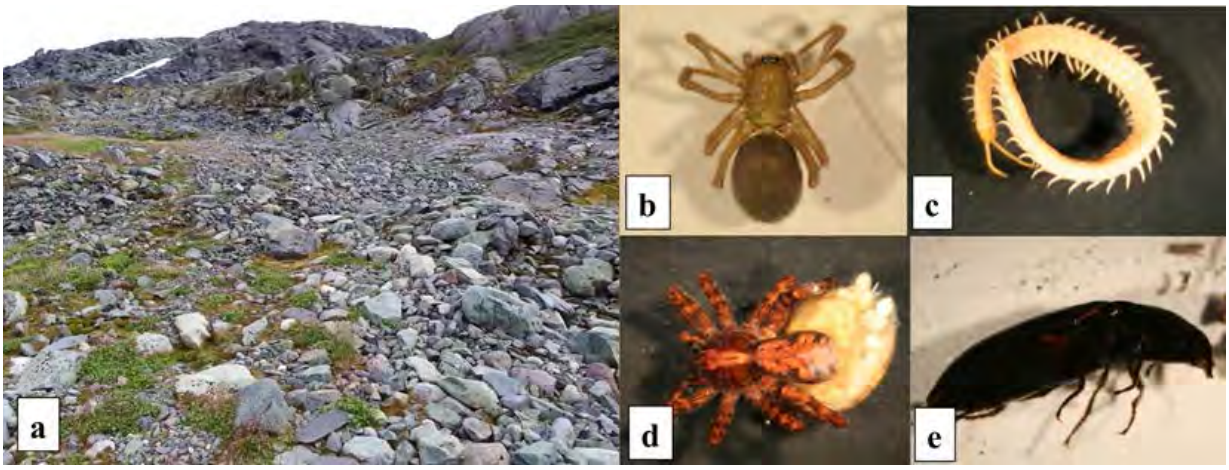

Figure 7-6 – Representative talus slope habitat, with little vegetation, comprised of primarily of cobble and boulder (a), and associated invertebrates; (b) Arachnida (linyphiid spider), (c) Chilopoda (centipede), (d) Arachnida (lycosid spider with egg sac), and (e) Coleoptera (beetle).

The last main sampling method used on Nimbus was sweep netting, conducted mainly in alpine meadow and dwarf shrub habitats (Figure 7-7aSweep netting is an active collecting method used to collect invertebrates associated with vegetation including pollinators (e.g., bees and flies), or those that feed on plants, such as plant bugs (Hemiptera: Coccinellidae) and aphids (Hemiptera: Aphididae). Specimens were collected using either single sweeps of the net (to capture specific insects observed while in the field), or using multiple sweeps (number recorded) through a specific habitat type when sufficiently large patches were available to allow numerous strides through a dominant vegetation type (i.e., dwarf heather and alpine meadow). Sweep methods were used at each site when conditions were sufficiently warm and calm enough for insects to be active. Dominant taxa obtained using this method included bees and wasps (Hymenoptera), adult flies (Diptera), and true bugs (Hemiptera) (Figure 7-7b to Figure 7-7e).

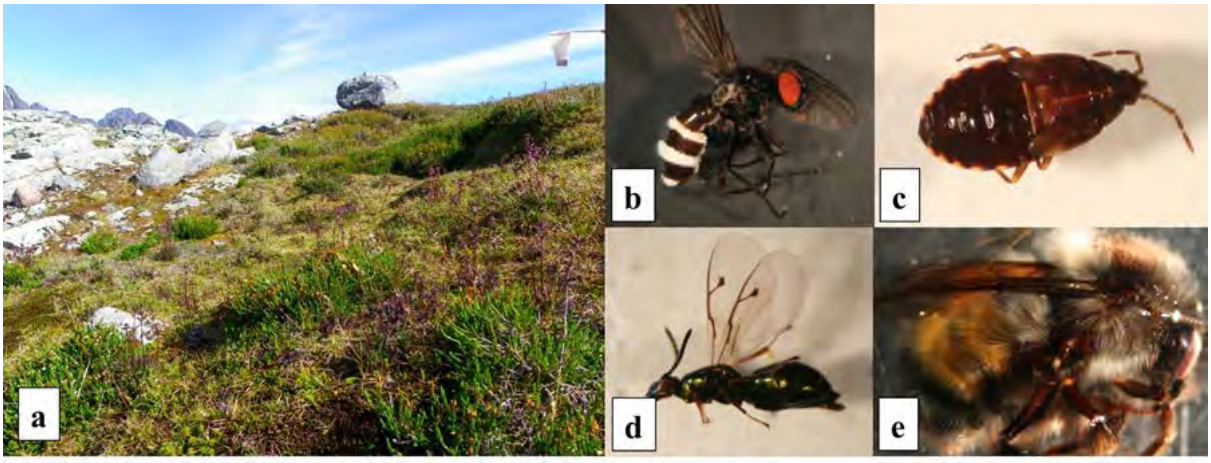

Figure 7-7 – Representative alpine dwarf shrub, comprised of heather and flowering plants (a) and associated invertebrates; (b) flies (Diptera), (c) true bugs (Hemiptera), (d) wasps (Hymenoptera) and (e) bumble bees (Hymenoptera).

Invertebrate identification

Most invertebrate samples were processed in two stages. Specimens within samples were first identified using available taxonomic keys and sorted by Order into 20-mL glass scintillation vials with fresh 95 % ethanol. Sweep-net samples did not undergo an initial sorting step and instead targeted pollinator taxa (i.e., flies and bees) were identified to Family using standard taxonomic keys (McAlpine et al. 1981). Collection and determination labels for specimen vials were printed on Resistall paper, which retains ink when stored in ethanol (BioQuip, California, USA, 1223RB). Once initial processing was complete, invertebrates from multiple sampling types (e.g., spider identifications from arachnid aspiration and pitfall trap samples) were identified to Family when possible using available taxonomic keys (Triplehorn and Johnson 2005; Ubick et al. 2005; Marshall 2007).

A small reference collection of specimens will be maintained at the University of Northern British Columbia, but the majority of specimens will be vouchered and deposited in entomological collections such as the Royal British Columbia Museum in Victoria, BC, the Spencer Entomological Collection at the Beatty Biodiversity Museum in Vancouver, and the Canadian National Collection of Insects in Ottawa. Specimens that were identified using DNA barcoding (see below) are vouchered at the Biodiversity Institute of Ontario.

DNA barcoding representative specimens

During sorting and identification of samples, representative specimens of the range of taxa obtained through this study were selected and identified using DNA barcoding methods to confirm morphological identifications and to obtain Genus- and Species-level identifications when possible. Specimens were sent to the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph in Ontario for DNA barcoding and digital imaging services. Following barcoding, species identifications were assigned by comparing the resulting barcode sequences to those existing annotations in nucleotide databases such as the Barcode of Life Database (BOLD) and GenBank. See Section 2.6 for details on DNA Barcoding methods.

Results

Soil core samples

A total of 2012 invertebrate specimens were obtained from 70 soil-core samples and sorted into one of five main groups: spiders (Arachnida: Araneae), mites (Arachnida: Acari), springtails (Collembola), proturans (Protura) and others (Table 7-3). The most abundant taxa were mites (n = 911), followed by springtails (n = 684). Although soil-core sampling does not target spiders and beetles, both groups were found in low numbers (n = 20 and n = 16, respectively).

Other taxa (Others, n = 341) present in soil-core samples included fly larvae (Diptera), worms (Annelida: Clitellata) and snails (Mollusca: Gastropoda), as well as some specimens whose identity could not determined (Table 7-3). Dipteran larvae were expected to be common in soil-core samples, but were found in very low numbers (n = 16), making them unsuitable for future studies of soil-dwelling invertebrates. Worms and snails were more abundant than dipteran larvae (n = 48), but most of the “Other” specimens were either unknown or not identified further due to time constraints (n = 277).

Pitfall traps

A total of 6045 invertebrate specimens were obtained from 25 pitfall-trap samples collected from Nimbus. Samples were sorted into one of five main groups: spiders (Arachnida: Araneae), beetles (Coleoptera), mites (Arachnida: Acari), springtails (Collembola), and others

Table 7-3 – Results of soil-core samples taken at Nimbus Mountain during 2014, including sampling site, date of collection, number of soil-core samples and of specimens belonging to each main group of invertebrates.

Site	Date Collected	No. Samples	Spiders	Mites	Beetles	Collembola	Protura	Others	Total
NIM1	22-Jul-14	7	1	89	1	18	11	14	**134**
NIM2	22-Jul-14	4	1	265	0	38	1	30	**335**
NIM4	23-Jul-14	8	0	44	0	16	9	69	**138**
NIM5	23-Jul-14	5	0	12	0	26	10	10	**58**
NIM6	9-Aug-14	5	1	37	0	57	0	7	**102**
NIM11	11-Aug-14	5	0	19	1	62	0	29	**111**
NIM8	17-Aug-14	5	0	31	0	17	2	6	**56**
NIM9	17-Aug-14	6	4	84	0	106	7	10	**211**
NIM10	19-Aug-14	5	1	31	1	18	0	4	**55**
NIM7	8-Sep-14	10	2	97	2	235	0	128	**464**
NIM12	9-Sep-14	5	6	48	3	50	0	32	**139**
NIM13	9-Sep-14	5	4	154	8	41	0	2	**209**
	TOTAL	**70**	**20**	**911**	**16**	**684**	**40**	**341**	**2012**

(Table 7-4). All five pitfall-trap samples at the NIM6 site were destroyed by an animal, which, based on the teeth marks, was likely the wolverine (*Gulo gulo*) that had been seen at a nearby site on a subsequent visit. Although not specifically targeted by our sampling, the most abundant taxa were springtails (n = 4319), which comprised 71 % of all invertebrates obtained by pitfall trapping. Pitfall trapping was successful in collecting target taxa (i.e., spiders and beetles) with similar numbers of each group obtained (n = 328 and n = 494, respectively). Also present in pitfall-trap samples were mites (n = 382) and other taxa considered as by-catch. Of the other taxa present in pitfall samples (n = 523), most abundant were adult flies (Diptera; n = 381) and bees or wasps (Hymenoptera; n = 90), comprising 7.8 % of all invertebrates obtained in pitfall-trap samples. Additional taxa were obtained in pitfall-trap samples including members of Orders Diplura and Protura (soil-dwellers and close relatives of springtails), as well as worms (Clitellata), caddisflies (Trichoptera), and snails (Gastropoda).

Table 7-4 – Results of pitfall-trap samples, sorted by collection date, taken at Nimbus during 2014, including sampling site, date of collection, number of days traps were out, number of each main group sorted and total per site and per taxonomic group.

Site	Date Collected	No. Samples	Days Out	Spiders	Beetles	Mites	Springtails	Others	TOTAL
NIM6	19-Aug-14	5	20	0	0	0	0	0	**0**
NIM8	8-Sep-14	5	21	79	112	202	2151	318	**2862**
NIM9	8-Sep-14	5	23	85	147	112	1393	81	**1818**
NIM10	9-Sep-14	5	21	54	186	63	716	77	**1096**
NIM11	8-Sep-14	5	28	110	49	5	59	38	**261**
			Totals:	**328**	**494**	**382**	**4319**	**514**	**6037**

Arachnid aspirations

A total of 239 invertebrates were identified from 10 arachnid-aspiration samples obtained from Nimbus, and were sorted into one of five main groups: spiders (Arachnida: Araneae), mites (Arachnida: Acari), beetles (Coleoptera), springtails (Collembola), and others (Table 7-5). All arachnid aspiration samples were obtained from talus slopes in each main sampling site. Spiders were the taxa targeted by this sampling method, and were the most abundant group obtained in aspiration samples (n = 116), but all invertebrates encountered during arachnid aspiration sampling were collected. Other taxa obtained by aspiration sampling included flies (Diptera), wasps (Hymenoptera), caddisflies (Trichoptera), true bugs (Hemiptera), worms (Clitellata), proturans (Protura), and snails (Gastropoda).

Spider identifications

A total of 407 spiders obtained in pitfall-trap samples (n = 247) and arachnid aspiration samples (n = 160) were identified to the Family level. Many caught in pitfall traps were damaged and unidentifiable (n = 35), and another 32% (n = 130) were in immature stages and identifiable only to Order (Table 7-6). Most abundant in samples were members of the Family Cybaeidae (soft spiders), comprising 38% of all spider specimens identified. Also common in aspiration samples were members of the families Lycosidae (wolf spiders; n = 27), and Pisauridae (nursery web spiders; n = 13), which are larger, ground-dwelling predatory spiders.

Table 7-5 – Results of arachnid-aspiration samples, sorted by date, obtained at Nimbus during 2014, including site code, date of collection, number of each main group sorted and totals per site and per taxonomic group.

Site	Date Collected	Spiders	Egg sacs	Mites	Beetles	Springtails	Diptera	Homoptera	Hemiptera	Hymenoptera	Trichoptera	Lepidoptera	Chiloptera	Castropoda	Clitellata	Unknown	Total
NIM5	2014-07-24	10	5	1	0	0	1	0	0	0	0	0	0	0	0	0	**17**
NIM6	2014-08-09	14	1	2	4	2	1	1	0	0	0	0	0	6	0	1	**32**
NIM11	2014-08-11	7	2	0	1	0	0	1	0	0	0	0	1	4	1	0	**25**
NIM8	2014-08-17	9	0	5	3	4	2	0	0	0	0	0	0	0	0	3	**26**
NIM9	2014-08-17	12	0	0	1	2	3	1	0	0	0	1	0	0	0	0	**24**
NIM10	2014-08-19	4	0	2	2	1	2	0	2	0	0	0	0	0	0	0	**18**
NIM6	2014-08-19	10	0	6	0	5	1	0	0	1	1	0	0	0	0	5	**21**
NIM7	2014-09-08	11	0	3	3	0	0	0	0	0	0	0	0	0	0	0	**8**
NIM12	2014-09-09	20	0	22	5	0	0	1	1	0	0	0	0	0	0	0	**49**
NIM13	2014-09-09	19	0	0	4	3	0	0	0	0	0	0	1	0	0	0	**27**
	TOTAL	**116**	**8**	**41**	**23**	**17**	**10**	**4**	**3**	**1**	**1**	**1**	**2**	**10**	**1**	**9**	**247**

Common in pitfall-trap samples were members of the Family Linyphiidae (sheetweb weavers; n = 15), tiny spiders comprising the second largest spider Family with more than 4,300 species described, and Pisauridae (nursery web spiders; n = 11). Additional spider families obtained in pitfall trap samples in low numbers included Hahniidae (dwarf sheet spiders), Lycosidae (wolf spiders), Metidae (orb-weaving spiders) and Tetragnathidae (long-jawed orb weavers). In aspiration samples, additional families included Linyphiidae (sheetweb weavers) and Hahniidae (dwarf sheet spiders).

Pollinator identifications

A total of 721 invertebrate specimens were identified from 52 sweep-net samples, comprised mainly of adult flies (Diptera) and bees (Hymenoptera) (Table 7-7). Other taxa were collected, but we focused on identifying these pollinating taxa because of their roles in maintaining plant community composition. Most abundant were house flies (Muscidae; n = 377), followed by black flies (Simuliidae; n = 69), mosquitoes (Culicidae; n = 67) and dance flies (Empididae; n = 59). Other fly families collected in lower numbers (n = 9 – 24) included midges (Chironomidae), march flies (Bibionidae), scuttle flies (Phoridae), horse flies (Tabanidae), flower flies (Syrphidae), crane flies (Tipulidae), and fungus gnats (Mycetophilidae). Ten additional

families were identified, which combined comprised only 3% of all invertebrates collected by sweep netting, including watersnipe flies (Athericidae), fruit flies (Tephritidae), root-maggot flies (Anthomyiidae), snipe flies (Rhagionidae), dung flies (Scathophagidae), dung midge (Scatopsidae), dark-winged fungus gnats (Sciaridae), wood gnats (Anisopodidae), Quasimodo flies (Curtonotidae), meniscus midges (Dixidae).

Table 7-6 – Identifications of spiders collected on Nimbus in 2014 by arachnid-aspiration and pitfall-trap methods.

Site	Date collected	Unidentifiable	Immature	Juvenile	Cybaeidae	Hahniidae	Linyphiidae	Lycosidae	Metidae	Pisauridae	Tetragnathidae	Total
NIM11	11-Aug-14	25	1	0	9	0	3	1	1	4	0	**44**
NIM8	8-Sep-14	2	18	0	35	0	3	1	0	3	0	**62**
NIM10	17-Aug-14	0	4	0	9	0	0	0	0	1	0	**14**
NIM9	17-Aug-14	4	10	4	23	0	7	0	0	0	1	**49**
NIM9	17-Aug-14	2	5	0	13	1	2	0	0	1	0	**24**
NIM6	19-Aug-14	0	7	0	18	0	0	0	0	0	0	**25**
NIM10	9-Sep-14	2	4	0	18	2	0	0	0	2	1	**29**
Subtotal Pitfalls		**35**	**49**	**4**	**125**	**3**	**15**	**2**	**1**	**11**	**2**	**247**
NIM5	23-Jul-14	1	1	0	1	0	0	4	0	1	0	**8**
NIM6	9-Aug-14	0	50	0	0	0	0	7	0	3	0	**60**
NIM6	19-Aug-14	0	4	0	1	0	1	5	0	0	0	**11**
NIM7	8-Sep-14	0	0	0	9	0	0	0	0	2	0	**11**
NIM9	17-Aug-14	0	7	0	10	0	0	0	0	3	0	**20**
NIM10	19-Aug-14	0	0	0	3	1	0	0	0	0	0	**4**
NIM11	11-Aug-14	0	1	0	5	0	0	0	0	1	0	**7**
NIM12	9-Sep-14	0	14	0	2	0	0	2	0	3	0	**21**
NIM13	11-Sep-14	0	0	0	0	0	9	9	0	0	0	**18**
Subtotal Aspirations		**1**	**77**	**0**	**31**	**1**	**10**	**27**	**0**	**13**	**0**	**160**
TOTAL		**36**	**126**	**4**	**156**	**4**	**25**	**29**	**1**	**24**	**2**	**407**

Few bees (Hymenoptera: Apidae) were identified from samples collected at Nimbus (n = 8), all but one of which were bumblebees, members of the Genus *Apidae* (Table 7-7). Also collected were adult beetles (Coleoptera; n = 8) belonging to two families, Elateridae (click beetles) and Elmidae (riffle beetles).

DNA barcoding

Representative specimens of invertebrates obtained through this study were analysed using DNA barcoding to confirm morphological identifications and increase taxonomic

Table 7-7 – Identifications of invertebrates collected on Nimbus in 2014 by sweep netting (O = Order, D = Diptera, H = Hymenoptera, C = Coleoptera).

	Site code	NIM1	NIM3	NIM4	NIM5	NIM6	NIM6	NIM8	NIM9	NIM9	NIM11	NIM12	NIM13	
	Date collected	22-Jul-14	22-Jul-14	23-Jul-14	23-Jul-14	9-Aug-14	19-Aug-14	17-Aug-14	8-Sep-14	17-Aug-14	11-Aug-14	9-Sep-14	9-Sep-14	Total
	No. samples	5	1	19	4	3	7	2	1	6	7	1	1	57
Flies (Diptera)	Diptera	0	0	0	0	0	0	0	0	0	0	0	0	0
	Syrphidae	0	0	6	0	1	3	0	0	1	2	0	0	13
	Bibionidae	0	0	0	0	1	15	0	0	0	1	1	3	21
	Phoridae	2	2	1	0	0	10	0	2	1	0	2	0	20
	Muscidae	32	10	35	2	44	80	7	19	78	28	40	2	377
	Tabanidae	0	0	8	0	0	0	1	0	4	4	0	0	17
	Simuliidae	26	0	11	8	1	0	0	15	5	3	0	0	69
	Culicidae	34	0	2	2	8	5	0	0	14	2	0	0	67
	Empididae	7	20	17	0	5	0	0	6	0	4	0	0	59
	Anisopodidae	0	0	0	0	0	0	0	0	0	0	0	0	0
	Chironomidae	0	14	0	0	0	0	0	0	0	0	3	7	24
	Mycetophilidae	0	3	0	0	0	0	0	4	0	0	0	2	9
	Sciaridae	0	0	0	0	0	0	1	0	0	0	0	0	1
	Tipulidae	1	1	3	0	2	1	0	4	0	0	0	0	12
	Scathophagidae	0	0	0	0	0	1	0	0	0	1	0	0	2
	Tephritidae	0	0	0	0	0	0	0	0	0	0	3	0	3
	Scatopsidae	0	0	0	0	0	0	0	0	0	0	2	0	2
	Anthomyiidae	0	0	0	0	1	0	0	0	0	0	1	0	2
	Dixidae	1	0	0	0	0	0	0	0	0	0	0	0	1
	Curtonotidae	0	0	1	0	0	0	0	0	0	0	0	0	1
	Rhagionidae	0	0	0	2	0	0	0	0	0	0	0	0	2
	Athericidae	0	0	0	1	0	0	0	0	3	0	0	0	4
Bees	Apidae	0	0	3	2	0	1	0	0	0	1	0	0	7
	Hymenoptera	0	0	0	0	0	0	0	1	0	0	0	0	1
Others	Coleoptera	0	0	2	0	0	0	0	0	0	0	0	0	2
	Elmidae	0	0	5	0	0	0	0	0	0	0	0	0	5
	Elateridae	0	0	1	0	0	0	0	0	0	0	0	0	1
	Scairidae	0	0	0	0	0	0	0	1	0	0	0	0	1
	Unknown	0	0	0	0	0	5	0	0	0	0	0	0	5
Total		**103**	**50**	**92**	**17**	**64**	**48**	**9**	**158**	**68**	**46**	**52**	**14**	**721**

resolution for taxa present in the study area. cytochrome oxidase I sequence data were successfully obtained for 278 specimens, including 152 ground predators (spiders and beetles), 68 pollinators (flies and bees), and 50 soil-dwellers (springtails and mites) (Table 7-8).

Taxonomic resolution allowed the identification of 48 specimens to Family, 73 to Genus, and 79 to the species level. All spider specimens (n = 111) were identifiable to the species level, whereas seven of the 11 mites sequenced could not be identified beyond class and four beyond Order. The lack of Family, Genus and species level identifications for mites suggests a lack of previous DNA barcoding studies of alpine mites.

Table 7-8 – Number of specimens identified based on COI sequence data (n), and the number of Family, Genus and species level taxonomic assignments per group.

Class	Order	Group	n	Family	Genus	Species
Arachnida	Araneae	Spiders	111	7	20	26
Arachnida	Mesostigmata	Mites	2	2	1	
Arachnida	Sarcoptiformes	Mites	2	1	1	1
Arachnida	Unknown	Mites	7			
Collembola	Entomobryomorpha	Springtails	27	2		
Collembola	Unknown	Springtails	12			
Insecta	Coleoptera	Beetles	41	8	18	21
Insecta	Diptera	Flies	58	19	25	26
Insecta	Hemiptera	True bugs	1	1	1	1
Insecta	Hymenoptera	Bees, wasps	10	4	4	4
Insecta	Orthoptera	Grasshopper	1	1	1	1
Insecta	Psocoptera	Barklice	1	1		
Insecta	Thysanoptera	Thrips	1	1		
Insecta	Trichoptera	Caddisflies	4	1	2	
		TOTAL:	**278**	**48**	**73**	**79**

Of the 58 fly specimens analysed by DNA barcoding, about 26 were identifiable to the species level, 46 to Genus level, and 13 to Family level only. All 10 hymenoptera specimens were identifiable to the Genus level and six to species. Almost all beetles were identifiable to the species level (39 of 41), with the remaining two specimens identifiable only to Family level. In addition to these targeted taxa, COI sequences were obtained for specimens of other insect Orders including caddisflies, true bugs, barklice, thrips, and a grasshopper.

Discussion

Our preliminary survey of the alpine invertebrates present in the high-elevation areas of Nimbus provide baseline information of the dominant taxa found in this study area along the proposed RoW. As expected, the most abundant groups of invertebrate included mites and springtails (soil and ground-dwelling taxa), flies (pollinators), and spiders and beetles (ground

predators). Most samples collected from Nimbus were identified to the Order or Family level. In addition, representative specimens of the taxa present in this study area were identified using DNA barcoding, a molecular method of species identification that provides increased taxonomic resolution, often to the Genus or level, and greater insight into baseline biodiversity in this relatively undisturbed and environmentally sensitive section of the proposed RoW.

Various invertebrate sampling methods were employed on Nimbus, including active methods (soil-core sampling, arachnid aspirations and sweep netting) and passive methods (pitfall traps). Each of the methods used in this study had advantages and disadvantages for the collection of a sufficient number of insects from such a remote area with limited access to sites (i.e., helicopter access only) in order for rigorous statistical analyses to be possible.

Soil-core sampling was a relatively simple method, successfully obtained a large number of specimens, and did not require substantial time in the field. Subsequent extraction of invertebrates from the soil using Berlese funnels, however, required a significant amount of lab time (~10–14 days per sample). Pitfall trapping obtained large numbers of invertebrates with relatively little effort in the field once traps were in place, but the use of various preservatives (e.g., hand sanitizer gel, mineral oil) in place of propylene glycol — containing a bittering agent to reduce loss due to wildlife — resulted in considerable damage to specimens and may reduce the DNA quality for downstream barcoding. Pitfall traps obtained taxa similar to those collected by arachnid aspiration but with far less per unit effort. Arachnid aspiration samples allowed the collection high quality ground-dwelling predators from talus slopes whose ruggedness makes other sampling methods difficult, but required considerable field time and did not obtain large numbers of specimens. Sweep netting allowed the collection of particular taxa from specific habitats or vegetation types, but required significant field time, was difficult to standardize due to high degree of patchiness of vegetation types, often resulted in damage to specimens, and obtained fewer specimens than other methods.

The use of an additional passive trapping method, Malaise trapping to collect flying invertebrates (pollinators such as flies and bees), was planned but not conducted because of uncertainty around return sampling intervals and their conspicuousness. Malaise traps typically collect large numbers of diverse insects and require little effort to maintain throughout the

season once in place (other than replacing trap bottles and monitoring to ensure the trap is not disturbed by wind or animals), and this method should be included in studies of high-elevation sections along the RoW should further invertebrate assessments be needed.

DNA barcoding allowed the identification of many taxa to the Genus or species level, providing far greater insight into baseline biodiversity and likely ecosystem services and functions than was possible, efficiently, based on morphological identifications alone. Unlike aquatic invertebrates, comprehensive taxonomic identification keys for multiple Orders of terrestrial insects are not available and the development of expertise in the identification of such a broad range of taxa much more difficult obtain. In addition to providing greater taxonomic resolution of taxa present in the study area, DNA barcoding revealed errors in morphological identifications of certain groups, particularly spiders, which would require the development of considerable taxonomic expertise before confidence in accurate morphological identifications would be possible.

Because many of the taxa identified using DNA barcoding were present in the Barcode of Life databases (BOLD, http://www.barcodeoflife.org/) and could be identified to the species level assuming correct underlying annotations, the use of next-generation sequencing methods (see Section 2.7), combined with passive trapping that collect large numbers of specimens, would vastly improve the use of invertebrates as bioindicators of ecosystem function and for monitoring changes in soil and water chemistry, as well as changes in vegetation community composition, along the high elevation sections of the RoW.

In summary, this study contributed to establishing baseline invertebrate diversity in the high elevation plateau of Nimbus Mountain many of which could be important in the maintenance of ecological function. The results also contribute information necessary for developing a protocol for the use of alpine invertebrates as bioindicators of ecosystem function, which could be used to monitor and assess changes in taxa richness or composition related to pipeline construction and vegetation restoration. Due to remote nature and limited access to sites, passive trapping methods, such as pitfall and Malaise traps, would be more suitable for future studies. Specimen identification that incorporates next-generation

sequencing technologies would supplement the ability to monitor and assess changes in community composition for such a broad range of taxa.

7.4. Restoration of Ecological Function using Biocrusts: Greenhouse Experiment

Annie-Claude Letendre, Katherine Stewart*, Darwyn Coxson

Introduction

Anthropogenic disturbances, such as trenching, can have lasting effects on alpine plant communities, inducing changes such as a decrease in plant and microbial diversity, native species abundance, soil organic carbon and nitrogen and mineralization rates (Capers and Taylor 2014). Furthermore, characteristics common to most alpine environments, such as nutrient poor soils, cause these ecosystems to have slow recovery rates following disturbance (Jorgenson and Joyce 1994).

The ecological restoration approach seeks to facilitate the recovery of disturbed sites by kick-starting early successional processes, and therefore is a practical tool to ensure restoration success (Burton 1991; Bullock 1998; Bulot et al. 2014). One of the key groups of organisms in these early successional processes is biological soil crusts (biocrusts), communities of organisms forming a thin layer at the uppermost surface of soils. They are typically composed of a mixture of algae, lichens, mosses, liverworts, and cyanobacteria. Biocrusts reach their greatest abundance in extreme environments such as arctic and alpine tundra (Gold et al. 2001; Belnap and Lange 2003; Kuske 2012; Sancho et al. 2014,), where they play an important role in stabilizing and enhancing soil structural properties and facilitating the establishment of soil-nutrient cycling (Belnap et al. 2008; Bowker et al. 2008; Maestre et al. 2011).

Biocrusts play a key role in supporting fundamental processes such as nitrogen and carbon cycling and are important for their ability to stabilize the soil surface environment through production of extracellular polysaccharides (Mager and Thomas 2011), which also has a strong influence on hydrological processes (Kindron 2001). Recovery of biocrusts can be significantly accelerated by active restoration methods (e.g., Belnap 2008; Xu et al. 2008; Liu et al. 2012). Restoration methods vary widely and optimal methodologies for restoring biocrusts

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have yet to be developed. Furthermore, to our knowledge, no studies have focused on facilitating restoration of biocrusts in alpine environments.

An important driver of success in restoration is site-preparation techniques that create variation in topography or microtopography. Spatial heterogeneity increases the number of niches in an ecosystem thereby enriching species diversity (Tilman 1993; Baer et al. 2004). Alpine environments are highly variable and provide a variety of niches for diverse plant communities. In particular, topography is a major driver of variability in alpine environments and is also known to be an important component of successful ecological restoration.

In this study, we conducted a biocrust inoculation experiment under controlled greenhouse conditions, investigated the effects of microtopography and biocrust inoculation on the recovery of biocrust function, expressed by soil surface area covered by biocrusts, by the development of soil surface photosynthetic activity (chlorophyll fluorescence) and nitrogen fixation activity (acetylene reduction assay) as well as other soil properties.

Methods

Greenhouse conditions

One of the goals of the greenhouse experiment was to develop a proof-of-concept for field application at our study sites located on Trapper and Nimbus Mountains, BC. The Trapper Mountain sampling site was located on a subalpine ridge characterized by scattered *Abies lasiocarpa* clumps interspersed with *Cassiope mertensiana* heath (9U 535260 6040565, 1187 m elevation). The Nimbus Mountain site was located on the southeast flank of Andesite peak on an east-west trending ridge top. The substrate was volcanic in origin (meta-basalts and meta-andesite) with alpine tundra habitat on slight north facing aspect; the site had 1-3 m microsite relief with a mosaic of small rocky outcrops and wet late-snowmelt bed depressions. The vegetation on outcrops was dominated by *Cassiope mertensiana* and *Stereocaulon alpinum* heath with abundant *Saxifraga tolmiei* and *Ranunculus cooleyae* in wet depressions (9U 564044 6008321 , 1640 m elevation). Greenhouse conditions were maintained at typical summer conditions (16-h photoperiod at 150 µmol × m^{-2} × sec^{-1} during light hours; 20 °C daytime

temperature, 10 °C nighttime temperature). The experiment ran for 12 weeks, which corresponds with the approximate snow free period on Trapper Mountain.

Experimental Design

The experiment examined biocrust development under four site-preparation treatments: 1) soil with flat, slightly compacted surfaces to emulate no site pretreatment; 2) soil with flat surfaces and repeating microridges across the surface (gullies separated by ridges 1 cm in height and width — simulating raking); 3) pit and mound, resulting in a single hummock-hollow complex; and 4) coarse gravel without soil to emulate an absence of site preparation or top soil (Figure 7-8).

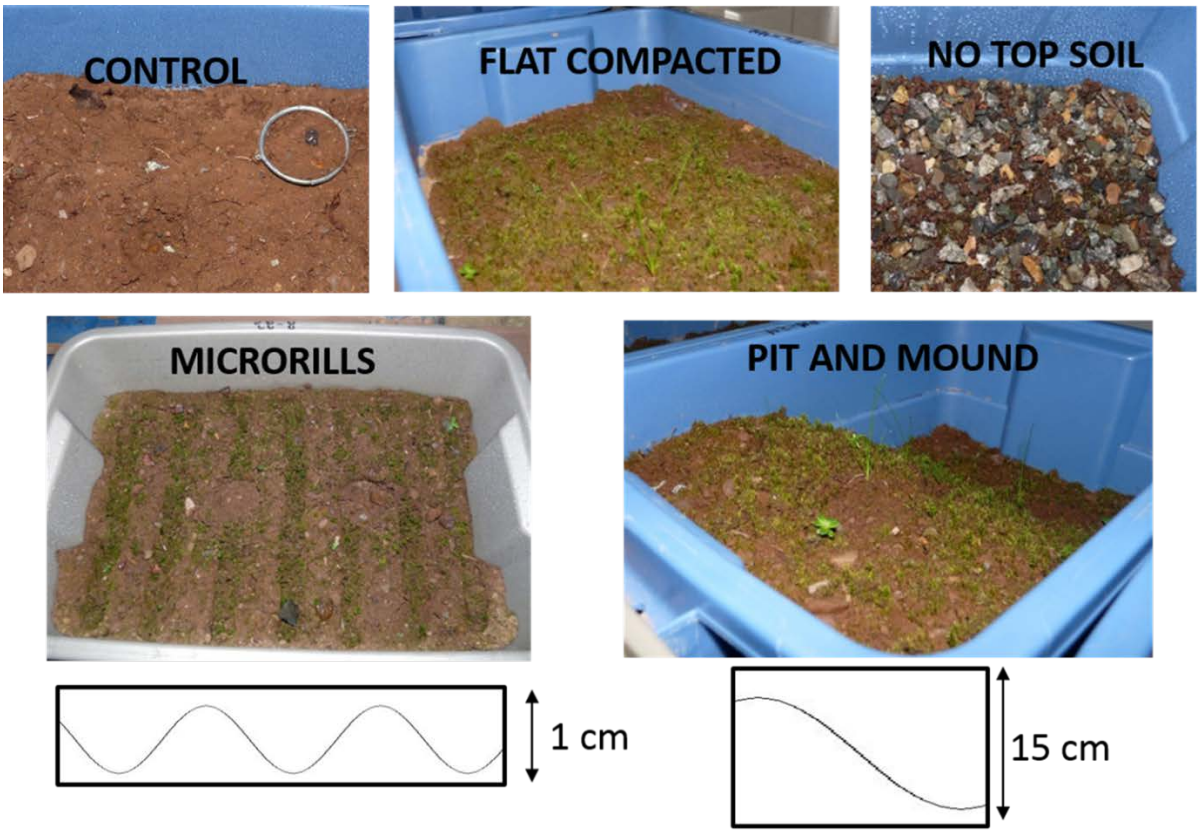

Figure 7-8 – The greenhouse experiment design included four microtopographical treatments: flat, pit and mound, microrills, and gravel with no top soil, as well as, a control.

Each site preparation treatment was replicated 10 times and each replicate consisted of a shallow plastic box of 760 cm^2 (0.076m^2 surface area) filled with 3 cm of crushed gravel and

topped with 2 cm of soil. Soil used in the experiment was sampled on August 16, 2014 at the Trapper mountain site. The top 2–10 cm of the soil surface was taken from areas with little vegetation, after being lightly scrapped (top 1–2 cm) with a shovel to remove any vegetation present. Site preparation was then be applied to each replicate (n = 36).

Each tray was wetted to field capacity and inoculated with the equivalent of 10% surface area of biocrust fragments (i.e., 0.0076 m^2) created by homogenizing a mixture of mature biocrusts fragments homogenized through a 4-mm sieve (Belnap et al. 2008, Maestre et al. 2008). Because the collected soils likely have free-living cyanobacteria, we included a control treatment (n = 9) that did not receive any inoculation with the biocrust. After inoculation with crusts fragments, each plot was watered with deionized water to ensure fragment contact with the underlying substrate. Each replicate was watered three times per week (Monday, Wednesday and Friday) with 150 mL of deionized water. Clear plastic lids were placed on the boxes to maintain relative humidity at approximately 70 %. Gravimetric soil surface moisture content (top three cm) was recorded weekly throughout the experiment.

Biocrust function

At six weeks and at 12 weeks after inoculation, two 14.5-cm^2 randomly selected samples were taken from each replicate. In the pit-and-mound treatment, three sets of two samples were taken on: top of the mound, midslope, and pit. For each sample, we determined chlorophyll fluorescence, a measure of photosynthesis, which was measured with a Hansatech FMS2 Field Fluorescence Monitoring System (Maxwell and Johnson 2000 in Ladron et al. 2004; Sancho et al. 2014) and acetylene reduction, a proxy of nitrogenase activity using acetylene reduction assays (ARAs) after Stewart et al. (1967) on a portable gas chromatograph (SRI 8610A, Wennick Scientific Corporation, Ottawa, ON) fitted with a Porapak column (Alltech Canada, Guelph, ON) and a flame ionization detector. Hydrogen, used as the carrier gas, was held at a constant pressure of 32 psi while column temperature was held at 65 °C. The chlorophyll fluorescence measurement was taken within one h of sampling and samples were then immediately contained and injected with acetylene at 10 % volume. Containers were then incubated in a growth chamber (Conviron Adaptis CMP6010, Winnipeg, Manitoba) under summer growing conditions (150 µmol × m^{-2} × sec^{-1}; 20 °C). After these measurements, one

sample was frozen at -20 °C for subsequent quantitative polymerase chain reaction (qPCR) analysis and one for exopolysaccharide (EPS) content.

The EPS content was determined using the phenol-sulfuric acid method (Crayton 1987; Li et al. 2009) and quantified with a microplate reader (Varioskan, Thermo Fisher Scientific, Walthman, MA). A 0.25 g sub-sample was used for DNA extraction. DNA extractions were carried out using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) and following the manufacturer's instructions. Extracted DNA was stored at -20 °C. Each 12.5 µL reaction was carried out in triplicate (samples, standards, and no template controls) and contained 6.25 µL Power Syber Green Mix (Applied Biosystems, Carlsbad, CA), 0.25 µL *nif*HF primer (0.2µM) and 0.25 µL *nif*HR (0.2 µM) (Life Technologies, Carlsbad, CA) (sequences as per Rosch et al. 2002), as well as 3.75 µL of deionized water and 2 µL of standards, nuclease-free water (no template control) or DNA extract from samples (diluted 1 µL to 100 µL). For qPCR, following the *nif*H quantitative PCR method from Meyer et al. (2013), a 7300 Real Time PCR machine (Applied Biosystems, Germany) was used under the following thermal cycling conditions: hot start (95 °C for 10 min); amplification (95 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 45 sec) for 40 repetitions; dissociation (95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec). *nif*H gene copies were quantified against a standard calibration curve obtained by serial dilution ranging from 10^2 to 10^8 *nif*H gene copies.

Biocrust establishment

Percent cover was visually estimated for each replicate at six weeks and 12 weeks. To ensure accuracy, all assessments were conducted by the same observer and with frames representing 1 % and 10 % surface area.

Soil properties

Available nitrogen was measured through determination of potentially mineralizable nitrogen using the anaerobic incubation method (Waring and Bremner, 1964) and subsequently analyzed with a discrete analyzer (SmartChem 200, Unity Scientific, Brookfield, CT). Dissolved organic carbon was measured from a soil extract (1:10 soil to water ratio shaken for one h on a reciprocating shaker), filtered to 0.45 µm, with a total organic carbon analyzer (Formacs HT, Skalar Analytical B.V., Breda, Netherlands). Dissolved organic carbon and potentially

mineralizable nitrogen were measured for the soil prior to inoculation (n = 2) and at 12 weeks for each replicate, with the exception of the gravel only treatment.

Data Analysis

Comparisons were done using ANOVA with Tukey post-hoc test and Kruskal-Wallis Rank Sum Test for the chlorophyll fluorescence data with a Mann Whitney post hoc test. All statistical analyses were conducted in R (R Core Team, 2014).

Results

Biocrust function

Comparisons of acetylene reduction between treatments revealed that the microrill treatment had significantly higher rates of nitrogen fixation than were seen in the control treatment and no top soil treatment (ANOVA, Tukey HSD posthoc, $p < 0.001$ and $p = 0.3$, respectively) at 12 weeks after inoculation (Figure 7-9).

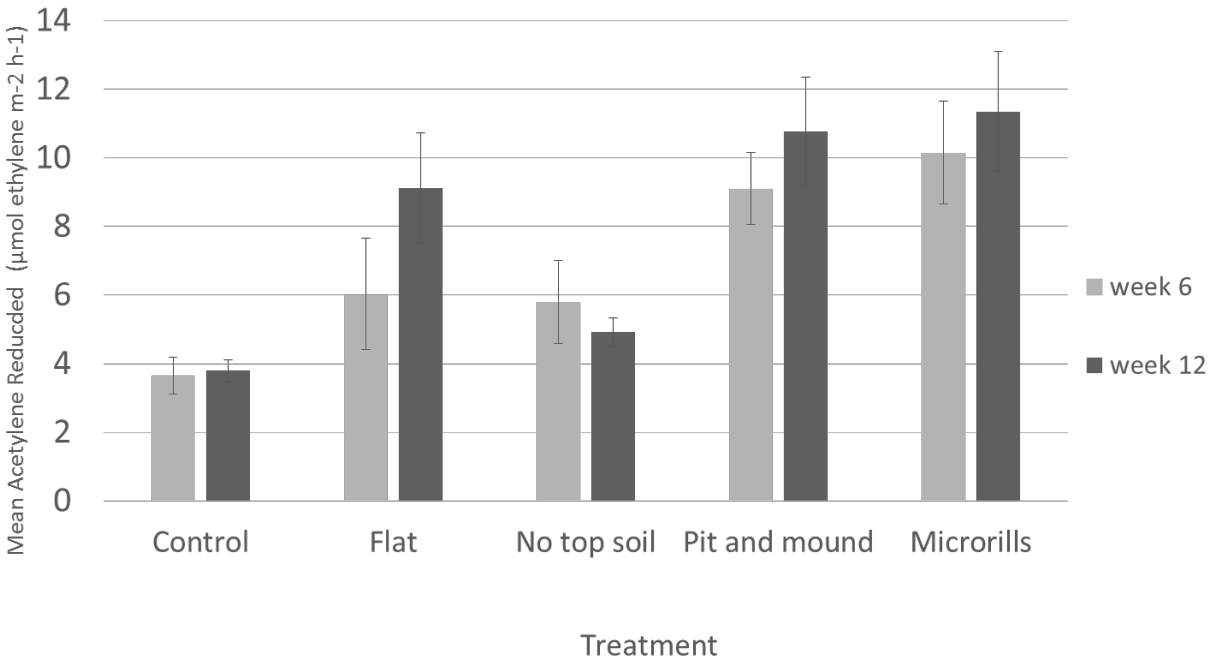

Figure 7-9 – Nitrogenase activity (± 1 SE) detected via Acetylene Reduction Assays measured 12 weeks following inoculation of different site preparation treatments. Microrills had the highest rates of acetylene reduced and were significantly higher than both the control and no top soil treatments.

*nif*H gene copy number (i.e., gene associated with nitrogen fixation) was not significantly different between treatments. Higher *nif*H gene copy numbers, however, were detected in the treatments receiving biocrust. The inoculant had a significantly more *nif*H (copies per g of soil) than the control and all treatments (ANOVA, all $p < 0.04$) (Figure 7-10).

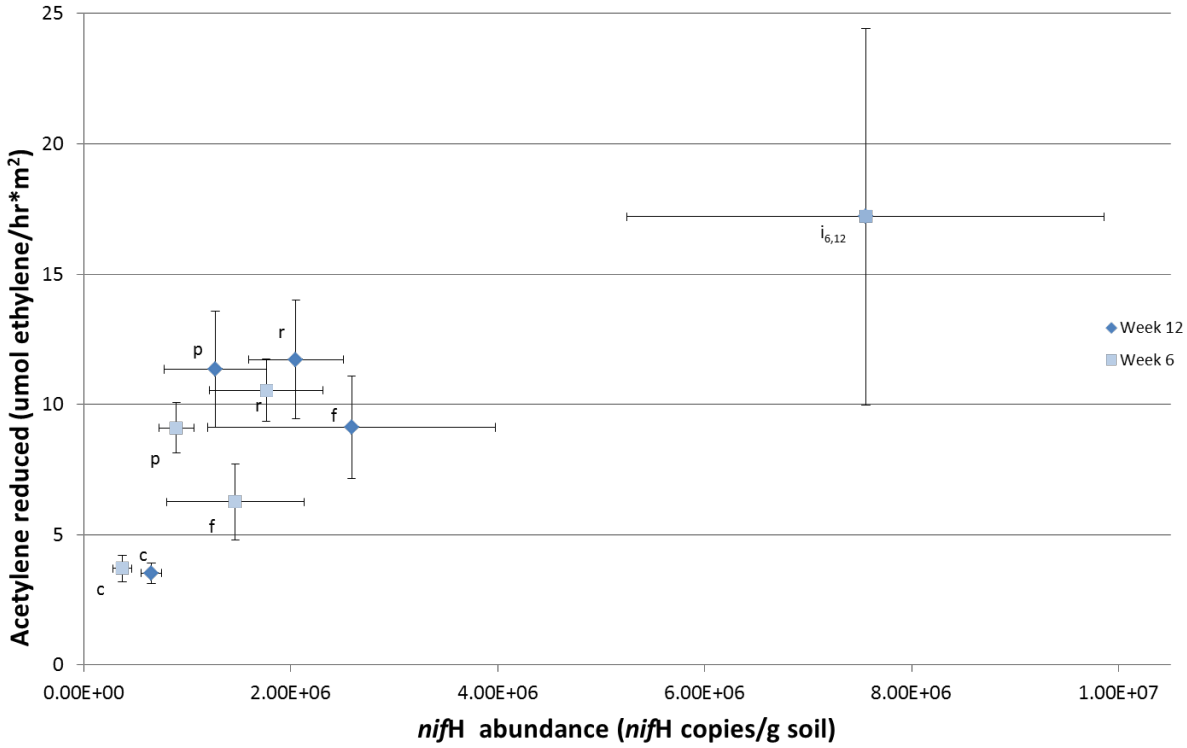

Figure 7-10 – Mean number of *nif*H copies per gram of soil and acetylene reduction (± 1 SE) for the biocrust inoculant (i), the control (c), and the three treatments: flat (f), pit and mound (p), and microrills (r) six weeks (light blue) and 12 weeks (dark blue) following inoculation in a greenhouse experiment.

Extracellular polysaccharide content (measured as $\mu g \times g^{-1}$ glucose) was significantly higher in the microrills treatment than in the control at six weeks ($p < 0.03$) (Figure 7-11). Despite a lack of other significant differences between treatments, we observed a higher EPS content in microrills at 12 weeks. Furthermore, EPS content was significantly higher at 12 weeks than at six weeks (ANOVA, $p < 0.01$).

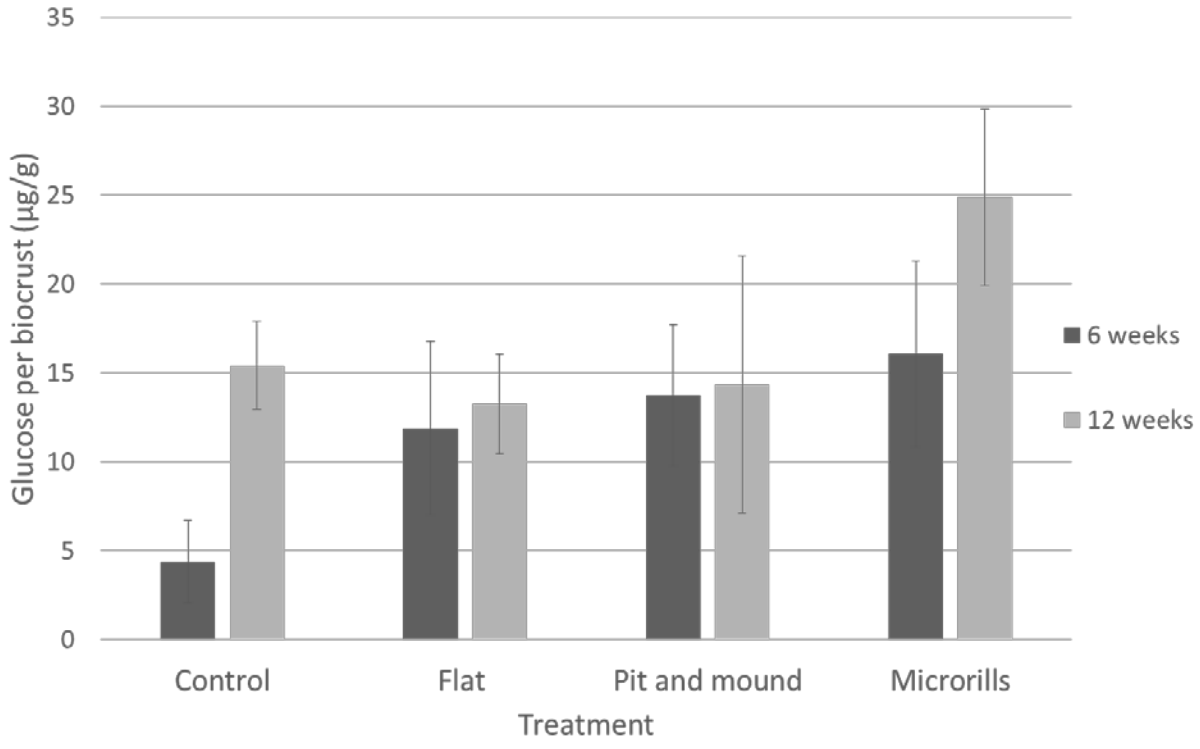

Figure 7-11 – Extracellular Polysaccharide (EPS) content of biocrust or soil surface measured as glucose per biocrust (± 1 SE) at six weeks (light bars) and 12 weeks (dark bars) following inoculation in a greenhouse experiment.

Twelve weeks after inoculation, chlorophyll fluorescence was significantly higher in the flat, pit and mound, and microrills treatments than in the no top soil treatment and control (Kruskal-Wallis, $p < 0.04$).

Establishment

Biocrust established on all treatments with top soil which received inoculant (i.e., flat, microrill and pit and mound). Furthermore, the percent cover of these same treatments increased between six and 12 weeks from inoculation. The highest biocrust cover was observed in the flat compacted and microrills treatments on week 12 (52% for both) (Figure 7-12).

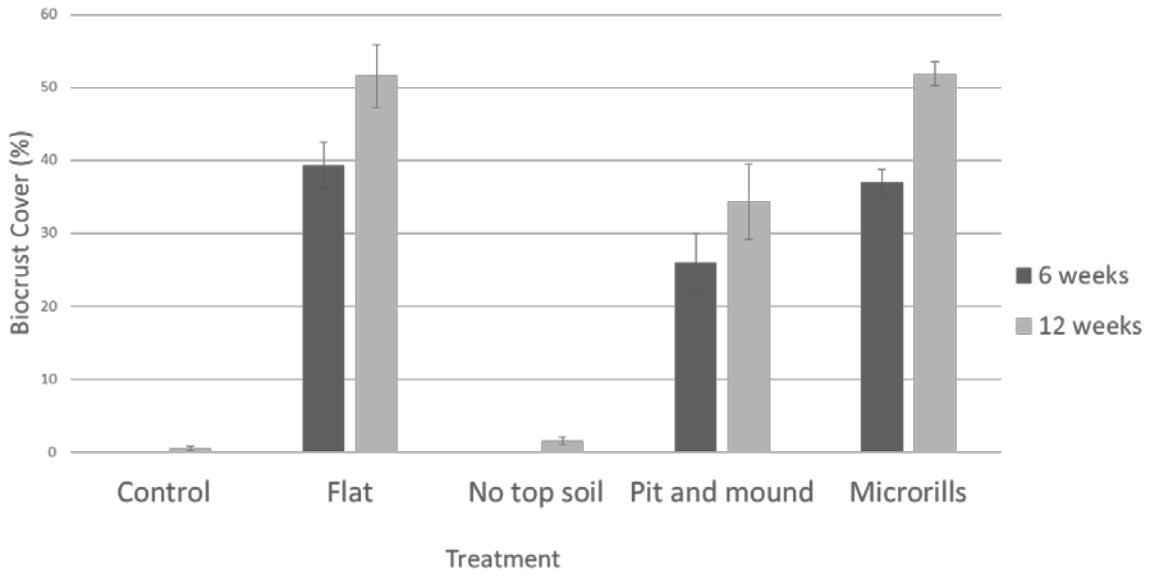

Figure 7-12 – Biocrust percent cover measured 6 weeks (light bars) and 12 weeks (dark bars) following inoculation of different site preparation treatments.

Soil properties

Mineralizable nitrogen measured in soil samples taken directly below biocrusts was found to be similar between treatments; however, higher mineralizable N was detected in the treatments receiving inoculation (Table 7-9). We found no significant differences in dissolved organic carbon.

Table 7-9 – Properties of soils underlying experimental plots 12 weeks following inoculation. Dissolved organic carbon (DOC) and potentially mineralizable nitrogen (Min N) values ± 1 SE.

	Control	Flat	Pit and Mound	Microrills
DOC (ppm)	154.2 ±12.1	118.5 ±21.1	133.4 ±12.2	162.6 ±21.3
Min N (ppm)	10.8 ±0.5	10.9 ±0.5	12.1 ±0.7	11.7 ±0.4

Discussion

Our results suggest that biocrust establishment was enhanced by site preparation. Unexpectedly, we observed higher biocrust cover in both the flat and microrill treatments. The similar biocrust cover between treatments may reflect the lack of compaction in the flat treatment, at least compared to the degree of compaction often seen in disturbed field sites. This can also explain the lack of differences between the flat treatment and other treatments in other measured variables (e.g., nitrogenase activity). Microtopography is generally regarded as a factor enhancing rehabilitation (e.g., Bowker et al. 2010). In the microrills treatment, we observed higher biocrusts colonization in the gullies than in the ridges. This shows that moisture is an important factor in controlling biocrust development. Similarly, Belnap (2001) found moisture to have a significant effect on biocrust nitrogenase activity. Microrills could thereby represent a practical restoration tool to facilitate biocrust establishment and function.

Given the importance of moisture for biocrust community growth and development, it is important to recognize that any deviation from field moisture levels may induce community changes. Li et al. (2009) found that increased soil moisture resulted in increased moss and lichen growth prompting important shifts in community structure and potentially functions. Although effort were made to replicate field conditions, the greenhouse conditions of this study were most likely poor representative of dry site conditions and may have caused some community shifts. Regardless, inoculation can undoubtedly accelerate the recovery of biocrusts communities.

Our results suggest that artificial inoculation with biocrusts increases soil surface nitrogen- fixation rates. Similarly, others (e.g., Belnap et al. 1993; Liu et al. 2002; Hawkes et al. 2004) found that artificial inoculation of soils with biocrust was an effective way of increasing nitrogen fixation. Although the microrills site preparation technique was the most effective at promoting restoration of nitrogenase activity, there was no significant difference in *nif*H copy numbers when compared with the other site preparation techniques we used. This lack of differentiation could be attributed to the high variability observed in *nif*H copy numbers. In addition, qPCR does not distinguish between viable and non-viable genes which may have obscured differences in viable gene copy numbers. Furthermore, expression of a gene does not

unequivocally imply that the enzyme is active (Zehr et al. 1993; Stewart et al. 2011). Nonetheless, despite these limitations, our results demonstrate that both *nif*H copy numbers and nitrogenase activity increased over time, suggesting that biocrust inoculation is effective at restoring nitrogen-fixation capability.

Although differences in the amount of extracellular polysaccharide extracts between treatments at 12 weeks were not statistically significant, our results show that the microrills treatment had higher EPS content. Furthermore, we observed that the difference between week six and week 12 EPS content was high in both microrill and control treatments. Although this result may be an artifact of variability in the data set, the microrill treatment not only had higher EPS but also higher fixation which likely indicates biocrust recovery following inoculation. In addition, the lack of differentiation between treatments could also be influenced by the relatively short duration of our study.

The short duration of our study could also explain the apparent homogeneity between treatment for both the dissolved organic carbon and the mineralizable nitrogen levels. However, since we observed higher photosynthetic activity as well as higher nitrogen-fixation rates in the microrills, we suggest that after a longer period these additional carbon and nitrogen inputs could be reflected in the soil properties.

Conclusions

Given the key role biocrusts play in ecosystem recovery from disturbance by reducing erosion, providing nitrogen inputs, as well as, promoting early succession processes, their reestablishment is a practical tool to ensure essential nutrient input into disturbed environments. This study provides insight into ways by which microtopography can create successful restoration conditions hence facilitating biocrust establishment. Our data suggests that salvaging biocrust to later reapply onto a disturbed site as an inoculant may be an effective means of restoring ecosystem function and ultimately leading to successful restoration practices in sensitive alpine environments.

7.5. Restoration of Ecological Function with Biocrusts: Field Experiment

Annie-Claude Letendre, Katherine Stewart*, Darwyn Coxson

Introduction

Anthropogenic disturbances, such as linear corridors, can have a profound impact on alpine vegetation and soils (Bayramov et al. 2012; Desserud and Naeth 2013; Bulot et al. 2014). Alpine environments can have particularly slow rates of recovery following a disturbance (Jorgenson and Joyce 1994) and restoration success can be challenging due to harsh environmental conditions such as short and cool growing seasons. We can, however, assume that the native, naturally occurring species, from the pre-disturbance ecosystem, are well adapted to local site conditions and therefore are appropriate to restore natural succession processes (Clewell et al. 2005).

Biocrusts are well adapted to restoring alpine sites because they readily colonize disturbed soils. Areas not occupied by vascular plants are particularly well suited to biocrust growth because they provide maximum sunlight and precipitations. Inoculation of soils with biocrusts can speed up restoration processes notably by increasing available nitrogen (Hawkes 2004), a nutrient that commonly limits plant productivity in alpine environments (Schmidt et al. 2012). Furthermore, in nutrient-limited soils, fertilizer addition may increase growth rates (Xu et al. 2008; Chen et al. 2011).

In addition to fertilization of disturbed sites, site-preparation techniques that create variation in topography can be an important driver of successful ecological restoration. We found that microrill microtopography was a site preparation technique that was effective at increasing biocrust nitrogen-fixation, as well as, creating wet-hollows heavily colonized by biocrust (see Section 7.4). Furthermore, the procedure to create microrills is easily implementable in the field; by simple raking of the soil surface.

In this study, we applied microrill microtopography to a field experiment in which we investigated the effects of fertilizer addition on the active restoration of disturbed biocrust

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communities through artificial inoculation. In addition, we evaluated the recovery of nitrogenase activity in comparison to undisturbed mature crust.

Methods

Site description

On Nimbus Mountain (9U 563555 6009633), at an elevation of approximately 1645 m, four treatments were applied to artificially disturbed plots following a randomized block design to account for the moisture gradient, with nine blocks. The four treatments were biocrust with fertilizer (BF), biocrust only (B), fertilizer only (F), and a control (C) with neither biocrust nor fertilizer (Figure 7-11). Each treatment applied to a 1-m^2 area was randomly assigned a location within each 3.5-m^2 block.

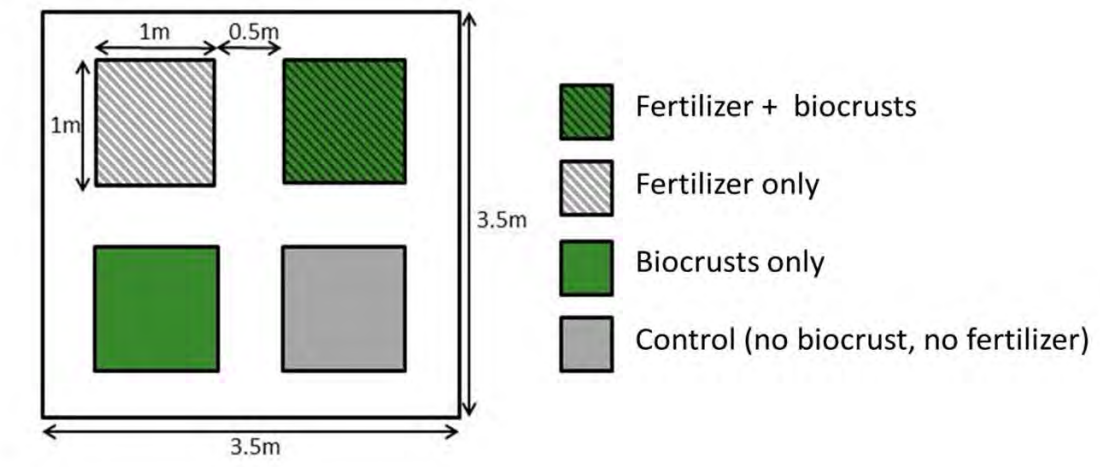

Figure 7-13 – Each block (n = 9) contained each treatment once. The treatments were biocrust and fertilizer (BF), fertilizer only (F), biocrust only (B) and a control with neither biocrust nor fertilizer (C). Each block measured 3.5m^2 and contained four plots of 1 m^2.

Biocrusts were collected from the areas delineated for treatment plots. Collection of biocrusts involved removing the biocrusts (0.5–3 cm depth) and approximately 0.5 cm of underlying soil. Biocrusts were then sieved to 4.5 mm and homogenized. Once the biocrusts were removed from all the plots, a site pre-treatment, which consisted of raking to an approximate depth of 3 cm, was applied to the plots in order to emulate surface disturbance. Raking was selected because it was deemed the most effective site preparation techniques

from the greenhouse experiment we previously conducted (see Section 7.4). The homogenized biocrust was reapplied onto treatments B and BF (n = 18, areas in green in Figure 7-13 in an amount equal to 10% of the surface area (i.e., 0.1 m^2 per plot) while the other treatments (F and C) didn't receive any biocrust (n = 18, areas in grey in Figure 7-11). A powdered 19:19:19 fertilizer was applied (110 kg × ha^{-1}) onto BF and F treatments (n = 18, hashed areas in Figure 7-11). The experiment ran for 12 weeks (from May 25th, 2015 – September 24th, 2015), which roughly correspond to the length of the snow-free period.

During the same period, we collected samples of undisturbed mature biocrust (0.5 cm – 3 cm depth and approximately 0.5 cm of underlying soil) from eight harvest locations on site. The harvest sites were chosen to be representative of the diversity of mature biocrust communities present at the site.

Biocrust function and establishment

At each sampling period (week 0, 6, and 12), Acetylene Reduction Assays (ARAs) were performed on a composite sample for each plot (n = 40), as well as, on 10 undisturbed biocrusts samples from eight harvest locations (n = 80). Prior to ARA incubations, measurement of chlorophyll fluorescence was taken. After the ARAs, samples were frozen at -20 °C for later EPS analysis. All analytical methods followed the procedures outlined in Section 7.4. Percent cover was estimated 12 weeks after the start of the experiment.

Soil properties

Potentially mineralizable nitrogen, quantified as a measure of available nitrogen, and dissolved organic carbon were measured for each plot (n = 36) at the start and at the end of the experiment. Analytical methods followed the procedures outlines in Section 7.4.

GeoChip

For each treatment, a composite biocrust sample, encompassing each replicate of that treatment, was taken 12 weeks after the start of the experiment. Samples were promptly stored at -20 °C for subsequent DNA extraction. Details of DNA extraction procedure can be found in the Section 7.4. Extracted DNA was sent to Glomics Inc. (Norman, OK) for analysis with GeoChip 5.0S.

Microclimate monitoring

We set up a microclimate monitoring station at (N 54.2305°, W 128.0243°), a data-logger (CR1000, Campbell Scientific Inc., Logan, UT) coupled to a multiplexer (AM416, Campbell Scientific Inc., Logan, UT) with a temperature and relative humidity probe (HMP45, Campbell Scientific Inc., Logan, UT), two moisture probes (EC-5, Decagon Devices Inc., Pullman, WA), as well as, 12 thermocouples inserted at the surface of mature biocrust (top 5 mm).

Data analysis

Comparisons were done using ANOVA with Tukey post-hoc tests. All statistical analyses were conducted in R (R Core Team, 2014). GeoChip data was analyzed based on methods by Zhang et al. (2014). We quantified total potential *nif*H in samples by obtaining the total signal detected for *nif*H genes from all organismal sources for each sample relative to the total signal intensity for that same sample.

Results

Biocrust function

Table 7-10 is a description of taxa diversity found on mature biocrusts from Nimbus Mountain. We identified over 65 species of lichen, liverworts, and moss.

Table 7-10 – Soil crust taxa on Nimbus Mountain.

Species	Taxonomic group	Substrate
Amygdalaria panaeola	lichen	Saxicolous
Baeomyces carneus	lichen	Terricolous
Cetraria commixta	lichen	Saxicolous
Cetraria delisei	lichen	Terricolous
Cetraria islandica ssp. islandica	lichen	Terricolous
Cladina arbuscula	lichen	Terricolous
Cladina rangiferina	lichen	Terricolous
Cladonia bellidiflora	lichen	Terricolous
Cladonia borealis	lichen	Terricolous
Cladonia borealis	lichen	Terricolous
Cladonia cervicornis	lichen	Terricolous
Cladonia ecmocyna ssp. occidentalis	lichen	Terricolous
Cladonia gracilis group	lichen	Terricolous
Cladonia macrophyllodes	lichen	Terricolous
Cladonia pleurota	lichen	Terricolous
Cladonia singularis	lichen	Terricolous
Cladonia uncialis var. uncialis	lichen	Terricolous
Frutidella caesioatra	lichen	Terricolous
Lecanora zosterae	lichen	On detritus

Species	Taxonomic group	Substrate
Lecidea hypnorum	lichen	Terricolous
Lecidoma demissum	lichen	Terricolous
Lepraria alpina	lichen	Terricolous
Leprocaulon albicans	lichen	Terricolous
Ochrolechia androgyna	lichen	Terricolous
Pertusaraia geminipara	lichen	Terricolous
Pertusaria oculata	lichen	Terricolous
Placidiopsis sp.	lichen	Terricolous
Placynthiella icmalea	lichen	Terricolous
Placynthiella uliginosa	lichen	Terricolous
Polyblastia gelatinosa	lichen	Terricolous
Porpidia contraponenda	lichen	On pebble
Psoroma tenue var. boreale	lichen	Terricolous
Rhizocarpon expallescens	lichen	Saxicolous
Solorina crocea	lichen	Terricolous
Stereocaulon alpinum	lichen	Terricolous
Stereocaulon botryosum	lichen	Terricolous
Stereocaulon vesuvianum	lichen	Terricolous
Thamnolia vermicularis	lichen	Terricolous
Thelenella muscorum var. muscorum	lichen	Terricolous
Umbilicaria hyperborea var. radicicola	lichen	Saxicolous
Anastrophyllum minutum var. minutum	liverwort	Terricolous
Blepharostoma trichophyllum	liverwort	Terricolous
Cephaloziella divaricata	liverwort	Terricolous
Diplophyllum taxifolium	liverwort	Terricolous
Gymnomitrion concinnatum	liverwort	Terricolous
Marsupella brevissima	liverwort	Terricolous
Marsupella ustulata	liverwort	Terricolous
Moerckia blyttii	liverwort	Terricolous
Nardia geoscyphus	liverwort	Terricolous
Pleurocladula albescens	liverwort	Terricolous
Ptilidium ciliare	liverwort	Terricolous
Tritomaria quinquedentata	liverwort	Terricolous
Andreaea rupestris	moss	Saxicolous
Anthelia juratzkana	moss	Terricolous
Bucklandiella microcarpa	moss	Terricolous
Bucklandiella sudetica	moss	Terricolous
Conostomum tetragonum	moss	Terricolous
Cynodontium tenellum	moss	Terricolous
Dicranoweisia crispula	moss	Terricolous
Dicranum elongatum	moss	Terricolous
Dicranum spadiceum	moss	Terricolous
Kiaeria blyttii	moss	Terricolous
Plagiothecium laetum	moss	Terricolous
Pogonatum contortum	moss	Terricolous
Pohlia sp.	moss	Terricolous
Polytrichum piliferum	moss	Terricolous
Ptychostomum sp.	moss	Terricolous
Racomitrium lanuginosum	moss	Terricolous
Arthrorhaphis sp.		Terricolous

Comparisons of acetylene reduction between treatments revealed that at week 12 the treatments had significantly higher rates of nitrogen fixation than at week six and that week six values were also significantly higher than ones from week 0 (ANOVA, Tukey HSD posthoc, all $p < 0.01$; Figure 7-14). No significant differences were found between treatments for acetylene reduction. We found no significant difference between treatments, control, and the inoculant for the relative signal intensity of *nif*H genes.

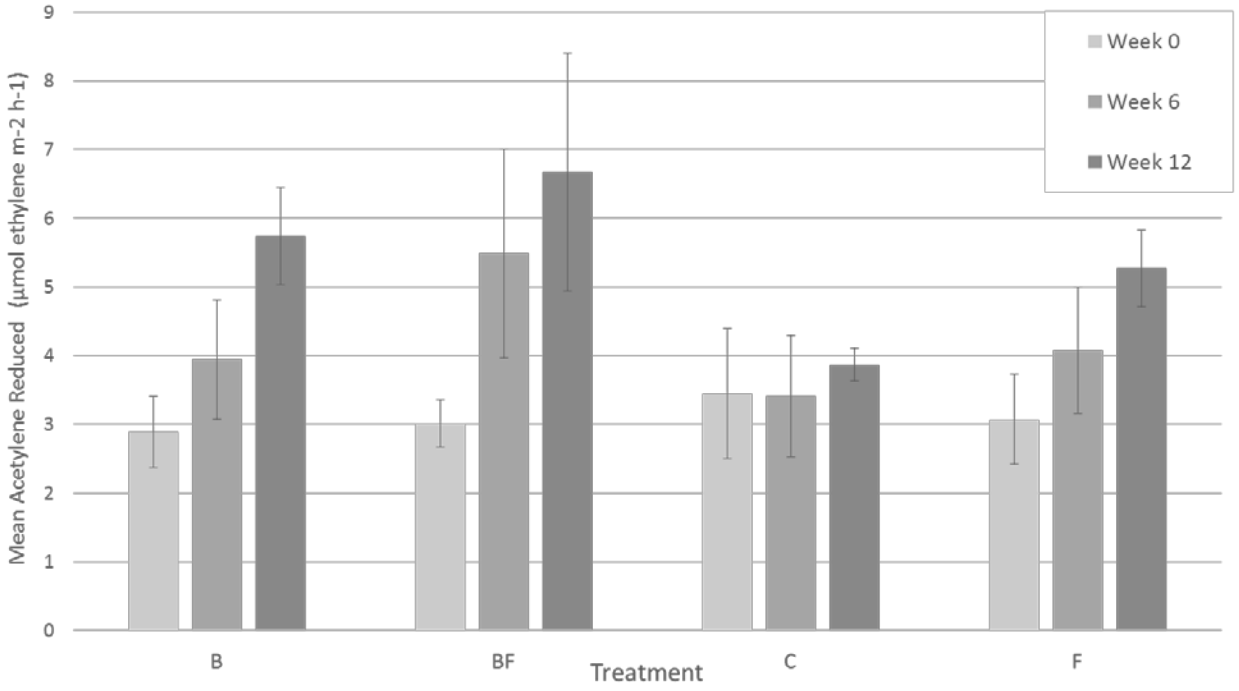

Figure 7-14 – Nitrogenase activity (± 1 SE) detected via Acetylene Reduction Assays measured 0, 6 and 12 weeks following the start of the experiment.

Extracellular polysaccharide (EPS) content was significantly lower in all treatments than in the inoculant (ANOVA, Tukey HSD posthoc, all $p < 0.01$) (Figure 7-15). Despite a lack of significant differences between treatments, we observed a decline in EPS content for the fertilizer only treatment between week 0 and 12.

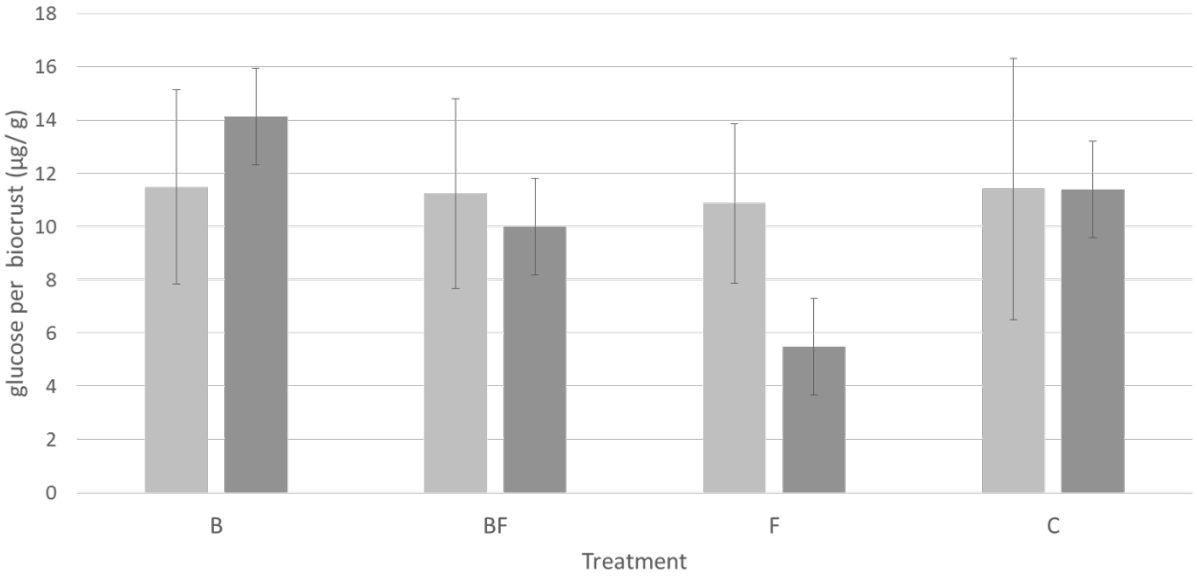

Figure 7-15 – Extracellular Polysaccharide (EPS) content of biocrust or soil surface (± 1 SE) 0 (light bars) and 12 weeks (dark bars) following the start of the field experiment.

Chlorophyll fluorescence (CF) was recorded in approximately half of the mature biocrust samples each in early summer (week 0), mid-summer (week 6) and late-summer (week 12) (Table 7-11). For mature biocrust samples with CF, week 12 samples had a significantly higher Fv to Fm ratio than week 0 and 6 (ANOVA, Tukey HSD posthoc, all $p < 0.05$). For the inoculation experiment, CF was solely recorded at week 12 from three samples, all from the biocrust and fertilizer treatment.

Table 7-11 – Chlorophyll fluorescence (± 1 SE) and nitrogenase activity (± 1 SE) of mature biocrusts in early summer (week 0), mid-summer (week 6) and late summer (week 12).

Time Period	Early Summer	Mid-summer	Late Summer
Mean acetylene reduced (µmol ethylene m^{-2} h^{-1})	15.1 ± 1.5	8.1 ± 1.7	7.4 ± 0.7
Chlorophyll fluorescence (Fv/Fm)	0.84 ± 0.01	0.85 ± 0.01	0.89 ± 0.01

Establishment

Biocrust percent cover was significantly higher in the biocrust only, and biocrust and fertilizer treatments than in the fertilizer only and the control treatment (ANOVA, Tukey HSD posthoc, all $p < 0.01$) (Table 7-12).

Table 7-12 – Biocrust percent cover (± 1 SE) of experimental plots measured in late summer, 12 weeks after the start of the field experiment.

Treatment	B	BF	C	F
Mean percent cover (%)	33.2 ± 5.0	31.8 ± 5.5	3.3 ± 1.0	10.1 ± 4.8

Soil properties

We found no significant difference in potentially mineralizable nitrogen (min N). However, between week 0 and 12, min N declined in the biocrust only treatment and the control, whereas it stayed at the same level for the fertilizer only, as well as, for biocrust and fertilizer treatments (Figure 7-16).

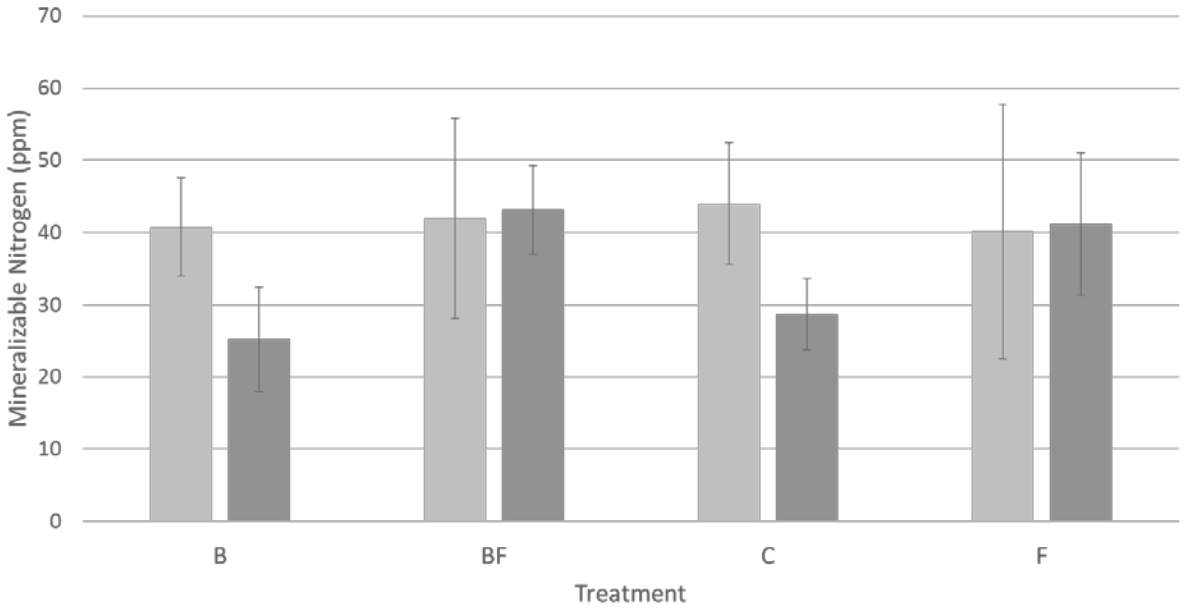

Figure 7-16 – Potentially mineralizable nitrogen as a measure of available nitrogen (± 1 SE) 0 (light bars) and 12 weeks (dark bars) following the start of the field experiment.

For dissolved organic carbon (DOC), we found a significant interaction effect between time and treatment (ANOVA, $p < 0.05$) (Figure 7-17). We also found that at week 12, the

biocrust and fertilizer treatment had a significantly higher DOC content than the fertilizer only treatment (ANOVA, $p < 0.05$).

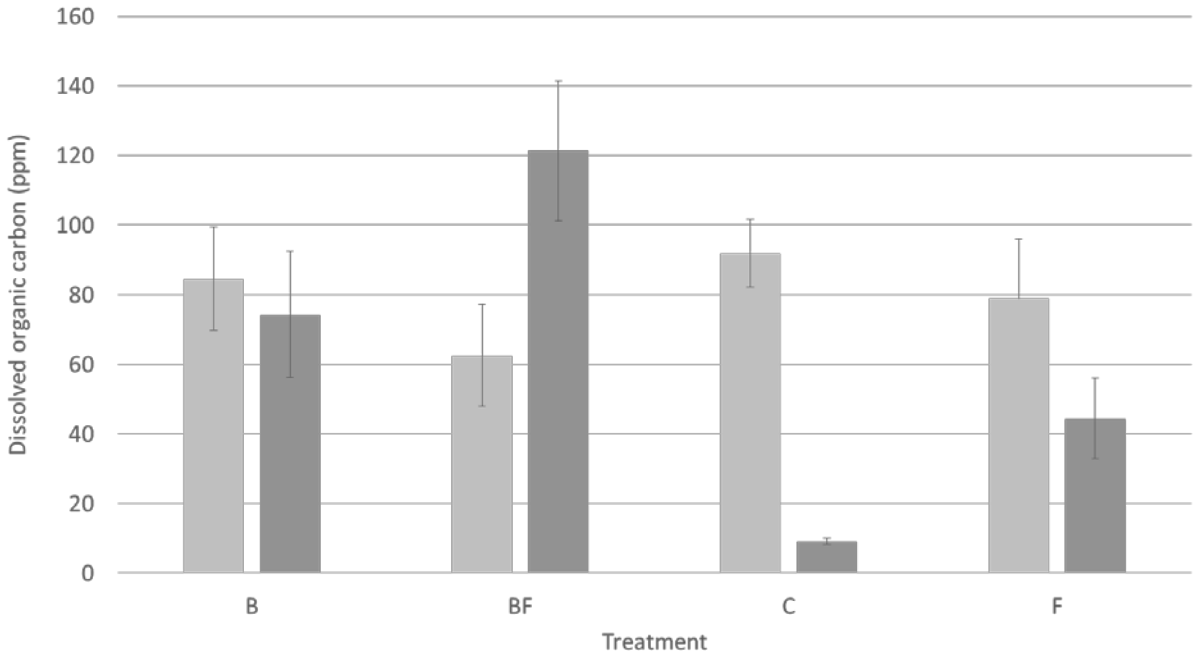

Figure 7-17 – Dissolved organic carbon (± 1 SE) 0 (light bars) and 12 weeks (dark bars) following the start of the field experiment.

Discussion

Our results suggest that following a disturbance, artificial inoculation with biocrust could increase soil surface nitrogen-fixation rates. Similarly, others (e.g., Liu et al. 2002, Hawkes et al. 2004) found that artificial inoculation of soils with biocrust was an effective way of increasing nitrogen fixation. Although the differences in nitrogenase activity between the inoculated treatments (B and BF) and the treatments without biocrust (C and F) are not statistically significant, our results show a distinct trend that the former have increasing nitrogenase activity over time from 0 – 6 – 12 weeks. Though less pronounced, there is also an increase in nitrogenase activity over time for the fertilizer only (F) treatment. Although addition of large amount of nitrogen may limit nitrogen-fixation, other studies have demonstrated a positive

relationship between moderate fertilizer addition and nitrogenase activity (e.g., Maestre et al. 2006). Hence, because our fertilization rate was relatively low (19:19:19 fertilizer at 110 kg × ha^{-1}) it is not surprising that we observe only a slight increase in nitrogen-fixation over time and only marginally superior to the control that may have some level of natural recovery.

Comparing the mean nitrogenase activity at week 12 of the plots inoculated with biocrusts (B and BF) to that of undisturbed mature biocrust at the same time period (late-summer, week 12)(6.2 and 9.1 µmol of ethylene $m^{-2} \times h^{-1}$ respectively) we obtain a 68% nitrogenase activity recovery rate. These numbers seems particularly high given the typically slow disturbance recovery rates of biocrust (Belnap 2002). In a recent study combining fertilizer addition and watering, however, Antoninka et al. (2015) successfully produced fully functional moss-dominated biocrusts. Likewise, the mesic nature of Nimbus Mountain combined to our fertilizer addition may have facilitated the establishment of biocrust biota dominated by such mosses. Signal intensity derived from microarrays of GeoChip 5.0S correspond to DNA hybridization matches, which indirectly indicate that the *nif*H gene is present in the sample. The sum of the total signal detected for *nif*H genes from all organismal sources allows us to represent total potential *nif*H in the sample. We did not detect any significant differences in the relativized signal intensities for *nif*H between our field treatments. Due to the limited duration of our study, changes in the *nif*H abundance may not have been observed. Due to the relatively high rates of biocrust colonization on Nimbus Mountain, the prevalence of *nif*H within the surface soils is likely quite high and detection of *nif*H legacy (i.e., non-functional DNA residing in the environment) may have masked treatment differences.

Our results suggest that biocrust establishment was enhanced by biocrust inoculation. In addition, while fertilizer addition had no effect on percent cover of treatments that received biocrust inoculation, it appears to have promoted at least some establishment on the fertilizer only treatment when compared to the control. Similarly, for the EPS content, there also seemed to be an interaction effect between fertilizer addition and the biocrust inoculation. While the EPS content stayed constant over time in the control as well as in the biocrust+fertilizer treatment, it declined sharply in the fertilizer only treatment. At the same time, there was an

increase in the biocrust only treatment. This may indicate that biocrust inoculation stimulated EPS secretion, while fertilizer addition impeded it.

Mature biocrust nitrogenase activity was significantly higher in early spring (week 0) and in late summer (week 12) than in mid-summer (week six). Seasonality of nitrogenase activity has been widely reported in the literature (e.g., Halvorson et al. 1992). In addition, late spring and late summer are generally wetter and because nitrogenase activity is highly dependent on moisture availability, it is not surprising that biocrust would be more active at those times of higher moisture (Belnap 2002). Similarly, chlorophyll fluorescence also exhibited seasonality but with a significantly higher Fv:Fm in late summer than in late spring and mid-summer.

There was no difference in mineralizable nitrogen between treatments nor in dissolved organic carbon (DOC). Over time, however, there was a decrease in DOC for the fertilizer treatment, whereas there is an increase in the biocrust+fertilizer treatment. Canton et al. (2014) found that DOC was higher (while TOC was lower) in soils where biocrust had been removed when compared to soils with biocrusts, and attributed this to the easier dilution of organic carbon in soils lacking biocrust. The low structural development of biocrusts on our treatment plots may have cause low organic carbon trapping resulting in overall lower DOC than expected under mature biocrust. Finally, the short duration of our study could explain the lack of clear differentiation between treatments for soil properties.

The time frame of our study was dictated by the short length of the snow-free period which is typical of high montane environments and depended on the spring 2015 precipitation volume. The short growing season typical of alpine environments contributes to the slow recovery rates. This highlights a need for multi-year studies to better assess long-term restoration success.

Conclusion

Undoubtedly, this study demonstrates that inoculation and fertilizer addition can successfully accelerate the recovery of biocrusts communities. Biocrust can play a key role in ecosystem recovery from disturbance notably by reducing erosion, providing nitrogen and carbon inputs, as well as, promoting early succession processes. Hence, their restoration is a

practical tool to ensure successful ecological restoration. Future research could assess the effects fertilization and inoculant rates on biocrust recovery to develop cost-effective methodologies adapted to alpine sites.

7.6. High Elevation Protocol: findings and recommendations

In summary, the study of high elevation invertebrates contributed to establishing baseline invertebrate diversity in the high elevation plateau of Nimbus Mountain, and provided information necessary for developing a protocol for the use of alpine invertebrates as bioindicators of ecosystem function that could be used to monitor and assess changes in taxa richness or composition related to pipeline construction and vegetation restoration. In particular we collected specimens of: (1) mites, collembolans, and dipteran larvae which we expect will be involved in soil cycling, decomposition and nutrient availability, and will influence vegetative and soil crust structure; (2) bees and flies, many of which are involved in pollination of native plants, influencing the vegetative assemblage; and (3) spiders, which through predation act to regulate the levels of other fauna. Our species list of recovered arthropods provides a substantial starting point for further natural historical investigations into interactions between arthropods and between arthropods and plants and lichens in this portion of the planned pipeline route. Due to remote nature and limited access to sites, passive trapping methods, such as pitfall and Malaise traps, should be considered for future survey-based studies. Because many ponds and streams are present throughout the study area, and aquatic invertebrates are particularly sensitive to environmental changes and provide considerable ecological input into the surrounding terrestrial areas, methods for sampling aquatic invertebrates should be included in future studies. Next-generation sequencing can also contribute immensely to the ability to monitor and assess changes in community composition for such a broad range of taxa since it allows simultaneous identification, often to the species level, of all taxa present in bulk samples without the need for taxonomic expertise.

Biocrusts are a practical tool to ensure successful ecological restoration. Our studies of high-elevation lichen communities demonstrate that inoculation and fertilizer addition can successfully enhance the recovery of biocrust communities under the harsh conditions of coastal alpine tundra habitats. Biocrusts play a key role in ecosystem recovery from disturbance — notably by reducing erosion, providing nitrogen and carbon inputs, as well as, promoting early succession processes. Hence, their inoculation is a practical tool to ensure successful

ecological restoration. Future research should be directed at assessing the effects of different fertilization and inoculant rates on biocrust recovery, in order to develop cost-effective methodologies for alpine sites. Additionally, the long-term assessment of current study plots would determine how the recovery of these disturbed soil surfaces proceeds over a multiyear period.

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Glossary of Terms

16S – 16S ribosomal RNA gene. A marker gene used for surveying bacterial/archaeal taxonomic diversity.

A horizon – Upper mineral horizons that exhibit loss of some constituents (e.g., clay; iron; organic matter), or *in situ* accumulation of organic matter (leading to a darkening of colour).

AP – Acid Phosphatase. A soil enzyme activity hydrolyzes inorganic phosphates from organic matter.

ARA – Acetylene Reduction Assay. An experimental technique used as a proxy for nitrogen fixing potential.

B horizon – Mineral horizons (usually below A horizons) that show accumulations of clay or minerals such as iron or aluminum oxides or organic material moved there by leaching. They often have a distinctly different structure, colour or consistency to the A horizon above and the horizons below.

bp – base pairs of DNA (ACTG). This is a measure of the length of a DNA sequence.

BACI – Before-After-Control-Impact. This is a scientific study design where sampling occurs before and after an impact and at both an impacted and control site.

BEC – Biogeoclimatic Ecosystem Classification. A classification system used by the BC Ministry of Forests for BC's various ecosystems. A biogeoclimatic zone is defined as "a geographic area having similar patterns of energy flow, vegetation and soils as a result of a broadly homogenous macroclimate."

BG – β-glucosidase. A soil enzyme activity hydrolyzes cellobiose to release glucose units.

Biological crusts – complex communities of bacteria, cyanobacteria, algae, mosses, liverworts, fungi and lichens.

Bray-Curtis cluster analysis – A statistical method of grouping data objects into similar clusters based on the Bray Curtis distance, a statistic used to quantify the compositional dissimilarity between treatments based on count data.

C – carbon.

C horizon – Largely unaltered parent geologic material — it is simply named so it comes after A and B within the soil profile. This layer is little affected by soil forming processes (weathering and genesis), and the lack of pedological development is one of the defining attributes.

CABIN – The Canadian Aquatic Biomonitoring Network is an aquatic biological monitoring program for assessing the health of freshwater ecosystems in Canada. (Environment Canada 2012)

CBH – Cellobiohydrolase. A soil enzyme activity that hydrolyzes 2-4 sugar units off the ends of cellulose molecules.

Diazotrophic – nitrogen fixing organisms.

eDNA – Environmental DNA.

Emergent macroinvertebrates – All invertebrate organisms (for example insects, worms, clams, crabs, octopus, snails, and starfish) that pass part of their life cycle in an aquatic environment.

EPT – Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) are the three insect Orders commonly used to test water quality.

EU – Ecosystem Units.

FSR – Forest Service Road.

F_{ST} – Fixation index is a measure of population differentiation due to genetic structure.

Gill Na^+,K^+-ATPase activity – Sodium-potassium adenosine triphosphatase (also known as Na+/K+ pump, sodium-potassium pump, or sodium pump) is an antiporter enzyme located in the plasma membrane of all animal cells.

Hardy-Weinberg equilibrium – A biological principle that states that both allele and genotype frequencies in a population remain constant, that is, they are in equilibrium from generation to generation unless specific disturbing influences are introduced.

HDD – Horizontal directional drilling is a steerable trenchless method of installing underground pipes, conduits and cables in a shallow arc along a prescribed bore path by using a surface-launched drilling rig, with minimal effects on the surrounding area.

High elevation – areas above treeline.

Index of preponderance – A metric for quantifying the size and abundance of prey items during diet analysis of fishes.

ITS2 – Internal transcribed spacer 2 gene. A marker gene used for surveying fungal taxonomic diversity.

LA-ICP-MS – Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry – is a high technology method that analyzes trace matter samples. A pulsed laser focuses on and vaporizes a very small amount of a solid sample. A gas stream transports the resultant vapor into high temperature plasma where the vapor sample is ionized before being extracted into a mass spectrometer for analysis.

Linkage disequilibrium – In population genetics, it is the non-random association of alleles at two or more loci, indicating that they may be on the same chromosome.

LFH – Organic surface horizons common in well drained forest soils (e.g., duff layers).

MAP – Mean Annual Precipitation. The average depth of rain and/or snow for a given area during a one year period.

MAT – Mean Annual Temperature. The average of the minimum and maximum temperatures for a specific area for a given year.

Massive parallel sequencing – Second generation sequencing – encompasses several high-throughput approaches to DNA sequencing, which is the process of determining the precise order of nucleotides within a DNA molecule.

Mehlich III – A widely used extractant for evaluating plant available phosphorus (P) in soils.

Meiobenthic fauna – Small benthic invertebrates that live in both marine and fresh water environments.

Microsatellites – Repeating sequences of 2-6 base pairs of DNA. They are used as molecular markers in genetics, for kinship, population and other studies.

MRPP – Multiple Response Permutation Procedure. A non-parametric test for the null hypothesis of no difference between two or more groups of entities (i.e., in the context of this report, predefined groups of samples containing a list of species abundance data).

MUB – 4-methylumbelliferone. A fluorescent chemical linked to synthetic substrates used in soil enzyme assays. When hydrolysis occurs, the MUB group is released allowing its fluorescence to be detected under proper excitation wavelengths of light in a spectrofluorometer.

Mycorrhizal – Is a symbiotic association between a fungus and the roots of a plant.

n – sample size.

N – Nitrogen.

NAG – 1,4-β-N-acetylglucosaminidase . A soil enzyme activity involved in chitin degradation.

Neutral DNA polymorphism – DNA variation not thought to be influenced by natural selection.

NextGen sequencing – Next generation sequencing or second generation sequencing – encompasses several high-throughput approaches to DNA sequencing, which is the process of determining the precise order of nucleotides within a DNA molecule.

***nif*H** – The nif gene is the gene responsible for the coding of proteins related and associated with the fixation of atmospheric nitrogen into a form of nitrogen available to plants. These genes are found in nitrogen fixing bacteria and cyanobacteria.

NMDS – Non-Metric Multidimensional Scaling. A gradient analysis method of comparing objects in a distance matrix (in ecology, usually these objects are species count data from different samples/environments) to compare community similarity in low-dimensional ordinations (i.e., graphing community data points in 2- or 3-dimensional space).

NMS Ordination – Nonmetric multidimensional scaling refers to an entire Family of ordination techniques that use rank order information to identify similarity in a data set. It is a nonparametric ordination method which seeks to best reduce space portrayal of relationships.

nosZ – Nitrous oxide reductase gene is a functional gene specific to denitrification and is largely unique to denitrifying bacteria.

ODC – Ornithine decarboxylase is the enzyme that catalyzes the decarboxylation (a chemical reaction that removes a carboxyl group and releases carbon dioxide) of ornithine (a product of the urea cycle) to form putrescine. This reaction is the committed step in polyamine synthesis.

OTU – Operational Taxonomic Unit. An operational definition for species, usually employed when DNA sequence data is the main criteria. The definition is usually based on a minimum percentage of DNA sequence identity that distinguishes species from each other.

P – Phosphorus.

PCR – a laboratory technique used to amplify small segments of extracted DNA.

PGM – Parent geological materials.

PIT Tags – Passive Integrated Transponder tags – a tag attached to an object (i.e., fish), for the purposes of automatic identification and tracking using a wireless non-contact system and radio-frequency electromagnetic fields to transfer data.

Polymorphic microsatellite loci – Microsatellite markers that contain more than one variant form or allele.

PRS probes – Plant Root Simulator probes. Devices installed directly into soils for measurement of diffusible ionic nutrients.

Pyrosequencing – A method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing by synthesis" principle. This involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically.

qPCR – Quantitative Polymerase Chain Reaction. A laboratory technique based on a PCR (polymerase chain reaction) used to amplify and simultaneously quantify the amount of a targeted region of a DNA molecule. An amplified region of DNA is known as an amplicon. PCR is a molecular biological method for in vitro enzymatically amplifying (creating multiple copies of) DNA.

RMP – PTP restoration management plan or restoration plan.

RNA – Ribonucleic acid is a ubiquitous Family of large biological molecules that perform multiple vital roles in the coding, decoding, regulation and expression of genes. Together with DNA, RNA comprises the nucleic acids, which, along with proteins, constitute the three major macromolecules essential for all known forms of life.

RoW – Right-of-Way.

S – Sulphur.

SE – standard error.

Second generation sequencing – Encompasses several high-throughput approaches to DNA sequencing, which is the process of determining the precise order of nucleotides within a DNA molecule.

Seed rain – Deposition of seeds by parent plants, animals, or wind

SMT probes – Soil-Moisture-Temperature probes. Electronic devices installed directly into soils for frequent monitoring of volumetric water content and soil temperature.

SOM – Soil organic matter.

STR – Simple tandem repeat loci (microsatellites) are repeating sequences of 2-6 base pairs of DNA. They are used as molecular markers use to generate individual specific genotypes. These are used in ecology to assess relationships among individuals (i.e., kinship) and populations (i.e., population structure and to make inferences on gene flow among populations).

Subsoil – Soil horizons that are below those moved during cultivation operations; often not as fertile as topsoil.

Topsoil – Layer of soil moved during cultivation operations (i.e., surface soil); often fertile if composed of an organic-rich A horizon.

UV inductively coupled plasma optical emission spectrometer – An analytical technique used for the detection of trace metals. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element. The intensity of this emission is indicative of the concentration of the element within the sample.

%VWC – % Volumetric Water Concentration. Soil moisture content expressed by weight as the ratio of the volume of water present to the volume of host material (soil) + water volume + air space.

WMC – Water Moisture Content (gravimetric). Soil moisture content expressed by weight as the ratio of the mass of water present to the dry weight of the soil sample.

XYL – β-xylosidase. A soil enzyme activity that removes xylose units from hemicellulose.

Young-of-the-Year (YOY) – Age-0 fish, or those animals born within the past year, which have not yet reached one year of age.