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Distribution and Relative Abundance of Reintroduced Atlantic Salmon, Salmo salar, in Lake Ontario Tributaries Using Environmental DNA

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DISTRIBUTION AND RELATIVE ABUNDANCE OF REINTRODUCED ATLANTIC SALMON, *SALMO SALAR*, IN LAKE ONTARIO TRIBUTARIES USING ENVIRONMENTAL DNA

by

Nabeelah Lulat

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DECLARATION OF CO-AUTHORSHIP

I. Co- Authorship

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: I am the sole author of Chapter 1 and Chapter 4, and I am the primary author of Chapters 2 and 3. Chapter 2 and Chapter 3 of this thesis is co-authored with my supervisor, Dr. Daniel Heath. In all cases, the key ideas, primary contribution, experimental designs, data analysis and interpretation were performed by the author (myself), with additional contributions on data analysis, interpretation of data, written discussion, editing and providing funding by co-author.

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ABSTRACT

Plans to reintroduce Atlantic salmon in Lake Ontario tributaries consists of stocking hatchery-reared fish yearly which will help to achieve a self-sustaining population. The issue with reintroduction remains in understanding the distribution of fishes after stocking. Environmental DNA (eDNA) provides a sensitive approach for monitoring that can offer inferences into fish distribution. I determined the distribution of stocked Atlantic salmon downstream from stocking sites using qRT-PCR and metabarcoding. I found that Atlantic salmon eDNA detection was more sensitive using qRT-PCR (51%) versus metabarcoding HTS (18.3%). However, metabarcoding provided data on fish community assemblages, which can help to monitor ecological interactions. I also found that eDNA and microsatellite markers genotyped and assigned an estimate number of individuals to 68.3% of the positive Atlantic salmon NGS data. This data indicates that eDNA and microsatellites can be used as a non-invasive method to quantify and monitor communities.

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CHAPTER 1

GENERAL INTRODUCTION

Environmental changes are not rare because ecosystems evolve (Oliver et al., 2015), but anthropogenic activities, such as overexploitation, habitat destruction, and climate change, all associated with human activities, have rapidly changed abiotic environmental factors, leading to declines in biodiversity (Ceballos et al., 2015; Mandrak & Cudmore 2010; Minamoto et al., 2012). Additionally, the invasion of non-indigenous species (NIS) via human mediated transport, such as ballast water discharge and recreational activities, have the potential to affect biotic factors of an ecosystem and cause further species loss (Lodge et al., 2006). Biodiversity loss is a global issue and is damaging ecosystem function and services across all ecoregions (He et al., 2019). Ecosystems are affected by these abiotic and biotic stressors differentially, some more than others.

Studies have shown that freshwater biodiversity, especially in North America, is declining at a faster rate than some of the most stressed terrestrial ecosystems, including tropical forests (Ricciardi & Rasmussen 1999; Dudgeon et al., 2006). The key reason for the higher rates of biodiversity loss in freshwater ecosystems is the disparate richness of freshwater habitats for aquatic species (Dudgeon et al., 2006). Freshwater habitats occupy less than 1% of the Earth's surface, yet they support approximately 10% of known species and 66% of aquatic vertebrate species (Strayer & Dudgeon 2010). Freshwaters are also exposed to many persistent and changing stressors because such waterbodies are

often close to urbanized and industrial areas (Keppeler et al., 2018). Although the Laurentian Great Lakes are one of the largest freshwater reservoirs in the world, and are thus of great economic, social and cultural importance to humans, they have experienced marked losses in native fish biodiversity (and gains in invasive species) resulting from human influences (Smith 1995). Of the 169 freshwater fish species historically native to the Great Lakes basin, 3 are globally extinct, 18 species have been extirpated and 82 species are at risk (Mandrak & Cudmore 2010). It is thus evident that freshwater ecosystems are losing species rapidly and are facing intense anthropogenic impacts.

Species at risk, extirpated or extinct can cause instability and cascading effects in ecosystems (Seddon et al., 2014). To protect and preserve vulnerable species, conservation efforts must use multidisciplinary approaches (environmental monitoring, genetics, population biology, biogeography, social sciences, etc.) that include both natural science and management research and applications to help reduce threats to biodiversity (Soulé et al., 1985). Conservation practices includes strategies to reverse the loss of species that are endangered or extirpated, and one such strategy is reintroduction of individuals to their native, but extirpated, habitat, potentially restoring viable populations (Muths & Dreitz 2008).

Reintroduction of Extirpated Species

Reintroduction programs, where wild or captive-bred individuals are translocated to their historical native ranges, have become an increasingly popular tool in conservation to address extirpations and to reduce species loss prior to extirpation or extinction

(Armstrong & Seddon 2008; Muths & Dreitz 2008; IUCN 2013). The goal of reintroduction is to re-establish self-sustaining viable populations (Muths & Dreitz 2008; IUCN 2013; Seddon et al., 2014). The use of reintroduction is not a novel approach for conservation management, as it has been applied for almost a century to address conservation objectives (Seddon et al., 2014). There were approximately 126 species reintroduction programs in the 1900s, which increased to 489 programs by 2005, reflecting both an increase in human impact over time as well a greater acceptance of reintroduction as a viable conservation option (Seddon et al., 2007; Muths & Dreitz 2008). Although reintroductions are common, the success of establishment of viable populations is low. For example, Fischer & Lindenmayer (2000) found that under 26% of animal reintroduction programs were successful. On the other hand, Cochran-Biederman et al., (2015) reported a success rate of 58% for freshwater fish reintroductions, although this could be an overestimate due to publication bias for successful reintroductions (Fischer & Lindenmayer 2000; Cochran-Biederman et al., 2015). Many factors can affect the success or failure of reintroductions, including dispersal, habitat quality, reproductive success, genetic diversity and community interactions. To increase reintroduction success, a thorough characterization of the factors affecting reintroduction outcomes is needed through experiments, monitoring, and integration of scientific evidence to allow conservation managers to make decisions that will meet reintroduction program goals.

One of the factors contributing to low success of many reintroduction efforts is the challenge of monitoring the organisms during and after reintroductions (Griffith et al., 1989; Muths & Dreitz 2008). This information is essential to provide an evaluation of

reintroduction efficacy. Generally, long-term monitoring of the released organisms is rarely implemented (Muths & Dreitz 2008). Post-release monitoring must be considered over an appropriate period to determine the establishment of the organism and identify ways for conservation managers to adapt and improve reintroduction strategies (Koelewijn et al., 2010; Bernardo et al., 2011; Riaz et al., 2019). Post-release monitoring data can identify preferred habitat and species co-occurrences, both of which are important for successful establishment (IUCN 2013; Lamothe et al., 2019). Monitoring species abundance and distribution, coupled with habitat assessment and mapping can provide data on whether reintroduced organisms will thrive or whether action is needed to improve habitat conditions, or other barriers to success need to be addressed, to achieve the conservation goals.

Conventional Monitoring Methods

Monitoring reintroduced species by physical sampling can provide data on life history variation, population biomass, movement patterns, resource use and distribution. However, those types of data may be difficult to acquire in aquatic ecosystems because aquatic habitats are hard to sample and some species are cryptic (Cooke et al., 2013). Monitoring aquatic species has generally relied on invasive sampling methods such as electrofishing and seining; however, telemetry techniques (acoustic and radio), provide detailed data on species distribution and movement and have become more common (Baldwin et al., 1996; Cooke et al., 2013; Thomsen & Willerslev 2015). However, capture-based monitoring can adversely impact the target species as well as their habitat, in

addition to potentially having low capture/detection rates (Baldwin et al., 1996; Ficetola et al., 2008). For example, electrofishing can cause spinal injuries or muscular hemorrhages (Cho et al., 2002; Snyder 2003). Additionally, monitoring through tagging organisms for telemetry, is expensive due to infrastructure needs and labour, which generally leads to small sample sizes (DeCelles & Zemeckis 2014). These limitations of capture-based monitoring may provide misleading data on reintroduced organism distribution and status. More recently, reintroduction efforts have incorporated molecular genetic techniques to monitor and manage reintroduced species at risk (DeSalle & Amato 2004). More specifically, environmental DNA (eDNA) has been used to non-invasively detect rare and at-risk species (Pilliod et al., 2013; Thomsen & Willerslev 2015).

Environmental DNA

Target species presence (among other information) can be obtained by analyses of genetic material (eDNA) from environmental samples such as water, sediment, air or faeces (Bohmann et al., 2014; Thomsen & Willerslev 2015). The use of eDNA was originally to study microbial communities, but has since been expanded to include the analysis of macroorganisms, for example, one of the first studies was the detection of the invasive American bullfrog, *Lithobates catesbeianus* (Ficetola et al., 2008). Due to the difficulty of monitoring elusive aquatic species using traditional surveillance methods, coupled with their sometimes cryptic life histories, eDNA has become an increasingly popular tool to detect the presence of aquatic species, specifically, fish species (Tsuji et al., 2019). Aquatic

eDNA is released from fish via blood, faeces, urine, sperm, eggs, and shed skin cells, among others (Dejean et al., 2011; Bohmann et al., 2014). Many eDNA studies have focused on detecting invasive species, these studies included amphibians, invertebrates, and fishes (Ficetola et al., 2008; Jerde et al., 2011; Macheler et al., 2014; Klymus et al., 2015; Klymus et al., 2017; Balasingham et al., 2018; Nevers et al., 2018; Mychek-Londer et al., 2020). However, eDNA has also been applied successfully to the detection of rare and endangered species (Pilliod et al., 2013; Janosik & Johnston 2015; Laramie et al., 2015; Sigsgaard et al., 2015; Bylemans et al., 2017; Balasingham et al., 2018; Bracken et al., 2019). Detection using eDNA analyses is now widely used to monitor the presence/absence of target species to provide valuable information on their distribution, abundance and community interactions (Adams et al., 2019; Ruppert et al., 2019).

Multiple studies have compared eDNA to conventional capture methods in aquatic ecosystems, showing that eDNA is more sensitive for detecting elusive, at-risk species (Shaw et al., 2017; Balasingham et al., 2018; Nevers et al., 2018; Berger et al., 2020). This sensitivity may be due, in part, to eDNA persisting in the environment for days to weeks (Dejean et al., 2011; Balasingham et al., 2017; Hinlo et al., 2018). However, the persistence of eDNA in the environment depends on extrinsic factors such as dilution, water chemistry, temperature, UV radiation, and microbial activity (Dejean et al., 2011; Takahara et al., 2012; Shogren et al., 2017; Bylemans et al., 2018; Tillotson et al., 2018). Additionally, intrinsic factors, such as target species body size, behaviour, biomass, life stage, and diet, can also influence the DNA shedding rate (Klymus et al., 2015). While eDNA transport in lentic systems is likely diffusion-related, eDNA in lotic systems is

complex because of water flow, which can affect the distribution and retention of eDNA (Jerde et al., 2011; Olson et al., 2012; Balasingham et al 2017; Tillotson et al., 2018). Specifically, the movement of eDNA downstream in lotic systems is expected to result in species detections downstream from their actual location. Identifying the source location of eDNA thus can be problematic; however, local retention of genetic material can help to locate areas with few individuals and identify areas that the species may no longer inhabit (Balasingham et al., 2017; Wood et al., 2020). Thus, it is important to collect samples upstream and at various intermediate sites, and also over multiple time periods to get a clear picture of species distribution patterns.

Quantitative Analysis of eDNA Signal Strength

Originally, eDNA would be amplified using end-point PCR followed by PCR product visualized on agarose gels for verification of species detection (Jerde et al., 2011; Turner et al., 2014). However, this method has a decreased detection probability (Turner et al., 2014; Harper et al., 2018; Wood et al., 2019). To increase sensitivity and detection of target species, quantitative real-time polymerase chain reaction (qRT-PCR) can identify target species using species-specific primers and provide an estimate of DNA quantity that may be present in an environmental sample (Takahara et al., 2012; Pilliod et al., 2013). A qRT-PCR assay is a form of PCR that detects and monitors the amplification of nucleic acids at each cycle by measuring the intensity of fluorescence, producing a C_T (cycle threshold) value (Ellison et al., 2006). Previous eDNA studies used qRT-PCR methods to quantify detections and found that eDNA concentration in water samples was related to

target species abundance (Takahara et al., 2012; Thomsen et al., 2012; Levi et al., 2019; Yates et al., 2019). However, qRT-PCR does not give an estimate of the absolute number of the target species, rather it can provide an estimate of the amount of eDNA available for amplification ("template eDNA"); however, many factors affect the concentration of target eDNA in a sample (Ficetola et al., 2008). Because there are extrinsic and intrinsic factors (as discussed above) that affect eDNA persistence, the concentration of viable target eDNA can be highly variable. Specifically, eDNA movement in lotic systems is difficult to quantify because of natural conditions (dilution, flow rate, etc.) decreasing eDNA persistence, which may result in very low to zero eDNA signal strength. If the target species is spatially dispersed, eDNA may continue to replenish within the environment, thus artificially increasing eDNA signal strength. Data obtained through qRT-PCR analyses of eDNA can be used to determine and map the spatial and temporal distribution of species that are of conservation concern (Barnes et al., 2014; Balasingham et al., 2017), but very few studies have applied eDNA to reintroduced species. One study (Riaz et al., 2019) successfully detected reintroduced riffle minnow (*Alburnoides bipunctatus*) eDNA in central Germany rivers using qRT-PCR. That study determined the detection distribution patterns along sampling transects to infer the minnow's dispersal and habitat colonization patterns. It is clear that the high sensitivity of qRT-PCR can help to monitor and evaluate eDNA signal within the environment and hence potentially allow long-term reintroduction success monitoring.

Characterization of Fish Community

Initially, eDNA studies focused on identifying one, or, at most, a few species, using species-specific PCR assays, followed by Sanger DNA sequencing of the PCR amplicon to confirm species identification (Taberlet et al., 2012). Alternatively, the development of high-throughput sequencing (HTS), also known as next-generation sequencing (NGS), now allows the detection of all members of a biotic community simultaneously using a single environmental sample (Lodge et al., 2012; Thomsen et al., 2012; Deiner et al., 2017) and "universal" PCR primers. This approach is known as "metabarcoding", which uses universal primers, rather than species-specific primers, to amplify small regions of the genome across all target species present in a habitat (Taberlet et al., 2012; Klymus et al., 2017). Since eDNA from all organisms present is mixed in aquatic environments, PCR amplification should generate amplicons from all target species, these amplicons can then be sequenced using HTS (Wilcox et al., 2018). After HTS, the metabarcode sequences are compared to a reference database (such as GenBank) and can be assigned to known species based on DNA sequence similarity (Taberlet et al., 2012; Wilcox et al., 2018). Previous studies targeting invertebrate and fish communities in aquatic environments have implemented eDNA metabarcoding to determine species diversity, the number of sequences for each species and the distribution of detections (Klymus et al., 2017; Yamamoto et al., 2017; Balasingham et al., 2018; Nguyen et al., 2019). One serious caveat of metabarcoding is that species identification may be incorrect due to the presence of closely related species. This can create bias in diversity analyses and lead to misidentification of rare and at-risk species; thus it is important to increase identity threshold confidence when assigning eDNA metabarcode sequences. A few eDNA studies

have compared the sensitivity and specificity of qRT-PCR and metabarcoding, and they found that metabarcoding is less sensitive than qRT-PCR for single species monitoring because of limitations of sequencing depth in HTS (Harper et al., 2018; Wood et al., 2019). However, only metabarcoding can provide whole community data, which can be used to infer species interactions. Thus, combination of both qRT-PCR and metabarcoding eDNA detection platforms has the potential for a powerful passive approach to monitoring reintroduced species.

Microsatellite DNA Analysis

While qRT-PCR applications and metabarcoding can be used to detect the presence and estimated signal strength of the target species (Takahara et al., 2012; Pilliod et al., 2013), it cannot provide a reliable estimate of the absolute numbers of individuals present. Microsatellite DNA markers, unlike barcoding sequencing (such as COI), provide a distinct genetic profile unique for each individual (Mills et al., 2000; Adams et al., 2019). Microsatellite data used in traditional population genetic studies provide information on genetic diversity, effects of inbreeding, gene flow, and population structure within and among populations (Selkoe & Toonen 2006), and have been widely used in forensic applications (Algee-Hewitt et al., 2016). A novel application of microsatellite DNA markers in stomach content eDNA has been shown to determine the number of target species prey present in a pool of DNA extracted from stomach contents (Carreon-Martinez et al., 2014). Microsatellite genetic markers have also been used as a monitoring tool to distinguish an individual species' identity via non-invasive sampling. A study conducted by Wheat et al., (2016) was able to detect the number of brown bear (*Ursus arctos*)

individuals present within a sampling area via DNA extracted from scat droppings and residual saliva on consumed salmon and amplified with microsatellite markers Another study by Monge et al., (2018) applied microsatellite markers on DNA isolated from almond fruits eaten by scarlet macaws (*Ara macao*) to determine the individual's sex for information on population genetics parameters. The information from these studies would be useful for conservation and management of the species' populations. Based on the success of these previous studies, microsatellite eDNA holds promise for the quantification of the number of individuals as well as their distribution based on their abundance for reintroduction and conservation monitoring purposes.

Atlantic salmon in Lake Ontario

Atlantic salmon (*Salmo salar*) are widely distributed in rivers in eastern North America and Europe, but their populations have declined, and many sub-populations have been extirpated over the last 200 years (Parrish et. 1998). Atlantic salmon was historically a native species in Lake Ontario (Dunfield 1985; Smith 1995); however, they were extirpated by 1896 following European settlement, likely due to habitat loss. Their loss caused a decline in Lake Ontario fishing with important economic effects (Scott et al., 2005). Habitat loss was due to construction of dams that blocked access to natal spawning areas, forcing the adults to spawn downstream or search for another site that may have unfavourable conditions (Parsons 1973; Solomon et al., 1999). Also, deforestation and pollution from agriculture increased runoff, siltation, stream temperature, and decreased flow, affecting river conditions and making them unsuitable for spawning and egg/larval survival (Parsons 1973). Lastly, Lake Ontario Atlantic salmon were overexploited due to

their richness and high value, removing adults that had the potential to reproduce (Smith 1995). Thus, reintroduction of Atlantic salmon is an important management and conservation goal, to bring back a highly valued species.

Government agencies bordering Lake Ontario began to stock Pacific salmonids in the early 1960s to control invasive species and improve recreational fisheries (Crawford 2001; Scott et al., 2005). The successful establishment of spawning Pacific salmonids indicated that stream habitats in Lake Ontario had recovered, and that Atlantic salmon may be now able to form self-sustaining populations (Scott et al., 2005). The Ontario Ministry of Natural Resources and Forestry (OMNRF) has been reintroducing various strains of Atlantic salmon to Canadian Lake Ontario streams since the late 1980s using hatchery-reared fish at various life stages (OMNRF 2018). However, no self-sustaining population has yet resulted (Dimond & Smitka 2005; COSEWIC 2010). While environmental conditions in Lake Ontario and its streams have improved over time, they are significantly different compared to their historical conditions. Early research indicated possible reasons for unsuccessful reintroduction of Atlantic salmon include the presence of introduced non-native salmonid species and invasive, high-thiaminase-containing prey (Dimond & Smitka 2005; COSEWIC 2010; OMNRF 2018). Recent research on reintroduced Atlantic salmon has focused on interactions with non-native salmonids, gene transcription variance among Atlantic salmon strains, the effects of a high thiaminase diet, and survival and migration patterns (Scott et al., 2005; Houde et al, 2015a; He et al., 2015; Houde et al., 2015b; Larocque et al., 2019). However, while those studies contributed to my knowledge of factors likely affecting reintroduction success, little work

has been done using eDNA to passively monitor the released fish. Therefore, developing a non-invasive monitoring protocol is a critical need to assess how well the stocked Atlantic salmon are acclimating to their new environment and determine any changes to their abundance, distribution, and habitat use.

Objectives and Thesis Structure

The overall objective of my graduate research was to evaluate the use of eDNA to detect reintroduced Atlantic salmon juveniles. While I focused on the reintroduction of Atlantic salmon, my work will inform reintroduction monitoring efforts across fish species.

The aim of Chapter 2 was to determine the distribution of Atlantic salmon eDNA signal strength along with characterizing the associated fish community at multiple sample sites around reintroduction sites. This information was collected by combining the high sensitivity of Atlantic salmon-specific qRT-PCR (and its quantitative capacity) with COI metabarcoding to detect Atlantic salmon presence and assess fish community composition. I mapped the presence of Atlantic salmon before and after reintroduction to detect DNA persistence, therefore providing data on Atlantic salmon presence and absence from stocking sites and downstream. My presence/absence data was compared to the patterns of eDNA presence of other fish species in the system to determine species co-occurrences with Atlantic salmon.

The aim of Chapter 3 was to quantify the number of reintroduced Atlantic salmon at sample sites identified as having Atlantic salmon (Chapter 2) using novel microsatellite

eDNA methods. The eDNA extracted as part of Chapter 2 was used with microsatellite marker PCRs to determine the allele frequencies at three microsatellites loci for the reintroduced Atlantic salmon. The eDNA genotype allele frequencies were compared to population genotype data, and using allele counting methods, I estimated the likely number of Atlantic salmon present in eDNA samples. The results from this work will provide new insight into monitoring rare fish species non-invasively and specifically to address conservation issues for reintroduced Atlantic salmon into Lake Ontario tributaries. Also, the combination of eDNA and microsatellite markers can be used as a suitable tool to quantify target species in environmental samples.

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CHAPTER 2

DISTRIBUTION AND ASSOCIATED FISH COMMUNITY OF REINTRODUCED ATLANTIC SALMON (*SALMO SALAR*) IN LAKE ONTARIO TRIBUTARIES USING ENVIRONMENTAL DNA

INTRODUCTION

In recent decades, there has been substantial biodiversity declines due to anthropogenic activities, including habitat alteration and degradation, overexploitation, pollution, and introduction of non-native species (Suski & Cooke 2007; Ceballos et al., 2015). Furthermore, biodiversity loss is a major concern for conservation and management because biodiversity is critical for ecosystem productivity and stability (Worm & Duffy 2003). Conservation strategies for restoring biodiversity include the creation and management of protected areas, habitat restoration, reduction of exploitation/predation, removal of non-native species and translocation of individuals to supplement existing populations (Seddon et al., 2014; Cochran-Biederman et al., 2015). Although these methods address biodiversity conservation in existing (but declining) populations, additional measures are required when native species experience local extirpation.

Reintroduction, the intentional transfer and release of organisms into areas of previous occupation, has become an increasingly important tool in conservation biology to restore populations of severely reduced or extirpated species to recover their functionality in impacted ecosystems (Armstrong & Seddon 2008; Muths & Dreitz 2008).

Some examples of reintroduction programs include the release of white-tailed eagles (*Haliaeetus albicilla*) in western Scotland, gray wolves (*Canis lupus*) in Yellowstone National Park, pool frogs (*Pelophylax lessonae*) in England and Macquarie perch (*Macquaria australasica*) in eastern Australia (Whitfield et al., 2009; Ripple et al., 2014; Sainsbury et al., 2016; Lutz et al., 2021). Regardless of the tremendous efforts focused on reintroduction, many fail to establish self-sustaining populations, the main goal of reintroduction (Wolf et al., 1998; White et al., 2021). Reasons for the lack of successful reintroductions vary among studies, and include unsuitable release sites, poor release strategies, ineffective post-release monitoring, high mortality, and the logistics and cost of the reintroduction (Griffith et al., 1989; Bearlin et al., 2002; Muths & Dreitz 2008; Lamothe et al., 2019; White et al., 2021).

Biodiversity is declining at a faster rate in freshwater ecosystems than in terrestrial ecosystems because they support a disproportionately higher number of species, coupled with a narrow range of habitats for many aquatic species (Ricciardi & Rasmussen 1999; Dudgeon et al., 2006). Specifically, freshwater fishes are the most diverse and threatened group of vertebrates, making them vulnerable to human activities, ultimately leading to biodiversity loss (Bruton 1995; He et al., 2019). Consequently, reintroduction of freshwater fishes is an important tool for the conservation of locally extirpated populations and the management of exploited species. For example, stocking of hatchery-reared fish to increase population numbers to supplement fisheries has become common across many fish species (Brown & Day 2002). Prominent examples of aquatic fish reintroductions for conservation include the release of trout cod (*Maccullochella*

macquariensis), Pacific lamprey (*Entosphenus tridentatus*) and bull trout (*Salvelinus confluentus*) (Bearlin et al., 2002; Close et al., 2009; Brignon et al., 2018). Although monitoring of released aquatic species is challenging due to field logistics and costs, it is vital to determine whether reintroduction strategies are effective or need to be modified and improved (Muths & Dreitz 2008).

Monitoring of aquatic species populations has traditionally relied on direct sampling such as electrofishing, trapping and other capture methods, sometimes coupled with tracking techniques (e.g. acoustic or radio telemetry); such capture-based approaches provide data on species identification, abundance, distribution and movement (Baldwin et al., 1996; Cooke et al., 2013; Thomsen & Willerslev 2015). However, such methods are expensive, time-consuming and can potentially harm the target individuals and their habitat (Thomsen & Willerslev 2015; Jerde et al., 2016). Additionally, these methods are not effective at detecting individuals that are rare, cryptic or elusive (Deiner et al., 2017). The limitations of capture-based monitoring can thus provide misleading or incomplete information on reintroduced organism status, movements and habitat use. Conversely, the use of molecular genetic methods can reduce labour costs and the effects associated with traditional capture-based monitoring methods. Environmental DNA (eDNA) analysis is a non-invasive surveillance method that has been developed to detect and monitor cryptic, elusive or low-density species, and provide important information for the management and conservation of freshwater fishes (Jerde et al., 2011; Thomsen et al., 2012). The method consists of collecting extraorganismal genetic material (e.g. sloughed cells, urine, faeces, gametes, etc.) that

persist in the environment with the purpose of extracting DNA and using molecular genetic markers to provide evidence of species presence and distribution (Ficetola et al., 2008; Dejean et al., 2011). Several studies have proven eDNA analysis as a powerful, sensitive tool in detecting endangered aquatic species (Laramie et al., 2015; Sigsgaard et al., 2015; Balasingham et al., 2018), but only a few have been applied to monitoring an aquatic reintroduction program. One such study successfully detected a declining population of riffle minnow (*Alburnoides bipunctatus*) that has been reintroduced to rivers in central Germany (Riaz et al., 2019). The reported persistence of downstream eDNA in that study corroborates other studies that have detected target species eDNA up to several kilometres downstream from a known source location, such eDNA "flow" can help with detecting a target species' presence in lotic systems, but can make it difficult to pinpoint the targets' precise location (Deiner & Altermatt 2014; Jane et al., 2015; Balasingham et al., 2017; Wood et al., 2020).

To quantify the success of fish reintroduction programs, and to thus determine possible causes of failures, it is crucial to monitor the distribution and possible community interactions of the reintroduced organisms. eDNA monitoring typically incorporates quantitative real-time PCR (qRT-PCR) or high throughput sequencing (HTS) methodology (metabarcoding). qRT-PCR is commonly used in eDNA studies because it provides a fast, targeted method for processing large eDNA sample sizes and is highly sensitive to detecting low DNA concentrations (Jerde et al., 2011; Wilcox et al., 2013). Furthermore, qRT-PCR can be used not only to infer specific species presence, but also provides quantitative estimate of signal strength (Takahara et al., 2012; Pilliod et al., 2013; Yates

et al., 2021). On the other hand, HTS metabarcoding is widely used in eDNA studies to determine fish community composition (Valentini et al., 2016; Klymus et al., 2017). Metabarcoding can provide estimates of community diversity and richness by determining not only species presence (based on amplicon sequence) but also relative signal strength based on sequence read depth (Valentini et al., 2016). Some studies have compared the sensitivity and specificity of the two approaches and found that metabarcoding is less sensitive than qRT-PCR for single species monitoring because of limitations in sequencing depth and replication (Harper et al., 2018; Wood et al., 2019). However, those studies did not analyze the community data to indicate species presence and absence, hence perhaps did not explore the eDNA metabarcoding data to its full extent. Ideally, a combination of both eDNA detection platforms has the potential for a holistic monitoring approach that can provide management with data on multi-species distribution.

Atlantic salmon (*Salmo salar*) was, historically, an abundant native species in Lake Ontario, but disappeared in the late 1800s, causing a decline in the Lake Ontario fishing industry, an important economic contributor to the region (Smith 1995; Scott et al., 2005). Extirpation was mainly caused by habitat degradation, pollution, and overfishing (Parsons 1973). With the establishment of viable Pacific salmonid populations in the early 1960s, it seemed possible that Atlantic salmon could be re-established (Scott et al., 2005). The Ontario Ministry of Natural Resources and Forestry (OMNRF) has been reintroducing hatchery-reared Atlantic salmon to Lake Ontario streams every year since the late 1980s (OMNRF 2018); however, few adults return to spawn providing little evidence of self-

sustaining populations (Dimond & Smitka 2005). Therefore, a robust non-invasive monitoring protocol applied during and after reintroduction is critical to assess how the stocked Atlantic salmon are using their new environment and thus possibly inform changes in stocking methods to improve success.

Here, I present a monitoring study using eDNA collected from three Ontario streams stocked with Atlantic salmon; I sampled the streams before, immediately after and 3 months after stocking. I analyzed the eDNA using a combination of qRT-PCR and CO1 metabarcoding to evaluate the distribution of the reintroduced salmon as well as their associated fish communities. Specifically, I sampled sites in close proximity to the Atlantic salmon release sites to determine the spatial distribution of their eDNA signal strength using qRT-PCR and characterize the fish community using CO1 metabarcoding. My objectives were to answer the following questions: (1) Determine the distribution of reintroduced Atlantic salmon in three streams at two weeks and three months after stocking. I expect that before stocking eDNA detection will be low, immediately post-stocking would be higher and three months post-stocking would be intermediate. (2) Test for specific fish community assemblages associated with the presence of reintroduced Atlantic salmon. This combination of qRT-PCR and metabarcoding is an effective approach for reintroduction program evaluation and monitoring due to the diverse information provided and minimal habitat/target species disruption.

MATERIALS AND METHODS

Stocking and Water Sampling

In 2018, ~890,000 hatchery-reared Atlantic salmon were stocked into Lake Ontario tributaries during three time periods: ~450,000 spring yearings (early April), ~450,000 spring juvenile (early May to June) and ~115,000 fall juveniles (October). Stocking rivers included Credit River, Duffins Creek, and Cobourg Brook, all of which are located in southern Ontario, draining into Lake Ontario (Figure 2.1). Stocking sites within these rivers were selected by the OMNRF to release hatchery-reared Atlantic salmon based on access and suitable habitat. Water sampling (for eDNA) occurred at three different times relative to spring stocking of the juveniles (May 7 to June 1): 1) before May stocking (sampling on 5, 6, and 7 May), 2) three weeks post-stocking (23, 24 and 25 June) and 3) three months post-stocking of spring juvenile (7, 8 and 9 October). Although fall juvenile stocking and three months post-stocking sampling occurred simultaneously, the sites selected for fall stocking did not overlap with the sites from spring juvenile stocking.

Surface water samples were collected using 500 mL Nalgene bottles (bleach sterilized and rinsed) with a bottle holder, approximately every 100 to 250 m (collected from accessible sites) over ~2 km of the stream. Sampling started downstream, moving upstream, wading into the stream at each site and reaching upstream to collect a sample to reduce the possibility of sampling contaminated downstream samples. Three samples (field replicates) were collected at each site, for a total of 618 samples. Nine field blank controls were taken, one for each sampling day, to confirm sterility of sampling equipment. The field blank controls consisted of distilled water in sterilized bottles filled before sample collection each day, opened during collection and transferred to Nalgene collection bottles to replicate possible airborne contamination. The field blank controls

were placed in the cooler, along with the other water samples that were collected throughout the day.

Filtration and Extraction

Water samples were filtered within 24 hours of collection using 1.2 µm pore size, 47 mm diameter glass microfiber filters (Whatman®, Maidstone, UK). After filtration, each filter was cut in half using sterile forceps and scissors (one half for eDNA extraction and the other half for future use). All half filters were placed in 2 mL storage tubes filled with RNAlater to preserve eDNA and were stored at -20 °C until further processing.

For DNA extraction, filters were washed with ddH_2O to remove residual RNAlater and placed into 2 mL screwcap tubes containing 400 µL of 1.0 mm glass beads (BioSpec Cat. No. 11079110). The DNA extraction method followed that described in Shahraki et al., (2018). To each tube, 400 µL of sucrose lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 9.0) was added and homogenized for 1 minute (three times) at 3000 strokes/minute (Mini-Beadbeater-24, BioSec Cat. No. 112011). Samples were incubated with 50 µL of lysozyme (10 mg/mL) and 70 µL of sodium dodecyl sulfate (1%) overnight at 37 °C, and then 2 µL of proteinase K (20 mg/mL) overnight at room temperature. After treatment, proteinase K was deactivated by placing samples in a 95 °C water bath for 10 minutes. DNA was purified in a 96-well plate using the solid-phase reversible immobilization (SPRI) paramagnetic bead-based method on an automated liquid handling workstation (Tecan Freedom Evo150 Liquid Handling Platform, Perkin Elmer, USA) (Vo & Jedlicka 2014; Shahraki et al., 2018). To

extract eDNA, 150 µL of the lysed sample was mixed with 225 µL of SPRI bead solution and incubated at room temperature for 5 minutes to bind the DNA. The beads were separated on a magnetic plate for 5 minutes then the clear solution was removed, after which, the beads were washed with 70% ethanol twice and air-dried for 5 minutes. Samples were eluted with 50 µL of TE buffer, transferred to a new 96-well plate and stored at -20 °C.

Detection of Atlantic salmon

qRT-PCR assay

Primer3 software (Rozen & Skaletsky 2000) was used to design species-specific primers (forward primer: 5'-TTCTCCTCCTGGCCTCATCT-3' and reverse primer: 5'-CTGCGTGGGCAAGATTACCT-3') for Atlantic salmon which targeted the mtDNA COI region (sequence available on NCBI: KF597049). The primers resulted in a total amplicon size of 90 bp. Basic Local Alignment Search Tool (BLASTn) was used to align the amplicon with sequences from closely related and possibly sympatric fish species: brown trout (*Salmo trutta*), Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), and lake trout (*Salvelinus namaycush*) (Figure 2.2). Also, the primers were tested in vitro with brown trout, chinook and coho salmon, to ensure the primers were Atlantic salmon-specific and did not amplify these species.

All eDNA field replicates, including the field blank controls, were amplified in triplicate (lab/technical replicates) using qRT-PCR in 12 µL reactions as follows: 6.0 µL of

PowerUp SYBR Green Master Mix (Life Technologies Inc, Burlington, ON, CA), 3.4 µL of ddH_2O, 0.6 µL of primer mix (0.2 µM of each primer) and 2.0 µL of eDNA template. qRT-PCR conditions were set to 95 °C for 10 minutes, followed by 40 cycles of 30 seconds at 94 °C and 60 seconds at 60 °C. All qRT-PCRs were run and analyzed on the QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies Inc. Burlington, ON, CA). Each 96-well plate included triplicates of the positive control (fin clip DNA), Atlantic salmon hatchery tank eDNA (positive control), negative control (ddH_2O), and Detroit River eDNA (no historical evidence of Atlantic salmon presence, negative control).

qRT-PCR assay development and application

To estimate the limit of detection (LOD) of the Atlantic salmon-specific (sensitivity analysis) and field-collected eDNA (interference analysis) qRT-PCR assay, a 10-fold dilution series of Atlantic salmon DNA was performed (from 2.2×10^{1} to 2.2×10^{-8} ng/µL) based on initial quantification using a NanoVue spectrophotometer (MA, USA). When running each assay for the sensitivity analysis, nine technical replicates were used for each dilution step, including six negative controls and three positive control dilutions from 2.2×10^{1} ng/µL. The LOD is defined as the lowest concentration in which 95% of positive samples were detected and the highest cycle threshold (C_T) value detected before the standard curve plateaus. (Bustin et al., 2009). The LOD was used to assign a threshold for whether Atlantic salmon eDNA is present or absent for each qRT-PCR. The resulting standard curve from the dilution series was also used to estimate the primer efficiency of the assay, using the efficiency equation (Yun et al., 2006).

I used hierarchical criteria to determine presence/absence of Atlantic salmon at a given field sample site to provide a conservative distribution of Atlantic salmon eDNA. The mean C_T of each field replicate was calculated by finding the average of the three lab replicates. Atlantic salmon were identified as detected when three or two out of three lab replicates were below the LOD C_T threshold. Atlantic salmon were identified as not detected when three or two out of three lab replicates were above the LOD threshold or an undetermined value.

I estimated target Atlantic salmon eDNA concentration at each site to quantify Atlantic salmon eDNA signal strength along each stream sampling transect. I used the qRT-PCR C_T data with the dilution series regression to estimate the concentration of Atlantic salmon eDNA for all lab replicates. Undetermined C_T values were set to an Atlantic salmon eDNA concentration of zero. To view the difference in eDNA concentration downstream from release sites, the mean concentration for the field and lab replicates were calculated for each site by averaging the concentration values for all replicates (nine values). The effect of sampling date on mean eDNA concentration was analyzed using univariate GLM analyses for each stream. In addition, linear regression models were used to examine the relationship between the distance to the nearest upstream stocking site and mean eDNA concentration for each stream per sampling period. All statistical analyses were conducted in SPSS software (IBM Corp.).

COI metabarcoding: fish community

PCR Amplification

All eDNA samples were PCR amplified for COI metabarcoding using nested PCR. For the first PCR, universal CO1 fish primers ("Fish"; Table 2.1; Ward et al., 2005) were used to amplify a 655 bp mtDNA COI fragment. For the second PCR, primers designed to target the freshwater fish species of the Great Lakes basin were used ("PS1"; Table 2.1; Balasingham et al., 2018). The primers were modified with 5' tails for HTS library preparation. PS1 primers target the COI region of 119 fish species present in the Great Lakes, resulting in an amplicon size of 247 bp.

PCRs were carried out in 25 µL volumes containing 17.4 µL ddH_2O, 2.5 µL 10x Taq reaction buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 200 nM of forward and reverse primers, 0.5 U of Taq polymerase (Bio Basic Canada Inc., Markham, ON, Canada) and 1 µL of eDNA template. The following cycling conditions were used: an initial 95 °C denaturation for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds and extension at 72 °C for 30 seconds, ending with a final extension of 10 minutes at 72 °C. Then 2 µL of undiluted first-round PCR products were used in the second (nested) PCRs, following the same temperature profile as above and 20 cycles of the 3 step-cycles. Each PCR plate included a positive control (Atlantic salmon fin clip) and two negative lab controls (ddH_2O; to ensure no laboratory contamination). The field controls were also metabarcoded.

Library Preparation and Sequencing

All second-round PCR products were cleaned with Sera-Mag Magnetic Beads (GE Healthcare Life Science, UK) to remove primer dimers and fragments less than 100 bp.

The cleaned PCR product was used as a template for another (third) short-cycle (6 cycles) PCR to ligate the adapters and individual barcode sequences to the amplicons for HTS library preparation (see He et al., 2017; Table 2.1). The third-round ligation PCRs consisted of 2.8 µL ddH_2O, 2 µL of 10x Taq reaction buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 0.5 µM Uni B primer, 0.5 µM Uni A primer, 0.5 U of Taq polymerase and 10 µL cleaned PCR product, for a 20 µL reaction. Thermocycling conditions used for the ligation PCR were as follows: initial 95 °C denaturation for 2 minutes, 6 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds, followed by a final extension of 5 minutes at 72 °C. Library PCR products were pooled, run on an agarose gel, gel-extracted and cleaned using GenCatch Gel Extraction Kit (Epoch Life Science Inc.). To determine the size and concentration of the products, the pooled amplicons were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). The resulting library was diluted to 55 pmol/µL and sequenced on an Ion Torrent NGS Personalized Genome Machine (Thermo Fisher Scientific).

Sequence Analyses

Quantitative Insights into Microbial Ecology (QIIME I) software was used to process raw HTS sequence data (Caporaso et al., 2010). QIIME I was used to remove sequences shorter than 200 bp or with more than three primer mismatches, and then to trim the adaptor, barcode and primer sequences from the sequence reads. All Great Lakes fish species COI sequences were downloaded to create a reference database. Taxonomy was assigned using BLASTn by comparing the filtered sequences against the reference

database. BLASTn parameters included an expected value of 10 to 10^{-60} and an identity threshold of 97%, to ensure the correct identification of species (Balasingham et al., 2018). Singletons (one hit) and doubletons (two hits) per sample were removed from the resulting BLAST table to reduce the potential bias of sequencing errors or contamination of samples (Flynn et al., 2015). Manual inspection of sequences was done for species that were rare or that had only three sequences by BLASTing sequences in GenBank to determine whether the species identification was accurate or if it was another closely related species. Disregarding one or two sequences avoids overestimation of diversity made by PCR or sequence artefacts (Zhan et al., 2014; Balasingham et al., 2018).

Species co-occurrences at the community level were assessed for each stream for each sampling period using the R package "cooccur" (Griffith et al., 2016; Veech 2013) in R Studio version 1.2.5033, to determine whether Atlantic salmon were detected at higher or lower frequencies than expected by chance in association with other species. Using presence (> 2 sequence reads) and absence (< 3 sequence reads) data for the field replicates, the package carries out statistical pair-wise comparisons to compare the probability that the observed co-occurrence frequency is greater than the expected neutral frequency (positive co-occurrence), less than the expected neutral frequency (negative co-occurrence), or random. The default threshold was kept as TRUE to remove species pairs with insufficient data (Veech 2013).

The signal strength for Atlantic salmon eDNA at a sample site was calculated by taking the total number of Atlantic salmon eDNA sequences for all three field replicates

at a sample site and dividing the total by the total number of eDNA sequence reads for that site.

RESULTS

Validation of qRT-PCR

Two qRT-PCR sensitivity analyses were conducted, ddH_2O (sensitivity) and field-collected eDNA spiked with serial diluted tissue DNA (interference) and a standard curve was generated for both experiments. A qRT-PCR efficiency of 101% (slope of -3.3) was calculated for the ddH_2O reactions and 116% (slope of -3) for the eDNA reactions (Figure 2.3). Yun et al., (2006) recommend utilizing primer with PCR efficiency between 80 to 120%. The qRT-PCR LOD for the ddH_2O reactions was at a mean C_T of 40, whereas LOD was at a mean C_T of about 36 for the eDNA reactions. Dilutions past these C_T values plateaued after reaching the LOD. The LOD value sets a threshold for all samples; a C_T value less than 36 was defined as positive for Atlantic salmon DNA and a C_T value of 36 to 40 or "undetermined" was considered negative for Atlantic salmon DNA because of low target DNA detection.

All negative controls (lab controls: ddH_2O and Detroit River eDNA) and field blank controls) for each sampling period and stream were negative for Atlantic salmon eDNA (lab controls produced undetermined C_T values and field controls produced a mean C_T of 38.2 (± SE 0.4) cycles). Positive control samples (eDNA extracted from a tank holding Atlantic salmon) produced a mean C_T of 23.7 (± SE 0.2) cycles. Atlantic salmon eDNA was detected in all three streams over the three sampling periods, resulting in 329 samples of

positive detections out of 618 samples collected (51%). The concentration estimates of Atlantic salmon eDNA varied along the sampling sites of each stream (Figures 2.4 to 2.6). Analyses of sampling period effect on eDNA detection indicated no significant effect on the mean eDNA concentrations of each stream: Cobourg Brook (F = 1.1, d.f. = 2, p = 0.4), Credit River (F = 2.2, d.f. = 2, p = 0.1) and Duffins Creek (F = 2.3, d.f. = 2, p = 0.1). The results of the regression analysis indicated that distance from the most upstream stocking site did not significantly explain the variation in mean eDNA concentrations across all three sampling periods for each stream: Cobourg Brook (R^2 = 0.001, F = 0.01, p = 0.99), Credit River (R^2 = 0.057, F = 2.4, p = 0.1) and Duffins Creek (R^2 = 0.041, F = 2.3, p = 0.1).

Validation of metabarcoding

Across the 645 samples, including field blank controls, I recovered 26,256,792 sequence reads. After fastq quality filtering, removing tag and adapter sequences, and BLASTing sequences against the COI reference database, 6,101,667 sequence reads remained, including the field blank controls. One of the nine field blank controls returned 31 reads; 3 rainbow trout, 25 Chinook salmon and 3 creek chub. These fish species are known to be present at the sites sampled, which could suggest field or likely laboratory contamination. The laboratory and other eight field blank controls were consistent with low levels of contamination (0 to 1 sequence read), so the sequence reads detected in one field control does not reflect systematic contamination across all samples. After removing field blank and laboratory control reads and species within each sample with

fewer than three sequence reads, 3,039,423 reads were retained for 555 samples. The average number of sequence reads per sample was 5,767 (3 to 107,727).

Atlantic salmon was detected in 115 out of 618 field replicate samples (85 out of 204 sites) using metabarcoding. In total 38,258 sequence reads were identified as Atlantic salmon (1.11% of total reads), ranging from 3 to 5296 sequence reads per site. The proportion of eDNA metabarcoding reads for Atlantic salmon relative to the other fish detected is illustrated in Figures 2.7 to 2.9.

Species co-occurrence analysis

Four sampling periods contained significant species associations with Atlantic salmon (Figure 2.10). Using presence/absence data from May sampling in Credit River, species co-occurrence analysis included 23 species across 58 field replicates, leaving 67 pairs that were analyzed. There were 0 negative, 6 positive (9%) and 61 random (91%) co-occurrences (Figure 2.10a). In Duffins Creek during May sampling, 21 species across 87 field replicates were analyzed, leaving 92 pairs. There were 4 negative (4.4%), 14 positive (15.2%) and 74 non (80.4%) species co-occurrences (Figure 2.10b). Duffins Creek during June sampling included 21 species across 83 field replicates were analyzed, leaving 94 pairs. There was 1 negative (1.1%), 12 positive (12.8%) and 81 non (86.2%) species co-occurrences (Figure 2.10c). In Duffins Creek during October sampling, 20 species across 91 field replicates were analyzed, leaving 130 pairs. There were 10 negative (7.7%), 19 positive (14.6%) and 101 non (77.7%) species co-occurrences (Figure 2.10d).

Species co-occurrence analysis indicated that species composition of fish communities largely followed random patterns of community assembly. In Credit River in May, Atlantic salmon had no negative co-occurrences, but a positive co-occurrence with white sucker (*Catostomus commersonii*). Atlantic salmon in Duffins Creek during May had no negative co-occurrences, but a positive co-occurrence with brook trout (*Salvelinus fontinalis*). In Duffins Creek during June, Atlantic salmon had no negative co-occurrences and a positive co-occurrence with brook stickleback (*Culaea inconstans*). Atlantic salmon in Duffins Creek during October had no negative co-occurrences, but a positive co-occurrence with rainbow trout (*Oncorhynchus mykiss*).

DISCUSSION

My work showed the efficacy and potential of using qRT-PCR and metabarcoding to detect Atlantic salmon eDNA in streams for reintroduction monitoring purposes. My qRT-PCR analyses found eDNA detection not to be influenced by the abundance of target species based on sampling period and there was no effect of dilution on the distance eDNA was transported downstream. I also found Atlantic salmon eDNA detection via qRT-PCR was more sensitive than metabarcoding; however, despite the reduced detection success, eDNA metabarcoding provided additional valuable information on fish community structure associated with stocked Atlantic salmon. Such data provides species co-occurrences and allows inferences concerning species presence or absence that may affect reintroduction success. My co-occurrence analysis resulted in some positive associations between Atlantic salmon and other species in Credit River and Duffins Creek; however, patterns of random co-occurrences were prevalent in all sampled streams.

Previous work suggests recurring disturbances or re-colonization events can produce random co-occurrences (McCreadie & Bedwell 2012). In this study, the regular stocking of Atlantic salmon multiple times each year may have acted as a disturbance to the fish community, reducing the potential for normal community interactions to develop – an important consideration for future stocking efforts.

Detection of Atlantic salmon

Seasonal change in target detection can vary based on eDNA production which is influenced by endogenous factors such as fish activity levels, metabolic rate and behaviour (such as predation and spawning; Stewart 2019). Exogenous factors also play a role in the availability of eDNA for detection, they include water temperature and chemistry, flow rate in lotic systems, and, specifically for this study, the density of introduced individuals. My qRT-PCR analysis detected the presence and persistence of Atlantic salmon eDNA several weeks and months after reintroduction. However, there was no significant relationship between the sampling periods and eDNA concentrations in each stream. It is important to note that positive detections do not provide enough information to assume Atlantic salmon is successfully reproducing, thus restocking is conducted multiple times a year to support reintroduced populations, increasing the chances of higher salmon returns to streams. Therefore, the low eDNA signal prior to the release of the salmon may have originated from overwintered salmon that survived previous releases but had not yet migrated to Lake Ontario or from yearling stocking that occurred the month before. Sampling in June, after OMNRF completed two weeks of

stocking all streams, resulted in a few detections with very strong signal strength. Previous studies have measured concentration of eDNA and found a positive correlation between eDNA concentration/detections and biomass (Dejean et al., 2011; Jerde et al., 2011; Thomsen et al., 2012; Yates et al., 2021, 2022). Although the addition of Atlantic salmon biomass was thought to considerably increase the chances of detection after stocking, the movement or death of the stocked fish may have contributed to lower detections at stocking sites. It was expected that sampling three months after stocking would result in fewer detections, consistent with some salmon still present in the stream, but most of the introduced fish have likely either dispersed or died. However, there was a spike of eDNA detection signal strength in October (even greater than detected in June) in the Credit River (Figure 2.5). This could possibly be due to accumulation of eDNA from upstream sites that were sampled or a favourable habitat that resulted in a concentration of released salmon. Studies conducted in lentic systems (Dejean et al., 2011; Thomsen et al., 2012; Piaggio et al., 2014) detected eDNA for at least 2 to 14 days after removal. However, the distribution of eDNA in lotic systems would be different due to complex, unpredictable factors such as flow rate, temperature and biological degradation influencing the retention of eDNA (Strickler et al., 2015; Jerde et al., 2016). Based on a field study conducted by Balasingham et al., (2017), Atlantic salmon eDNA water were released from holding tanks in a lotic system and they found a depletion in eDNA signal within 2 days, indicating that once the source is removed, long-term persistence of eDNA in flowing systems is unlikely due to dilution and degradation, which also prevail at spatial scales.

Spatial detection of eDNA is important because eDNA is likely to be transported quickly in lotic systems by water influx and flow rate. My results showed substantial spatial variation in Atlantic salmon eDNA detection signal strength along each stream, with obvious signal strength peaks and areas of no detection. However, my analysis of distance to the stocking sites and signal strength does not support a simple distance-decay model of eDNA flow and dilution since eDNA concentration was not statistically correlated with downstream transport of eDNA from stocking sites across all sampling periods. Although eDNA is expected to be transported downstream and subject to dilution and degradation, studies have generally found that eDNA reflects individuals (the source) present nearby (Spear et al." 201'; Wilcox et al., 2016; Balasingham et al., 2017). Many stocking sites were close to one another, about 3 to 10 km, which can explain the fluctuations in concentrations observed downstream. Generally, studies have compared upstream and downstream eDNA detections and found a functional decrease in eDNA concentration (Deiner & Altermatt 2014; Laramie et al., 2015; Jerde et al., 2016; Balasingham et al., 2017; Wood et al., 2020). These studies were able to detect eDNA from 30 m up to 12 km downstream but found no consistent relationship between detection and distance as they found variable concentrations as distance increased downstream from the source. The variability I observed in eDNA concentrations can be explained by the movement of a "breakout phase" at each release site, where large DNA fragments originated from sloughed cells and mixed into small fragments. Physical processes (i.e. water flow) disperse the smaller fragments downstream from the source, increasing the likelihood of detecting eDNA; however, after this point, detections

decrease due to dilution, degradation and cell settling (Barnes et al., 2014; Strickler et al., 2015; Wood et al., 2020). In this study, this process likely occurred numerous times due to the close proximity of the stocking sites resulting in overlapping plumes. eDNA should have been detected downstream because the addition of biomass has previously increased chances of detection (Dejean et al., 2011; Jerde et al., 2011; Thomsen et al., 2012). Several months after stocking, I predicted concentrations would be lower and less variable because this would have given individuals enough time to equilibrate in their new environment and colonize habitats elsewhere. An obstacle often seen in reintroductions is that released individuals reject habitats near release sites and travel long distances before acclimating, reducing their chances of survival (Stamps & Swaisgood 2007; Germano & Bishop 2009). Sampling further upstream and downstream should be included in eDNA sampling protocols as eDNA persistence can inform if individuals sought out novel areas to inhabit. Although eDNA cannot pinpoint the exact location of a target species, the key advantage of eDNA is that the precise location is not needed to identify their presence as quantified eDNA can be mapped to view the strength spatial distribution.

Detection of the fish community

The presence and distribution of native species with which reintroduced species can have unpredictable effects on reintroductions due to interspecific interactions. Introducing a species to an existing fish community can lead to competition, facilitation or nothing if there is no niche overlap. In my study, only a few significant co-occurrences

were detected. Atlantic salmon are frequently found sympatric with white suckers, which is seen in Credit River eDNA samples collected in May (Symons 1976). Symons (1976) observed the behaviour of Atlantic salmon and other stream fishes in an artificial stream and found that although Atlantic salmon preferred fast water habitats and white suckers preferred areas with slow flow, when both species occupied the same space, salmon were more successful in competing for space due to their aggressive behaviour. Generally, when similar species coexist, resource partitioning occurs to avoid overlapping of niches (diet, space and time). Atlantic salmon and brook trout frequently inhabit the same habitat because brook trout occupies a wider variety of habitat conditions, increasing the chances of salmon occupying the same area (Gibson 1966). Samples collected from Duffins Creek in May had Atlantic salmon and brook trout distributions that exhibited positive co-occurrence, indicating that stocked Atlantic salmon had acclimated to the same habitat conditions as brook trout. Mookerji et al., (2104) observed the feeding habits and diet of Atlantic salmon and brook trout. They found no difference in foraging times to avoid interspecific interactions but found that trout were generalist feeders (wider prey breadth) and salmon were specialized feeders (certain prey). This may reflect an adaptive response to coexisting through the trout exploiting a greater range of available resources. There was a positive co-occurrence between Atlantic salmon and brook stickleback in Duffins Creek in May; however, there is, to my knowledge, no published literature or previous known coexistence of the two fishes. Sympatric native species are not necessarily deleterious for the successful reintroduction of a species, but because of their lack of adaptation and experience with abundant generalist competitors,

the additional stress can make them vulnerable to predation and poor performance due to limited resources (Griffin et al., 2000).

Although the environmental conditions of Lake Ontario streams have improved, there are still significant differences compared to the historical conditions of Lake Ontario Atlantic salmon, such as the presence of non-native salmonids. The presence of non-native salmonids has previously been shown to limit the success of native salmonid reintroductions, threatening survival, growth, and reproduction through competition for resources and predation (e.g., Houde et al., 2015a, b). This is because native and non-native salmonids do not share co-evolutionary history and have not experienced selective pressures to evolve niches, resulting in competitive interactions (Coghlan et al., 2007). For example, the reintroduction of greenback cutthroat trout to streams in eastern Colorado were successful when non-native rainbow trout and brook trout were absent (Harig et al., 2000). Samples collected from Duffins Creek in October had Atlantic salmon and rainbow trout distributions that exhibited positive co-occurrences, inferring aggregation among the pair of species. Metabarcoding results also showed the presence of multiple non-native salmonids such as brown trout (*Salmo trutta*), Chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*), but they did not have any significant occurrence patterns with Atlantic salmon. Rainbow trout is known to be more aggressive than Atlantic salmon and share similar ecological niches (Gibson 1981; Hearn & Kynard 1986). Houde et al., (2015a) tested for competitive interactions between two strains of reintroduced Atlantic salmon and non-native salmonids and found that survival and fitness-related traits (length, mass, and condition) were reduced when Atlantic

salmon were in the presence of rainbow trout. Additionally, Coghlan et al., (2007) collected diet samples of the two salmonids and observed an overlap in diet when both salmonids were sympatric, with salmon feeding as specialists and trout feeding as generalists. If trout are generalist feeders, a positive co-occurrence with Atlantic salmon could possibly be due to trout feeding on salmon. Rainbow trout has a competitive advantage over reintroduced Atlantic salmon because of their prior residency and establishment of territories (Volpe et al., 2001). These studies collectively indicate that overlapping niches and competitive interactions with rainbow trout can potentially impede the establishment of Atlantic salmon.

The presence of Atlantic salmon eDNA using metabarcoding can be viewed as a semiquantitative proportion based on the total number of eDNA reads produced per site. The sequence read proportions provide a measure of eDNA signal strength to infer detection probability (Balasingham et al., 2018). The eDNA proportions among streams and sampling periods were generally low (less than 10%), indicating Atlantic salmon contributed little eDNA to the water samples relative to the other detected species, and most likely occurred at low densities. However, Atlantic salmon eDNA read proportions at some sites were higher than 30%, indicating a greater abundance of Atlantic salmon. In some cases stocking and neighbouring sites had higher eDNA read proportions than sites further away from the stocking site, although it was not consistent. This was the case during June sampling in all three streams and during October in Credit River and Duffins Creek. Released salmon after stocking in June likely settled near their release site because

of natal cues that possibly encouraged them to seek habitat of origin instead of novel areas (Stamps & Swaisgood 2007).

Conclusion

In this study, I extracted eDNA from filtered water samples to detect reintroduced Atlantic salmon in three Lake Ontario tributaries, before and after stocking using both qRT-PCR and metabarcoding. My qRT-PCR results provided evidence that Atlantic salmon eDNA is detected both before and after stocking, suggesting low dispersal in each stream. The uneven spatial distribution of the Atlantic salmon eDNA signal was consistent with areas of preferred salmon habitat, although other possibilities exists due to complex eDNA flow patterns in the lotic systems sampled. I found that Atlantic salmon eDNA detection was more sensitive using qRT-PCR (51% of samples with positive detections) versus metabarcoding HTS (18.3% of samples with positive detections), although this result may reflect the limitations of the metabarcoding primers. However, despite low detection success with metabarcoding, I was able to use the metabarcoding data of species composition to assess species co-occurrences, which reflects possible ecological interactions. Overall, my results based on the combined results of the eDNA detection platforms holds promise as a holistic monitoring tool for the management and conservation of reintroduced species. I recommend that eDNA studies involve continuous monitoring over multiple days and seasons to observe temporal distribution patterns because variable conditions can cause differences in eDNA persistence, affecting target species signals and community composition.

TABLES

Table 2.1 Primers and PCR conditions for PCRs used to create HTS library for metabarcoding eDNA.

	Primers (5' – 3')	**PCR cycles**	**T_A**
First PCR	**FishF1**; TCAACCAACCACAAAGACATTGGCAC **FishR1**; TAGACTTCTGGGTGGCCAAAGAATCA	30	52 ℃
Second PCR	**UniA-PS1F**; acctgcctgccg-TATTTGGYGCYTGRGCCGGRATAGT **UniB-PS1R**; acgccaccgagc-CARAARCTYATRTTRTTYATTCG	20	52 ℃
Third PCR	**UniA**; CATCTCATCCCTGCGTGTCTCCGACTCAGXXXXXXXXXXGATacctgcctgccg **UniB**; CCTCTCTATGGGCAGTCGGTGATacgccaccgagc	6	60 ℃

FIGURES

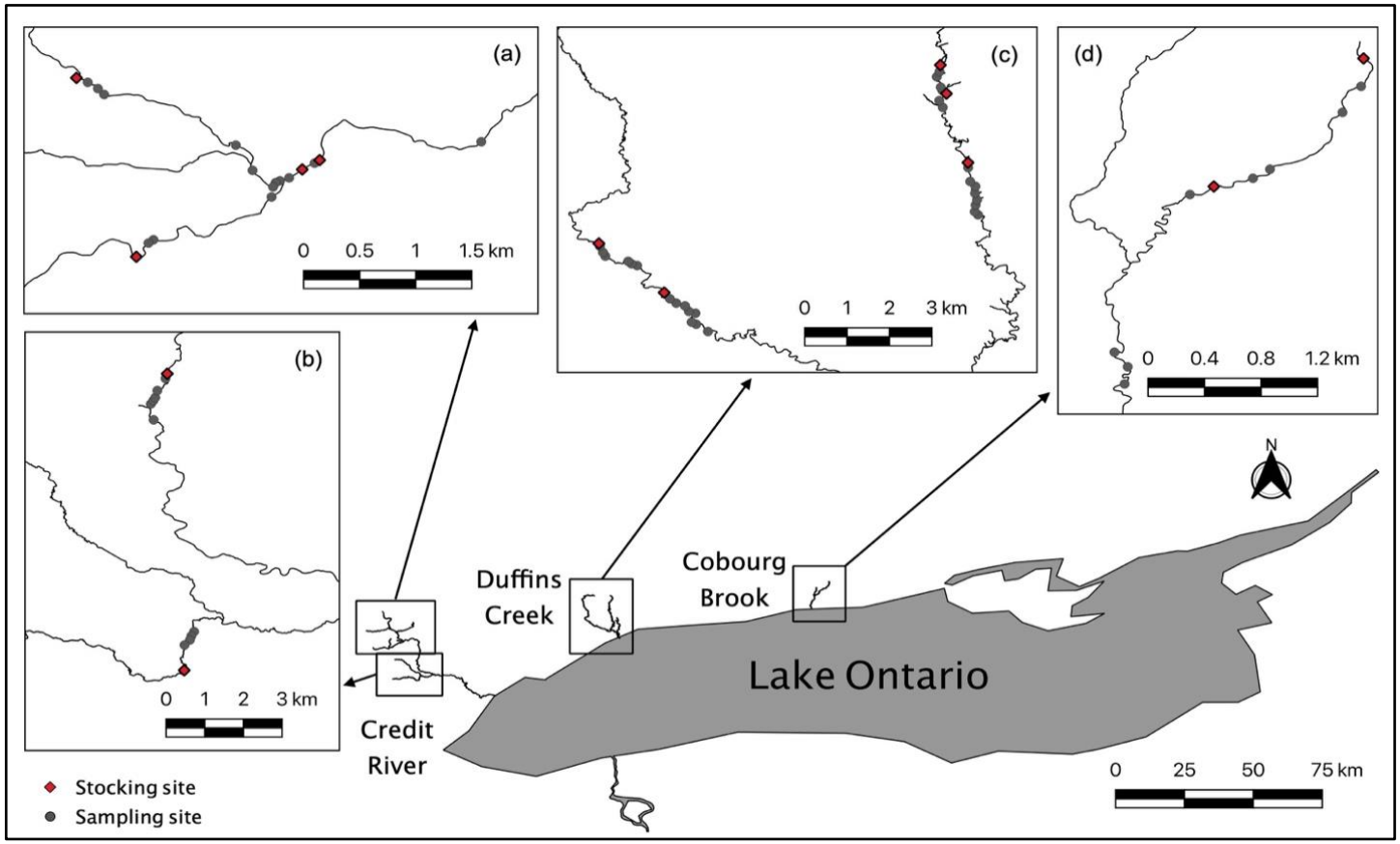

Figure 2.1 eDNA sampling sites in three Lake Ontario tributaries (Credit River, Duffins Creek and Cobourg Brook). Stocking sites (red diamonds) of Atlantic salmon spring juveniles were determined by the Ontario Ministry of Natural Resources (Credit River = 7 sites, Duffins Creek = 5 sites, Cobourg Brook = 3 sites). Sampling began 2 km downstream and moved upstream towards each stocking site. Sampling at these sites occurred at three different times: May (before stocking), June (weeks after stocking) and October (3 months post-stocking).

	Forward primer (5′-3′)	Reverse primer (3′-5′)
Atlantic salmon	TTCTCCTCCTGGCCTCATCT	AGGTAATCTTGCCCACGCAG
Brown trout	TTCTCCTCCTAGCCTCGTCT	CGGCAATCTTGCCCACGCAG
Brook trout	TACTTCTCCTGGCTTCGTCC	TGGGAACCTCGCCCACGCAG
Chinook salmon	TCCTCCTCCTATCTTCCTCT	CGGCAATCTAGCCCACGCAG
Coho salmon	TCCTCCTCCTATCTTCCTCT	CGGCAACCTCGCCCACGCAG
Lake trout	TCCTTCTCCTGGCTTCGTCC	CGGGAACCTCGCCCACGCAG
Rainbow trout	TCCTCCTCCTGTCTTCATCA	CGGCAACCTCGCCCACGCAG

Figure 2.2 COI primer sequence alignment of Atlantic salmon (NCBI ID: MK216626.1) with related salmonid species: brown trout (NCBI ID: EU524354.1), coho salmon (NCBI ID: KF918886.1), rainbow trout (NCBI ID: FJ999122.1), Chinook salmon (NCBI ID: KP720599.1), brook trout (NCBI ID: HQ960598.1) and lake trout (NCBI ID: KX145595.1). Highlighted nucleotides show base differences with *Salmo salar* primer sequence.

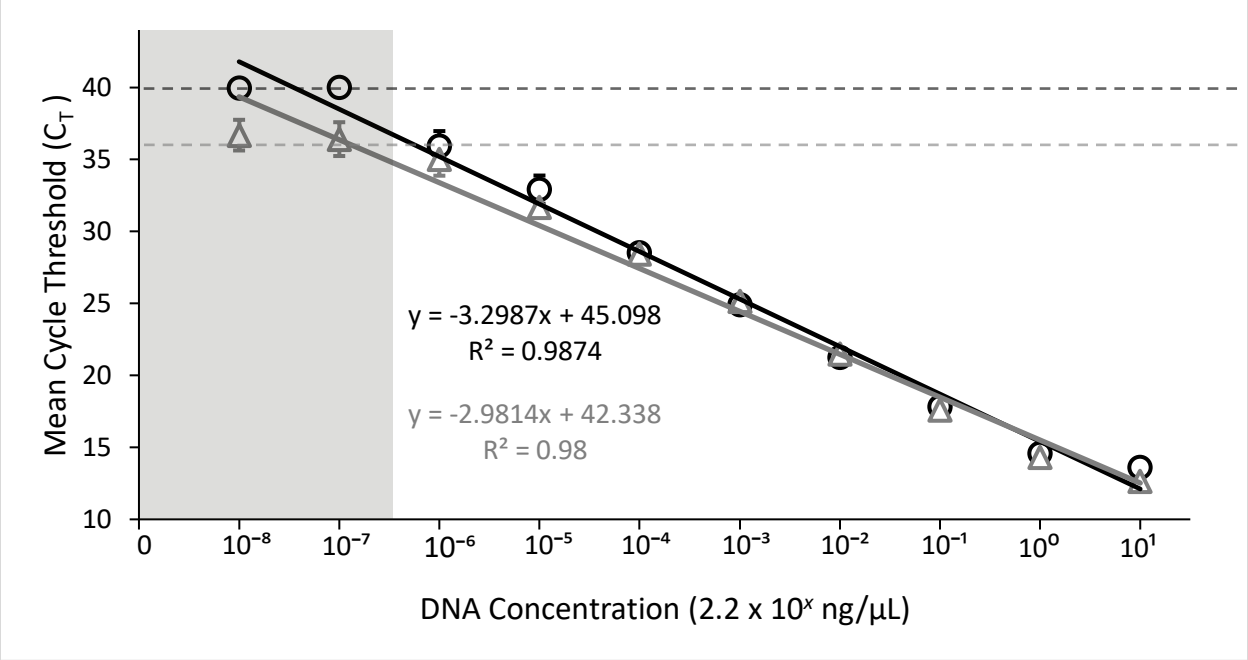

Figure 2.3 Results of the sensitivity and interference analyses for Atlantic salmon-specific qRT-PCR primers using SyBr green assays. The Atlantic salmon DNA was diluted with water (circles, black, solid line = senstivity) and field-collected eDNA (triangle, grey, dashed line = interference). All symbols show mean C_T ± 1.0 SEM. A ten-fold template DNA dilution series was used with reactions run in nine replicates at each dilution. Initial template DNA concentration was 22 ng/µL.

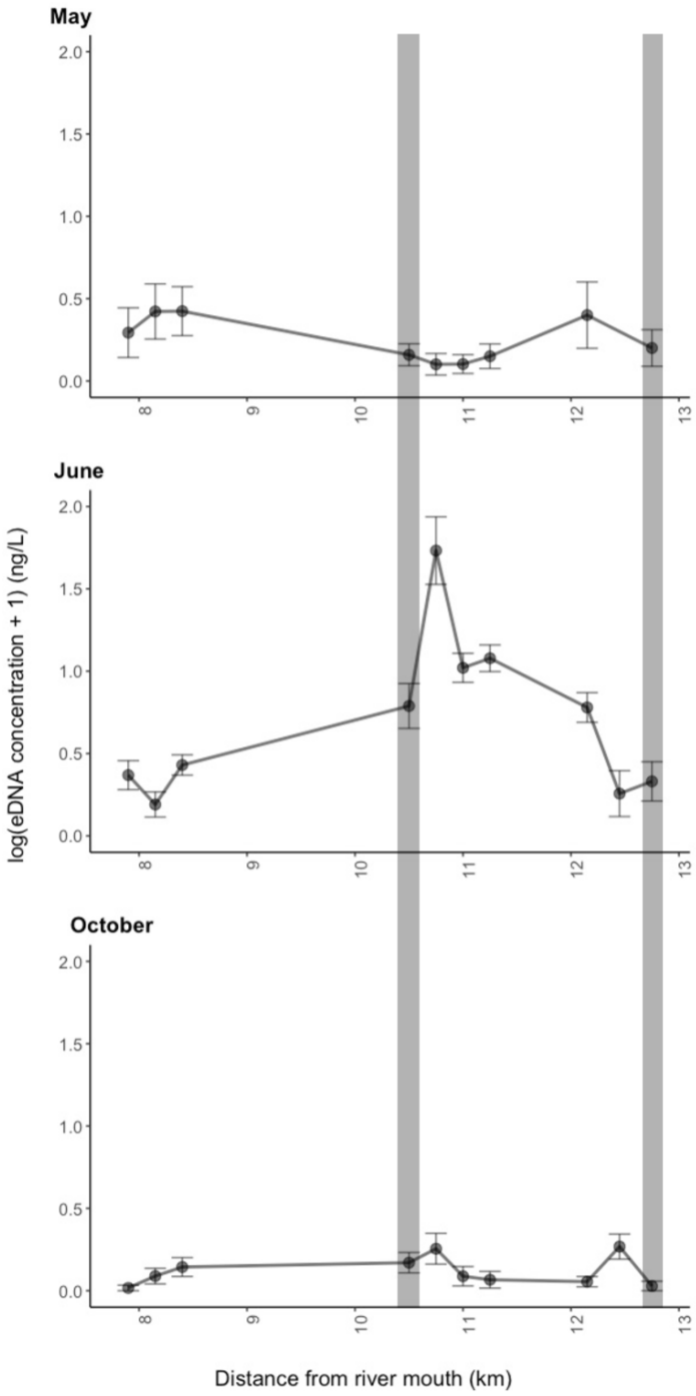

Figure 2.4 Variation in mean eDNA concentration (ng/L ± SEM) estimates of Atlantic salmon plotted against distance from the river mouth for sampling sites in Cobourg Brook during each sampling period. Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Average eDNA concentration of each site was calculated using all field and lab replicates of each site (9 values were used to calculate the mean).

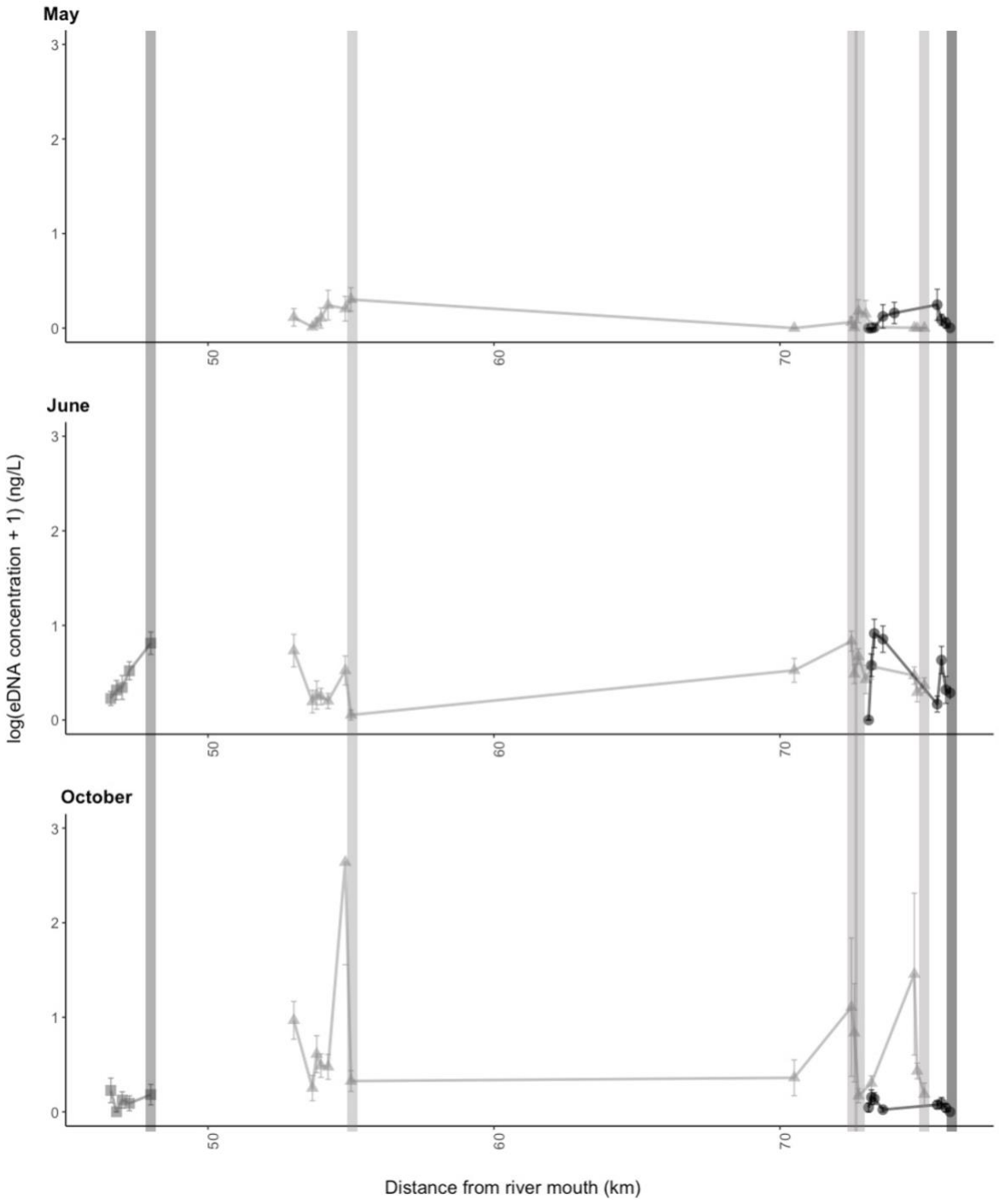

Figure 2.5 Variation in mean eDNA concentration (ng/L ± SEM) estimates of Atlantic salmon plotted against distance from the river mouth for sampling sites in Credit River during each sampling period. Lower Credit River (square, dark grey), Credit River (triangle, light grey) and Upper Credit River (circle, black). Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Average eDNA concentration of each site was calculated using all field and lab replicates of each site (9 values were used to calculate the mean).

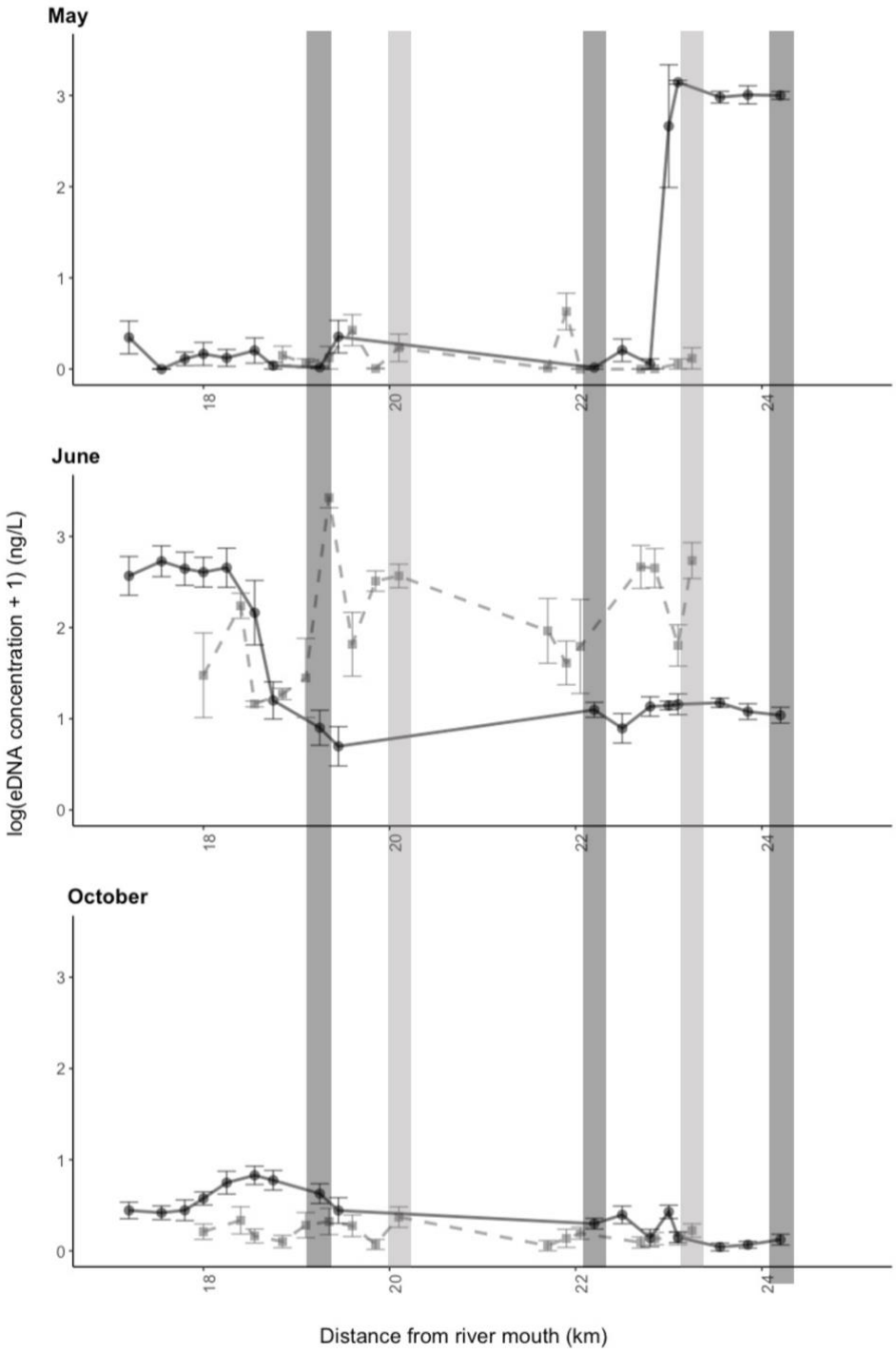

Figure 2.6 Variation in mean eDNA concentration (ng/L ± SEM) estimates of Atlantic salmon plotted against distance from the river mouth for sampling sites in Duffins Creek during each sampling period. East Duffins Creek (circle, black, solid line) and West Duffins Creek (square, grey, dashed line). Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Average eDNA concentration of each site was calculated using all field and lab replicates of each site (9 values were used to calculate the mean).

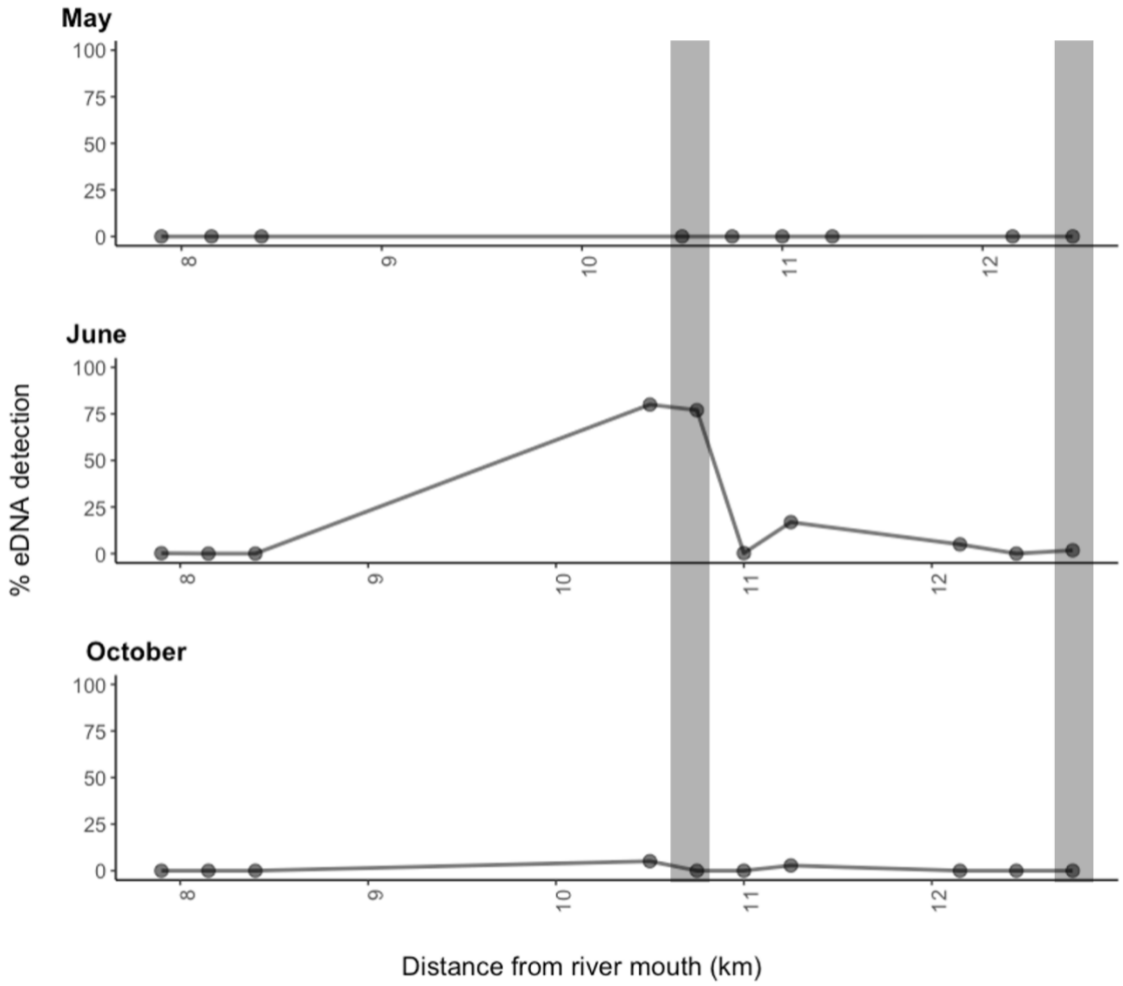

Figure 2.7 Metabarcoding sequence read proportion (%) of Atlantic salmon sequences relative to all sequence read numbers recovered from eDNA metabarcoding data at sampling sites in Cobourg Brook during each sampling period plotted against distance from river mouth. Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Percentage total of Atlantic salmon eDNA was calculated by taking the number of eDNA sequence reads of each species divided by the total number of reads from each site and multiplied by 100.

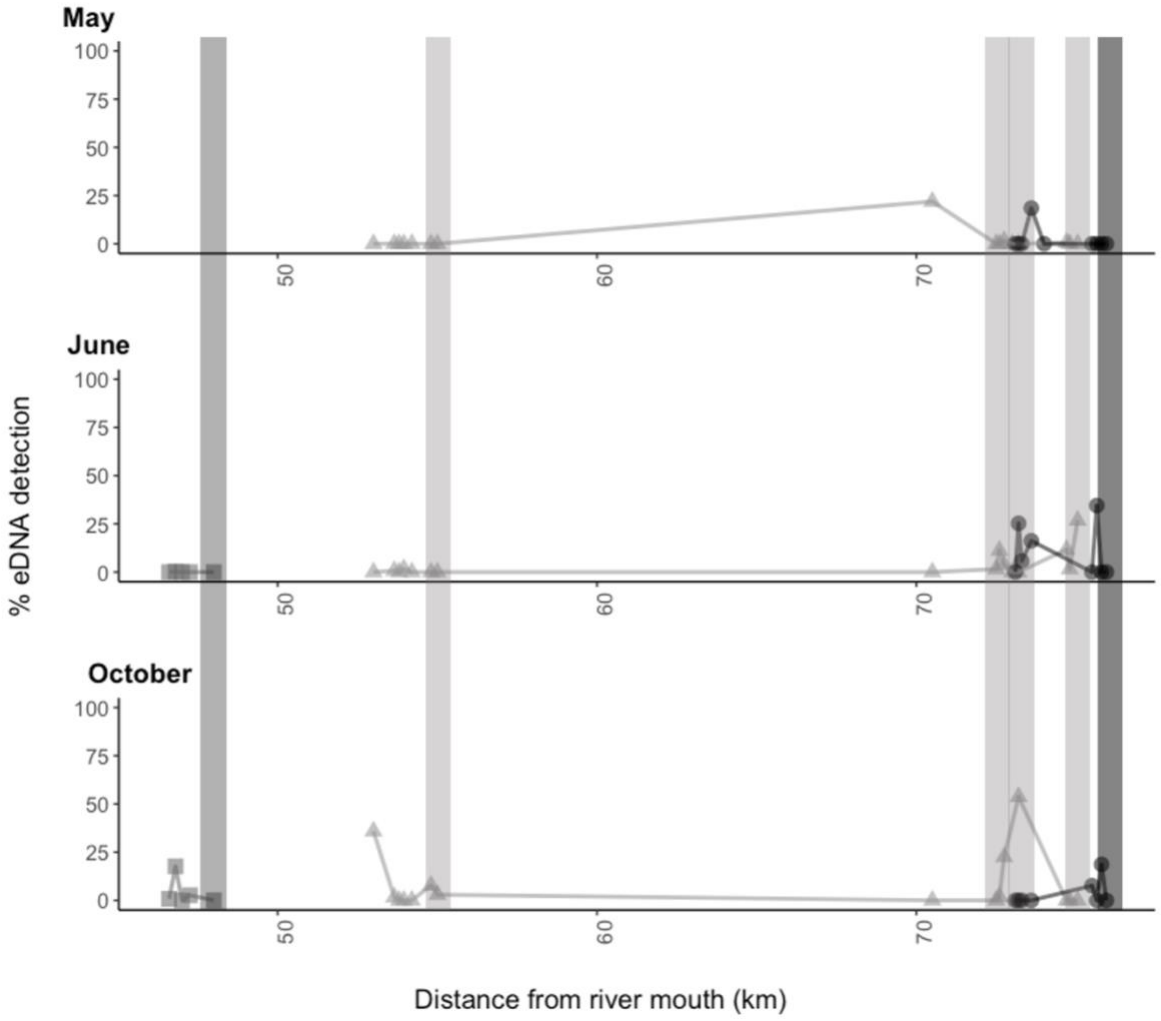

Figure 2.8 Metabarcoding sequence read proportion (%) of Atlantic salmon sequences relative to all sequence read numbers recovered from eDNA metabarcoding data at sampling sites in Credit River during each sampling period plotted against distance from river mouth. Lower Credit River (square, dark grey), Credit River (triangle, light grey) and Upper Credit River (circle, black). Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Percentage total of Atlantic salmon eDNA was calculated by taking the number of eDNA sequence reads of each species divided by the total number of reads from each site and multiplied by 100.

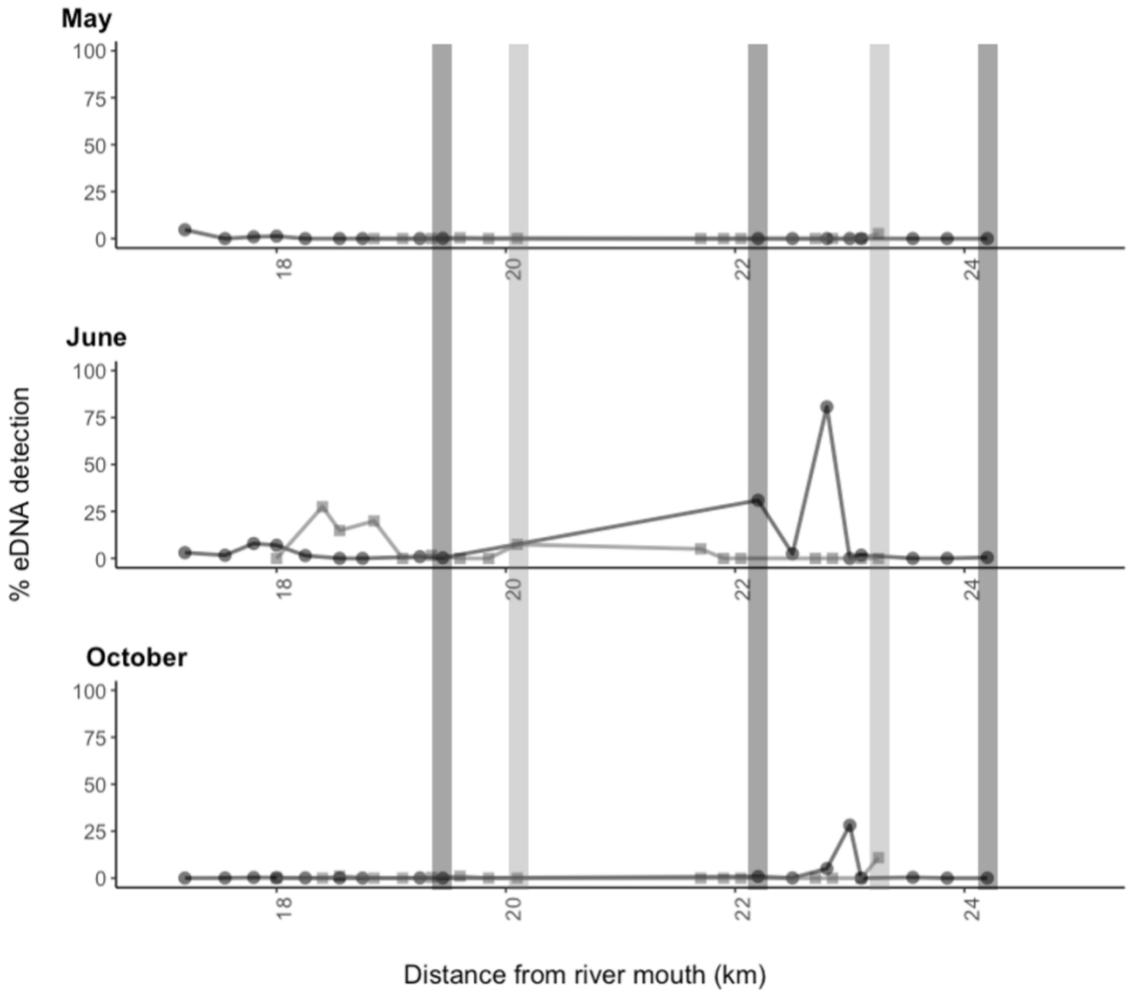

Figure 2.9 Metabarcoding sequence read proportion (%) of Atlantic salmon sequences relative to all sequence read numbers recovered from eDNA metabarcoding data at sampling sites in Duffins Creek during each sampling period plotted against distance from river mouth. East Duffins Creek (circle, black) and West Duffins Creek (square, grey). Shaded area indicates a stocking site chosen by the OMNRF to release Atlantic salmon juveniles. **Note:** Percentage total of Atlantic salmon eDNA was calculated by taking the number of eDNA sequence reads of each species divided by the total number of reads from each site and multiplied by 100.

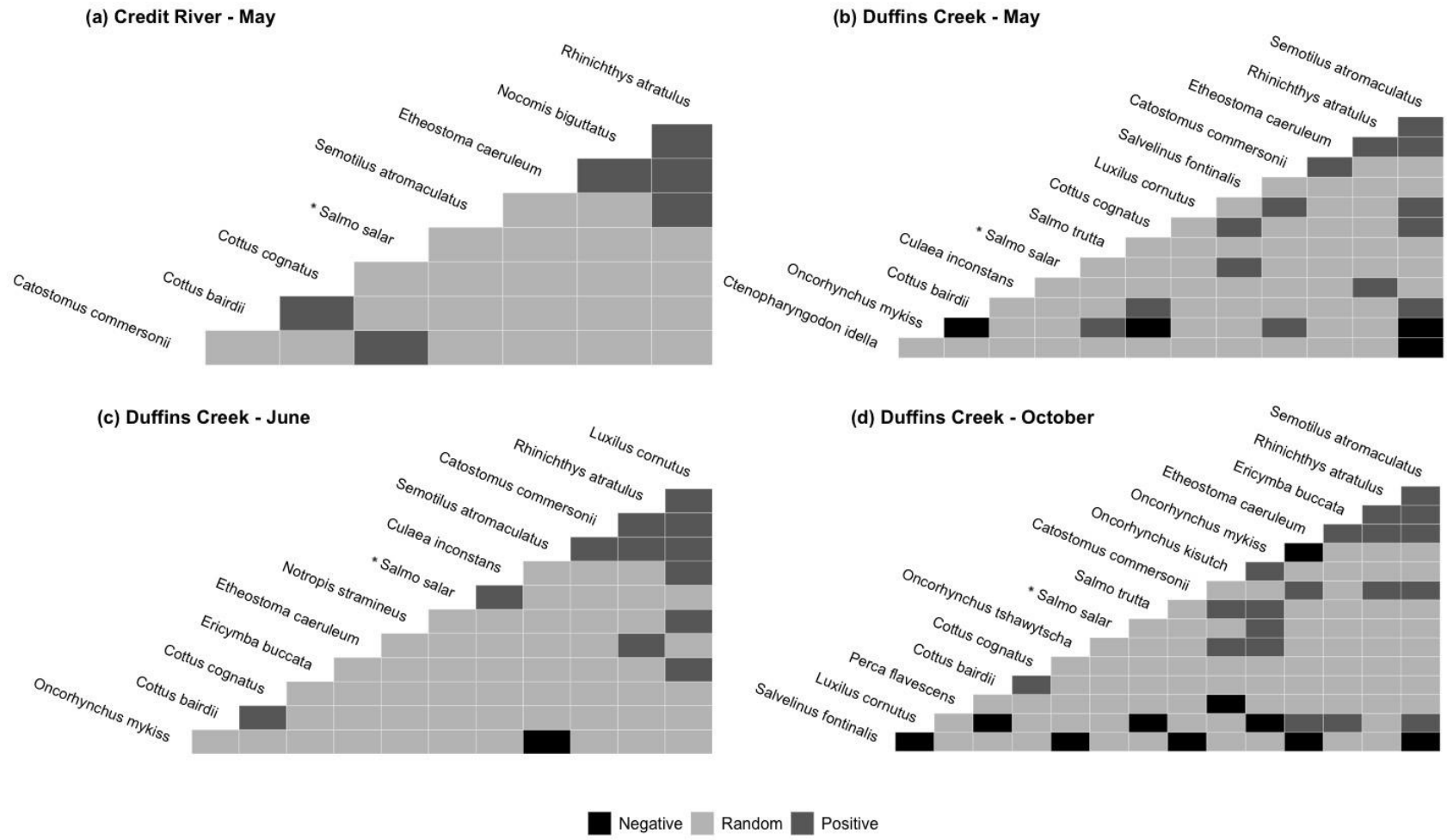

Figure 2.10 Pairwise cooccurrence patterns among key species (including Atlantic salmon). Note that only cooccurrence plots are shown for those which had significant effects for Atlantic salmon with at least one other species. Species that co-occur more frequently than by chance ($p < 0.05$) are positive interactions (dark grey) whereas species that co-occur less frequently than by chance ($p < 0.05$) are negative interactions (black). Random co-occurrences (light grey) reflect when species cooccurrence did not differ from random chance ($p > 0.05$). * indicates target species, Atlantic salmon (*Salmo salar).*

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CHAPTER 3

ABUNDANCE OF REINTRODUCED ATLANTIC SALMON (*SALMO SALAR*) IN LAKE ONTARIO TRIBUTARIES USING MICROSATELLITE ENVIRONMENTAL DNA (emDNA)

INTRODUCTION

As anthropogenic changes continue to persist in freshwater ecosystems, it is becoming increasingly important to monitor the consequences of those changes. The use of molecular genetic markers is becoming common in conservation and management as they can provide valuable information for monitoring species abundance and distribution. Tissue samples for DNA analyses from individuals are typically collected through invasive methods, but it can be difficult to obtain samples from elusive, cryptic, or protected aquatic species (Thomsen & Willerslev 2015). However, the analysis of environmental DNA (eDNA) has been established as a non-invasive genetic method for species detection (Ficetola et al., 2008; Thomsen et al., 2012). Sources of extra-organismal eDNA in the aquatic environment include blood, scat, urine, gametes, and shed skin (and other) cells (Bohmann et al., 2014). Although traditional eDNA analyses such as qRT-PCR and metabarcoding applications (see Chapter 2) are used to detect the presence of aquatic species, measure community diversity and estimate relative species abundance (Jerde et al., 2011; Thomsen et al., 2012; Yates et al., 2021), it cannot provide an estimate of the absolute number of individuals present (but see Yates et al., 2022). Information on target species abundance can provide insights into population decline, stability, or recovery,

perhaps informing conservation priorities. In the long run, a quantitative approach would yield a more reliable assessment tool (Bohmann et al., 2014).

Isolating mitochondrial and nuclear DNA from environmental samples has the potential to provide population-specific information (Bohmann et al., 2014). Studies that use eDNA commonly target mitochondrial DNA (mtDNA) because there are several copies of the mitochondrial genome in each cell and because it degrades at a slower rate than nuclear DNA (Bylemans et al., 2018). However, mtDNA provides only limited insight into the dynamics and evolutionary history of a population due to acting as a single evolutionary locus (Sigsgaard et al., 2020). Although mitochondrial eDNA markers can improve detection probability, eDNA can potentially include sufficient nuclear DNA for a more detailed genetic survey of eDNA. Nuclear DNA is commonly used in genetic studies as it provides more robust data on divergence within and among populations than mtDNA (Adams et al., 2019). Specifically, nuclear microsatellite DNA markers provide the opportunity to differentiate among individuals even those with related genetic signatures (Wheat et al., 2016; Monge et al., 2018).

Microsatellite marker data used in traditional population genetic studies provide information on genetic diversity, effects of inbreeding, gene flow, and population structure within and among sample sites (Selkoe & Toonen 2006). However, it can also be used as a non-invasive monitoring tool, and with highly variable allele frequencies, emDNA can provide estimates of individual genotype numbers. For example, Wheat et al., (2016) was able to detect the number of brown bear (*Ursus arctos*) individuals present

within a sampling area via DNA extracted from scat droppings and residual saliva on consumed salmon genotyped at microsatellite markers. Similarly, Monge et al., (2018) applied microsatellite markers using saliva DNA isolated from almond fruits eaten by scarlet macaws (*Ara macao*) to determine the individual's sex using sex-specific markers. A different application of microsatellite eDNA markers has been shown to estimate the number of yellow perch (*Perca flavescens*) prey present in a pool of DNA extracted from predator stomach contents (Carreon-Martinez et al., 2014). Recently, eDNA studies in fish species have explored on the potential for emDNA analyses; for example a mesocosm and field sample analysis of emDNA in round gobies (*Neogobius melanostomus*; Andres et al., 2021). Although emDNA is still being develop as a fishery and conservation tool, it is theoretically possible to use microsatellite eDNA markers to determine the minimum number of individuals of a target species in an eDNA sample.

The objective of this study was to determine if eDNA collected from freshwater samples is an effective and feasible source of template for microsatellite DNA genotyping, and ultimately, for estimating abundance. I used reintroduced Atlantic salmon (*Salmo salar*) in Lake Ontario tributaries as a model system to assess feasibility. I used qRT-PCR says to identify sites with Atlantic salmon present, then genotyped the eDNA with microsatellite DNA markers and used a microsatellite allele counting approach to quantify the number of Atlantic salmon contributing to each sample. While considerable additional resercah and development will be needed to optimize this approach, my study shows the potential of emDNA to utilize the additional information content in eDNA for population genetic applications.

MATERIALS AND METHODS

Sampling Site and Water Sampling

About 890,000 hatchery-reared Atlantic salmon were stocked into Lake Ontario tributaries in 2018 during three time periods: ~450,000 spring yearlings (early April), ~450,000 spring juvenile (early May to June) and ~115,000 fall juveniles (October). Stocking rivers included three Lake Ontario tributaries: Credit River, Duffins Creek, and Cobourg Brook specific stocking sites within these tributaries were selected by the OMNRF (see Chapter 2). Water for eDNA was sampled at three different times before and after spring juvenile stocking period: before stocking (5, 6, and 7 May), post-stocking (23, 24 and 25 June), and three months post-stocking (7, 8 and 9 October).

Surface water samples were collected as described in Chapter 2, those same samples were used here. Briefly, sample sites were approximately every 100 to 250 m apart over ~2 km of the stream, downstream of the top release site. Sampling started downstream and moved upstream towards stocking sites, wading into the stream at each site and reaching upstream to collect the sample. Three samples (field replicates) were collected at each site, for a total of 618 samples. Nine field blank controls were taken, one for each sampling day, to confirm sterility of sampling equipment.

Filtration and Extraction

As described in Chapter 2, water samples were filtered within 24 hours of collection using 1.2 µm pore size, 47 mm diameter glass microfiber filters (Whatman®, Maidstone, UK). After filtration, each filter was cut in half using sterile forceps and scissors

and were placed in 2 mL storage tubes filled with RNAlater and stored at -20 °C until further processing.

For DNA extraction, half filters were rinsed with ddH_2O to remove residual RNAlater and placed into 2 mL screwcap tubes containing 400 µL of 1.0 mm glass beads (BioSpec Cat. No. 11079110). The DNA extraction method followed is described in Shahraki et al., (2018; see Chapter 2).

Detection of Atlantic salmon

qRT-PCR assay

A species-specific qRT-PCR assay was used to determine which samples contained Atlantic salmon DNA before applying microsatellite markers (see Chapter 2). I used the results from Chapter 2 to identify detection positive samples, following the conservative detection criteria described there.

Microsatellite eDNA

Primer Modification and PCR Amplification

All eDNA samples that had positive hits for Atlantic salmon were PCR amplified at three Atlantic salmon microsatellite DNA loci: *Ssa*A86, *Ssa*A119 and *Ssa*A124 (King et al., 2005). The published microsatellite marker primers were modified by designing new primers closer to the repeat sequence (to reduce the amplified flanking region of the sequence to target a smaller fragment (~ 250 bp) due to the degradation of eDNA in lotic systems; Rees et al., 2014). Multiple modified primers were designed for each locus and

each were tested with eDNA samples containing Atlantic salmon to determine which primer pairs amplified DNA (Table 3.1).

I used my extracted eDNA as template to amplify the three microsatellite markers using a two-step PCR process. The first pre-amplification PCR consisted of 2.5 µL of 10x Taq reaction buffer, $MgSO_4$ (volume optimized for each locus, Table 3.1), 200 µM of each dNTP, 200 nM of forward and reverse primers, 0.5 U of Taq polymerase (Bio Basic Canada Inc., Markham, ON, Canada) and 2 µL of eDNA template, for a total volume of 7.6 µL. The thermal cycling conditions were: an initial denaturation at 94 °C for 2 minutes, followed by 15 cycles of 3 step-cycling, including denaturation at 94 °C for 40 seconds, annealing at optimized temperatures for each locus (Table 3.1) for 40 seconds and extension at 72 °C for 1 minute, ending with a final extension at 72 °C for 5 minutes. The second PCR consisted of the same reagents described above, plus 1 µL of first PCR product and ddH_2O, for a total reaction volume of 25 µL. The second PCR thermocycling conditions were the same as above, but the 3 step-cycling ran for 30 cycles.

Library Preparation and Sequencing

Second-round PCR products for the three microsatellite loci were combined within sample and cleaned with Sera-Mag Magnetic Beads (GE Healthcare Life Science, UK) to remove primer dimers and fragments less than 100 bp. The cleaned PCR product was used as a template for a third, short-cycle PCR to ligate the adapters and individual barcode sequences for HTS library preparation (He et al., 2017). Ligation PCRs consisted of 2.8 µL of ddH_2O, 2 µL of 10x Taq reaction buffer, 2 mM $MgSO_4$, 200 µM of each dNTP, 0.5 µM

Uni B primer, 0.5 µM Uni A primer, 0.5 units of Taq polymerase and 10 µL cleaned PCR product, for a total of 20 µL reaction. Thermocycling conditions used for the barcoding PCR were: 95 °C for 2 minutes followed by 7 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, ending with 72 °C for 5 minutes. The barcoded PCR products were pooled, gel-extracted with a GenCatch Gel Extraction Kit (Epoch Life Science Inc.). To assess the size and concentration of the products, the pooled amplicons were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and diluted to 55 pmol/µL. The diluted library was then sequenced on an Ion GeneStudio S5 Next Gen Sequencer (Thermo Fisher Scientific) to determine microsatellite allele sequence and allow repeat number determination.

Genotyping and Data Analyses

I used Quantitative Insights into Microbial Ecology (QIIME I) software to demultiplex sequence data by individual eDNA samples and separate microsatellite marker sequences based on locus primer sequences, using the split libraries script (Caporaso et al., 2010). R Studio v. 1.2.5033 was then used to run NGS-usat (accessed at https://github.com/denisroy1/NGS-usat), to determine the number of alleles in each sample. The R-based script counts the number of repeat motifs within each sequence and creates frequency-based plots on the number of repeats (x-axis) versus the number of reads (y-axis). Genotypes were automatically generated; however, I verified the number and size of the alleles (peaks) visually.

An allele counting bootstrap method (as described in Carreon-Martinez et al., 2014) used known Atlantic salmon microsatellite genotypes (dataset provided by Dr. C Wilson, Ontario Ministry of Natural Resources and Forests) to estimate the number of individuals at each sample site. Briefly, 25 OMNRF genotypes were selected at random for each locus and the relationship between the number of genotypes (fish) and the number of unique alleles observed was generated. This method was (bootstrapped) replicated 25 times and the outcome (number of unique alleles) averaged across the 25 replicates for each locus (Figure 3.1; Supplementary Table 3.1). This relationship was used to calculate the average number of Atlantic salmon present in eDNA samples based on the number of microsatellite alleles detected in each emDNA sample. The number of fish estimated at each locus was averaged across the three loci to estimate the total number of Atlantic salmon contributing to each eDNA sample. Samples that did not amplify at all three loci were rejected as failed PCR.

The effect of sampling date on mean eDNA concentrations was analyzed using univariate GLM analyses within each stream. To test for temporal and spatial differences in the estimated numbers of Atlantic salmon detected, non-parametric Kruskal-Wallis tests were used to test for differences among the three sampling periods (temporal) and at the sample sites within each stream (spatial). If a significant effect was detected, unplanned pairwise comparisons were carried out using Dunn's multiple comparison tests with Bonferroni correction to determine which levels of independent variables differ from other levels and account for multiple simultaneous significant tests.

Additionally, the allele frequency for all three microsatellite markers per sampling stream were visualized semi-quantitatively as the number of eDNA sequences for each repeat motif divided by the total number of eDNA sequences returned. The total frequency of each eDNA sequence was obtained by combining all the eDNA microsatellite repeat sequences for each stream. This method illustrates the frequency of alleles at each stream based on how many sequences were returned for each repeat motif. If there is a high allele frequency at a specific number of repeats, this indicates the allele is common in the population. Non-parametric Kruskal-Wallis tests were used to investigate differences between the microsatellite allele frequency distributions of each stream across the three sampling periods.

RESULTS

A total of 192, 215 and 211 eDNA field samples were analyzed using the Atlantic salmon specific qRT-PCR assay for samples collected in May, June and October, respectively (Chapter 2). Of the 618 samples, 33, 180 and 106 field samples tested positive for Atlantic salmon eDNA using the species specific qRT-PCR at each of the 3 sampling periods, respectively (Chapter 2). These 319 samples were selected for further microsatellite eDNA analyses.

Tandem repeat of 3 motifs or less were not included in my analyses since amplification of short dinucleotide motifs are prone to errors and non-specific amplification, resulting in high likelihood of PCR artefacts. The observed microsatellite alleles from the emDNA were compared to the cumulative allele curves derived from

OMNRF Atlantic salmon genotypes to determine a predicted number of contributing salmon. Those individual counts were averaged across the three loci (Figure 3.1). Of the 319 samples analyzed, 68.3% (218 samples) were successfully genotyped and assigned an estimated number of individuals (Table 3.2). Of the remaining samples, 24.5% (78 samples) failed to produce results at all three loci and 7.2% (23 samples) resulted in the detection of 1 individual at one locus giving an average of 0 fish. The probable number of Atlantic salmon at each sample site for the 3 seasonal samples were used to draw frequency of occurrence maps (Figure. 3.2). The mean number of Atlantic salmon per successfully genotyped eDNA sample was estimated among the sampling periods in each stream (Figure 3.3).

The results of the univariate analyses indicated that there was a significant difference in the mean estimated number of fish in the Credit River ($F = 12.9$, d.f. = 2, $p < 0.05$) and Duffins Creek ($F = 14.8$, d.f. = 2, $p < 0.05$) across the three sampling dates; however, there was no significant effect in Cobourg Brook ($F = 0.4$, d.f. = 2, $p = 0.7$). Kruskal-Wallis tests between sampling time and stream were conducted for subsequent analyses (Table 3.3). Among the three sampling dates, a difference in fish abundance among streams was only observed in June. Pairwise comparisons using Dunn's test indicated that Duffins Creek abundance estimates were significantly different from Cobourg Brook ($P = 0.027$) and Credit River ($P < 0.0001$). Moreover, there were significant differences in fish detection between sampling periods at Credit River and Duffins Creek sites. Additional Dunn's tests indicated samples collected in June were significantly

different from October collection for both Credit River ($P < 0.0001$) and Duffins Creek ($P < 0.0001$). No other differences were statistically significant.

Of the 319 samples PCR amplified, 59.6% were genotyped with *Ssa*A86, 24.1% with *Ssa*A119 and 44.2% with *Ssa*A124. Figure 3.4 shows the frequency of alleles with different CA repeat lengths for each stream at the three loci analyzed, per sampling period. Using the Kruskal-Wallis test, I found that the distributions of allele frequencies at locus *Ssa*A119 were significantly different between the three stocking streams in October, while allele frequencies at locus *Ssa*A124 varied significantly among streams in June and October.

DISCUSSION

My study represents a preliminary quantitative test of emDNA applications in a reintroduced population of Atlantic salmon. While previous studies have taken samples directly from individual remains (e.g. feeding traces, fecal matter or hair) to trace samples to specific individuals (Gillett et al., 2008; Wheat et al., 2016; Aylward et al., 2018; Monge et al., 2018), the present study relies on samples collected from the environment (i.e. eDNA) to identify allele frequency distributions. To my knowledge, this is the first application of emDNA analyses to a conservation goal.

I was able to generate an estimate of the number of Atlantic salmon per sampling period across the three streams. I did not find a significant effect of sampling date on estimated fish numbers in Cobourg Brook, perhaps reflecting the lower sample size for Cobourg Brook. Since some statistical tests such as the Kruskal-Wallis test are sensitive to

small sample sizes, it would be helpful to increase the number of samples in future studies to increase the number of positive detections and hence increase statistical power.

With my abundance estimates dispersed along the sampling transects of each stream, it would be difficult to determine the location of individual Atlantic salmon due to the complex dispersion of eDNA (and emDNA) in lotic systems (Barnes et al., 2014; Strickler et al., 2015). The number of individuals at downstream sites was generally low compared to the sites upstream, it is unlikely that emDNA flow contributed substantially to my counts. Some upstream sites had estimates of more than 6 individuals, indicating likely suitable habitat for juvenile Atlantic salmon. This suggests that released salmon may not be straying too far downstream, although I cannot rule out eDNA flow and dilution contributing to the low fish counts downstream. It is important to note that although I was able to estimate the number of fish at each site, the estimates do not account for fish detection at multiple sites.

Limitations and recommendations

Results from this study demonstrate that aquatic eDNA samples can be used for more than species-specific detection and metabarcoding, that the information content of eDNA can be explored using hypervariable DNA sequences or perhaps other loci. The application of emDNA allele counting could provide a useful framework to quantify fish distribution; however, large population sizes would tend to swamp the allele counting approach (i.e., the observed number of alleles may fall above the plateau of the fish number to allele number relationship). As this is a novel approach using eDNA samples

for individual count estimates, there are some limitations and recommendations. First, eDNA samples contain a mixture of DNA material from many different organisms present in the environment, which may interfere with the detection of a target species that may exist at low abundance relative to others. Small amounts of target emDNA may be collected, limiting genotyping and population estimation. Alternatively, large amounts of non-target DNA present in eDNA samples could intefere in the amplification of microsatellite markers (Gillett et al., 2008). In this study, only 1.5 L (three 500 mL samples) of water were collected per site. Perhaps for future studies, more water, at least 3 to 6 L, should be collected per site to increase the likelihood of obtaining detections.

My genotyping success rate for emDNA was ~68%. While this is a promising level of success, I feel it could be improved on. For example, a better, microsatellite DNA optimized, DNA extraction method could improve the amplification efficiency of microsatellite markers. Although the sucrose lysis-robotic bead method used in this study is known to be quick, safe, environmentally friendly and high-throughput (Shahraki et al., 2019), eDNA extraction protocols using cetyltrimethylammonium bromide (CTAB) and phenol-chloroform-isoamyl alcohol (PCI) have been shown to increase the yield of eDNA (Barnes et al., 2014; Hunter et al., 2019; Shahraki et al., 2019). The development of targeted eDNA extraction methods is needed to maximize the recovery of microsatellite eDNA and its quality. Secondly, the size of the amplicon may also be limiting genotyping success. Markers *Ssa*A86 and *Ssa*A124 had moderate amplification success, considering the primers were modified to produce a smaller amplicon to accommodate possibly degraded emDNA. Alternatively, the marker *Ssa*A119 was not modified and had the

lowest amplification and scoring success. The long size of the marker could have affected the amplification of eDNA. For future studies, microsatellite primers should be redesigned to amplicons of less than 200 bp to amplify degraded eDNA and improve PCR reactions (Rees et al., 2014; Monge et al., 2018). Another possibility for low genotyping success is the lack of microsatellite loci used. The individual counts using the allele counting model were generally inconsistent among the markers for each sample. Only three markers were used in this study, however, other population genetic studies use multiple markers to target other parts of the genome to improve the robustness of population diversity estimates (Selkoe & Toonen 2006). For this reason, additional markers should be used when analysing eDNA samples to increase the success of genotyping and assigning number of individuals to samples (Adams et al., 2019). Lastly, because emDNA targets nuclear DNA, it is possible that the nuclear eDNA present was either too dilute or too degraded (or both) for reliable PCR amplification, explaining why the overall low estimates of fish numbers. Furthermore, as is the case for eDNA, the persistence of emDNA would have been affected by dilution, water chemistry, temperature, UV radiation, and microbial activity (Shogren et al., 2017; Bylemans et al., 2018; Tillotson et al., 2018).

One important issue for emDNA is that as eDNA samples contain DNA from multiple species, including possibly closely-related species, it is possible that emDNA alleles may reflect other closely related species present in the system (for example, brown trout *Salmo trutta*). For future studies, researchers should ensure that PCR replicates give

consistent results or use species-specific markers to reduce overlap among the species (Wheat et al., 2016).

Lastly, microsatellite markers occur less often in the environment than mitochondrial markers because of factors such as degradation of DNA resulting in smaller fragments, reducing detectability (Strickler et al., 2014; Adams et al., 2019; Sigsgaard et al., 2020). When low DNA quantity and quality of microsatellite eDNA are obtained to count individuals, there are challenges during the amplification process which bias estimates (Taberlet et al., 1999). These include the presence of false alleles due to missing alleles in samples and allelic dropout due to the loss of allelic variation (Taberlet et al., 1996; Taberlet et al., 1999; Adams et al., 2019). Because target eDNA is diluted in a mixture of non-targeted DNA, errors in PCR can amplify non-specific DNA and be misinterpreted as true alleles. This is especially the case when dinucleotide microsatellites are used (Taberlet et al., 1999). This perhaps explains the high frequency of shorter allele repeats as a result of non-specific products being represented at markers *Ssa*A119 and *Ssa*A124. Additionally, amplified diluted microsatellites can result in poor to no amplification of multiple alleles that could be present in eDNA samples, reducing allelic diversity and abundance estimates. I increased the number of PCR cycles which may have amplified non-specific products and reduced allele variation, hence low allele counts. I also noticed multiple samples had positive detections using the qRT-PCR assay, but PCR assays with microsatellite markers failed. Therefore, using trinucleotide or tetranucleotide microsatellite markers and running samples in replicates can often mitigate these challenges and possibly provide reliable genotypes of eDNA samples

(Taberlet et al., 1996; Taberlet et al., 1999). Furthermore, the R package I used to genotype samples is commonly used to genotype a single diploid organism, limiting the detection of allelic variation in eDNA. Developing statistical programs specific to analysing eDNA data or having a stricter allele scoring criteria could reduce the chance of scoring a false allele at each locus (Gillett et al., 2008).

Conclusion

My study used a novel and simple molecular genetics application of microsatellite allele counting to quantify Atlantic salmon present at sites where eDNA was collected. The combination of a qRT-PCR assay for presence and absence detection coupled with microsatellite analysis for estimating species numbers provides a promising, rapid non-invasive tool in conservation for quantitative estimates of abundance over traditional monitoring studies. Compared to the thousands of fish released, it is noteworthy that the number of Atlantic salmon estimated using the microsatellite allele counting model is an underestimate likely due to the multiple factors that degrade eDNA and the sampling protocol. Future eDNA studies need to include microsatellite markers to diversify our understanding of population genetics through a non-invasive approach. This type of analysis can only provide a minimum number of fish detected, but the accuracy should increase if more microsatellite markers are used. Microsatellites provide a greater resolution of population structure than mitochondrial markers that are commonly used in eDNA studies and have the advantage of recovering unique traces of alleles to identify the number of individuals in a sample. This study provides a useful monitoring framework

for the conservation and management strategies of other species to quantify the abundance and determine distribution based on the aggregation of species.

TABLES

Table 3.1 Characteristics of primers used for amplification of microsatellite markers selected for genotyping *Salmo salar* in eDNA samples: locus name, modified primer sequences, repeat motif, PCR conditions ($MgSO_4$ and annealing temperature), approximate amplicon size and GenBank accession numbers.

Name	Primer sequence (5' - 3')	Motif	$MgSO_4$ (µL)	T_A	Amplicon Size (bp)	Accession no.
*Ssa*A86	**F**; CCCAGTGGTTCTAGATGAGTGA **R**; GCCTCTCCCACCTCCAAT	CA	2	58 ℃	173	AF525200
*Ssa*A119	**F**; TCTGGAAGTTTCCCTACTTCTG **R**; TCTTTAACTGTTGCCTTAACGAC	CA	2.5	50 ℃	216	AF525201
*Ssa*A124	**F**; CTCCTGCACCTGACTTCTATTC **R**; ACTGGGCCACAGGCTATCAC	CA	2.5	58 ℃	211	AF525202

Table 3.2 Number of eDNA samples with positive Atlantic salmon (AS) DNA detections, the number of samples successfully genotyped and the estimated number of fish in relation to the observed number of alleles at each stream sampled over the 3 sampling periods.

	AS positive samples	Successfully genotyped	Estimated no. fish
May			
Cobourg Brook	8	5	8
Credit River	3	2	2
Duffins Creek	22	18	36
June			
Cobourg Brook	23	12	28
Credit River	60	28	27
Duffins Creek	97	74	109
October			
Cobourg Brook	10	7	10
Credit River	40	29	72
Duffins Creek	56	43	115

Table 3.3 Significance of sampling period (between streams) and stream (between sampling periods) on the number of Atlantic salmon detected per eDNA sample collected from Lake Ontario tributaries. Bolded P values are significant.

	χ^2	*P*-value
Sampling Period		
May	2.21	0.33
June	26.98	**1.39e-06**
October	5.45	0.066
Stocking Stream		
Cobourg Brook	1.63	0.44
Credit River	22.18	**1.53e-05**
Duffins Creek	17.85	**0.00013**

FIGURES

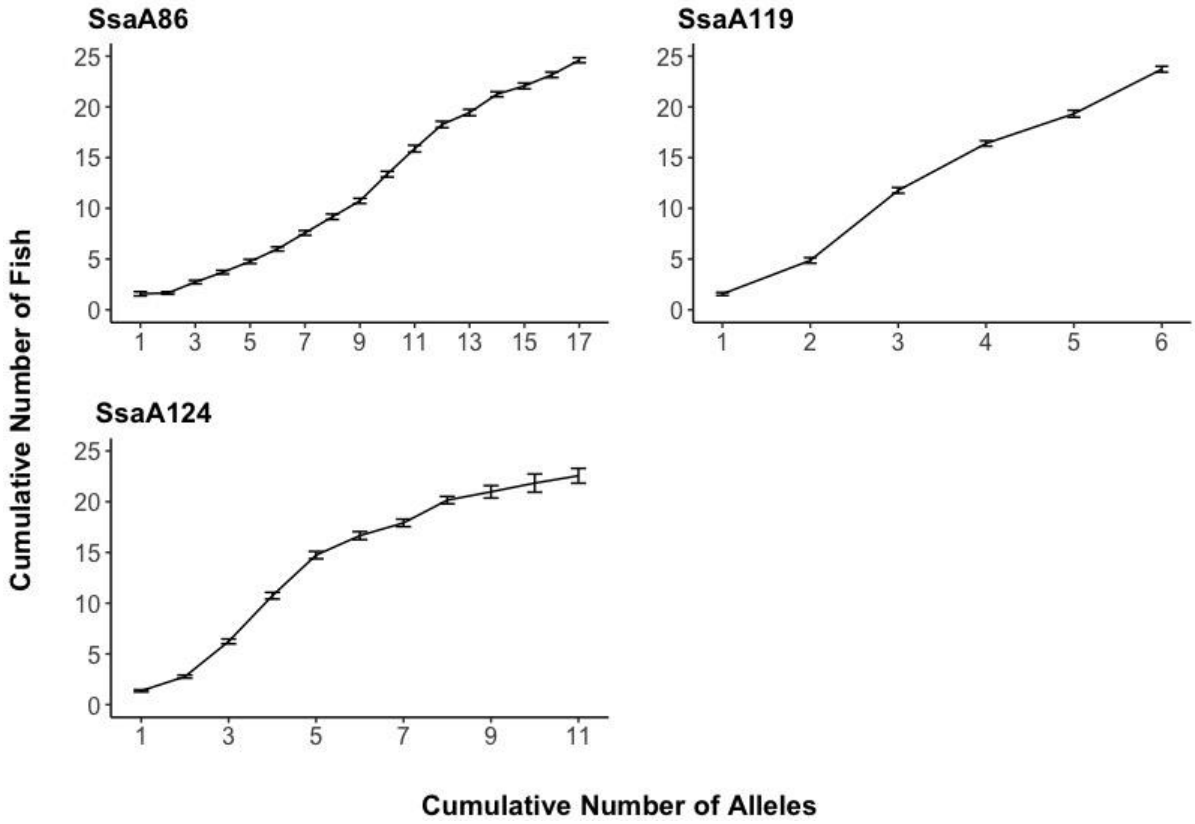

Figure 3.4 Relationship between the cumulative number of alleles detected in the genotypes of adult broodstock OMNRF Atlantic salmon (X-axis) and the cumulative numbers of fish (genotypes) selected for the three microsatellite loci. Note that random draws of genotypes from the OMNRF genotype database were replicated 25 times and the mean and SEM of those 25 replicates are shown on the graph.

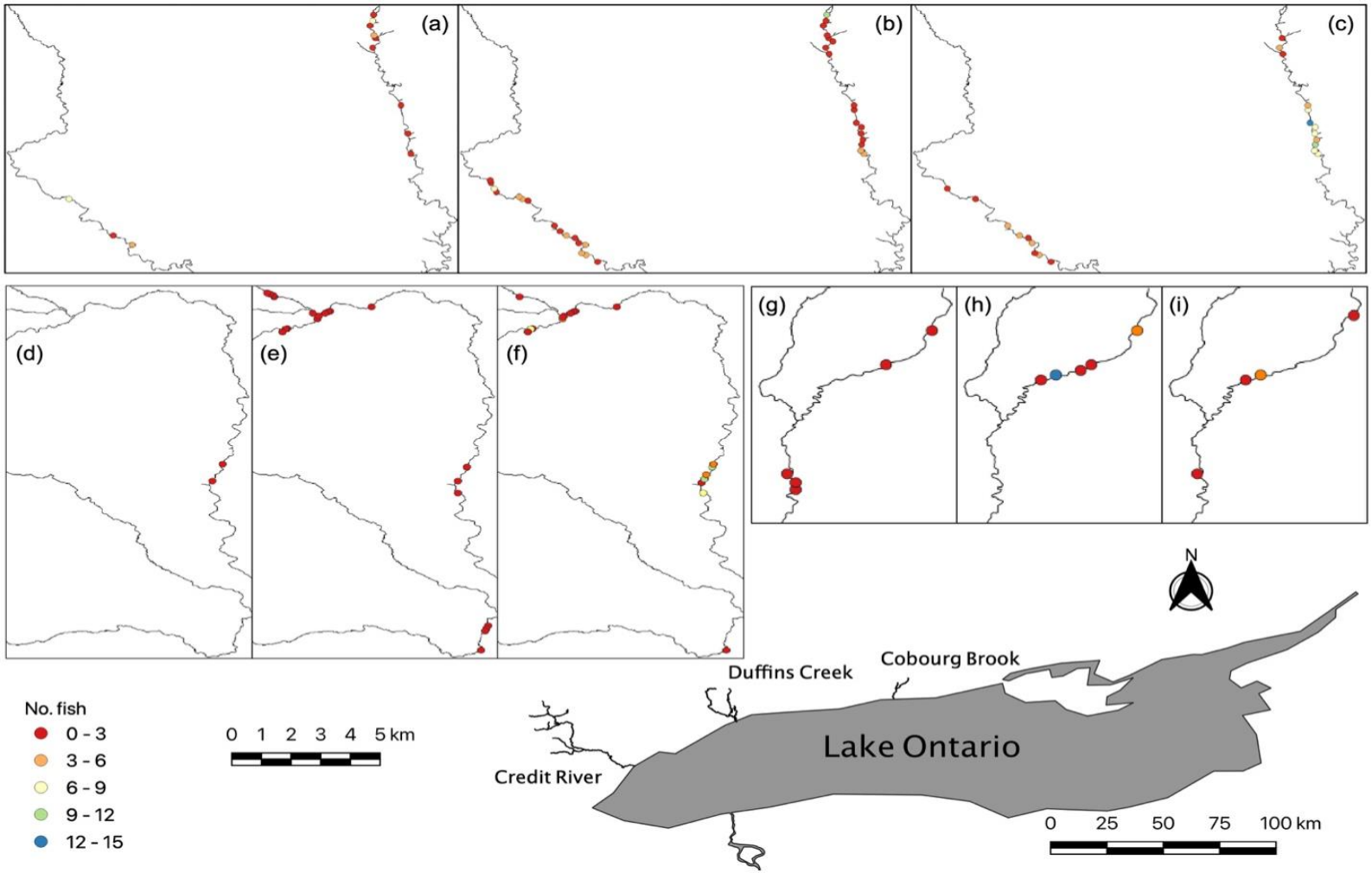

Figure 3.2 Distribution of eDNA sampling sites along 3 Lake Ontario tributaries stocked with Atlantic salmon across 3 sampling periods. Circle colour indicates the number of salmon detected at each sampling site based on the allele counting at the three microsatellite markers. (a) Duffins Creek during May, (b) Duffins Creek during June, (c) Duffins Creek during October, (d) Credit River during May, (e) Credit River during June, (f) Credit River during October, (g) Cobourg Brook during May, (h) Cobourg Brook during June and (i) Cobourg Brook during October.

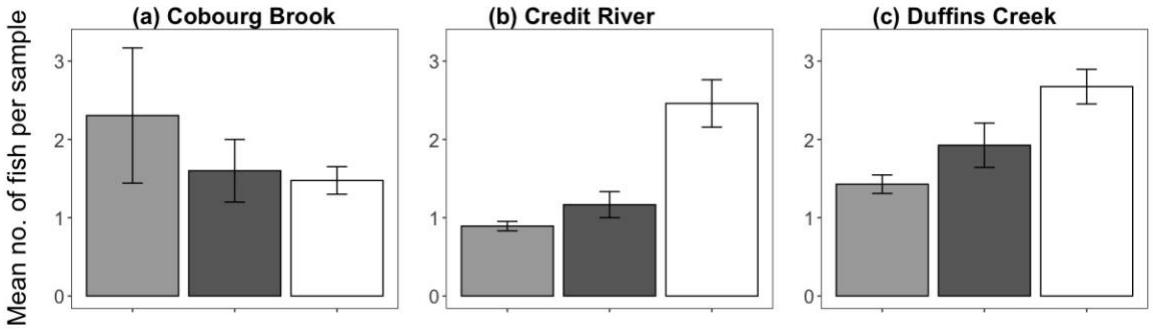

Figure 5.3 Mean estimated numbers (± SEM) of Atlantic salmon detected at single sample sites (averaged across all sampled sites) using emDNA analyses and eDNA collected in May (dark grey), June (light grey) and October (white) from (a) Cobourg Brook, (b) Credit River, (c) Duffins Creek. No significant differences were detected using GLMs

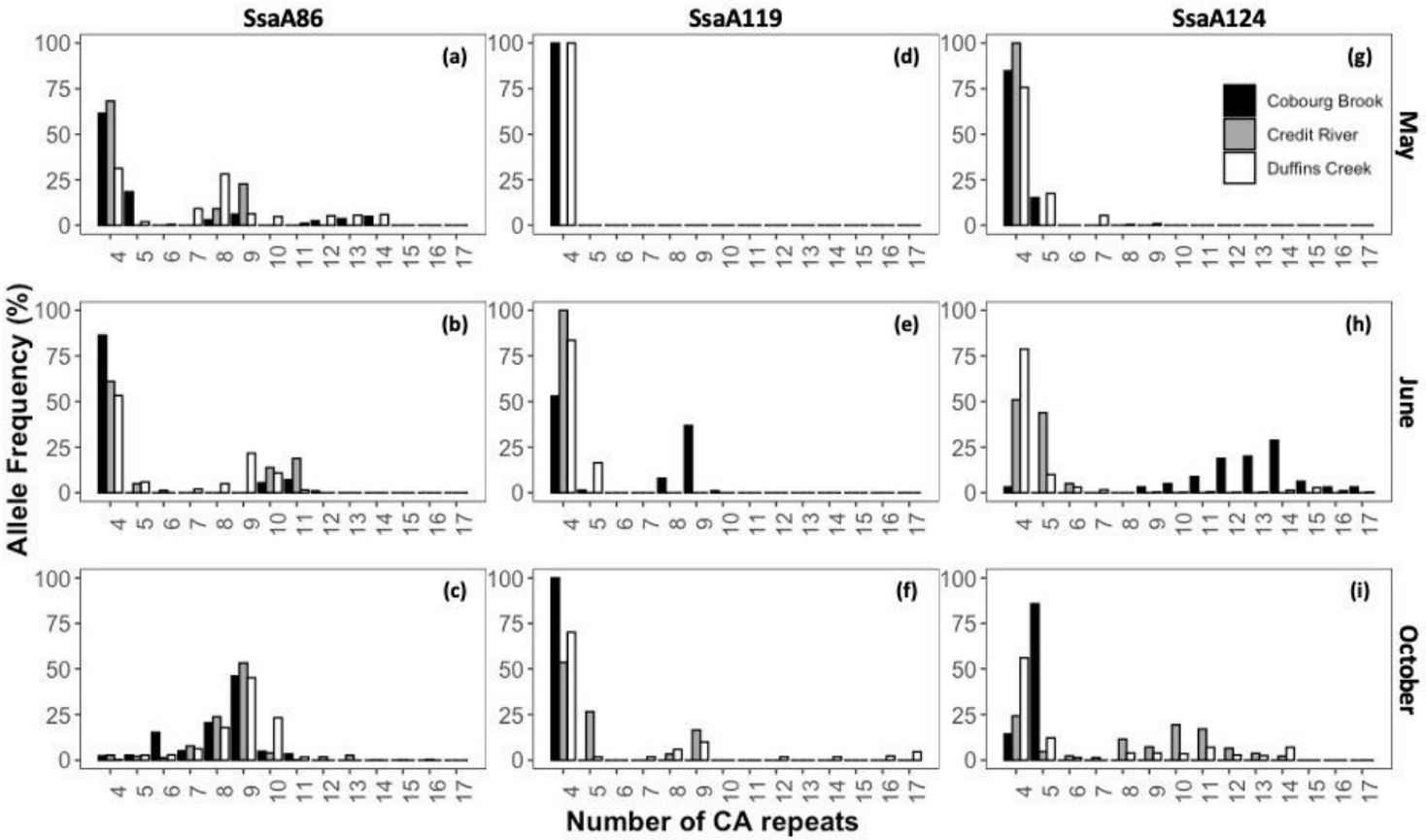

Figure 3.4 Allele frequency distribution of "CA" microsatellite repeat lengths in eDNA samples using three microsatellite markers. The number of occurrences of alleles at each CA repeat length is plotted as a percent of the total within each stream per sampling period. Allele size is reported as the number of CA repeats. Itested for allele frequency differences among the 3 streams for each time point for each allele (reported as χ^2) **(a)** *Ssa*A86 and May sampling (χ^2 = 5.04, *P* = 0.08). **(b)** *Ssa*A86 and June sampling (χ^2 = 1.19, *P* = 0.55). **(c)** *Ssa*A86 and October sampling (χ^2 = 0.54, *P* = 0.76). **(d)** *Ssa*A119 and May sampling (χ^2 = 1.03, *P* = 0.60). **(e)** *Ssa*A119 and June sampling (χ^2 = 3.06, *P* = 0.22). **(f)** *Ssa*A119 and October sampling (χ^2 = 7.22, *P* = 0.03). **(g)** *Ssa*A124 and May sampling (χ^2 = 3.06, *P* = 0.22). **(h)** *Ssa*A124 and June sampling (χ^2 = 6.97, *P* = 0.03). **(i)** *Ssa*A124 and October sampling (χ^2 = 8.50, *P* = 0.01)

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SUPPLEMENTARY TABLE 3.1

The most probable number of individuals necessary to explain the cumulative number of distinct alleles across the three microsatellite loci. Lower and upper values based on 95% confidence intervals.

Observed alleles	**Number of possible individuals sampled**		
	Most likely	Lower	Upper
SsaA86			
1	2	1	2
2	2	1	2
3	3	2	3
4	4	3	4
5	5	4	5
6	6	6	6
7	8	7	8
8	9	9	10
9	11	10	11
10	13	13	14
11	16	15	17
12	18	18	19
13	19	19	20
14	21	21	22
15	22	21	23
16	23	23	24

17	25	24	25
SsaA119			
1	2	1	2
2	5	4	5
3	12	11	12
4	16	16	17
5	19	19	20
6	24	23	24
SsaA124			
1	1	1	2
2	3	2	3
3	6	6	7
4	11	10	11
5	15	14	15
6	17	16	17
7	18	17	19
8	20	19	21
9	21	20	22
10	22	20	24
11	23	21	24

CHAPTER 4

GENERAL DISCUSSION

Amidst continuously increasing chances of extirpation of populations and species, reintroduction has become an increasingly important conservation practice. However, the implementation of an effective monitoring program is key for reintroduction success as otherwise reintroduction failures do not provide information for improvement (Griffith et al., 1989; Armstrong & Seddon 2008). Environmental DNA analysis is a monitoring technique with broad applications in research, but especially in management and conservation. My overall objective was to evaluate the use of eDNA as a monitoring tool to determine the fate of Atlantic salmon (*Salmo salar*) reintroduced into Lake Ontario tributaries. Specifically, I detected Atlantic salmon using: (1) a COI qRT-PCR assay to identify positive detections, (2) a universal primer set for eDNA metabarcoding to determine salmon presence and their associated fish community and (3) emDNA analyses to estimate the number of individual salmon contributing eDNA to each sample.

Chapter 2 results demonstrated the efficacy of eDNA for detecting the presence and distribution of Atlantic salmon before and after reintroduction. I showed that Atlantic salmon could be detected several months after reintroduction, suggesting they are surviving and dispersing in the three study systems. My co-occurrence analyses demonstrated several interactions with non-native salmonids and other fishes. Using the two detection methods simultaneously can provide incrementally more information useful for conservation and management. For example, the two approaches increase the

likelihood of detection when they are rare, provide two semi-quantitative estimates of relative abundance, and identify community interactions among fishes.

Chapter 3 showed the feasibility of developing estimates of individuals present at a site using emDNA analyses through allele counting and emDNA allele frequency distributions. While I used an allele counting model driven by genotyped Atlantic salmon stocked into Lake Ontario tributaries (OMNRF); more sophisticated Bayesian approaches would provide robust confidence limits on the abundance estimates. The eDNA research community is working hard to develop quantitative methods, since presence/absence data have limited value for conservation and management applications. My emDNA feasibility study hold great promise for a different approach to making eDNA estimates more quantitative.

Despite the fact that I used the same samples for both qRT-PCR and metabarcoding in Chapter 2, there were substantial differences in the Atlantic salmon detection patterns. First, I found that the qRT-PCR assay outperformed the metabarcoding in detecting Atlantic salmon. The qRT-PCR assay using the species-specific primer set had a short amplicon, perhaps driving higher sensitivity and hence higher detection rates. Other possible contributing factors include; to low eDNA concentrations (Pilliod et al., 2014; Lacoursière-Roussel et al., 2016), inefficient eDNA extraction method (Deiner et al., 2015; Piggott 2016; Shahraki et al., 2019), lack of “universal” primer specificity (Pompanon et al., 2012; Wilcox et al., 2013) or PCR inhibition from organic material (Hunter et al., 2019). However, many of those factors should equally affect both

platforms. Secondly, I observed a few positive detections using metabarcoding where qRT-PCR failed to detect Atlantic salmon. Because universal primers target multiple species, they are subject to primer affinities across species and detection biases by variable eDNA concentrations (Klymus et al., 2017; Kelly et al., 2019). However, my work has shown that the two methods are capable of generating data not limited to target species presence but also the assessment of community interactions.

The identification of the minimum number of Atlantic salmon individuals per eDNA sample (Chapter 3) is a significant contribution towards species monitoring efforts. My study is among the first to apply microsatellite markers to aquatic eDNA samples for quantitative data. Most previous emDNA studies used eDNA taken from organismal remains such as stomach content, feeding traces, fecal matter or hair (Carreon-Martinez et al., 2014; Wheat et al., 2016; Monge et al., 2018). Spatial data of fish counts along the sampling transects in each stream indicated high counts upstream and lower counts downstream. This follows the theory of downstream transport in lotic systems leading to diluted and degraded eDNA (Barnes et al., 2014; Goldberg et al., 2015). Based on these results, eDNA strategies for monitoring a species should incorporate microsatellite markers to broaden the scope of population genetic questions.

The presence/absence results from Chapter 2 and individual count data from Chapter 3 collectively provide important information for surveying reintroduced Atlantic salmon. It is interesting to note that sites with high detections (Chapter 2) did not always produce a high count of individuals (Chapter 3). The source of these inconsistencies is

not clear and need additional investigation. Mitochondrial and microsatellite data can be used in tandem for reintroduction monitoring assessment without disturbing populations that are not yet self-sustained and hence vulnerable. For reintroduction efforts, the combination of signal distributions and relative abundance estimates are important to identify "hotspots" where the species may be concentrated, indicative of preferred habitat – information valuable for future release planning. Monitoring target species before and after a reintroduction can provide information on the retention of eDNA which can infer whether the species persists in a certain habitat or migrated to another area to search for new resources. It is noteworthy that this is not enough data to determine if the reintroduction was a success, but rather be used to passively monitor and assess fish persistence.

Future Directions

My thesis has made a significant contribution to the assessment of reintroduced Atlantic salmon distribution by evaluating eDNA as an monitoring tool. Specifically, in Chapter 3 I implemented a novel molecular genetic method with eDNA samples which was initially applied by Carreon-Martinez et al., (2014) with predator stomach content DNA. Environmental DNA can be used for long-term monitoring of target species and its community interactions (Chapter 2) and the quantitative analysis can provide a baseline for future conservation efforts (Chapter 3).

Several potential biases may interfere with the presence/absence detections and abundance estimates. Therefore, it is important to follow recommendations set out by

other studies to maximize eDNA recovery by optimizing sampling and extraction methods and determining the best PCR amplification strategies to reduce eDNA detection variability. I suggest that future studies include more sampling points in a study system and sample during several seasons (e.g. different time points after reintroduction) to get a better understanding of the movement and distribution of species and determine whether the population is growing or declining based on eDNA signal strength. The allele counting model in Chapter 3 can identify a minimum number of individuals which may be useful for a more quantitative approach to establish hotspot sites. Moreover, incorporating multiple markers in eDNA studies can be useful for finer-scale assessments of species at risk or invasive species. It can also help to use allele frequency distributions to estimate effective population size and possible inbreeding. Additional COI or other mitochondrial markers can help to increase the chances of positive detection rates whereas additional microsatellite markers can increase the success of genotyping samples (Adams et al., 2019). When selecting or designing markers, they should be species-specific to minimize contamination and non-specific amplification. Additionally, developing eRNA assays can help to assess what genes released fish are expressing. Lastly, I recommend that eDNA analysis for presence/absence and relative abundance data be used in combination with traditional methods for increased species detections that may not be observed in one method and reveal additional information on community interactions. These suggestions can help to better profile the distribution of target species using eDNA. Although there are limitations to eDNA analyses, the results presented in this thesis are a stride towards using eDNA as a rapid non-invasive monitoring tool to assess

biodiversity and quantify species. An effective monitoring program will help maximize the progress of conservation and reintroduction programs towards meeting its ecological goals, determine needs for additional management and decrease uncertainty about any effects on management.

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