PILOTING ENVIRONMENTAL DNA FOR FISHERIES MONITORING IN THE UPPER PEACE RIVER WATERSHED

by

Andrew McDermot-Fouts

B.Sc. University of Northern British Columbia, 2014

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
NATURAL RESOURCES AND ENVIRONMENTAL STUDIES

UNIVERSITY OF NORTHERN BRITISH COLUMBIA

March 2024

ABSTRACT

Applications of environmental DNA (eDNA) represent one of the most significant recent advances in aquatic species monitoring. eDNA has the potential to dramatically increase the effectiveness and efficiency of fisheries monitoring across large geographic areas and particularly for species that have traditionally been difficult to survey. The Williston Reservoir and its surrounding watershed in northern British Columbia is a vast and heavily disturbed aquatic ecosystem where fish stocks have been difficult to monitor with traditional survey techniques. Although eDNA has many potential benefits, the validation process required in a novel environment can be a barrier to adoption. Primer choice and validation, study design and laboratory workflows are critical considerations for eDNA studies and must be evaluated when developing a novel approach in a particular geographic area. My research advanced the validation of species-specific eDNA assays for six prominent pelagic species in the Williston Reservoir and a generic, metabarcoding primer set that was effective in detecting the diversity of fish in the surrounding streams and rivers. The species-specific approach included the validation of four published assays and the development of novel assays for detecting lake trout (*Salvelinus namaycush*) and peamouth (*Mylocheilus caurinus*). Lake trout and bull trout (*Salvelinus confluentus*) are very closely related species that are sympatric throughout much of northwestern North America. The development of a novel lake trout assay that did not cross amplify bull trout eDNA is an important achievement for species-specific eDNA monitoring. Reservoir samples were collected from above and below the thermocline during the summer when the reservoir was stratified to identify species-specific patterns of eDNA distribution throughout the water column. Species detections in the reservoir were comparable for species-specific and metabarcoding assays

when compared against gillnet catches in the reservoir. Abundant species that exhibit diel vertical migrations were detected by eDNA and gillnets at all depths. eDNA copy numbers detected were higher for samples with greater abundance and biomass of each species caught in the gillnets. Samples collected from tributaries around the reservoir were tested with metabarcoding primers and species detections were compared to results from a species-specific assay for Arctic grayling and a snorkel survey. Metabarcoding results were comparable to those from the Arctic grayling eDNA study and the snorkels survey, although the metabarcoding methods in this study were less sensitive due to a reduced amount of sample replication. Piloting eDNA in the Williston Reservoir and surrounding watershed has provided valuable insights into eDNA sampling design and the overall workflow for future eDNA studies in the region.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENS

I would like to thank my supervisor Dr. J. Mark Shrimpton for all of his help and guidance throughout my graduate research. I would also like to thank the other members of my research committee, Dr. Margaret Docker from the University of Manitoba, and Dr. Brent Murray from the University of Northern British Columbia for their help with my project and their careful review of my thesis. I would like to thank Dr. Heath from the University of Windsor for serving as my external examiner and for the thoughtful comments he made. I am grateful for the help that Caitlin Pitt provided in the lab at UNBC for my ddPCR analysis. I owe a special thank-you to Shelby Toews, Jonathon Leblanc and James Watkins from the University of Windsor for their help with my metabarcoding sample processing and with the bioinformatics analysis. I would like to thank Bryce O'Connor and staff from Chu Cho Environmental for their help with data collection in the Williston Reservoir. I would alsso like to thank Mike Stamford for sharing his eDNA samples from around the Williston Watershed and John Haggen for helping with the snorkel survey data. I would never have been able to complete a graduate degree without the personal funding provided by Veterans Affairs Canada, the Natural Sciences and Engineering Research Council of Canada, and UNBC. This project was made possible with funding from the Fish and Wildlife Compensation Program, the Genomic Network for Fish Identification, Stress and Health, and Genome Canada.

PROLOGUE

Effective management of a fish stock requires an understanding of fish abundance, species composition, size and age composition, spatial distribution patterns, and an assessment of the accuracy and precision of the information gathered (Kubečka et al., 2009). Large lakes and reservoirs are difficult to study because sampling methods can be limited in their effectiveness and even cost-prohibitive on a large scale. The approaches used to monitor species within the aquatic environment often require intensive effort to determine species presence and considerably more effort to accurately assess population size. Multiple sampling techniques are often used to compensate for gear biases, and the approach may also differ depending on the type or "size" of habitat (Kubečka et al., 2009; Rudstam et al., 2012; Winfield et al., 2009). Fisheries scientists are often forced to settle for an estimate of the "true picture" of a stock because the resources needed for an accurate assessment are seldom available (Kubečka et al., 2009). Stock assessments provide a snapshot that is only valid for a particular moment in time and multiple snapshots are needed to truly understand fish population dynamics. Knowledge of temporal variation in numbers of fish and use of habitat is essential when habitat is altered through anthropogenic disturbance.

The creation of large reservoirs by the impoundment of river systems causes profound environmental changes which can drastically affect fish populations (Blackman, 1992; Ross, 1997). Many kilometres of lotic habitat are flooded and converted to hectares of lentic habitat. Reservoirs usually experience a short period of high productivity shortly after they are created, followed by a gradual loss of nutrients and productivity over time (Coxson et al., 2018; Schindler et al., 2009). Species composition in a reservoir can change dramatically as the original riverine species decline and lacustrine species become dominant in the reservoir and as reservoir

productivity fluctuates (Blackman, 1992). The effects on riverine species can extend far beyond the reservoir if much of the remaining fluvial habitat is restricted to higher gradient headwaters and if the reservoir becomes a migration barrier, isolating critical habitats and populations (Clarke et al., 2007; Stamford & O'Connor, 2022). Reservoirs often present many unique challenges for fisheries sampling that are not encountered in natural lakes. For example, the flooded forests that underly many temperate reservoirs can snag gears with nets or anchor lines and can hide fish that might otherwise be detected by hydroacoustic methods (Pillipow & Langston, 2002; Sebastian et al., 2003, 2009). Monitoring fish stocks in the hundreds of tributary streams that drain into a reservoir can be an even more daunting task. Sampling approaches in streams can be even more varied and resource intensive than those designed for a reservoir due to factors such as the variety of habitat types, access issues, and the amount of effort required. Conventional approaches to fisheries inventories in streams and reservoirs are resource intensive, costly and have many limitations, often leaving fisheries managers to make difficult decisions based on information that may be outdated, inaccurate, or simply unavailable.

Combining extensive hydroacoustic surveys with limited gillnetting has become an internationally adopted method for fisheries assessments in large reservoirs and lakes (Kubečka et al., 2009; Winfield et al., 2009). Acoustic methods measure the distribution and abundance of fish by transmitting sound pulses (pings) through the water column and analyzing the return echoes. The time delay between the transmission of a ping and the reception of a return echo is analyzed to determine fish distribution. Simultaneously, the intensity of the returning echo is analyzed to determine fish size (Rudstam et al., 2012). Because hydroacoustic methods only determine the size of individual fish, they cannot easily differentiate between species without the biological data that is provided by traditional fishing gears. Gillnetting surveys are most commonly used in

combination with hydroacoustic surveys to determine the biological characteristics of each species in the surveyed environment (Kubečka et al., 2009; Rudstam et al., 2012). Every sampling technique has limitations and combining more than one technique is often required to assess fish stocks in lakes and reservoirs (Kubečka et al., 2009).

Combining hydroacoustic methods with traditional gears can improve the accuracy and detail of fisheries surveys but there are strengths and weaknesses to any combination of techniques. For example, the first few metres of the water column is a blind spot for downward looking hydroacoustic transducers and can be problematic if fish are concentrated near the surface (Rudstam et al., 2012; Sebastian et al., 2003). Gillnetting results have been relied upon to characterize the distribution and abundance of fish near the surface that cannot be effectively targeted by hydroacoustic surveys, but gillnetting can be highly selective (Olin et al., 2009; Zale et al., 2012). Any biases associated with traditional gears are incorporated into the acoustic estimates (Rudstam et al., 2012). The probability that fish will encounter a gillnet and be retained depends a variety of factors including their behaviour and activity, swimming speed, size and morphology (Olin et al., 2009). Gillnets do not efficiently capture rare species (Sard et al., 2019; Shelton et al., 2019; Zale et al., 2012), fish that are too small or too large for the mesh sizes in the net, fish that swim too slowly to become entangled and fish who are not active at the times, locations and depths where the nets are set (Olin et al., 2009; Vašek et al., 2009). Furthermore, gillnet catch rates decline over time as they accumulate fish (Olin et al., 2009). Incorporating an active sampling gear, such as trawling, can overcome many of the pitfalls of gillnetting and hydroacoustic methods (Kubečka et al., 2009) but may still fall short of providing a true picture of the stock being assessed. Fast swimming fish, such as large piscivores, tend to avoid trawls (Bethke et al., 1999) and the depth of the trawl can select for certain species over others (Olin et al., 2009).

A considerable drawback of hydroacoustic and trawling surveys are their associated cost and complexity (Kubečka et al., 2009). Hydroacoustic surveys require specialized equipment and a sampling crew with expert knowledge and skills. Trawling is expensive and requires a specialized boat and a trained crew. Qualified crews and appropriate equipment for hydroacoustic and trawling surveys are often a limited resource (see Sebastian et al., 2003) and may contribute to the omission of trawls for long term surveys of lentic environments.

The Potential for eDNA

Genetic analysis of fish DNA obtained directly from water samples (environmental DNA) has become a powerful tool for biodiversity monitoring (Jerde, 2019; Schenekar, 2023). Two approaches to detecting species with environmental DNA (eDNA) are methods designed to detect a single species or methods that detect multiple species (Thomsen & Willerslev, 2015). Single species detection uses the polymerase chain reaction (PCR) or quantitative PCR (qPCR) to identify one or a few known species in a sample with species-specific primers and probes. Multi-species detection (eDNA metabarcoding) uses next generation sequencing (NGS) in combination with generic primers to identify a group of species in an eDNA sample by analyzing genetic barcodes. Single species methods have greater specificity, sensitivity, and quantification ability but can only detect one target species at a time (Bylemans et al., 2019; Thomsen & Willerslev, 2015). Metabarcoding is more expensive and time consuming overall compared to species-specific methods but is much more efficient and cost effective for diverse systems with many known species or in systems where information about species composition is lacking. Metabarcoding is also useful for comparing habitat use, species interactions and in identifying potentially critical habitats (biodiversity hotspots) (Balasingham et al., 2018).

eDNA has the potential to supplement or even replace traditional methods of fisheries inventory and improve the quality of data gathered for a relatively low cost. eDNA can usually detect rare species with greater efficiency and sensitivity compared to conventional sampling gears such as gill netting (Evans, Shirey, et al., 2017; Jerde et al., 2011; Thomsen et al., 2012) and has proven to be particularly useful for monitoring lake trout (*Salvelinus namaycush*) (Lacoursière-Roussel, Rosabal, et al., 2016; Littlefair et al., 2020). Fish eDNA tends to have a smoother distribution in space and time than the fish themselves, and integrates information about fish presence and abundance over a larger area than most traditional methods of fish capture (Pont et al., 2018; Shelton et al., 2019). eDNA metabarcoding can provide a more accurate and efficient measure of community diversity, detecting more species with fewer samples and less effort (Sard et al., 2019).

The distribution of eDNA in a waterbody can provide valuable information about the distribution of species. Seasonal thermal stratification and the thermal preferences of fish species influences the distribution of eDNA in lakes (Klobucar et al., 2017; Littlefair et al., 2020). Thermal stratification in lakes separates the epilimnion and hypolimnion in summer (Wetzel, 2001). Fish distribute throughout the water column in accordance with their thermal niche, and in response to predators and prey. Littlefair et al. (2020) found that eDNA distributions also became stratified in summer. The eDNA of species such as lake trout that remain below the thermocline were only detected in water samples taken from the hypolimnion and species with a preference for warmer, shallower water were only detected in samples taken from the epilimnion. However, collecting eDNA samples at various depths throughout the water column presents several challenges with respect to sample contamination and potential depth limits of sampling equipment. At least two

studies have employed long hoses and electric pumps to sample at specific depths in the water column (Klobucar et al., 2017; Littlefair et al., 2020).

Many studies have demonstrated that eDNA particle concentrations are positively correlated with species abundance (Klobucar et al., 2017; Lacoursière-Roussel, Rosabal, et al., 2016; Sard et al., 2019; Shelton et al., 2019; Stoeckle et al., 2017; Takahara et al., 2012; Yates et al., 2020) and could provide a rapid, cost-effective indicator of abundance or biomass for fisheries stock assessment (Rourke et al., 2021). However, the eDNA particle concentration at a particular sample location depends on a variety of biotic and abiotic factors that influence the amount of genetic material being shed into the environment, how and where it is transported, and how long it persists before it is degraded (Hansen et al., 2018; Klymus et al., 2015; Rourke et al., 2021; Takahara et al., 2012). The stochasticity of biotic and abiotic processes in natural environments complicates the relationship between eDNA copy number and fish abundance, but recent approaches have overcome enough of this uncertainty to provide a reasonably accurate measure of fish abundance with eDNA by standardizing against estimates with traditional gears (Chambert et al., 2018; Lacoursière-Roussel, Rosabal, et al., 2016; Shelton et al., 2019).

Research Objectives

My graduate research has piloted and validated the use of eDNA survey methods for monitoring fish species. I conducted my research in the Williston Watershed in north-central British Columbia. I outline the study area and knowledge gaps for the watershed in Chapter 1. In Chapter 2, I identified existing qPCR assays that were developed to detect kokanee (*Oncorhynchus nerka*), rainbow trout (*Oncorhynchus mykiss*), lake whitefish (*Coregonus clupeiformis*), and bull trout in other geographic regions and validated them for their use in the Williston Reservoir. I developed and validated a novel qPCR assay for lake trout because previously published qPCR

assays were not specific enough to discriminate between sympatric lake trout and bull trout. There were no previously developed qPCR assays for peamouth, so I developed and validated a novel peamouth assay for use in the Williston Reservoir. By modeling my sampling design and eDNA analysis on those published in Littlefair et al. (2020), I was able to analyze the vertical distribution of eDNA in the Williston Reservoir during summer stratification. My eDNA sampling in the Williston Reservoir was conducted concurrently with the 2021 pelagic fisheries survey led by the BC Ministry of Forest Lands and Natural Resource Operations and Rural Development (FLNRORD). In Chapter 3, I compared eDNA metabarcoding and species-specific qPCR methods for detecting fish in the Williston Reservoir and in a variety of the surrounding tributaries. Both eDNA methods were compared against conventional sampling gears in the reservoir and in streams, and I investigated the relationship between eDNA concentration and fish abundance in the Williston Reservoir. I re-analyzed many eDNA samples collected by Stamford et al. (2020, 2022) from streams in the Williston Watershed with eDNA metabarcoding and compared those results to the original eDNA detections for Arctic grayling using qPCR.

CHAPTER 1: STUDY AREA

Williston Reservoir Limnology

The Williston Reservoir is British Columbia's largest body of freshwater (surface area of 1779 km^2, Stockner et al., 2005). The reservoir was created in 1968 by the construction of the W.A.C. Bennett Dam on the upper Peace River and is comprised of three reaches (Figure 1. 1): the Finlay Reach which is the flooded valley of the south flowing Finlay River, the Parsnip Reach which is the flooded valley of the north flowing Parsnip River and the Peace Reach which encompasses the east flowing Peace River valley between the confluence of the Parsnip and Finlay tributaries and the dam. The Finlay and Parsnip reaches lie within a wide, flat bottomed rocky mountain trench bounded by the Omineca Mountains to the west and the Rocky Mountains to the east. The Peace Reach is a V-shaped basin and is much narrower and deeper than the Finlay and Parsnip reaches. The Williston Reservoir is a dimictic system with two periods of deep mixing (May and November) and two periods of stratification (June to October and February to April). During summer stratification, the epilimnion depth varies between reaches and depending on the duration and strength of wind events. Average summer epilimnion depths measured in 2000 ranged from 20 m to 30 m in the Finlay reach, 15 m to 20 m in the Parsnip Reach, and 30 m to 40 m in the Peace Reach (Stockner et al., 2005). The Williston Reservoir experienced an initial increase in productivity during the first decade after impoundment, but reservoir wide productivity has since declined dramatically to an ultra-oligotrophic state (Harris et al., 2006; Stockner et al., 2005). The Finlay Reach had the highest measures of productivity among the three reaches in 2000, followed by the Parsnip Reach and Peace Reach (Stockner et al., 2005).

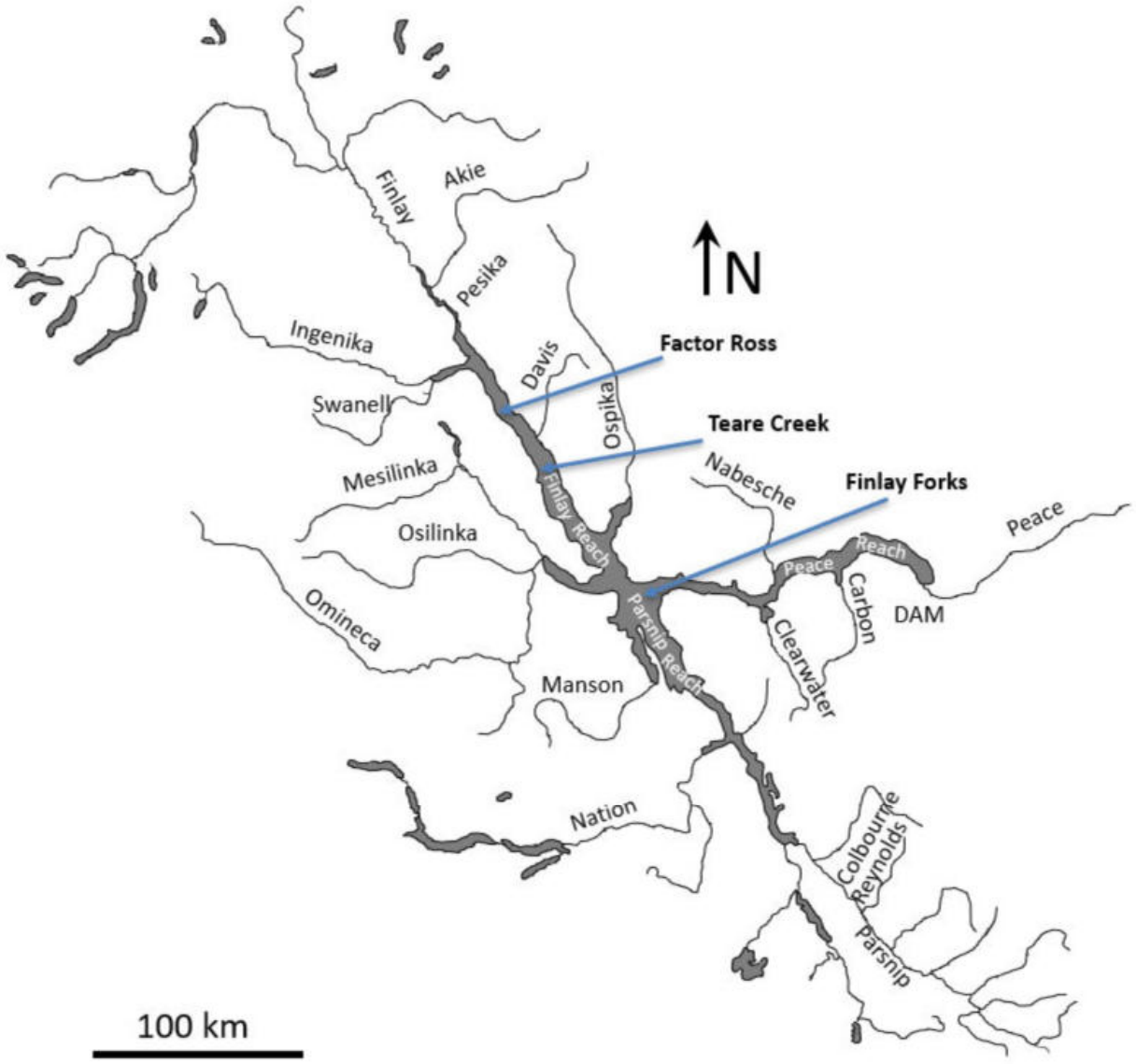

Figure 1. 1. The Williston Reservoir watershed in northern British Columbia. The Finlay Reach is the flooded valley of the south flowing Finlay River, the Parsnip Reach is the flooded valley of the north flowing Parsnip River, and the Peace Reach encompasses the east flowing Peace River valley between the confluence of the Parsnip and Finlay tributaries and the W.A.C. Bennet dam near Hudson's Hope, BC. Arrows indicate the Factor Ross and Teare Creek sample locations in the Finlay Reach where eDNA samples were collected. Gillnet and trawl surveys were conducted at the Teare Creek and Finlay Forks locations.

Tributaries of the Williston Reservoir

The Williston Watershed encompasses all the tributaries that drain into the Williston Reservoir upstream of the W.A.C. Bennett Dam and includes 10 major rivers (drainage >1,500 km^2), seven large rivers (400-1,500 km^2), 49 medium rivers (50-400 km^2), and 262 small rivers (<50 km^2) (Langston & Blackman, 1993). The creation of the Williston Reservoir drastically altered the lotic systems within the basin by inundating a large portion of many streams and rivers, limiting the availability and connectivity of stream habitats for fish (Fish and Wildlife Compensation Program, 2020).

Fish Species in the Williston Watershed

A diverse assemblage of 19 fishes belonging to four orders and five families have been identified in the streams, rivers, lakes and reservoir that are encompassed within the Williston Watershed (Table 1. 1)(Blackman, 1992; Cowie & Blackman, 2003, 2004; Fish and Wildlife Compensation Program, 2020; Langston & Blackman, 1993; Langston & Murphy, 2008; Province of British Columbia, 2020; Sebastian et al., 2003). Lentic habitats in the watershed are more diverse, and often more productive than those in the reservoir. Similarly, the diversity of species in lentic habitats is greater than in the reservoir. Diversity in local species assemblages often only includes a small subset of the species in the entire watershed and are frequently dominated by a few, very abundant species. Many of the species present in the watershed are not widely distributed and are considered rare.

Table 1. 1. The 19 fish species known to occur in the Williston Watershed and their general habitat preferences.

Common Name	Family	Scientific Name	Habitat preference
Prickly sculpin	Cottidae	*Cottus asper*	lotic/lentic (littoral, benthic)
Slimy sculpin	Cottidae	*Cottus cognatus*	lotic/lentic (littoral, benthic)
Lake trout	Salmonidae	*Salvelinus namaycush*	lentic (pelagic)
Bull trout	Salmonidae	*Salvelinus confluentus*	lotic/lentic
Rainbow trout	Salmonidae	*Oncorhynchus mykiss*	lotic
Kokanee	Salmonidae	*Oncorhynchus nerka*	lentic
Lake whitefish	Salmonidae	*Coregonus clupeiformis*	lentic
Mountain whitefish	Salmonidae	*Prosopium williamsoni*	lotic
Pygmy whitefish	Salmonidae	*Prosopium coulterii*	lentic/lotic
Arctic grayling	Salmonidae	*Thymallus arcticus*	lotic
Longnose sucker	Catostomidae	*Catostomus catostomus*	lentic/lotic
White sucker	Catostomidae	*Catostomus commersonii*	lentic/lotic
Largescale sucker	Catostomidae	*Catostomus macrocheilus*	lotic
Peamouth	Leuciscidae	*Mylocheilus caurinus*	lentic
Northern pikeminnow	Leuciscidae	*Ptychocheilus oregonensis*	lentic (littoral)/lotic (large, slow)
Redside shiner	Leuciscidae	*Richardsonius balteatus*	lotic/lentic
Lake chub	Leuciscidae	*Couesius plumbeus*	lentic
Longnose dace	Leuciscidae	*Rhinichthys cataractae*	lotic
Burbot	Gadidae	*Lota lota*	lentic/lotic

Pelagic Fish Species of the Williston Reservoir

The fish community assemblage in the Williston Reservoir has changed dramatically over the last 50 years (Barrett & Halsey, 1975; Blackman, 1992; Pillipow & Langston, 2002; Sebastian et al., 2003; Stockner et al., 2005), and recent surveys indicate it is still quite dynamic (DWB Consulting Services Ltd., 2019, 2020, 2021; Langston, 2012; Plate et al., 2012; Sebastian et al., 2009). The Williston Reservoir converted more than 600 km of mainstem and large river tributary habitat to reservoir environment and changed the fish community composition in favour of species adapted to lacustrine habitats (Blackman, 1992). The littoral zone in the reservoir is essentially abiotic because extensive annual drawdowns for hydroelectric power limit the growth of aquatic plants and associated aquatic insect communities. As a result, plankton is the primary food source in this ultra-oligotrophic system (Blackman, 1992; Stockner et al., 2005). The Williston Reservoir's fish community has historically been dominated by planktivores, but species

composition and relative abundances have fluctuated in the decades since impoundment (Barrett & Halsey, 1975; Blackman, 1992; Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2003, 2009). Recent surveys indicate that lake whitefish, kokanee, and peamouth are the most abundant pelagic fish in the Williston Reservoir (O'Connor, 2022; Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2003, 2009). Piscivorous species in the reservoir are much less abundant in the pelagic zone and include rainbow trout, bull trout, and lake trout (O'Connor, 2022; Pillipow & Langston, 2002; Sebastian et al., 2003, 2009). Bull trout and lake trout are apex predators in the reservoir and exist at very low densities. Lake trout and bull trout populations have fluctuated since the reservoir was created (Barrett & Halsey, 1975; Blackman, 1992; Pillipow & Langston, 2002; Sebastian et al., 2003), and lake trout are a potential threat to the long-term persistence of bull trout in pelagic habitats (Hagen & Weber, 2019). Recent pelagic species inventories of the Williston Reservoir have been completed approximately every 8-10 years (O'Connor, 2022; Pillipow & Langston, 2002; Sebastian et al., 2009). The status of the pelagic fish community is largely unknown in the intervals between inventory years, but other studies have suggested that significant changes in population dynamics can occur over shorter time spans. For example, aerial enumerations of kokanee spawning in the Williston Watershed suggested that kokanee abundance declined sharply in the years before the 2021 pelagic survey (DWB Consulting Services Ltd., 2019, 2020, 2021), in contrast to findings from the pelagic survey (Weir et al., 2023). The most abundant pelagic species documented in the Williston Reservoir and the population changes observed in past surveys are summarized in the following sections.

Lake Whitefish

Lake whitefish were the dominant species in fisheries inventories of the Williston Reservoir in 1974, 1988, and 2000 (Blackman, 1992; Pillipow & Langston, 2002; Sebastian et al.,

2003), but were overtaken by kokanee in 2008 and 2021 (O'Connor, 2022; Sebastian et al., 2009). Lake whitefish have an optimal temperature range of 8 to 14°C (McPhail, 2007), and pelagic adults are known to actively select depths where water temperatures are below 16°C (Gorsky et al., 2012). Juvenile lake whitefish are more tolerant of warmer temperatures than adults and may occupy water as warm as 19.5°C (McPhail, 2007). Divergence into limnetic and benthic forms has been described in British Columbia and the Yukon (McPhail, 2007; Roberge & Slaney, 2001). Limnetic lake whitefish feed on zooplankton near the surface and are known to exhibit diel vertical migrations throughout the spring and summer (Gorsky et al., 2012). Lake whitefish in the Williston Reservoir appear to prefer near-surface habitats in summer (< 7.5 m) and have been caught in similar numbers in surface nets set in both near and off-shore areas (Sebastian et al., 2003). Surveys in the Peace Reach have detected lake whitefish at both shallow (< 15 m) and mid-water depths (15-30 m) at night (O'Connor, 2022; Sebastian et al., 2003, 2009).

Kokanee

Kokanee are native to Thutade Lake at the headwaters of the Finlay River, and to Arctic and Tacheeda Lakes in the headwaters of the Parsnip River. A small population of native kokanee established themselves in the Williston Reservoir shortly after impoundment (Langston, 2012) and recent genetic research has identified the Thutade Lake population as their source (Wilson & Shrimpton, 2020). Kokanee originating from Hill Creek (Arrow Reservoir) and Meadow Creek (Kootenay Lake) of the Columbia River were introduced into the Williston Reservoir between 1990 and 1998 (Langston & Murphy, 2008; Langston, 2012). Kokanee populations increased dramatically following the introduction of non-native stocks to the reservoir. Pelagic fish surveys in 2008 and 2012 identified kokanee as the new dominant planktivorous species in the reservoir, surpassing lake whitefish (Plate et al., 2012; Sebastian et al., 2009). Aerial enumerations identified

a sharp increase in the number and distribution of spawning kokanee between 2002 to 2006 (Langston, 2012) to an apparent peak abundance in 2010 when kokanee were observed spawning in the majority of tributaries to the Williston Reservoir (DWB Consulting Services Ltd., 2019). Recent spawner enumerations suggested that kokanee populations have declined since 2010 (DWB Consulting Services Ltd., 2019, 2020, 2021). In contrast, the pelagic survey conducted in 2021 suggests kokanee abundance in the reservoir may be double that of the 2008 survey (Weir et al., 2023).

Kokanee behaviour and distribution in the water column is characterized by diel vertical migrations that balance feeding opportunities and predation risks. In many lakes and reservoirs, kokanee ascend to the food rich middle and upper strata of the water column to feed on zooplankton during low light periods at dusk and dawn, remaining in shallow waters overnight. At dawn, kokanee descend to depths of 60 metre or more where they remain during the day to avoid predators (Clark & Levy, 1988; Hardiman et al., 2004; McPhail, 2007; Scheuerell & Schindler, 2003). All age groups of rearing kokanee (ages 0-4) are found almost exclusively in the pelagic zones of the Williston Reservoir (Sebastian et al., 2009) and have an optimal temperature range of 6 to 13°C (McPhail, 2007). Despite these temperature preferences, kokanee in the Williston Reservoir have been particularly vulnerable to capture in overnight gillnets set at the surface (0-2.4 m). Catch rates have declined steeply in nets set below 5 m, although deep gillnetting sets have rarely been used in pelagic surveys of the Williston Reservoir (O'Connor, 2022; Sebastian et al., 2003, 2009).

Peamouth

Peamouth travel in schools and juveniles are often abundant in shallow weedy zones of lakes (McPhail, 2007). Little is known about peamouth temperature preferences but they appear

to be more tolerant of warmer temperatures than other pelagic species (McPhail, 2007; Roberge & Slaney, 2001). Adults are known to utilize the pelagic habitats of the Williston Reservoir and have rivaled lake whitefish and kokanee for abundance in fisheries surveys between 1988 and 2012 (Blackman, 1992; Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2009). In the summer months, peamouth exhibit a diel migration pattern between surface and inshore waters in the evenings to deeper, offshore habitats in the morning (McPhail, 2007). Peamouth in the Williston Reservoir are abundant near the bottom in near-shore habitats (Barrett & Halsey, 1975; Blackman, 1992). Pelagic surveys have caught high numbers of peamouth near the surface in gillnets (Pillipow & Langston, 2002), but trawling and gillnetting results suggest that they are not abundant in the pelagic zone at depths greater than 5 m (Sebastian et al., 2003). Peamouth are a member of the minnow family which has recently undergone a taxonomic reclassification from Cyprinidae to Leuciscidae (Mandrak et al., 2023; Schönhuth et al., 2018; Tan & Armbruster, 2018).

Rainbow Trout

Rainbow trout populations in the Williston Reservoir have declined since its creation (Blackman, 1992; Plate et al., 2012) and now only constitute a small portion of the pelagic fish community (Sebastian et al., 2009). In large lakes and reservoirs, rainbow trout usually remain within 50 m of shore and below the 18°C isotherm (McPhail, 2007). Sub-adult and adult rainbow trout have been caught in the pelagic zone of the Williston Reservoir, but most frequently near the surface (< 7.5 m) in transition zones between near-shore and off-shore habitat (Sebastian et al., 2003, 2009).

Bull Trout

Bull trout in large, oligotrophic lakes may forage in the littoral zone during spring and fall, but avoid water temperatures above 15 °C in summer by moving below the thermocline and into the pelagic zone (McPhail & Baxter, 1996). Large bull trout in the Williston Reservoir prey heavily on kokanee (Hagen & Weber, 2019) but constitute a small proportion (< 5 % in 2008) of the pelagic fish community (Sebastian et al., 2009). Bull trout in the Williston Reservoir are part of the Western Arctic population, which is listed as a species of Special Concern under the federal Species at Risk Act (Species At Risk Public Registry, 2019). Bull trout are a provincially blue listed species in B.C. (B.C. Conservation Data Center, 2021) due to their low abundance and high vulnerability to a variety of threats. Although bull trout abundance in the Parsnip Reach appears to have declined recently, populations in the Peace Reach appear to be stable and populations in the Finlay Reach appear to have increased in abundance (Hagen & Weber, 2019). Bull trout have been caught in gillnets set at all depths from the surface to 30 m, but have been caught most abundantly in surface nets (< 7.5 m) (Sebastian et al., 2003).

Lake Trout

Lake trout are native to the Upper Peace Watershed and colonized the Williston Reservoir naturally by dispersing from populations upstream (Hagen & Weber, 2019). Lake trout prefer cool water and will thermoregulate by selecting depths with temperatures below 10 °C (Hardiman et al., 2004; McPhail, 2007). Recent acoustic tagging studies have determined that lake trout make extensive seasonal migrations throughout the Williston Reservoir ranging between 50 to 115 km in length and remain at depths below the thermocline (between 20-30 m) in summer (Culling & Euchner, 2018). Lake trout are top predators that prey heavily upon kokanee and lake whitefish but have not been considered a major contributor to the reservoir's pelagic fish community (< 1 %

in 2008) in the past (Blackman, 1992; Sebastian et al., 2003, 2009). Lake trout data have been nearly or completely absent from most of the past pelagic surveys in the Williston Reservoir because gillnets have rarely been set at depths below 20 m (Sebastian et al., 2003) and large piscivores are often able to evade trawl nets (Bethke et al., 1999; Olin et al., 2009). Some researchers have speculated that lake trout populations may have experienced a recent increase but better monitoring is needed to understand how their populations are changing (Culling & Euchner, 2019; Hagen & Weber, 2019).

Knowledge Gaps in the Williston Watershed

The Williston Reservoir is an extraordinarily large and heavily perturbed lacustrine ecosystem that has experienced dramatic physical and ecological changes since its creation. Several studies have provided critical information on the ecology of the fish community in the Williston Reservoir but substantial knowledge gaps still exist (Fish and Wildlife Compensation Program, 2020). Monitoring of introduced kokanee revealed a rapid increase in their abundance and spawning range throughout the reservoir to its apparent peak in 2010 (DWB Consulting Services Ltd., 2019, 2020, 2021; Langston, 2012). The influence that fluctuations in kokanee abundance have had on the fish community in the Williston Reservoir and their causes are not well understood. Kokanee, lake whitefish and peamouth occupy a similar ecological niche in the reservoir and changes in the distribution and abundance of one species will undoubtedly affect the others. Kokanee are also an important forage species for lake trout and bull trout, and may have competitive interactions with stream dwelling species.

Lake trout and bull trout are top predators in the reservoir with a high degree of niche overlap, including their preference for other salmonids as a prey source (Culling & Euchner, 2019; Donald & Alger, 1993; Hagen & Weber, 2019; Johnson & Martinez, 2000; McPhail & Baxter,

1996; Sebastian et al., 2009). Lake trout are the more dominant of the two species and can displace native bull trout in lakes and reservoirs after a relatively short period of co-existence (Donald & Alger, 1993; Fredenberg, 2002). Hagen and Weber (2019) completed a comprehensive information synthesis on the conservation status of bull trout in the Williston Reservoir and identified a potentially increasing lake trout population as a threat. A better understanding of lake trout abundance and habitat use is a major information gap for this system. The structure and complexity of the aquatic food web is an important factor influencing the outcomes of lake trout and bull trout competition (Fredenberg, 2002).

Overall, there is limited information available on historical fish abundance and distribution within the tributary streams of the Williston Reservoir (Fish and Wildlife Compensation Program, 2020). The inventories and life history studies conducted in the Williston Watershed have focused largely on Arctic grayling (e.g., Blackman, 2002; Cowie & Blackman, 2003, 2004; Stamford et al., 2020, 2022; Strohm et al., 2020), bull trout (e.g., Hagen et al., 2020; Hagen & Spendlow, 2019; Hagen & Weber, 2019), and kokanee (e.g., Coxson et al., 2018; DWB Consulting Services Ltd., 2019, 2020, 2021; Wilson & Shrimpton, 2020). However, large-scale inventories of entire fish communities have rarely been completed, and the status and trends of many fish species are unknown.

The Fish and Wildlife Compensation Program (FWCP) is a partnership between BC Hydro, the province of British Columbia, Fisheries and Oceans Canada, Indigenous Nations and public stakeholders with a mandate to conserve and enhance fish and wildlife in the upper Peace River Basin Ecosystem (Fish and Wildlife Compensation Program, 2020). A few of the research priorities for the FWCPs Peace Region Rivers, Lakes and Reservoirs Action Plan include:

- to better understand the relationships between reservoir productivity, kokanee, lake trout and other fish populations;
- to develop a cost-effective method for monitoring lake trout populations to understand the potential competitive effects on bull trout;
- to conduct research on priority, but lesser-known, fish populations related to conservation status, critical habitats, and key limiting factors; and
- work with Indigenous Nations and stakeholder communities to characterize culturally important fish species in priority freshwater habitat locations.

Conventional Survey Approaches for Pelagic Fish in the Williston Reservoir

Fish inventories of the Williston Reservoir since 2000 have assessed the distribution and abundance of pelagic species with a combination of hydroacoustic surveys and gillnetting (O'Connor, 2022; Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2003, 2009). Mid-water (5-35 m) trawl surveys were included in the 2000 and 2021 surveys, but not in any other survey before or after. An accurate and complete picture of the fish community in the Williston Reservoir has been difficult to produce due to the limitations of conventional survey methods and an apparent focus on the most abundant species and the shallow water habitats they frequent. Hydroacoustic surveys have had difficulty distinguishing between similarly sized species that occur together in the water column (Sebastian et al., 2009). Lake whitefish, kokanee, and peamouth in the Williston Reservoir have overlapping size ranges and are abundantly distributed in surface and mid-water depths to 30 m during night surveys. Bull trout and lake trout are also difficult to differentiate because of similarities in body sizes and distributions. Gillnets have primarily been set at shallow depths to target abundant species such as lake whitefish, kokanee and peamouth. Bull trout have been caught in surface gillnets but lake trout were not caught in a

gillnet in a pelagic survey between 1990 and 2012 (Blackman, 1992; Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2003, 2009). Mid-water (5-35 m) trawling in 2000 did not capture any bull trout and only one lake trout (Sebastian et al., 2003). Bull trout and lake trout are both assumed to be relatively rare in the Williston Reservoir, but pelagic surveys have poorly defined their relative abundances despite the potential value of this information for managing these high priority species. Fisheries inventories of the Williston Reservoir would benefit greatly from survey methods with improved species resolution and detection sensitivity at a reasonable cost.

Conventional Survey Approaches in Streams and Rivers

A variety of survey techniques have been applied in the tributaries around the Williston Reservoir including electrofishing (Coxson et al., 2018; Stamford & O'Connor, 2022), snorkel surveys (Cowie & Blackman, 2003, 2004; Strohm et al., 2020), beach seines (Blackman, 2002; Stamford & O'Connor, 2022), redd counts (Hagen & Spendlow, 2019), angling (Stamford & O'Connor, 2022), and aerial enumeration by helicopters (DWB Consulting Services Ltd., 2019, 2020, 2021; Langston, 2012). Stream surveys have often targeted a few focal species for a specific purpose, but inventories that include all species across a wide range of habitats have rarely been completed and would be very difficult and costly to do with conventional survey methods.

CHAPTER 2:
DEVELOPMENT AND VALIDATION OF SPECIES-SPECIFIC eDNA ASSAYS FOR PELAGIC FISH IN THE WILLISTON RESERVOIR

INTRODUCTION

The Williston Reservoir is an extraordinarily large and heavily perturbed lacustrine ecosystem that has experienced dramatic physical and ecological changes since its creation in 1968. Recent surveys have found that kokanee, lake whitefish and peamouth, all planktivores, are the most abundant fish species in the pelagic zone of the Williston Reservoir. Piscivorous species are much less abundant in the pelagic zone, and include rainbow trout, bull trout and lake trout (O'Connor, 2022; Plate et al., 2012; Sebastian et al., 2009). An accurate and complete picture of the fish community in the Williston Reservoir has been difficult to produce due to the challenges associated with studying such a large inland waterbody, the limitations of conventional survey methods, and an apparent focus on the most abundant species. Fish inventories of the Williston Reservoir since 2000 have assessed the distribution and abundance of pelagic species with a combination of hydroacoustic surveys, gillnetting and trawls (Pillipow & Langston, 2002; Plate et al., 2012; Sebastian et al., 2003, 2009). These types of conventional pelagic surveys are costly, have occurred relatively infrequently, and poorly characterize the status of rare, difficult to capture species such as bull trout and lake trout. The shortcomings of past approaches to fisheries inventories have been recognized and addressed in recently updated research priorities for the Williston Reservoir (Fish and Wildlife Compensation Program, 2020). Fisheries priorities include a better understanding of relationships between kokanee and lake trout, maximizing the population viability of bull trout through monitoring and developing a cost-effective method for monitoring lake trout populations.

Organisms continuously shed DNA into their surrounding environments in the form of fur, scales, skin, mucus, waste, and other tissues. The genetic material that an organism leaves behind in its environment is referred to as environmental DNA or eDNA. The eDNA of aquatic animals can be collected in a water sample, extracted, and analyzed to identify the species that occur in those habitats without the need to observe or capture them (Thomsen et al., 2012; Thomsen & Willerslev, 2015). A useful feature of eDNA in aquatic environments is its smoother and more continuous distribution in space and time than the organisms themselves (Shelton et al., 2019). At the same time, eDNA either settles out of the water column (Canals et al., 2021) or degrades over a period of days to weeks (Rourke et al., 2021; Takahara et al., 2012). The rapid degradation of eDNA in fresh water means that detections indicate the recent presence of an animal, and potentially misleading signals from the past are generally avoided (Thomsen & Willerslev, 2015). One of the first successful applications of eDNA was in the early detection of invasive species (Jerde et al., 2011). Many studies have demonstrated that eDNA can detect rare or cryptic aquatic species with higher sensitivity than conventional sampling gears, and often at a lower cost (Deiner et al., 2017; Evans, Shirey, et al., 2017; Jerde et al., 2011; Jerde, 2019; Thomsen et al., 2012). Early eDNA research focused on detecting species presence, and determining the distributions and habitat preferences of a species (Thomsen & Willerslev, 2015). Over a decade ago, researchers began to explore the correlation between aquatic species abundance and/or biomass and eDNA concentrations (Takahara et al., 2012). Since then, a great deal of research has investigated the correlation between fish abundance and/or biomass and eDNA concentrations to improve the accuracy and precision of eDNA methods that may soon be a cost-effective alternative, or at least a valuable supplement, to traditional inventory methods (Rourke et al., 2021).

There is tremendous potential for eDNA in the upper Peace River Watershed and molecular methods for species detection have already been used successfully to monitor the distribution of Arctic grayling in tributaries of the Williston Reservoir (Stamford et al., 2020, 2022; Strohm et al., 2019). Detecting fish species using eDNA methods, rather than directly sampling them, can reduce impacts on sensitive species and increases the power of field surveys for rare and elusive species (Goldberg et al., 2016). eDNA methods involve the careful design and validation of molecular markers, thoughtful sampling design, care in avoiding contamination, complex laboratory procedures, and an interpretation of data that requires careful consideration of multiple factors. There are several methods used to detect eDNA but the most accessible and simplest to use is a method known as quantitative polymerase chain reaction (abbreviated as qPCR). Developing a robust, species-specific, qPCR based assay for eDNA detection takes a great deal of time and expertise to do properly (Langlois et al., 2021), and this may discourage the use of eDNA in situations where it would otherwise be appealing. Fortunately, eDNA research has developed an ever-expanding catalogue of qPCR assays, and groups like the Genomic Network for Fish Identification, Stress and Health (GEN-FISH) are working towards the goal of developing and validating eDNA assays for the detection all freshwater fish species in Canada (GEN-FISH, 2020). However, the performance of published qPCR assays is dependent upon a variety of factors and assays must be tested and optimized when being used by a different laboratory, with different reaction conditions (Bustin & Huggett, 2017; Klymus et al., 2020), and when used in a different geographic region than for which they were originally designed (Goldberg et al., 2016; Thalinger et al., 2021). The results of an eDNA study can be interpreted with confidence after the entire workflow used to detect a species' DNA from an environmental sample has been validated (Thalinger et al., 2021).

In this study, I sought to pilot targeted eDNA methods for surveying the pelagic fish community in the Williston Reservoir. This included the validation of previously published qPCR assays for kokanee, rainbow trout, lake whitefish and bull trout as well as the design and validation of novel qPCR assays for lake trout and peamouth. Assays were validated in the lab and on environmental samples that were collected from the Finlay Reach of the Williston Reservoir. eDNA sampling in the Finlay Reach was conducted concurrently with a pelagic fisheries inventory of the entire Williston Reservoir in the summer of 2021. The eDNA results were compared to the gillnet catch results in the Finlay Reach.

The final validation state of each qPCR assay was evaluated based on the criteria and principles described by Thalinger et al. (2021) to determine if eDNA assays are ready for use in routine species monitoring. Five levels of the validation scale incorporate the entire workflow used to detect a species' DNA in an environmental sample, from sample collection through to the interpretation of PCR results. Levels 1 and 2 include assay design, basic *in silico* analysis, *in vitro* optimizations, testing on target species tissue and specificity testing on closely related, non-target species. eDNA results from assays that have only been validated to level 2 cannot be interpreted with confidence until further levels of validation have been achieved. Level 3 and 4 validation criteria includes testing on environmental samples (*in situ* validations). More extensive specificity testing that includes co-occurring non-target species and sensitivity testing that establishes detection limits are considered important criteria for assay validation at level 4. Level 5 validation includes statistical modelling of detection probability and investigations of ecological and physical factors that may influence eDNA in the environment. Level 3 validation is considered essential and the results from assays validated to level 4 and 5 can be interpreted with confidence (Thalinger et al., 2021).

METHODS

Creating a Mitochondrial DNA Sequence Database

A diverse assemblage of fishes belonging to four orders and five families inhabit the Williston Watershed. I created a reference sequence database of mitochondrial genes for 19 species (Table 1. 1) that have been identified in the Williston Reservoir and its surrounding watershed (Province of British Columbia, 2020; Sebastian et al., 2009). Available sequences from the cytochrome oxidase subunit 1 (COI) gene, NADH dehydrogenase subunit 1 (NADH) gene, and cytochrome b (CYTB) gene were downloaded from the National Center for Biotechnology Information's (NCBI) Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide/) and imported to Geneious Prime version 2021.2.2 (www.geneious.com). Sequences were organized in Geneious Prime by gene and by species and aligned using the default parameters in the MUSCLE alignment algorithm. Intraspecific genetic variation was incorporated into a consensus sequence for each species by coding polymorphic sites with International Union of Pure and Applied Chemistry (IUPAC) codes. Published assays for lake trout, bull trout, rainbow trout, kokanee, lake whitefish and peamouth were evaluated based on the alignments of consensus sequences.

In Silico Screening of Published Assays

Published qPCR assays were available for lake trout, bull trout, kokanee, rainbow trout, and lake whitefish but not for peamouth. Published lake trout assays were only available for the COI gene (Hernandez et al., 2020; Lacoursière-Roussel, Rosabal, et al., 2016) and were a nearly complete match with bull trout DNA. Candidate assays were screened for potential cross-amplification of non-target DNA from species in the Williston Watershed. Primer and probe sequences for each assay were compared against the consensus sequence alignments of the 19 fish species from Table 1. 1 using Geneious Prime. The minimum criteria for *in silico* specificity were

two mismatches with all non-target species in at least two of the three component sequences (the two primers, or a primer and a probe) and at least one mismatch in the other (Currier et al., 2018). Published assays for bull trout, kokanee, rainbow trout and lake whitefish were selected for *in vitro* validation based on these criteria (Table 2. 1).

Table 2. 1. Published qPCR assays for bull trout, kokanee and rainbow trout selected for validation and novel assays for lake trout and peamouth that were developed in this study for use in the Williston Watershed.

Species and Assay Name	Target Gene	Primers/ Probe	Sequence 5' to 3'	Amplicon length (bp)	Citation
Kokanee	COI	Forward	GGAAACCTTGCCCACGCG	152	Rasmussen Hellberg et al. (2010)
Sock_COI		Reverse	AAAAGTGGGGTCTGGTACTGAG		
		Probe	CTCTGTTGACTTAACCATC		
Rainbow trout	COI	Forward	ACCATTATTAACATAAAACCTCCAG	121	Rasmussen Hellberg et al. (2010)
Rain_COI		Reverse	GTAATGCCTGCTGCCAGGA		
		Probe	CGTTTGAGCCGTGCTA		
Lake whitefish	CYTB	Forward	CAAACCTCCTTTCTGCCGTG	198	Hernandez et al. (2020)
COCL_CYTB		Reverse	AGTTGATCCCTGCTGGGTTG		
		Probe	TTGTGCAGTGAATCTGA		
Bull trout	CYTB	Forward	AGTACTTCACCTTCTGTTTCTGCATG	134	Wilcox et al. (2013)
BUT1_CYTB		Reverse	CAATATAGCTACGAAACCGAGGAGG		
		Probe	CCGACAAAATCTCA		
Lake trout	CYTB	Forward	GCCTTCCACTTCCTATTCCCA	191	This study
LT_PCC		Reverse	GCTAGGGTTGTTAGGCCAAGTAG		
		Probe	ACGCCGATAAAATCTCGTTCCACCC		
Peamouth	CYTB	Forward	GCACTCACATCACTGACATTG	165	This study
PCC_CYTB		Reverse	AGGACCCCTCCTAATTTGTTC		
		Probe	TACTCCCCCACACATCCAACCAGAG		

Novel Assay Design

I designed novel qPCR assays to detect lake trout and peamouth (Table 2. 1). Both assays were developed for the CYTB gene due to its divergence among species in the Williston

Watershed. The CYTB gene was the only mitochondrial gene examined with enough sequence divergence for a targeted assay that could discriminate between lake trout and bull trout. Integrated DNA Technologies' (IDT) PrimerQuest Tool (https://www.idtdna.com/) was used as a starting point for designing qPCR primers and probes. The primer and probe sets generated by the PrimerQuest Tool were transferred to Geneious Prime for assessment against target and non-target species sequences. The IDT generated assays identified promising regions for primers and probes and were modified to optimize specificity.

Assay specificity was achieved primarily by maximizing the number and influence of base-pair mismatches between target and non-target species (Currier et al., 2018; Klymus et al., 2020; So et al., 2020). The number of mismatches between primer and probe sequences is generally considered proportional to an assay's specificity, but the effect of a mismatch can vary depending on its type and position (Klymus et al., 2020; So et al., 2020; Wilcox et al., 2013). Mismatches were prioritized in the primer sequences because they influence assay specificity more than mismatches in the probe (Wilcox et al., 2013). Mismatches were positioned as close as possible to the 3' end of primers and as close as possible to the 5' end of probes to maximize their effect (Klymus et al., 2020; Langlois et al., 2021; So et al., 2020; Wilcox et al., 2013). Purine-purine mismatch pairings (i.e., A-A, A-G, or G-A) and C-C mismatch pairings, referred to as "critical" mismatches by So et al. (2020), were incorporated into primers and probes where possible because they reduce amplification efficiency of non-target DNA more than other mismatch types. Optimizing the influence of mismatches was particularly important for the lake trout assay because it was not possible to incorporate more than two base pair mismatches with bull trout into any of the primers or probes (Table 2. 2).

Multiple candidate primer and probe sequences for each species were assessed individually and in combination for characteristics and interactions that could affect assay sensitivity, PCR conditions and efficiency, and that may have had some additional impact on specificity (Table 2. 4 and Table A3). Primer and probe melting temperatures (T_m) were analyzed using IDT's OligoAnalyzer Tool (https://www.idtdna.com/) with the default parameter settings for qPCR (Klymus et al., 2020; Prediger, 2013). Forward and reverse primers were designed to have a T_m between 60-64 °C and to be within 2 °C of one another. Probe melting temperatures were designed to have a T_m 4-8 °C higher than the primers. The GC content of primers and probes were kept between 35-65%, and regions with 4 or more consecutive Gs were avoided. The number of Gs and Cs in the last 5 bases of the 3' end of primers, known as a GC clamp (Thornton & Basu, 2011), were noted for their potential influence on primer binding and assay specificity, but was considered less important than incorporating mismatches. G bases were avoided at the 5' end of probes because of their potential to dampen the signal from green and yellow dyes (Klymus et al., 2020; Prediger, 2013). Probes were designed to be compatible with IDT's double quenched probes (3IABkFQ and ZEN) and FAM or HEX fluorophores (https://www.idtdna.com/). Amplicon lengths were kept between 100 and 250 base pairs to maintain high PCR efficiency while also allowing for verification by Sanger sequencing (Hernandez et al., 2020; Klymus et al., 2020).

Table 2. 2. Primer and probe mismatches of the *LT_CYTB* assay with closely related, non-target species from the Williston Watershed. Mismatches between the primer and probe and non-target species sequences are underlined and coloured. Critical mismatches (So et al., 2020) are coloured red and other mismatches are coloured blue.

Assay/Species	F-primer Mismatches (5'-3')	R-primer Mismatches (5'-3')	Probe Mismatches (5'-3')
LT_CYTB	GCCTTCCACTTCCTATTCCCA	GCTAGGGTTGTTAGGCCAAGTAG	ACGCCGATAAAATCTCGTTCCACCC
Bull trout	GCCTTTCACTTCCTATTCCCT	GCTAGGGTTGTTAGGCCAAGCAA	ACGCCGACAAAATCTCATTCCACCC
Lake whitefish	GCCTTTCACTTCTTATTCCCC	GCCAAGGATGTTAGTCCTAGTAG	ATGCCGATAAAATCTCATTCCACCC
Kokanee	GCCTTTCACTTCCTGTTCCCT	GCTAAGGATGTTAGACCAAGAAG	ATGCCGATAAAATCTCGTTCCACCC

Table 2. 3. Primer and probe mismatches of the *PCC_CYTB* assays with closely related, non-target species from the Williston Watershed. Mismatches between the primer and probe and non-target species sequences are underlined and coloured. Critical mismatches (So et al., 2020) are coloured red and other mismatches are coloured blue. Mismatches with polymorphic sites (for longnose dace) were also noted, although they may not always be a mismatch.

Assay/Species	F-primer Mismatches (5'-3')	R-primer Mismatches (5'-3')	Probe Mismatches (5'-3')
PCC_CYTB	GCACTCACATCACTGACATTG	AGGACCCCTCCTAATTTGTTC	TACTCCCCCACACATCCAACCAGAG
Redside shiner	GCTCTTACATCACTAACTCTA	AGGACTCCCCCCAGTTTGTTT	TACCCCACCACATATCCAACCAGAG
N. pikeminnow	GCCCTTACATCATTAGCACTA	AGGACCCCTCCTAGTTTATTT	CACTCCCCCACATATCCAGCCTGAA
Lake chub	GCTCTCACATCATTAGCCCTA	AGAACCCCTCCCAGCTTGTTA	CACCCCGCCACACATCCAACCGGAG
Longnose dace	RCTCTCRCATCCCTYACRYTA	AGKACYCCTCCYARYTTGTTW	TACCCCRCCRCACATCCARCCRGAR

The potential for secondary structure formation between primers and probe was assessed with the OligoAnalyzer Tool (Integrated DNA Technologies, 2021). Hairpin structures were ignored if a predicted T_m was lower than the anticipated PCR annealing temperatures. Primers and probes that produced hairpin structures with a T_m at or above the anticipated annealing temperature were rejected or redesigned. All possible combinations of dimer structures between primers and probes were analyzed and evaluated with respect to the difference in free energy, or ΔG. Dimer structures with a predicted ΔG of -9 kcal/mol or greater were considered problematic. Primers and probes that had the potential to create strong dimer structures were redesigned if possible. In situations where primer or probe design was constrained by other criteria, the potential for secondary structures was weighted against other priorities, such as assay specificity. Multiple candidate assays were designed and tested for each species, but the body of this study only describes the successful assays that were validated on environmental DNA. The unsuccessful assay candidates are described in Appendix B.

Table 2. 4. Characteristics and secondary structure analysis of the *LT_CYTB* and *PCC_CYTB* assays as calculated with IDT's OligoAnalyzer Tool.

					Dimer Structures ΔG (kcal/mol)			
Assay	Primer/ Probe	Sequence (5'-3')	T_m (°C)	Hairpin T_m (°C)	Self	F/R Primers	F/ Probe	R/ Probe
*LT_*	Forward	GCCTTCCACTTCCTATTCCCA	63.7	None	-3.14	-7.81	-3.42	-7.48
CYTB	Reverse	GCTAGGGTTGTTAGGCCAAGTAG	64.2	52.8	-9.28			
	Probe	ACGCCGATAAAATCTCGTTCCACCC	68.2	36.4	-5.19			
*PCC_*	Forward	GCACTCACATCACTGACATTG	61.4	33.8	-3.53	-3.42	-3.9	-4.64
CYTB	Reverse	AGGACCCCTCCTAATTTGTTC	61.5	50.6	-6.24			
	Probe	TACTCCCCCACACATCCAACCAGAG	68.7	25.2	-3.17			

Final screening of assays included testing through NCBI's Primer-Blast program (Ye et al., 2012) to identify potential non-targets in the NCBI nt/nr database that might amplify with the assay. Any relevant non-target species that were identified as being a close match were included in *in vitro* specificity testing.

In Vitro Assay Validation

Assay Optimization

The published *Sock_COI*, *Rain_COI*, *COCL_CYTB*, and *BUT1_CYTB* primer/probe sets were purchased from Applied Biosystems (https://www.thermofisher.com) in the TaqMan format with Minor Groove Binding (MGB) probe chemistry and VIC fluorophores. The novel primers and probes for lake trout (*LT_CYTB*) and peamouth (*PCC_CYTB*) were purchased from IDT in the PrimeTime® qPCR Assay format with double quenched probes (3IABkFQ and ZEN) and FAM fluorophores (Integrated DNA Technologies Inc., Coralville, IA). Assays were ordered with default primer and probe concentrations and were added to all PCR reactions at a final 1x concentration. The 1x concentrations for TaqMan assays were 900 nM of primers and 250 nM of probe. The 1x concentrations for IDT assays were 500 nM primers and 250 nM probe.

Optimal PCR annealing temperatures were determined for each qPCR assay by running a temperature gradient test on the Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) at eight annealing temperatures between 50 and 60 °C. Assays were tested against synthetic DNA templates (Integrated DNA Technologies Inc.)(Appendix A).

Assay Specificity

All six of the assays in Table 2. 1 were tested for amplification of their target species and for cross-amplification of closely related and representative species from the Williston Watershed (Table 2. 5). Genomic DNA used for specificity testing was extracted from the fin tissues of 15 fish species with a DNeasy Blood and Tissue kit (Qiagen, Mississauga, ON) following the manufacturer's protocol. Assays were tested against extracted DNA from at least two individuals from non-target species in the same family. Some assays were tested against three to six samples from non-target species with a higher risk of cross-amplification due to factors such as a low number of primer mismatches (e.g., *LT_CYTB* assay and bull trout) (Table 2. 5). Assays were tested on the QX200 Droplet Digital PCR system in 20 µL final reaction volumes that included 10 µL of 2x ddPCR Supermix for Probes (no dUTPs) (Life Science, Mississauga, ON), 1 µL of 20x concentrated assay, 5 µL of extracted DNA (2-6 ng) and 4 µL of nuclease free water (IDT). The thermocycling protocols for all assays were: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 seconds and annealing at 58 °C for 60 seconds, followed by a final incubation at 98 °C for 10 min.

Table 2. 5. List of species whose extracted DNA was included in the specificity testing of each qPCR assay. Numbers represent the number of samples per species that each assay was tested against.

	Assay Name					
Species	***Sock_COI***	***Rain_COI***	***COCL_CYTB***	***BUT1_CYTB***	***LT_CYTB***	***PCC_CYTB***
Slimy sculpin			1	1	1	1
Lake trout	2	2	4	6	6	1
Bull trout	2	2	3	6	6	1
Rainbow trout	7	7	2	4	4	1
Kokanee	6	6	2	4	4	1
Lake whitefish	4	4	6	5	5	1
Mountain whitefish	4	4	2	3	3	1
Arctic grayling	2	2	1	2	2	1
White sucker			1	2	2	1
Peamouth chub	1	1	1	2	2	3
Northern pikeminnow	1	1	1	2	2	3
Redside shiner						3
Lake chub						3
Longnose dace						3
Burbot			1	1	1	1

Assay Sensitivity

Sensitivity was measured on the Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) with dilution series experiments. A synthetic DNA template (gBlock, Integrated DNA Technologies Inc.) of each target amplicon was used to create 1:5 standard serial dilutions at eight dilution points with six molecular replicates of each standard. Synthetic templates were based on consensus sequences generated in Geneious Prime from the NCBI sequence database. Use of synthetic templates enabled an accurate estimation of template concentrations. The gBlock sequences can also incorporate multiple species' templates into the same synthetic DNA molecule. A gBlock with multiple templates can be used in duplex reactions with two assays and the equimolar ratio of the templates is useful for evaluating the performance of both assays relative to one another. Targeted concentrations for each standard dilution point were between 10,000 and 0.13 copies/20 µL ddPCR reaction. However, the end-point ddPCR measurements of synthetic template concentrations varied for each batch of gBlock used. The standard

concentrations for each dilution series (Table A1) were calculated from the mean concentration of gBlock detected by ddPCR at the most concentrated dilution point and dividing by five for each subsequent standard. The limit of detection (LOD) was defined and assessed in two ways: standard and modeled LOD. Standard LOD was defined as the lowest concentration of target analyte that was detected with 95% confidence (Klymus et al., 2019). Detections were required in all six replicates of a dilution level with ddPCR to achieve 95% confidence level or better for this study. Modeled LOD was assessed with a statistical model for digital PCR developed by Hunter et al. (2017). The modeled LOD was defined as the lowest amount of analyte that could be both detected and distinguished from a source of overestimation bias in qPCR known as the concentration plateau; as DNA concentrations approach zero, measured concentrations may be higher than expected and result in a nonlinear standard dilution series (Hunter et al., 2017). The modeled LOD was determined by analyzing the dilution series data with R code provided by Hunter et al. (2017).

In Situ Assay Testing in the Williston Reservoir

eDNA Field Sampling

I sampled eDNA in the Finlay Reach of the Williston Reservoir near the Teare Creek and Factor Ross sampling stations established by previous pelagic fish surveys (Blackman, 1992; Pillipow & Langston, 2002; Sebastian et al., 2003, 2009). Teare Creek samples were collected at Latitude: 56.40, Longitude: -124.47 and Factor Ross samples were collected at Latitude: 56.59, Longitude: -124.65 (Figure 1. 1). The Teare Creek station was sampled on August 16, 2021 and the Factor Ross station was sampled on August 17, 2021. Sampling dates were chosen to coincide with summer stratification of the water column and to avoid periods when mature rainbow trout or bull trout and kokanee migrate out of the pelagic zone of the reservoir to spawn. Temperature and dissolved oxygen profiles were measured at Teare Creek from the surface to a depth of 50 m

to characterize and confirm lake stratification in the Finlay Reach at the time of sampling. The thermocline extended from 10 m depth down to 25 m (Figure 2. 1). Temperatures above 10 m ranged from 17.1 °C to 17.9 °C. Temperatures between 25 and 50 m depths ranged from 6.8 to 5.3 °C.

Water samples were taken at five depths dispersed between the surface and a maximum depth of 30 m at the Teare Creek and Factor Ross locations. Three 1-L replicate water samples were taken at each depth. Water was sampled at the surface by submerging sterilized plastic sample bottles until filled. Water was sampled at 5, 10, 20 and 30 m with a Van Dorn water sampler (Wildco, Yulee, FL) and then transferred into sterilized sample bottles. Each of the three 1-L sample replicates from a given depth were collected from separate deployments of the Van Dorn to that depth. Samples collected at the surface (0 m) and at depths of 5 and 10 m were taken from above the thermocline. Samples collected at 20 m were near the bottom of the thermocline where temperatures were approximately 10 °C. Samples collected from 30 m were below the thermocline where temperatures were between 5 and 6 °C (Figure 2. 1).

To prevent contamination, the Van Dorn sampler was cleaned before use at each site by submerging it in a bucket of 30% bleach, followed by submersion in two separate buckets of sterile fresh water to rinse away the bleach. Two 1-L bottles of distilled water were taken to and from the field as non-template control samples in the same cooler where water samples were stored. Water samples were stored in a cooler on the boat or in a fridge at 4 °C until they were filtered.

Water samples were filtered onto 47 mm, 1.5 µm pore glass microfiber filters (Cytiva, Marlborough, MA). Filters were placed in a Nalgene™ Single Use Analytical Filter Funnel (ThermoFisher Scientific) that was attached to a sidearm Erlenmeyer flask. Negative pressure to draw water through the filter quickly was created with a peristaltic pump (Geotech Environmental

Equipment Inc., Denver, CO) attached to the side arm of the flask. Teare Creek samples were filtered 24 hours after collection and Factor Ross samples were filtered within 5 hours of collection. Teare Creek sample filtration was delayed due to a windstorm that forced the field crew to seek shelter on the reservoir. Samples were kept in a cooler during the day, transferred to a fridge overnight and filtered the next morning. Filters were stored in individual coin envelopes and all three replicates from each depth were preserved together in plastic specimen bags filled with 90 g of self-indicating silica beads (Fisher Scientific, Ottawa, ON). All negative controls of distilled water were filtered, stored, and preserved in the same way as field samples from each location. Preserved filters were stored at room temperature for 72 hours and then transferred to a -80 °C freezer until DNA was extracted.

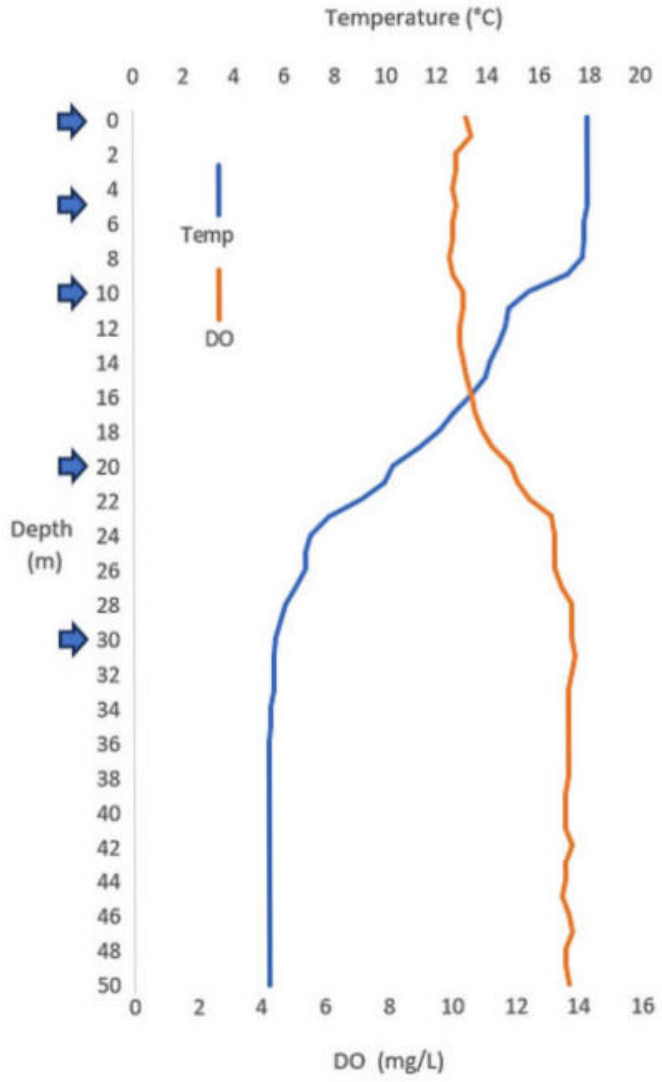

Figure 2. 1. Dissolved oxygen (DO) and temperature profile measured at the Teare Creek sample location on 16 August 2021. eDNA sample depths are indicated by arrows.

eDNA Extraction

DNA was extracted in a room dedicated to low quality DNA sources and a negative control was included with each batch of extractions to monitor for contamination. DNA was extracted from filters using a DNeasy Blood and Tissue kit. Filters were rolled and cut into 1 mm slices with sterile tweezers and scissors and then placed in a sterile 2 mL centrifuge tube with two 5/32-inch steel grinding beads. The tubes were shaken in a Genogrinder 2000 (SPEX SamplePrep, Metuchen,

NJ) at 1500 oscillations/minutes for 90 s. Tubes were centrifuged at 6000 x g for 1 minute before adding 870 µL of Buffer ATL and 30 µL of Proteinase K. Tubes were incubated at 56 °C with 150 rpm shaking for 30 min, followed by a second shake in the Genogrinder at 1000 oscillations/minute for 60 s. Tubes were incubated at 56 °C and 150 rpm shaking for another 90 min. After incubation, tubes were vortexed for 15 seconds and centrifuged for 3 minutes at 10 000 x g. Supernatant was transferred to a sterile 1.5 mL centrifuge tube. Buffer AL and 95% ethanol were added to the 1.5-mL centrifuge tube in volumes equal to the volume of the supernatant. The remainder of the extraction procedure followed the DNeasy Blood and Tissue Kit's spin column procedure with the modification of using 2 x 50 µL AE buffer for the final elution step. Eluted DNA samples were stored at -20 °C until used for polymerase chain reaction (PCR) analysis.

ddPCR Analysis of eDNA

Extracted eDNA samples were analyzed on a Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) according to the manufacturer's instructions (Bio-Rad Labratories, Inc., 2022). The 20 µL ddPCR reactions contained 1x ddPCR Supermix for Probes (Life Science, Mississauga, ON), 1 µL of each assay (primers and probe) at 20x concentration, and 5 µL of eDNA sample. Each reaction was partitioned into nanoliter sized water-in-oil micro-droplets by a Droplet Generator (Bio-Rad) and the prepared droplets were transferred into a 96-well PCR plate. The PCR plate was subsequently heat-sealed with pierceable foil using a PX1™ PCR plate sealer (Bio-Rad) and then amplified in a C1000 Touch™ deep-well thermal cycler (Bio-Rad). The thermocycling protocol was: initial denaturation at 95 °C for 10 minutes, then 45 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s, followed by a final incubation at 98 °C for 10 min. After cycling, the 96-well plate was placed into a Droplet Reader (Bio-Rad). Droplets of each sample were analyzed sequentially, and fluorescent signals of each droplet were

measured individually by a detector. The first elutions (i-elutions) of all eDNA samples were amplified in two PCR replicates with the *Sock_COI*, *Rain_COI*, *COCL_CYTB*, *BUT1_CYTB* and *LT_CYTB* assays. The *PCC_CYTB* assay only assessed a single PCR replicate of the second elution (ii-elution) of eDNA samples, due to the limited volume of sample available. Filtration and extraction negative controls were amplified with all assays and negative PCR controls were included on each plate by substituting nuclease free water (IDT) for DNA.

The potential for samples to produce false negative results was evaluated by testing for DNA integrity and inhibition. DNA integrity was evaluated by testing for plant DNA with the ePlant assay (i.e., IntegritE-DNA™; Veldhoen et al., 2016). Inhibition was evaluated by detecting a known quantity of Lambda DNA that was added to the ddPCR mastermix as a positive control with a Lambda assay (Murray, 2021). One replicate from each sample depth at the Teare Creek and Factor Ross locations were tested for integrity and inhibition in a single 20 µL final reaction volume. Each reaction included 10 µL of 2x ddPCR Supermix for Probes (no dUTPs) (Life Science, ON, Canada), 0.5 µL of 40x concentrated lambda assay, 0.5 µL of 40x concentrated ePlant assay (Veldhoen et al., 2016), 5 µL of the first elution of each eDNA sample, and 4 µL of nuclease free water spiked with lambda DNA at a concentration of 2 copies per µL. Integrity and inhibition tests used a similar thermocycling protocol as for other environmental samples but with an annealing temperature of 56 °C.

The minimum criterion for a positive species detection in eDNA samples was set at 3 positive droplets between all PCR replicates of a sample, following the recommendations from Bio Rad's Droplet Digital PCR Applications Guide (Bio Rad). Positive droplets were pooled from the two PCR replicates of each sample tested with every assay except *PCC_CYTB*. Species

detections were evaluated independently for each of the three eDNA sample replicates collected from each depth in the water column.

Gillnet Sampling

Pelagic gillnets were used to validate the eDNA results. Floating and sinking monofilament "experimental" gillnets were set at the Teare Creek Station between August 14 and 15, 2021 (O'Connor, 2022). Gillnetting was also planned for the Factor Ross Station, but many days of high winds prevented the survey team from getting out on the reservoir. Gillnets were set at other locations around the reservoir and the one set at Finlay Forks on August 4, 2021 was the closest to Teare Creek and Factor Ross (Figure 1. 1). Each net was comprised of six panels, 2.4 m deep and 15.3 m long connected end-to-end for a total length of 94.1 m. Stretch mesh sizes of the six panels were 25 mm, 76 mm, 51 mm, 89 mm, 38 mm, and 64 mm. At Teare Creek, one floating net was set at the surface and five sinking nets were set at 5-metre depth increments from the surface to a maximum depth of 25 m. At Finlay Forks, nets were only set at 5-metre increments between 5 and 20 m depth. Nets were deployed parallel to the shoreline and in locations where the depth of the reservoir was at least 70 m to avoid littoral species (O'Connor, 2022). High wind and waves on August 15 prevented the recovery of the Teare Creek gillnets until that evening, resulting in a 24-hour soak time. The high wind and waves during the day also released the anchor buoy that held the deep end of the chain of nets. This caused the net set at 25 m to sink and become entangled in sunken debris, ultimately preventing its recovery. All fish captured in the nets were identified to species, weighed, and measured for fork length.

RESULTS

In Silico Screening and Novel Assay Design

The published assays for kokanee, rainbow trout, lake whitefish and bull trout in Table 2. 1 were all successfully validated *in silico* for their application in the Williston Reservoir. The corresponding qPCR assays for kokanee and rainbow trout were designed to be specific enough to differentiate between one another as well as other sympatric salmonids. The *Sock_COI* and *Rain_COI* assays both had a total of seven mismatches with their congener and at least eight with all the other salmonids in the Williston Watershed. Primer mismatches were often closer to the 3' end and the *Sock_COI* assay and had a single critical mismatch with rainbow trout and lake whitefish near the 5' end of the forward primer. The *COCL_CYTB* lake whitefish assay had the fewest total mismatches with lake trout (seven) and bull trout (eight). Both *COCL_CYTB* primers had mismatches at the 3' ends with lake trout and bull trout, but no critical mismatches. The *BUT1_CYTB* bull trout assay had the fewest mismatches with lake trout, the other species within the genus *Salvelinus*: one mismatch at the fifth position from the 3' end of the forward primer, three mismatches near the middle and 5' end of the reverse primer, and two mismatches in the probe. Interestingly, the forward primer of the *BUT1_CYTB* assay included two sites of intraspecific polymorphism for bull trout. Individual sequences downloaded from NCBI for bull trout indicated that the polymorphic sites were both either G or A and both haplotypes appeared to be common. The *BUT1_CYTB* assay was designed with G bases at both polymorphic sites in the forward primer, one of which was the only mismatch with lake trout. The *BUT1_CYTB* assay narrowly met the minimum *in silico* mismatch criteria for inclusion in this study and had two mismatches with some bull trout haplotypes.

Multiple primer and probe combinations were assessed and tested for lake trout and peamouth (Table 2. 4 and Table A2), and the most successful assay candidates for both species were chosen for further validation (Table 2. 4). Specificity was the most important design aspect for the lake trout assay, and mismatches between lake trout and bull trout were prioritized. No more than two mismatches with bull trout could be incorporated into each primer and probe sequence (Table 2. 2). Mismatch influence was optimized by including a critical A-G mismatch in the forward primer and by positioning all mismatches as close to the 3' end of primers as possible. The risk of cross-amplification with the most abundant pelagic salmonids in the Williston Reservoir was also low due to the number of total mismatches with the *LT_CYTB* primers and their relative influence due to mismatch position and type (Table 2. 2). Prioritizing assay specificity constrained the options for primer locations and resulted in trade-offs with the potential for secondary structures in the reverse primer (Table 2. 4). The hairpin T_m of the *LT_CYTB* reverse primer was relatively high, and dimer structures that involved the reverse primer had a moderately high ΔG values. Secondary structures were formed primarily with the GGCC sequence located in the middle of the reverse primer. Options were limited for redesigning the reverse primer while still incorporating key mismatches, and often resulted in higher ΔG values for potential secondary structures, or substantially reduced assay specificity. The *LT_CYTB* primer T_m was designed to be high enough to avoid optimal PCR annealing temperatures that would overlap with the high hairpin T_m of the reverse primer.

Peamouth and other leuciscid species in the Williston Watershed have highly divergent CYTB sequences which made designing the *PCC_CYTB* assay relatively uncomplicated. Hairpin T_ms and ΔG values for dimer structures were low (Table 2. 4). Primer and probe mismatches with the three other leuciscid species in the Williston Watershed were numerous, and their positions

were biased towards the 3' end of the primers (Table 2. 3). As many as two A-G critical mismatches were incorporated into the forward primer, and near the 3' end with two of the three other leuciscid species.

In Vitro Validation

Annealing Temperature Optimization

Temperature gradient tests revealed the optimal annealing temperatures for each assay (Table 2. 6), although most assays performed well at all temperatures above 52°C (Appendix A). The *BUT1_CYTB* bull trout assay was particularly robust, performing similarly at all temperatures. The kokanee assay performed best at 54 °C, but droplet amplitude was only slightly reduced at 56 °C and 58 °C. The lake trout assay performed well at or above 58 °C. Below 58 °C, the average amplitude of positive droplets was reduced, and the range of amplitudes for positive droplets increased (Figure A 9). An annealing temperature of 58 °C was suitable for all the assays tested and considered to be appropriate for duplexing assays in a single reaction, or when using more than one assay on the same PCR plate.

Table 2. 6. Optimal annealing temperatures determined from temperature gradient tests with each assay and a synthetic DNA template (gBlock).

Assay	Target Species	Optimal Annealing Temperature (°C)
Sock_COI	kokanee	54
Rain_COI	rainbow trout	56-58
COCL_CYTB	lake whitefish	56-58
BUT1_CYTB	bull trout	50-60
LT_CYTB	lake trout	60
PCC_CYTB	peamouth	54-56

Assay Specificity

All six qPCR assays in this study demonstrated species specificity when tested against DNA extracted from the tissues of non-target species. The baseline amplitude for lake trout

template was slightly higher than for other species when tested with the *COCL_CYTB* and *BUT1_CYTB* assays (Figure A 13 and A 14). However, the difference in droplet amplitude was negligible and droplets were unambiguously identified as negative. The *COCL_CYTB* and *BUT1_CYTB* assays had relatively few primer and probe mismatches with lake trout DNA, but both assays were specific enough to discriminate between lake trout and their target species. The novel lake trout assay did not show any evidence of cross-reaction with bull trout DNA despite only having two mismatches in each primer and the probe (Figure A 15).

Assay Sensitivity

The six assays tested had Standard LODs ranging from concentrations of 0.86 to 9.12 copies/µL in the 5 µL of sample that was added to the ddPCR reaction, translating to a range of 4.32 to 45.6 total copies in a 20 µL ddPCR reaction (Table 2. 7). Modeled LODs did not differ from Standard LODs for the *Rain_COI, COCL_CYTB,* and *LT_CYTB* assays. Modeled LODs for the *Sock_COI* and *BUT1_CYTB* assays could not be calculated because their dilution series remained linear to the lowest dilution level. The Standard and Modeled LODs for the *PCC_CYTB* assay differed substantially. Standard LOD for the *PCC_CYTB* assay was relatively high because the assay only detected template in 5 out of 6 of the replicates at the next dilution point that represented 1.82 copies/µL in the original sample, or 9.12 copies/20 µL ddPCR reaction.

Table 2. 7. Limits of detection (LOD) for the six assays tested in this study. Concentrations of the synthetic DNA standards were adjusted after comparison with ddPCR results from the dilution of highest concentration in the series. Standard LOD is the lowest dilution level with >95% detection amongst replicates. Modeled LOD was assessed with a statistical model for digital PCR developed by Hunter et al. (2017). Modeled LOD was not applicable (N/A) when the relationship between expected and measured DNA concentrations remained linear and a "concentration plateau" was not observed at low concentrations. For both Standard and Modeled LOD, the DNA concentration of the sample and the concentration in the 20 µL PCR reaction mixture are shown.

Assay	Standard LOD (copies/µL of sample)	Standard LOD (copies/20 µL rxn)	Modeled LOD (copies/µL of sample)	Modeled LOD (copies/20 µL rxn)
Sock_COI	1.22	6.08	N/A	N/A
Rain_COI	1.28	6.40	1.28	6.40
COCL_CYTB	0.86	4.32	0.86	4.32
BUT1_CYTB	0.99	4.96	N/A	N/A
LT_CYTB	0.99	4.96	0.99	4.96
PCC_CYTB	9.12	45.6	0.36	1.82

In Situ Assay Testing in the Williston Reservoir

ddPCR Analysis of eDNA

All the samples collected from the Williston Reservoir had low concentrations of eDNA and the detected concentrations varied between species. Most species detections came from eDNA samples collected above the thermocline. The number of samples with positive detections, eDNA concentrations, and the diversity of detected species all declined with increasing depth. However, these observations were more pronounced at Factor Ross than at Teare Creek. Tests with lambda and ePlant assays indicated that Factor Ross samples from 20 m may have been inhibited (Figure 2. 2). The lambda assay detected lambda DNA that was included in each reaction as a positive control at a concentration of 2 copies per µL. The ePlant assay detected chloroplast DNA present in water. Inhibition was indicated in the Factor Ross samples from 20 m by the low amplitude of positive droplets for lambda and the absence of positive droplets for ePlant (Figure 2. 2). Despite this evidence for inhibition, kokanee were detected in two out of three replicate samples from Factor Ross at 20 m and at concentrations as high as 15 copies per reaction.

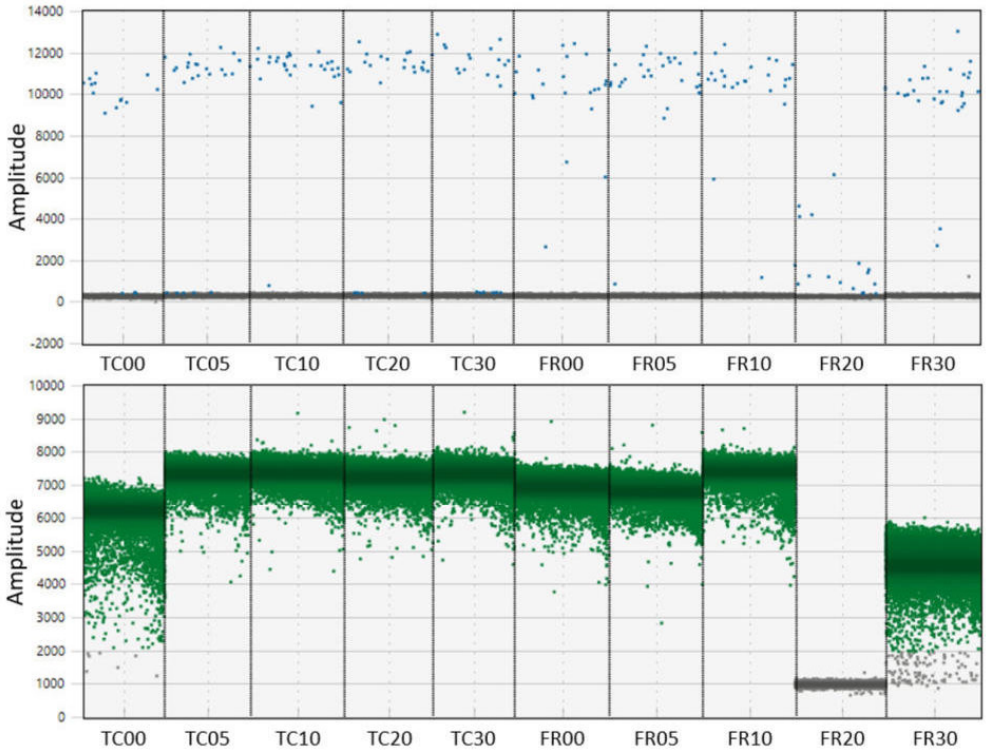

Figure 2. 2. Tests for sample inhibition and integrity with the lambda (top) and ePlant (bottom) assays. Droplet charts are from tests of first elution samples from Teare Creek (TC) and Factor Ross (FR) at sample depths that are indicated by numbers in the sample codes. The master mix for all samples was spiked with lambda DNA at a concentration of 2 copies per µL. The low amplitude of positive droplets for lambda and the absence of positive droplets for ePlant at FR20 is indicative of inhibition. Droplets that were positive for PCR amplification are coloured and negative droplets are grey.

The total number of eDNA copies detected from all samples at Factor Ross and at Teare Creek demonstrated similar trends of eDNA abundance for most species. Kokanee eDNA was by far the most abundant at Factor Ross, followed by eDNA from lake whitefish, bull trout and rainbow trout (Figure 2. 3). Compared to Factor Ross, kokanee eDNA was much less abundant at Teare Creek and was nearly identical to lake whitefish in total eDNA copies detected at that location. The total abundance of lake whitefish, bull trout and rainbow trout eDNA was comparable between sites. The amount of bull trout and rainbow trout eDNA was always much less, relative to the amount of kokanee and lake whitefish eDNA.

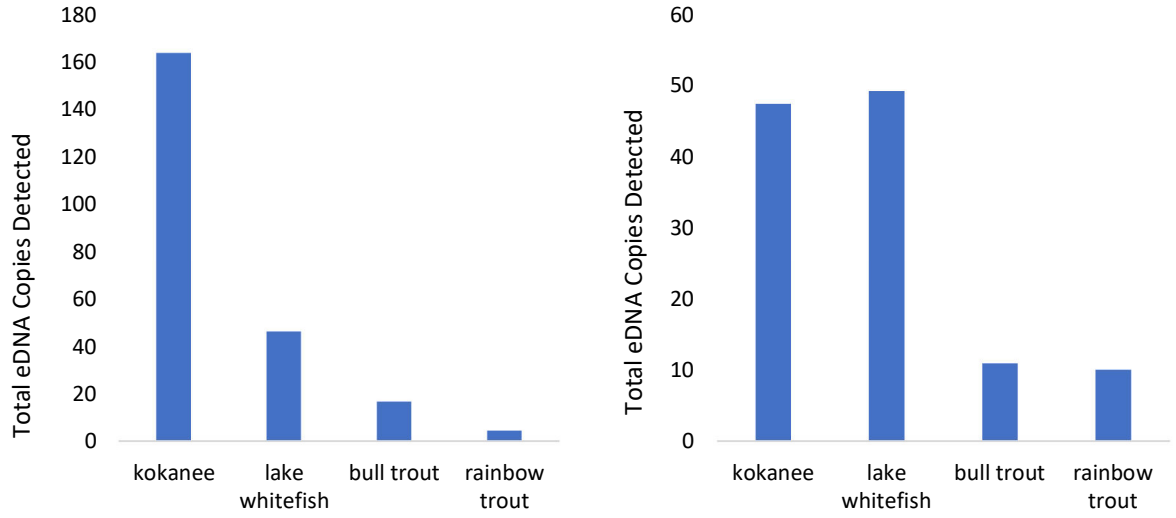

Figure 2. 3. Total number of eDNA copies from each species detected in 10 µL of the first elution of all samples at Factor Ross (left) and Teare Creek (right). Counts of peamouth eDNA copies are omitted because tests of the *PCC_CYTB* assay were not comparable.

Kokanee were detected in the greatest number of samples at Teare Creek and Factor Ross and had the highest concentrations of eDNA in individual samples. Kokanee were detected at every sample depth other than 10 m at Teare Creek (Figure 2. 4). The number of samples that kokanee were detected in declined with depth, and so did the sample eDNA concentrations. Kokanee eDNA concentrations were as high as 21 copies per reaction (16 positive droplets) in samples above the thermocline at Factor Ross but were lower than 10 copies per reaction (8 positive droplets) elsewhere. Lake whitefish were the second most detected species and were also detected more frequently and with higher eDNA concentrations at sample depths above the thermocline. Lake whitefish eDNA concentrations were as high as 15 copies per reaction (10 positive droplets) in one surface sample from Factor Ross, but positive samples from other depths had lower concentrations. Lake whitefish eDNA concentrations from Teare Creek did not vary greatly in any of the positive samples.

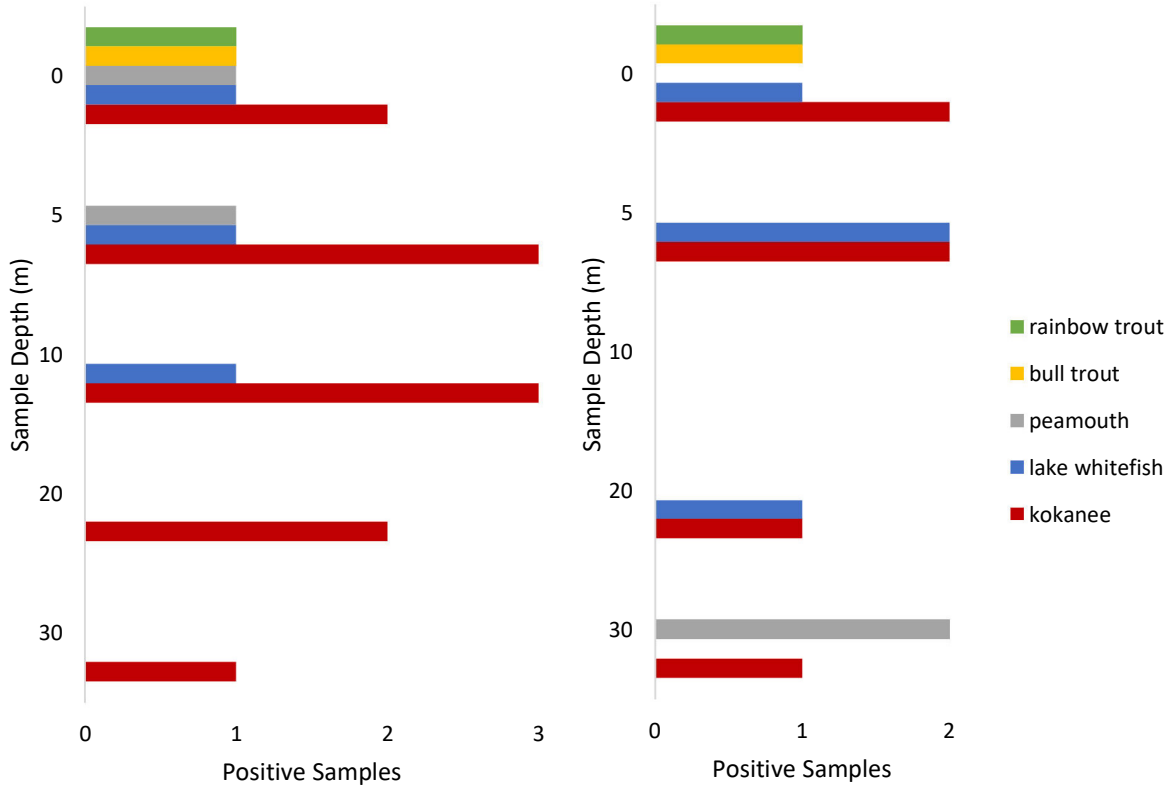

Figure 2. 4. Species detections using eDNA by depth at Factor Ross (left) and Teare Creek (right) sample locations. A total of three 1-litre eDNA samples were collected from each depth and their extractions were tested with six species-specific qPCR assays.

Peamouth, bull trout, rainbow trout and lake trout were detected in few or no samples, and eDNA concentrations were low in positive samples for those species. The peamouth assay was only tested on a single PCR replicate of the second elution of eDNA extractions, but peamouth were still the third most frequently detected species at both sample locations. Peamouth were only detected from Teare Creek at 30 m, and only above the thermocline at Factor Ross. Bull trout and rainbow trout were detected at the surface, but the positive bull trout sample from Teare Creek and both positive rainbow trout samples only contained the minimum concentration of eDNA required for a positive detection (3 positive droplets between both PCR replicates of a sample). Lake trout were not detected in any samples.

Gillnet Survey

Gillnets at Teare Creek were set above and throughout the thermocline but not below it. Gillnets set at the surface and at 5 metres were above the thermocline where temperatures were warmest. Depths between 10 and 23 m were fished with three gillnets where water temperatures transitioned from approximately 16 °C down to 7 °C (Figure 2. 1). A small number of peamouth and kokanee were caught in gillnets set at the surface and all other fish were caught in the nets at 15 m. Kokanee were the smallest but most numerous fish caught in the nets, averaging 65 g each. Kokanee made up 67% of the individual count, but only 41% of the total biomass (Figure 2. 6). Lake whitefish were the second most abundant species in the gillnets at 21% of the total count. Lake whitefish had an average mass of 186 g and made up 37% of the total biomass. Peamouth were the third most numerous species caught, but they only made up 5.5% of the total biomass, averaging 76 g each. A single bull trout that weighed 880 g was caught in the gillnet at 15 m and a single rainbow trout weighing 96 g was caught at 5 metres. Lake trout were not caught in gillnets at Teare Creek.

The gillnets at Finlay Forks were only set at four depths between 5 and 20 m deep (Figure 2. 5). Kokanee were the most numerous species in the nets at Finlay Forks. Although most were caught at 10 and 15 m, there was not much of a difference in catch abundance between all four depths. Lake trout were caught at all four depths at Finlay Forks and were the second most abundant species in the nets. Few lake whitefish were caught and only at 5 and 10 m depth.

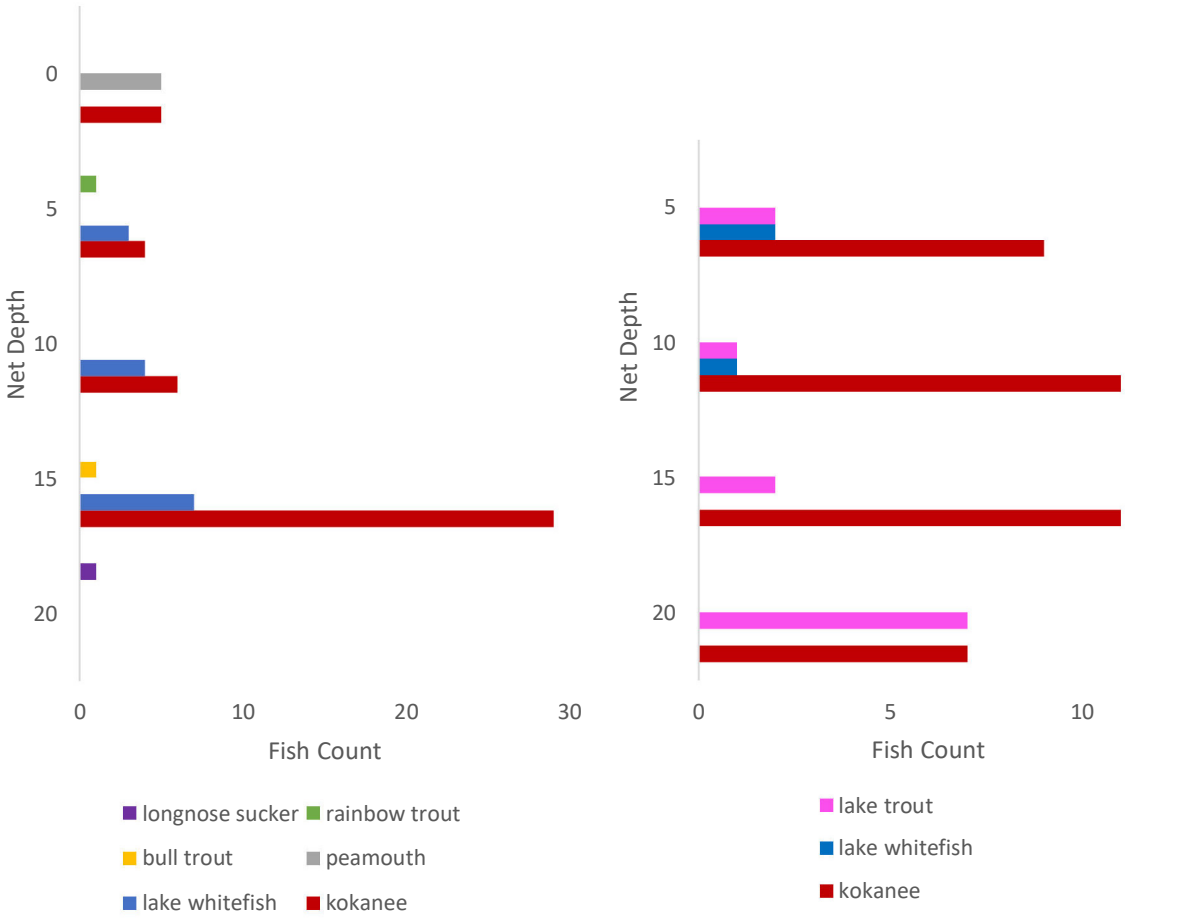

Figure 2. 5. Gillnet catches by species at each set depth between the surface and 20 m at Teare Creek (left) and Finlay Forks (right). Each net panel spanned two vertical metres in the water column.

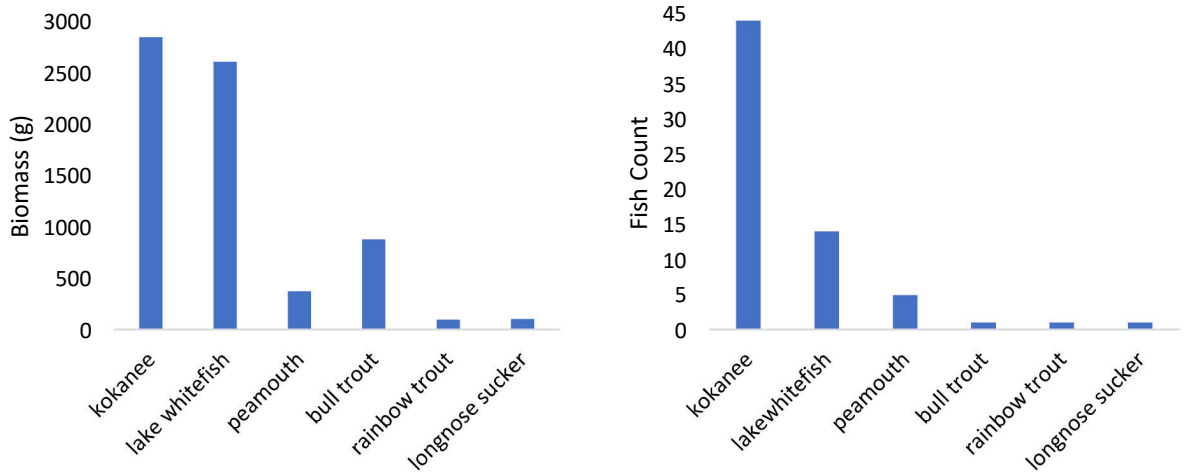

Figure 2. 6. Total biomass (left) and total count (right) of each species caught in gillnets at Teare Creek.

DISCUSSION

Assay Design and *In Vitro* Validation

I successfully developed two novel qPCR assays and validated them alongside four published assays to pilot species-specific eDNA methods for monitoring the pelagic fish community in the Williston Reservoir. Published assays for lake whitefish, kokanee, rainbow trout and bull trout were developed by others for use outside of northern British Columbia on real-time qPCR platforms. This study validated the published assays so that they can be used with confidence in the Williston Reservoir and with ddPCR. The development of novel lake trout and peamouth assays were necessary for the implementation of species-specific eDNA surveys in the Williston Reservoir. Lake trout monitoring is a priority for fisheries managers (Fish and Wildlife Compensation Program, 2020), and developing a specific assay that could differentiate lake trout from bull trout was essential. The *LT_CYTB* assay performed well in this study, and additional field testing will increase confidence in detection results. Peamouth are not a high priority species for monitoring in the Williston Reservoir, but eDNA results would not be as comparable to traditional surveys without the ability to detect all six of the dominant fish species in the pelagic community. The peamouth assay performed well but also requires additional field testing to increase detection confidence.

Species-specific eDNA monitoring relies upon qPCR assays that amplify only the DNA of their target species. The qPCR assays tested in this study demonstrated that they could reliably distinguish between target and non-target species from the Williston Watershed. Assay specificity is situation specific and heavily influenced by the design of primers and probes (Klymus et al., 2020; Thalinger et al., 2021). The *Sock_COI, Rain_COI* and *COCL_CYTB* assays were originally designed and validated by others against most of the same species that co-occur in the Williston

Watershed (Hernandez et al., 2020; Rasmussen Hellberg et al., 2010) and included species-specific point mutations close to the 3' ends of primers. This study validated these three assays for use in the Williston Reservoir and with the ddPCR platform. The *BUT1_CYTB* assay was designed primarily to discriminate between bull trout and invasive brook trout (*S. fontinalis*) (Wilcox et al., 2013). Although there were five mismatches between the *BUT1_CYTB* primers and lake trout DNA, they were not located near the 3' end. Wilcox et al. (2013) observed some minor cross-reactivity between the *BUT1_CYTB* assay and lake trout DNA with real-time PCR, but my results with ddPCR were unambiguous. The ddPCR platform is not dependent upon reaction efficiency and the digital format of presence and absence enhances the delineation of a true positive from false positives (Burns et al., 2010; dMIQE Group, 2020; Doi et al., 2015). The slight increase in negative droplet amplitude I observed when testing the *BUT1_CYTB* assay against lake trout DNA (Figure A 14) is likely due to similar low-level cross-reactions observed by Wilcox et al. (2013). Despite this small but noticeable difference, there was still a clear separation between the droplet amplitudes achieved when the *BUT1_CYTB* assay reacted with bull trout DNA versus lake trout DNA (Figure A 14). The *LT_CYTB* and *PCC_CYTB* assays were developed with primers that maximized the influence of the available mismatches with closely related species from the Williston Reservoir. This was especially important for the *LT_CYTB* assay as there were very few mitochondrial DNA sequence differences between lake trout and bull trout. *In vitro* testing of the *LT_CYTB* and *PCC_CYTB* assays demonstrated that they were highly specific (Appendix A). The *in vitro* success of these novel assays is a testament to the importance and value of carefully designing and thoroughly analyzing the performance of novel assays *in silico*.

Sensitivity is an essential aspect of assay performance and validation and is particularly important for detecting some of the rare species in the Williston Reservoir. The standard LOD

definition from Klymus et al. (2019) used in this study is the most widely accepted definition for qPCR assays used in eDNA studies (Thalinger et al., 2021). However, Hunter et al. (2017) argued that the standard definitions and strategies for assessing the LOD of an eDNA assay can be either too stringent or subjective and may result in biased estimates of species presence. Klymus et al. (2019) acknowledged that detections below the LOD are expected in eDNA studies and may still represent true positives. Detections below the LOD are important and should not be disregarded, but the level of confidence in detections below the LOD is less than desired (Klymus et al., 2019). PCR assays may also overestimate low DNA concentrations due to fixed instrumental responses that cannot be monitored or corrected with experimental controls (Hunter et al., 2017). The "concentration plateau" describes the nonlinear relationship between expected and measured DNA concentrations observed in dilution series experiments. The statistically modeled LOD described by Hunter et al. (2017) accounts for nonlinear responses in a serial dilution experiment and was used as a double check for validating assay sensitivity.

Five of the six assays tested in this study demonstrated high sensitivity. LOD values for the kokanee, rainbow trout, lake whitefish, bull trout and lake trout assays ranged from 4.32 to 6.40 copies per 20 µL reaction (Table 2. 7). These values are very close to the most sensitive LOD theoretically possible for real-time qPCR at 3 copies per reaction, which assumes a Poisson distribution, a 95% chance of including at least one copy in the PCR and single copy detection (Bustin et al. 2009). Digital PCR provides greater precision than real-time qPCR (dMIQE Group, 2020) and the theoretical LOD on the Bio-Rad QX200 Droplet Digital PCR system is one copy per reaction (Bio-Rad Labratories, Inc., 2022). The Modeled LOD is based on a mathematically determined value that accounts for instrumental responses that may otherwise overestimate the sensitivity of an assay. Modeled LOD values could not be determined for the *Sock_COI* and

BUT1_CYTB assays because a concentration plateau was not observed. However, the dilution series results for both assays remained linear throughout the range tested, including the dilution level that defined Standard LOD.

The peamouth assay did not demonstrate the same level of sensitivity as the other assays. Five out of six PCR replicates were successful at the dilution level corresponding to 9.12 copies per reaction, and single replicate failed to detect any DNA. As a result, the lowest concentration that we tested that could meet the Standard LOD criterion of 95% confidence was five times higher (Table 2. 7). However, the accuracy of LOD values depends on the serial dilution ratio, the number of replicates tested at each dilution level, and can be affected by inconsistent lab procedures and simple human errors. A smaller dilution ratio, like 1:2 instead of 1:5, would have allowed for more accurate LOD determinations by reducing the incremental changes in copy numbers between concentration levels. Studies that have tested assays with real-time qPCR platforms often include many more than six replicates (Klymus et al., 2019). A downside of ddPCR is the increased cost compared to real-time qPCR: a factor that limited the number of replicates that were used in dilution series experiments for this study. Factors such as the stochastic distribution of DNA molecules in very low concentration samples, pipetting errors, inhibition of the PCR or even degraded target DNA are all possible causes for a single PCR replicate to fail to detect low concentrations of DNA (Hunter et al., 2017). The Standard and Modeled LOD values differed by two dilution levels for the peamouth assay. The Modeled LOD is an unbiased estimate of sensitivity that prevents an overestimation of the LOD due to instrumental factors or artifacts in ddPCR, but provides a less stringent definition than the Standard LOD described by Klymus et al. (2019). It seems likely that the true LOD of the *PCC_CYTB* assay lies between the values

determined by the Standard and Modeled LODs reported by this study, and repeating the dilution series experiment would provide clarity.

The performance of the eDNA assays tested in this study were affected by the entire workflow, including the PCR platform and reaction conditions for which the assays were optimized. Droplet digital qPCR has many advantages over real-time qPCR and is particularly well suited to eDNA studies because it can detect trace levels of target DNA with high confidence (dMIQE Group, 2020). The increased sensitivity of ddPCR has been demonstrated in other studies (Burns et al., 2010; Doi et al., 2015; Sahu et al., 2021). The LOD for the *COCL_CYTB* assay published by Hernandez et al. (2020) was 8 copies per reaction on a real-time qPCR platform, and 4.32 copies per reaction in this study (Table 2. 7). ddPCR is an end-point measurement that is independent of reaction efficiency and is less influenced by matrix materials such as other DNA and inhibitors (Bio-Rad Labratories, Inc., 2022; Burns et al., 2010; dMIQE Group, 2020; Doi et al., 2015). Reactions that produce droplets with intermediate fluorescence that do not separate nicely into positive or negative partitions (a phenomenon known as "rain") may not affect the ultimate quantification of DNA with ddPCR, but it is a sign of poor reaction efficiency, sub-optimal annealing temperatures or mismatches between the assay and the target sequence (dMIQE Group, 2020). The *LT_CYTB* assay had a relatively high potential for forming dimer structures with the reverse primer (Table 2. 4) but performed well on the ddPCR platform. The temperature gradient tests clearly indicated that the *LT_CYTB* assay performs best at temperatures at or above 58 °C, but the potential for poor reaction efficiency likely contributed to the increased range in droplet amplitudes observed at lower annealing temperatures (Figure A 9). Wilcox et al. (2013) found that PCR cycle threshold (Ct) values (the cycle number that indicates the detection of target DNA) for the *BUT1_CYTB* assay were strongly skewed downwards when bull trout DNA was

diluted into lake trout DNA at ratios of 1:10 and 1:100. However, because DNA molecules are partitioned in ddPCR, the likelihood of lake trout DNA influencing the quantification of bull trout DNA would have been minimal. The ddPCR platform and the reaction conditions used in this study have optimized the six assays tested, and the workflow I followed has validated their future use in eDNA studies of the Williston Reservoir.

In Situ Assay Validation in the Williston Reservoir

The eDNA and gillnet results from the Finlay Reach demonstrate that molecular methods are at least as sensitive as traditional survey gears, especially for rare species. Aside from a single longnose sucker which was not targeted with an eDNA assay, all species caught in the gillnets at Teare Creek were also detected by eDNA. Abundant species like kokanee, lake whitefish and peamouth should not have been difficult to detect with either method, and agreement between gillnets and eDNA on those species was expected. Lake trout, bull trout and rainbow trout are rare species in the Williston Reservoir, and detecting them with pelagic gillnets has required a significant amount of sampling effort in the past (Pillipow & Langston, 2002; Sebastian et al., 2003, 2009). The gillnets set at Teare Creek for this study were also part of a larger pelagic survey of the entire reservoir in 2021, but Teare Creek was the only location where bull trout were caught in a gillnet (O'Connor, 2022). eDNA has proven to be a very sensitive and efficient method for detecting rare species compared to conventional sampling gears (Evans, Shirey, et al., 2017; Jerde et al., 2011; Thomsen et al., 2012). Bull trout and rainbow trout were detected with eDNA at both Factor Ross and Teare Creek. Their eDNA was detected in very few samples and at very low concentrations per sample, but this is not unexpected for rare species. The *LT_CYTB* assay was tested on environmental samples for the first time in this study, and the results must be interpreted cautiously. However, it is reassuring that lake trout were undetected by both eDNA and gillnets in

the Finlay Reach, and the results provide partial *in situ* validation for the novel lake trout assay (Thalinger et al., 2021). Of all the gillnets set around the Williston Reservoir in 2021, only one location in the Peace Reach and the nets set at Finlay Forks caught lake trout (O'Connor, 2022). A total of 12 lake trout were caught at Finlay Forks (Figure 2. 5), which suggests that lake trout are easily detected with gillnets when they are present. The gillnet results also suggest that lake trout were not abundant outside of the Finlay Forks and Peace Reaches of the reservoir in early August of 2021. The agreement between eDNA and gillnet results in the Finlay Reach gives confidence in eDNA as a sensitive survey tool for presence and absence of species and advances the validation of the individual assays used in this study.

The vertical distribution of species detections with eDNA were consistent with the gillnet results, but eDNA integrates information about fish presence over larger areas and timescales than traditional sampling methods (Shelton et al., 2019). Kokanee had the broadest ranging vertical distribution of any species in the Finlay Reach as detected with both eDNA and gillnets. Although the gillnets only sampled to a depth of 20 m, a nighttime trawl survey conducted at Teare Creek on August 10, 2021 only caught kokanee and only at depths between 23 and 30 m (Weir et al., 2023). The gillnet at Teare Creek was left in place for 24 hours due to poor weather, but the catch is likely more representative of the nighttime distribution of fish. Gillnet catch rates are typically highest at dusk and dawn (Vašek et al., 2009) and are reduced as fish accumulate in the nets (Olin et al., 2009). The gillnet and trawl catches demonstrate that kokanee were distributed both above and below the thermocline from the surface to a depth of 30 m. These results were biased towards nighttime fish distributions and suggest that kokanee preferred greater depths at night. eDNA has a smoother distribution in space and time than the fish themselves and should be detectable at all of the depths that a fish has frequented in the hours to days prior to sample collection (Pont et al.,

2018; Shelton et al., 2019). This was exemplified in the Williston Reservoir by the mostly continuous detection of kokanee and lake whitefish eDNA throughout the water column. The eDNA results suggest that kokanee, lake whitefish, and perhaps even peamouth utilize depths above and below the thermocline during late summer and are not confined to any zone in the water column. The gillnet results offer an incomplete characterization of vertical fish distributions, but one that overlaps with the distribution interpreted from eDNA. Characterizing the vertical distributions of rare species like bull trout and rainbow trout is difficult given the limited dataset of a few chance detections with eDNA and gillnets.

The distribution and abundance of fish eDNA in the Williston Reservoir is influenced by the ecology and behaviour of each species as well as the ecology of eDNA itself. Kokanee, lake whitefish and peamouth were all detected in at least one sample from depths below the thermocline, but most species detections were in samples collected from the epilimnion. Kokanee, lake whitefish and peamouth are planktivorous species that are known to exhibit diel vertical migrations in summer (Clark & Levy, 1988; Gorsky et al., 2012; Hardiman et al., 2004; McPhail, 2007; Scheuerell & Schindler, 2003). Although these three species spend time above and below the thermocline, eDNA production is positively correlated with metabolic rates and feeding (Klymus et al., 2019; Rourke et al., 2021), which are both increased for planktivores when they are in the warmer waters of the epilimnion. Fish metabolism is sensitive to temperature (Chabot et al., 2016) and would be elevated in the warmer waters of the epilimnion compared to the cooler hypolimnion water. Kokanee, lake whitefish and peamouth migrate to shallower depths above the thermocline to feed on plankton, and actively feeding fish can shed as much as 10 times more DNA than non-feeding fish (Klymus et al., 2015).

Rainbow trout do not stray far from shoreline features in large reservoirs (McPhail, 2007) and have been caught most frequently in gillnets near the surface and near to shore in the Williston Reservoir (Pillipow & Langston, 2002; Sebastian et al., 2009). The detection of rainbow trout eDNA at the surface is consistent with historical gillnet results and likely an indication of feeding in this location. The eDNA sample locations were further from shore than is typical for rainbow trout, but the Williston Reservoir is subject to heavy wind which may transport eDNA near the surface of the water long distances from where it originated. At the same time, eDNA at the surface degrades faster than eDNA at depth because of increased exposure to UV radiation from sunlight, elevated temperatures, and an increase in both the abundance and metabolism of microbes that break down DNA (Takahara et al., 2012; Thomsen et al., 2012). eDNA production is elevated in the warm and productive waters of the epilimnion where many species of fish feed actively. The distribution of eDNA above the thermocline is likely broadened by the mixing action of heavy winds, increasing detection sensitivity of eDNA but potentially reducing the spatial accuracy of detections.

Factors affecting the distribution and abundance of eDNA in the hypolimnion can make detecting species below more difficult than above the thermocline. Bull trout and lake trout are cold water stenotherms and avoid the warm surface waters in summer, although they may exhibit vertical migrations into the epilimnion to pursue prey (McPhail & Baxter, 1996; Riley et al., 2021). Bull trout and lake trout metabolic rates would be slowed by the cooler temperatures in deeper waters, and their larger body size would result in a lower metabolic rate relative to mass compared to smaller species. Fish metabolic rates scale nonlinearly with body size and larger fish release less eDNA into the environment per unit of mass compared to smaller bodied fish (Chabot et al., 2016; Yates et al., 2020). If bull trout move into the epilimnion to feed, they could release more eDNA

at the surface than they do at depth, even if they spent most of their time in deeper water. This may explain why gillnets caught bull trout at 15 m, but eDNA only detected them at the surface. eDNA produced below the thermocline may have a patchier distribution than eDNA in the wind mixed epilimnion because the water column below the thermocline is subject to much less movement and internal mixing (Wetzel, 2001). In addition, eDNA will sink in the water column like any other particulate organic matter, and especially if it is attached to or part of larger particulates such as feces, carcasses, or scales (Canals et al., 2021). Consequently, the probability of detection may be greater for eDNA in the epilimnion where it is suspended longer due to wind mixing, while eDNA in the hypolimnion may sink relatively quickly and only be detectable in the water column for a short time before it settles to the bottom. Acoustic monitoring studies in the Williston Reservoir have observed that lake trout tend to congregate around discrete habitat features (Culling & Euchner, 2019) which would further limit the distribution of their eDNA. The 2021 gillnet results from the entire reservoir (O'Connor, 2022) also provide evidence for a patchy lake trout distribution. Bull trout and lake trout are rare species in the Williston Reservoir, and their eDNA is expected to be sparsely distributed. If either species venture into the surface waters to feed, they may release a disproportionate amount of eDNA there compared to below the thermocline. Wind mixing would likely disperse any eDNA left at the surface and reduce its concentration, but potentially increase the likelihood of detection. Bull trout and lake trout eDNA below the thermocline is also likely to be found in low concentration, but likely has a patchier distribution. Klobucar et al. (2017) found that reliably detecting species that remain below the thermocline in summer requires a substantial amount of sampling effort, including a large number and spatial distribution of samples as well as a large volume of water filtered per sample. The distribution of

eDNA in the hypolimnion is likely more contracted than in the epilimnion but should more accurately represent the recent presence of fish in the area sampled.

A recent review supported the findings for a positive correlation between fish abundance and the concentration of eDNA (Rourke et al., 2021). The correlation between eDNA concentration and species abundance is complicated by the same factors that affect the detection and distribution of eDNA (Yates et al., 2019), although the relationship can be improved with careful study design and data modeling (Chambert et al., 2018; Fukaya et al., 2018; Shelton et al., 2019; Yates et al., 2020). It is reassuring to see a similarity between the relative abundance and biomass of the species caught in gillnets and eDNA concentrations detected (Figure 2. 3 and Figure 2. 6). Kokanee were the dominant species caught in the gillnets as measured by the number of individuals and by total biomass. Similarly, kokanee eDNA was more concentrated in the water column than eDNA from other species at Factor Ross. eDNA from lake whitefish, bull trout and rainbow trout were detected at concentrations that were comparable between Factor Ross and Teare Creek, and each species ranked similarly in relative eDNA concentration compared to the gillnet catch abundance and biomass. However, the relative concentration of kokanee eDNA at Teare Creek differed from Factor Ross and was more like the concentration of lake whitefish eDNA at Teare Creek. This appears to be a slight departure from the relationship between eDNA concentration and gillnet catch abundance observed for other species and at Factor Ross. It is difficult to explain what may have caused this difference, but the relationship between kokanee eDNA concentration and relative abundance appears to be reduced at Teare Creek.

The eDNA results from Teare Creek are discontinuous and less comparable to the gillnet catch, but these differences may have been caused by sample degradation and the timing of the sampling event. Water samples were collected from Teare Creek in the morning on August 16 but

were not filtered and preserved until the following day. This delay in sample filtration was due to a windstorm that forced the field crew to seek shelter on the reservoir and delayed the return to camp until late in the evening. Every effort was made to keep the samples cool during the day but conditions were not optimal on the boat. The water samples were kept in a fridge overnight and filtered the next morning. The eDNA samples were also collected at Teare Creek the day after the gill net was set in the same location. The sequencing of eDNA sample collection after the gillnetting was not ideal and may have increased the amount of eDNA in the water column at Teare Creek. Water samples from Factor Ross were collected early in the morning and filtered hours later that same day. Although the Factor Ross samples were collected more than 20 km from Teare Creek, the eDNA results from Factor Ross compare better to the Teare Creek gillnet catch, and eDNA detections were more continuous throughout the water column. eDNA degradation in the water samples from Teare Creek may explain the lack of any detections at 10 m, and the reduction in kokanee detections relative to those at Factor Ross.

The results of this study have demonstrated that detecting rare species in an enormous, unproductive waterbody like the Williston Reservoir can be challenging and provides insights into how eDNA methods for large, unproductive lakes could be improved. Increasing the volume of water filtered per sample, as well as the number and distribution of samples would increase detection probabilities and confidence. eDNA studies of lake trout in smaller, presumably more productive lakes in Ontario and Quebec were able to detect fish from 500-1000 mL volumes per replicate collected below the thermocline in late summer (Lacoursière-Roussel, Côté, et al., 2016; Littlefair et al., 2020). However, Klobucar et al. (2017) filtered 5 L per replicate to detect Arctic char (*Salvelinus alpinus*) that were in low abundance in relatively unproductive Alaskan lakes. Stamford et al. (2020) filtered volumes of 3-5 L per replicate to detect Arctic grayling from

tributaries to the Williston Reservoir. Sampling larger volumes of water is highly recommended for future eDNA studies in the Williston Reservoir. The 1-L replicate samples collected for this study confidently detected relatively abundant species like kokanee and lake whitefish, but eDNA concentrations were low, very low, or non-existent for rare species like bull trout and lake trout. The clear, ultra-oligotrophic water sampled from the Williston Reservoir did not clog any of the filters or reduce filtering efficiency and larger sample volumes than the 1-L used in this study could easily have been processed through a single filter. Schabacker et al. (2020) recently developed an eDNA collection method for lentic systems that has improved detection sensitivity and requires fewer samples than the traditional filter methods used in this study. The method uses a 64-µm mesh tow net to filter more than 3000 L of water per sample. Although the present study was only meant to pilot eDNA methods, increasing the number and distribution of sample locations would also have increased the probability of detecting rare species. When the Williston Reservoir stratifies in late summer, the thermocline sets up very deep in the water column, making sampling efforts below it more difficult. Furthermore, the eDNA of rare species like bull trout and lake trout may be difficult to detect below the thermocline. Sampling eDNA in summer when a lake stratifies can provide insight into fish distribution and habitat usage, but sampling during spring or fall turnover in a lake may simplify sampling efforts and improve detection probabilities for rare species. Studies that collected eDNA samples in early spring (Lacoursière-Roussel, Côté, et al., 2016) and fall (Klobucar et al., 2017; Littlefair et al., 2020) when lakes were isothermal found that the eDNA of coldwater stenotherms like lake trout was distributed evenly throughout the water column. Samples collected under isothermal conditions also had higher concentrations of rare species eDNA compared to samples collected during stratification in summer (Klobucar et al., 2017; Littlefair et al., 2020).

CHAPTER 3:
VALIDATION OF eDNA METABARCODING ASSAYS FOR FISH SPECIES IN THE WILLISTON WATERSHED

INTRODUCTION

Rapid biodiversity monitoring is critical to conservation management in ecosystems that are affected by climate change and other anthropogenic pressures. Freshwater habitats have been classified as one of the most vulnerable ecosystems worldwide (WWF, 2020). Traditional sampling methods for monitoring fish have various forms of bias (Kubečka et al., 2009; Zale et al., 2012), are not easily scalable across vast geographic ranges and habitat types, and often fail to detect rare or cryptic species (Evans, Shirey, et al., 2017; Jerde et al., 2011; Thomsen et al., 2012). Biodiversity monitoring with molecular techniques that can detect the DNA released by organisms into their environment, referred to as environmental DNA (eDNA), is a recent development that is revolutionizing the way biodiversity is monitored, particularly in aquatic habitats (Beng & Corlett, 2020; Cristescu & Hebert, 2018; Deiner et al., 2017; Jerde, 2019; Ruppert et al., 2019). Although eDNA-based methods have been developed relatively recently, they are now frequently applied in biodiversity monitoring, especially for fish in aquatic environments (Schenekar, 2023).

Two approaches are commonly used to detect species with eDNA: single species detection and multi-species detection, also known as eDNA metabarcoding. Single species detection involves amplifying eDNA in a polymerase chain reaction (PCR) or quantitative PCR (qPCR) using species-specific primers. Species are detected if their template DNA is present and is amplified in the PCR reaction. Sampled water should contain DNA from all the species present in the environment, but characterizing community composition by detecting one species at a time is cost-inefficient and limited by the amount of sample available (Thomsen & Willerslev, 2015).

Metabarcoding assays employ generic primers that target highly conserved gene regions that flank polymorphic regions (Balasingham et al., 2018). In a metabarcoding study, community DNA is PCR-amplified and then sequenced on a Next Generation Sequencing (NGS) platform. Species are detected and differentiated based on the identification of unique amplicon sequences, or species barcodes.

eDNA metabarcoding is powerful and cost-efficient but the method has some limitations and a complex process for analyzing results that must be considered before it is implemented in a particular situation. Although metabarcoding primers are designed to amplify a wide range of taxa, they may not detect all species present in the sampling environment due to biases in primer affinity for certain species (Deagle et al., 2014; Morey et al., 2020; Olds et al., 2016). A multiple assay approach is a popular solution to the shortfalls of individual markers and can maximize species detections and confidence (Evans, Li, et al., 2017; Hänfling et al., 2016; Morey et al., 2020; Olds et al., 2016; Snyder et al., 2020). However, analyzing environmental samples with multiple metabarcoding primer sets increases the costs and time associated with analysis and may not be necessary or appropriate if a single primer set is sufficient to identify all the target species in a particular environment (Morey et al., 2020). Next Generation Sequencing produces a massive number of sequences that must be filtered through a bioinformatics pipeline before the results can be interpreted. Bioinformatic filtering removes sequencing errors and attempts to assign the diversity of sequence reads to individual species. Matching sequences to species is complicated if there is a limited amount of genetic variation in the amplicon for closely related species. Metabarcoding primers are designed for highly conserved genes so they limit the number of primer mismatches across taxa, but they sacrifice amplicon sequence diversity. Primers can be designed

for less conserved genes with more amplicon diversity and better species resolution, but this may affect primer bias between species.

Aquatic species conservation in the rivers, lakes and reservoirs of the Upper Peace River Basin is constrained by limits on the time and resources available to monitor such a vast and often remote area (Fish and Wildlife Compensation Program, 2020). Species-specific eDNA methods have been used to monitor Arctic grayling (*Thymallus arcticus*) in rivers and streams that drain into the Williston Reservoir since 2018 (Stamford et al., 2020, 2022; Strohm et al., 2019). eDNA has a great deal of potential for monitoring aquatic species in the Williston Watershed, and metabarcoding methods could provide a rapid and cost-effective means of investigating fish distributions and community compositions. In this study, I evaluated two metabarcoding primer sets for use on the fish species in the Williston Reservoir and its surrounding watersheds. eDNA metabarcoding results were compared against gillnet sampling in the Williston Reservoir and a snorkel survey that was conducted in the Ingenika River. eDNA samples from other tributaries around the reservoir were also tested with metabarcoding primers. All reservoir samples were tested with species-specific primers for six dominant pelagic species, and tributary samples were tested with species-specific primers for Arctic grayling. Detection results from the metabarcoding analysis were compared against the species-specific eDNA analysis.

METHODS

Creating a Mitochondrial DNA Sequence Database

A reference sequence database of mitochondrial genes was created for 19 fish species (Table 1. 1) that have been identified in the Williston Reservoir and its surrounding watershed (Province of British Columbia, 2020; Sebastian et al., 2009). Available mitochondrial DNA (mtDNA) sequences from the 12S gene, cytochrome oxidase subunit 1 gene (COI), and

cytochrome b (CYTB) gene were downloaded from the National Center for Biotechnology Information's (NCBI) Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide/) and imported to Geneious Prime version 2021.2.2 (www.geneious.com). Sequences were organized in Geneious Prime by gene and by species and aligned using the default parameters in the MUSCLE alignment algorithm. Intraspecific genetic variation was incorporated into a consensus sequence for each species by coding polymorphic sites with IUPAC codes.

In Silico Screening

A diverse assemblage of fishes belonging to four orders and seven families inhabit the Williston Watershed. Primer sequences from published metabarcoding assays that were validated in other freshwater fish studies were uploaded into Geneious Prime and screened for use with the fish species in the Williston Watershed. Primer sequences were compared against the consensus sequences in the Geneious database, and base pair mismatches with forward and reverse primers were tallied. Assays with the fewest number of mismatches with the species in the Williston Watershed were favoured during the selection process. Genetic distances between the amplicons for each species were calculated in Geneious Prime with the Tamura-Nei distance model and visually assessed on a neighbour-joining tree. Consensus trees were created based on the entire amplicon, including the primer sequences. Genetic distances between amplicon sequences were visualized in the consensus trees to assess an assay's ability to resolve each of the 19 species in the Williston Watershed. No single metabarcoding assay was able to resolve all species in the Williston Watershed, but many eDNA metabarcoding studies have overcome this problem by testing their samples with multiple assays (Evans, Li, et al., 2017; Hänfling et al., 2016; Morey et al., 2020; Olds et al., 2016). The MiFish-U (MiFish; M) and FishCYTB-L (FishCYTB; F) assays were selected for testing in the Williston Watershed (Table 3. 1) because they had the lowest

number of primer mismatches among all of the assays considered and when used in tandem, the genetic distances between species amplicons was assessed to be sufficient for resolving at least most of the species in the Williston Watershed. The MiFish primer set amplifies a portion of the 12S gene. The highly conserved nature of the 12S gene minimized the number of mismatches for all species. The MiFish forward primer had a single mismatch at the second base position with sequences from every species in the Williston Reservoir. This was corrected by changing that base from a thymine (T) to a cytosine (C) so that the primer set perfectly matched every species. The downside to a highly conserved target gene like 12S is that species resolution can be more difficult. The FishCYTB assay targeted a segment of the Cytochrome B gene (CYTB), which has greater interspecific sequence divergence than the 12S gene. However, the primer regions for the CYTB gene were more likely to include mismatches. The MiFish and FishCYTB were chosen because the in silico analysis predicted that they would be able to amplify and correctly identify the DNA for all of the species in the Williston Watershed and together.

Table 3. 1. Primer sequences of the metabarcoding assays selected for testing on the fish species in the Williston Watershed. The underlined "C" base in the MiFish-U_F primer was changed from the "T" in the original sequence to perfectly match all 19 fish species considered in this study. UniA sequences were added to the 5' ends of forward primers and UniB sequences were added to 5' ends of reverse primers for barcoding and sequencing on the Ion Torrent platform.

Primer Set	Primer Name	Primer Sequence (5'-3')	mtDNA Marker	Amplicon size (bp)	Citation
MiFish-U	MiFish-U_F (modified)	GCCGGTAAAACTCGTGCCAGC	12S	238-242	Miya et al. (2015)
	MiFish-U_R	CATAGTGGGGTATCTAATCCCAGTTTG			
FishCYTB-L	FishCYTB-L_F	GCCTACGCYATYCTHCGMTCHATYCC	CYTB	233	Snyder et al. (2020)
	FishCYTB-L_R	GGGTGTTCNACNGGYATNCCNCCAATTCA			
Ion Torrent	UniA	ACCTGCCTGCCG			
	UniB	ACGCCACCGAGC			

In Vitro Validation

Metabarcoding assay performance was evaluated with a DNA mock community. The mock community contained DNA from 12 fish species from the Williston Watershed, including six salmonids (lake trout, bull trout, rainbow trout, kokanee, lake whitefish, mountain whitefish, Arctic grayling), two minnows (northern pikeminnow, peamouth), and a representative species from 3 other families (white sucker, slimy sculpin, and burbot). DNA used in the mock community was extracted from fin tissues using a Qiagen DNeasy Blood and Tissue kit (Qiagen, ON, Canada) following the manufacturer's instructions and quantified with a Qubit Fluorometer (ThermoFisher Scientific). The DNA from each species was diluted to between 0.05 and 0.11 ng/µL and 10 µL of each was added to the mock community sample. The final concentration of each species' DNA in the mock community was between 4.88 and 11.8 pg/µL.

In Situ Assay Testing in the Williston Watershed

Pelagic eDNA Sampling from the Williston Reservoir

Pelagic eDNA sampling was conducted as described in Chapter 2.

eDNA Sampling from Tributaries of the Williston Reservoir

The tributary eDNA samples tested in this study were a subsample of those collected from streams in the Finlay, Nation and Ospika regions in 2019, and from the Parsnip and Peace regions in 2020 and 2021 (Stamford et al., 2020, 2022). Stamford et al. (2020; 2022) analyzed the eDNA samples for Arctic grayling using a species-specific qPCR assay. Collection methods for eDNA samples from the tributaries are presented in Stamford et al. (2020) and Stamford et al. (2022). In this study, I analyzed a total of 44 replicates from 29 sample locations in representative regions of

the Williston watershed (Table 3. 2). The negative control samples that were included with the original field samples were incorporated into the metabarcoding analysis.

Table 3. 2. Location, year and collection information for the eDNA samples collected from around the Williston watershed and analyzed with the metabarcoding primers in this study (Stamford et al., 2020, 2022).

Location	Sample Region	Sample Year	No. of Sites Sampled	Total No. of Replicates
Akie Creek	Finlay	2019	1	2
Pesika Creek	Finlay	2019	1	2
Ingenika River – middle	Finlay	2019	3	6 (2 for each site)
Ingenika River – upper	Finlay	2019	4	8 (2 for each site)
Ingenika River – headwaters	Finlay	2019	2	4 (2 for each site)
Ospika River	Ospika	2019	7	10
Manson River	Nation	2019	2	2
Carbon Creek	Peace	2020	2	2
Clearwater River	Peace	2020	2	2
Nabesche Creek	Peace	2020	2	2
Colbourne Creek	Parsnip	2021	1	2
Reynolds Creek	Parsnip	2021	2	2
TOTAL			**29**	**44**

eDNA Extraction

DNA from both reservoir and tributary eDNA samples was extracted at UNBC using the same modified protocol described in Chapter 2.

PCR-based Library Preparation and Next-generation Sequencing

A preliminary polymerase chain reaction (PCR1) was performed on all eDNA samples at UNBC to amplify target DNA. PCR1 samples were all amplified in 25 µL reaction volumes using a Qiagen Multiplex PCR kit (Cat. No. 206143). Reaction mixtures included 12.5 µL of Qiagen Multiplex PCR Master Mix (1x final concentration) and 2.5 µL of primers (0.3 µM for MiFish or 0.6 µM for FishCYTB). The remaining 10 µL volume varied among samples. The mock community and contrasting dilution series samples contained 2 µL of extracted DNA mixture and

8 µL of nuclease free water (IDT). Tributary eDNA samples contained 3 µL of sample (i-elutions) and 7 µL of nuclease free water. Reservoir eDNA samples (from Teare Creek and Factor Ross locations) contained 10 µL of the ii-elution. Negative PCR controls contained 10 µL of nuclease free water (IDT) only. Thermocycler protocols for the reaction mixtures with MiFish primers was: initial denaturation at 95 °C for 15 minutes, then 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 63 °C for 90 seconds and elongation at 72 °C for 30 seconds followed by a final incubation at 72 °C for 10 minutes. The thermocycler protocol for reaction mixtures with FishCYTB primers was: initial denaturation at 95 °C for 15 minutes, then 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 90 seconds and elongation at 72 °C for 30 seconds followed by a final incubation at 72 °C for 10 min. All reservoir samples were run for an additional 10 PCR cycles, for a total of 45 cycles. PCR1 products were visualized on 2% agarose gels (E-Gel precast agarose gels, ThermoFisher Scientific) to verify amplification success. PCR1 samples were submitted to the Environmental Genomics Facility at the Great Lakes Institute for Environmental Research Laboratory at the University of Windsor (Windsor, Ontario, Canada).

PCR1 samples were further processed and sequenced unidirectionally on the Ion GeneStudio S5 sequencer (Applied Biosystems, Burlington, Ontario) at the University of Windsor. A PCR clean up was performed on PCR1 samples using Sera-Mag beads (Cytiva, Marlborough, MA) following an in-house protocol (Appendix D). A second polymerase chain reaction (PCR2) incorporated the IonA adaptor (required for the Ion Torrent sequencing reaction), a unique 10-12 base-pair sequence tag (barcode) to distinguish each individual sample, and the P1 adaptor required for attaching the prepared amplicons to the Ion Spheres. PCR2 samples were amplified in 25 µL reaction volumes that contained 8.4 µL of ultrapure water, 2.5 µL of both 10X Taq buffer and $MgSO_4$, 1 µM of UniA barcode, 1 µM of UniB adaptor, 1 µM of dNTPs, 0.1 µL of Taq DNA

Polymerase High Purity (Bio Basics), and 10 µL of the bead-cleaned product from PCR1. The PCR2 reactions were amplified with an initial denaturation at 95 °C for 1 minute, followed by 8 cycles of: 95 °C for 15 seconds, 55 °C for 15 seconds, and 72 °C for 30 seconds, and a final elongation of 72 °C for 7 minutes.

Samples were normalised by pooling varying amounts based on band intensities after running a 2% agarose gel; 2 µL were added for samples with strong bands, 5 µL were added for samples with faint bands, and 10 µL was added for samples that did not appear to have bands. The final two normalised amplicon libraries, 12S (MiFish) and CYTB (FishCYTB), were then run on 2% TAE agarose gels stained with gel red at approximately 96 V for 4 hours. Run time was increased while using a low voltage at this step to allow for sufficient separation of the 233 base-pair bands for the MiFish amplicons and 238-242 base-pair FishCYTB (amplicons plus all required adaptor sequences) from those containing amplicons with only one adaptor sequence attached. The 233 base-pair MiFish amplicons and 238-242 base-pair FishCYTB amplicons were extracted from the gel by visualising the bands on the GelDoc Imaging System (BioRad) using a clean, sterile scalpel, and purified using the GenCatch Gel Extraction Kit (Epoch Life Sciences), following the manufacturer's protocol. The quality and concentration of the 12S and CYTB amplicon libraries were analysed using a High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer. All concentrations were normalized to 60 pmol/L and the libraries were loaded on to the Ion Torrent Chef for templating onto the 530 chip (Life Technologies). The following day, the loaded 530 chip was put onto the Ion GeneStudio S5 sequencer along with the Ion 510 & 520 & 530 Chef Kit chemistries (Thermo Fisher) for sequencing.

Bioinformatic Analysis of Next-generation Sequencing Data

Bioinformatics analysis were performed with Mothur version 1.48.0 (Schloss et al., 2009) and QIIME2 version 2023.9 (Bolyen et al., 2019). Raw sequences were demultiplexed in Mothur, and a manifest file was imported into QIIME2. Primers and adapters were trimmed from sequences with the Cutadapt plugin (Martin, 2011) followed by denoising with DADA 2 (Callahan et al., 2016). Filtering parameters included a minimum sequence length of 100 bp, and a quality score cutoff of 2. All amplicon sequence variants (ASVs) were compared against the entire NCBI nucleotide database with the Basic Local Alignment Search Tool (BLASTn). BLAST formatters included were a minimum percent identity match of 97%, a maximum number of alignments per ASV set to 100, and a single High-scoring Segment Pair (HSP).

The resulting ASVs were manually filtered by reviewing the BLAST results. Any ASVs that were matched to non-fish species (e.g., birds, mammals, invertebrates, and bacteria) were discarded. ASVs with unidentified species hits or no hits were also discarded. The BLAST results were analyzed for all ASVs that were matched to fish species to determine the most appropriate species assignment. Species assignments that were unambiguously identified as one of the 19 species known to occur in the Williston Watershed (100% identity, or only one species result) were retained. The top hits for some ASVs were for fish species that were not native to the study area. In many cases, BLAST results identified multiple species with the same or very similar values for identity, E-value, and bit scores. In these cases, the ASV was assigned to the highest scoring species from the Williston Watershed, if one was identified. If multiple species from the same genus that occur or had potential to occur in the Williston Watershed were identified with the same BLAST scores, the ASV was only classified to the genus level. Several ASVs produced by both metabarcoding assays were only resolved to the genus level for *Salvelinus*. Confidence in species

assignments for an ASV were improved by comparison against the results from the mock community samples and by comparing the species results from both primers. Any ASVs that were only matched to fish species that do not occur in the Williston Watershed were discarded.

Species detections were evaluated based on three levels of bioinformatic stringency and their related detection certainty in a similar manner to Evans, Li, et al. (2017). Bioinformatic stringency is related to the level of certainty that a detection with eDNA represents the true presence of a species in the environment. Under the low-stringency and low confidence scenario, a species was considered detected if it was found in at least one sample using at least one of the two metabarcoding assays. For the moderate-stringency, moderate confidence scenario, species presence was inferred by the detection of its DNA in at least two samples, or if the DNA was detected by both assays in a single sample. The high-stringency and high confidence scenario required the detection of a species in a minimum of two samples by both assays.

qPCR Data Comparison

All the eDNA samples assessed with metabarcoding primers in this study were previously analysed with species-specific qPCR assays. The reservoir samples from Teare Creek and Factor Ross were analysed with species-specific assays for kokanee, rainbow trout, lake whitefish, bull trout, lake trout and peamouth (Chapter 2). PCR protocols for the reservoir samples differed slightly between qPCR and metabarcoding analysis. Most notably, qPCR analysis was performed on two 5 µL replicates of the i-elution for each sample, while PCR1 for metabarcoding samples was performed on a single replicate of the ii-elution. The eDNA samples from tributaries of the Williston Reservoir were previously tested for Arctic grayling by Stamford et al. (2020) and Stamford et al. (2022). Summaries of the qPCR data are provided in this study for comparison between the two eDNA methods. The PCR protocols for the qPCR and metabarcoding approach

also differed for samples collected from tributary streams to the Williston Reservoir. The qPCR protocols for Arctic grayling utilized two 5 µL replicates of the i-elution for each sample, while PCR1 for metabarcoding samples was performed on a single 3 µL replicate of the same sample for each metabarcoding assay.

Gillnet Sampling in the Williston Reservoir

The eDNA results from the Williston Reservoir were compared with catch results from pelagic gill nets set at Teare Creek. Gillnet sampling methods are described in Chapter 2.

Snorkel Surveys of the Ingenika River

In 2019, a snorkel survey of the Ingenika River was paired with eDNA sampling for Arctic Grayling. A detailed description of the Ingenika snorkel survey is provided in Strohm et al. (2020), and the eDNA sampling procedure that accompanied it is described in Stamford et al. (2020). Poor visibility in 2019 reduced the number of sites that could be snorkeled compared to those where eDNA was collected. eDNA samples selected for metabarcoding analysis in this study were from the middle and upper river sections that were snorkeled, as well as four replicates from two headwater sites that were not paired with snorkeling.

RESULTS

In Silico Validation

The MiFish and FishCYTB assays were selected for use in tandem because their strengths are complementary, their weaknesses in species resolution have minimal overlap, and they are both well suited to the species assemblage in the Williston Watershed. The modified MiFish primers did not have any mismatches with the species in the Williston Watershed, but the amplicons for closely related species had low genetic distances between them. Largescale sucker

were the only species without a sequence in the NCBI nucleotide database that covered the MiFish region of the 12S gene and were excluded from the *in silico* analysis for that assay. MiFish amplicon divergence was low between closely related species and only differed by a single base pair for kokanee and rainbow trout, as well as between prickly and slimy sculpins (Figure 3. 1). The FishCYTB primers include six degenerate bases in the forward primer and five in the reverse primer (Table 3. 1). There were few mismatches with the FishCYTB primers across all species (Table 3. 3). Most species mismatches occur in the middle or at the 5' end of the primers and none are within five positions from the 3' end. Mismatches are most significant for kokanee and rainbow trout. Both species have a single mismatch at the sixth base position from the 3' end of the reverse primer, and the total number of mismatches are slightly higher than for other species (Table 3. 3). FishCYTB amplicons differed greatly between most species, including those belonging to the same genus (Figure 3. 2). The one exception was the low genetic distance between bull trout and lake trout amplicons.

Table 3. 3. FishCYTB primer mismatches with the CYTB sequences of 19 species from the Williston Watershed.

	FishCYTB Primer Mismatches		
Species	**Forward**	**Reverse**	**Total**
Prickly sculpin	0	0	0
Slimy sculpin	0	0	0
Lake trout	1	0	1
Bull trout	2	0	2
Rainbow trout	2	1	3
Kokanee	2	2	4
Lake whitefish	1	0	1
Mountain whitefish	0	0	0
Pygmy whitefish	2	0	2
Arctic grayling	1	0	1
Longnose sucker	1	1	2
White sucker	0	1	1
Largescale sucker	0	1	1
Peamouth chub	1	1	2
Northern pikeminnow	2	1	3
Redside shiner	1	1	2
Lake chub	0	1	1
Longnose dace	0	0	0
Burbot	2	0	2

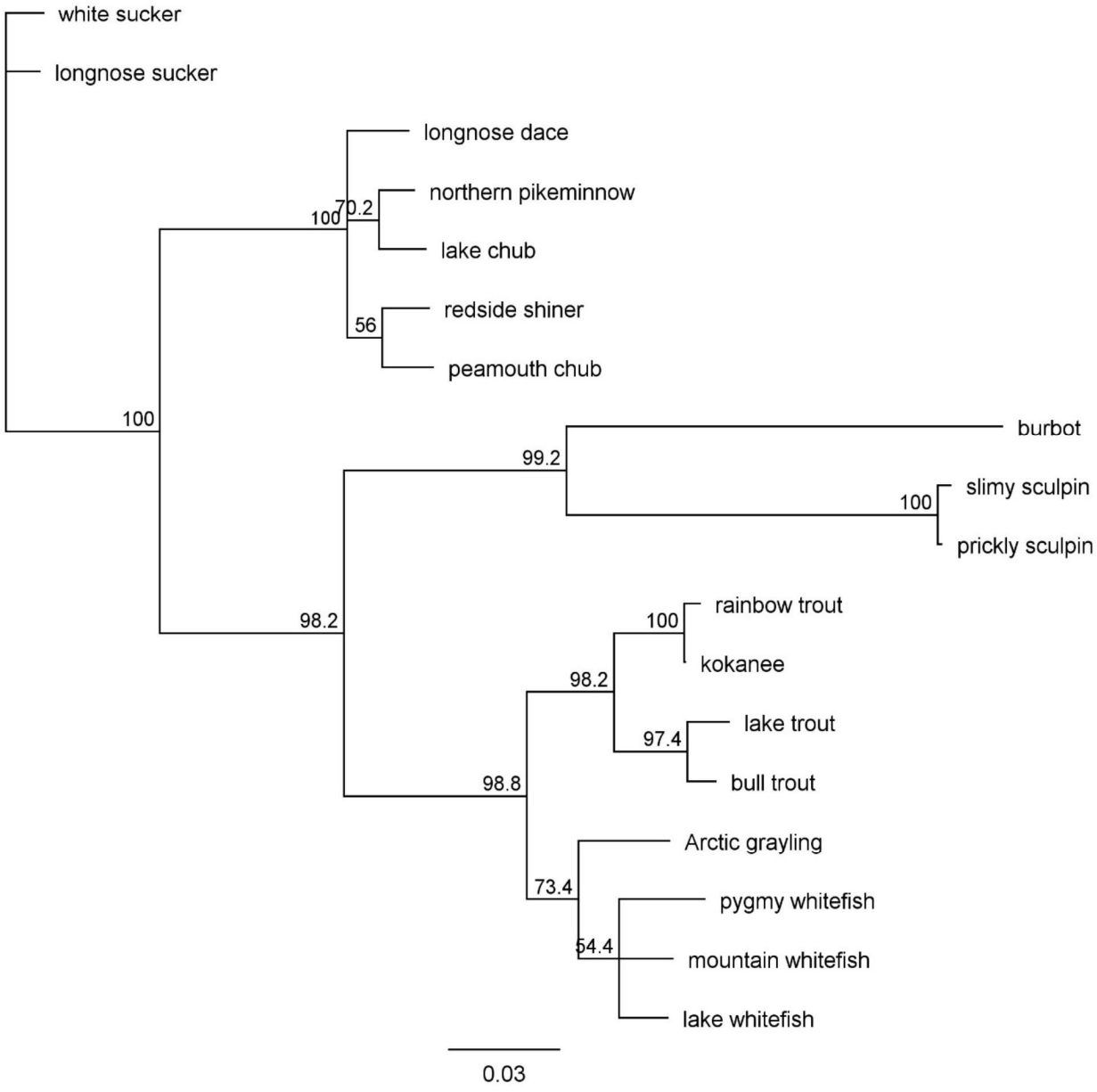

Figure 3. 1. Neighbour joining tree for the MiFish amplicons (214-218 bp) from 18 species in the Williston Reservoir created using the Tamura-Nei distance model. Percent consensus based on 500 bootstrap iterations is shown at the nodes. The scale bar indicates the relative genetic distance between amplicon sequences for each species.

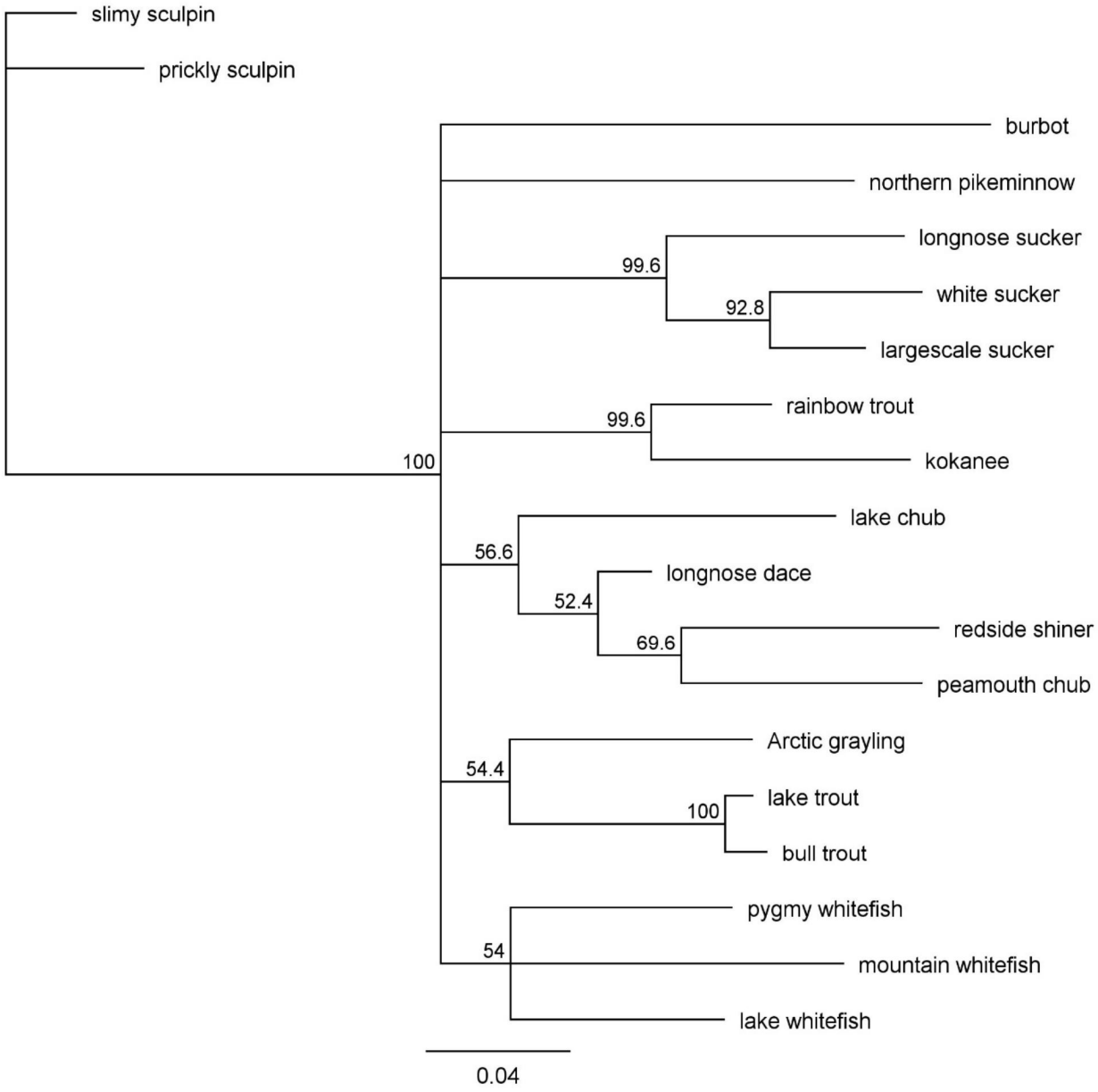

Figure 3. 2. Neighbour joining tree for the FishCYTB amplicons (209 bp) from 19 species in the Williston Reservoir created using the Tamura-Nei distance model. Percent consensus based on 500 bootstrap iterations is shown at the nodes. The scale bar indicates the relative genetic distance between amplicon sequences for each species.

ASV Assignment and Species Resolution

A total of 54,845 reads were generated from all samples amplified with the MiFish assay. Only 8,338 reads from 28 ASVs were retained for final analysis after discarding ASVs that did not have any matches with fish species from the Williston Watershed. The FishCYTB assay generated 1,118,323 total reads from the same samples. Of those, 80,706 FishCYTB amplicon reads were retained from 51 ASVs that matched fish species in the Williston Watershed.

The MiFish (12S) assay was unable to discriminate between kokanee and rainbow trout, however both species were unambiguously matched to ASVs produced with the FishCYTB assay. Five MiFish ASVs had identical BLAST scores for both kokanee and rainbow trout. Neither species was detected with the MiFish assay in any of the tributary samples despite multiple detections of rainbow trout with the FishCYTB assay, and by snorkeling observations that confirmed the presence of rainbow trout in the Ingenika River. Detections for the MiFish rainbow trout/kokanee ASVs in the reservoir samples were assumed to be kokanee because they were much more abundant in the gillnet catch and because the FishCYTB assay only detected kokanee in the same samples.

Many ASVs from both metabarcoding assays identified species from the genus *Salvelinus*, but identification to species was not always clear. Three MiFish ASVs and four FishCYTB assays were unambiguously identified as bull trout. Two MiFish ASVs and one FishCYTB ASV were matched with identical BLAST scores to lake trout, Dolly Varden (*Salvelinus malma*), Arctic char (*Salvelinus alpinus*), as well as other species from the same genus that are not native to North America. Bull trout were either not identified in the top 100 hits for these ASVs or they received lower BLAST scores than the other species. These *Salvelinus* ASVs were detected in the mock community samples, reservoir samples from Teare Creek and Factor Ross, as well as in some of

the Ingenika River headwaters samples. Three MiFish ASVs and six FishCYTB ASVs had equal BLAST scores for Dolly Varden, Arctic char, brook trout (*Salvelinus fontinalis*) and bull trout, but not for lake trout. All the ASVs that could not be unambiguously identified to a single species were classified to the genus level only.

Sculpin were not as easily identified to species with the MiFish assay as they were with FishCYTB. Spoonhead sculpin (*Cottus ricei*) received the highest BLAST scores for all sculpin ASVs produced by the MiFish assay. Spoonhead sculpin are not known to occur in the Williston Watershed upstream of the W.A.C. Bennet Dam on the Peace River, but they have been observed downstream of it (McPhail, 2007). Slimy or prickly sculpin were the next best match to the MiFish ASVs and only one of those two species was identified for each OUT. The BLAST scores for the slimy or prickly sculpins were only slightly lower than the scores for spoonhead sculpin. The FishCYTB assay unambiguously matched ASVs to either slimy or prickly sculpins but not to spoonhead sculpin. Cross referencing the MiFish results with the FishCYTB results suggested that slimy or prickly sculpin was a more appropriate species assignment for the MiFish ASVs.

Non-fish Sequences

BLAST results for the MiFish and FishCYTB sequences included matches to a wide range of non-fish species, many of which are associated with aquatic environments. The number and diversity of non-fish species identified was much higher for the FishCYTB assay than for MiFish. A large portion of non-fish sequences from both assays were attributed to bacteria, protists, and aquatic invertebrates. Mammal species included beavers (*Castor canadensis* – identified by both assays) and a variety of other rodent species, porcupine (*Erethizon dorsatum*) least weasel (*Mustela nivalis*), little brown myotis (*Myotis lucifugus*), moose (*Alces alces*), caribou (*Rangifer tarandus*), elk (*Cervus canadensis nannodes*), snowshoe hare (*Lepus americanus*), and wolf

(*Canis lupis*). Bird species included the hermit thrush (*Catharus guttatus*) and wren (*Tryoglodytes* sp.).

Mock Community

At least nine of the 12 species included in the mock community samples were detected by at least one of the metabarcoding assays (Table 3. 4). Although two replicates of the mock community were tested with each assay, only one of the MiFish replicates was sequenced successfully. Slimy sculpin and rainbow trout were not detected by either of the assays. Bull trout and kokanee were only detected by FishCYTB. The read count for many species was low for all samples, but the MiFish amplified mock community sample had a much lower read count compared to the FishCYTB amplified mock community samples. Lake trout and bull trout DNA were both included in the mock community samples. BLAST results for one MiFish ASV and one FishCYTB ASV matched many species from the genus *Salvelinus* including lake trout and bull trout. Because the mock community included only lake trout and bull trout DNA and bull trout were more confidently identified with a FishCYTB ASV, the ambiguous *Salvelinus* FishCYTB ASV was suspected to represent potential detections of lake trout DNA. However, only the ambiguous, genus level *Salvelinus* ASV was detected in the MiFish mock community sample and may have been an amplification of DNA from bull trout or lake trout.

Table 3. 4. Read counts for each of the 12 species included in the mock community samples.

Species	MiFish	FishCYTB-1	FishCYTB-2
Slimy sculpin			
Salvelinus sp.	41	186	212
Bull trout		23	47
Rainbow trout			
Kokanee		47	35
Lake whitefish	14	97	141
Mountain whitefish	39	449	468
Arctic grayling		21	87
White sucker	4	26	25
Peamouth	21	269	280
Northern pikeminnow	20	92	80
Burbot	10	31	48

In Situ Assay Testing on Reservoir eDNA Samples

Kokanee and lake whitefish were detected throughout the water column by both the MiFish and FishCYTB assays at Teare Creek and Factor Ross with high confidence (Table 3. 5). Peamouth were detected in fewer samples, but detection confidence was still high at Teare Creek and Factor Ross. Kokanee, lake whitefish and peamouth were the most abundantly caught species in gillnets at Teare Creek. The gillnet catch also included one rainbow trout, one bull trout and one longnose sucker (Table 3. 5). Bull trout and longnose sucker were detected with moderate confidence by the metabarcoding primers, but rainbow trout was not detected at either location. Species detected by eDNA metabarcoding but not by gillnets at Teare Creek included mountain whitefish and slimy sculpin (both moderate confidence). Four species (white sucker, prickly sculpin, pygmy whitefish and redside shiner) were detected at Factor Ross in addition to those detected at Teare Creek, but with low confidence. Multiple ASVs that could be confidently identified only to genus level for *Salvelinus* were detected at both locations. All reservoir samples were tested for the presence of lake trout and Arctic grayling with species-specific qPCR assays but neither species was detected.

Table 3. 5. Species detections above (<10 m depth) and below (> 10 m depth) the thermocline in the Williston Reservoir at the Teare Creek and Factor Ross stations with eDNA and gillnets. eDNA metabarcoding species detections with a single assay are noted as M for MiFish or F for FishCYTB. Detections with both metabarcoding assays are noted as B. Detection confidence with eDNA metabarcoding is indicated as high (green), moderate (yellow) or low (red). Species-specific detections with qPCR assays and species caught by gill net are noted as X.Species not tested for with qPCR are noted with a dash (-).

	FACTOR ROSS eDNA				TEARE CREEK eDNA				TEARE CREEK Gillnet	
	Metabarcoding		qPCR		Metabarcoding		qPCR			
Species	<10 m	>10 m	<10 m	>10 m	<10 m	>10m	<10 m	>10 m	<10 m	>10m
Prickly sculpin	M		-	-			-	-		
Slimy sculpin	M	B	-	-	M	B	-	-		
Bull trout	B		X		F		X			X
Lake trout										
Salvelinus sp.	B	B	-	-	M	F	-	-		
Rainbow trout			X				X		X	
Kokanee	B	B	X	X	B	B	X	X	X	X
Lake whitefish	B	B	X		B	B	X	X	X	X
Mountain whitefish	M	F	-	-	M	F	-	-		
Pygmy whitefish		M	-	-			-	-		
Arctic grayling										
Longnose sucker	M	M	-	-	F		-	-		X
White sucker	M		-	-			-	-		
Peamouth	B	F	X		F	B		X	X	
Redside shiner	F		-	-			-	-		

Species detections for eDNA differed somewhat between qPCR and metabarcoding methods. Kokanee, rainbow trout, bull trout, lake trout, lake whitefish, Arctic grayling and peamouth were the only species tested for with qPCR. Results were similar between the two eDNA methods for kokanee, lake whitefish, peamouth and bull trout (Table 3. 5). Multiple ASVs that could only be confidently attributed to genus level for *Salvelinus* were detected above and below the thermocline at Teare Creek and Factor Ross. Lake trout were not detected in the gillnets or with the novel qPCR assay that was being evaluated for use in the Williston Reservoir (Chapter 2). Rainbow trout were not detected with either metabarcoding assay. Rainbow trout were detected by qPCR, but the number of eDNA copies detected was very low (See Chapter 2).

In Situ Assay Testing on Williston Tributary eDNA Samples

A total of 15 species and ASVs that matched the *Salvelinus* genus were detected in samples from tributary streams around the Williston Reservoir. Slimy sculpin, mountain whitefish and bull trout were detected with at least moderate confidence in every stream that was tested (Table 3. 6). Longnose sucker and rainbow trout were detected in most streams, and all other species were detected to a lesser extent.

Table 3. 6. Species detections with the MiFish (M), FishCYTB (F), or both (B) metabarcoding assays in tributary rivers and creeks to the Williston Reservoir. Detection confidence is indicated as high (green), moderate (yellow) or low (red).

Sample Location	Prickly sculpin	Slimy sculpin	Longnose sucker	White sucker	Largescale sucker	Mountain whitefish	Pygmy whitefish	Bull trout	*Salvelinus* **sp.**	Rainbow trout	Kokanee	Arctic grayling	Longnose dace	Northern pikeminnow	Redside shiner	Burbot	Total species detected by confidence level (High:Mod:Low)
Akie Creek		B	B			B	B	B			F	F				F	4:6:8
Pesika Creek		B	F			B		B		F		F[2]					3:4:6
Middle Ingenika		B				B		F		F		F[2]					2:5:5
Upper Ingenika		B	F			B		B	M	F							3:5:6
Ingenika Headwaters		B	F			B		F	B	F							2:5:6
Ospika River		B	B	F	F	B		B				B[2]	F				4:5:8
Manson River	F	B	F	B		B		B		F			B	F	F	F	1:5:11
Carbon Creek		B	F			B		B	F								2:2:5
Clearwater Creek		B				B		B	F	F							3:4:5
Nabesche Creek		B	F			B		B		F							3:4:5
Colbourne Creek		B	F			B		F		F		[2]				B	2:5:5
Reynolds Creek[1]		B	F			B		F	F	F		F[2]	F			B	0:8:9

[1]One of the MiFish amplified samples from Reynolds Creek was not successfully sequenced.
[2]Streams where Arctic grayling was detected with qPCR.

The FishCYTB assay detected more species and had a greater number of detections per species than the MiFish assay did. The most abundant and ubiquitously distributed species (slimy sculpin, mountain whitefish and bull trout) were almost always detected with both metabarcoding assays. Most of the other detections for a species were with the FishCYTB assay. Rainbow trout were

frequently detected, but only by the FishCYTB assay. The only instance where the MiFish assay had a detection and the FishCYTB did not, was for one of the *Salvelinus* ASVs in the Upper Ingenika River (Table 3. 6 and Table 3. 7).

eDNA Metabarcoding Comparison with Snorkel Surveys

The large number of samples collected from the Ingenika River allowed me to assess distribution of species along the length of a river and make direct comparisons to the snorkel surveys that were performed in the same reaches. Snorkel surveys in the middle and upper reaches of the Ingenika River observed mountain whitefish, bull trout, Arctic grayling and rainbow trout, but did not record observations of other species (Strohm et al., 2020). Counts of bull trout, rainbow trout and Arctic grayling ranged from 18 to 37 individuals per reach. Mountain whitefish counts were considerably higher with 279 and 259 individuals in the middle and upper reaches, respectively. Both eDNA metabarcoding assays detected slimy sculpin and mountain whitefish in every sample from the Ingenika River (Table 3. 6). Rainbow trout and either bull trout or *Salvelinus* sp. were detected in nearly every sample. Bull trout were detected in all three reaches of the Ingenika, while *Salvelinus* sp. ASVs were detected almost exclusively in the headwaters reach (Table 3. 7). Arctic grayling were detected in two out of six samples from the middle Ingenika reach. Longnose suckers were only detected with FishCYTB and only in the upper river and headwaters samples. Burbot were detected with both assays in the middle reach and longnose dace were detected in a single sample from the upper reach.

eDNA Metabarcoding Comparison with qPCR

All the tributary samples I analyzed with eDNA metabarcoding were originally tested with a qPCR assay for Arctic grayling by Stamford et al. (2020; 2022). Arctic grayling were detected by metabarcoding and qPCR in both samples from Pesika Creek and Reynolds Creek (Table 3. 6).

Metabarcoding detected Arctic grayling in two out of six samples from the middle Ingenika River where qPCR detected them (Table 3. 7), and in two out of four samples where qPCR detected them from the Ospika River. Metabarcoding detected Arctic grayling in one sample from Akie Creek when qPCR did not, and qPCR detected Arctic grayling in one sample from Reynolds Creek when metabarcoding did not (Table 3. 6). The MiFish assay only detected Arctic grayling in a single sample from the Ospika River and all other metabarcoding detections were with the FishCYTB assay.

Table 3. 7. Species detections with the MiFish (M), FishCYTB (F) or both (B) metabarcoding assays from the middle, upper and headwaters reaches of the Ingenika River. Snorkel counts for the middle Ingenika River were 279 mountain whitefish, 18 bull trout, 19 rainbow trout, 27 Arctic grayling. Snorkel counts for the upper Ingenika River were 259 mountain whitefish, 37 bull trout, 19 rainbow trout, 23 Arctic grayling.

Sample	Slimy sculpin	Longnose sucker	Mountain whitefish	Rainbow trout	Bull trout	*Salvelinus* sp.	Arctic grayling	Longnose dace	Burbot
Mid 1A	B		B	F	F		*		M
Mid 1B	B		B	F	F		*		
Mid 2A	B		B	F			*		
Mid 2B	B		B	F	F		F*		B
Mid 3A	B		B	F	F		F*		
Mid 3B	B		B	F	F		*		
Upper 1A	B		B		B				
Upper 1B	B	F	B	F	B	M			
Upper 2A	B		B	F	F				
Upper 2B	B		B	F					
Upper 4A	B		B	F	B				
Upper 4B	B	F	B	F	B			F	
Upper 5A	B	F	B		F				
Upper 5B	B		B	F	F				
Headwater 1A	B	F	B	F		B			
Headwater 1B	B	F	B	F	F	F			
Headwater 1C	B	F	B	F	F	B			
Headwater 2A	B		B			B			
Headwater 2B	B	F	B	F		B			

*Samples where Arctic grayling was detected with species-specific qPCR analysis.

DISCUSSION

eDNA Metabarcoding in the Williston Reservoir

eDNA metabarcoding was effective in detecting most fish species in pelagic habitats of the Williston Reservoir when compared to qPCR and gillnetting surveys. Rainbow trout were the only species detected by qPCR and gillnetting but not by either metabarcoding assay. Although the MiFish assay was unable to differentiate between rainbow trout and kokanee, signal detection with this assay in the reservoir was likely kokanee as rainbow trout were detected by the FishCYTB in many of the tributary samples. Metabarcoding detection probability would have been reduced compared to qPCR due to the types of samples both methods analyzed. The qPCR analyses were conducted on i-elutions of the reservoir samples and metabarcoding was conducted on the ii-elutions of the same samples. Subsequent elutions of the same sample typically yield less DNA than the first (Qiagen, 2020) and since rainbow trout were barely detected by qPCR in the first elution, it is not surprising that they were not detected by metabarcoding on the ii-elutions. Like the qPCR analysis, species detection confidence with metabarcoding was proportional to the catch abundance in the gillnets. Kokanee and lake whitefish were the most abundant species caught in the gillnets and were detected with high confidence by eDNA metabarcoding. The Williston Reservoir is both enormous and unproductive; consequently, detection confidence with eDNA could be improved by increasing the sampling effort. This could be done by filtering larger volumes per sample, collecting more samples, and by using innovative sample techniques that capture higher concentrations of eDNA, like the mesh tow net method described by Schabacker et al. (2020). Detection probabilities are also affected by the level of replication during other stages of the workflow such as PCR and sequencing (Ficetola et al., 2015). Increasing the number of

PCR1 replicates in this study would likely have increased our detection probabilities for rare reservoir species like rainbow trout.

The metabarcoding analysis detected multiple species that were absent from any of the gillnet surveys conducted throughout the Williston Reservoir (O'Connor, 2022) including slimy sculpin, mountain whitefish, pygmy whitefish and redside shiner (Table 3. 5). Slimy sculpin and redside shiner are less susceptible to being captured in gillnets because of their small body size and behaviour (Kubečka et al., 2009; Olin et al., 2009). Rare species such as pygmy whitefish would have a very low probability of capture in traditional gears but may be readily detected by eDNA due to its increased sensitivity (Evans, Shirey, et al., 2017; Jerde et al., 2011; Thomsen et al., 2012). Pygmy whitefish and redside shiner were detected with low confidence using eDNA metabarcoding, but slimy sculpin and mountain whitefish were detected with moderate confidence. Mountain whitefish would have been susceptible to capture in gillnets but were absent from any of the nets set in the reservoir in 2021 (O'Connor, 2022). Therefore, the detection of mountain whitefish eDNA in the pelagic zone does not appear to be the result of their presence in the same area. It is possible that some of the unexpected species detections were the result of eDNA that was transported into the pelagic zone of the reservoir from elsewhere. Mountain whitefish and slimy sculpin were detected in every tributary of the reservoir that I tested (Table 3. 6), and it is possible that their DNA was transported into the reservoir by streamflow (Evans, Li, et al., 2017) and could have been transported further by wind and water currents in the reservoir itself. However, eDNA will only remain detectable in the environment for a relatively short period that ranges from a few days to a couple of weeks before decaying (Rourke et al., 2021; Takahara et al., 2012) or settling out of the water column (Canals et al., 2021; Jane et al., 2015; Stamford et al., 2020). The distance that eDNA can be transported from any tributary into the pelagic zone of the

Williston Reservoir is unknown, but other studies in lakes have demonstrated that eDNA dispersal is limited by summer stratification (Littlefair et al., 2020). Mountain whitefish and other species may utilize near-shore areas in the reservoir that were not targeted with gillnets but that were close enough to the eDNA sample sites to facilitate detection of transported eDNA. The distance that eDNA could be dispersed in the epilimnion of the Williston Reservoir may be quite large when strong winds persist for days, as they did immediately prior to the collection of samples for this study.

The difficulty that the MiFish and FishCYTB assays had in resolving species from the *Salvelinus* genus is problematic for the use of eDNA metabarcoding in the Williston Reservoir. Some ASVs were unambiguously matched to bull trout, but many could not be confidently identified beyond the genus level. Lake trout and bull trout are high priority species for fisheries management in the Williston Reservoir (Fish and Wildlife Compensation Program, 2020) and effective eDNA monitoring should be able to differentiate the two species. Lake trout DNA was included in the mock community but did not produce any ASVs that were unambiguously matched to lake trout in the BLAST results. At least one ASV from both metabarcoding assays that was detected in the mock community was suspected to have been the result of lake trout DNA. These same ASVs were detected in some of the reservoir samples and in the headwater samples from the Ingenika River. Lake trout were not identified in any of the reservoir samples with a novel qPCR assay (although the assay was being validated for that purpose at the time), and they were not caught in any of the gillnets set at Teare Creek. It is possible that the *Salvelinus* ASVs were detecting lake trout in the reservoir, but there is no compelling evidence to support this idea. It appears equally likely that those same *Salvelinus* ASVs could have been detecting bull trout eDNA. The utility of eDNA metabarcoding in the Williston Reservoir could be improved if an

alternate combination of primers were able to resolve all the pelagic species that are priorities for fisheries management.

eDNA Metabarcoding in Williston Tributaries

The probability of detection with eDNA metabarcoding was proportional to the relative abundance of each species in the sampling environment. Mountain whitefish were much more abundant than any of the other species observed during the 2019 snorkel surveys in the Ingenika River (Strohm et al., 2020) and accounted for 80% of the species captured in a 2021 survey of the Ospika River (Stamford & O'Connor, 2022). Mountain whitefish were detected in every eDNA sample I tested with high confidence (Table 3. 7). Detections of less abundant species were often reduced with metabarcoding compared to qPCR and snorkel observations. Bull trout and rainbow trout were detected in most samples, although to a lesser extent than mountain whitefish. This result was consistent with the relative abundance of each species observed in the Ingenika River snorkel surveys and in surveys of the Ospika River (Stamford & O'Connor, 2022). Arctic grayling were observed more frequently than rainbow trout and bull trout in the middle reach of the Ingenika River during the snorkel survey, but Arctic grayling were detected in fewer samples than the other two species with eDNA metabarcoding. The reduced detection confidence with eDNA metabarcoding can be partially explained by the poor performance of the MiFish assay. Across all the tributary samples tested, the MiFish assay never detected rainbow trout while bull trout and Arctic grayling were detected much less frequently with MiFish than they were with the FishCYTB assay. Arctic grayling were also detected less often with metabarcoding in the Ingenika River and Ospika River compared to the qPCR analysis of the same samples. Although Arctic grayling were detected in more samples with qPCR than with metabarcoding, the difference in sensitivity between the two methods was not substantial. Differences in lab procedures likely

contributed to the discrepancy between metabarcoding and qPCR detection results. The qPCR workflow that was used for detecting Arctic grayling in the tributary samples included amplifying eDNA on a droplet digital PCR (ddPCR) platform. ddPCR has many advantages over traditional PCR including increased sensitivity, and it is less influenced by matrix materials such as non-target DNA and inhibitors (Bio-Rad Labratories, Inc., 2022; Burns et al., 2010; dMIQE Group, 2020; Doi et al., 2015). The small amount of sample that was tested in the metabarcoding analysis relative to qPCR would have reduced the probability of detection for metabarcoding. The PCR1 reactions for the metabarcoding analysis were performed on a single replicate for the MiFish and FishCYTB assays that tested a total of 6 µL of sample. The qPCR reactions were amplified in two PCR replicates that included a total of 10 µL of the sample. Increasing the number of PCR replicates has demonstrated a reduction in the rate of false negatives and to increase the detection probability for rare species in metabarcoding studies (Ficetola et al., 2015). Increasing the number of PCR1 replicates could have increased the detection probability for eDNA metabarcoding in this study to a level that would have been comparable to qPCR. This was supported in the analysis of reservoir samples from this study where the total sample volumes tested by qPCR and metabarcoding were the same (albeit from different elutions) and the detection results from both methods were similar.

The eDNA sample distribution and number of samples that were analyzed in this study was not sufficient to describe the full diversity of species accurately and confidently in any single watercourse and may have contributed to the discrepancy between species detections with metabarcoding and snorkeling in the Ingenika River. The eDNA of very abundant and evenly distributed species like mountain whitefish and slimy sculpin should likewise be abundant and evenly distributed in the environment. This is supported by the detection of mountain whitefish and slimy sculpin in every sample analyzed by eDNA metabarcoding. However, relatively rare

species like Arctic grayling often have a patchier distribution resulting in eDNA concentrations that vary throughout the environment (Eichmiller et al., 2014; Rourke et al., 2021). eDNA samples collected by Stamford et al. (2020) in the Ingenika River were spaced 1.5 km apart based on the assumption that Arctic grayling eDNA would remain suspended in the water column over that distance. That assumption proved to be reasonable for Arctic grayling, although false negatives in some sample replicates suggested that eDNA may not always travel that far, and concentrations are reduced with distance. The 1.5 km distance between samples may not be an appropriate spacing for all species or at all times of the day or year. Species-specific behaviour can affect the rates of eDNA shedding (Klymus et al., 2015) and habitat preferences or environmental factors may affect eDNA shedding rates and downstream transport of eDNA (Barnes et al., 2014; Barnes & Turner, 2016; Jane et al., 2015). Sample size is an important determinant of the accuracy and precision with which eDNA metabarcoding can estimate species richness in any system (Evans, Li, et al., 2017). The distribution and size of samples selected for this exploratory study of eDNA metabarcoding in the Williston Watershed are insufficient to confidently detect all the species present. When an appropriate sample design is implemented, eDNA metabarcoding has proven effective in determining species richness in environments that can be difficult to sample with traditional fish-capture methods (Balasingham et al., 2018; Evans, Shirey, et al., 2017; Hänfling et al., 2016).

The issue of resolving closely related species with metabarcoding was not as problematic in the tributaries as it was in the reservoir due to the difference in species assemblages between the two environments. The inability of the MiFish assay to differentiate between kokanee and rainbow trout was overshadowed by the fact that the MiFish rainbow trout/kokanee ASV was never detected in any of the tributaries, even when rainbow trout were observed near sample

locations. Kokanee and rainbow trout eDNA would be present together in many of the same stream habitats around the Williston Reservoir from the time kokanee adults begin migrating to spawn in the fall until fry migrate out of the same streams in the spring. Although differentiating between species would not be possible with the MiFish assay during that period, it would not be a problem with the FishCYTB assay. Both assays had some difficulty resolving species of the genus *Salvelinus,* but bull trout are likely the only species of that genus present in streams and rivers of the Williston Watershed. Lake trout complete their entire life cycle in lacustrine environments (McPhail, 2007; Riley et al., 2021) and have never been observed in streams or river habitats around the Williston Reservoir (Culling & Euchner, 2019; Zemlack & Langston, 1994). Therefore, it would be safe to assume that any *Salvelinus* ASVs detected in tributary samples were for bull trout. In the headwaters reach of the Ingenika River, genus level *Salvelinus* ASVs were detected with high confidence while ASVs that unambiguously matched to bull trout were detected less (Table 3. 7). This result contrasted with other reaches of the Ingenika River where bull trout ASVs were detected frequently and there was only a single *Salvelinus* detection. This contrasting pattern of detections is an interesting observation which may indicate some genetic differences between bull trout that occupy the headwaters of the Ingenika River and those that are present elsewhere. Bull trout in the Ingenika headwaters may be a stream-resident population that has become at least partially isolated from bull trout with fluvial or adfluvial life histories. Little is known about the distribution of Dolly Varden in the Williston Watershed but they have been identified in tributaries of Thutade Lake at the headwaters of the Finlay River (Hagen, 2000) and more recently in the headwaters of the Ingenika River (J. Hagen, personal communication, 15 April 2024). Bull trout and Dolly Varden are sympatric in the Thutade watershed and although hybridization has been confirmed there, it appears to occur infrequently (Hagen, 2000; Redenbach & Taylor, 2003).

Differentiating between bull trout and Dolly Varden with metabarcoding or species-specific eDNA methods would be challenging due to the genetic similarity between the two species and their tendency to hybridize (Redenbach & Taylor, 2003; Taylor et al., 2001).

Evaluating eDNA Metabarcoding for use in the Williston Watershed

MiFish and FishCYTB Assay Performance

I chose to evaluate the MiFish and FishCYTB metabarcoding assays for their potential applications in the Williston Reservoir and the surrounding watershed. In this study, the FishCYTB assay outperformed MiFish assay and was effective enough to be used on its own in the tributaries. The FishCYTB assay generated approximately 20 times more raw reads, 10 times more reads that were matched to fish species in the Williston Watershed, and nearly twice as many final ASVs compared to MiFish. Both assays failed to detect slimy sculpin and rainbow trout in the mock community, but the results from the *in situ* samples suggest that this may not have been an issue with the assays. Slimy sculpin were detected by both assays, and rainbow trout were detected by FishCYTB in the Williston samples. The FishCYTB assay did not consistently fail to detect any species like the MiFish assay did with rainbow trout. The FishCYTB assay detected more species in more samples compared to the MiFish assay, and the MiFish results added little to the FishCYTB results. Littlefair et al. (2020) used the MiFish assay successfully on its own in the Experimental Lakes Area of Ontario with many of the same species. The species assemblage there may not have been comprised of as many closely related species, and the absence of bull trout would have made lake trout detections less ambiguous. Littlefair et al. (2020) also analyzed more samples and more technical PCR replicates than I did and used a different bioinformatics pipeline. Metabarcoding studies have often used multiple genetic markers to increase overall detection probability. The 12S, 16S and CYTB genes are commonly used in combination, and CYTB

markers have often detected more species than those designed for 12S (Evans et al., 2016; Evans, Li, et al., 2017; Hänfling et al., 2016; Morey et al., 2020; Olds et al., 2016). The performance of the 12S and CYTB markers relative to one another varied in those studies due to a variety of factors that included the relative length of the amplicons for each marker (Hänfling et al., 2016), the diversity of the species assemblage being tested (Morey et al., 2020) and the sampling effort applied (Evans, Li, et al., 2017). Amplicon lengths for the MiFish and FishCYTB amplicons were nearly identical (Table 3. 1), the diversity of the species assemblage in the Williston Watershed is relatively low, and the sampling effort applied in this study was small relative to the size of the reservoir. The results of this study are exploratory and not comparable to those from larger applied studies with more comprehensive sample designs and more sophisticated bioinformatics analysis. However, the same methods were applied with the MiFish and FishCYTB assay in this study and their performance can be evaluated relative to one another.

A significant shortfall of both the MiFish and FishCYTB assays was the inability to resolve bull trout and lake trout. Substituting MiFish for an assay that could discriminate between these two species would improve the overall performance of eDNA metabarcoding for the Williston Watershed, but this level of species resolution may not be possible with any metabarcoding marker. Metabarcoding primers are usually designed for highly conserved genes to minimize primer mismatches and maximize species coverage, often at the expense of specificity. It is important to note, however, that an eDNA metabarcoding assay includes the entire workflow from field sampling to bioinformatics analysis and the sensitivity and specificity of an assay is determined by more than just the primers used (Deiner et al., 2017; Ficetola et al., 2015; Goldberg et al., 2016). The CYTB gene has a relatively high degree of interspecific species diversity and was a good candidate for differentiating between lake trout and bull trout. The CYTB gene was

chosen as the best candidate for that purpose when I designed a species-specific lake trout assay for the Williston Reservoir (Chapter 2), although it was still quite challenging. The FishCYTB assay has proven effective enough to use on its own to detect at least 15 of the 17 species that exist in the tributaries of the Williston Reservoir, and species-specific qPCR methods appear to be better suited to differentiating between such closely related species as bull trout and lake trout.

Metabarcoding vs. qPCR

eDNA metabarcoding is particularly well suited to large-scale inventories of streams in the Williston Watershed and could efficiently characterize changes in community structure across space and time. Compared to traditional sampling methods, eDNA metabarcoding can be cost effectively scaled up to inventory vast geographic ranges with minimal effort, has increased sensitivity for detecting rare and cryptic species, and is easily adapted to all habitat types (Jerde et al., 2013; Olds et al., 2016; Sard et al., 2019). Although I only tested a small number of samples from 13 streams and rivers around the reservoir, the detection results have revealed interesting patterns in species distribution and co-occurrence (Table 3. 6). Species occurrence data for entire fish communities provided by eDNA metabarcoding can be used to screen for critical habitats, biodiversity hotspots, and species interactions that may provide more insights to fisheries managers than species-specific approaches (Balasingham et al., 2018). Monitoring with eDNA metabarcoding is also well suited to the early detection of invasive species (Jerde et al., 2013). The Williston Watershed is a massive geographic region that has changed dramatically since the creation of the reservoir and will continue to change in the future because of climate change, industrial development, forestry, and angling pressure, among other factors. This study has provided several insights into the potential for eDNA metabarcoding as a method for large-scale

fisheries monitoring in the Williston Watershed as well as many lessons learned that will inform any future applications of the methodology.

Metabarcoding has advantages over a species-specific approach when the monitoring goal is to characterise diverse and dynamic fish communities, but species-specific methods appear to be more appropriate for monitoring focal species. The pelagic fish community in the reservoir is dominated by only six species, three of which are relatively abundant (kokanee, lake whitefish and peamouth), and three that are relatively rare (bull trout, lake trout and rainbow trout) (O'Connor, 2022; Plate et al., 2012; Sebastian et al., 2009). Species-specific qPCR assays are preferred for detecting a small number of rare species because the analysis is faster, not complicated by bioinformatics pipelines, highly specific, and relatively inexpensive (Bylemans et al., 2019; Morey et al., 2020). My results from the Williston Reservoir do not show a substantial difference in sensitivity between methods for the species that were detected, but differentiating between bull trout and lake trout detections is an important requirement for pelagic species monitoring that was not addressed well with the metabarcoding assays. The metabarcoding assays tested in this study appeared to have a lower probability of detecting Arctic grayling in tributary samples, although sensitivity could be increased by testing more PCR1 replicates (Ficetola et al., 2015). Species-specific assays appear to have an advantage over metabarcoding in the relationship they produce between eDNA concentrations and species abundance or biomass (Rourke et al., 2021), which would be a useful application for eDNA in the reservoir. Metabarcoding has many potential applications in the Williston Watershed, but a species-specific approach is better suited to studying the pelagic species in the Williston Reservoir.

EPILOGUE

Assay validation is critical for eDNA studies and even previously developed assays must be validated for their application in a particular geographic area and with a particular workflow (Goldberg et al., 2016; Thalinger et al., 2021). My research advanced the validation of species-specific qPCR assays for the six prominent pelagic species in the Williston Reservoir and a metabarcoding assay that was effective in detecting fish in the surrounding streams and rivers. The assays for kokanee, rainbow trout and bull trout have been used previously in Northern British Columbia (Dr. Brent Murray, pers. comm.), but the lake whitefish assay from Hernandez et al. (2020) was only developed recently, and this study may be the first to validate it in western North American. Novel qPCR assays were developed for lake trout and peamouth to cover all six of the most abundant pelagic species in the Williston Reservoir but have also addressed challenges for eDNA that are not unique to this situation. Bull trout and lake trout are high priority species for fisheries management in the Williston Reservoir (Hagen & Weber, 2019) and throughout western North America (Donald & Alger, 1993; Fredenberg, 2002; Martinez et al., 2009; Riley et al., 2021; Species At Risk Public Registry, 2019). The bull trout assay used in this study was previously validated for specificity with other closely related species, including lake trout (Wilcox et al., 2013). However, *in silico* validations of published lake trout assays developed in eastern Canada (Hernandez et al., 2020; Lacoursière-Roussel, Côté, et al., 2016) indicated that they would cross-amplify bull trout DNA. The development and validation of a lake trout assay that can discriminate between bull trout is an important achievement for eDNA monitoring in lakes and reservoirs across western North America where these two species are sympatric. This study found that the FishCYTB metabarcoding assay could detect at least 15 of the 19 species in the Upper Peace River Watershed reliably enough that it could be used on its own. Having validated eDNA assays for species in the Williston Reservoir and the surrounding watershed is a prerequisite for any future

eDNA studies and will reduce the barriers to the application of eDNA methods in fisheries management in the region.

Piloting eDNA in the Williston Reservoir and surrounding watershed has provided valuable insights into eDNA sampling design and the overall workflow for future eDNA studies. Detecting and characterizing the distribution of pelagic fish within the Williston Reservoir is challenging with any means of sampling because of the enormous size of the reservoir and the relatively low abundance of pelagic fish. Although my research affirmed the sensitivity of eDNA, my results demonstrated that pelagic eDNA concentrations are very low, especially for rare species like bull trout and lake trout. Filtering larger volumes of water is recommended to increase detection confidence and minimize false negative results, but the methods used in my research have practical limits. Collecting and storing large volumes of water for filtration on shore can be logistically challenging and filter efficiency can be reduced quickly as particles accumulate and reduce filter porosity. The high-volume eDNA water sampling method developed by Schabacker et al. (2020) is an alternative to traditional eDNA sampling techniques that could address the challenges with sampling logistics and eDNA sensitivity in the Williston Reservoir, and has the potential to increase pelagic eDNA sampling efficiency.

By modeling my sampling design and eDNA analysis on those published by Littlefair et al. (2020), I was able to analyze the vertical distribution of eDNA in the Williston Reservoir during summer stratification. Seasonal thermal stratification and the thermal preferences of fish species influences the distribution of eDNA in lakes (Klobucar et al., 2017; Littlefair et al., 2020). Although a stratified water column can limit the transportation and distribution of eDNA, many of the pelagic species in the Williston Reservoir exhibit diel vertical migrations. This resulted in eDNA detections above and below the thermocline for species who used habitats throughout the

water column. eDNA appeared to be distributed more homogeneously and abundantly above the thermocline, particularly for the relatively abundant planktivorous species. Piscivorous species were relatively rare and were not detected frequently enough to make any assumptions about their vertical distribution. I suggested that the distribution of eDNA below the thermocline is likely to be patchier but may provide a more precise representation of fish distributions due to the reduced potential for transportation relative to the wind mixed layers above the thermocline. Most of the pelagic species in the reservoir appear to be more detectable above the thermocline during summer stratification, while sampling below the thermocline is likely to provide more useful information about the distribution of lake trout. The distribution of eDNA for all species is likely to be more homogenous and easily detectable from a single depth when the reservoir water column is isothermal in spring and fall (Klobucar et al., 2017; Lacoursière-Roussel, Côté, et al., 2016; Littlefair et al., 2020). Sampling during those periods would require less effort to detect all species, but it may not be possible to draw as many conclusions about distribution.

eDNA has the potential to supplement traditional methods of fisheries inventory in the Williston Reservoir and may be a cost-effective and efficient solution for monitoring rare species across extremely large geographic areas. eDNA can detect rare species with greater efficiency and sensitivity compared to conventional sampling gears such as gill netting (Evans, Shirey, et al., 2017; Jerde et al., 2011; Thomsen et al., 2012) and has proven to be particularly useful for monitoring lake trout (Lacoursière-Roussel, Rosabal, et al., 2016; Littlefair et al., 2020). Developing a cost-effective method for monitoring of lake trout is a priority action for the Williston Reservoir (Fish and Wildlife Compensation Program, 2020) and traditional methods for monitoring lake trout are unrealistic for this waterbody. Two common survey methods for monitoring lake trout include Spring Littoral Index Netting (SLIN) and Summer Profundal Index

Netting (SPIN) (Jessup & Millar, 2011). Both methods would require hundreds of gillnet sets on a waterbody the size of the Williston Reservoir. Gillnetting in the Williston Reservoir is challenging due to several factors that include the flooded forests on the bottom of the reservoir that are difficult to anchor in and can entangle nets, and extreme weather that can be difficult to impossible to work in safely. Gillnetting at such high intensity across the reservoir would also result in large numbers of fish mortalities. A great deal of recent eDNA research has focused on understanding the relationship between the concentration of eDNA and the abundance of fish to develop eDNA as a method of stock assessment (Rourke et al., 2021). Several studies have demonstrated that eDNA particle concentrations are positively correlated with species abundance (Klobucar et al., 2017; Lacoursière-Roussel, Rosabal, et al., 2016; Sard et al., 2019; Shelton et al., 2019; Stoeckle et al., 2017; Takahara et al., 2012; Yates et al., 2020). eDNA research is actively pursuing the development of protocols that will provide reliable estimations of abundance (Rourke et al., 2021; Schenekar, 2023). Once achieved, eDNA will provide a rapid, cost-effective estimate of abundance for fisheries stock assessment in large waterbodies like the Williston Reservoir, where traditional means of stock assessment are challenging, if not impossible.

Although methods are still being developed, standardized, and refined, eDNA is a valuable tool with current utility and incredible potential for fisheries monitoring in the Williston Watershed. eDNA has been used successfully for monitoring Arctic grayling in the tributaries of the Williston Reservoir and has helped to better understand the extent of their current distribution (Stamford et al., 2020, 2022; Stamford & O'Connor, 2022). eDNA could be used in a similar way to effectively monitor other rare or important species but would not necessarily require the same sampling effort. eDNA analyses consume small amounts of the total sample collected and any unused volumes can be stored for long periods and analyzed for other purposes later. My

metabarcoding analysis reused several of the eDNA samples collected for Arctic grayling research, highlighting the potential cost savings and efficiency of eDNA for addressing multiple research objectives. eDNA has been collected from many of the streams from around the Williston Reservoir and reanalyzing those samples with a metabarcoding assay, like in this study, would be an effective means of conducting a large-scale inventory of tributary streams without any additional sampling costs. Expanding further, standardizing eDNA sampling throughout the Upper Peace River Watershed would be beneficial as a means of addressing multiple monitoring objectives. eDNA is revolutionizing biodiversity monitoring in aquatic environments and future applications of this technology are likely to become standard practices (Schenekar, 2023). This study has laid the groundwork for the future of eDNA in the Upper Peace River Watershed and will help to facilitate its adoption as eDNA-based methods are added to the standard toolbox for fisheries management.

As the number and magnitude of threats to aquatic environments increases, it is imperative that technologies for monitoring aquatic species evolve to identify problems and solutions more efficiently. Applications of eDNA represent one of the most significant recent advances in aquatic species monitoring that has many advantages over traditional survey methods (Jerde, 2019; Schenekar, 2023). eDNA is a rapidly growing field of research and is increasingly becoming a standard tool for biodiversity monitoring around the world (Schenekar, 2023). In Canada, research groups such as the Genomic Network for Fish Identification, Stress and Health (GEN-FISH, 2020) are developing and validating eDNA "toolkits" that will make eDNA methods quick, easy, reliable, inexpensive and broadly applicable across Canada for all freshwater species. Although eDNA methods are unlikely to completely replace traditional sampling methods, they are currently able to provide a substantial amount of the information required for a "complete picture" of a fish

population with much fewer resources. eDNA can identify species composition and spatial distribution, but traditional methods that capture individual fish will likely always be required to gather information about fish size and age. A great deal of research has focused on understanding how eDNA can provide a reliable estimate of fish abundance and biomass (Rourke et al., 2021), and it may only be a matter of time before this is achieved. eDNA and similar genetic methods for species identification have been broadly integrated into biodiversity monitoring and conservation programs across Canada. For example, Parks Canada uses eDNA to monitor aquatic species across the country (Parks Canada Agency, 2023) and programs like the Canadian Aquatic Biomonitoring Network are implementing a technique similar to metabarcoding to replace traditional taxonomic analysis (Canada, 2014). Like any developing technology, eDNA will continue to improve and new applications will be developed, but it is clearly a technology that can solve many of the challenges that fisheries managers are facing today.

BIBLIOGRAPHY

Balasingham, K. D., Walter, R. P., Mandrak, N. E., & Heath, D. D. (2018). Environmental DNA detection of rare and invasive fish species in two Great Lakes tributaries. *Molecular Ecology*, *27*(1), 112–127. https://doi.org/10.1111/mec.14395

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, *48*(3), 1819–1827. https://doi.org/10.1021/es404734p

Barrett, D. T., & Halsey, T. G. (1975). *Fishery resources of Williston Reservoir: A preliminary report* (54411). https://a100.gov.bc.ca/pub/acat/documents/r54411/Fishery_Williston_1531173190351_1170898330.pdf

B.C. Conservation Data Center. (2021). *Species Summary: Salvelinus confluentus pop. 10, bull trout—Western Arctic populations*. B.C. Ministry of Environment. https://a100.gov.bc.ca/pub/eswp/speciesSummary.do?id=312190

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: Opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Bethke, E., Arrhenius, F., Cardinale, M., & Håkansson, N. (1999). Comparison of the selectivity of three pelagic sampling trawls in a hydroacoustic survey. *Fisheries Research*, *44*(1), 15–23. https://doi.org/10.1016/S0165-7836(99)00054-5

Bio-Rad Labratories, Inc. (2022). *Droplet digitial PCR applications guide*. https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf

Blackman, B. G. (1992). *Fisheries resources of Williston Reservoir twenty years after impoundment* (239; p. 35). Peace/Williston Fish and Wildlife Compensation Program. https://a100.gov.bc.ca/pub/acat/documents/r20547/Blackman1992pwfwcp_report_no_239_1306777274296_dcd031b80f9bf542c185a57ea06fab0ea4cc48cc03ec8e9efcc883468de66e88.pdf

Blackman, B. G. (2002). *The distribution and relative abundance of Arctic grayling (*Thymallus arcticus*) in the Parsnip, Anzac and Rable Rivers* (PWFWCP Report No. 254). https://a100.gov.bc.ca/pub/acat/documents/r35844/pwfwcp_report_no_254_1363355935405_e2062bc3ef9224c80e01f7c590775e3a71bd5a459de5bd248f57e7106b055d6b.pdf

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), Article 8. https://doi.org/10.1038/s41587-019-0209-9

Burns, M. J., Burrell, A. M., & Foy, C. A. (2010). The applicability of digital PCR for the assessment of detection limits in GMO analysis. *European Food Research and Technology*, *231*(3), 353–362. https://doi.org/10.1007/s00217-010-1277-8

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular Detection and Quantification*, *14*, 19–28. https://doi.org/10.1016/j.bdq.2017.11.001

Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M., & Furlan, E. M. (2019). A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA*, *1*(4), 402–414. https://doi.org/10.1002/edn3.41

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Canada, E. and C. C. (2014, August 12). *CABIN news: Assessing freshwater health through DNA and community based moinitoring* [Guidance]. https://www.canada.ca/en/environment-climate-change/services/canadian-aquatic-biomonitoring-network/news-events.html

Canals, O., Mendibil, I., Santos, M., Irigoien, X., & Rodríguez-Ezpeleta, N. (2021). Vertical stratification of environmental DNA in the open ocean captures ecological patterns and behavior of deep-sea fishes. *Limnology and Oceanography Letters*, *n/a*(n/a). https://doi.org/10.1002/lol2.10213

Chabot, D., Steffensen, J. F., & Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *Journal of Fish Biology*, *88*(1), 81–121. https://doi.org/10.1111/jfb.12845

Chambert, T., Pilliod, D. S., Goldberg, C. S., Doi, H., & Takahara, T. (2018). An analytical framework for estimating aquatic species density from environmental DNA. *Ecology and Evolution*, *8*(6), 3468–3477. https://doi.org/10.1002/ece3.3764

Clark, C. W., & Levy, D. A. (1988). Diel vertical migrations by juvenile sockeye salmon and the antipredation window. *The American Naturalist*, *131*(2), 271–290.

Clarke, A. D., Telmer, K. H., & Shrimpton, J. M. (2007). Habitat use and movement patterns for a fluvial species, the Arctic grayling, in a watershed impacted by a large reservoir: Evidence from otolith microchemistry. *Journal of Applied Ecology*, *44*(6), 1156–1165. https://doi.org/10.1111/j.1365-2664.2007.01350.x

Cowie, D. M., & Blackman, B. G. (2003). *2001-2002 Arctic grayling (*Thymallus arcticus*) fry surveys in the Omineca and Osilinka Rivers* (PWFWCP Report No. 276).

Cowie, D. M., & Blackman, B. G. (2004). *2003 Actic grayling (*Thymallus arcticus*) fry surveys in the Ingenika River* (PWFWCP Report No. 291; p. 19).

Coxson, D. S., Huber, D. P. W., Shrimpton, J. M., Theilman, A. C., & Wiensczyk, A. (2018). *Ecosystem impact of nutrient enrichment by Kokanee in the Williston Reservoir Watershed* (Pea-F18-F-2296). Natural Resources and Environmental Studies Institute. http://a100.gov.bc.ca/appsdata/acat/documents/r55493/PEA_F18_F_2296_1547227412356_7224430300.pdf

Cristescu, M., & Hebert, P. (2018). Uses and misuses of environmental DNA in biodiversity science and conservation. *Annual Review of Ecology, Evolution, and Systematics*, *49*. https://doi.org/10.1146/annurev-ecolsys-110617-062306

Culling, B., & Euchner, T. (2018). *Peace Reach lake trout movements—Year 2* (PEA-F18-F-2313; p. 30). Fish and Wildlife Compensation Program - Peace.

Culling, B., & Euchner, T. (2019). *Peace Reach lake trout movements—Year 3* (Peace Project No. PEA-F19-F-2622; p. 27). Fish and Wildlife Compensation Program - Peace.

Currier, C. A., Morris, T. J., Wilson, C. C., & Freeland, J. R. (2018). Validation of environmental DNA (eDNA) as a detection tool for at-risk freshwater pearly mussel species (Bivalvia: Unionidae). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *28*(3), 545–558. https://doi.org/10.1002/aqc.2869

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: Not a perfect match. *Biology Letters*, *10*(9), 20140562. https://doi.org/10.1098/rsbl.2014.0562

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., Vere, N. de, Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

dMIQE Group. (2020). The digital MIQE guidelines update: Minimum information for publication of quantitative digital PCR experiments for 2020. *Clinical Chemistry*, *66*(8), 1012–1029. https://doi.org/10.1093/clinchem/hvaa125

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., & Minamoto, T. (2015). Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLOS ONE*, *10*(3), e0122763. https://doi.org/10.1371/journal.pone.0122763

Donald, D. B., & Alger, D. J. (1993). Geographic distribution, species displacement, and niche overlap for lake trout and bull trout in mountain lakes. *Canadian Journal of Zoology*, *71*(2), 238–247. https://doi.org/10.1139/z93-034

DWB Consulting Services Ltd. (2019). *Williston Watershed kokanee spawner distribution and aerial enumeration surveys (2018)* (PEA-F19-F-2895-DC-103364). Fish and Wildlife Compensation Program - Peace Region.

DWB Consulting Services Ltd. (2020). *Williston Watershed kokanee spawner distribution and aerial enumeration survey (2019)* (PEA-F20-F-3359-DC-106374; p. 51).

DWB Consulting Services Ltd. (2021). *Williston Watershed kokanee spawner distribution and aerial enumeration surveys (2020)* (PEA-F21-F-3363-DC109837; p. 57). Fish and Wildlife Compensation Program - Peace.

Eichmiller, J. J., Bajer, P. G., & Sorensen, P. W. (2014). The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLOS ONE*, *9*(11), e112611. https://doi.org/10.1371/journal.pone.0112611

Evans, N. T., Li, Y., Renshaw, M. A., Olds, B. P., Deiner, K., Turner, C. R., Jerde, C. L., Lodge, D. M., Lamberti, G. A., & Pfrender, M. E. (2017). Fish community assessment with eDNA metabarcoding: Effects of sampling design and bioinformatic filtering. *Canadian Journal of Fisheries & Aquatic Sciences*, *74*(9), 1362–1374. https://doi.org/10.1139/cjfas-2016-0306

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., Mahon, A. R., Pfrender, M. E., Lamberti, G. A., & Lodge, D. M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 29–41. https://doi.org/10.1111/1755-0998.12433

Evans, N. T., Shirey, P. D., Wieringa, J. G., Mahon, A. R., & Lamberti, G. A. (2017). Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries*, *42*(2), 90–99. https://doi.org/10.1080/03632415.2017.1276329

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Rayé, G., & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, *15*(3), 543–556. https://doi.org/10.1111/1755-0998.12338

Fish and Wildlife Compensation Program. (2020). *Peace Region rivers, lakes and reservoirs action plan*. https://fwcp.ca/app/uploads/2020/08/Action-Plan-Peace-Region-Rivers-Lakes-Reservoirs-Aug-11-2020.pdf

Fredenberg, W. (2002). Further evidence that lake trout displace bull trout in mountain lakes. *Intermountain Journal of Science*, *8*(3), 10.

Fukaya, K., Murakami, H., Yoon, S., Minami, K., Osada, Y., Yamamoto, S., Masuda, R., Kasai, A., Miyashita, K., Minamoto, T., & Kondoh, M. (2018). Estimating fish population abundance by integrating quantitative data on environmental DNA and hydrodynamic modelling. *Molecular Ecology*, *n/a*(n/a). https://doi.org/10.1111/mec.15530

GEN-FISH. (2020). *About GEN-FISH*. GEN-FISH. https://gen-fish.ca/about/

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299–1307. https://doi.org/10.1111/2041-210X.12595

Gorsky, D., Zydlewski, J., & Basley, D. (2012). Characterizing seasonal habitat use and diel vertical activity of lake whitefish in Clear Lake, Maine, as determined with acoustic telemetry. *Transactions of the American Fisheries Society*, *141*(3), 761–771. https://doi.org/10.1080/00028487.2012.675905

Hagen, J. (2000). *Reproductive isolation between Dolly Varden (salvelinus malma) and bull trout (s. confluentus) in sympatry: The role of ecological factors* [University of British Columbia]. https://doi.org/10.14288/1.0099497

Hagen, J., & Spendlow, I. (2019). *Bull trout spawner abundance within the Williston Reservoir Watershed 2018, and critical spawning habitats of the Omineca River Watershed* (FWCP Project No. PEA-F19-F-2626).

Hagen, J., Spendlow, I., & Pillipow, R. (2020). *Critical spawning habitats and abundance of bull trout in the Williston Reservoir watershed, 2019* (PEA-F20-F-2956; p. 57). Fish and Wildlife Compensation Program - Peace.

Hagen, J., & Weber, S. (2019). *Limiting factors, enhancement potential, critical habitats, and conservation status for bull trout of the Williston Reservoir Watershed: Information synthesis and recommended monitoring framework* (p. 153). FWCP.

Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver, A., & Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, *25*(13), 3101–3119. https://doi.org/10.1111/mec.13660

Hansen, B. K., Bekkevold, D., Clausen, L. W., & Nielsen, E. E. (2018). The sceptical optimist: Challenges and perspectives for the application of environmental DNA in marine fisheries. *Fish and Fisheries*, *19*(5), 751–768. https://doi.org/10.1111/faf.12286

Hardiman, J. M., Johnson, B. M., & Martinez, P. J. (2004). Do predators influence the distribution of age-0 kokanee in a Colorado reservoir? *Transactions of the American Fisheries Society*, *133*(6), 1366–1378. https://doi.org/10.1577/T03-1234.1

Harris, S. L., Langston, A. R., Stockner, J. G., & Vidmanic, L. (2006). *A limnological assessment of four Williston reservoir embayments, 2004.* (p. 62).

Hernandez, C., Bougas, B., Perreault-Payette, A., Simard, A., Côté, G., & Bernatchez, L. (2020). 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. *Environmental DNA*, *2*(3), 373–386. https://doi.org/10.1002/edn3.89

Hunter, M. E., Dorazio, R. M., Butterfield, J. S. S., Meigs-Friend, G., Nico, L. G., & Ferrante, J. A. (2017). Detection limits of quantitative and digital PCR assays and their influence in presence–absence surveys of environmental DNA. *Molecular Ecology Resources*, *17*(2), 221–229. https://doi.org/10.1111/1755-0998.12619

Integrated DNA Technologies. (2021). *Using OligoAnalyzer Tool to analyze hairpins and dimers | IDT*. Integrated DNA Technologies. https://www.idtdna.com/pages/support/faqs/how-do-i-use-the-oligoanalyzer-tool-to-analyze-possible-hairpins-and-dimers-formed-by-my-oligo

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, *15*(1), 216–227. https://doi.org/10.1111/1755-0998.12285

Jerde, C. L. (2019). Can we manage fisheries with the inherent uncertainty from eDNA? *Journal of Fish Biology*, 1–13. https://doi.org/10.1111/jfb.14218

Jerde, C. L., Chadderton, W. L., Mahon, A. R., Renshaw, M. A., Corush, J., Budny, M. L., Mysorekar, S., & David M., L. (2013). Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences*. https://doi.org/10.1139/cjfas-2012-0478

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jessup, L., & Millar, N. (2011). *Application of a new method for monitoring lake trout abundance in Yukon: Summer profundal index netting (SPIN)* (Yukon Fish and Wildlife Branch Report TR-11-11). https://open.yukon.ca/sites/default/files/new_method_to_monitor_lake_trout_SPIN_1.pdf

Johnson, B. M., & Martinez, P. J. (2000). Trophic economics of lake trout management in Reservoirs of differing productivity. *North American Journal of Fisheries Management*, *20*(1), 127–143. https://doi.org/10.1577/1548-8675(2000)020<0127:TEOLTM>2.0.CO;2

Klobucar, S. L., Rodgers, T. W., & Budy, P. (2017). At the forefront: Evidence of the applicability of using environmental DNA to quantify the abundance of fish populations in natural lentic waters with additional sampling considerations. *Canadian Journal of Fisheries and Aquatic Sciences*. https://doi.org/10.1139/cjfas-2017-0114

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2019). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282. https://doi.org/10.1002/edn3.29

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, *183*, 77–84. https://doi.org/10.1016/j.biocon.2014.11.020

Klymus, K. E., Ruiz Ramos, D. V., Thompson, N. L., & Richter, C. A. (2020). Development and testing of species-specific quantitative PCR assays for environmental DNA applications. *Journal of Visualized Experiments*, *165*, 61825. https://doi.org/10.3791/61825

Kubečka, J., Hohausová, E., Matěna, J., Peterka, J., Amarasinghe, U. S., Bonar, S. A., Hateley, J., Hickley, P., Suuronen, P., Tereschenko, V., Welcomme, R., & Winfield, I. J. (2009). The true picture of a lake or reservoir fish stock: A review of needs and progress. *Fisheries Research*, *96*(1), 1–5. https://doi.org/10.1016/j.fishres.2008.09.021

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., & Hedgpeth, J. (2000). 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research*, *28*(2), 655–661.

Lacoursière-Roussel, A., Côté, G., Leclerc, V., & Bernatchez, L. (2016). Quantifying relative fish abundance with eDNA: A promising tool for fisheries management. *Journal of Applied Ecology*, *53*(4), 1148–1157. https://doi.org/10.1111/1365-2664.12598

Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish abundance and biomass from eDNA concentrations: Variability among capture methods and environmental conditions. *Molecular Ecology Resources*, *16*(6), 1401–1414. https://doi.org/10.1111/1755-0998.12522

Langlois, V. S., Allison, M. J., Bergman, L. C., To, T. A., & Helbing, C. C. (2021). The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. *Environmental DNA*, *3*(3), 519–527. https://doi.org/10.1002/edn3.164

Langston, A. R. (2012). *Williston Watershed kokanee spawner distribution and enumeration surveys (2002 – 2006)* (Peace Region Report No. 357; p. 22). FWCP.

Langston, A. R., & Blackman, B. G. (1993). *Fisheries resources and enhancement potentials of selected tributaries of the Williston Reservoir Volume II* (Report No. 70; p. 185). Peace/Williston Fish and Wildlife Compensation Program. https://a100.gov.bc.ca/pub/acat/documents/r35366/pwfwcp_report_no_070_1360354625130_ab5d1d2216f01b7cbdb42136e10249e8fdb1d3d173d713a69571c05c9f2d0dea.pdf

Langston, A. R., & Murphy, E. B. (2008). *The history of fish introductions (to 2005) in the Peace/Williston Fish and Wildlife Compensation Program area.* (PWFWCP Report No. 325; p. 62). FWCP.

Littlefair, J. E., Hrenchuk, L. E., Blanchfield, P. J., Rennie, M. D., & Cristescu, M. E. (2020). Thermal stratification and fish thermal preference explain vertical eDNA distributions in lakes. *bioRxiv*, 2020.04.21.042820. https://doi.org/10.1101/2020.04.21.042820

Mandrak, N. E., Curry, A. R., Dumont, P., Reist, J. D., Taylor, E. B., & Watkinson, D. A. (Eds.). (2023). Freshwater fisheries in Canada: Historical and contemporary perspectives on the resources and their management, Chapter 1. In *Freshwater Fisheries in Canada: Historical and Contemporary Perspectives on the Resources and Their Management*. American Fisheries Society. https://doi.org/10.47886/9781934874707.ch1

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), Article 1. https://doi.org/10.14806/ej.17.1.200

Martinez, P. J., Bigelow, P. E., Deleray, M. A., Fredenberg, W. A., Hansen, B. S., Horner, N. J., Lehr, S. K., Schneidervin, R. W., Tolentino, S. A., & Viola, A. E. (2009). Western lake trout woes. *Fisheries*, *34*(9), 424–442. https://doi.org/10.1577/1548-8446-34.9.424

McPhail, J. D. (2007). *The freshwater fishes of British Columbia*. University of Alberta Press.

McPhail, J. D., & Baxter, J. S. (1996). *A review of bull trout (Salvelinus confluentus) life-history and habitat use in relation to compensation and improvement opportunities* (Fisheries Management Report No. 104, 35 p.; p. 39). Department of Zoology, U.B.C.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(7), 150088. https://doi.org/10.1098/rsos.150088

Morey, K. C., Bartley, T. J., & Hanner, R. H. (2020). Validating environmental DNA metabarcoding for marine fishes in diverse ecosystems using a public aquarium. *Environmental DNA*, *2*(3), 330–342. https://doi.org/10.1002/edn3.76

Murray, B. W. (2021). *FWCP Report on developing an aquatic survey eDNA degradation rate assay* (PEA-F21-F-3217).

O'Connor, B. (2022). *Williston pelagic fish survey gillnetting summary 2021* (PEA-F22-F-3587-DCA; Prepared for the Peace Region Fish and Wildlife Compensation Program, p. 13).

Olds, B. P., Jerde, C. L., Renshaw, M. A., Li, Y., Evans, N. T., Turner, C. R., Deiner, K., Mahon, A. R., Brueseke, M. A., Shirey, P. D., Pfrender, M. E., Lodge, D. M., & Lamberti, G. A. (2016). Estimating species richness using environmental DNA. *Ecology and Evolution*, *6*(12), 4214–4226. https://doi.org/10.1002/ece3.2186

Olin, M., Malinen, T., & Ruuhijärvi, J. (2009). Gillnet catch in estimating the density and structure of fish community—Comparison of gillnet and trawl samples in a eutrophic lake. *Fisheries Research*, *96*(1), 88–94. https://doi.org/10.1016/j.fishres.2008.09.007

Parks Canada Agency, G. of C. (2023, July 24). *Environmental DNA at Parks Canada—Nature and science*. https://parks.canada.ca/nature/science/especes-species/adne-edna

Pillipow, R. A., & Langston, A. R. (2002). *Williston Reservoir fish assessments 2000, pelagic netting summary* (PWFWCP Report No. 261; p. 47).

Plate, E. M., Bocking, R. C., & Degan, D. J. (2012). *Williston fish index in the vicinity of W.A.C. Bennett Dam. Peace Water Use Plan: Monitoring programs for the Peace spill protocol (GMSMON-4 WAC Bennett Dam Entrainment Study)* (p. 61). BC Hydro.

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., & Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, *8*(1), Article 1. https://doi.org/10.1038/s41598-018-28424-8

Prediger, E. (2013). *How to design primers and probes for PCR and qPCR | IDT*. Integrated DNA Technologies. https://www.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes

Province of British Columbia. (2020). *Habitat wizard lakes report: Williston Lake*.

Qiagen. (2020). *DNeasy blood & tissue handbook—QIAGEN*. https://www.qiagen.com/us/resources/resourcedetail?id=68f29296-5a9f-40fa-8b3d-1c148d0b3030&lang=en

Rasmussen Hellberg, R. S., Morrissey, M. T., & Hanner, R. H. (2010). A multiplex PCR method for the identification of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) in North America. *Journal of Food Science*, *75*(7), C595–C606. https://doi.org/10.1111/j.1750-3841.2010.01752.x

Redenbach, Z., & Taylor, E. B. (2003). Evidence for bimodal hybrid zones between two species of char (Pisces: Salvelinus) in northwestern North America. *Journal of Evolutionary Biology*, *16*(6), 1135–1148. https://doi.org/10.1046/j.1420-9101.2003.00619.x

Riley, S., Hansen, M., Krueger, C., Noakes, D., & Muir, A. (2021). *The lake charr: Biology, ecology, distribution, and management* (pp. 1–12). https://doi.org/10.1007/978-3-030-62259-6_1

Roberge, M., & Slaney, T. (2001). Life history characteristics of freshwater fishes occuring in British Columbia, with major emphasis on Lake habitat requirements. *Fisheries and Aquatic Sciencs*, *2574*, 189 pp.

Ross, M. R. (1997). *Fisheries conservation and management*. Prentice Hall.

Rourke, M. L., Fowler, A. M., Hughes, J. M., Broadhurst, M. K., DiBattista, J. D., Fielder, S., Walburn, J. W., & Furlan, E. M. (2021). Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA*, *00*, 1–25. https://doi.org/10.1002/edn3.185

Rudstam, L. G., Jech, J. M., Parker-Stetter, S. L., Horne, J. K., Sullivan, P. J., & Mason, D. M. (2012). Chapter 13: Fisheries acoustics. In *Fisheries Techniques* (3rd ed., pp. 597–636). American Fisheries Society.

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Sahu, R., Vishnuraj, M. R., Srinivas, C., Dadimi, B., Megha, G. K., Pollumahanti, N., Malik, S. S., Vaithiyanathan, S., Rawool, D. B., & Barbuddhe, S. B. (2021). Development and comparative evaluation of droplet digital PCR and quantitative PCR for the detection and quantification of *Chlamydia psittaci*. *Journal of Microbiological Methods*, *190*, 106318. https://doi.org/10.1016/j.mimet.2021.106318

Sard, N. M., Herbst, S. J., Nathan, L., Uhrig, G., Kanefsky, J., Robinson, J. D., & Scribner, K. T. (2019). Comparison of fish detections, community diversity, and relative abundance using environmental DNA metabarcoding and traditional gears. *Environmental DNA*, *1*(4), 368–384. https://doi.org/10.1002/edn3.38

Schabacker, J. C., Amish, S. J., Ellis, B. K., Gardner, B., Miller, D. L., Rutledge, E. A., Sepulveda, A. J., & Luikart, G. (2020). Increased eDNA detection sensitivity using a novel high-volume water sampling method. *Environmental DNA*, *2*(2), 244–251. https://doi.org/10.1002/edn3.63

Schenekar, T. (2023). The current state of eDNA research in freshwater ecosystems: Are we shifting from the developmental phase to standard application in biomonitoring? *Hydrobiologia*, *850*(6), 1263–1282. https://doi.org/10.1007/s10750-022-04891-z

Scheuerell, M. D., & Schindler, D. E. (2003). Diel vertical migration by juvenile sockeye salmon: Empirical evidence for the antipredation window. *Ecology*, *84*(7), 1713–1720. https://doi.org/10.1890/0012-9658(2003)084[1713:DVMBJS]2.0.CO;2

Schindler, E. U., Sebastian, D., Vidmanic, L., Andrusak, H., Stockner, J., Bassett, M., & Ashley, K. I. (2009). *Arrow Lakes Reservoir fertilization experiment, Year 8 (2006) report* (Fisheries Project Report No. RD 125). Fish and Wildlife Science and Allocation, Ministry of Environment, Province of British Columbia.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/AEM.01541-09

Schönhuth, S., Vukić, J., Šanda, R., Yang, L., & Mayden, R. L. (2018). Phylogenetic relationships and classification of the Holarctic family Leuciscidae (Cypriniformes: Cyprinoidei). *Molecular Phylogenetics and Evolution*, *127*, 781–799. https://doi.org/10.1016/j.ympev.2018.06.026

Sebastian, D. C., Andrusak, G., Scholten, G., & Langston, A. (2009). *An index of fish distribution and abundance in Peace Arm of Williston Reservoir based on hydroacoustic and gillnet surveys* (GMSMON #13; p. 98).

Sebastian, D. C., Scholten, G. H., & Woodruff, P. E. (2003). *Williston Reservoir fish assessment: Results of hydroacoustic, trawl and gill net surveys in August, 2000* (p. 72) [Stock Management Report No. 16]. Province of BC.

Shelton, A. O., Kelly, R. P., O'Donnell, J. L., Park, L., Schwenke, P., Greene, C., Henderson, R. A., & Beamer, E. M. (2019). Environmental DNA provides quantitative estimates of a threatened salmon species. *Biological Conservation*, *237*, 383–391. https://doi.org/10.1016/j.biocon.2019.07.003

Snyder, M. R., Stepien, C. A., Marshall, N. T., Scheppler, H. B., Black, C. L., & Czajkowski, K. P. (2020). Detecting aquatic invasive species in bait and pond stores with targeted environmental (e)DNA high-throughput sequencing metabarcode assays: Angler, retailer, and manager implications. *Biological Conservation*, *245*, 108430. https://doi.org/10.1016/j.biocon.2020.108430

So, K. Y. K., Fong, J. J., Lam, I. P. Y., & Dudgeon, D. (2020). Pitfalls during in silico prediction of primer specificity for eDNA surveillance. *Ecosphere*, *11*(7). https://doi.org/10.1002/ecs2.3193

Species At Risk Public Registry. (2019). *Species profile: Bull trout western Arctic populations*. Government of Canada. https://wildlife-species.canada.ca/species-risk-registry/species/speciesDetails_e.cfm?sid=1202

Stamford, M., & O'Connor, B. (2022). *Fish sampling at eDNA sites for Finlay Reach Arctic grayling* (FWCP Report No. PEA-F22-F-3408).

Stamford, M., Strohm, J., Euchner, T., Pitt, C., & Murray, B. (2022). *Williston grayling distribution: Parsnip, Peace, Dinosaur* (FWCP Project Number PEA-F21-F-3198).

Stamford, M., Strohm, J., Pitt, C., & Murray, B. (2020). *2019 Williston Arctic grayling distribution: eDNA monitoring* (PEA-F20-F-2965). https://a100.gov.bc.ca/pub/acat/documents/r59037/PEA_F20_F_2965_1611779660377_D0D7943A93.pdf

Stockner, J., Langston, A., Sebastian, D., & Wilson, G. (2005). The limnology of Williston Reservoir: British Columbia's largest lacustrine ecosystem. *Water Quality Research Journal*, *40*(1), 28–50. https://doi.org/10.2166/wqrj.2005.003

Stoeckle, M. Y., Soboleva, L., & Charlop-Powers, Z. (2017). Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PLOS ONE*, *12*(4), e0175186. https://doi.org/10.1371/journal.pone.0175186

Strohm, J., Bonderud, E., & Stamford, M. (2019). *2018 Williston Arctic grayling distribution: eDNA monitoring* (PEA-F19-F-2659; p. 42).

Strohm, J., Hagen, J., & Stamford, M. (2020). *Ingenika River Arctic grayling 46 years post-flooding: 2019 snorkeling counts in index sites* (FWCP Project No. PEA-F20-F-2963; p. 42). Chu Cho Environmental LLP.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. *PLOS ONE*, *7*(4), e35868. https://doi.org/10.1371/journal.pone.0035868

Tan, M., & Armbruster, J. W. (2018). Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysi). *Zootaxa*, *4476*(1), Article 1. https://doi.org/10.11646/zootaxa.4476.1.4

Taylor, E. B., Redenbach, Z., Costello, A. B., Pollard, S. M., & Pacas, C. J. (2001). Nested analysis of genetic diversity in northwestern North American char, Dolly Varden (Salvelinus malma) and bull trout (Salvelinus confluentus). *Canadian Journal of Fisheries & Aquatic Sciences*, *58*(2), 406. https://doi.org/10.1139/f00-262

Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., Traugott, M., Goldberg, C. S., & Bruce, K. (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *bioRxiv*, 2020.04.27.063990. https://doi.org/10.1101/2020.04.27.063990

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Thornton, B., & Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, *39*(2), 145–154. https://doi.org/10.1002/bmb.20461

Vašek, M., Kubečka, J., Čech, M., Draštík, V., Matěna, J., Mrkvička, T., Peterka, J., & Prchalová, M. (2009). Diel variation in gillnet catches and vertical distribution of pelagic fishes in a stratified European reservoir. *Fisheries Research*, *96*(1), 64–69. https://doi.org/10.1016/j.fishres.2008.09.010

Veldhoen, N., Hobbs, J., Ikonomou, G., Hii, M., Lesperance, M., & Helbing, C. C. (2016). Implementation of novel design features for qPCR-Based eDNA assessment. *PLOS ONE*, *11*(11), e0164907. https://doi.org/10.1371/journal.pone.0164907

Weir, T., Peck, K., & Johner, D. (2023). *Williston Reservoir 2021 hydroacoustic, trawl and gillnet survey—Synthesis report*. Province of British Columbia, Ministry of Forests, FIsh & Wildlife Branch.

Wetzel, R. G. (2001). *Limnology: Lake and river ecosystems* (3rd ed). Academic Press.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLOS ONE*, *8*(3), e59520. https://doi.org/10.1371/journal.pone.0059520

Wilson, P. N., & Shrimpton, J. M. (2020). *Genetic population structure and demographics of kokanee introduced to the Williston Reservoir* (PEA-F19-F-2870-DCA; p. 41). Fish and Wildlife Compensation Program - Peace. https://a100.gov.bc.ca/pub/acat/documents/r58750/PEA_F9_F_2870_DCA_1594143450750_4139699754.pdf

Winfield, I. J., Fletcher, J. M., James, J. B., & Bean, C. W. (2009). Assessment of fish populations in still waters using hydroacoustics and survey gill netting: Experiences with Arctic charr (*Salvelinus alpinus*) in the UK. *Fisheries Research*, *96*(1), 30–38. https://doi.org/10.1016/j.fishres.2008.09.013

WWF. (2020). *Living planet report 2020—Bending the curve of biodiversity loss.*

Yates, M. C., Fraser, D. J., & Derry, A. M. (2019). Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environmental DNA*, *1*(1), 5–13. https://doi.org/10.1002/edn3.7

Yates, M. C., Glaser, D. M., Post, J. R., Cristescu, M. E., Fraser, D. J., & Derry, A. M. (2020). The relationship between eDNA particle concentration and organism abundance in nature is strengthened by allometric scaling. *Molecular Ecology*, *n/a*(n/a). https://doi.org/10.1111/mec.15543

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*, 134. https://doi.org/10.1186/1471-2105-13-134

Zale, A. V., Parrish, D. L., Sutton, T. M., & American Fisheries Society (Eds.). (2012). *Fisheries techniques* (3rd ed). American Fisheries Society.

Zemlack, R. J., & Langston, A. R. (1994). *Finlay River lake trout spawning investigation* (PWFWCP Report No. 80; p. 6pp plus appendices). Peace/Williston Fish and Wildlife Compensation Program. http://a100.gov.bc.ca/appsdata/acat/documents/r35380/pwfwcp_report_no_080_1360361338672_fced2e50d9abf765520f00550f7bc885edc696f901cbea99139799182d018168.pdf

APPENDIX A:
IN VITRO qPCR ASSAY VALIDATION

Synthetic DNA Templates

Synthetic DNA templates were designed for each qPCR assay based on consensus sequences for each species. Synthetic DNA templates were purchased from Integrated DNA Technologies Inc. as gBlocks® Gene Fragments. Synthetic DNA templates were used in the temperature gradient and sensitivity testing of qPCR assays. The synthetic template sequences that all the qPCR assays in this study were tested on are shown in the following four figures (Figures A1 to A4).

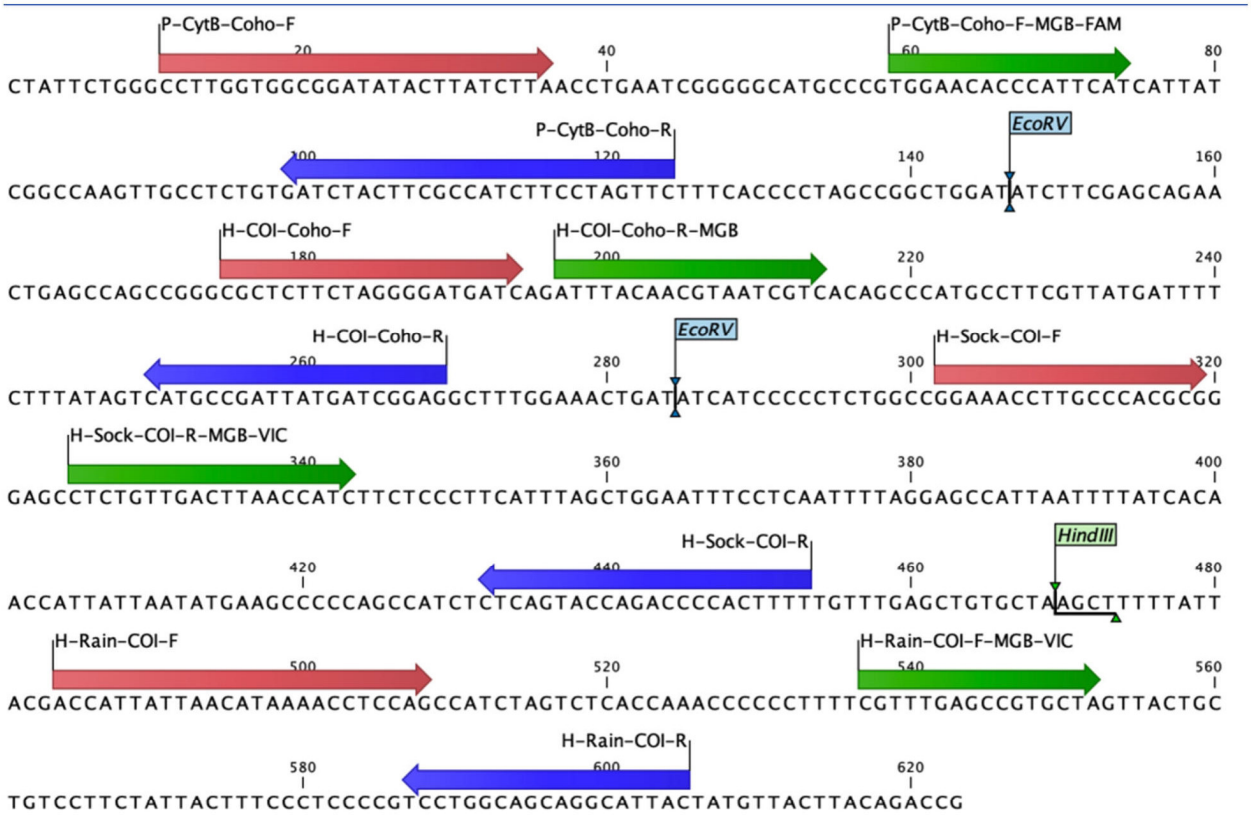

Figure A 1. Synthetic DNA template sequence used to test the *Sock_COI* and *Rain_COI* assays. Primers and probes are annotated for the *Sock_COI* and *Rain_COI* assays as H-Sock-COI and H-Rain-COI, respectively. The template also included sequences for other salmon species not tested for in this study.

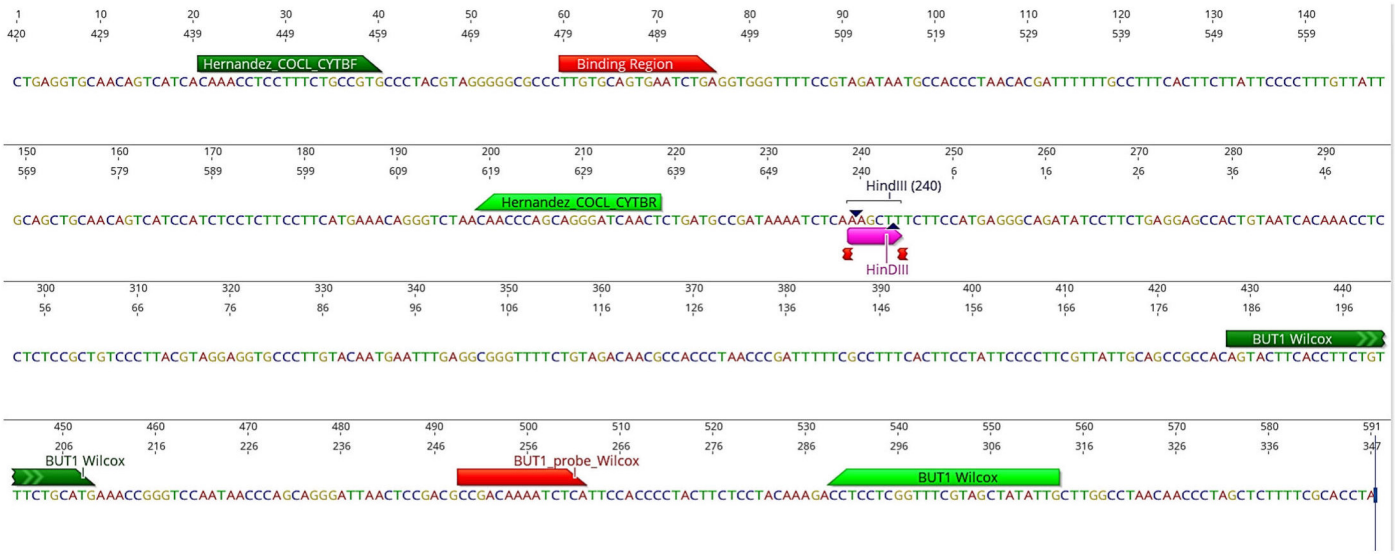

Figure A 2. Synthetic DNA template sequence used to test the *COCL_CYTB* and *BUT1_COCL* assays. Forward primers are coloured dark green, reverse primers are coloured light green, and probes are coloured red.

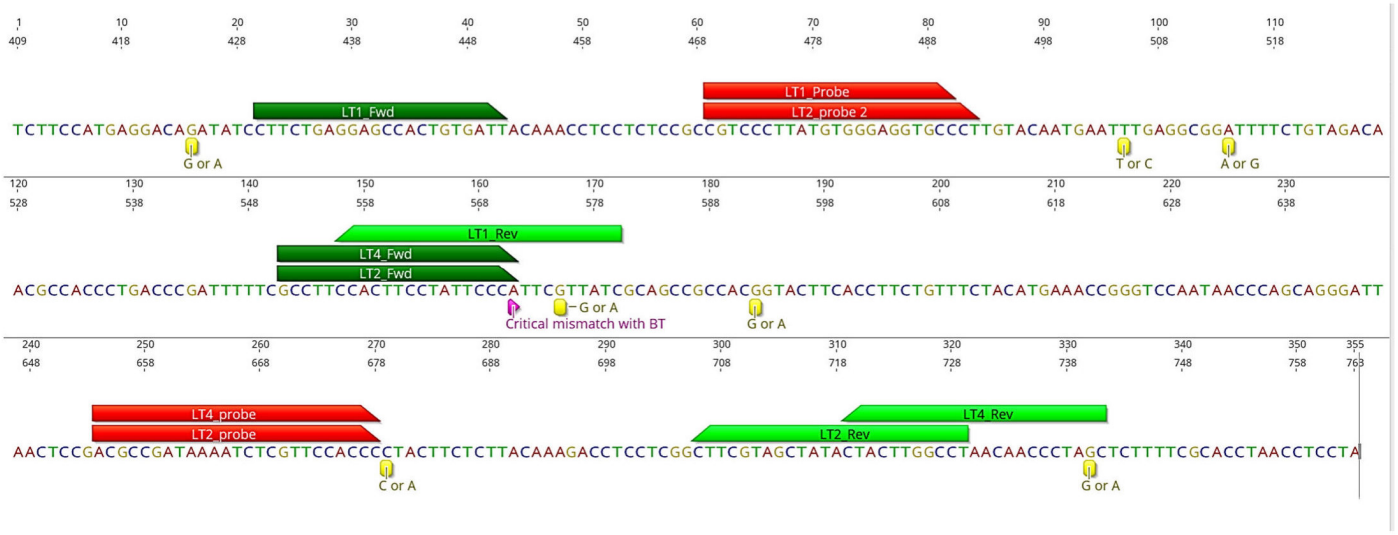

Figure A 3. Synthetic DNA template sequence used to test all the candidate assays for lake trout. Forward primers are coloured dark green, reverse primers are coloured light green, and probes are coloured red. The primers and probe labeled LT4 were the most successful candidates and became the novel *LT_CYTB* assay featured in this study.

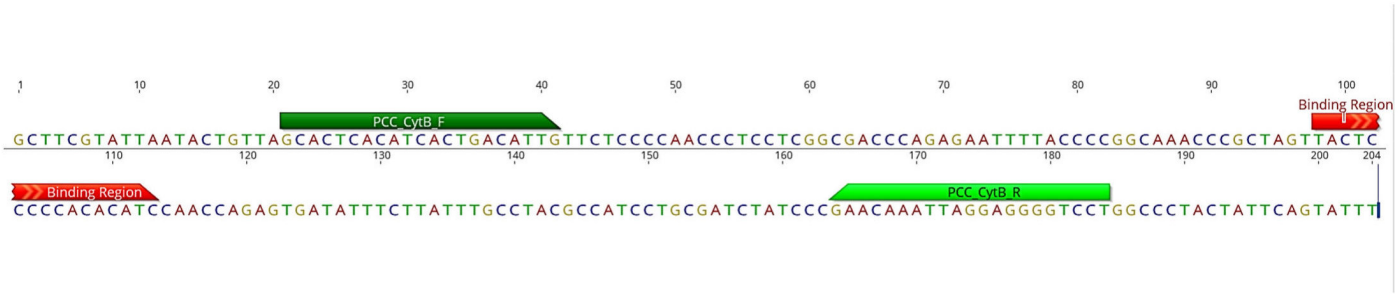

Figure A 4. Synthetic DNA template sequence used to test the novel peamouth assay, *PCC_CYTB*. Forward primers are coloured dark green, reverse primers are coloured light green, and probes are coloured red.

Temperature Gradient Tests

Optimal PCR annealing temperatures were determined for each qPCR assay by running a temperature gradient test on the Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) at eight annealing temperatures between 50 and 60 °C. Assays were tested against synthetic DNA templates (Integrated DNA Technologies Inc.).

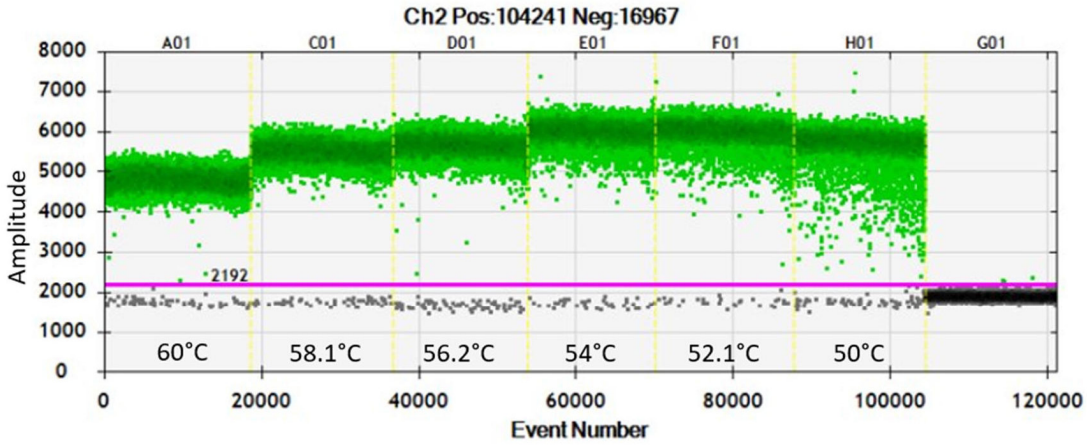

Figure A 5. Temperature gradient results for the kokanee assay, *Sock_COI*.

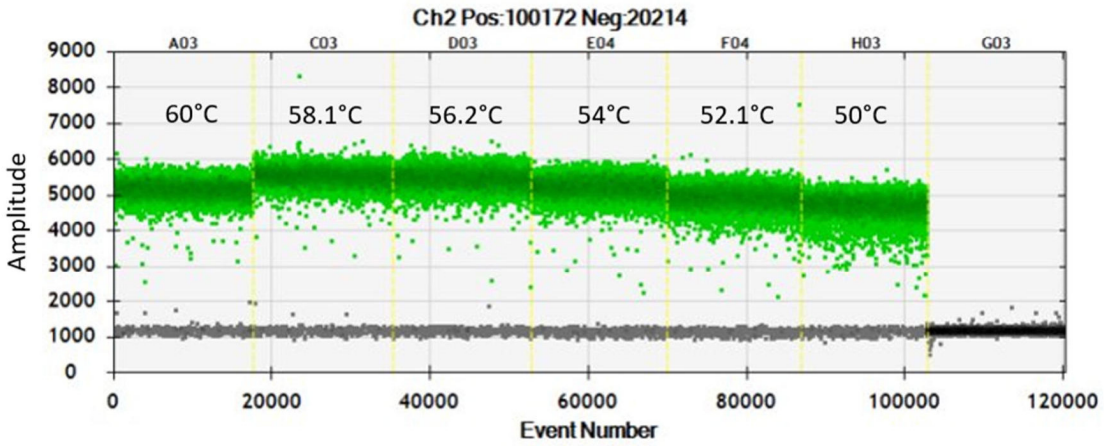

Figure A 6. Temperature gradient results for the rainbow trout assay, *Rain_COI*.

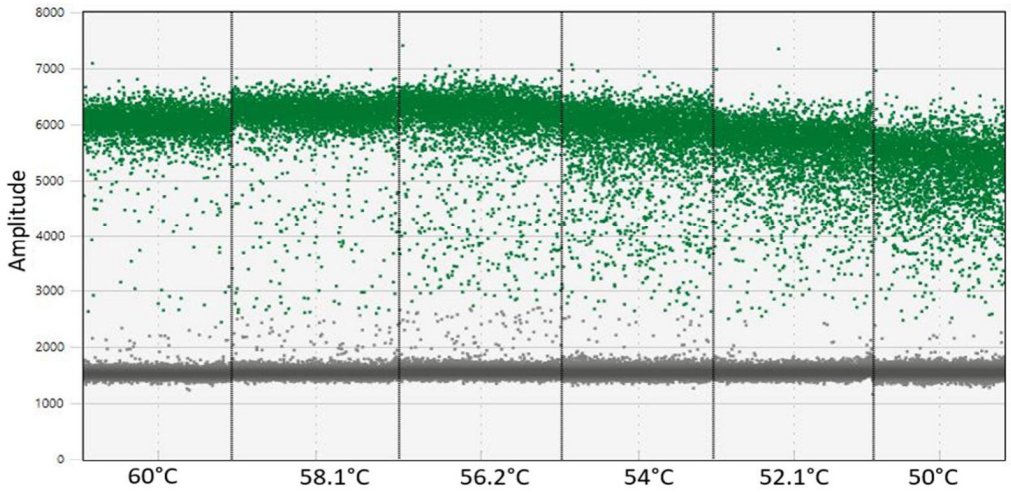

Figure A 7. Temperature gradient results for the lake whitefish assay, *COCL_CYTB*.

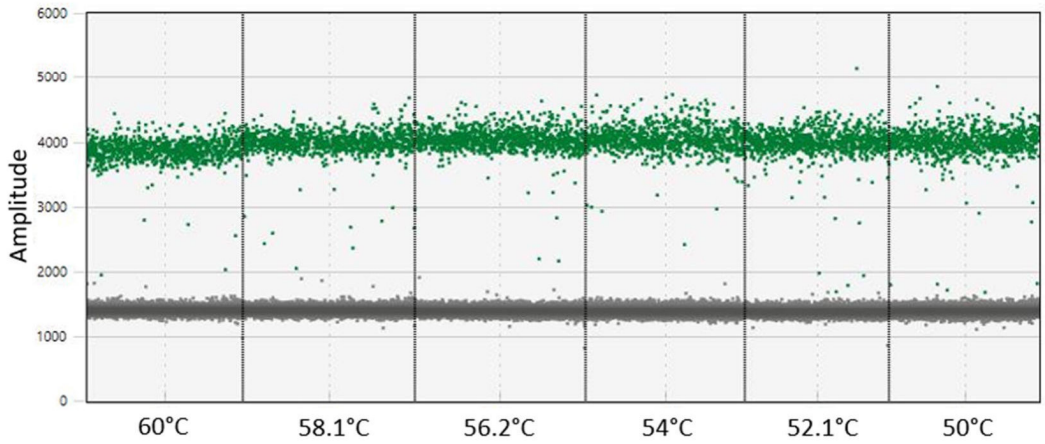

Figure A 8. Temperature gradient results for the bull trout assay, *BUT1_CYTB*.

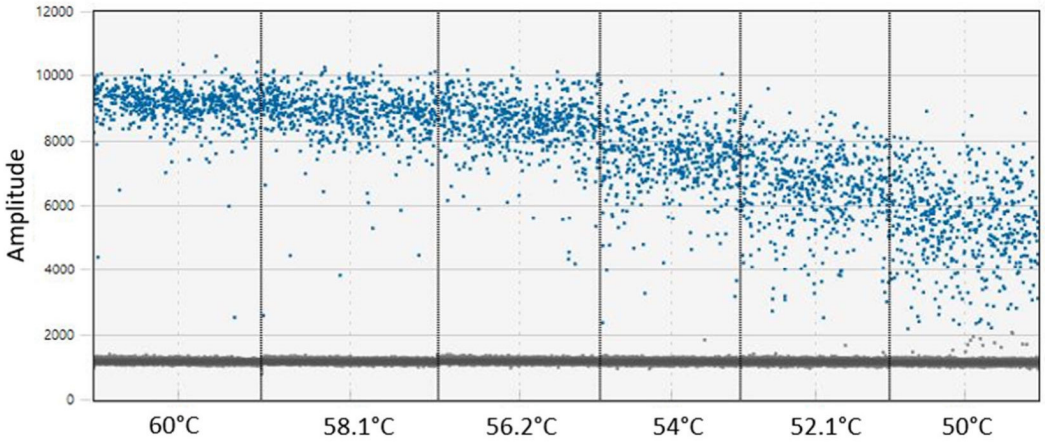

Figure A 9. Temperature gradient results for the lake trout assay, *LT_CYTB*.

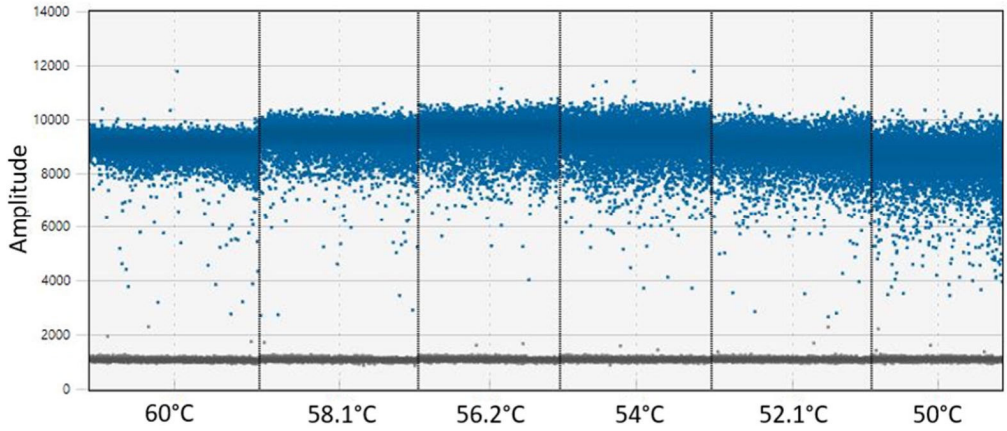

Figure A 10. Temperature gradient results for the peamouth assay, *PCC_CYTB*.

Specificity Tests

qPCR assays were tested for amplification of their target species and for cross-amplification of closely related and representative species from the Williston Watershed. Genomic DNA used for specificity testing was extracted from the fin tissues of 15 fish species using the DNeasy Blood and Tissue kit (Qiagen, Mississauga, ON) following the manufacturer's protocol. Assays were tested against extracted DNA from at least two separate individuals from all non-target species in the same family. Assays were tested on the QX200 Droplet Digital PCR system in 20 µL final reaction volumes that included 10 µL of 2x ddPCR Supermix for Probes (no dUTPs)

(Life Science, Mississauga, ON), 1 µL of 20x concentrated assay, 5 µL of extracted DNA (2-6 ng) and 4 µL of nuclease free water (IDT). The thermocycling protocols for all assays were: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 seconds and annealing at 58 °C for 60 s, followed by a final incubation at 98 °C for 10 minutes. The following figures are representative results from species in the same family found in the Williston Reservoir.

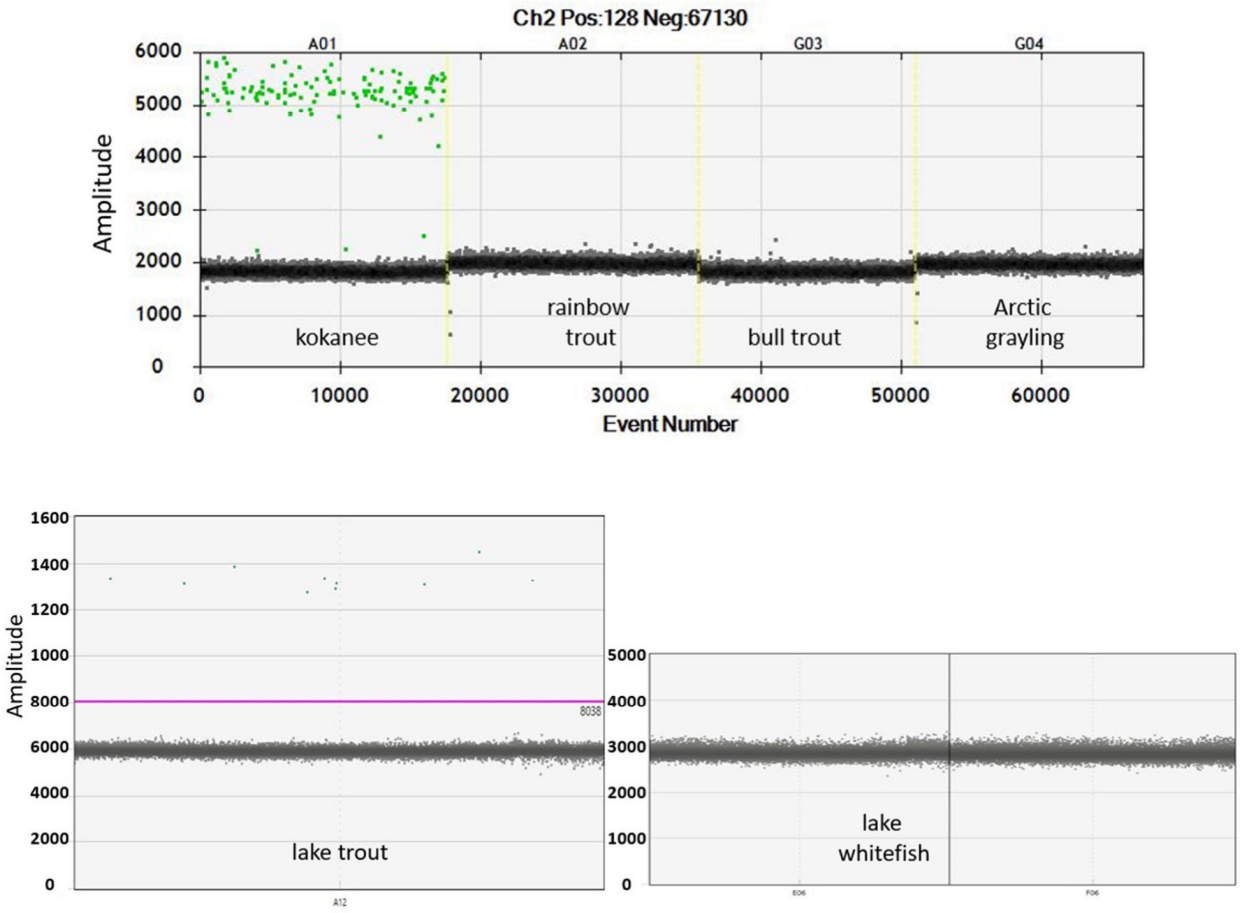

Figure A 11. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target salmonid species for the kokanee assay, *Sock_COI*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey. Trace amounts of lake whitefish contamination were observed with lake trout samples collected from gillnets in the Williston Reservoir.

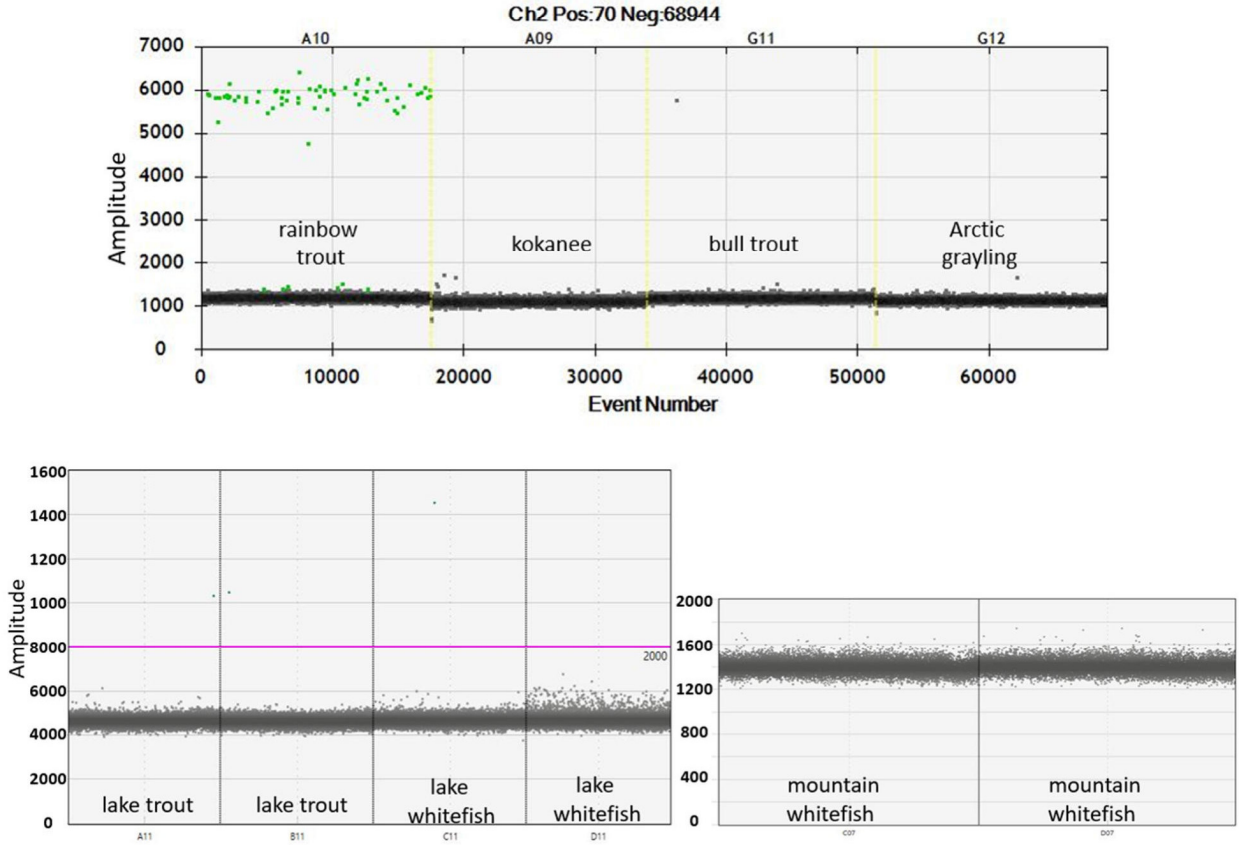

Figure A 12. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target salmonid species for the rainbow trout assay, *Rain_COI*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey.

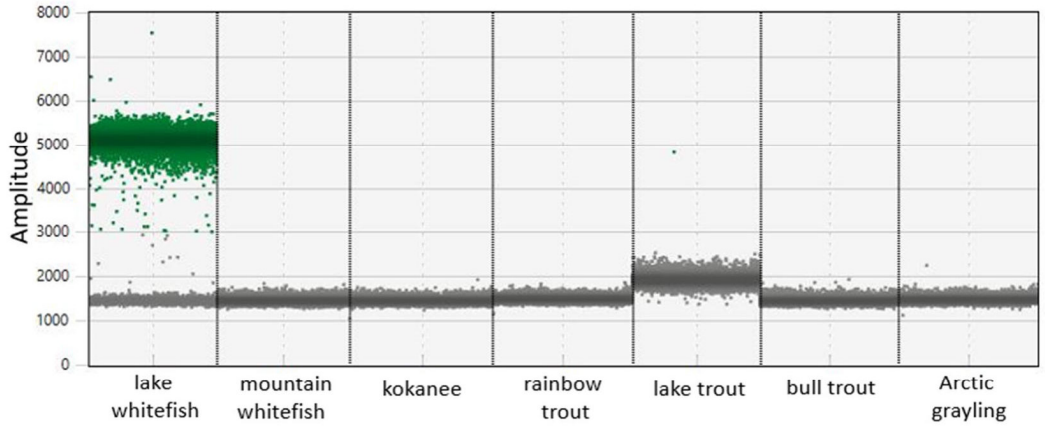

Figure A 13. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target salmonid species for the lake whitefish assay, *COCL_CYTB*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey.

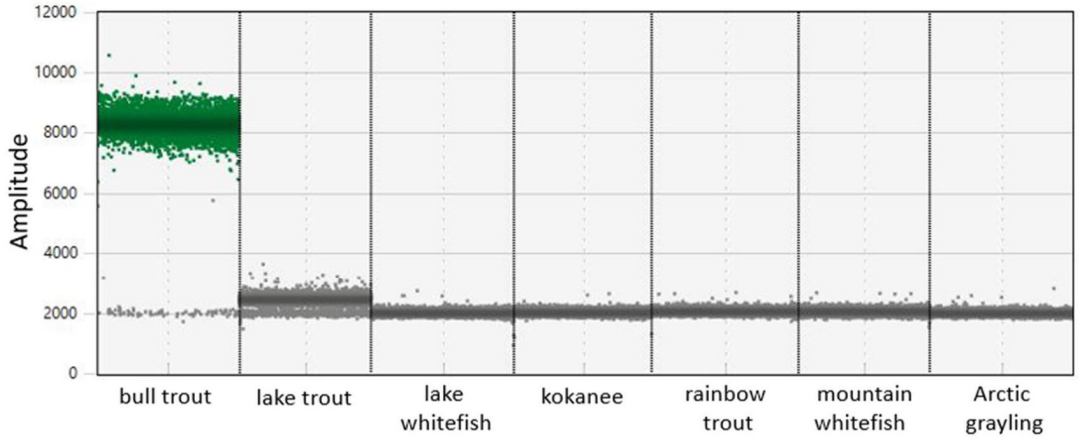

Figure A 14. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target salmonid species for the bull trout assay, *BUT1_CYTB*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey.

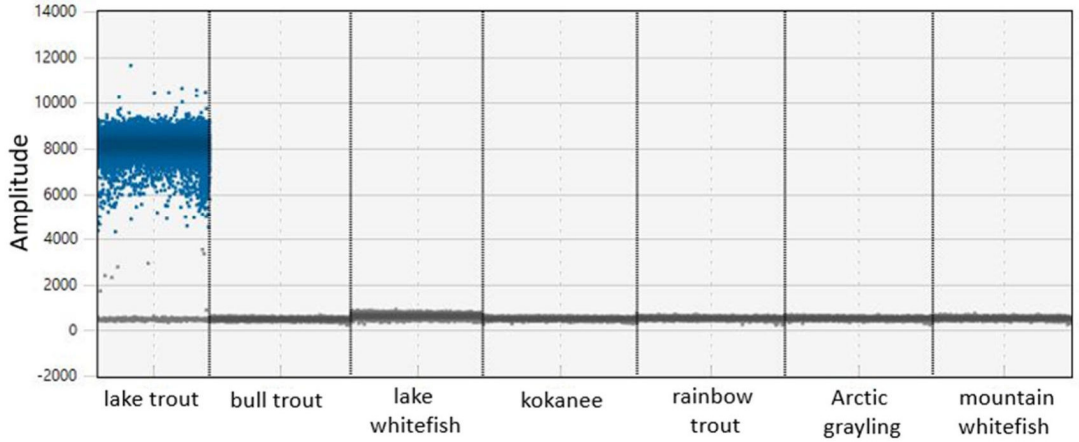

Figure A 15. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target salmonid species for the lake trout assay, *LT_CYTB*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey.

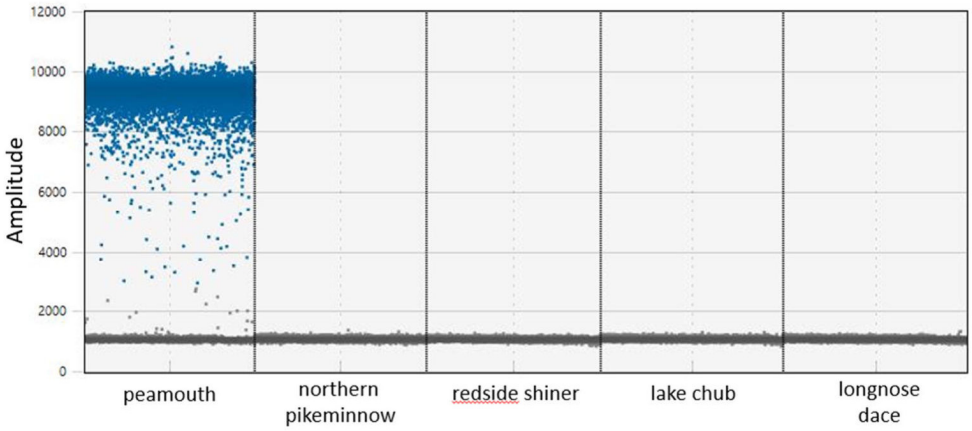

Figure A 16. Fluorescence amplitude of droplets plotted against DNA samples of target and non-target Leuciscidae species for the peamouth assay, *PCC_CYTB*. Droplets that were positive for PCR amplification are coloured and negative droplets are coloured grey.

Sensitivity Tests

Sensitivity was measured for on the Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) with dilution series experiments. A synthetic DNA template (gBlock, Integrated DNA Technologies Inc.) of each target amplicon was used to create 1:5 standard serial dilutions at eight dilution points with six molecular replicates of each standard. Synthetic templates were based on consensus sequences generated in Geneious Prime from the NCBI sequence database. Targeted concentrations for each standard dilution point were between 10 000 and 0.13 copies/20 µL ddPCR reaction. However, the end-point ddPCR measurements of synthetic template concentrations varied for each batch of gBlock used. The standard concentrations for each dilution series (Table A1) were calculated from the mean concentration of gBlock detected by ddPCR at the most concentrated dilution point and dividing by five for each subsequent standard. The limit of detection (LOD) was defined and assessed in two ways. Standard LOD was defined as the lowest concentration of target analyte that was detected with 95% confidence (Klymus et al., 2019). Detections were required in all six replicates of a dilution level to achieve 95% confidence level for this study. Modeled LOD was assessed with a statistical model for digital PCR developed

by Hunter et al. (2017). The modeled LOD was defined as the lowest amount of analyte that could be both detected and distinguished from a source of overestimation bias in qPCR known as the concentration plateau; as DNA concentrations approach zero, measured concentrations may be higher than expected and result in a nonlinear standard dilution series (Hunter et al., 2017). The modeled LOD was determined by analyzing the dilution series data with R code provided by Hunter et al. (2017). Modeled LOD could not be calculated for the *Sock_COI* and *BUT1_CYTB* assays because their dilution series data remained linear and detections were completely absent at the lowest dilution levels.

Table A 1. Limits of detection for the six assays tested in this study. Concentrations of the synthetic DNA standards were adjusted after comparison with ddPCR. Standard LOD is the lowest dilution level with >95% detection amongst replicates. Modeled LOD was assessed with a statistical model for digital PCR developed by Hunter et al. (2017). Modeled LOD was not applicable (N/A) when the relationship between expected and measured DNA concentrations remained linear and a "concentration plateau" was not detected at low concentrations.

Assay	Dilution Series Standard Concentrations (copies/rxn)	Standard LOD (copies/rxn)	Modeled LOD (copies/rxn)
Sock_COI	3800, 760, 152, 30.4, 6.08, 1.22, 0.24, 0.05	6.08	N/A
Rain_COI	4000, 800, 160, 32, 6.40, 1.28, 0.26, 0.05	6.40	6.40
COCL_CYTB	2700, 540, 108, 21.6, 4.32, 0.86, 0.17, 0.03	4.32	4.32
BUT1_CYTB	3100, 620, 124, 24.8, 4.96, 0.99, 0.20, 0.04	4.96	N/A
LT_CYTB	15500, 3100, 620, 124, 24.8, 4.96, 0.99, 0.20	4.96	4.96
PCC_CYTB	5700, 1140, 228, 45.6, 9.12, 1.82, 0.37, 0.07	45.6	1.82

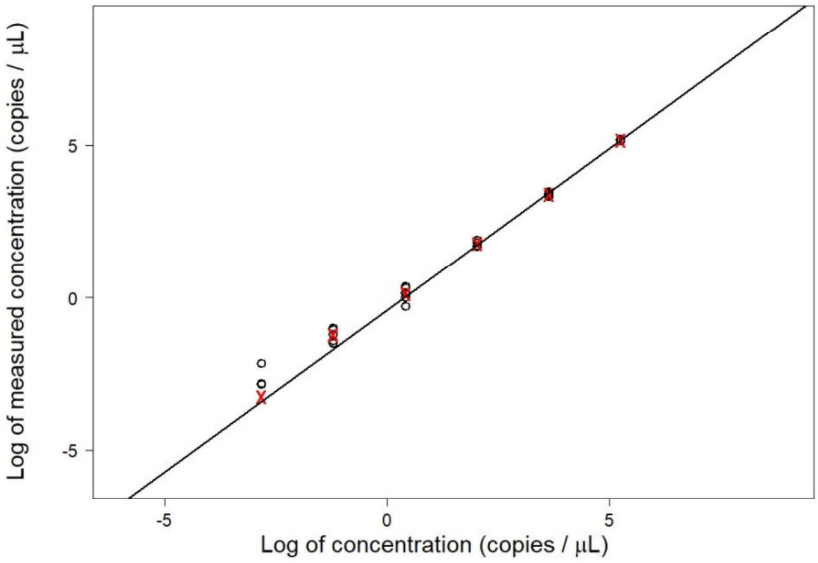

Figure A 17. ddPCR-based LOD assessment of the kokanee assay, *Sock_COI,* with a dilution series of synthetic kokanee DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD could not be calculated for the *Sock_COI* assay because the dilution series did not exhibit a concentration plateau.

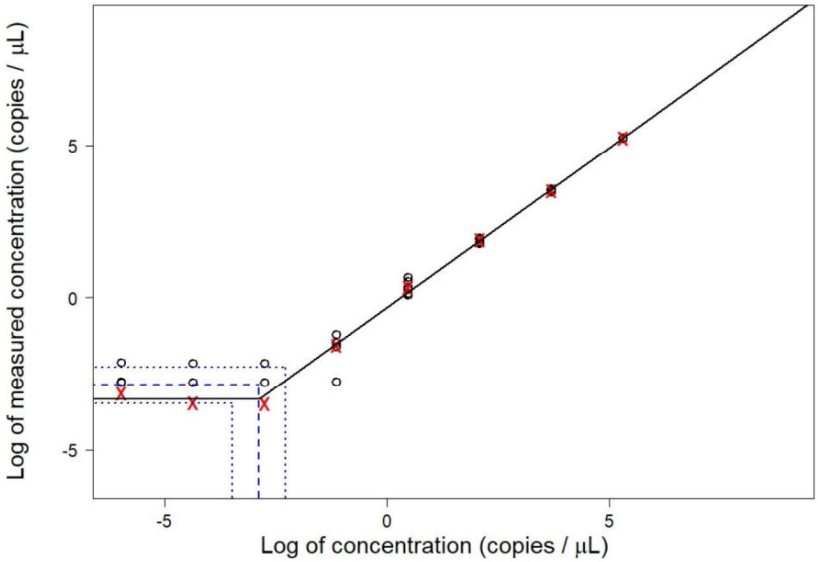

Figure A 18. ddPCR-based LOD assessment of the rainbow trout assay, *Rain_COI,* with a dilution series of synthetic rainbow trout DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD is defined at the point of intersection between linearly related standard concentrations (solid line) and instrumental responses that do not vary with concentration (concentration plateau; dashed line). Upper and lower 95% confidence limits are shown (dotted lines). The upper 95% confidence limit was used as a conservative bound for the Modeled LOD (Hunter et al. 2017).

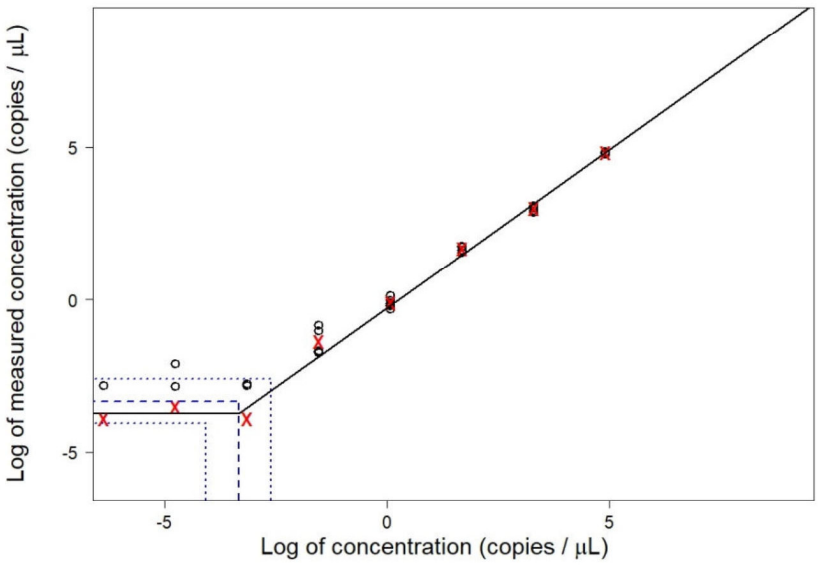

Figure A 19. ddPCR-based LOD assessment of the lake whitefish assay, *COCL_CYTB,* with a dilution series of synthetic lake whitefish DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD is defined at the point of intersection between linearly related standard concentrations (solid line) and instrumental responses that do not vary with concentration (concentration plateau; dashed line). Upper and lower 95% confidence limits are shown (dotted lines). The upper 95% confidence limit was used as a conservative bound for the Modeled LOD (Hunter et al. 2017).

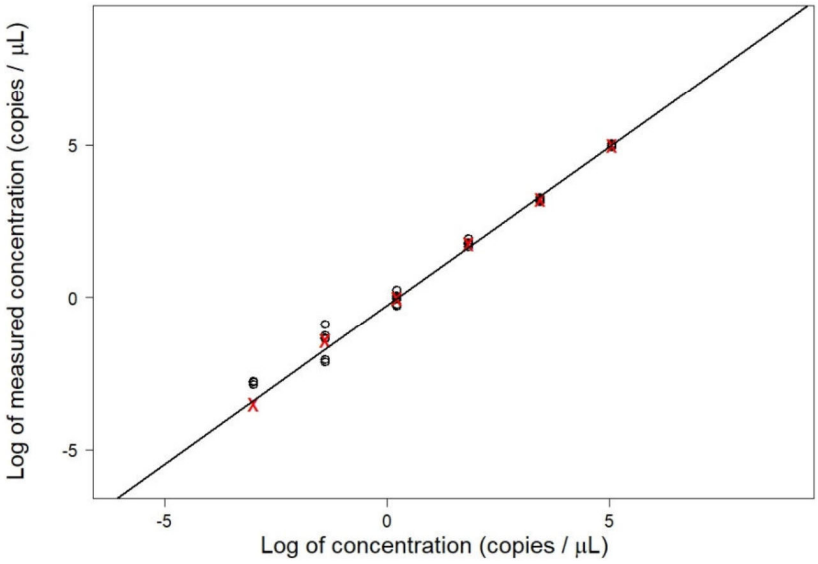

Figure A 20. ddPCR-based limit of detection (LOD) assessment of the bull trout assay, *BUT1_CYTB,* with a dilution series of synthetic bull trout DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD could not be calculated for the *Sock_COI* assay because the dilution series did not exhibit a concentration plateau.

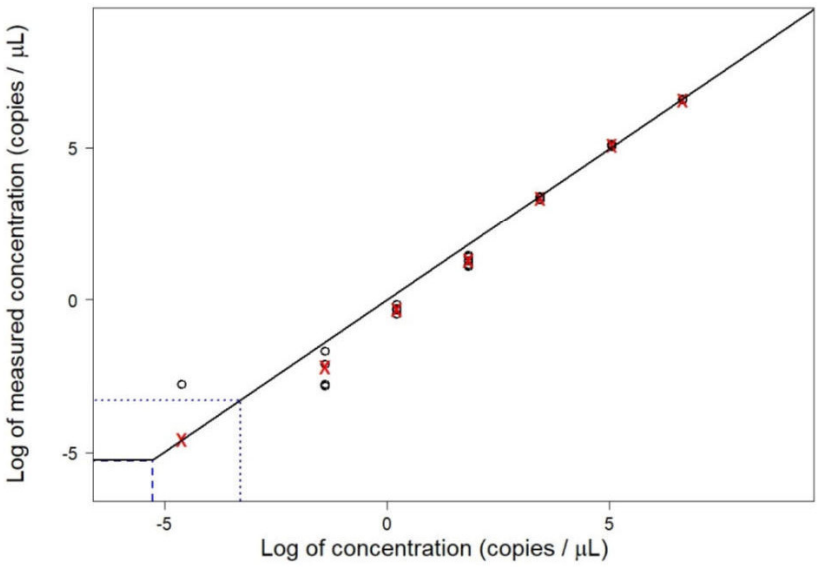

Figure A 21. ddPCR-based LOD assessment of the lake trout assay, *LT_CYTB,* with a dilution series of synthetic lake trout DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD is defined at the point of intersection between linearly related standard concentrations (solid line) and instrumental responses that do not vary with concentration (concentration plateau; dashed line). Upper and lower 95% confidence limits are shown (dotted lines). The upper 95% confidence limit was used as a conservative bound for the Modeled LOD (Hunter et al. 2017).

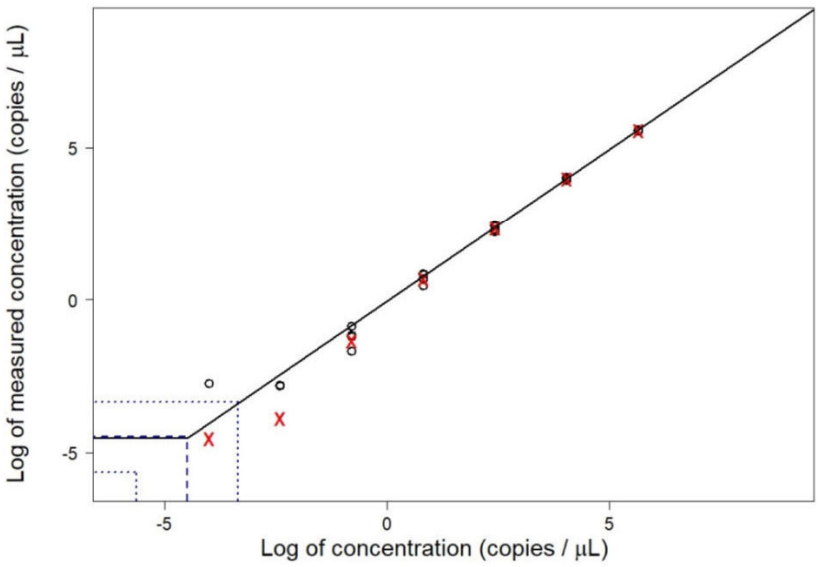

Figure A 22. ddPCR-based limit of detection (LOD) assessment of the peamouth assay, *PCC_CYTB,* with a dilution series of synthetic peamouth DNA. Synthetic DNA concentrations along the x-axis include a 1:5 standard dilution with eight points and 6 replicates each. The log of measured concentrations is along the y-axis. Replicate averages (including zeros) are denoted by a red X. The Standard LOD is the lowest dilution level with 100% detection across all replicates. The Modeled LOD is defined at the point of intersection between linearly related standard concentrations (solid line) and instrumental responses that do not vary with concentration (concentration plateau; dashed line). Upper and lower 95% confidence limits are shown (dotted lines). The upper 95% confidence limit was used as a conservative bound for the Modeled LOD (Hunter et al. 2017).

APPENDIX B:
CANDIDATE qPCR ASSAYS

I developed and tested a single candidate qPCR assay for peamouth (Table 2. 1), two candidate assays for bull trout (Table A2) and three for lake trout (Table 2. 1 and Table A2). Table A2 describes the characteristics of candidate assays that did not perform well during preliminary screening and that were rejected. Assay performance during *in vitro* specificity testing was the primary consideration for determining assay success. Assays were rejected if they amplified the extracted DNA from non-target species. The performance of the two candidate bull trout assays were compared against the published *BUT1_CYTB* assay from Wilcox et al. (2013). The *LT2_CYTB* lake trout assay shared the same forward primer and probe sequence as the successful lake trout assay that was eventually named *LT_CYTB* (Table 2. 1) but had a different reverse primer sequence. The *LT2_CYTB* assay was developed as an alternate to the *LT_CYTB* assay if it did not perform well during *in vitro* testing. Due to the success of the *LT_CYTB*, the *LT2_CYTB* assay was never tested *in vitro*.

Table A 2. Characteristics and secondary structure analysis of the unsuccessful candidate assays for bull trout and lake trout as calculated with IDT's OligoAnalyzer Tool.

Species/ Assay Name	Target Gene	Primers/ Probe	Sequence 5' to 3'	(bp)	T_m (°C)	Hairpin T_m (°C)	Dimer Structures ΔG (kcal/mol)			
							Self	F/R Primers	F/ Probe	R/ Probe
Bull trout *BT2_CYTB*	CYTB	Forward	CCTTCTGAGGAGCCACTGTAATC	23	63.9	47.7	-4.67	-8.19	-4.67	-9.68
		Reverse	AATAACGAAGGGGAATAGGAAGTGA	25	63.7	1.6	-3.61			
		Probe	TGTCCCTTACGTAGGAGGTGCCCTT	25	70.0	50.9	-8.22			
Bull trout *BT3_CYTB*	CYTB	Forward	CGCCTTTCACTTCCTATTCCCC	22	64.6	None	-3.61	-5.46	-3.89	-3.61
		Reverse	GCCAAGCAATATAGCTACGAAACC	24	63.7	53.8	-8.26			
		Probe	ACGCCGACAAAATCTCATTCCACCC	25	68.7	23.8	-3.61			
Lake trout *LT1_CYTB*	CYTB	Forward	CTTCTGAGGAGCCACTGTGATT	22	63.9	14.6	-3.3	-7.96	-6.37	-7.71
		Reverse	GATAACGAATGGGAATAGGAAGTGG	25	62.9	25.9	-3.61			
		Probe	CGTCCCTTATGTGGGAGGTGCCCT	24	70.9	57.3	-7.71			
Lake trout *LT2_CYTB*	CYTB	Forward	GCCTTCCACTTCCTATTCCCA	21	63.7	None	-3.14	-7.81	-3.42	-6.53
		Reverse	AGGCCAAGTAGTATAGCTACGAAG	24	63.2	44.5	-9.28			
		Probe	ACGCCGATAAAATCTCGTTCCACCC	25	68.2	34.4	-5.19			

Candidate Assay Design and Optimization

eDNA assays frequently incorporate conjugated minor groove binder (MGB) groups at the 3' end of probes because an MGB probe can be shorter and more specific than an unmodified probe (Kutyavin et al., 2000). Addition of the MGB stabilizes the DNA duplex between probe and template, especially at A+T rich sites, reducing the length of probe needed to achieve the high T_m required for PCR. MGB probes increase specificity because mismatches within the MGB region are more destabilizing than mismatches in non-MGB probes, resulting in a greater reduction in T_m. All four of the published qPCR assays tested in this study (Table 2. 1) were designed with MGB probes. The MGB probes likely contributed in varying degrees to the specificity of those assays but the *BUT1_CYTB* assay may not have been as specific without it, due to the reduced influence of primer mismatches with lake trout DNA. The *LT_CYTB* and *PCC_CYTB* assays were designed without MGB probes because the benefits of the MGB chemistry for those assays were not deemed necessary. Primer mismatches have a much larger influence on assay specificity than mismatches in the probe (Wilcox et al., 2013). The *LT_CYTB* and *PCC_CYTB* assays were designed with a focus on primer specificity and *in vitro* testing demonstrated that the assays were specific enough without an MGB probe. The novel assays also had the advantage of being designed specifically for the species assemblage in the Williston Watershed. Probe length was not a problem for the *LT_CYTB* and *PCC_CYTB* assays because the probe regions were relatively G+C rich and appropriate T_ms were achieved with 25mer non-MGB probes. A shorter MGB probe also reduces the distance between the fluorophore and quencher, reducing background fluorescence and potentially providing increased assay sensitivity (Kutyavin et al., 2000). The *LT_CYTB* and *PCC_CYTB* assays were both designed with double quenched probes, which is an alternative method for reducing background fluorescence in a longer probe (Prediger, 2013). A considerable downside of MGB assays is the increased cost compared to non-MGB assays. The TaqMan MGB

assays purchased for this study were sold in 360-reaction quantities from ThermoFisher Scientific for $434 CAD. The IDT PrimeTime® Std qPCR Assays (non-MGB) with double quenched probes purchased for this study were sold in 500-reaction quantities for $170 CAD. All the assays tested in this study demonstrated that they were species-specific, regardless of their probe chemistry. The *LT_CYTB* and *PCC_CYTB* assays could easily be modified to incorporate MGB probes, but the benefits to assay specificity do not appear to justify the added costs for eDNA monitoring in the Williston Reservoir.

Optimal PCR annealing temperatures were determined for each candidate qPCR assay by running a temperature gradient test on the Bio-Rad QX200 Droplet Digital PCR system (Life Science, Mississauga, ON) at eight annealing temperatures between 50-60 °C. Assays were tested against synthetic DNA templates (Integrated DNA Technologies Inc.). An annealing temperature of 58 °C was suitable for all the assays tested and was the annealing temperature used in all the subsequent *in vitro* testing for specificity.

Candidate Assay Specificity

The *BT2_CYTB* bull trout assay that I developed demonstrated similar specificity to the *BUT1_CYTB* assay developed by Wilcox et al. (2013) (Figure A 23). However, the variance in droplet amplitude for the *BUT1_CYTB* assay was less than that for the *BT2_CYTB* assay. The large variance in droplet fluorescence observed in the *BT2_CYTB* assay may have made it more difficult to clearly identify a positive detection compared to the *BUT1_CYTB* assay. The *BT3_CYTB* assay was also reasonably specific, but there was a visible amount of cross amplification with lake trout DNA (Figure A 25). Lake trout DNA was not amplified as efficiently as bull trout DNA and the difference in fluorescence amplitude was enough to differentiate between the extracted DNA of both species. However, this difference may not have

been as great in an environmental sample that contained the DNA of both species. The *BUT1_CYTB* assay outperformed both candidate bull trout assays that I developed and was the only bull trout assay chosen for further validation in this study.

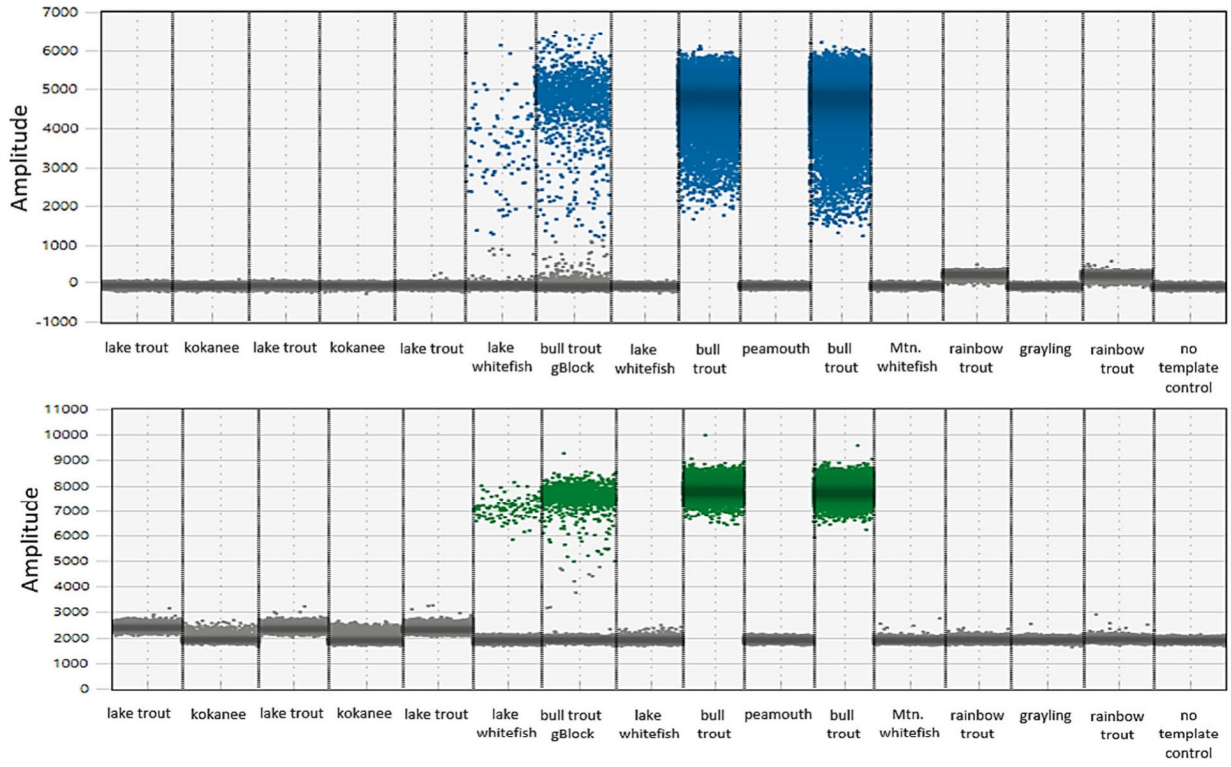

Figure A 23. Fluorescence amplitude of ddPCR droplets plotted against DNA samples of target and non-target species from the Williston Reservoir for the *BT2_CYTB* candidate bull trout assay (top) and the BUT1_CYTB assay (bottom) from Wilcox et al. (2013). Some of the lake whitefish tissue samples that DNA was extracted from were contaminated with bull trout DNA. This is evident from the small number of positive droplets in some but not all lake whitefish samples.

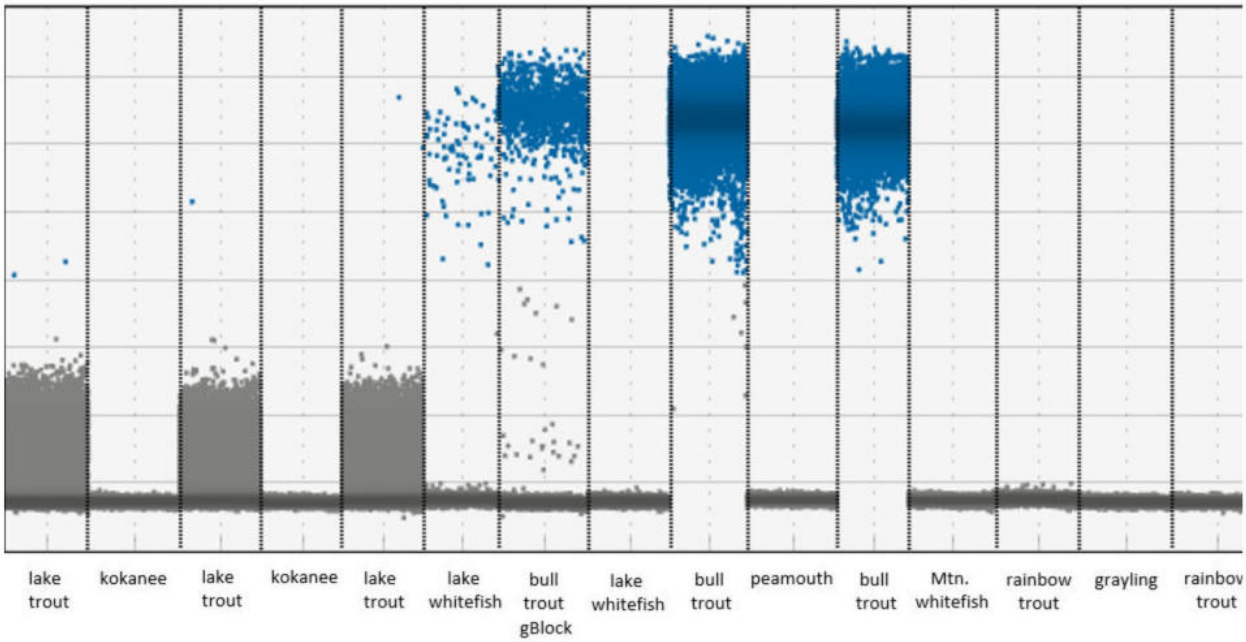

Figure A 24. Fluorescence amplitude of ddPCR droplets plotted against DNA samples of target and non-target species from the Williston Reservoir for the *BT3_CYTB* candidate bull trout assay. Some of the lake whitefish tissue samples that DNA was extracted from were contaminated with bull trout DNA. This is evident from the small number of positive droplets in some but not all lake whitefish samples.

The *LT1_CYTB* candidate lake trout assay performed similarly in the specificity tests to the *BT3_CYTB* assay. The *LT1_CYTB* assay demonstrated low amplitude cross amplification with extracted bull trout DNA and although the difference in fluorescence amplitude was enough to differentiate between the extracted DNA of both species, it was not ideal (Figure A 25). The *LT1_CYTB* assay was therefore not selected for further validation in this study.

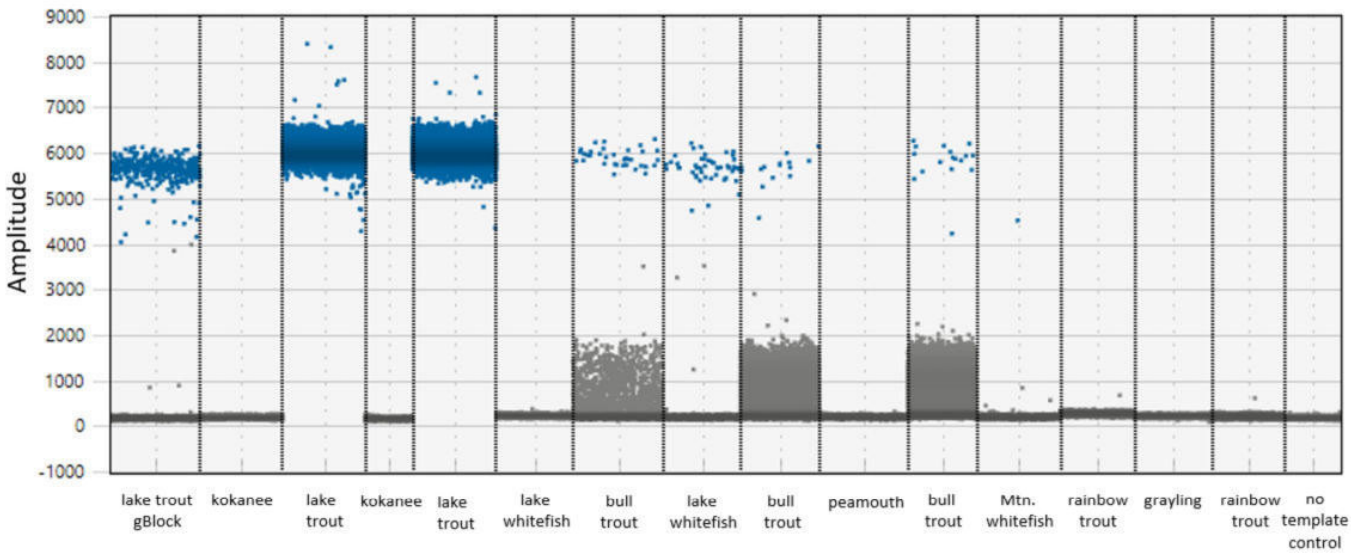

Figure A 25. Fluorescence amplitude of ddPCR droplets plotted against DNA samples of target and non-target species from the Williston Reservoir for the *LT1_CYTB* candidate lake trout assay. Some of the lake whitefish and bull trout tissue samples that DNA was extracted from were contaminated with lake trout DNA. This is evident from the small number of positive droplets in some but not all lake whitefish samples.

APPENDIX C:
PELAGIC SPECIES QPCR ASSAY VALIDATIONS FOR THE WILLISTON RESERVOIR

A validation scale devised by Thalinger et al. (2021) provides criteria for determining if eDNA assays are ready for use in routine species monitoring. The five levels of the validation scale incorporate the entire workflow used to detect a species' DNA in an environmental sample, from sample collection through to the interpretation of PCR results (Table A3). Levels 1 and 2 include assay design, basic *in silico* analysis, *in vitro* optimizations, testing on target species tissue and specificity testing on closely related, non-target species. eDNA results from assays that have only been validated to level 2 cannot be interpreted with any confidence until further levels of validation have been achieved.

More extensive specificity testing that includes co-occurring non-target species and sensitivity testing that establishes an LOD are considered important criteria for assay validation at level 4. The *in silico* assay design and screening and *in vitro* testing completed for the six assays in this study meet the criteria for validation at level 1, 2 and much of level 4. Level three and four validation criteria includes testing on environmental samples (*in situ* validations). Confidence in eDNA results build with the level of validation. Level three validation is considered essential and the results from assays validated to level four and five can be interpreted with confidence (Thalinger et al., 2021).

The novel lake trout assay, *LT_CYTB,* could not be validated beyond level two because it did not successfully detect lake trout eDNA in samples collected from the Williston Reservoir. However, the eDNA and gillnet results from Teare Creek and the eDNA results from Factor Ross all agreed with one another, but a verified negative result is not the same as a verified positive result. Collecting and analyzing samples from other locations in the Williston Reservoir where

lake trout are known to be abundant, such as Finlay Forks, would likely result in a positive detection and help to validate the *LT_CYTB* to at least level three.

The novel peamouth assay, *PCC_CYTB,* achieved a few positive detections from the eDNA samples taken in the Finlay Reach, but the quality and quantity of those eDNA samples was poor. The presence of peamouth in the area was confirmed by the gillnet results and that adds confidence to the positive eDNA detections. However, more extensive field testing of the *PCC_CYTB* assay is needed to be confident in the results.

Although *in vitro* specificity testing for this study was robust, I did not determine detection probabilities of any of the assays with statistical models. Further, this was only a pilot study and did not produce enough data to rigorously investigate the ecological and physical factors influencing eDNA in the Williston Reservoir. These are key criteria that must be met to validate an eDNA assay to level five and should be the focus of future eDNA studies in the Upper Peace River watershed.

Table A 3. The five validation levels described by Thalinger et al. (2021) and the validation level achieved by each of the six assays tested in this study.

			Assays					
Level	**Description**	**Criteria**	***Sock _COI***	***Rain _COI***	***COCL_ CYTB***	***BUT1_ CYTB***	***LT_ CYTB***	***PCC_ CYTB***
1	Incomplete	Basic *in silico* analysis.	X	X	X	X	X	X
2	Partial	*In vitro* specificity testing.	X	X	X	X	X	X
3	Essential	Target species successfully detected from an eDNA sample. Specifics of eDNA extraction and concentration reported.	X	X	X	X	Partial	Partial
4	Substantial	LOD determined. Extensive field testing and *in vitro* testing on co-occurring, non-target species.	X	X	X	X	Partial	Partial
5	Operational	Comprehensive specificity testing, detection probability from statistical modelling and investigations of ecological and physical factors potentially influencing eDNA in the environment.						

APPENDIX D:
ENVIRONMENTAL GENOMICS FACILITY (GREAT LAKES INSTITUTE OF ENVIRONMENTAL RESEARCH) IN-HOUSE PCR CLEAN-UP PROCEDURE

SPRI (Sera-Mag) bead solution recipe for PCR clean-up

Table A 4. Reagents and amounts used in bead solution recipe for PCR clean-up.

Reagent	Mass	Volume
PEG-8000 powder	10g	-
5M NaCl	-	25 mL
Washed Sera-Mag Beads	-	1 mL
ddH_2O	-	24 mL

1. Add 10g of PEG-8000 to a 50mL Falcon tube.
2. To the powder, add NaCl and fill with ddH_2O up to the 50 mL mark.
3. Shake/vortex Falcon tube until all PEG has dissolved. Note: Shaking for five minutes and then letting it sit for a few minutes usually works the best.
4. Resuspend stock solution of Sera-Mag beads by shaking until there is no longer "sediment."
5. Wash the beads: Transfer 1mL bead suspension to a 1.7mL tube and pellet the beads on a magnetic rack (~3 min). Keeping the tube on the magnetic rack, remove the storage buffer and add 1mL of TE. Remove the tube from the magnetic rack, vortex to mix well and place back on magnetic rack. Remove TE buffer and repeat the TE wash one more time. Fully resuspend the beads in 1mL TE and mix well.
6. Add bead suspension to the Falcon tube and mix immediately. Wrap Falcon tube in aluminum foil and store at 4°C.

Bead-Cleaning Steps

1. Mix 15uL of the bead solution with 10uL of PCR product thoroughly by pipetting 10 times.
    - This step binds PCR products 100bp and larger to the magnetic beads.
    - Let the mixed samples incubate for 3-5 minutes at room temperature for maximum recovery.
2. Place the reaction plate onto a magnetic plate for 5-10 minutes to separate beads from the solution.
    - The separation time is dependent on the size of the reaction.
    - Wait for the solution to clear before proceeding to the next step.
3. Aspirate the cleared solution form the reaction plate and discard.
    - This step must be performed while the reaction plate is situated on the magnetic plate.

- Do not disturb the collection of magnetic beads.

4. Dispense 200uL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature.
    - Aspirate out the ethanol and discard.
    - Repeat for a total of two washes. It is important to perform these steps with the reaction plate situated on the magnetic plate.
    - Do not disturb the magnetic beads.
    - Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
5. Place the reaction plate on bench top to air-dry.
    - Be sure to allow the plate to dry completely.
    - The plate should be left to air-dry for 10-20 minutes on a bench top to allow complete evaporation of residual ethanol.
6. Add 40uL of elution buffer (ddH_2O or TE) to each well of the reaction plate and mix the solution with beads by pipetting 10 times.
    - Put the reaction on the magnetic plate for 5 minutes and wait for the solution to become clear.
    - Remove all solution to a new plate and keep it at -20°C.