The detectability of Atlantic salmon (Salmo salar) microsatellite and mitochondrial environmental DNA

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Abstract

Little is known about what information can be gathered from microsatellite eDNA. It is important to gain a deeper understanding of the detectability and analysis of microsatellite eDNA because it could provide information about population size that mitochondrial eDNA cannot. Water samples were collected from tank and river experiments, and rivers known to contain Atlantic salmon (*Salmo salar*) and analyzed for Atlantic salmon mitochondrial and microsatellite eDNA. Mitochondrial eDNA was detected from all the tank experiments and 10 out of 15 rivers known to contain Atlantic salmon. Microsatellite eDNA was detected from all the tank experiments and none of the river experiments. The microsatellite alleles detected from the water were not solely representative of the genotypes of the fish in the tanks, thus individuals could not be clearly identified. The inconsistent detectability of nonrepresentative microsatellite alleles from water suggests that microsatellite eDNA cannot presently provide population-level information about Atlantic salmon.
Keywords

Environmental DNA (eDNA), microsatellite DNA, mitochondrial DNA, Atlantic salmon, non-invasive population monitoring method, reintroduction
Summary for Lay Audience

The analysis of environmental DNA (eDNA), or DNA from animals detected from water and soil, is a non-invasive tool to determine information about animals. Mitochondrial eDNA, mitochondrial DNA detected from water, is used to determine the presence of different fish species. Mitochondrial eDNA can determine the presence of fishes because each species of fish has different mitochondrial DNA, so mitochondrial DNA detected from an eDNA sample can be separated by fish species, and the number of species can be counted. Nuclear eDNA is another type of DNA that can be detected from water and could potentially provide information about how many individuals of a fish species are present in the water. One type of nuclear DNA being investigated is microsatellite DNA because microsatellite DNA typically differs from individual to individual, so if microsatellite DNA can be detected from eDNA samples the DNA could be separated by individual and the number of individuals could be counted. Past research has found that microsatellite eDNA is detectable from water, but a study has not been done to see if the microsatellite eDNA found in water matches the DNA of individuals present in the water. Thus, water samples were collected from tank and field experiments, and rivers containing Atlantic salmon, and I analyzed the samples for mitochondrial and microsatellite eDNA. Mitochondrial eDNA was detected from all the tank experiments, confirming that Atlantic salmon DNA was present in the tank water. Additionally, mitochondrial eDNA was detected from 1/7 of the adult return, 7/12 of the naturalized watershed, and 7/16 of the stocked river sites containing Atlantic salmon, thus Atlantic salmon were at those sites. Microsatellite eDNA was detected from all the tank water samples and compared to the DNA detected from the fish in the tanks. The microsatellite DNA from the water was not a good enough match to the fish DNA to identify individuals. Overall, this study was the first to directly compare microsatellite DNA detected from water with the DNA of the fish in that water, and it does not support using microsatellite eDNA to identify individuals.
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<th>Meaning</th>
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<tr>
<td>ATL</td>
<td>Animal tissue lysis</td>
</tr>
<tr>
<td>COI</td>
<td>Cytochrome oxidase subunit I</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eDNA</td>
<td>Environmental DNA</td>
</tr>
<tr>
<td>GEN-FISH</td>
<td>Genomic network for fish identification, stress, and health</td>
</tr>
<tr>
<td>GPS</td>
<td>Global positioning system</td>
</tr>
<tr>
<td>MNRF</td>
<td>Ontario Ministry of Northern Developments, Mines, Natural Resources, and Forestry</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NYSDEC</td>
<td>New York State Department of Environmental Conservation</td>
</tr>
<tr>
<td>OFAH</td>
<td>Ontario Federation of Anglers and Hunters</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIT</td>
<td>Passive integrated transponder</td>
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Chapter 1

1 Introduction

1.1 Biodiversity

Despite its importance, global biodiversity has experienced a substantial decline over the past 50 years due to habitat loss, global warming, habitat fragmentation, pollution, overexploitation, and human encroachment, resulting in a tens-to-hundreds-fold increase in the species extinction rate compared to the last ten million years (Hautier et al., 2015). Indeed, over 140,000 species are currently classified as threatened, underscoring the urgency of preserving vital populations and their habitats (ICUN, 2022). A key element to inform conservation programs is accurately assessing species abundance, which has led to the widespread adoption of population monitoring practices.

1.2 Population monitoring

Population monitoring encompasses a wide range of scientific methods for tracking changes in the abundance, distribution, and genetic composition of a species over time. The choice of population monitoring method depends on the biological characteristics of the species, its abundance and distribution, and the questions being addressed. For example, mist netting is a common method for monitoring bird populations because it allows researchers to capture and release individuals easily (Dunn and Ralph, 2004). GPS sensors and other remote tracking technologies are used to track animal movement and behaviors, enabling researchers to monitor populations over large spatial and temporal scales (Prosekov et al., 2020). Ultimately, it is important to use reliable and effective methods to ensure that monitoring efforts provide meaningful data that can be used to inform conservation and management decisions.

Traditional fish population monitoring methods most commonly consist of mark and recapture, net fishing, trapping, and electrofishing. Mark and recapture consists of three steps: 1) capture fish with a net, 2) tag the individuals and release them, and 3) return to the place of capture and recapture. The number of recaptured individuals can then be
used to estimate population size (Hamel et al., 2015). Netting and trapping also require the capturing and handling of the study species to determine species presence and population size. Electrofishing is a method used to capture a large number of individuals quickly and efficiently, by releasing an electric current into the water (Bohlin et al., 1989). All of the traditional monitoring methods are relatively invasive as they require direct contact with fish (Radinger et al., 2018). These direct interactions have moderate to high adverse welfare effects. For example, utilizing mark and recapture or netting increases individual stress and predation risk. Similarly, the current from electrofishing can cause damage to delicate tissues such as gills, eyes, and fins. The shock can also disrupt physiological processes, including respiration and ion regulation, leading to rapid exhaustion and death (Mesa and Schreck, 1989). Additionally, electrofishing can affect other aquatic organisms that are not targeted for sampling. The electric field generated by the electrofishing equipment may interfere with the communication, navigation, and feeding behaviors of non-target species, including migratory fishes and invertebrates (Underwood, Bromaghin, and Klosiewski, 2011). Aside from the negative affect on fish, traditional population monitoring methods can also be costly and labour-intensive. These costs can lead to low sampling effort, so samples may not be representative of the larger population (Sigsgaard et al., 2015; Spear et al., 2021; Radinger et al., 2018). Therefore, alternative population monitoring methods that do not require direct interactions with fishes are needed to address these challenges (Radinger et al., 2018).

There are currently three non-invasive population monitoring techniques for fishes: video recording, snorkeling, and the analysis of environmental DNA (eDNA). Video recording can reveal repeated activities and habitats of a particular species or help identify new behaviors that were previously unknown (Castaneda et al., 2020). Moreover, video recording can capture rare and elusive events such as courtship rituals, predation, and territorial displays (Struthers et al., 2015). However, there are drawbacks to using video recording as a population monitoring tool. The first drawback of video recording is that it requires electricity and data storage, which may be a challenge in remote locations that require sustained monitoring. Another drawback of video recording is its reliance on water clarity to monitor populations. Placing the video recording device in a location with a clearer view avoids certain areas, which causes important behaviors to be missed in the
recording (Mallet and Pelletier, 2014). The second non-invasive fish population monitoring method is visual observation by snorkeling. Snorkeling as a population monitoring method is as effective as conventional methods for non-cryptic species in determining community composition and population size. Snorkeling may also be more cost-effective than traditional monitoring methods (Brock, 1982; Macnaughton et al., 2015). However, snorkeling as a population monitoring tool is limited by snorkeler skill and experience, water depth, and water clarity (Thurow and Schill, 1996). The third non-invasive monitoring method is eDNA analysis. The analysis of eDNA involves collecting and analyzing DNA from organisms that is shed into the environment, such as water and sediment. In comparison to visual methods eDNA analysis is advantageous because information about species presence can be acquired without the need to see the species of interest, thus eDNA analysis can more easily provide information about evasive and endangered species. Additionally, eDNA water sample collection can be done on a larger scale, as water samples can be collected quickly by individuals with less training than that of snorkelers. However, relative to video recording and snorkeling, eDNA analysis is a relatively new population monitoring method. Thus, further research is required to assess how much information can be gathered about fishes using eDNA analysis.

1.3 Environmental DNA

1.3.1 A brief history of eDNA

Environmental sampling of genetic material was first applied to microbial research in the late 1980s. At the time microbial community makeup was primarily determined using cell cultures and morphological identification. However, alternative methods were being explored, as not all microbial species can be grown in cell cultures. Thus, several studies used microbial ribosomal RNA detected from soil samples to provide more information on the genetic makeup of microbial communities (Brock et al., 1987; Olsen et al., 1986; Orgam et al., 1987; Pace et al., 1986). Further research was conducted into the detectability of microbial ribosomal RNA, and in 1999 microbial ribosomal RNA was detected from freshwater (Fisher and Triplett, 1999). The detectability of ribosomal RNA
from environmental samples in microbial research led to the investigation of the detectability of eukaryotic DNA from environmental samples.

The first study to successfully detect eukaryotic DNA from environmental samples was Martellini et al., 2005. In that study, species-specific primers were designed to amplify mitochondrial DNA from humans (*Homo sapiens*), cows (*Bos taurus*), pigs (*Sus domesticus*), or sheep (*Ovis aries*). PCR amplification was then used to determine if the DNA detected from wastewater solids included DNA from each species. The success of that study helped establish the basis for other studies to use mitochondrial DNA as a tool to differentiate between species in environmental samples.

A second significant study in the development of eDNA analysis was that of Ficetola et al. (2008), which was the first study to detect DNA from freshwater samples, determine invasive species presence, and use the term environmental DNA. That study investigated the detectability of mitochondrial eDNA of the invasive American bullfrog (*Lithobates catesbeianus*) in lab and field settings using a cytochrome oxidase subunit 1 (COI) mitochondrial marker. In both the lab and field settings, frog DNA was detected from all the systems containing American bullfrogs, and no DNA was detected from the systems that did not contain American bullfrogs. Ficetola et al. (2008) showed that mitochondrial DNA detectability from environment samples was possible and can provide valuable information about the presence of a species in a given area.

Subsequent eDNA research firmly established mitochondrial eDNA analysis as a tool to determine the presence of low-density species. In 2011, Goldberg et al., detected mitochondrial eDNA corresponding to the evasive rocky mountain tailed frog (*Ascaphus montanus*) from multiple rivers, and Jerde et al., 2011 detected mitochondrial eDNA corresponding to the invasive Asian carp (*Cyprinus carpio*) in the Great Lakes Basin. Together these studies provided critical confirmation that mitochondrial eDNA was detectable from wetlands, rivers, and lakes. Another study was conducted comparing the traditional population monitoring methods of the invasive American bullfrog (nocturnal and diurnal surveys) to mitochondrial eDNA analysis of water samples (Dejean et al., 2011). The study found that mitochondrial eDNA analysis was able to detect American
bullfrog presence in all the locations where presence was found using traditional methods, and none of the negative control locations (Dejean et al., 2011). The aforementioned studies helped establish mitochondrial eDNA analysis of water samples as a tool to determine species’ presence in aquatic systems.

One of the most common applications of eDNA analysis is for the detection of invasive aquatic species (Erikson et al., 2016; Diaz-Ferguson et al., 2014; Piaggio et al., 2014; Takahara et al., 2013). For example, in 2013 Goldberg et al. conducted a series of experiments assessing the detectability of invasive New Zealand mudsnail (Potamopyrgus antidorum) mitochondrial eDNA at varying population densities. The study found that as few as one individual could be detected using mitochondrial eDNA analysis of water samples. These findings were subsequently verified in field studies where the presence of invasive species was successfully detected (Dubreuil et al., 2022; Thomas et al., 2020; Xia et al., 2021).

Mitochondrial eDNA analysis is also used as a replacement for traditional population monitoring methods to detect the presence of endangered species. The main difficulty encountered when using traditional methods is false negatives for species presence, as many endangered species persist at a low abundance. The first study to assess the use of mitochondrial eDNA to determine the presence of an endangered aquatic species was Olson et al., 2012. In the study, researchers collected eDNA water samples from different sites with known densities of endangered salamander, the eastern hellbender (Cryptobranchus alleganiensis). Olson et al. (2012) were able to detect Eastern hellbender eDNA from all locations where it was known to be present regardless of population density. Subsequent studies have used mitochondrial eDNA as a tool to determine the presence of aquatic endangered species in multiple systems (Atkinson et al., 2019; Bylemans et al., 2018; Cardas et al., 2020; Simpfendorfer et al., 2016).

In addition to detecting the presence of a single aquatic species, mitochondrial eDNA analysis can be used to determine the species composition of aquatic communities. Minamoto et al. (2012) were the first to examine the mitochondrial eDNA of multiple species from a single water sample. They collected water samples from a controlled lab
experiment and several field locations. The researchers used universal mitochondrial primers that can amplify multiple species in the same PCR reaction. They used Next-Generation Sequencing (NGS) to identify the species present within the sample.

Ultimately, the paper demonstrated that multiple species can be detected in eDNA water samples using mitochondrial primer pairs. Following that paper, Thomsen et al., 2012 tested the ability of mitochondrial eDNA analysis to determine fish community biodiversity against multiple traditional methods: angling, beach-seine, fyke netting, fish pots, gillnetting, snorkeling, and trawling. The study developed generic mitochondrial primer sets to detect the mitochondrial gene cytochrome b in a variety of local fishes. Through the use of these mitochondrial primer sets, they showed that the eDNA approach detected DNA from all of the species identified using traditional population monitoring methods. A number of studies have similarly validated the capacity of mitochondrial eDNA to determine the species composition of an aquatic community that corresponds to the community determined using traditional methods (Evans et al., 2017; Hanfling et al., 2016; Shaw et al., 2016; Thomsen et al., 2012). Mitochondrial eDNA has also been shown to be a highly sensitive and specific approach, which for example can distinguish between closely related endangered brook trout (Salvelinus fontinalis) and bull trout (Salvelinus confluentus) (Wilcox et al., 2013). Ultimately, mitochondrial eDNA analysis is now widely used to determine community composition using either (1) primer pairs designed for each species of interest (Bylemans et al., 2018; Rodgers et al., 2015) or (2) universal primers designed to detect multiple species (Egan et al., 2013; Kelly et al., 2014).

1.3.1.1 Species abundance

Beyond determining species presence, mitochondrial eDNA analysis is also being assessed as a potential tool for determining species abundance. The first investigation into the ability of mitochondrial eDNA to provide abundance information was conducted in 2012 by Takahara et al., who assessed the ability of mitochondrial eDNA concentration in water to reflect the biomass of common carp (Cyprinus carpio). Experiments were conducted using multiple densities of Common carp in aquarium and pond settings and collected water samples from field locations known to contain common
carp. A positive correlation between fish biomass and mitochondrial eDNA concentration (read copy number / volume of water) was found for both the lab and pond experiments. However, there was no correlation between mitochondrial eDNA concentration and fish biomass from the field sampling. Subsequent studies have defined biomass and abundance differently, so for clarity in my thesis I will define biomass as the total mass of fish and abundance as the total number of fish in a given area. Perez et al., 2017 assessed the relationship between mitochondrial eDNA copy number and fish abundance and biomass for largemouth bass (*Micropterus salmoides*) and American shad (*Alosa sapidissima*) (Perez et al., 2017). Traditional population monitoring methods were utilized to determine the abundance and biomass of the species at different field locations, and water samples were collected alongside the traditional monitoring. No relationship between the abundance or biomass and the number of eDNA copies detected was found for either species (Perez et al., 2017). Another study examined the relationship between the number of ayu sweetfish (*Plecoglossus altivelis*) recorded during a snorkeling survey and the number of mitochondrial eDNA copies detected from water samples. A significant correlation between fish abundance and eDNA concentration per volume of water was found (Doi et al., 2017). A third study (Coutler et al., 2019) assessed the invasive Asian silver carp’s (*Hypophthalmichthys nobilis*) abundance and biomass through hydroacoustic surveys and mitochondrial eDNA concentration (total read count detected for each fish species) through water sample collection in the field. They found a positive relationship between biomass and density and mitochondrial eDNA concentration. A fourth study (Muri et al., 2020) looked at a community of fish and compared the biomass and abundance to detected eDNA concentration (copy number) from mitochondrial eDNA in a pond. The study found that species abundance and biomass were significantly positively correlated to eDNA read count for every species. Similarly, other studies focusing solely on abundance and eDNA concentration have found mixed results. Positive correlations were reported for bony fishes, brook trout, lake trout (*Salvelinus namaycush*), Ryukyu ayu (*Plecoglossus altivelis*) (Akamatsu et al., 2020; Baldigo et al., 2017; Lacoursiere-Roussel et al., 2016b; Kelly et al., 2014), while no correlations were reported for Arctic char (*Salvelinus alpinus*) or brown trout (*Salmo trutta*) (Capo et al., 2019). Overall, there is some evidence that eDNA
concentration covaries with abundance, but the predictive power of the relationship is low, particularly due to the many factors that have been found to affect eDNA concentration. Tillotson et al. (2018) found a positive correlation between eDNA concentration and abundance but also found a positive correlation between eDNA concentration and temperature or live/dead fish ratio. Thus, future research is needed to help standardize measurements of biomass and abundance in the field of eDNA and to determine the affect abiotic factors have on the relationship between mitochondrial eDNA concentration and relative species abundance. Moreover, the sensitivity of mitochondrial eDNA concentration to factors other than species abundance suggests that it is not the ideal eDNA measure to provide population level information.

1.3.1.2 Nuclear eDNA

Nuclear eDNA, particularly microsatellites, could potentially be used in place of mitochondrial eDNA to determine species abundance. Microsatellites are non-coding regions that mutate as a result of slippage during DNA replication, instead of substitution (Putman and Carbone, 2014). The slippage causes microsatellites to form a series of one to six repeated nucleotides that form motifs of repeated regions (Field and Wills, 1998). Additionally, the flanking regions of microsatellites are highly conserved within a species, for both prokaryotes and eukaryotes (Jarne and Lagoda, 1996). These attributes result in the high likelihood that microsatellite genotypes vary between individuals in a population. The high variability and conservation of microsatellites enables them to discriminate between relative number of individuals. It is this ability that may allow microsatellites to fill in the population level informational gaps left by mitochondrial analysis in eDNA research.

The potential use of microsatellites in eDNA analysis was first suggested by Olson et al. (2012) based on the widespread use of microsatellite markers in population genetics to determine migration events and effective population size. Migration events occur when individuals of a population geographically separate to form a new population and persist in the new population. Microsatellite markers can be used to assess the migration of individuals directly by identifying genotypes of migrant individuals. First, the microsatellite genotypes of individuals in the new and original population are determined.
Then, the repeat regions of the microsatellites can be compared to determine how closely related the individuals of the original population are to individuals of the new population (Haasl and Payseur, 2011). Thus, microsatellite markers have the ability to differentiate between individuals in a given population. This ability could potentially be applied to identifying the microsatellite alleles of specific individuals from eDNA samples. In addition to providing information about migration, microsatellites are used in population genetics to determine effective population size. Effective population size is the number of individuals in an ideal population that would lose genetic variation at the same rate as the observed population (Haasl and Payseur, 2011). Creel et al. (2003) is particularly relevant to the potential use of microsatellites in eDNA analysis. They assessed the ability of microsatellites to determine effective population size from trace samples in a wolf population of a known size, recovered from a multi-year census. Importantly, the study used a threshold to determine the probability of obtaining a certain set of matching alleles by chance from two unique individuals. The threshold was defined as the ratio of the number of individuals detected compared to the known number of individuals sampled. This threshold is directly related to microsatellites’ potential use in eDNA analysis because it sets a precedent for a potential threshold to be used to identify microsatellite alleles detected from eDNA samples as “true” or unique alleles that represent a unique individual. Overall, the use of microsatellites in population genetics to determine migration events and effective population size provides a basis for the use of microsatellites in eDNA analysis to provide more population level information about species.

Olson et al. (2012) was the first study to detect microsatellite alleles successfully from eDNA water samples. The authors used a dilution series of eDNA extracted from the water of tanks containing Eastern hellbenders to determine the minimum concentration of eDNA needed for analysis, showing that microsatellite eDNA could be amplified as bands on an agarose gel at a concentration as low as 2 ng µL⁻¹, compared to that of the amplified concentration of mitochondrial eDNA, 2 x 10⁻⁶ ng µL⁻¹. Presently, there is only one additional study that investigated microsatellite eDNA and water samples. Andres et al. (2021) compared the microsatellite allele frequencies detected from eDNA water samples to the allele frequencies detected from DNA mixtures (pooled samples of
DNA collected directly from the fins of the fish in the water). They found that the microsatellite allele frequencies detected in the eDNA water samples and DNA mixtures were highly correlated, and that DNA mixtures reliably estimated the number of genetic contributors in the eDNA water samples. However, the study provided little information about the reliability and consistency of the genetic information obtained from the water samples. Specifically, Andres et al. (2021) does not provide a direct comparison of the alleles detected from the DNA collected directly from the individuals in an experiment and the associated eDNA water samples from that experiment. Beyond the above findings, a number of challenges and uncertainties remain surrounding the potential use of nuclear eDNA as a population monitoring tool. Specifically, nuclear eDNA persists at lower concentrations than mitochondrial eDNA; thus consistently amplifying microsatellite loci from eDNA samples may be challenging (Olson et al., 2012). Moreover, information needs to be gathered about the ability of microsatellite loci detected from eDNA samples to accurately represent the microsatellite loci of the individuals present in the water. If these challenges can be addressed, the combined use of nuclear and mitochondrial eDNA has the potential to increase the non-invasive spatial-temporal monitoring of fishes in a more cost and labour effective way.

1.4 Study species

Atlantic salmon (Salmo salar) are ecologically, culturally, and economically important fish. Atlantic salmon are predators in both marine and freshwater environments, contributing to the regulation of energy flow through food webs (Fisheries & Oceans Canada, 2009). They also play a role in nutrient cycling, as the migration of salmon from the ocean to freshwater systems allows for the transport of marine-derived nutrients into freshwater environments, which can have a significant effect on nutrient cycling and productivity (Watz et al., 2022). Atlantic salmon have long-standing cultural and spiritual significance to Indigenous peoples in North America. Historically, Atlantic salmon were an important food and cultural resource for Indigenous peoples and have played a central role in their traditions, ceremonies, and livelihoods. The sustainable management and protection of Atlantic salmon populations are therefore critical to preserving Indigenous cultures and ways of life (Fisheries & Oceans Canada, 2009). Recreational fishing for
Atlantic salmon generates substantial economic activity in many communities (Pinfold, 2011). The Atlantic salmon farming industry is also a significant contributor to the economy globally grossing over $15 billion in 2021 (Shahbandeh, 2023). On a smaller scale, in North America, the Atlantic salmon farming industry grosses an estimated $26 million annually. The financial significance of the Atlantic salmon industry consequently provides jobs, supports global and local businesses, and contributes to the food supply (Fisheries & Oceans Canada, 2005). However, salmon farming can also have a negative effect on the environment and wild salmon populations, including the spread of disease and parasites, genetic introgression, and pollution. Overall, Atlantic salmon have ecological, economic, and cultural importance, and their conservation and management require a balanced approach that considers the needs of multiple stakeholders and the sustainability of the resource.

Despite their overall importance, Atlantic salmon populations have faced significant declines and continue to face threats from human activities, climate change, and other factors. It is important to protect and conserve these populations to ensure that they continue to play their important ecological, economic, and cultural roles. Thus, monitoring and reintroduction programs are important tools for conserving and restoring Atlantic salmon populations. Monitoring programs can help track the status and trends of populations, identify threats, and inform management and conservation efforts (Watz et al., 2022). Reintroduction programs can help supplement wild populations, restore extirpated populations, and enhance genetic diversity. There have been various efforts to reintroduce Atlantic salmon into historically occupied habitats. These efforts have involved stocking hatchery-raised salmon, as well as implementing habitat restoration and conservation measures to support the survival and reproduction of wild and stocked fish (Fisheries & Oceans Canada, 2005). Reintroduction efforts can be complex and require careful planning and management to be successful. Factors such as habitat availability and quality, predation, competition, and genetic diversity must be carefully considered when designing and implementing reintroduction programs (Thorstad et al., 2021). All in all, monitoring and reintroduction programs are important components of broader conservation and management strategies aimed at protecting and restoring Atlantic salmon populations.
Historically, Ontario was home to the largest freshwater population of Atlantic salmon in the world. The landlocked population in Ontario is thought to have originated from anadromous (migratory) Atlantic salmon that became landlocked after being isolated from the ocean by the formation of the St. Lawrence River thousands of years ago. Although the landlocked Atlantic salmon population in Ontario has faced significant declines in recent decades, efforts to restore and conserve this population have been underway, including the release of hatchery-raised salmon into the wild, habitat restoration, and conservation measures to protect and manage the remaining wild populations. Conservation and restoration efforts for this unique population of Atlantic salmon are important not only for the survival of the species in Ontario but also for the preservation of an important example of the evolutionary and ecological processes that shape freshwater ecosystems.

Government-led efforts to restore the population began in the 1940s by the Ontario Department of Lands and Forests. In that first effort, Atlantic salmon from Eastern Canada were brought to Ontario and stocked in several rivers. However, a combination of high mortality rates of stocked individuals and sporadic stocking failed to produce a self-sustaining population (Parsons, 1973). The next attempt to stock Lake Ontario with Atlantic salmon was led by the New York State Department of Environmental Conservation (NYSDEC) in 1985. Although the NYSDEC stocking program did not continue into the present day it led to the creation of the current restoration program: The Bring Back the Salmon Program. The Bring Back the Salmon Program is run by the Ontario Ministry of Northern Developments, Mines, Natural Resources, and Forestry (MNRF), the Ontario Federation of Anglers and Hunters (OFAH), and other partners. The program is made of four phases: (1) initial Atlantic salmon stocking in the three main Lake Ontario tributaries, (2) continued stocking within additional tributaries, (3) habitat restoration, and (4) tracking the movements of stocked Atlantic salmon (Bring Back the Salmon, 2023). Currently, the Bring Back the Salmon program is focusing on the later three phases of the program with an emphasis on the presence and movement of Atlantic salmon in different river management regimes. The program defines three of the key river management regimes as adult return (rivers where adult Atlantic salmon have been visually reported), naturalized watershed (rivers where Atlantic salmon populations are
known to inhabit) and stocked (rivers that are actively stocked with Atlantic salmon fry). It is important to understand if differences in Atlantic salmon presence exist in different river management regimes as it provides information on what river management regimes allow restoration to be the most successful. Moreover, it is important to monitor Ontario Atlantic salmon using non-invasive methods to minimize any negative effects monitoring could have on the reintroduction success of the species. Therefore, utilizing non-invasive monitoring technologies is key to understanding and assessing the success of the program in different river management regimes.

1.5 Rationale and objectives

My research aims to address the remaining uncertainties of the potential use of nuclear eDNA as a population monitoring tool by determining if: (1) if eDNA from water samples taken from a tank containing fish with known microsatellite genotypes can be used to detect the identity and frequency of the microsatellite alleles present in those individuals; (2) if the number of unique microsatellite alleles detected from eDNA will increase with the number of fish in the tank in a predictive way; (3) if nuclear eDNA results under lab conditions can be replicated in field conditions; and (4) if these eDNA approaches can be applied to assess the survival of Atlantic salmon stocked in the Bring Back the Salmon program.
Chapter 2

2 Methods

2.1 Microsatellite eDNA experiments

2.1.1 Lab experiments

Using juvenile Atlantic salmon, trials were conducted to determine if microsatellite alleles could be successfully detected from eDNA water samples in controlled laboratory conditions. The lab experiments consisted of five trials at three densities (n = 1, 2, 4 individuals per 40 L of water) and a negative control (no fish present in a tank) (Finstad et al., 2009 and Kaspersson, et al., 2010). The Atlantic salmon used in the lab experiments were obtained as fertilized eggs from the MNRF Normandale Research Facility (Vittoria, Ontario) in the Fall of 2017. The Atlantic salmon descended from the LaHave River population (Nova Scotia, Canada; 44.4°N, 64.5°W). Twenty-four hours after fertilization, eggs were transported to a hatchery facility at Western University and housed in egg trays at 7 ± 0.5°C. Once the endogenous yolk sacs of the fish were nearly absorbed, approximately four months post-fertilization, the alevin were transferred to 40 L Rubbermaid totes, and the rearing temperature was raised to 11 ± 0.5°C. Each tank was covered with mesh to ensure the fish did not jump out during the trials. During each trial, the bottom of each tank was covered with pebbles, and filled with 15°C London Municipal water. Before each trial, the tank was drained and then rinsed and scrubbed, with a gloved hand, twice with a 10-fold diluted Lavo Pro 6 bleach solution then rinsed and scrubbed, with a gloved hand, ten times with London Municipal water. Additionally, the pebbles present in each tank were rinsed with bleach and water using the above cleaning method. For each trial, the Atlantic salmon were transferred from a central holding tank to the trial tanks and held for a 96-hour habitation period with flow-through water delivered at 100 mL / minute (Guivas and Brammell, 2020). The fish were transferred using handheld nets, that were rinsed with DI water between each use. Then, the water flow was cut off for a 24-hour period (Sassoubre et al., 2016). After which, three 1 L water samples were collected from the surface water of each tank using DNA-
free 3 L containers and a gloved hand. After sample collection, the fish were anesthetized using buffered tricaine methanesulphonate (Syndel, BC), weighed, and the adipose fin was removed to determine the genotype of each fish. The fin clip was immediately placed into a 1.5 mL Eppendorf tube containing 95% ethanol. Individual fish were marked with a PIT (passive integrated transponder) tag inserted into the abdominal, to ensure that fish were not used for multiple trials. Then, the fish were returned to the central holding tank.

2.1.2 Field experiments

Field experiments mirroring the densities used in the lab experiments were conducted to compare the ability to detect microsatellite alleles from eDNA water samples in the field and lab. Field experiments were conducted at two sites along Duffins Creek in the Greenwood Conservation Area, Ajax, Ontario, Canada (Site 1: 43°894180”N,-79°065878”W; Site 2: 43°54’09.3”N, 79°04’12.4”W) (Figure 2.1).

Duffins Creek in the Greenwood Conservation Area was selected as the site for the field experiments because it is a stocking site for the Bring Back the Salmon program. MNRF stocking approval was contingent upon conducting the field experiments in a river where Atlantic salmon were already stocked because it ensured that a new species was not being introduced to the ecosystem. Stocking coordinates were known, and experimental sites were selected to be upstream of the stocking sites to reduce the likelihood that eDNA of other Atlantic salmon would be detected; as previous research has shown that stocked Atlantic salmon are more likely to move downstream from the stocking location (Mokdad et al., 2022). Furthermore, eDNA water samples were collected the previous summer from Duffins Creek in the Greenwood Conservation Area. These water samples found that Atlantic salmon eDNA was not detectable from the stocked population during June and early July. Thus, the field experiments could be conducted during the months when the eDNA of the stocked population was not detectable (June and early July), further reducing the potential detectability of Atlantic salmon eDNA from individuals not used in the field experiments.
Figure 2.1 Map and images of the field experiment two net pen sites in Duffins Creek (Site 1: 43°89'41.8"N, -79°06'58.78"W; Site 2: 43°54'09.3"N, 79°04'12.4"W). Map produced with ArcGIS.

The Atlantic salmon juveniles used in the field experiments were obtained from the MNRF Normandale Research Facility (Vittoria, Ontario) in May 2022. The Atlantic
salmon descended from the LaHave River population (Nova Scotia, Canada; 44.4°N, 64.5°W). Fish were transported from the Normandale Research Facility to the hatchery facility at Western University in a large plastic bag filled 20% with water from the Normandale Research Facility and 80% with oxygen. To ensure the water did not warm during transport the plastic bag, containing the fish, was placed in a cooler containing ice. Additionally, to ensure that the fish did not freeze a barrier of cardboard was placed between the ice and plastic bag within the cooler. Once at the hatchery facility at Western University, the fish, remaining in the plastic bag, were placed in the water of the holding tank for ten minutes to acclimate the fish to the water temperature of the holding tank. Then, the plastic bag was opened, and the fish were transferred into the water of the holding tank. The fish were held in the holding tank, until transport for field experiments.

Juvenile Atlantic salmon were transported from the hatchery facility at Western University to Duffins Creek in a fish transport cooler. The cooler was filled halfway with water from the holding tank and a one Quart plastic bag was filled with ice, to reduce the rate of water warming during transport. An Airstone was placed into the cooler water and connected to a portable pump through tubing run through the lid of the cooler. This allowed the cooler water to be aerated during transport. After the water, ice, and air pump were set up in the transport cooler the fish were transferred from the holding tank to the transport cooler using a handheld net. The handheld net was then rinsed with DI water. Next, the transport cooler was secured to the bed of the truck using bungie cords.

Once at the field site, the juvenile Atlantic salmon were deployed in 15 cm × 50 cm × 50 cm net pens made of mesh, tubing, and zip-ties. At each site, two steel poles were secured to the riverbed. Then, the net pen was secured to the poles using zips-ties. The net pen was submerged under the water, but it did not touch the riverbed. Fish were held in a bag of water, from the fish transport cooler, and partially submerged in the river for five minutes, to allow the fish to acclimate to the temperature of the river (Figure 2.2). Next, the fish were deployed into the net pen, and the opening of the net pen was closed and secured. The field experiments consisted of three density variations n = 1, 2, and 4 fish per net pen. Each density variation was repeated three times at Site 1 and Site 2. Once the fish were deployed the time of day and surface temperature of the water was recorded at
the net pen. Personnel then exited the river upstream of the net pen location, to avoid disturbing any eDNA stored in the sediment downstream from the net pen. The fish were deployed for a 1-hour period before sample collection. Relative to the net pen location, water samples were collected starting from the most downstream to the most upstream locations to avoid the capture of resuspended eDNA from sediment. Three 1 L water samples were collected at four locations at each site: 100 m and 25 m downstream of the net pen, directly behind the net pen, and directly upstream of the net pen. The samples collected upstream of the net pens acted as a background sample from eDNA already present in the stream (Figure 2.2). Water samples were collected using unopened 500 mL plastic water bottles. Each water bottle was opened and emptied directly before it was used for sample collection. At each sampling location, the time of day and surface water temperature were recorded, and the water flow was measured using a Flow Meter (Figure 2.2).

Figure 2.2 Images of the field experiment net pen and water sample collection protocol. Images from left to right: (1) the steel poles were secured to the riverbed, (2) the net was attached to the steel poles with zip ties and submerged in the river, (3) the fish were held in the water to acclimate to the river temperature, (4) water samples were collected, (5) the water flow rate, temperature, and time were recorded.
2.2 OFAH Atlantic salmon stocking and eDNA sample collection

In collaboration with OFAH and the Bring Back the Salmon Program, eDNA water samples were collected in Ontario rivers to determine where Atlantic salmon may be present. Samples were collected from three different river management regimes sites: adult return (sites where adult Atlantic salmon had previously been visually identified), naturalized watershed (sites where populations of Atlantic salmon were known to live), and stocked (sites where Atlantic salmon fry were stocked in 2021). A total of 36 sites (7 adult return, 12 naturalized watershed, 13 stocked, and 4 adult return/stocked) were sampled between May-July 2021 (Figure 2.3). The sites were further categorized as single-sample (sites sampled one time) and multiple-sample (sites sampled multiple times and at multiple locations). There were 30 single-sample sites and 5 multiple-sample sites (Appendix C). The multiple-sample sites were all stocked sites and were sampled three times to assess the effect of stocking on the detectability of Atlantic salmon eDNA over time. For these five sites sampling occurred two to four days pre-stocking, four days post-stocking, and six weeks post-stocking. In addition to sampling water at the stocking site, samples were collected from multiple locations surrounding the site to assess the movement of Atlantic salmon over time. Samples were collected 100 m upstream and 300-700 m downstream of the stocking sites (see Wood et al., 2022).

For this work, samples were collected using a Halltech automated OSMOS eDNA sampler (OSMOS), provided by the Genomic Network for Fish Identification, Stress, and Health (GEN-FISH) (GEN-FISH eDNA Sampling Protocol). The OSMOS was used as outlined in the Gen-Fish eDNA Sampling Protocol (Gen-Fish OSMOS Field Use Manual, 2021). The OSMOS consists of four key components: the backpack unit, a telescope pole, a tripod, and a filter housing unit (Figure 2.4). Before entering the field, the filter housing units were cleaned using a series of 100% ethanol and 10-fold diluted bleach (LAVA PRO 6) washes. Next, a 1.5 µm glass fiber filter membrane was placed into each filter housing unit. The filter housing unit was kept in an unused Ziploc bag until used in the field.
The first step in the field was setting up the four key components of the OSMOS. First, the tripod was secured to the ground, then the backpack unit was hung on the tripod. Second, tubing was used to connect the backpack unit to the telescope pole. Third, the telescope pole was secured to the top of the tripod and the filter housing unit was attached to the end of the telescope pole. After the OSMOS was set up at a sampling site, the backpack unit was turned on and programmed to run 1 L of water. To collect a water sample, the end of the filter housing unit holding the filter was placed into the water and the backpack unit was run, allowing 1 L of water to flow through the filter. Forceps, sterilized in 100% ethanol and a flame, were used to remove and place the filter into a 15 mL Falcon tube containing 100% ethanol. Then, the water flow rate and volume of water were recorded (Figure 2.4). At each location, 1 L of bottled water was run through the OSMOS as a negative control. Then, three replications of 1 L of river water were run over three new filters. The 15 mL falcon tubes containing 100% ethanol and the filters were stored at -20°C until DNA extraction.

Figure 2.3 Map of the sites sampled in collaboration with the OFAH monitoring and stocking of Atlantic salmon in Ontario. Depiction of the locations where eDNA water samples were collected in Summer 2021. Blue points denote adult return sites, yellow points denote naturalized watersheds, and orange points denote stocked sites. Map produced with ArcGIS.
Figure 2.4 Parts of the OSMOS and field sample collection steps. The upper image depicts the OSMOS in the field and its parts. (A) is the backpack unit, (B) is the telescope pole, (C) is the tripod, (D) is the filter housing unit. The lower image (from left to right) depicts the removal of the filter from the filter housing unit after water is run over the filter.
2.3 DNA filtration and extraction

The lab and field experiment water samples were filtered to collect the eDNA using a Two Stage Edwards vacuum pump (Bluffton, Indiana) and a 500 mL NALGENE Filter Holder and Receiver (Rochester, NY). For each water sample, a 0.45 µm nitrocellulose fiber membrane was placed on the Support Plate of the Filter Holder. Then, 500 mL of the sample water was transferred into the Upper Chamber of the Filter Holder. Next, the Two Stage Edwards vacuum pump was connected to the Filter Holder and Receiver and turned on. After the 500 mL of the sample water was filtered the Receiver was emptied and an additional 500 mL of the sample water was filtered. The vacuum pump ran for an additional 20 minutes after filtration to dry the filter. The dry filter was then removed from the Support Plate using forceps. The forceps were cleaned to reduce DNA contamination. First, forceps were placed in a 10-fold diluted Lavo Pro 6 bleach solution for one minute, then rinsed with DI water. Next, the forceps were placed in DI water for three minutes and rinsed with DI water again. The cleaning process was conducted twice between the use of the forceps. The filter was folded into quarters and cut in half using scissors rinsed in a 10-fold diluted Lavo Pro 6 bleach solution three times then three rinses with DI water, then placed in a container of DI water for three minutes and rinsed with DI water three times. Each half acted as a technical replicate. Each replicate was cut into strips and placed into a labeled 1.5 mL Eppendorf tube. The vacuum pump was cleaned after each sample was filtered. For cleaning, the Upper Chamber of the Filter Holder and Support Plate was removed from the Receiver, and both were rinsed with a 10-fold diluted Lavo Pro 6 bleach solution three times followed by ten rinses with DI water. The same filtration and cleaning process was conducted for all three water samples and a negative control (1 L of DI water run over a filter).

The DNA from the fin clips and all filters was extracted using a Dneasy Blood & Tissue Kit (Qiagen), where the filters were treated as “tissue” (Goldberg et al., 2016). The filters collected in the OFAH sampling required pre-processing steps before DNA extraction and altered buffer concentrations because they were stored in ethanol. Prior to DNA extraction the sample filters were removed from the 15 mL Falcon tubes using DNA-free forceps and placed into unused Petri dishes in the laminar flow hood. The filters were left
in the petri dishes until completely dry, to remove any excess ethanol. The filter paper was then suspended in 500 µL of ATL buffer and 50 µL of Proteinase K and incubated at 56°C for at least 8 hours (Majaneva et al., 2018). After incubation, the DNA was extracted from the filters following the steps outlined in the Dneasy Blood & Tissue Kit (Qiagen) protocol for “tissue” after the lysis step (Figure 2.5).

Figure 2.5 Sample preparation for DNA extraction. From left to right. (A) is the lab collected and run over a filter that captures the DNA, then the DNA is extracted. (B) is the field collected and run over a filter that captures the DNA, then the DNA is extracted. (C) is the OFAH field filter samples are removed from the ethanol and dried, then the DNA is extracted.

2.4 PCR analysis

The COI mitochondrial primer pair, COI 188, previously designed for Atlantic salmon (Dalvin et al., 2010), was used to assess the samples collected for this thesis. COI 188 was originally used in the Neff lab by an undergraduate thesis student, Amanda Nurse, and successfully amplified mitochondrial Atlantic salmon DNA from fin clips and water samples. The amplification of mitochondrial DNA from tank/river eDNA water samples was used as a positive control for DNA template integrity in the lab and field.
experiments. In the OFAH eDNA water samples, amplification of mitochondrial DNA was used to determine the presence of Atlantic salmon.

Each PCR assay for the OFAH field samples contained 1.0 µL of template DNA, 1.0 µL of 10x PCR buffer (containing 15 mM MgCl₂), 0.2-0.6 µL of MgCl₂ (25 mM), 0.2 µL of the forward and reverse primers (10 µM), 0.3 µL of nucleotides (10 mM), and 0.05 µL of GenScript Green Taq DNA Polymerase (Cat. No. E00043) (5 units / µL) with distilled water added to reach a total reaction volume of 10 µL. The thermocycler conditions previously optimized by a Neff Lab undergraduate student, Amanda Nurse, were used (35 cycles with a 30-second denaturation step at 94°C, a 30-second annealing step at 56°C, and a 30-second extension step at 72°C). Additionally, a positive control of Atlantic salmon DNA from a fin clip, and a negative control of DI water were used for each run. The subsequent PCR products were analyzed on 1% agarose gels stained with ethidium bromide using Tris-borate-EDTA buffer. For each gel, a 1,200 bp ladder was loaded into the first well followed by PCR product samples. Gels were run at 100 v for 40-50 minutes. Gels were analyzed under UV light and images of the gel were taken using Quantity One (Version 4.4.0).

A scoring system was used to determine if mitochondrial DNA was successfully amplified from eDNA water samples. Successful amplification of mitochondrial DNA was defined as band amplification above background intensity at approximately 188 bp in length. Background intensity was determined for each gel image, as the UV light exposure varied between gels. Background intensity was defined using three factors. Factor one was the intensity (brightness) of the area of the gel where no product was loaded. Factor two was the intensity of the area where the ladder had been loaded at the largest band (1,200 bp). Factor three was the intensity of the area where the positive control had been loaded at approximately 188 bp. Thus, successful amplification of mitochondrial DNA from an eDNA water sample was defined as an eDNA water sample that had an intensity greater than factor one, and similar in intensity to either factor two or three at approximately 188 bp in length. Unsuccessful amplification of mitochondrial DNA from an eDNA water sample was defined as either (1) an eDNA water sample that had an intensity greater than factor one, and similar to factor two and three but not at the
expected length in base pairs, or (2) an eDNA water sample that did not have an intensity greater than factor one. Figure 2.6 shows examples of gel images where eDNA water samples were identified as successful. The top row of Figure 2.7 (A-B) shows gels containing OFAH eDNA water samples. The bottom row (C) is a gel that contains tank eDNA water samples from a lab experiment.

Figure 2.6 Agarose gel electrophoresis showing amplification of mitochondrial DNA from eDNA water and fin clip samples. A 1,200 bp ladder was loaded in the left most lane in each gel image. On the right side of each gel image is a column of three smaller images taken from the left gel image (the top image is the band of the positive control of Atlantic salmon DNA amplified from a fin clip sample, the center image is 1,200 bp band from the ladder, the bottom image is an area of the gel where no product was loaded). (A-B) are OFAH eDNA water samples. (C) is tank eDNA water samples from lab experiments. In each gel electrophoresis image the “Tank eDNA water” lanes are tank eDNA water samples from a tank containing fish, “River eDNA water” lanes are river eDNA water from rivers containing fish, the “DI” lane is a negative control for filtration and DNA extraction protocols using DI water, the “Fin clip” lanes are positive controls of Atlantic salmon DNA, and the (-) lane is the PCR negative control where no template DNA is present.
Primer pairs previously designed to amplify microsatellite Atlantic salmon DNA, were tested to assess their ability to detect microsatellite alleles present in water samples. A total of 35 different primer pairs were used (King et al., 2005; O’Reilly et al., 1996; Paterson et al., 2004). The primer pairs were categorized into three types: 17 previously designed (microsatellite primer pairs that had been previously designed for Atlantic salmon and were not altered), 12 modified (previously designed microsatellite primer pairs that had been altered using NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify for shorter DNA strands), and 6 Windsor primers (microsatellite primer pairs altered by MSc student Nabeelah Lulat) (Table 2.1). First, PCR assays were used to assess the ability of each primer pair to amplify microsatellite DNA from eDNA water and fin clip samples. Each PCR contained 1.0 µL of template DNA, 1.0 µL of 10x PCR buffer (containing 15 mM MgCl₂), 0.2-0.6 µL of MgCl₂ (25 mM), 0.2 µL each of forward and reverse primers (10 µM), 0.3 µL of nucleotides (10 mM), and 0.05 µL of Green Taq DNA Polymerase (5 units / µL) with distilled water added to reach a total reaction volume of 10 µL. The primer pairs were optimized by altering the MgCl₂ concentration (1.5mM, 2mM, and 2.5mM), the annealing temperature (58˚C/60˚C or 50˚C/52˚C), and the cycle length (35, 40, 43, 45 cycles). PCRs were with a 30-second denaturation step at 94˚C, a 30-second annealing step, and a 30-second extension step at 72˚C. If the primer pairs consistently amplified microsatellite DNA from fin clip samples, but not from tank eDNA water samples under the above conditions a nested PCR was run. The nested PCR consisted of two rounds of PCR. Both rounds of PCR used the conditions that successfully amplified microsatellite DNA from fin clips for that set of primer pairs. The first PCR was 1.0 µL of template DNA directly from the DNA samples, for both the fin clips and tank eDNA water samples. The second PCR then used 1.0 µL of the PCR product from the first PCR was the template DNA. The subsequent PCR products were analyzed on 1% agarose gels stained with ethidium bromide using Tris-borate-EDTA buffer. For each gel, a 1,200 bp ladder was loaded into the first well followed by PCR product samples. Gels were run at 100 v for 40-50 minutes. Gels were analyzed under UV light and images of the gel were taken using Quantity One (Version 4.4.0).
Table 2.1 The microsatellite primer pairs assessed on their ability to amplify DNA from fin clip and eDNA water samples.

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2.5 Microsatellite eDNA Experiments Next-generation sequencing (NGS) analysis

2.5.1 NGS library preparation

PCR assays were performed for the lab and field experiment samples using microsatellite primer pairs that successfully detected fin clip and eDNA water samples on an agarose gel. The mitochondrial gene, COI, was used as a positive control for DNA template integrity for the fin clip and water samples. Four microsatellite primer pairs (SSsp 2201, SSsp 2213, SSsp 1605-2, and SSsp 1605-4) were used to amplify Atlantic salmon DNA. Each microsatellite primer pair was optimized using fin clip and water sample DNA. Table 2.2 summarizes the optimal PCR conditions for each primer pair. PCRs were conducted to prepare the samples for Next-generation sequencing (NGS). PCRs
contained 1.0 µL of template DNA, 2.0 µL of 10x PCR buffer (containing 15 mM MgCl₂), 0.2-0.4 µL of MgCl₂ (25 mM), 0.4 µL each of forward and reverse primers (10 µM), 0.6 µL of nucleotides (10 mM), and 0.1 µL of Taq DNA polymerase (5 units / µL) with distilled water added to reach a total reaction volume of 20 µL. PCRs were run for 28-37 cycles with a 30-second denaturation step at 94°C, 30-second annealing step at a temperature specific to each primer pair (Table 2.2), and a 30-second extension step at 72°C.

**Table 2.2 PCR conditions for microsatellite primer pairs to amplify Atlantic salmon DNA from fin clips and lab water samples.**

<table>
<thead>
<tr>
<th>Primer Pair ID</th>
<th>Amplicon Size (bp)</th>
<th>Optimal Annealing Temperature (°C)</th>
<th>Optimal Cycle Length</th>
<th>Optimal MgCl₂ Content (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSsp1605-2</td>
<td>106-240</td>
<td>58</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>SSsp1605-4</td>
<td>115-250</td>
<td>58</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>SSsp2201</td>
<td>259-371</td>
<td>58</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>SSsp2213</td>
<td>151-191</td>
<td>58</td>
<td>45</td>
<td>1</td>
</tr>
</tbody>
</table>

PCR products were analyzed on 2% agarose gels stained with ethidium bromide using Tris-borate-EDTA buffer. For each gel, a 1,200 bp ladder was loaded into the first well followed by PCR product samples. Gels were run at 100 v for 40-50 minutes. Gels were analyzed under UV light and images of the gel were taken using Quantity One (Version 4.4.0). A ladder scale indicating the number of base pairs represented by each band was added to each image using Image Lab (version 6.0.1).

PCR products were cleaned using SPRI beads. First, 10 µL of PCR product was mixed with 15 µL of SPRI bead suspension in a 96-well tray and incubated for five minutes. Second, the mix was placed onto a 96-well magnetic reaction plate for 10 minutes to allow the beads to separate from the solution. The solution was removed from the tubes and discarded. Then, 200 µL of 70% ethanol was added and incubated for 30 seconds, the ethanol was then removed, and this step was repeated twice. Next, the 96-well tray was removed from the magnetic reaction plate and dried for 20 minutes. When the tubes were dry, 40 µL of ddH₂O was added to each tube and pipetted ten times to mix with the bead suspension. Then, the 96-well tray was placed on the magnetic reaction plate for five
minutes. After the solution and beads separated the solution was removed and pipetted into a new 96-well tray.

Then, PCRs were conducted, using the cleaned PCR product as the template DNA and the UniB primer of each primer pair and a unique UniA barcode. Each PCR contained 1.0 µL of template DNA, 1.0 µL of 10x PCR buffer (containing 15 mM MgCl₂), 0.2-0.4 µL of MgCl₂ (25 mM), 0.5 µL of the UniA and UniB primer (10 µM), 0.3 µL of nucleotides (10 mM), and 0.05 µL of Green Taq DNA Polymerase (5 units/µL) with distilled water added to reach a total reaction volume of 10 µL. PCRs were run for 8 cycles with a 30-second denaturation step at 94°C, a 30-second annealing step at 55°C, and a 30-second extension step at 72°C. PCR products were analyzed on 2% agarose gels stained with ethidium bromide using Tris-borate-EDTA buffer. For each gel, a 1,200 bp ladder was loaded into the first well followed by PCR product samples. Gels were run at 100 v for 40-50 minutes. At this stage, primer pairs SSsp 1605-4 and SSsp 2213 failed to amplify DNA consistently and thus no samples were sent for Next Generation Sequencing (NGS).

The PCR products for each microsatellite primer pair were then pooled together. The volume of each PCR product added to the pool ranged between 2-5 µL. The PCR products with the brightest banding had 2 µL added to the pool, and the PCR products with the dimmest banding had 5 µL added to the pool. Then, 10 µL of the pooled PCR products and 7 µL of the loading dye were mixed. The mix was analyzed on a 2% agarose gel stained with ethidium bromide using Tris-acetate-EDTA buffer. For each gel, a 1,200 bp ladder was loaded into the first well followed by pooled PCR product. Gels were run at 96 v for 3.5-4 hours. Gels were analyzed under UV light and images of the gel were taken using Quantity One (Version 4.4.0). A gel extraction was then conducted according to the DNeasy Gel Extraction Kit. The Gel Extraction product was subsequently sent to the University of Windsor for NGS with Ion Torrent.

2.5.2 Ion Torrent Analysis

NGS by Ion Torrent was conducted using a semiconductor chip; CMOS (Complementary metal-oxide semiconductor). The semiconductor chip contains millions of micro-wells that cover a sensory layer of pixels. First, a series of chemicals were used to cut the
sample DNA into millions of fragments. Second, fragments were attached to beads. Third, the beads were flowed in a plane across the micro-chip and a single bead was deposited into each well. Then, the chip was flooded by one nucleotide, either adenine, cytosine, guanine, or thymine. If a single base was integrated into a single strand of DNA a hydrogen ion was released. A hydrogen ion was only released if the nucleotide was complementary to the next base on the strand. The release of the hydrogen ion changes the pH of the liquid, this change was detected and measured by the ion-sensitive layer below the well. If no change in pH is detected, then no hydrogen ion was released, and the nucleotide was not found on the next base on the strand of DNA. The above process was repeated every 15 seconds with each nucleotide. The micro-wells function as a method to capture and translate the chemical information released from DNA sequencing into digital information known as base calling. After all the bases are called the digital DNA information (sequences) is stored on a *.fastq file. The sequences in the *.fastq file were demultiplexed according to the unique barcode attached to each sample during library preparation. Thus, the sequences were separated into different *.fastq files according to the barcode associated with each sample.

### 2.5.3 Bioinformatics

First, the fin clip fastq samples were called to establish that the allele calling program worked sufficiently for each microsatellite primer pair. Initially, the NGS-usat 1.0 script ([https://github.com/denisroy1/NGS-usat](https://github.com/denisroy1/NGS-usat)) was used to call the alleles in the fin clip *.fastq samples. The repeat pattern and forward flanking region of each microsatellite primer pair were inputted into the script. The NGS-usat 1.0 script was able to call alleles in the samples but the parameters for allele calling in the script were determined to be too stringent. Specifically, the allele-calling parameters were too stringent because the script failed to call reads that contained the pattern of interest if a substitution error occurred. Thus, I created a new pipeline was created using Excel to allow for substitution error called Excel allele-calling program version one. This pipeline allowed a single base pair substitution within the repeat region. The Excel pipeline counted the length of each read detected from 0-250 bases for each fin clip sample. Thus, the allele of each read was called based on the length (number of bases) of the read. Then, the frequency of each
allele (length from 0-250 bases) was recorded and inputted into an allele frequency plot (Figure 2.7). However, the signal of the microsatellite peaks in the allele frequency graphs were not clear. More specifically, peaks did not have the expected stutter peak pattern associated with microsatellites. Thus, the Excel pipeline was altered to require a minimum of three contiguous repeats of the microsatellite motif for each read to be considered (Excel allele-calling program version two). The allele-calling programs NGSusat 1.0, Excel allele-calling program version one and two are compared using one of the sequenced fin clip samples from the lab trials in Figure 2.7. The subsequent allele frequency graphs for the fin clip samples had a clearer microsatellite peak signal (Figure 2.7).

Additionally, an analysis was conducted to assess if the proportion of reads called to an allele changed as the minimum number of contiguous repeats for a read to be called to a particular allele changed, and if a specific minimum number of contiguous repeats performed best. First, the Excel pipeline was run requiring a minimum of 0-10 contiguous repeats of the microsatellite pattern for reads 1-250 bases. Second, the proportion of reads attributed to each of the three most frequent alleles compared to the proportion of total reads called was determined for each minimum number of contiguous repeats. The proportion of reads attributed to the three most frequent alleles was then used to determine if the fin clip came from a heterozygous (an individual had two alleles that were consistently more frequent than the third allele, as the minimum number of contiguous repeats increased) or homozygous (an individual had one allele that was consistently more frequent than the second and third allele, as the minimum number of contiguous repeats increased) individual. Third, the proportion of reads attributed to the “true” alleles (alleles determined to be representative of a heterozygous or homozygous individual) compared to the total proportion of reads called was determined for each minimum number of contiguous repeats. Fourth, the read depth (total number of reads called), as the minimum number of contiguous repeats increased, was determined. The above steps were completed for all the fin clips, and subsequent plots were made to show the findings for each fin clip.
After individuals were confidently labeled as heterozygotes or homozygotes, the above steps were applied to the reads detected for the tank eDNA water samples. Next, plots were created for each trial to compare the proportion and length of reads detected for the tank eDNA water samples to the reads detected for the fin clips of the individuals present in the tank. Additionally, for densities $n = 2$ or $n = 4$ fish / tank plots were made comparing the proportion and length of reads detected for the tank eDNA water samples to the reads detected for each fin clip of each individual present in the tank.

**Figure 2.7** Sample Allele Frequency graphs for SSsp 1605-2 using three methods. (A) depicts the allele frequency graph of a fin clip sample generated using the original allele-calling program NGS-usat 1.0. (B) depicts the fin clip sample allele frequency graph generated using the Excel allele-calling program version one. (C) depicts the fin clip allele frequency graph generated using the Excel allele-calling program version two, which required a minimum of three contiguous repeats.
Chapter 3

3 Results

3.1 Lab experiments

The Atlantic salmon COI gene (mitochondrial) was consistently amplified from tissue samples and water samples collected from tanks containing 1, 2, or 4 Atlantic salmon (Figure 3.1). There was no amplification of the COI gene for the filtration and DNA extraction negative control sample (DI water was instead substituted in the filtration and DNA extraction protocol). Similarly, there was no amplification of the COI gene when water was collected from tanks that did not contain Atlantic salmon (not shown). There was no amplification of the COI gene in the PCR negative controls (samples containing the master mic and no template DNA) for any of the PCRs. These observations are consistent with the successful detectability of Atlantic salmon eDNA from the water samples.

Figure 3.1 Agarose gel electrophoresis showing amplification of DNA samples from fin clip and tank water samples using a mitochondrial or microsatellite primer pair. A 1,200 bp ladder was loaded in the leftmost lane in each gel image. (A) shows mitochondrial primer pair COI 188. (B) shows microsatellite locus SSsp 1605-2. In each gel electrophoresis image the “Tank eDNA water” lanes are tank eDNA water samples from a tank containing fish, the “DI” lane is a negative control for filtration and DNA extraction using DI water, the “Fin clip” lanes are positive control of Atlantic salmon DNA, the (-) lane is PCR negative control where no template DNA is present.
In this study, PCR amplification for 35 microsatellite loci was assessed for DNA obtained in fin clip and water samples. All microsatellite primer pairs assessed successfully amplified microsatellite DNA from fin clip samples. Only two of the primer pairs, however, consistently amplified DNA from water samples: SSsp 2201 and SSsp 1605-2. Loci SSsp 1605-2 is shown successfully amplified from all tank eDNA water samples e.g., Figure 3.1. There was no amplification of either locus when DI water was instead substituted in the filtration and DNA extraction protocol. Similarly, there was no amplification of either locus when water was collected from tanks that did not contain Atlantic salmon. These data suggest the PCR was able to amplify eDNA extracted from the water samples for these loci.

For loci SSsp 1605-2 and 2201 the microsatellite sequences were characterized for tank eDNA water samples, fin clips, and negative controls using Ion Torrent sequencing. No sequences were detected from the fin clip and tank eDNA water samples for the SSsp 2201 locus, during the first Ion Torrent run. Thus, the lab experiment samples were characterized for a second time for SSsp 2201 using Ion Torrent sequencing. To ensure that the lack of sequence detectability was not a result of a technical error the GLIER Ion Torrent sequencing facility was consulted on each step of library preparation. Each step was determined to be successful, and the samples were characterized for a second time. Locus SSsp 2201 sequences were again not detected from the Ion Torrent sequencing run. Therefore, only microsatellite sequences detected for locus SSsp 1605-2 were analyzed, as detailed below.

3.1.1 Microsatellite locus SSsp 1605-2 NGS sequence analysis

First, the negative control samples were assessed to identify any contamination. No amplification was observed, and no amplicons were detected from NGS sequencing from samples where DI water was substituted in the filtration and DNA extraction protocol. An amplicon was detected from the negative control trials where no fish were present in the tanks. This amplicon was sequenced and run through NCBI BLAST (https://blast.ncbi.nlm.nih.gov) and was not identifiable as Atlantic salmon DNA. It
matched uncultured bacterium DNA or had “no significant similarity” to a known genetic sequence.

Second, the fin clip sequences were analyzed to determine the best protocol for calling alleles. Initially, previously created allele calling program NGS-usat 1.0 was used to call the alleles in the fin clip samples. The program labelled alleles by the length of the repeat region instead of the length of the read. Additionally, when single base substitutions occurred in the repeat region a read that contained eight repeats, for example, would be incorrectly called to contain seven repeats. As a result, the program did not allow for alleles detected from the fin clip samples to be confidently called. Thus, an alternative protocol for allele calling was used. The protocol included only reads that (1) contained the forward primer and (2) included some minimum number of contiguous repeats of the motif for that locus (GATA); hereafter contiguous repeats is defined as the forward primer pair followed by some number of repeats of the motif for locus SSsp 1605-2. The alleles were then characterized based on the total sequence length of the read. Analyses were conducted first to assess the effect of the minimum number of contiguous repeats on (1) read depth and (2) the proportion of reads called to either of the two most abundant allele sizes for that sample. The number of reads included for a sample dropped substantially when the minimum number of contiguous repeats, required for an allele to be called, increased from 0 (forward primer pair followed by no repeats) to 1 (forward primer pair followed by a single repeat) (Figure 3.2). This suggests that this initial filter was highly selective in removing non-target sequences. Figure 3.2 demonstrates that the read depth has a steep decrease from zero to one minimum contiguous repeat and remains relatively constant as the minimum number of contiguous repeats increased from one to nine.

A slow decrease in read depth with increasing filter length was observed up to 8-10 contiguous repeats; at this threshold the program began to filter out the target alleles. The proportion of reads attributed to the two most frequent alleles was relatively steady from 1-8 contiguous repeats (Figure 3.3). The findings of the analysis were relatively consistent for all the fin clip samples. The analysis found that the proportion of reads attributed to the “true” alleles (two most frequent alleles) increased the most after one
contiguous repeat was required (an allele was only called if it contained the forward primer pair followed by at least one instance of the repeat motif for locus SSsp 1605-2). The graphs in Figure 3.3 are representative of the fin clip samples analyzed for the SSsp 1605-2 locus, as the majority of the reads detected for each individual represent ~ 80% of the true allele(s). The number of reads retained remained steady from 1-8 contiguous repeats (an allele was called if it contained the forward primer pair followed by at least 1-8 instances of the repeat motif for locus SSsp 1605-2). The analysis of the fin clip samples for locus SSsp 1605-2 illustrate that increasing the minimum number of contiguous repeats from 0 to 1 has the greatest effect on the proportion of reads attributed to an allele. Moreover, the proportion of reads attributed to an allele and the total number of reads detected in a fin clip sample did not show any large fluctuation from 1-8 contiguous repeats. A minimum of three contiguous repeats was determined to be favourable to call alleles from eDNA tank water samples for microsatellite locus SSsp 1605-2. Three contiguous repeats were set as the minimum for two reasons. First, the lack of fluctuation of the total read depth and proportion of alleles observed in the fin clip samples from 1-8 contiguous repeats depicted in Figures 3.2 and 3.3. Second, at least three repeats of the pattern would reduce the likelihood of incorrectly attributing a read to an allele.

Figure 3.2 Average change in the read depth for fin clips, as a function of the minimum number of contiguous repeats required for an allele to be called, for microsatellite locus SSsp 1605-2. The vertical axis of the graph is the total read depth (total number of reads detected). The horizontal axis of the graph is the
minimum number of contiguous repeats required by the Excel pipeline to call an allele in a fin clip sample. The orange line represents the average read depth for the fin clips. The yellow band represents a 95% confidence interval.

Figure 3.3 Change in the proportion of reads attributed to “true” allele(s), in eight fin clip samples, as the minimum number of contiguous repeats required for an allele to be called increases for microsatellite locus SSsp 1605-2. The vertical axis of each graph is the proportion of reads (frequency of read X / total reads detected) for a fin clip sample (the “X” denotes reads of the true alleles). The horizontal axis of
each graph is the minimum number of contiguous repeats required by the Excel pipeline to call an allele in a fin clip sample. “True” alleles are defined as alleles that comprise the majority of the reads in a fin clip sample and are representative of the SSsp 1605-2 locus for the individual associated with the fin clip sample.

A total of 35 fin clip samples were sequenced for locus SSsp 1605-2. Four alleles were observed for all the fin clip samples: 114, 118, 122, and 126 bases. The alleles called in order from most to least frequent was 122 (32% of reads (17,075 reads)), 114 (29% of reads (16,736 reads)), 118 (21% of reads (13,949 reads)), and 126 (18% of reads (10,358 reads)). All the fin clip samples were identified as heterozygotes. Five genotypes were observed for all the fin clip samples: 114/122, 114/126, 118/122, 118/126, and 122/114. The genotypes detected in order from most to least frequent were 114/122 (35%), 118/122 (26%), 114/126 (16%), 118/126 (16%), and 122/144 (7%).

Next, the proportion of reads attributed to each allele in the fin clip and tank eDNA water samples were compared for each density and trial using line graphs (Figure 3.4). In both the fin clip and tank eDNA water samples, the majority of reads range from 110-130 bases. In these figures, the length in bases is referred to as allele size. The fin clip samples have 1-2 distinct peaks at either allele sizes 114, 118, 122, or 126. The tank eDNA water samples have 2-4 peaks ranging from allele sizes 114-126. The depiction of the tank eDNA water and fin clip samples for tanks containing 1-4 fish, do not show a clear visual similarity between the tank eDNA water samples and the associated fin clip samples (Figure 3.4). Additionally, the number of peaks observed in the tank eDNA water samples increases as the number of fish present in the tanks increase (Figure 3.4). No clear similarity in peaks is observed between the tank eDNA water samples and their associated fin clips for any density of fish in the lab experiments.
Figure 3.4 The proportion of reads attributed to alleles 110-130 bases in length for the fin clip and associated tank eDNA water samples for three tank experiments. The vertical axis of each graph is the proportion of reads (number of read X / total reads detected) for a sample (the “X” denotes reads of the true alleles). The horizontal axis of each graph is the length in bases of each read that had a minimum number of three contiguous repeats. The blue line of each graph, fin genotype, is the proportion of reads attributed to each allele size detected from the fin clip sample associated with each trial. In (B) and (E) the fin genotype, is the average fin
genotype for the number of fish in the tank ((sum of the proportion of reads associated from each fin clip sample) / number of fish in the trial). The red (tank H$_2$O sample 1), orange (tank H$_2$O sample 2), and yellow line (tank H$_2$O sample 3) in each graph are the proportion of reads attributed to each allele size detected from the water for that trial. (A) is a lab trial where one fish was present in the tank. (B) is a lab trial where two fish were present in the tank. (E) is a lab trial where four fish were present in the tank. (C-D) are the genotype of each fish present in the tank for the n = 2 fish / tank depicted in Graph B. (F-I) are the genotype of each fish present in the tank for the n = 4 fish / tank depicted in Graph E.

In addition to visual peak similarity between tank eDNA water and fin clip samples, the most common (top) alleles detected in both types of samples were compared. Table 3.1 shows the alleles detected in the fin clip and tank eDNA water samples in order from most to least common (left to right), and if the most common allele detected is the same for the fin clip and associated tank eDNA water samples. The table illustrates that the most common allele detected in the fin clip and the associated tank eDNA water samples are only the same when all four alleles are present in the fin clip samples. Thus, the factor that affect if the most common alleles detected in the tank eDNA water and fin clips are the same is the presence of all four alleles in the fin clips instead of the alleles present in the water uniquely representing an individual in the tank.

The percent of alleles out of the range (percent of alleles detected excluding alleles 114, 118, 122, and 126) was calculated (Table 3.1). In trials where 2 or 4 fish were present in the tank, the mean of the percent of alleles out of the range was calculated for the fin clip samples, for example (“percent of alleles out of the range for Fish 1” + “percent of alleles out of the range for Fish 2”) / total number of fish in the tank. The column shows that the fin clip samples detected on average 10-29% of alleles out of the range and the tank eDNA water samples detected 21-41% of alleles out of the range. Thus, in addition to the alleles of the fish present in the tank there was an additional factor that affects what alleles were detected from the tank eDNA water samples.
Table 3.1 The most common alleles and percent of uncommon alleles detected from fin clip and tank eDNA water samples for locus SSsp 1605-2.

<table>
<thead>
<tr>
<th>Number of Fish</th>
<th>Trial</th>
<th>Sample type</th>
<th>Allele Present</th>
<th>Top Alleles Match</th>
<th>% of Alleles out of range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>Water</td>
<td>118 114 126 122</td>
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<tr>
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</tr>
<tr>
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<td></td>
<td>Water</td>
<td>118 114 126 122</td>
<td>yes</td>
<td>30%</td>
</tr>
<tr>
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</tr>
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<td></td>
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<td>yes</td>
<td>30%</td>
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<td></td>
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<td>118 114 126 122</td>
<td>yes</td>
<td>30%</td>
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<td>13</td>
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<td></td>
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<td>30%</td>
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<tr>
<td></td>
<td>15</td>
<td>Water</td>
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<td>yes</td>
<td>30%</td>
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</table>

3.2 Field experiments

The Atlantic salmon COI gene (mitochondrial) was successfully amplified from all fin clip samples, but only from some of the river eDNA water samples (Figure 3.5). There
was no amplification of the COI gene when DI water or bottled water were instead substituted in the filtration and DNA extraction protocol.

Figure 3.5 Agarose gel electrophoresis showing PCR amplification results of DNA from fin clip and river eDNA water samples using a mitochondrial primer pair. A 1,200 bp ladder was loaded in the left most lane in each gel image. (A-B) depict agarose gels where no mitochondrial DNA was amplified from river eDNA water samples. (C) depicts agarose gels where mitochondrial DNA was amplified from river eDNA water samples. In each gel electrophoresis image the “River eDNA water – Up/Behind/25 m/100 m” lanes are river eDNA water samples collected upstream, directly behind, 25 m, or 100 m downstream the net pen, respectively, the “B” lane is a water sample of bottled water, the “(-)” lane is PCR negative control where no template DNA was present, the “Fin clip” lane is a positive control for Atlantic salmon DNA.

The Atlantic salmon locus SSsp 2201 (microsatellite) was successfully amplified from all fin clip samples, but not from any river eDNA water samples (Figure 3.6). There was no amplification of the microsatellite locus SSsp 2201 when DI water or bottled water were substituted in the filtration and DNA extraction protocol. The Atlantic salmon locus SSsp 1605-2 (microsatellite) was successfully amplified from all fin clip samples, but not from any river eDNA water samples (Figure 3.6). There was no amplification of the
microsatellite locus SSsp 1605-2 when DI water or bottled water were substituted in the filtration and DNA extraction protocol.

Figure 3.6 Agarose gel electrophoresis showing amplification of DNA from fin clip samples and no amplification from any river eDNA water samples using microsatellite primer pairs SSsp 1605-2 and SSsp 2201. A 1,200 bp ladder was loaded in the left most lane in each gel image. (A) is DNA amplified with microsatellite locus SSsp 1605-2. (B) is DNA amplified with microsatellite locus SSsp 2201. In each gel electrophoresis image the “River eDNA water – Up/Behind/25 m/100 m” lanes are river eDNA water samples collected upstream, directly behind, 25 m, or 100 m downstream the net pen, respectively, the “B” lane is a water sample of bottled water, the “(-)” lane is PCR negative control no template DNA is present, and the “Fin clip” and “Fin” lanes are positive controls of Atlantic salmon DNA.

3.3 The detection of Atlantic salmon presence in OFAH stocking

A total of 36 sites were sampled in 15 rivers in Ontario in Summer 2021. Of the 36 sites Atlantic salmon presence was detected at 10 single-sample sites (7 naturalized watershed, 1 adult return, 1 stocked, and 1 adult return/stocked) and 5 multiple-sample sites (Figure 3.7). At the single-sample sites, presence was typically only detected at the most upstream sites in a river. The multiple-sample sites were sampled upstream, downstream, and at the stocking sites days pre-stock, four days post-stock, and six weeks post-stock. Table 3.2 depicts the detection of Atlantic salmon mitochondrial DNA from the multiple sample sites grouped by river (3 sites in Duffins Creek and 2 sites in Credit River). No Atlantic salmon mitochondrial DNA was detected from any of the upstream locations pre-stock, four days post-stock, or six weeks post-stock. No Atlantic
salmon mitochondrial DNA was detected from any of the locations at the stocking site pre-stocking, four days post-stocking, and six weeks post-stocking. No Atlantic salmon mitochondrial DNA was detected from any of the downstream locations pre-stocking or four days post-stocking. Atlantic salmon mitochondrial DNA was detected from every downstream location six weeks post-stocking (Table 3.2).

Figure 3.7 Atlantic salmon COI DNA detected from eDNA water samples at sites monitored by the OFAH. The map depicts 36 sites where eDNA water samples were collected. Sites denoted with a blue circle represent sites where COI Atlantic salmon DNA was detected. Sites denoted with an orange X represent sites where COI Atlantic salmon DNA was not detected.

Table 3.2 Atlantic salmon COI DNA detected from eDNA water samples at multiple-sample sites monitored by the OFAH. The blue squares represent sites where mitochondrial COI Atlantic salmon DNA was not detected. The orange squares represent sites where COI Atlantic salmon DNA was detected.
Chapter 4

4 Discussion

4.1 Experiments

A key validation of mitochondrial eDNA approaches is the ability to detect DNA specific to a species using water from a tank in which the species is housed. Multiple studies have demonstrated the capacity to detect mitochondrial DNA associated with different species of fish that were present in a tank (Shu, Ludwig, and Peng, 2021; Turanov and Rutenko, 2020; and Simpfendorfer et al., 2016). Similarly, in my study I successfully amplified Atlantic salmon mitochondrial DNA from all tank eDNA water samples. Across these studies 1-36 fish were held in volumes of water ranging from 40-300 L, for densities that ranged from 0.003-0.12 fish / L, compared to my study of 1-4 fish in 40 L of water, for densities ranging from 0.025-0.1 fish / L (Appendix A & B). Mitochondrial eDNA approaches are thus robust across a range of laboratory conditions.

A second useful validation of mitochondrial eDNA approaches is the ability to detect DNA from a natural water body in which species of interest occupy. Studies that held fish in enclosures within rivers have frequently shown that mitochondrial DNA can be detected from the river water downstream of where a study species is held (Driessche et al., 2023; Jane et al., 2015; Wacker et al., 2019; and Wood et al., 2022). In my study, Atlantic salmon mitochondrial DNA only amplified from 5.3% (8/150) of all the river eDNA water samples, which breaks down to 7.8% (4/51) of the samples collected 25 m downstream, 4.1% (2/48) of the samples collected directly downstream, 3.9% (2/51) of the samples collected 100 m downstream the net pens. Mitochondrial DNA was not consistently detected from river eDNA water samples, and not clearly influenced by distance from fish and fish density. In a similar study, Wood et al. (2022) found that mitochondrial DNA was only detected from 56% of the river eDNA water samples. Thus, the low detectability of mitochondrial DNA from river eDNA water samples is likely partially affected by similar environmental factors affecting eDNA persistence in river
environments. Moreover, my study deployed fish of a much lower biomass (1-4 fish ranging from 1.8-11.4 g) than previous studies (Appendix A). For example, Jane et al. (2015) collected river eDNA water samples downstream of Brook trout ranging from a biomass of 68.2-168.1 g. Wacker et al. (2019) collected river eDNA water samples downstream of freshwater pearl mussels (*Margaritifera margaritifera*) ranging from 50-100 individuals. Wood et al. (2022) collected river eDNA water samples downstream of adult Atlantic salmon ranging from 3-63 individuals. Finally, Driessche et al. (2023) collected eDNA river water samples downstream of a variety of fishes ranging from 122.91-989.34 g in biomass and a density of 9-50 individuals. Moreover, they found that biomass had a significantly positive relationship with mitochondrial eDNA concentrations detected. Thus, the inability to detect mitochondrial DNA from river eDNA water samples in my study is likely due to the smaller fish density (1-4 fish) and biomass (1.8-11.4 g) used compared to other studies. The ability to consistently detect mitochondrial DNA from tank water but not from river water in my study, suggests that fish biomass per volume of water and water type (river or tank) affect the detectability of eDNA. Overall, biomass appears to be a key determinant of the ability to detect mitochondrial DNA from river water.

A valuable line of inquiry is determining if mitochondrial DNA is more likely to be amplified than nuclear DNA from water in which a specific species is found. Several studies have compared the detectability of mitochondrial and nuclear eDNA (Andres et al., 2021; Bylemans et al., 2017; Jensen et al., 2022; Jo et al., 2020; Moushomi et al., 2019; Lulat, 2023; and Sigsgaare et al., 2020). In particular, a study investigated the shedding and decay rate of mitochondrial and nuclear eDNA in a lab setting for the Japanese Jack Mackerel (*Trachurus japonicus*) found that mitochondrial and nuclear eDNA have similar shedding and decay rates, but that nuclear eDNA persists in lower concentrations than mitochondrial eDNA (Jo et al., 2020). The authors suggested that mitochondrial eDNA will persist in the water longer because it exists in higher concentrations than nuclear eDNA. Lulat (2023) was able to detect mitochondrial and nuclear eDNA of Atlantic salmon from river eDNA water samples. In all the above studies the detectability of nuclear eDNA occurs in tandem with the detectability of mitochondrial eDNA, likely because mitochondrial eDNA is more persistent in water.
than nuclear eDNA. Similarly, in the lab experiments conducted in my study, nuclear eDNA was only detected in tank eDNA water samples where mitochondrial eDNA was also detected, while in the field experiments nuclear eDNA did not amplify at either microsatellite locus for river eDNA water samples. The experiments in my study support that the detectability of mitochondrial DNA from eDNA water samples is a predictor of the likelihood of nuclear DNA detectability from those samples; and that if mitochondrial DNA is not detected from eDNA water samples, nuclear DNA will similarly not be detected.

The first key step in the validation of the microsatellite eDNA approach is the ability to amplify microsatellite DNA specific to a species detected from water that housed the species. Presently, four studies have reported the successful amplification of microsatellite DNA from eDNA water samples (Andres et al., 2021, Oslen et al., 2012; Lulat, 2023; Buxton, 2018). Three of the studies concluded that the successful amplification of microsatellite DNA from eDNA water samples was evidence that microsatellite DNA was present in the water samples (Andres et al., 2021 and Oslen et al., 2012; Lulat, 2023). Contrary to these findings, Buxton (2018) observed inconsistency in the amplification of microsatellite DNA from eDNA water samples and suggested that the DNA was not high quality enough to expect microsatellite DNA to be detected. In my study, two microsatellite loci (SSsp 2201 and SSsp 1605-2) were amplified from all the tank eDNA water samples as detected on agarose gels, which suggests that microsatellite DNA is detectable from eDNA water samples.

The second key step in the validation of the microsatellite eDNA approach is the ability to detect microsatellite alleles, from water samples, that are representative of the individuals present in the water. In addition to amplifying microsatellite DNA, Andres et al. (2021) were also able to detect microsatellite alleles from mesocosm eDNA water samples. Similarly, Lulat (2023) was able to detect microsatellite alleles from river eDNA water samples. This led both studies to conclude that microsatellite alleles were detectable from eDNA water samples. Andres et al. (2021) concluded that DNA mixture analyses were able to estimate the number of individuals contributing to the microsatellite genetic material detected from eDNA water samples in mesocosm experiments. Lulat
(2023) concluded that microsatellite alleles detected from river eDNA water samples can be used to identify allele frequency distributions of Atlantic salmon in Ontario rivers. In my study, microsatellite alleles for locus SSsp 1605-2 were detected from all tank eDNA water samples when fish were present in the tank, and from none of the tank eDNA water samples when fish were absent. Furthermore, the alleles detected from the tank eDNA water and fin clip samples were in the same allele size range (114-126 bp). Despite the successful amplification and allele detectability, however the microsatellite alleles detectability from the tank eDNA water samples were not exclusive to those detected from the associated fin clips. Thus, successful amplification of microsatellite DNA from tank eDNA water samples does not necessarily reflect the successful detectability of only the desired microsatellite alleles.

One possible explanation for the inconsistency in microsatellite DNA amplification and allele detectability from tank eDNA water samples is contamination during the procedure. The methodology of this study consisted of tank set up, water collection and filtration, DNA extraction, PCR, and NGS. Contamination causing the observed results did not occur during the tank experiments because the negative control experiments (tanks that did not contain fish) never detected microsatellite Atlantic salmon eDNA, despite being conducted between trials containing fish. Additionally, the negative control samples (DI water run through the filtration process at the same time as the tank eDNA water samples) collected for each trial also did not lead to microsatellite amplification.

The remaining steps in the methodology were PCR and NGS by Ion Torrent. Although, PCRs for the tank eDNA water samples were conducted using varying aliquots of master mix ingredients and the PCR negative controls did not show amplification, PCR error was likely introduced at this step. Specifically, the low quality of the water sample template DNA likely resulted in the creation of artifact alleles during PCR. Prior to NGS by Ion Torrent, ABI genotyping (DNA samples are fluorescently labelled during PCR, separated by capillary electrophoresis, and sized by comparison to an internal comparison, resulting unique electropherograms per sample using an Applied Biosystems 3730XL 96-capillary DNA Analyzer) was conducted for a few tank eDNA water and fin clip samples and where one fish was present in a tank. In the resulting electropherogram
some stutter peaks, identified as background noise, were observed in all the tank eDNA water and fin clip samples. In order to reduce the background noise, observed in the ABI genotyping samples in the future Ion Torrent NGS analysis, a premade master mix containing Fisher Platinum II Taq Hot-Start DNA Polymerase was added to the PCR protocol, however no amplification was observed, so the original PCR master mix was used for NGS library prep. Similarly, to the ABI genotyping, ~ 20% of the detected reads from the fin clip samples were identified as error when using Ion Torrent sequencing. The observation of error from both platforms but no amplification in any of the negative controls, suggests that error occurred during the PCR step. This possibility is supported by the percent of alleles detected outside of the allele range, as between 21-41% of the alleles detected from the tank eDNA water samples were outside the desired range (114-126 bp). The findings of my study suggest that high PCR error from low template concentrations was the cause of the results seen in the tank eDNA water samples. My study found that microsatellite DNA detected from tank eDNA water samples was amplified and detected on agarose gels, but microsatellite alleles not exclusive to the alleles detected from the fish were detected from those same samples. The discrepancies between the microsatellite alleles detected from samples found in my study do not support the use of microsatellite loci to identify and assign unique alleles detected from tank eDNA water samples to the alleles of known individuals in the water.

The findings of my study provide a direct comparison between microsatellite alleles detected from individual fish and the water from a tank in which the fish were housed. The inability to detect representative alleles from tank eDNA water samples in my study contrasts the findings of Andres et al. (2021) and Lulat (2023). The difference may reflect differences in the validation of different study factors. There are two key differences between the findings of Andres et al. (2021) and those of my study, namely (1) the length of time the fish were housed in the water and (2) the internal validation of assigning specific alleles to the alleles of the fish present in each experimental trial. Andres et al. (2021) housed the fish in the experimental mesocosms for one hour. Previous studies have shown that it takes approximately 96 hours for eDNA release from fish to reach an equilibrium. Moreover, eDNA release is highest in the first hour after transferring fish into a new environment (Guivas and Brammell, 2020). Thus, the short
time between fish transfer and water sample collection may have led to an increase in eDNA concentration that is not reflective of natural eDNA concentrations, allowing for higher quality microsatellite alleles to be detected. In my study, tank eDNA water samples were collected after a 96-hour period to ensure that eDNA concentrations were as a representative of natural eDNA concentrations as possible. Additionally, Andres et al. (2021) collected water samples from mesocosms containing fish and specified which water samples were collected from which trials. They also collected fin clip samples from the fish in the mesocosms, but they did not specify which fin clip samples were collected from which trial. Thus, when comparing the microsatellite alleles detected from water and fin clip samples, the study compared the alleles detected from water samples collected from a specific trial to the alleles detected from all the fin clip samples from every trial. Therefore, a direct comparison between the alleles known to be in the water (alleles detected from the fin clip sample of the specific fish in that trial) and the alleles detected from the water samples from that trial could not be made. In contrast, I assessed the alleles detected from the tank eDNA water samples in each trial to the alleles detected from the fin clips of the individuals present in the tank of that particular trial. Thus, my study provides a more direct comparison of the ability of microsatellite alleles detected from eDNA water samples to be exclusively representative of the alleles of the known individuals in the water. The main difference between the findings of Lulat (2023) and those of my study is the validation of the microsatellite loci used in each study. Lulat (2023) assessed three dinucleotide microsatellite loci: SSsaA 86, 119, and 124, while my study assessed two tetranucleotide microsatellite loci: SSsp 2201 and 1605-2. Lulat (2023) found that the most frequent alleles detected from the river eDNA water samples had a maximum contiguous repeat length of 4 (8 bases) for each locus, which was inconsistent over sampling periods (some sampling periods detected a range of low frequency alleles at repeat lengths > 4). This affects the level of confidence in the conclusions made by Lulat (2023), as the conclusions drawn in the study can only be made if the allele calling program only requires a contiguous repeat length of \( \leq 4 \) for the eDNA water samples. For example, if alleles were only called from the water samples detected from Lulat (2023) if they contained a contiguous repeat length of 5 (10 bases) the majority of the alleles reported in the study would not be called. Comparatively, in
my study allele frequency was not greatly affected by the number of contiguous repeats required for an allele to be called. This is particularly evident in Figure 3.2 and 3.3 where the total read depth and proportion of reads detected remains steady as the number of contiguous repeats required for an allele to be called increases for the fin clip samples (similar consistencies were observed in the microsatellite alleles detected from the tank eDNA water samples although not shown). Specifically, similar proportions of alleles were called from the fin clip and tank eDNA water samples when the contiguous repeat length required for an allele to be called increased from 1-8 (4-32 bases). The consistency of allele detectability for the successful locus used in my study provides further validation of the conclusions made in my study compared to Lulat (2023). My study provides a better understanding of the detectability of microsatellite alleles from eDNA water samples, and how exclusively representative those alleles are of the microsatellite alleles of the fish in the water.

### 4.2 Atlantic salmon presence in Ontario rivers

Mitochondrial eDNA analysis is an emerging tool used to monitor Atlantic salmon in Ontario rivers. Thus far, one study assessed the detectability of mitochondrial DNA from Atlantic salmon stocked in three Ontario rivers (Credit River, Duffins Creek, and Cobourg Brook) using COI primer pairs previously designed for Atlantic salmon (Lulat, 2023). Samples were collected pre-stocking, three weeks post-stocking, and three months post-stocking. The study found that the strength of the eDNA detection signal of Atlantic salmon mitochondrial DNA was strongest at three months post-stocking, then three weeks post-stocking, and lowest at pre-stocking. This study established a basis for the detection of Atlantic salmon mitochondrial DNA from eDNA water samples of stocked rivers in Ontario (Lulat, 2023). In my study, Atlantic salmon presence was detected at 50% of the naturalized watershed, 25% of the adult return, and 12.5% of the stocked single-sample sites. Additionally, at seven out of eight of the rivers, where presence was detected, Atlantic salmon mitochondrial DNA was only detected at the most upstream site. To my knowledge, this is the first study to assess Atlantic salmon presence using eDNA water samples from rivers with different management regimes (adult return, naturalized watershed, stocked) on a large scale in Lake Ontario tributaries. The detection
of Atlantic salmon at the single-sample sites demonstrates a relationship between presence and management regime, and presence and location. The increased detection of Atlantic salmon mitochondrial eDNA at naturalized watershed sites is likely a result of more consistent Atlantic salmon activity at those sites. The key difference between the naturalized watershed sites, and the other regime management sites, is that naturalized watershed sites contain Atlantic salmon populations. The latter two site types do not necessarily maintain consistent densities of Atlantic salmon, and increased activity and population densities increase the concentration of eDNA in water. Thus, there is a decreased likelihood that Atlantic salmon mitochondrial DNA would be detectable at those locations. The increased detection of Atlantic salmon mitochondrial eDNA at the most upstream sites is likely a result of the similarity in land use and stream order of the more upstream sites. Many of the more upstream sites were surrounded by wider riparian zones (areas containing plant communities on the edge of the riverbank) which are known to have a positive effect on the species in the rivers they surround (Summers, Giles, and Stubbing, 2005; Xiang, Zhang, and Richardson, 2016). Additionally, the upstream sites were typically less populated. In comparison, most of the downstream sites were closer to more densely trafficked less vegetated areas. Therefore, the presence of Atlantic salmon in Ontario rivers is affected by the land use of the surrounding area. Additionally, the more upstream sites were typically in a lower stream order than the more downstream sites, meaning that the rivers were narrower at the more upstream sites. A smaller river has a lower volume of water in which eDNA is suspended, thus increasing the concentration of eDNA per volume of water. An increased eDNA concentration per volume of water increases the likelihood of detecting eDNA. Thus, it is highly likely that the smaller order rivers mainly observed in the more upstream sites positively affected the detectability of mitochondrial eDNA from those sites. Overall, my study provides insight into the effect site type has on the presence of Atlantic salmon in Ontario.

A key factor in re-establishing the Atlantic salmon population in Ontario is the monitoring of the movement of the stocked populations on a yearly basis. Presently, two studies and one assessment (conducted by the Lake Ontario Management Unit) have assessed the presence of Atlantic salmon in Ontario. All provided information about the
movement of Atlantic salmon in Ontario rivers. The first (Lulat 2023) also used eDNA analysis to assess the presence and abundance of stocked Atlantic salmon. The second study Larcoque et al. (2020) utilized acoustic telemetry to assess the success of hatchery and naturally reared Atlantic salmon smolts migrating from the upper Credit River to Lake Ontario. The authors found that the majority of smolts moved downstream, instead of upstream, over time. Finally, the Lake Ontario Management Unit conducted an assessment of Atlantic salmon migration using the Riverwatcher (a video fish counter technology) to monitor the movement of the stocked fish in the Ganaraska Fishway. Four Atlantic salmon were observed by the Riverwatcher, the first of which was recorded on July 13, 2021. The Atlantic salmon had visual markers indicating the year they were stocked, all the Atlantic salmon observed were stocked between 2016-2019. The low number of individuals observed upstream further indicates the preference for stocked Atlantic salmon to move downstream over time. In my study, Atlantic salmon presence was only detected downstream of the stocking sites six weeks post-stocking. The detection of Atlantic salmon presence at the multiple-sample sites demonstrates a relationship between presence and sampling time and location. The findings of the multiple-sample sites in this study further support the downstream movement of Atlantic salmon over time. As well, the observations recorded by the Riverwatcher are from the same year and sampling season as my study which allows for more direct inferences to be made. Specifically, both the Riverwatcher and my sample collection first detected Atlantic salmon in the middle of July. Moreover, the Riverwatcher only detected adult return Atlantic salmon from previous stocking years. Thus, the Atlantic salmon detected six weeks post-stocking (end of July) is likely representative of adult return Atlantic salmon from previous years, rather than the fingerlings stocked the year of sampling. Additionally, my study provides further insight into the findings of Lulat (2023) as that study was only able to detect Atlantic salmon presence six weeks post-stocking, and not four days post-stocking. All in all, my study provides information on how sampling time and location affect the likelihood of mitochondrial DNA detectability from eDNA water samples, thus providing a set of best practices for the successful monitoring of Atlantic salmon in Ontario using eDNA analysis.
4.3 Limitations and future directions

The findings of this thesis lead to several questions regarding the use of microsatellite DNA detected from eDNA water samples as a tool to provide population-level information about fish. First, what, if anything can be done to mitigate the challenges associated with microsatellite DNA amplification and allele detectability from eDNA water samples.

The first challenge in the use of microsatellite DNA in eDNA analysis is the consistent detectability of microsatellite DNA from different types of eDNA water samples. While previous studies have detected microsatellite DNA from mesocosm and river eDNA water samples, this study was only able to detect microsatellite DNA from tank eDNA water samples and not river eDNA water samples. A sufficient investigation has yet to be conducted assessing water sample type, species abundance and biomass, and success of microsatellite DNA detectability. Thus, future studies may focus on the relative detectability of microsatellite DNA from different types of water samples in relation to species abundance and biomass.

The second challenge in the use of microsatellite DNA in eDNA analysis is the detectability of representative microsatellite alleles from eDNA water samples. In this study, microsatellite alleles were detected from tank eDNA water samples, but the alleles were not representative of the alleles of the individuals in the tanks. The first potential solution to this problem that could have been applied in this study is adding steps prior to sequencing that increase the quality of the DNA in the samples, potentially increasing the detectability of representative alleles from eDNA water samples. Another potential solution is the use of more microsatellite primer pairs to detect alleles. To keep the scope of the project within that of an MSc Thesis, microsatellite primer pairs previously designed for Atlantic salmon were assessed, instead of new primer pairs being designed. The limits in time and scope of this project led to a microsatellite primer pair successfully amplifying DNA from eDNA water samples. Future studies may wish to investigate (1) detectability of microsatellite alleles from eDNA water samples using altered PCR and library preparation protocols, (2) the difference in ability of microsatellite primer pairs
designed using the DNA of the fish from the study and microsatellite primer pairs previously designed for the study species to detect representative alleles from eDNA water samples, and (3) a study with a longer time frame as more microsatellite primer pairs and PCR and library preparation protocols could be assessed. Studies of this nature may provide more information on best practices for microsatellite eDNA analysis in regard to primer pair design and PCR and library preparation protocols.

The successful amplification of microsatellite DNA from eDNA water samples in this and past studies, suggests that on a whole nuclear DNA is detectable from eDNA water samples. The low percentage of microsatellite primer pairs that successfully amplified microsatellite DNA from tank eDNA water samples (2 out of 35 primer pairs) and the lack of confidence in how representative microsatellite alleles detected from eDNA water samples are of the alleles known to be in the water, suggests that this type of nuclear DNA may not be best suited for eDNA analysis. A possible future direction is the investigation of the ability of other types of nuclear DNA to be detected and provide population level information about a species. Single nucleotide polymorphisms (SNP), in particular, could be used as an alternative to microsatellites. Similarly, to microsatellites, SNPs have been historically used in population genetics to provide information about the relationships within and between populations (Liu and Fu, 2015; Spitzer et al., 2016). Thus, future studies may wish to investigate the ability of SNP DNA and alleles to be detected from eDNA water samples.

### 4.4 Conclusion

With the continued push to monitor fishes using less invasive techniques the understanding and expansion of the analysis of mitochondrial and nuclear DNA from eDNA water samples is more relevant than ever. This study provides the first direct comparison of microsatellite alleles detected from eDNA water samples to the microsatellite alleles of the fish present in the water. I found that microsatellite alleles detected from tank eDNA water samples were not exclusively representative of the microsatellite alleles known to be in the water. These results provide new insight into the ability of microsatellite DNA detected from eDNA water samples to identify unique
individuals in the water. Additionally, this study provides information on the amplification of mitochondrial and microsatellite DNA from tank and river eDNA water samples at varying fish biomasses. This study found that both types of DNA were able to be consistently detected from eDNA water samples collected from tanks containing a fish biomass of $\geq 36.7$ g, while both types of DNA were not able to be consistently detected from eDNA water samples collected from a river containing a fish biomass of 1.8-11.4 g. This result provides evidence that fish biomass per volume of water is proportional to the detectability of DNA from eDNA water samples. Finally, this study provides information on the presence of Atlantic salmon in Ontario rivers. This study found that the detection of Atlantic salmon mitochondrial DNA from eDNA water samples was affected by site type. Specifically, Atlantic salmon mitochondrial DNA was most frequently detected at naturalized watershed sites. I also found that sampling time and location from the stocking site affect the detection of Atlantic salmon mitochondrial DNA from eDNA water samples. In particular, from sites sampled multiple times and at multiple locations surrounding the stocking site, mitochondrial DNA was only detected downstream stocking sites six weeks post-stocking. The variation in the detectability and detection of microsatellite and mitochondrial DNA from eDNA water samples observed in this study suggests that further investigation is needed into the factors affecting DNA detectability, and into other types of DNA that may provide a more detailed picture of populations using eDNA analysis.
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Appendix A Lab experiment sample collection information. From left to right the table includes, fish density, trial number, time of fish deployment, time of water flow cut off, eDNA tank water sample collection time, tank temperature on the day of sample collection, fish weight and length for each trial.

<table>
<thead>
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<th>Date</th>
<th>Density (# of fish)</th>
<th>Trial #</th>
<th>H2O temp (°C)</th>
<th>Fish deployment time</th>
<th>Sampling time</th>
<th>Water flow rate (m/s)</th>
<th>Weight (g)</th>
<th>Length (m)</th>
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<td>1</td>
<td>22.5</td>
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<td>100 m</td>
<td>2.07pm</td>
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<td>Fish 2</td>
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<td>1</td>
<td>23.2</td>
<td>1:32pm</td>
<td>25 m</td>
<td>2.04pm</td>
<td>Fish 3</td>
<td>Fish 4</td>
</tr>
<tr>
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<td>100 m</td>
<td>2:32pm</td>
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<td>25 m</td>
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<td>3.2</td>
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</table>
Appendix B Field experiment sample collection information. From left to right the table includes, date of the trial, the fish density, trial number, river water temperature at time of fish deployment, time of fish deployment, eDNA river water sample collection time and water flow rate at 100 m, 25 m, and directly behind the net pen, and fish weight and length for each trial, for pen 1 and pen 2, respectively.

<table>
<thead>
<tr>
<th>Density (# of fish)</th>
<th>Trial #</th>
<th>Fish Deployed</th>
<th>Water Flow turned off</th>
<th>H2O Collection</th>
<th>Weight (g)</th>
<th>Length (cm)</th>
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Appendix C OFAH sampling location information. From left to right the site number, latitude and longitude of the site, site name, the name of the river where the site is located, and the site type.

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<th>Site Number</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Site Name</th>
<th>River Name</th>
<th>Site Type</th>
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</tbody>
</table>
Curriculum Vitae

Name: Miklosi Simone

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2016-2020 B.Sc.

Honours and Awards:
The Western Scholarship of Excellence
2016

Faculty of Arts and Humanities Entrance Scholarship of Excellence
2016

Dean’s Honour List
2020

Biology Graduate Teaching Award
2022-2023

Related Work Experience:
Teaching Assistant
The University of Western Ontario
2020-2023