The Use of Environmental DNA to Characterize Fish Assemblages in Temperate Estuaries of Varying Levels of Nutrient Impact

by

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Author’s Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This dissertation was written entirely by the author. However, I acknowledge that Dr Simon Courtenay, Dr Michael van den Heuvel, Dr Michael Coffin, Dr Royce Steeves, Monica Boudreau, and Christina Pater-Watts all contributed to the study design used in **Chapter 3** and **Chapter 4**. Dr Simon Courtenay, Dr Michael van den Heuvel, Dr Royce Steeves, Jess Kidd, Nathanael Bergbusch, and Dr Felicitas Egunyu all provided editing throughout the dissertation. Statistical analyses were improved by consolation with Dr Marc Skinner, Dr Marti Anderson, and Kyle Knysh. Nathanael Bergbusch assisted with the creation the generalized additive model used in **Chapter 3.** I also acknowledge that Dr Royce Steeves performed the bioinformatics required for identifying sequenced DNA in **Chapter 4**.

Abstract

The inner region has been identified as an important area for evaluating the health of estuaries, as many riverine inputs, like excessive nutrients (eutrophication), are more concentrated here than in the lower reaches of the estuary. However, the inner estuary's fish assemblage is undersampled in Canada's southern Gulf of Saint Lawrence due to issue of accessibility, and avoidance due to high macroalgal biomass. To help address these issues, this dissertation investigated the inner estuary's fish assemblage with novel methods for evaluating these assemblages. **Chapter 3** assessed whether the inner estuarine region possessed distinct nearshore fish assemblages relative to the middle and outer estuarine regions. The abundance of northern mummichog (*Fundulus heteroclitus macrolepidotus*) was also investigated as a potential indicator of estuarine eutrophication to simplify the sampling effort. Three Prince Edward Island estuaries with varying levels of nutrient impact were sampled in August 2020 and again, along with one additional estuary, in June and August 2021. Each estuary was sampled in the inner, middle, and outer regions. Results from multivariate analyses suggest that the inner region is generally distinct from the middle and outer regions at all estuaries. Mummichogs were generally found in higher abundance in the inner region of most estuaries and displayed a strong, positive linear correlation with sea lettuce abundance. Nearshore fish assemblages were more similar between estuaries from the same shoreline (north vs south shore) than between estuaries with similar levels of nutrient impact (defined by eutrophic times). However, the inner region of estuaries with higher levels of nutrient impact were found to also have relatively higher mean mummichog abundance than inner regions of estuaries with lower nutrient impact. Thus, mummichog abundance may offer an indication of eutrophication within the inner region of estuaries. **Chapter 4** evaluated whether environmental DNA (eDNA) metabarcoding, could act as a complement or replacement to beach seining. Three stations (inner, middle, and outer estuary) were sampled using eDNA medium collection (1 L water samples) and beach seines across estuaries sampled in the previous data chapter. eDNA metabarcoding detected more fish species than beach seining, including deeper water species like striped bass (*Morone*

saxatilis) and the endangered winter skate (*Leucoraja ocellata*). eDNA metabarcoding also differentiated stations 0.4-3 km apart and detected the seasonal and interannual shifts in the fish assemblages suggested by beach seining. Most surprising was that the most abundant fish taxa detected by eDNA metabarcoding and beach seining often contributed similar percentages of the total composition. Thus, eDNA metabarcoding has not only the potential to act as a complement to beach seining (i.e., detect additional species/ genera) but could serve as a replacement in the sea lettuce-infested inner regions of eutrophic estuaries. This dissertation's primary findings, namely that mummichog abundance in the inner estuary may serve as an indicator of eutrophication and eDNA metabarcoding could serve as a complement and replacement for beach seining, may be directly used in assessing estuarine health across the southern Gulf of Saint Lawrence.

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List of Abbreviations

ANOVA	Analysis of Variance
CAMP	Community Aquatic Monitoring Program
dbRDA	Distance Based Redundance Analyses
DFO	Fisheries and Oceans Canada
DISTLM	Distance Based Linear Models
DO	Dissolved Oxygen
DR	Dunk River (Exclusive to **Chapter 3**)
eDNA	Environmental DNA
ER	Enmore River (Exclusive to **Chapter 3**)
FC	Freeland Creek (Exclusive to **Chapter 3**)
GAM	Generalized Additive Models
MEQ	Marine Environmental Quality
nMDS	non-metric Multidimensional Scaling
PEI	Prince Edward Island
PERMANOVA	Permutational Analyses of Variance
rRNA	Ribosomal RNA
RT-qPCR	Real-Time Quantitative Polymerase Chain Reactions
sGSL	Southern Gulf of Saint Lawrence
SIMPER	Similarity Percentage
WR	Wheatley River (Exclusive to **Chapter 3**)
YOY	Young-of-the-Year

Chapter 1: General Introduction

1.1 Background and Rationale

Temperate estuaries are the most degraded marine habitat due to historical and current anthropogenic activities (Jackson et al. 2001). Estuarine degradation is strongly associated with higher human population, agriculture, and industrial activities within an estuary's catchment (Lotze et al. 2006; Van Niekerk et al. 2013; Freeman et al. 2019; Zhai et al. 2019). The degradation of estuaries is a global concern as estuaries are both economically and ecologically valuable, offering many important ecosystem services (Martínez et al. 2007; Thrush et al. 2013). As such, developing techniques and indicators to assess the health or level of degradation of an estuary has become of increasing interest (Van Niekerk et al. 2013; Freeman et al. 2019; Coffin et al. 2021b).

The inner region, closest to the river, appears to be a critical area for assessing the overall health of an estuary (Coffin et al. 2021b; Niu et al. 2021). Pollutants are often more concentrated in the inner region as it is the first to receive riverine inputs, and this region experiences longer residence times than in regions closer to the open ocean (Schein et al. 2012; Bugden et al. 2014; Niu et al. 2021; Turner et al. 2021). As a result, pollutants' effects are more pronounced in the inner region (Hale et al. 2016; van den Heuvel et al. 2019; Coffin et al. 2021a; Niu et al. 2021; Turner et al. 2021).

The inner region has proven to be important for evaluating the effects of nutrient enrichment (eutrophication) on the health of estuaries, including in Canada's southern Gulf of Saint Lawrence (sGSL; Schein et al. 2012; Bugden et al. 2014; van den Heuvel et al. 2019; Coffin et al. 2021a). Prince Edward Island (PEI) is the province most afflicted by eutrophication in the sGSL due to its intensive potato agriculture industry, which accounts for 22 % and 30 % of Canada's potato acreage and production respectively (Grizard et al. 2020; StatsCan 2022). The elevated concentrations of nitrogen-based fertilizers (primarily nitrate) in the inner region fuels the overgrowth of opportunistic macroalgae, like sea lettuce (*Ulva* sp.; Howarth and Marino 2006; Bugden et al. 2014). Although sea lettuce is native to temperate estuaries in the North Atlantic, its increased abundance is problematic, as it overgrows

and outcompetes light-limited seagrasses, like eelgrass (*Zostera marina*) in the eutrophicated waters (Valiela et al. 1997; Hauxwell et al. 2001; Hauxwell et al. 2003; van den Heuvel et al. 2019). The loss of eelgrass is of ecological concern as eelgrass-dominated habitats support a more diverse faunal community than sea lettuce-dominated habitats, including many economically valuable species (Deegan et al. 2002; Hughes et al. 2002; Joseph et al. 2006; Schein et al. 2012; Joseph et al. 2013; Hale et al. 2016).

Fisheries and Oceans Canada (DFO) is developing a Marine Environmental Quality (MEQ) guideline to foster efforts to address eutrophication in estuaries of the southern Gulf of Saint Lawrence (Coffin et al. 2021b). MEQ guidelines have included developing indicators of estuarine eutrophication, such as dissolved oxygen variability and eelgrass loss, primarily focussed in the inner region of the sGSL's estuaries (Coffin et al. 2021b). DFO is currently investigating whether nearshore nekton communities (i.e., fish, crabs, shrimp) could be used as bioindicators of estuarine health, including eutrophication, through their stewardship program, the Community Aquatic Monitoring Program (CAMP; DFO 2011). However, faunal studies and nekton monitoring programs across the sGSL have generally excluded the inner region of estuaries, due to difficulty in operating beach seines in the dense macroalgae mats in many estuaries (e.g., Joseph et al. 2006; DFO 2011; Schein et al. 2012; Schmidt et al. 2017). As a result, how eutrophication has impacted the nekton of the inner region remains largely understudied in the sGSL.

The northern mummichog (*Fundulus heteroclitus macrolepidotus:* hereon mummichog) has been proposed as a single species indicator of eutrophication which could simplify the assessment of estuaries (Finley et al. 2009). Mummichog is one of the few truly residential estuarine fish found across the Atlantic coast of North America and has been used as a model organism in numerous pollution-related studies (Burnett et al. 2007). Mummichogs are well adapted to eutrophic estuaries and appear at higher abundances in sea lettuce-rich sites than eelgrass-dominated ones (Schein et al. 2012; Finley et al. 2013; Lockfield et al. 2013; Dixon et al. 2017). Thus, higher abundances of mummichog may indicated higher levels of estuarine eutrophication (Finley et al. 2009).

The molecular sampling technique known as environmental DNA (eDNA) metabarcoding may provide an effective alternative to beach seines for monitoring the sea lettuce-infested inner region of many estuaries in the sGSL. In eDNA metabarcoding, DNA fragments that enter the environment from shed skin/mucous, decaying tissue, or excrement are collected from the air, water, or sediment samples and typically analysed using regions of the mitochondrial genome that are highly variable among species but conserved within species to detect various taxa (Harrison et al. 2019; Caza-Allard et al. 2022; Miya 2022). eDNA metabarcoding has been shown to be both labour- and time-efficient by outperforming traditional physical sampling methods for detecting species (P.F. Thomsen et al. 2012; Shaw et al. 2016; Fujii et al. 2019). Additional advantages of eDNA metabarcoding include that it is non-invasive (i.e., requires no handling of the organism) and minimally disturbs the sampled habitat (Thomsen and Willerslev 2015; Afzali et al. 2021; He et al. 2022). However, eDNA metabarcoding's quantification capacity is still in its infancy, especially for marine and coastal systems, and questions remain over how well eDNA metabarcoding reflects the abundances of organisms present in the water (Liu et al. 2019; Afzali et al. 2021; Cole et al. 2022; He et al. 2022).

1.2 Thesis Outline

The central thesis of this work is that eDNA metabarcoding can be used to represent estuarine fish assemblages, which may then be used to complement or even replace beach seining in the inner estuarine region for use in monitoring programs. Samples from beach seining and eDNA metabarcoding (from water samples) were collected throughout the summers of 2020 and 2021 to capture interannual, seasonal, and spatial variability present in estuaries with varying levels of nutrient impact from PEI's north and south shores. In addition to furthering research in the field of ichthyofaunal ecology, this dissertation's results could directly inform monitoring programs, such as CAMP, which may be then contribute to the establishment of DFO's MEQ guideline.

The dissertation is comprised of a literature review (**Chapter 2**), two data chapters, and a brief discussion chapter as follows:

Chapter 3 investigated if the inner estuarine region's fish assemblages differ from those in the middle and outer estuarine regions using beach seining. Four estuaries of varying levels of nutrient impact (low-to-high) were sampled in June and August 2021 to observe seasonal shifts in the fish assemblage. Three estuaries sampled in August 2020 and August 2021 were compared to see if interannual variability is present in the observed assemblage. Two primary hypotheses were investigated: 1) The nearshore fish assemblage and/or mummichog abundance of the inner region of PEI estuaries differs from regions closer to the ocean. These results provided insight into whether CAMP's surveys capture the longitudinal variability throughout the estuaries in the sGSL. 2) The estuaries with similar overall levels of nutrient impact will have similar overall nearshore fish assemblage and/or mummichog abundance. These results will aid in determining whether nearshore fish can be used as indicators for assessing eutrophication.

Chapter 4 evaluated two recently developed 12S metabarcoding primer sets' ability to act as either a complement or replacement to beach seining. Thus, the following three primary hypotheses were tested: 1) The selected eDNA metabarcoding primer sets capture the seasonal, yearly, and spatial shifts in the fish assemblages that are detected with beach seining, 2) The selected eDNA metabarcoding primer sets detect more species of fish present within an estuary than beach seining, including non-nearshore species, and 3) The selected 12S eDNA metabarcoding primer sets can provide quantitative data on fish abundance similar to that of beach seining in terms of relative proportion of the composition. This chapter's results cumulatively provided insight into whether eDNA metabarcoding can complement or replace CAMP's existing beach seine-based sampling regime.

The concluding chapter (**Chapter 5**) synthesizes and integrates the key findings of the previous data chapters. In addition, it includes recommendations for implementing eDNA

in monitoring programs based on the cumulative findings of both data chapters. Finally, it also suggests areas for future research.

Chapter 2: Review of Relevant Literature and Methodologies

2.1 Defining Estuaries and Estuarine Health

Estuaries are hydrologically complex and geomorphologically diverse environments (Elliott and McLusky 2002; Babson et al. 2006; Thrush et al. 2013). As such defining the extent of the estuarine region is difficult and many different definitions exist (Elliott and McLusky 2002). For the purposes of this review, estuaries will be understood as semi-enclosed regions of transitionary habitat where fresh and saltwater mix resulting in a salinity gradient along the length of the estuary (Elliott and McLusky 2002; Telesh and Khlebovich 2010; Thrush et al. 2013). The upper estuarine limit, where the river becomes the estuary, is generally defined as where the water's salinity exceeds 0.5 PSU, and the estuary ends where the water's salinity exceeds 30 PSU, becoming the ocean (Elliott and McLusky 2002).

Estuarine health is also difficult to define definitively due to many competing definitions (see Van Niekerk et al. 2013 and references within). Perhaps the most succinct definition comes from Van Niekerk et al. (2013), defining estuarine health "…as the maintenance of ecosystem structure and function, including natural variability and resilience, on a landscape scale." Thus we could imagine degradation of estuaries, or any ecosystem, as factors reducing the overall ecosystem's health, leading to potential collapse (Bland et al. 2018).

2.2 Anthropogenic Impacts to Estuaries

Estuaries the world over have been excellent locations for human habitation due to the ease of navigation and transport along the coast, especially if sheltered harbours were present (Reepmeyer et al. 2016; Kuzmin 2017), abundant food sources and access to fresh water (Rick and Erlandson 2009; Jerardino 2010), and access to flat fertile land that is easily cultivated (Gedan et al. 2009). In modern times, humans still possess an affinity for the sea as the human population is greater near the coastline than in the interior (Roman et al. 2000; Small and Nicholls 2003; He and Silliman 2019). In fact, 40 % of the global population lives within 100 km of the shore, with some countries reaching 80-100 % of their population

within that radius (Martínez et al. 2007). This trend is expected to grow in both developed (Freeman et al. 2019) and developing (Zhai et al. 2019) countries for the foreseeable future.

Anthropogenic impacts on the coastal environment are not novel phenomena due to this historical inclination toward settling near estuaries. There is evidence that prehistoric hunter-gatherer cultures' local harvesting of coastal animals, especially shellfish, led to reductions in the population of those animals, which altered the structure and function of nearshore ecosystems (Rick and Erlandson 2009; Jerardino 2010). However, as societies became agrarian, and later industrial, the impact on estuaries has increased in parallel with the growing human population (Diaz 2001; Van Niekerk et al. 2013; Thrush et al. 2013; He et al. 2014; Freeman et al. 2019; Zhai et al. 2019). For the purposes of this review, I will be focusing on agriculture. As of 2016, agricultural land covers over 37% of the earth's total land (World Bank 2021), making it one of the largest "biomes" on the planet (Ellis et al. 2010). The following section will examine the dominant agricultural impacts on temperate estuaries, and how they negatively affect biodiversity.

2.3 Eutrophication of Temperate Estuaries

Agriculture causes many well-known impacts on estuaries ranging from decreasing freshwater inputs altering the estuary's salinity (Rodriguez et al. 2001), increasing sediment and turbidity (Sirabahenda et al. 2019), to pesticide runoff directly killing aquatic life (Fulton et al. 1999; Zhou et al. 2014). However, agriculture's greatest impact is introducing large amounts of exogenous nutrients to the estuarine environment (Diaz 2001; Lotze et al. 2006; Howarth et al. 2011; Bugden et al. 2014). In marine environments, such as estuaries, nitrogen is often considered the limiting nutrient for primary producer growth; however, in some marine systems, phosphorus may be the limiting nutrient (Howarth and Marino 2006). It is worth noting that managing nitrogen and phosphorus inputs has been suggested for meaningful long-term control improvements to degraded estuaries (Paerl 2009; Howarth et al. 2011). Despite this, for the purposes of this literature review, I will only focus on nitrogen.

There are two major pathways through which anthropogenic nitrogen enters the estuary. The first of these pathways is atmospheric deposition from nitrogen aerosol, which results from combustion (e.g., NO_x or ash), and erosion and is often the dominant supply of nitrogen in many coast systems (Paerl 1997; McIver et al. 2015; Kanakidou et al. 2016). Atmospheric nitrogen aerosols can be transported thousands of kilometres. For example, dust blown over 5000 km from the Sahara desert increases the productivity of the surface waters of the tropical west Atlantic (Jickells and Moore 2015). The second pathway results from excessive amounts of nitrogen-based fertilizers applied to fields slowly infiltrating into the groundwater (Jiang et al. 2011; Jiang et al. 2015). Eventually the nitrogen-laced water will enter nearby rivers and streams through springs, finally being transported to the downstream estuary (Jiang et al. 2011; Jiang et al. 2015).

Excessive amounts of nitrogen dramatically changes the estuaries' dominant macrophyte as slow-growing benthic flora, like seagrasses, are outcompeted by fast-growing macroalgae and phytoplankton (Duarte 1995; Valiela et al. 1997). Nitrogen is generally low in pristine temperate estuarine systems (Peralta et al. 2003). As a result, roots alone cannot supply all the nitrogen that seagrasses, like eelgrass (*Zostera marina*), need in order to grow (Peralta et al. 2003). Rapid absorption of nitrogen (primarily nitrate) through the leaves is useful to estuarine macrophytes, especially in intertidal zones, where tides routinely flush nutrients out of the estuary (Pedersen and Borum 1992; Pérez-Mayorga et al. 2011). This adaptation helps them take advantage of random pulse nutrient events (Hemminga et al. 1994; Phillips and Hurd 2004). However, short-lived opportunistic algae are more sensitive to changes in nutrients than perennial macroalgae and seagrasses (Pedersen and Borum 1996).

Opportunistic macroalgae like sea lettuce (e.g., *Ulva* species like *Ulva lactuca* and *Ulva intestinalis*) are more efficient than seagrasses at absorbing nitrogen species (e.g., nitrate and ammonium) directly from the water column with their high surface area to volume ratio (Littler and Littler 1980; Teichberg et al. 2007). Sea lettuces are very responsive

to sudden increases in nitrogen, as the enzyme nitrate reductase (also present in eelgrass) helps it to assimilate nitrate quickly into its tissues (Teichberg et al. 2007). Sea lettuce can also quickly increase its nitrate reductase levels to increase nitrogen assimilation rates if conditions are appropriate (Teichberg et al. 2007). However, sea lettuce and other opportunistic macroalgae require higher concentrations of nitrogen to sustain their rapid growth rates than slower-growing species like eelgrass (Pedersen and Borum 1996). Therefore, the continuous input of nitrogen from agriculture releases the sea lettuce from the naturally low nitrogen levels that regulated its growth (Littler and Littler 1980; Teichberg et al. 2007; Pérez-Mayorga et al. 2011).

The ecosystem cannot sustain the high levels of macrophytic or algal growth for long (Diaz 2001). Dense macroalgae mats alter the water's biogeochemistry, especially regarding dissolved oxygen (DO) levels (Hauxwell et al. 2001). Macroalgal growth within an estuary is self-limited, as once the biomass surpasses a certain density (often by mid-summer), newer growth begins to shade out and kill-off older growth (Lavaud et al. 2020). Bacteria begin to break down the algae as the warmer months progress, consuming most of the water's DO levels to the point of low to almost no DO (i.e., hypoxia (<4.0-2.0 mg/L) and anoxia (0.0 mg/L) respectively) left in the water (Duarte 1995, Bugden et al. 2014). This entire process is known as eutrophication, as it increases the rate of organic carbon production, in the form of macroalga tissues, in the environment (Nixon 1995). Eutrophic sites dominated by sea lettuce alternate between supersaturation (> 10 mg/L) during the day or early summer and to hypoxic/ anoxia conditions at night or late summer (< 4 mg/L; Coffin et al. 2018b). In comparison, seagrass sites tend to have stable DO levels through the year (Coffin et al. 2018b). Hypoxia tends to be restricted to the inner region of estuaries (up to 25% of surface area) and rarely extends to outer regions (Coffin et al. 2021a). The flux in DO levels may be energetically expensive for eelgrass, as it must translocate oxygen from tissues exposed to oxygenated environments, like stems, to its roots buried in the anoxic mud (Pregnall et al. 1984). Low DO levels in the water may interfere with this process and put stress on eelgrass (Hauxwell et al. 2001).

The excess of nutrients stimulates the over-proliferation of macroalgae which shade out and eventually kill eelgrass as eelgrass is light-limited in temperate latitudes (Short et al. 1995; Valiela et al. 1997; Hauxwell et al. 2001; Hauxwell et al. 2003; Peralta et al. 2003; M.S. Thomsen et al. 2012). Fast-growing, non-attached macroalgae are better suited to eutrophic estuaries than seagrasses as they can float higher in the water column (Duarte 1995; Coffin et al. 2017). Macroalgae growth, like sea lettuce, tends to be the densest in the inner region of estuaries, closest to river's freshwater input where nitrogen-species concentrations will be the highest (Bugden et al. 2014; Iriarte et al. 2015). This has resulted in eelgrass being excluded from the inner region of many eutrophic estuaries (Deegan 2002; van den Heuvel et al. 2019). For these reasons, along with other anthropogenic impacts, there has been a 65% loss in seagrass beds worldwide (Lotze et al. 2006).

The change in the dominant primary producer from seagrass to opportunistic macroalgae also shifts the animals that inhabit the estuary. The biggest problem eutrophic temperate estuaries pose to estuarine faunae is the seasonal hypoxia that generally occurs during summer (Coffin et al. 2018a). Brief, seasonal periods of hypoxia are common in estuaries, even those not impacted by eutrophication, and as such many of the estuarine faunae have adapted to intermediate occurrences (Rabalais et al. 2007; Rabalais et al. 2010; Coffin et al. 2018b). However, eutrophic estuaries can experience extended duration of hypoxia far beyond what the faunae can tolerate (Riedel et al. 2012; Coffin et al. 2018a). Mobile faunae, such as fishes and crabs, can vacate the hypoxic region and move into micro-habitats with more oxygen, move down or upstream of the hypoxia, or move into shallow water or closer to the surface where there is more oxygen interchange (Lenihan and Peterson 1998; Ritter and Montagna 1999; Shimps et al. 2005; Coffin et al. 2017; Dixon et al. 2017; Roloson et al. 2021). Mobile fauna may also escape hypoxia by using ebb tides to take them out of the inner regions where hypoxia is the most frequent (Brady and Targett 2013). Benthic or stationary animals cannot easily escape hypoxic areas and often succumb to oxygen deprivation (Lenihan and Peterson 1998; Ritter and Montagna 1999; Coffin et al. 2017). As a result, many eutrophic estuaries have seasonal losses in their benthic

communities and have fewer, hypoxia tolerant species (Kodama and Horiguchi 2011; Froehlich et al. 2015). Even if the faunae are capable of avoiding hypoxia or tolerating it, the low oxygen condition may alter the animals' behaviour, like reducing feeding resulting in decline in growth or fitness (Sagasti et al. 2001; Shimps et al. 2005). Faunae may also succumb to the high levels of hydrogen sulphide (H_2S) which forms from anaerobic respiration of certain bacteria during hypoxia (Riedel et al. 2012).

The physical loss of seagrass itself results in biodiversity losses because it is an important habitat for diverse species (Deegan et al. 2002; Joseph et al. 2006; Schein et al. 2012; Joseph et al. 2013). Often, seagrass-dominated estuaries are critical nurseries for many commercial and non-commercial fish species (Roman et al. 2000; Joseph et al. 2006; Joseph et al. 2013; Whitfield, 2017). As a result, estuaries and seagrass meadows are economically important (Barbier et al. 2011; Thrush et al. 2013). They have been estimated to have ecosystem service values of over 3 X that of terrestrial ecosystems combined and over 47 X that of agricultural and altered ecosystems (Martínez et al. 2007).

Seagrass beds increase habitat complexity by providing a more intricate habitat for prey species to hide in than sea lettuce can provide, thus reducing predation (Deegan 2002 and references within; Joseph et al. 2013; Reynolds et al. 2018). Therefore, increased seagrass habitat complexity increases fish biomass, richness and abundance over what is found in eutrophic estuaries (Deegan et al. 2002; Hughes et al. 2002; Schein et al. 2012; Hale et al. 2016). Opportunistic macroalgae, such as sea lettuce, still can provide a level of habitat complexity that bare sediment does not provide if seagrasses are absent (Wilson et al. 1990; Sogard and Able 1991; Coffin et al. 2017). However, eutrophic macroalgae-dominated habitats are generally less biodiverse relative to seagrass-dominated habitats (Hughes et al. 2002; Schein et al. 2012; Schmidt et al. 2017; Coffin et al. 2018a).

2.4 Indicators of Estuarine Eutrophication

Developing indicators to classify how eutrophic an estuary has become is a global priority for many environmental management agencies (Ferreira et al., 2011; Coffin et al.

2021b; EPA 2021). Promising estuarine indicators include DO profiles (Coffin et al. 2018b; see EPA 2021), nutrient loading within the catchment (Jiang et al. 2011; Bugden et al. 2014; McIver et al. 2015; Kelly et al. 2021), and eelgrass coverage (Hitchcock et al. 2017; van den Heuvel et al. 2019). Bioindicators for marine eutrophication have also been developed over the years. Benthic invertebrate communities offer useful indicators as they reflect a smaller spatial scale due to their limited mobility (Pearson and Rosenberg 1978; Ritter and Montagna 1999; Riedel et al. 2012; Coffin et al. 2017; Coffin et al. 2018a). For example, polychaete worms offer simple single taxon assessment as there is a variety of species that range in hypoxia tolerances offering an indication of succession (Pearson and Rosenberg 1978; Ritter and Montagna 1999; Cardoso et al. 2007).

Estuarine vertebrates', such as fishes, utility as indicators is less apparent. Estuarine fish assemblages appear to mainly be determined more by the estuaries' geomorphology than its state of degradation (Elliott and Quintino 2007; Harrison and Whitfield 2012; Baptista et al. 2014; Tweedley et al. 2017). This could be due to estuarine fishes adapting to live in a naturally stressful environment due to the high variability of everything from DO levels to salinity in estuaries (Elliott and Quintino 2007). Fishes are also highly mobile taxa, and as such often are able to detect and escape hypoxic region to areas with favourable oxygen conditions (Wannamaker and Rice 2000; Shimps et al. 2005; Brady and Targett 2013; Roloson et al. 2021). As such, fish-based indicators, whether it be a single species or metrics like richness, often cannot be used to distinguish eutrophication status of an estuary (Snigirov et al. 2019).

A cumulation of abiotic and biotic factors structure fish assemblages along the length of temperate estuaries. Physio-chemical conditions like salinity, temperature, and turbidity change vertically and horizontally within an estuary, creating ecological "barriers" that structure the fish assemblages found within certain regions or depths (Whitfield 2021). Biotic factors also contribute to the structuring of fishes within an estuary. Vegetation, or lack thereof, is a structuring force in most temperate estuaries, with certain species being associated with macrophytes, macroalgae, or sediment (Schein et al. 2012; Joseph et al.

2013; Whitfield 2017). Competitive exclusion and predation may also lead to niche partitioning within the estuary (Whitfield 2020), and overfishing from humans, and other anthropogenic activities, may result in extinctions or extirpations (Jackson et al. 2001; Lotze et al. 2006). It is worth stating the importance of the listed factors will depend on the specific region of the world the estuary is found (Whitfield and Elliott 2002).

Despite their shortcomings, fishes are generally preferred by many environmental monitoring organizations over invertebrates. Fishes are easier to identify than many invertebrates, thereby requiring less taxonomic training (Whitfield and Elliott 2002; Thériault et al. 2006). Fishes also generally garner higher public interest due to their known cultural and economic importance (Whitfield and Elliott 2002).

Studies across the north-eastern Atlantic have suggested the northern mummichogs (*Fundulus heteroclitus macrolepidotus*) to be a useful indicator of anthropogenic impacts, from industrial pollution to agriculture (Leblanc et al. 1997; Ferraro et al. 2001; Courtenay et al. 2002; Thériault et al. 2006). Mummichogs have several features that make them ideal for eutrophication studies in estuaries. For one, mummichogs have a broad range along the eastern Atlantic ranging from Newfoundland, Canada to Florida in the United States (Able and Fahay 1998). Mummichogs also are one of the few truly residential estuarine fish and have a high site fidelity, appearing to remain in the same estuary or section of an estuary for most of the year (Skinner et al. 2005; Lockfield et al. 2013). Mummichogs are capable of tolerating hypoxic conditions for over a week and can supplement oxygen need with surface respiration (Stierhoff et al. 2003; Dixon et al. 2017) and may not avoid hypoxia like other fish (Wannamaker and Rice 2000). Increased mummichog abundance, biomass and individual body mass is associated with nutrient enrichment due to increased food sources (Schein et al. 2012; Finley et al. 2013; Lockfield et al. 2013). As such, it was suggested that high abundances of adult and young-of-the year (YOY) mummichogs could be a useful single indicator of eutrophication for the southern Gulf of Saint Lawrence (sGSL; Finley et al. 2009).

2.5 The Community Aquatic Monitoring Program (CAMP) and Eutrophication in the Southern Gulf of Saint Lawrence

Eutrophication is a major concern in the sGSL, especially in the waters around Prince Edward Island (PEI), due to the province's intensive agriculture industry (Jiang et al. 2011; Bugden et al. 2014; Grizard et al. 2020). As a result, Fisheries and Oceans Canada (DFO) is developing a Marine Environmental Quality (MEQ) guideline to foster efforts to address eutrophication in estuaries of the sGSL (Coffin et al. 2021b). The primary goal will be to identify indicators that can assess the eutrophication status of estuaries (Coffin et al. 2021b). DFO is currently investigating nearshore fish assemblages' potential as a bioindicator for eutrophication that their stewardship program could monitor: the Community Aquatic Monitoring Program (CAMP).

Starting in 2003, CAMP was initially a collaboration between DFO and the Southern Gulf of St. Lawrence Coalition on Sustainability fostering relationships and stewardship activities with local non-government environmental groups, First Nations groups, and maritime universities (Weldon et al. 2005; DFO 2011). CAMP currently samples 35 estuaries throughout the sGSL chosen due to the presence of eelgrass, local industrial activity (e.g., presence of seafood processing facility or agriculture in the catchment), or special conservation status (e.g., Marine Protect Area). Six sampling stations are established in each estuary, generally having two stations from the inner, middle, and outer estuary with one station from each shoreline. Stations are generally accessible by road to simplify volunteer access and thus is the main factor in station selection (DFO 2011). Before CAMP's suspension in 2020 due to the ongoing COVID-19 pandemic, CAMP collected these samples in June, July, and August but historically has collected them from May to September (M. Boudreau, DFO Moncton, personal communication).

There is increasing interest to use CAMP's growing database as a means of assessing the anthropogenic impacts, including eutrophication, to estuaries in the region (Thériault and Courtenay 2010; DFO 2011). CAMP's sampling protocol involves sampling the nearshore habitat (<2 m depth) with a beach seine (net: 30 x 2 m, central bag: 2 x 1 m, mesh: 6 mm) by

walking the net 15 m perpendicular from the shore into the water, turning and walking 15 m parallel to the shore until the entire net is submerged in the water, and then circling back to the starting point on the shore (Weldon et al. 2005). Overall, this samples a 225 m^2 area. Captured fish, crabs, and shrimp are identified to species or genus, separated into age classes (adult vs YOY), enumerated, and then released. Fauna known to be fragile (e.g. Atlantic silverside (*Menidia menidia*), or gaspereau (*Alosa* species)) or predatory (e.g., green crab (*Carcinus maenas*)) are generally counted first to prevent unwanted mortalities. Captured fauna are enumerated up to 300 individuals, after which the remaining fauna's identities and abundances are estimated. However, the beach seine can get clogged with sea lettuce in the inner regions of eutrophic estuaries, impeding sampling (Weldon et al. 2005). Therefore, beach seining cannot effectively monitor the regions most impacted by eutrophication. As such, alternative sampling methods are being investigated, especially molecular techniques.

2.6 Emerging Biodiversity Monitoring Techniques: Environmental DNA (eDNA) Metabarcoding

Wildlife managers have been trying to develop more efficient methods to monitor the precipitous decline in global biodiversity, with molecular sampling, especially of DNA, being among the most promising (Pimm et al. 2015; Grant et al. 2021; Miya 2022). For this review, I will focus on an emerging DNA-based monitoring tools for fish in marine and estuarine environments. Animals release DNA fragments to water, soil, or air through their mucous, excrements, shed skin, or decomposing tissue: collectively referred to as environmental DNA (eDNA; Harrison et al. 2019). These genetic fragments can be detected and used to identify single-species via quantitative Polymerase Chain Reactions (qPCR) and multiple species via metabarcoding (Goldberg et al. 2016; Miya 2022; Xiong et al. 2022). Both qPCR-based detection and metabarcoding rely on short, standardized regions in the genome that can be used to identify species based on comparisons to known sequences in databases, such as the Barcode of Life Data Systems (Grant et al. 2021 and references within). Metabarcoding is particularly appealing as it allows for the detection of multiple species from a single complex environmental sample, often water in the case of fishes,

providing greater insight into the broader faunal community than a single barcode could provide (Cristescu 2014; H. Jo et al. 2019; Djurhuus et al. 2020; Shu et al. 2020; Miya 2022).

The genesis of eDNA as a field of study can be traced back to bacterial barcode studies that began in the late 1980s (Ogram et al. 1987). The inventions of the PCR (mid-1980's) and massively parallel high-throughput sequencing platforms (a.k.a, next-generation sequencing; early 2000's) would allow greater development in the field of genetics, as DNA replication became an automated process and vast quantities of sequences could be rapidly and simultaneously processed (Shendure and Ji 2008; Singh et al. 2014). The first vertebrate PCR-based study detected the invasive bullfrog (*Rana* (*Lithobates*) *catesbeiana*) in ponds in France (Ficetola et al. 2008), while four years later, the first marine studies on multiple fishes (i.e., metabarcoding) were performed (P.F. Thomsen et al. 2012). Since then, there has been a steady, almost exponential, growth in the number of scholarly publications using eDNA metabarcoding (Shu et al. 2020; Grant et al. 2021; Miya 2022; Xiong et al. 2022). As of 2021, nearly double the number of publications were published in freshwater environments (60.7%), than marine environments (30.4%) in ichthyofaunal metabarcoding studies (Xiong et al. 2022). Regardless of the water's salinity, a clear trend is emerging that metabarcoding is quickly becoming a common tool for monitoring fishes (Shu et al. 2020; Miya 2022; Xiong et al. 2022). As the cost of high-throughput sequencing declines due to technological improvements (Goodwin et al. 2016), eDNA metabarcoding holds many possibilities for monitoring fisheries or ecosystem health.

2.7 Barcoding Region

Mitochondrial DNA is generally preferred in most metabarcoding studies over nuclear DNA for a variety of reasons. Each cell has multiple mitochondria, and each mitochondrion can contain multiple mitochondrial DNA copies, offering dozens or even hundreds/thousands of copies per cell for detection, and the mitochondrial membrane may also protect the DNA by reducing degradation (Foran 2006). The mitochondrial 12S and 16S ribosomal RNA (rRNA) genes are the preferred loci for most ichthyofaunal metabarcoding

studies (Shu et al. 2020). The popularity of these genes is due to a combination factors including possessing highly conserved regions for primer binding that flank highly divergent regions that are required for taxonomic resolution (Di Finizio et al. 2007; Cawthorn et al. 2012; Collins et al. 2019). In addition, there are growing number of sequences in databases, universal primers, and bioinformatic software packages for these loci (Miya et al. 2015; Collins et al. 2019; Shu et al. 2020; Xiong et al. 2022). As such, these factors have helped to create a certain 'inertia' in research culture that sustains the popularity of these loci.

2.8 Production and Degradation of eDNA in the Marine and Coastal Environment

Multiple factors appear to drive ichthyofaunal eDNA production. Temperature appears to have the strongest effect on ichthyofaunal eDNA production (T. Jo et al. 2019; Caza-Allard et al. 2022). However, there is no clear linear relationship between higher or lower temperatures and eDNA concentration (Andruszkiewicz Allan et al. 2021).

Another factor to consider is that the eDNA of different species of fishes appear to shed and decay at different rates. There are multiple explanations proposed. Differences in shedding rates could be due to differences in the fishes' integument (i.e., scale type, absence of scale, and mucosal covering; Caza-Allard et al. 2022). Thalinger et al. (2021) identified a relationship between more shedding with increased activity, likely due to the fishes' physiology and behaviour. Life stage should also be considered. Ostberg and Chase (2022) found that eDNA production increases after Chinook salmon (*Oncorhynchus tshawytscha*) fry hatched. Smaller or younger fish appear to produce more eDNA per unit weight than larger or adult fish, likely due to increased surface area to volume ratios in the former (Spear et al. 2021; Yates et al. 2021). However, fishes of similar sizes and ecologies appear to shed eDNA at similar per unit weight rates and decay at similar rates, despite differences in eDNA origin (Sassoubre et al. 2016). There remains a great deal of variability in all the observations mentioned, with some studies finding the opposite trend (Andruszkiewicz Allan et al. 2021).

Multiple environmental and biological processes degrade eDNA in aquatic ecosystems. Ultraviolet (UV) radiation (especially UVB, 290-320 nm) promotes the nucleic acid thymine to dimerize with either itself or other nucleotides, which interferes with DNA replication (Ravanat et al. 2001). Despite this theoretical threat, studies investigating UV's contribution to eDNA degradation consistently indicate that UV plays a minor role in marine systems. Studies of eDNA in the marine environment in indoor shaded conditions and sunlight at different depths found that the eDNA decay rate was similar with a first-order rate constant of around 0.05 to 0.1 h^{-1} (Sassoubre et al. 2016; Andruszkiewicz et al. 2017). These results may be explained by the fact that the 10 % irradiance depth of UVB ranges between 0.2-5 m, due to higher amounts of suspended material filtering out the UV (Tedetti and Sempéré 2006). Thus, UV likely only damages eDNA near the surface of the water.

Microorganisms appear to be the most important factor in eDNA degradation. Conditions that promote or regulate microbial growth, such as warm water (25-30 °C) and neutral pH have been found to have higher rates of eDNA decomposition than non-conducive environments (Barnes et al. 2014; Strickler et al. 2015; T. Jo et al. 2019; Andruszkiewicz Allan et al. 2021). There is evidence that suggests coastal waters, where higher temperatures and nutrients are present, experience higher eDNA degradation rates than offshore waters (Collins et al. 2018). Even nutrients resulting from high fish biomass may spur increased microbial degradation of eDNA (Caruso et al. 2003; T. Jo et al. 2019). Degradation may also come from extracellular nuclease, which would behave similarly under similar conditions optimal for microbes (Barnes et al. 2014). As such, it has become common practice to add antimicrobials to water samples or place them on ice while in transport to limit both enzymatic and microbial degradation (e.g., Hunter et al. 2019; Liu et al. 2019).

Sediment particles play an important role in the sequestering and preservation of eDNA in aquatic environments. Anionic DNA molecules form an electrostatic attraction with cationic particles in sediments (Romanowski et al. 1991; Levy-Booth et al. 2007; Hou et al. 2014). This adsorption onto sediment particles may help protect the eDNA from microbial or enzymatic decomposition and heavy metal or UV damage, thus preserving the DNA (Lorenz

et al. 1981; Corinaldesi et al. 2008). As a result, fish eDNA is often found at higher concentrations in sediment than in the water column (Turner et al. 2015). However, highly turbid waters can complicate recovering eDNA from water samples. The electrostatic attraction with sediment removes eDNA from the water column, especially in low flow environments, and humic acids may inhibit PCR (B.C. Stoeckle et al. 2017). Resuspended sediment also could introduce eDNA produced several months to years ago, complicating species detection, as eDNA samples have been recovered from marine sediment dated to be over 10,000 years old (Corinaldesi et al. 2008; B.C. Stoeckle et al. 2017).

The persistence or transport of eDNA at and between aquatic locations may be influenced by currents and tides. Some of the earliest papers published on eDNA in the marine environment showed ocean currents' potential to transport eDNA from far distances into bays and estuaries (Foote et al. 2012). More recent evidence with water samples has demonstrated that incoming and outgoing tides do not appear to be a major driver in the communities revealed by eDNA within temperate estuaries (Kelly et al. 2018; van Bleijswijk et al. 2019). However, there remains evidence that tidal influences may be estuary specific, depending on hydrogeology (Ahn et al. 2020). It appears that eDNA metabarcoding water samples can differentiate community structure between spatially separated regions in tidal temperate estuarine or coastal areas and open water (Port et al. 2016; O'Donnell et al. 2017; H. Jo et al. 2019), and even in well-mixed arctic estuaries (Lacoursière-Roussel et al. 2018).

The eDNA in a coastal system changes daily and monthly (van Bleijswijk et al. 2019). It appears that eDNA concentration follows the diurnal patterns of the fish at the site, with the eDNA concentration changing every few hours (Jensen et al. 2022). Seasonal effects are less clearly understood. A growing number of recent studies has found that eDNA abundance often reflected the changes in abundances of coastal fishes as the seasons changed (Jia et al. 2020; Stoeckle et al. 2021). Data from Collins et al. (2018) suggest that seasons play no role in the abundance or degradation of eDNA in marine systems, while Salter (2018) found that season was important in the marine degradation rates, with increased microbial

degradation during the warming months of the year. Regardless of what factors are driving the production, decomposition or disappearance of eDNA, it appears eDNA can persist in the water column for either a few days or a few weeks depending on the environment (Collins et al. 2018; Harrison et al. 2019).

2.9 Biases, Limitations, and Future of eDNA Metabarcoding

All sampling methods have inherent biases and measurement errors, contributing to uncertainty (Regan et al. 2002), and eDNA metabarcoding is no different. For instance, controlling false positives and negatives is one of eDNA research's greatest challenges (Goldberg et al. 2016; Cristescu and Hebert 2018). Both qPCR and metabarcoding cannot currently differentiate DNA deriving from living or dead tissue, which may lead to an inaccurate impression of the organisms currently living in the water body (Thomsen and Willerslev 2015). As a result, DNA contamination from fish markets (Yamamoto et al. 2016), piscivorous bird faeces or decomposing corpses (Merkes et al. 2014), human sewage (Fujii et al. 2019), or past laboratory work (Goldberg et al. 2016) can distort the results. Therefore, developing a strict DNA decontamination protocol and a careful protocol of negative controls at each step from eDNA collection to sequencing is essential (Goldberg et al. 2016). Different laboratory methods ranging from selected extraction kit, filter material and pore size, and preservation may also influence results (Eichmiller et al. 2016; Djurhuus et al. 2017; Hermans et al. 2018).

Perhaps the most important challenges in ichthyofaunal eDNA metabarcoding are primer biases and limited barcode sequences in databases (Schenekar et al. 2020; Shu et al. 2020; Zhang et al. 2020). Well-designed primers will preferentially amplify the same locus of the taxa of interest over other taxa, resulting in an over-representation of the former at the expense of the latter (Schenekar et al. 2020; Zhang et al. 2020). Even if equal quantities of different species eDNA are present, primer bias often prevents equal proportion from being represented in eDNA metabarcoding's sequence reads (Andruszkiewicz et al. 2017). Poorly designed primers generally amplify non-target taxa, leading to wasted sequencing effort

(Collins et al. 2019). Even if the desirable primers can be obtained, there is often an issue of insufficient reference sequences for many fishes, especially for the 12S and 16S rRNA genes (Collins et al. 2019; Xiong et al. 2022). However, reference sequence database deficiencies are slowly resolving as each new study adds missing reference barcodes (Miya 2022 and references within).

Metabarcoding's biggest promise lies in offering a rapid and potentially cheaper means of species identification and quantification (Danovaro et al. 2016; Bush et al. 2019). There has been concerns raised over the past decade that there are not enough trained taxonomists, which is troubling as there is a growing need to track the precipitous decline in biodiversity (Bacher 2012; Paknia et al. 2015). Barcoding more broadly has begun to fill in the loss of physical identification (Danovaro et al. 2016; Bush et al. 2019). It has been well established that eDNA metabarcoding detects higher ichthyofaunal diversity than most traditional sampling techniques, like bottom trawling, fyke nets, and remote cameras (e.g., Shaw et al. 2016; Fujii et al. 2019; Liu et al. 2019; Zou et al. 2020; Afzali et al. 2021; Cole et al. 2022). Differences in the sensitivities of eDNA metabarcoding and traditional sampling methods result from the biases inherent to all sampling methods. Most traditional methods are designed to capture certain species, trophic levels, or ecological niches (Kelly et al., 2017). eDNA metabarcoding's reliance on DNA fragments instead of entire organisms allows for the simultaneous detection of multiple domains of life (depends on primer set(s)), or the detection of certain taxa that traditional methods are biased against, culminating in eDNA detecting more taxa per sampling effort (Djurhuus et al. 2020; Hallam et al. 2021). However, eDNA metabarcoding may increase the risk of false positive detections from exogenous DNA transported to the study location by currents, tides, or the faeces of piscivores (P.F. Thomsen et al. 2012; Merkes et al. 2014; Cheang et al. 2020). Nevertheless, complementing or replacing labour-intensive sampling methods with eDNA metabarcoding will undoubtedly streamline future ichthyofaunal assessments.

There is great interesting in exploring eDNA metabarcoding's capability to provide quantitative information for ichthyofaunal assessments (e.g., Knudsen et al. 2019; Afzali et al. 2021). Many single species experiments and studies have shown a relationship between eDNA concentrations in laboratory and field work at various salinities (Itakura et al. 2019; Spear et al. 2021; Stoeckle et al. 2021; Thalinger et al. 2021). It has been repeatedly demonstrated that eDNA metabarcoding read proportions provide quantitative information that relates to both density or total biomass in both aquaria and mesocosm experiments (Evans et al. 2016; Di Muri et al. 2020; Shu et al. 2021) and field experiments, even in estuaries (van Bleijswijk et al. 2019; Afzali et al. 2021; He et al. 2022). The chosen primer appears to play a major role in metabarcoding's quantitative abilities due to the previously mentioned issue of primer biases (Shu et al. 2021). Reliable, independent estimations of abundance or biomass are required to test the ability of eDNA metabarcoding to reflect species abundance (e.g., Di Muri et al. 2020). As a result, eDNA metabarcoding needs to be initially paired with a traditional sampling method for validation in aquatic ecosystems and has been increasingly investigated in estuaries (van Bleijswijk et al. 2019; Fujii et al. 2019; Zou et al. 2020; Afzali et al. 2021). However, traditional sampling methods are far from inerrant, and possess biases unique to each method that distort the fish assemblage they reveal. As such, all traditional sampling methods offer an imperfect, but still useful, means of validating eDNA metabarcoding.

2.10 eDNA as a Complementary Method for Beach Seining

Beach seining is a commonly used sampling technique for coastal faunae due to its ease of use and replicability (Weldon et al. 2005). It has been used extensively in monitoring the health of seagrass habitats (Weldon et al. 2005; Finley et al. 2009; Schein et al. 2012; Baker et al. 2016; Kidd et al. 2021). Results from beach seining nearshore species are often highly variable between samples due to the 'patchiness' of species distribution in their habitat (P.F. Thomsen et al. 2012; Baker et al. 2016; Nevers et al. 2018; Shelton et al. 2019). Another issue with seining is the systematic exclusion of deeper water species that rarely

inhabit the nearshore water where beach seines effectively operate (Weldon et al. 2005; Steele et al. 2006). It has long been shown that eDNA metabarcoding detects higher species richness in coastal marine environments than many physical sampling methods, including beach seining (P.F. Thomsen et al. 2012; Andres et al. 2022; He et al. 2022). The distribution of eDNA appears more homogenous in the water than the distribution of fishes and as a result, has greater consistency and less variability between samples than seining when detecting a single fish species (Nevers et al. 2018, Shelton et al. 2019). He et al. (2022) recently found that select 12S metabarcoding primers provide some insight into the abundance of fishes caught in nearshore seagrass beds. However, this study was from a single year and did not factor in seasonal changes; thus, future studies are needed to affirm these observations.

Chapter 3: Using Longitudinal Nearshore Fish Assemblages to Evaluate Varying Levels of Nutrient Impact in Temperate Estuaries.

3.1 Overview

The inner region has been recently identified as a key area in estuarine health assessments, especially with regards to eutrophication. However, the inner estuary's fish assemblage is undersampled in Canada's southern Gulf of Saint Lawrence (sGSL) due to issues of accessibility and avoidance due to high macroalgal biomass (i.e., *Ulva* sp.). High abundances of the northern mummichog (*Fundulus heteroclitus macrolepidotus*) have been proposed as a single species indicator of eutrophication that could simplify sampling efforts. This study had two objectives: 1) Assess whether the inner estuarine region possessed distinct nearshore fish assemblages and/or higher mummichog abundance relative to the middle and outer estuarine regions; and 2) Assess whether estuaries with similar levels of nutrient impact (high-to-low impact defined by eutrophic time) had similar nearshore fish assemblages and/or mummichog abundance. Beach seines were used to collect nearshore fish at the inner, middle, and outer regions of three estuaries in August 2020 and four estuaries in June and August 2021 in Prince Edward Island, Canada. The inner region's nearshore fish assemblages were generally significantly different from the middle and outer regions, often containing higher abundances of mummichogs and the young-of-the-year of many fishes, regardless of nutrient impact level, shoreline (north shore vs south shore), month, or year. Nearshore fish assemblages were generally more similar between estuaries on the same shoreline (north shore vs south shore) than similar levels of nutrient impact. When comparing only the inner regions between estuaries, inner regions with higher levels of nutrient impact were found to have relatively higher mean mummichog abundances than inner regions with lower eutrophic times. Mummichog abundance also displayed a strong, positive linear relationship with sea lettuce coverage. We conclude that relatively high mummichog abundance could offer a simple, reliable indicator for degraded estuarine health in the inner region due to eutrophication in the sGSL.

3.2 Introduction

Increased nitrogen pollution from agriculture has been identified as one of the major threats to the health of temperate estuaries worldwide due to the resulting eutrophication (Burkholder et al. 2007; Howarth et al. 2011). In estuaries, nitrogen stimulates the mass proliferation of fast-growing, opportunistic macroalgae, like sea lettuce (*Ulva sp.*), which shade out light-limited seagrasses, like eelgrass (*Zostera marina*), and cause hypoxia as it decomposes (Valiela et al. 1997; Hauxwell et al. 2001; Deegan et al. 2002; Coffin et al. 2018b). Both algal blooms and seagrass loss culminate in biodiversity declines (Hughes et al. 2002; Schein et al. 2012).

Recent studies have suggested that the inner region is important to understanding the health of estuaries. The inner estuarine region is the first to receive riverine inputs, like nutrients, and has a longer residence time than regions closer to the ocean (Coffin et al. 2018b; Niu et al. 2021). As a result, pollutants are generally more concentrated here than in the middle and outer regions (Schein et al. 2012; Turner et al. 2021). Studies in Canada's southern Gulf of Saint Lawrence (sGSL) have demonstrated that the inner estuarine region is most affected by eutrophication as a result of excessive nitrogen loading (namely nitrate), with dense mats of sea lettuce excluding eelgrass and frequent hypoxia often being restricted to this region in many estuaries (van den Heuvel et al. 2019; Coffin et al. 2021a). This is especially the case in the coastal waters surround Prince Edward Island (PEI), due to intensive agriculture practices in the province (Bugden et al. 2014). Regarding eutrophication's effects on estuarine biodiversity, past studies were mainly conducted in the middle and outer estuarine regions (e.g., Joseph et al. 2006; Schein et al. 2012; Schmidt et al. 2017) and as a result, the inner region's faunal communities remain poorly understood in the sGSL.

Fisheries and Oceans Canada (DFO)'s stewardship program, the Community Aquatic Monitoring Program (CAMP), may offer a unique opportunity to address the lack of information on faunal communities in the sGSL's inner estuarine region and aid in the assessment of eutrophication (DFO 2011). CAMP was developed in 2003 by DFO in collaboration with the Southern Gulf of St. Lawrence Coalition on Sustainability under the

Oceans Act-1996 (Weldon et al. 2005). CAMP's original purpose was to raise awareness about the coastal environment and allow DFO to engage with various non-governmental environmental organizations, but now is being evaluated for assessing estuarine health (DFO 2011). The program involves collecting six beach seines to monitor nearshore nekton (e.g., fish, crabs, shrimp), along with water parameters and aquatic vegetation coverage estimations, mainly from the middle and outer regions of 35 estuaries throughout the sGSL with volunteers from citizen science groups, universities, and First Nations (Weldon et al. 2005; DFO 2011). Therefore, CAMP presents a cost-effective means of obtaining estuarine nearshore nekton community data. However, CAMP's sampling regime often excludes the inner region of most of its estuaries due to difficulties operating beach seines in the thick sea lettuce mats (Weldon et al. 2005; DFO 2011; van den Heuvel et al. 2019). Estuaries are known to have strong spatial gradients resulting in longitudinally distinct faunal communities (Ysebaert et al. 2003; Whitfield 2021). However, little data currently exist to support this prediction in the sGSL and to justify the increased sampling effort required from volunteers to sample the inner region.

Fishes are known to be poorer bioindicators of estuarine degradation than benthic invertebrates, as many fishes can be quite tolerant to certain impacts like hypoxia (Whitfield and Elliott 2002; Shimps et al. 2005; McGowan et al. 2022). This fact has not escaped DFO's notice (DFO 2011). However, fishes are particularly interesting to DFO for use as indicators of estuarine health as it is easier to train volunteers to identify fishes than many invertebrates (Whitfield and Elliott 2002; Weldon et al. 2005; Thériault et al. 2006). Fish also garner high public interest due to their known economic and cultural connections (Whitfield and Elliott 2002). This factor may be important to capitalize on in temperate estuaries as they lack large charismatic animals that can be used to foster public awareness (Duarte et al. 2008).

Finley et al. (2009) proposed that high abundances of the northern subspecies of mummichog (*Fundulus heteroclitus macrolepidotus*; hereon mummichog) could potentially be a single species indicator to simplify eutrophication assessment of the sGSL's estuaries.

Mummichogs have been used in several anthropogenic impact studies across eastern North America, including the sGSL, due to their tolerance to pollutants and high site fidelity (see Burnett et al. 2007). Mummichogs are well adapted to eutrophic estuaries and appear at higher abundances in sea lettuce-rich sites than eelgrass-dominated ones (Schein et al. 2012; Finley et al. 2013; Lockfield et al. 2013; Dixon et al. 2017). For example, mummichogs are tolerant to hypoxic condition and are even capable of performing aquatic surface respiration, which may help them survive long periods of hypoxia (Wannamaker and Rice 2000; Dixon et al. 2017).

This study aimed to address two main objectives. The first objective was to investigate whether the nearshore fish assemblage and/or mummichog abundance in the inner region differed from those in the middle and outer regions of four estuaries in PEI, Canada. For the purposes of this comparison we define assemblage as the ray finned fish (i.e., Actinopterygii) occupying a defined geographic region at the same moment in time (*sensu* Fauth et al. 1996). The second objective was to investigate whether estuaries with similar levels of nutrient impact (high-to-low impact) based on the recently developed eutrophic time metric (*sensu* Coffin et al. 2021a) had similar nearshore fish assemblages and/or mummichog abundances.

3.3 Materials and Methods

3.3.1 Site Selection

Four estuaries in Prince Edward Island (PEI) were selected to include two north shore sites (Wheatley River (WR) and Freeland Creek (FC)) and two south shore sites (Dunk River (DR) and Enmore River (ER)) to account for variation in geography, nutrient loading, and dominant aquatic vegetation (Table 3.1; Figure 3.1A-B; Coffin et al. 2018b; van den Heuvel et al. 2019). The estuaries' eutrophication statuses were determined *a-priori* using data collected by Coffin et al. (2018b); van den Heuvel et al. (2019) and are displaed in Table 3.1. Estuaries classified *a-priori* as high nutrient impact (WR and DR) were located in the

Table 3.1: Summary of *a-priori* classification of eutrophication status of studied estuaries based on data collected by Coffin et al. (2018b) and van den Heuvel et al. (2019). Estuaries classified as having a low nutrient impact (Freeland Creek and Enmore River) had lower percentage of agriculture in watershed, lower nitrate loading, higher eelgrass coverage within estuary, and presence of eelgrass in the inner region (represented by 10 % of estuarine surface area) relative to the estuaries classified to having a high nutrient impact (Wheatley River and Dunk River).

	High Nutrient Impact	Low Nutrient Impact
North Shore (Lagoon-type)	**Wheatley River**	**Freeland Creek[1,2]**
Residence time (days)	1.74	2.13
Tidal Amplitude (m)[3]	0.9-0.1[4]	0.9-0.1[5]
Watershed area (km^2)	42.1	10.9[6]
Estuarine area (ha)	292	94.8[6]
Mean depth (m)	1.49	0.80
Agricultural land use (%)	66.4	17.4[6]
Nitrate loading (kg ha^{-1} yr^{-1})	149.0	1.4
***Z. marina* coverage of available habitat (%)**	20.9	49.4
Dominant vegetation at 10% Station	*Ulva* species	*Z. marina*
Sampled:	2020: August 2021: June, August	2020: August 2021: June, August
South Shore (Embayment-type)	**Dunk River[7]**	**Enmore River**
Residence time	0.46	0.56
Tidal Amplitude (m)[3]	1.7-0.4[8]	1.2-0.1[9]
Watershed area (km^2)	161.1	36.6
Estuarine area (ha)	973	130
Mean depth (m)	1.51	1.37
Agricultural land use (%)	68.0	10.5
Nitrate loading (kg ha^{-1} yr^{-1})	312.5	5.8
***Z. marina* coverage of available habitat (%)**	24.5	37.8
Dominant vegetation at 10% Station	*Ulva* species	*Z. marina*
Sampled:	2020: August 2021: June, August	2021: June, August

1. Data from Bideford was used to represent Freeland Creek unless specified
2. Freeland Creek would be later re-classified as having a mid nutrient impact based on dissolved oxygen variability profile and eutrophic time from data collected in 2021 relative to Wheatley River and Enmore River.
3. Peak-to-peak amplitude range calculated from tide charts.
4. Rustico Harbour was the closest tidal station to Wheatley River
5. Ellerslie was closest tidal station to Freeland Creek
6. Re-estimated for Freeland Creek in QGIS.
7. Dunk River would be later re-classified as having a mid nutrient impact based on dissolved oxygen variability profile and eutrophic time from data collected in in 2021 relative to Wheatley River and Enmore River.
8. Summerside Harbour was the closest tidal station to Dunk River
9. Egmont Bay was the closest tidal station to Enmore River

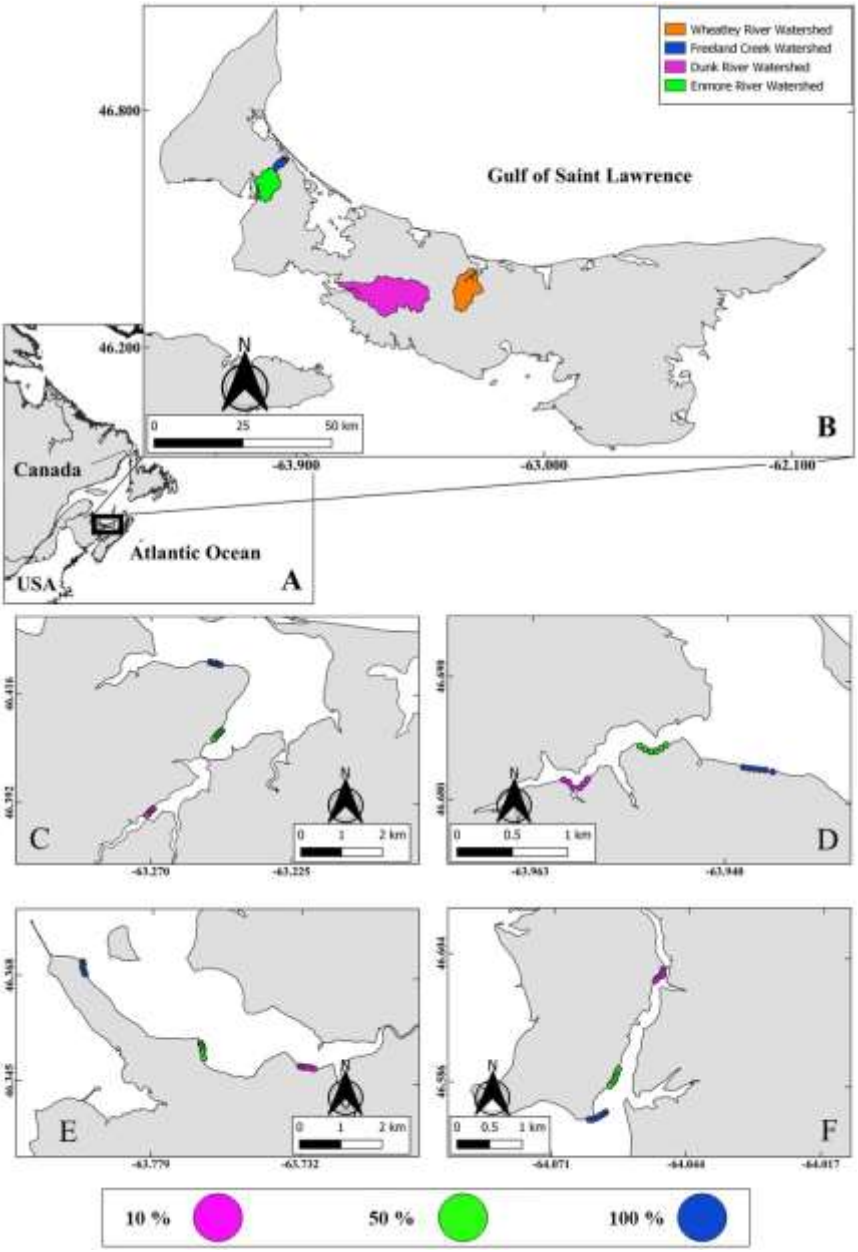

Figure 3.1: A) Study area in the context of north-eastern North America. B) Map of the sampled estuaries' watersheds in Prince Edward Island, Canada, during the summer of 2020 and 2021. Enmore River (lime green) was only sampled in 2021. The estuaries were Wheatley River (C), Freeland Creek (D), Dunk River (E), and Enmore River (F). Dissolved oxygen and salinity loggers were moored at the seaward boundary of the upstream 10% station in each estuary. Five or six beach seine nets were collected at the seaward boundary of each station. Image created using QGIS (64 Bit, Version: 3.16.11 Hannover).

province's highly agricultural central region (Table 3.1; Figure 3.1A-B; Grizard et al. 2020). In contrast, estuaries classified *a-priori* as low nutrient impact (FC and ER) were in the province's less-agricultural western portion (Table 3.1; Figure 3.1A-B; Grizard et al. 2020). All sampled estuaries receive relatively little freshwater inputs and are classified as well-mixed mesotidal with mixed semi-diurnal tides (Dohler 2007; Bugden et al. 2014). However, the lagoon-like estuaries on the north shore (WR and FC) are known to have longer residence times (<2 days vs 0.5 days) and lower tidal amplitudes (0.9-0.1 m vs 1.7-0.1 m peak-to-peak amplitude) than embayment type estuaries on the south shore (DR and ER; Table 3.1; Figure 3.1; see Coffin et al. 2017). WR, FC, and DR were sampled in 2020 and 2021, while ER was added in the summer of 2021 to include an additional south shore estuary (Table 3.1).

For this study, the upper estuarine boundaries was defined at 0.5 PSU (Coffin et al. 2018b; van den Heuvel et al. 2019) or where upstream causeways restricted saltwater to flow into the riverine portion of the estuary, as was the case of FC and DR. At the same time, geographic features marked the estuarine area's lower boundaries (Figure 3.1C-F). Each estuary was divided into three stations based on the estuary's surface area: 10 % of total surface area (closest to the river/ inner estuary), 50 % of total surface area (middle of the estuary), and 100 % of total surface area (closest to the ocean/ outer estuary). Five-to-six sampling sites were established at the seaward boundary of each station (Figure 3.1C-F). The 10 % station (19-27 PSU) tends to be the site most impacted by eutrophication on PEI and is often dominated by sea lettuce (*Ulva lactuca* and *U. intestinalis*; van den Heuvel et al. 2019). In contrast, the 50 % and 100 % stations (25-30 PSU) tend to be dominated by eelgrass (*Zostera marina*) or bare sediment.

3.3.2 Dissolved Oxygen

Dissolved oxygen (DO) variability has been determined to be one of the single best indicators of estuarine eutrophication status in the sGSL (Coffin et al. 2018b; Coffin et al. 2021a). As such, it was used to examine whether our *a-priori* classifications of the four estuaries' eutrophication status (i.e., high nutrient impact vs low nutrient impact) were appropriate. Oxygen sensor deployment followed methods set out by Coffin et al. (2018b). In short, Onset Hobo® optical dissolved oxygen loggers (accuracy of:±0.2 mg/L from 0 to 8

mg/L and ±0.5 mg/L from 8 to 20 mg/L and a resolution of 0.02 mg/L according to manufacturer, set to measure every 15 min) with antifouling guards (model 26-GUARD-2) and Onset Hobo® salinity loggers (Low Range: 100 to 10,000 µS/cm, High Range: 5,000 to 55,000 µS/cm, Model U24-002-C, set to measure every 30 min) were moored at 0.5 m from the estuaries' bottom in waters 1.0-2.5 m in depth between May and September 2021 to capture the summer's DO profile at the 10 % station's seaward boundary. The DO loggers were visited once in July, to remove any biofouling on the sensors, download data, and take YSI readings for calibration. To better analyse the DO variability, the eutrophic time (the proportion of time above 10 mg/L plus time below 4 mg/L), and the coefficient of variation were calculated between 1 June 2021 (0:00 h) and 25 August 2021 (23:45 h) and plotted in MiniTab® Statistical Software, V 21.1 (2021 Minitab, LLC, 64-Bits).

3.3.3 Beach Seining

Five or six beach seine hauls were performed at the seaward boundary of each station approximately 50 m apart between high and low tide during the morning to mid-afternoon throughout the sampling months. Tides do not appear to significantly shift the species composition, abundance, or the size distribution of each species in the sGSL between spring and neap tides (Landry et al. 2007 unpublished). Nevertheless, samples were collected near high tide in the morning to mid-afternoon for consistency. Sampling took between 1-2 days to complete at each estuary and occurred in August in 2020 and June and August in 2021. Hypoxic conditions tend to be more pronounced in August than in June (Coffin et al. 2018b), thus allowing for differences in fish assemblages pre and post hypoxia to be detected if they were present.

Sampling followed a beach seining protocol outlined in Schein et al. (2012). In short, nearshore fishes (<1.5 m water depth) were sampled with a beach seine (30 m × 1.5 m seine, 3 mm mesh and 1.2 m bag) by walking 15 m perpendicular to the shore into the water, then walking 15 m parallel to the shore before circling back to shore, capturing an area of

approximately 225 m^2 (Schein et al. 2012). Captured fishes were identified to the level of species and sorted into adults or young-of-the-year (YOY) based on size and numerated as such. Mummichogs (*Fundulus heteroclitus macrolepidotus*) and banded killifish (*F. diaphanous*), and threespine stickleback (*Gasterosteus aculeatus*) and blackspotted stickleback (*G. wheatlandi*) were combined into a single YOY variables (*Fundulus* sp. and *Gasterosteus* sp. respectively) due to difficulties distinguishing their YOY. Winter flounder (*Pseudopleuronectes americanus*) and smooth flounder (*Pleuronectes putnami*) were combined (*Pleuronectidae* sp.) at both the adult and YOY levels due to possible inconsistencies in their differentiation, as were all *Alosa* sp. Faunae known to be fragile (e.g., Atlantic silverside (*Menidia menidia*), and *Alosa* sp.) or predatory (e.g., green crab (*Carcinus maenas*)) were counted first to minimize the risk of unwanted mortalities. To minimize recaptures, captured faunae were released downstream of sampling or after the following seine haul was started.

Water parameters (i.e., temperature (°C), DO concentration (mg/L), and salinity (PSU)) were collected adjacent to the sampling area using a YSI 650 MDS multiparameter water quality meter before seining each time (Weldon et al. 2005). CAMP's protocol uses a quadrat thrown within the enclosed seine area to estimate vegetation coverage. This study deviated from CAMP vegetation protocol due to concerns over scaring fishes with the quadrat. Instead, a visual estimation of the percent coverage of sea lettuce, eelgrass, or bare sediment in the entire area enclosed by the beach seine was made before the net was hauled back into the shore. This helped to determine the dominant aquatic vegetation present at that seine site without entering the enclosed area.

3.3.4 Multivariate Analyses

All beach seining data were imported into the PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK). All singletons and doubletons were removed from all seine nets collected to remove the influence of rare, potentially arbitrarily distributed

species (Clarke et al. 2014). Next, a square root transformation was applied to down-weight the importance of abundant taxa (Clarke et al. 2014). Finally, the Bray-Curtis similarity index was selected for the resemblance measure as it ignores joint absences (Clarke et al. 2014). All analyses were performed on the total abundance for adults and YOY combined for each taxonomic group, and again with the adults and YOY as separate entities. Both analyses yielded similar results and trends were more visible with the adults and YOY separated, and as such only those results are presented.

A series of two-factor crossed permutational multivariate analysis of variances (PERMANOVA)s were performed for each estuary to analyse the differences in the nearshore fish assemblages between June and August in 2021 and then the differences between August 2020 and August 2021 (see Appendices A.1-A.2). Factors included either Month (levels: June, August) or Year (levels: August 2020 and August 2021) and Station (levels: 10 %, 50 %, and 100 %) using Type III sum of squares with fixed effects sum to zero for mixed terms, and a maximum of 9999 permutations. Pairwise tests were performed on significant factor interactions (see Appendices A.3-A.6). Balanced PERMANOVA designs, such as in this study, are robust against heterogeneity of multivariate dispersion, and as such PERMANOVAs were still performed on data that violated this assumption (Anderson and Walsh 2013). Two-factor similarity percentages (SIMPER) analyses based on the Bray-Curtis index were then used to estimate the average dissimilarity between factors (Month/Year, Station) within each estuary and the fishes that accounted for them (Clarke et al. 2014). Finally, non-metric multidimensional scaling (nMDS) plots were used to help visualize the patterns in the multivariate data.

Next in the analyses, distance based linear models (DISTLM) were performed on each estuary Month or Year combination separately to model the relationships of measured environmental parameters (i.e., temperature, DO, salinity, % coverage of *Ulva* sp., % coverage of *Z. marina*, and % coverage of sediment) with the variation in the fish assemblages observed. First, it was determined by visualizing with histograms that %

coverage of *Ulva* sp., % coverage of *Z. marina*, and % coverage of sediment needed to be transformed with the natural logarithm (log x+1) and salinity by square root to help minimize skewness. After that, all data were normalized. Draftsman plots suggested that none of the measured variables had significant co-correlation (r>0.9), and as such, none were removed, except for *Z. marina* coverage in DR, as we never observed eelgrass at this estuary (see Appendices A.7-A.8). Afterwards, the DISTLM's selection procedures using the BEST procedure were performed (Anderson et al. 2008). This identified the ordering of variables that best model the relationships using the Akaike's information criterion (AIC) selection criterion. The AIC's proportion of explained variability does not improve when the number of environmental predictor variables increases, instead trying to find the lowest number of variables needed to explain the model, with 9999 permutations. Finally, distance-based Redundancy Analysis (dbRDA) was used to visualize the relationships.

One-factor analysis of similarities (ANOSIM) tests were performed to examine whether estuaries with similar levels of nutrient impact had similar fish assemblages during August 2020 and August 2021 (Clarke et al. 2014). ANOSIM's R statistic allowed for the ability to quantify dissimilarities between high nutrient impact and low nutrient impact estuaries. August was selected as this was the month when hypoxia is most prevalent in nutrient-impacted estuaries (Coffin et al. 2018b). The fish assemblage data were analysed in two separate ways. First, all samples collected at the three stations (10 %, 50 %, 100 %) were pooled to compare how the overall fish assemblages differed between the estuaries. Next, only the fish assemblage data collected at the 10 % stations between estuaries were compared, as it was the most impacted region. The tests were performed with estuaries as unordered factors, 9999 permutations and Spearman rank correlations (see Appendix A.9 for pooled stations' results). Then one-factor SIMPERs based on the Bray-Curtis index were performed to estimate dissimilarity between estuaries. nMDS plots were used to visualize any patterns. It was found that the patterns revealed by the nMDS plots were similar whether the data were pooled by all three stations together or analysed only at the 10 % station (see

Appendix A.10). Thus, it was decided to present the pooled results as it encompasses all three stations in our nMDS plots.

3.3.5 Univariate Analyses

Previous studies in PEI have suggested that mummichogs abundance could be used as an indicator of eutrophication for the sGSL (Finley et al. 2009; Schein et al. 2012). It was assumed that the vast majority of *Fundulus* sp. captured (adult and YOY) would represent mummichogs due to so few adult banded killifish being captured.[1] To test if *Fundulus* sp. abundances could be used as an indicator of eutrophication, a generalized additive model (GAM; Wood et al. 2016) was constructed. This model examined differences in the abundances of *Fundulus* sp. between estuaries, stations, months, and whether measured environmental parameters (i.e., temperature, DO, salinity, % coverage of *Ulva* sp., % coverage of *Z. marina*, and % coverage of sediment) had a linear or non-linear relationship with *Fundulus* sp. abundances, using data collected in August 2020, and June and August 2021. Bias size, dispersion of residuals, homogeneity of variance, and the relationship between the observed and predicted response were assessed to verify that model assumptions were not violated. To help achieve a near normal distribution, the following parameters were transformed: *Ulva*, *Z. marina*, and sediment % coverages were natural logarithm (log (x+1)) transformed; salinity was square root transformed; DO (mg/L), and temperature had no transformations. It was determined that *Fundulus* sp. abundances were tweedie distributed (poisson-like, discrete distribution with high levels of zero inflation). The model was run in R (version 4.0.5; R Core Team 2021) using the mgcv routine (v. 1.8.39; see Appendix A.11), and model outputs were visualised using ggplot2 (v. 3.3.5), wplot (v. 1.1.1), and gratia (v. 0.7.0; Wickham 2009; Wood et al. 2016).

[1] The YOY of banded killifish and mummichog are indistinguishable. However, adults can be readily distinguished. During the entire duration of field sampling (August 2020 and June and August 2021), only 26 adult banded killifish were captured, compared to 50,084 adult mummichogs.

Each estuary was analysed using a series of ANOVAs to examine whether the GAM would obscure any estuary-, month-, or year-specific patterns relating to the abundance of *Fundulus* sp. In particular, we examined whether the mean *Fundulus* sp. abundance found at the 10 % station was consistently higher than the 50 % and 100 % station at all the estuaries we sampled. These ANOVAs also were done to test whether comparing only the 10 % station's mean *Fundulus* sp. abundance between the estuaries would produce a different pattern from pooling all three stations sampled within an estuary. These analyses provided the ability to examine whether the mean abundance of *Fundulus* sp. could distinguish estuaries identified as having a relatively higher nutrient impact (e.g., WR) from estuaries of lower nutrient impact (e.g., ER) at the region most susceptible to eutrophication. To prepare the data, the data were transformed using the natural logarithm (log+1) to help achieve the assumption of homogeneity of variance and normality among residuals. All univariate analyses were performed in MiniTab® Statistical Software.

In a similar design to the multivariable test, a series of two-factor ANOVAs were performed to compare the mean *Fundulus* sp. abundance between the three stations within each individual estuary between Month (June and August) or Year (August 2020 and August 2021). However, it was found that the patterns revealed by the two-factor ANOVAs' results generally matched those revealed in the GAM (see Appendices A12-A13). Thus, only the results from the GAM were presented to reduce redundancy.

A series of one-factor ANOVAs were performed to compare the mean *Fundulus* sp. abundance at the 10 % stations between the three estuaries sampled in August 2020, and four in August 2021 (see Appendix A.14). Tukey tests were used to identify which estuaries differed if a statistical significances were detected, as it is recommended for unplanned comparisons due to its reduced risk of type I errors relative to other post-hoc tests (Ruxton and Beauchamp 2008). The mean abundance per seine net and the 95% confidence intervals were back transformed to allow easier assessment and visualization. Alpha was set at $p=0.05$ for all multivariate tests, GAM, and ANOVAs.

3.4 Results

3.4.1 Summer 2021 Dissolved Oxygen Profiles

Dissolved oxygen (DO) variability profiles were taken at the 10 % station (inner region) of the four estuaries throughout the summer of 2021 to see if the *a-priori* classification of an estuaries' eutrophication status (i.e., high nutrient impact vs low nutrient impact) as appropriate. Wheatley River (WR) displayed characteristics of an estuary with high nutrient impacts identified by Coffin et al. (2018b), having extended periods of supersaturation (>10 mg/L) in early summer and hypoxia (<4 mg/L) later in the summer, resulting in a higher eutrophic time relative to the other estuaries (Figure 3.2A-B). Freeland Creek (FC) and Dunk River (DR) displayed higher and lower than expected periods of supersaturation and hypoxia throughout the summer respectively and had similar summer DO profiles and eutrophic times (Figure 3.2A-B). Therefore, our *a-priori* classification of DR as high nutrient impact and FR as low nutrient impact may not have been appropriate. Enmore River (ER) had the most stable oxygen profile through the summer and lowest eutrophic time (Figure 3.2A), which are characteristics of estuaries with low nutrient impact. As a result, it was decided to maintain the *a-priori* classification for WR and ER, while re-classifying FC and DR as having mid nutrient impacts relative to WR and ER.

3.4.2 Spatial, Seasonal, and Interannual Variation in Station's Nearshore Fish Assemblage

Next, it was investigated whether the nearshore fish assemblage at the 10 % station (inner region nearest to freshwater inputs) was distinct from the 50 % (middle region) and 100 % (outer region nearest to ocean) stations across four estuaries with varying levels of nutrient impact. Two temporal scales were investigated: first, to see whether the nearshore fish assemblages at the three stations differed between June and August during summer 2021, and second, between August 2020 and August 2021, to see if the late-summer assemblages are consistent between years. ER was excluded from this interannual analysis as it was

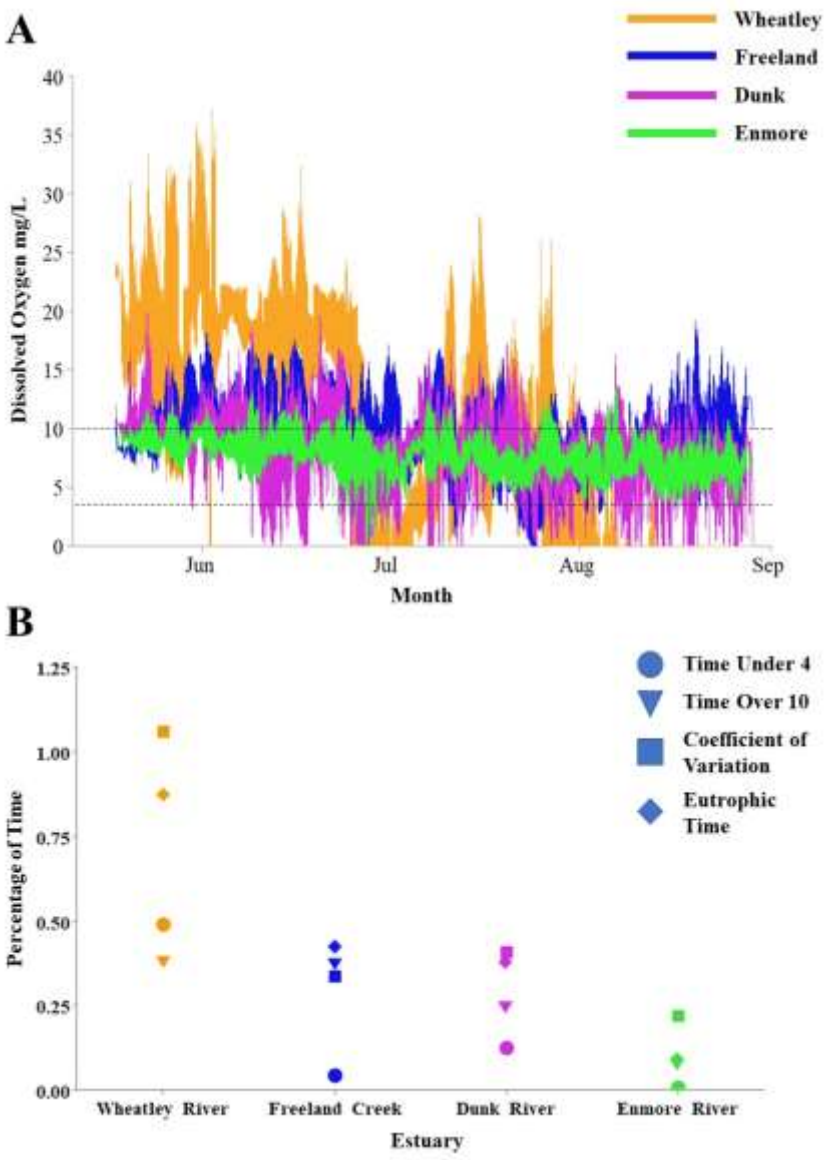

Figure 3.2: A) Summer oxygen profile for the four estuaries' 10 % station across Prince Edward Island, Canada, in 2021. Area between supersaturation (>10 mg/L) and hypoxia (<4 mg/L), as represented by the black dashed lines, indicate normoxic conditions. B) Mean values of the percentage of time under 4 mg/L (circles), over 10 mg/L (triangles), the coefficient of variation (squares), and for eutrophic time (diamonds) for all dissolved oxygen values over the course of the dissolved oxygen logger deployments. Estuaries are presented in descending order of highest (Wheatley River) to lowest (Enmore River) eutrophic times. Image created using MiniTab® Statistical Software, V 21.1 (2021 Minitab, LLC, 64-Bits).

sampled only in 2021 (Table 3.1).

It was found that the 10 % station of all estuaries tended to be significantly different from the 50 % and 100 % stations in June and August in 2021 ($P(MC)<0.05$ for most tests; Figure 3.3A.1-D.1). These significant differences appear to be consistent in August in both 2020 and 2021 for most estuaries ($P(MC)<0.05$ for most tests; Figure 3.3A.2-C.2). The only exception was in DR in August 2021, when the 10 % station was found to be statistically similar to the 50 % station ($P(MC)>0.05$; Appendix A.3C). The 50 % and the 100 % stations also tended to be statistically distinct from one another ($P(MC)<0.05$ for most tests; Figure 3.3). Significant shifts in each station's fish assemblages were observed between June and August in 2021 ($P(MC)<0.05$ for each test; Figure 3.3A.1-D.1) and August 2020 and August 2021 ($P(MC)<0.05$ for most tests: Figure 3.3A.2-C.2). Overall, these findings suggest that the 10 % station's fish assemblage is significantly distinct from the 50 % and 100 % stations' assemblages, and each station displayed high degrees of seasonal and interannual variability.

Adult mummichogs and *Fundulus* sp. YOY appear to be generally found at higher abundances in the 10 % station than the 50 % and 100 % stations at most estuaries, regardless of level of relative nutrient impact and were temporally consistent. SIMPER analyses suggested that higher *Fundulus* (adult and YOY combined) sp. abundances at the 10 % station contributed to its dissimilarities from the 50 % and 100 % stations in WR (52.71 and 46.36 %), FC (26.36 and 32.33 %) and DR (31.32 and 34.72 %) in summer 2021 (Table 3.2A-C). These observations appear consistent at WR and FC between August 2020 and 2021 (Figure 3.3A-B). *Fundulus* sp. were the second most influential species in discriminating stations after Atlantic silversides (*M. menidia)*, and still found at higher abundances in the 10 % station than in the two outer stations in ER (Table 3.2D). This was also the case in DR between August 2020 and August 2021 (Table 3.3C). It is also worth noting that many other fishes' YOY (namely Atlantic silversides, fourspine stickleback (*Apeltes quadracus*) and *Gasterosteus* sp.) were often more abundant in the 10 % stations than the other two stations, especially the 100 % station, across all estuaries (Table 3.2; Table 3.3).

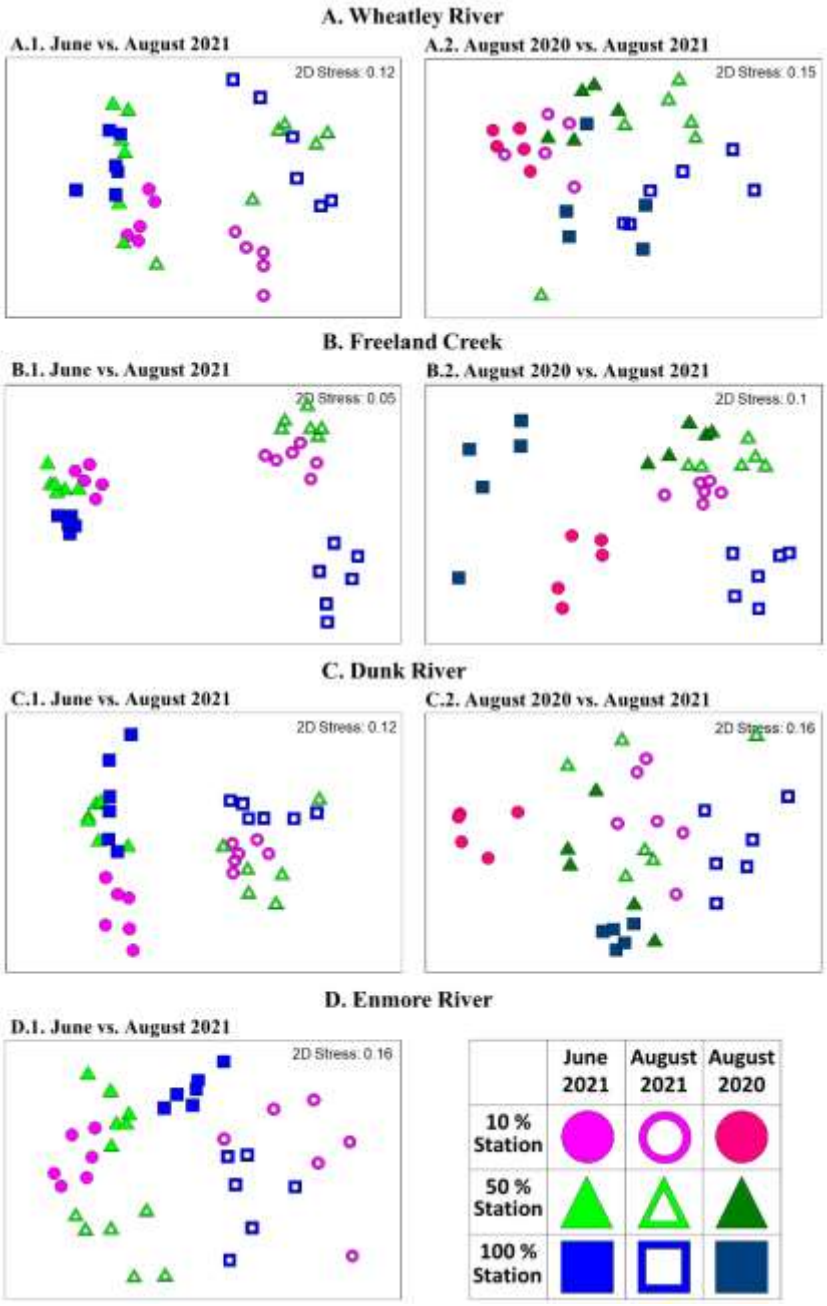

Figure 3.3: nMDS plots comparing the multivariate data cloud between 1) June and August 2021 and 2) August 2020 and August 2021with fish abundance separated by adults and young-of-the-year. Stress was found to be at or below 0.16, indicating most images offer a decent representation of each data cloud's shape. Square root transformations, Bray-Curtis similarity, 100 restarts. Images created using PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK).

Table 3.2: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all months (June and August) between stations within an estuary in sampled Prince Edward Island, Canada during 2021. Fish counts were separated into adults and young-of-the-year. Estuaries were analysed independently. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

A. Wheatley River

10 % and 50 %					
Av. Dissimilarity: 46.51 %					
Species	**10% Av. Abu**	**50% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
F. hetero-clitus (A)	25.3	16.7	14.8	1.62	31.75
Fundulus sp.(YOY)	18.4	4.65	9.75	0.92	20.96
M. menidia (YOY)	6.20	1.02	3.94	0.58	8.48
Gasterosteus sp. (YOY)	1.05	4.03	3.16	0.79	6.80
A. quadracus (YOY)	4.18	4.30	2.77	1.07	5.96
					73.94%

10 % and 100 %					
Av. Dissimilarity: 50.39 %					
Species	**10% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
F. hetero-clitus (A)	25.3	12.9	12.6	1.96	24.93
Fundulus sp.(YOY)	18.4	5.04	10.8	0.90	21.43
M. menidia (YOY)	6.20	3.20	5.47	0.83	10.86
G. aculeatus (A)	0.97	4.94	4.34	0.75	8.60
G. wheatlandi (A)	0.75	3.77	3.39	0.90	6.73
					72.55%

50 % and 100 %					
Av. Dissimilarity: 41.31 %					
Species	**50% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
F. hetero-clitus (A)	16.7	12.9	11.7	0.96	28.39
Gasterosteus sp.(YOY)	4.03	0.17	4.73	0.65	11.45
A. quadracus (YOY)	4.30	0.99	4.20	0.80	10.16
Fundulus sp. (YOY)	4.65	5.04	3.55	0.73	8.59
A. quadracus (A)	4.83	3.44	3.41	1.42	8.25
M. menidia (YOY)	1.02	3.20	3.27	0.89	7.91
					74.75%

Table 3.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all months (June and August) between stations within an estuary in sampled Prince Edward Island, Canada during 2021. Fish counts were separated into adults and young-of-the-year. Estuaries were analysed independently. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

B. Freeland Creek

10 % and 50 %

Av. Dissimilarity: 28.84 %

Species	10% Av. Abu	50% Av. Abu	Av. Diss	Diss/ SD	Cont. %
F. hetero-clitus (A)	16.1	14.4	5.07	1.34	17.57
A. quadracus (A)	4.01	9.39	4.13	1.10	14.33
Gasterosteus sp.(YOY)	6.77	5.65	3.09	1.78	10.73
Fundulus sp.(YOY)	8.01	5.18	2.54	0.89	8.79
G. aculeatus (A)	4.62	5.90	1.69	1.29	5.86
Tau. adspersus (YOY)	0.72	2.64	1.62	1.14	5.62
M. menidia (YOY)	1.78	0.00	1.58	0.84	5.49
Syn. fuscus (A)	1.50	0.60	1.35	1.11	4.68
					73.07%

10 % and 100 %

Av. Dissimilarity: 39.78 %

Species	10% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
F. hetero-clitus (A)	16.1	9.96	7.60	1.68	19.09
Gasterosteus sp.(YOY)	6.77	0.20	6.30	3.61	15.84
Fundulus sp.(YOY)	8.01	2.41	5.27	1.04	13.24
M. menidia (YOY)	2.33	6.01	3.54	1.40	8.90
A, quadracus (YOY)	6.53	4.22	2.20	0.74	5.52
G. wheatlandi (A)	2.00	4.49	2.01	0.77	5.05
A. quadracus (A)	4.01	3.58	1.85	1.40	4.66
					72.29%

50 % and 100 %

Av. Dissimilarity: 43.69 %

Species	50% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
Gasterosteus sp. (YOY)	5.65	0.20	6.10	1.07	13.96
A. quadracus (A)	9.39	3.58	5.32	1.75	12.18
F. hetero-clitus (A)	14.4	9.96	5.06	1.46	11.59
M. menidia (A)	1.83	6.01	3.94	1.56	9.01
M. menidia (YOY)	0.00	3.02	3.55	0.91	8.12
Fundulus sp.(YOY)	5.18	2.41	3.13	0.73	7.16
A. quadracus (YOY)	6.16	4.22	2.55	0.69	5.84
Syn. fuscus (YOY)	1.29	3.36	2.28	0.77	5.22
					73.07%

Table 3.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all months (June and August) between stations within an estuary in sampled Prince Edward Island, Canada during 2021. Fish counts were separated into adults and young-of-the-year. Estuaries were analysed independently. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

C. Dunk River

10 % and 50 %

Av. Dissimilarity: 50.42 %

Species	10% Av. Abu	50% Av. Abu	Av. Diss	Diss/ SD	Cont. %
F. hetero-clitus (A)	10.2	3.57	15.8	0.99	31.32
M. menidia (A)	5.22	7.51	12.3	1.05	24.42
Alosa sp.(YOY)	4.87	2.79	5.81	0.75	11.52
M. menidia (YOY)	6.23	6.95	3.72	0.70	7.37
					74.63%

10 % and 100 %

Av. Dissimilarity: 53.42 %

Species	10% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
F. hetero-clitus (A)	10.2	1.45	18.6	1.10	34.72
M. menidia (A)	5.22	6.47	9.24	1.06	17.30
Alosa sp.(YOY)	4.87	4.48	5.82	0.69	10.90
M. menidia (YOY)	6.23	7.87	3.92	0.71	7.33
					70.26%

50 % and 100 %

Av. Dissimilarity: 44.88 %

Species	50% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (A)	7.51	6.47	11.2	1.04	24.93
F. hetero-clitus (A)	3.57	1.45	6.33	1.64	14.11
Alosa sp.(YOY)	2.79	4.48	6.28	0.58	13.99
M. menidia\ (YOY)	6.95	7.87	3.87	0.74	8.62
P. pungitius (A)	2.35	0.99	3.31	0.82	7.38
Pseudo-pleuronectes sp. (A)	0.26	0.82	3.17	0.95	7.07
					76.10%

Table 3.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all months (June and August) between stations within an estuary in sampled Prince Edward Island, Canada during 2021. Fish counts were separated into adults and young-of-the-year. Estuaries were analysed independently. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

D. Enmore River

10 % and 50 %

Av. Dissimilarity: 59.75 %

Species	10% Av. Abu	50% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	8.20	12.8	26.3	1.63	43.99
F. hetero-clitus (A)	7.95	2.43	8.95	1.63	14.98
Gasterosteus sp.(YOY)	6.32	4.22	5.25	1.54	8.78
Fundulus sp.(YOY)	3.09	1.12	4.64	0.72	7.76
					75.52%

10 % and 100 %

Av. Dissimilarity: 58.85 %

Species	10% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	8.20	3.09	13.5	1.55	22.95
F. hetero-clitus (A)	7.95	4.93	9.27	1.35	15.75
Gasterosteus sp.(YOY)	6.32	0.75	9.17	1.46	15.58
Fundulus sp.(YOY)	3.09	0.95	7.16	0.74	12.17
Alosa sp.(YOY)	0.14	2.08	6.36	0.76	10.81
					77.25%

50 % and 100 %

Av. Dissimilarity: 59.90 %

Species	50% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	12.8	3.09	20.5	1.54	34.22
Gasterosteus sp.(YOY)	4.22	0.75	8.78	1.43	14.66
F. hetero-clitus (A)	2.43	4.93	6.83	1.45	11.40
M. menidia (A)	2.44	1.33	4.15	1.54	6.92
Alosa sp.(YOY)	0.44	2.08	3.77	0.84	6.30
					73.50%

Table 3.3: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all years (August 2020 and August 2021) between stations within an estuary in sampled Prince Edward Island, Canada. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

A. Wheatley River

10 % and 50 %					
Av. Dissimilarity: 42.73%					
Species	**10% Av. Abu**	**50% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
Fundulus sp.(YOY)	43.5	17.3	16.47	2.41	34.88
F. hetero-clitus (A)	19.6	16.3	8.41	1.51	17.82
M. menidia (YOY)	12.6	5.33	5.76	0.86	12.19
A. quadracus (YOY)	3.74	9.91	5.24	1.80	11.11
					76.00%

10 % and 100 %					
Av. Dissimilarity: 51.33%					
Species	**10% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
Fundulus sp.(YOY)	43.5	14.1	22.9	2.49	43.05
F. hetero-clitus (A)	19.6	10.3	10.3	1.78	19.34
M. menidia (YOY)	12.6	3.58	8.46	1.19	15.92
					78.31%

50 % and 100 %					
Av. Dissimilarity: 45.04%					
Species	**50% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
F. hetero-clitus (A)	16.3	10.3	9.42	0.76	19.36
A. quadracus (YOY)	9.91	1.58	8.17	1.93	16.80
Fundulus sp.(YOY)	17.3	14.1	7.43	1.64	15.27
Gasterosteus sp.(YOY)	6.97	0.63	7.18	1.06	14.77
A. quadracus (A))	7.59	2.41	5.29	1.98	10.87
					77.07%

Table 3.3 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all years (August 2020 and August 2021) between stations within an estuary in sampled Prince Edward Island, Canada. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

A. Freeland Creek

10% and 50%					
Av. Dissimilarity: 37.58 %					
Species	**10% Av. Abu**	**50% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
F. hetero-clitus (A)	23.5	6.11	11.7	0.96	28.58
Fundulus sp.(YOY)	17.2	11.0	5.95	1.43	14.53
M. menidia (YOY)	8.58	2.03	4.71	1.20	11.49
Gasterosteus sp.(YOY)	4.87	9.33	3.49	1.65	8.51
Tau. adspersus (YOY)	0.35	4.43	2.86	1.05	6.99
					70.10%

10% and 100%					
Av. Dissimilarity: 47.76 %					
Species	**10% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
Fundulus sp.(YOY)	17.2	3.33	12.8	1.69	23.69
F. hetero-clitus (A)	23.5	11.6	12.8	1.04	23.62
M. menidia (YOY)	8.58	3.30	7.15	1.07	13.23
Gasterosteus sp.(YOY)	4.87	0.22	4.74	1.44	8.76
M. menidia (A)	2.74	1.80	3.29	1.44	6.08
					75.37%

50% and 100%					
Av. Dissimilarity: 53.81 %					
Species	**50% Av. Abu**	**100% Av. Abu**	**Av. Diss**	**Diss/ SD**	**Cont. %**
Gasterosteus sp.(YOY)	9.33	0.22	10.8	4.09	16.86
F. hetero-clitus (A)	6.11	11.6	9.90	0.90	15.47
Fundulus sp.(YOY)	11.0	3.33	8.83	1.86	13.79
M. menidia (YOY)	2.03	3.30	6.46	3.11	10.09
A. quadracus (YOY)	9.57	4.61	6.21	1.81	9.70
Tau. adspersus (YOY)	4.43	0.00	4.97	0.96	7.77
					73.68%

Table 3.3 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across all years (August 2020 and August 2021) between stations within an estuary in sampled Prince Edward Island, Canada. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

C. Dunk River

10% and 50%

Av. Dissimilarity: 43.87 %

Species	10% Av. Abu	50% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	9.37	15.9	10.13	1.25	21.38
Fundulus sp.(YOY)	11.8	3.90	9.09	1.16	19.19
Alosa sp.(YOY)	5.41	3.14	7.00	0.89	14.78
M. menidia (A)	2.86	5.09	6.37	1.66	13.44
F. hetero-clitus (A)	9.37	6.18	5.21	1.15	11.00
					79.79%

10% and 100%

Av. Dissimilarity: 43.06 %

Species	10% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	9.37	23.1	15.1	1.49	29.80
Fundulus sp.(YOY)	11.8	0.47	11.3	1.87	22.34
Alosa sp.(YOY)	5.41	5.80	7.61	0.93	15.00
M. menidia (A)	2.86	5.89	5.64	2.25	11.12
					78.26%

50% and 100%

Av. Dissimilarity: 41.42 %

Species	50% Av. Abu	100% Av. Abu	Av. Diss	Diss/ SD	Cont. %
M. menidia (YOY)	15.9	23.1	10.6	1.26	23.65
Alosa sp. (YOY)	3.14	5.80	8.16	0.74	18.18
F. hetero-clitus (A)	6.18	7.75	7.21	1.66	16.06
M. menidia (A)	5.09	5.89	4.56	1.32	10.17
Fundulus sp.(YOY)	3.90	0.47	4.20	1.33	9.37
					77.43%

Nearshore fish assemblages changed alongside a combination of measured environmental parameters that varied between estuaries. Of particular interest was a significant linear relationship between sea lettuce (*Ulva* sp.) coverage and the nearshore fish assemblages observed at all estuaries (WR, FC, and DR) in June and August in 2021 ($p<0.05$), except ER. Eelgrass (*Z. marina)* coverage was found to be significant for WR and FC ($p<0.05$) and was found to be nearly significant in ER ($\sim p<0.05$). Temperature, DO concentrations, and salinity were strongly correlated with the first dbRDA axis, which tended to help separate June and August during the summer of 2021 (Figure 3.4A.1-D.1). At the same time, the second dbRDA axis tended to separate the stations and was often correlated with sea lettuce or sediment coverage (Figure 3.4A.1-D.1). The 10 % station was visibly distinct at WR and was associated with lower eelgrass and sediment coverage (due to high sea lettuce coverage) than the 50 % and 100 % stations (Figure 3.4A.1). The 10 % stations were less consistently distinct from the other two stations in DR, FC and ER (Figure 3.4B.1-D.1). It appeared WR experienced less interannual variation than FC or DR between August 2020 and August 2021 (Figure 3.4A.2). Eelgrass coverage and sea lettuce coverage were strongly correlated with WR's and FC's first, and second axis (Figure 3.4A.2, B.2). At DR, it was found that temperature was most strongly correlated to the first dbRDA axis, and salinity on the second axis (.2).

3.4.3 Comparison of the Average Nearshore Fish Assemblage between Estuaries in August Figure 3.4C

Next, we examined whether estuaries with similar levels of nutrient impact have similar nearshore fish assemblages. Moderately significant differences were found between the nearshore fish assemblages in each of the four estuaries in August 2021 ($R=0.59$; $P(perm)<0.05$; Figure 3.5). Pairwise comparisons suggest the nearshore fish assemblages were more similar between samples collected along the same shoreline ($0.245< R<0.323$) than similar relative nutrient impact levels ($0.68<R<0.922$), with the north shore versus south shore clustering visible in the nMDS plot (Figure 3.5A). August 2020 also showed significant differences ($R=0.59$; $P(perm)<0.05$). However, the three estuaries sampled in

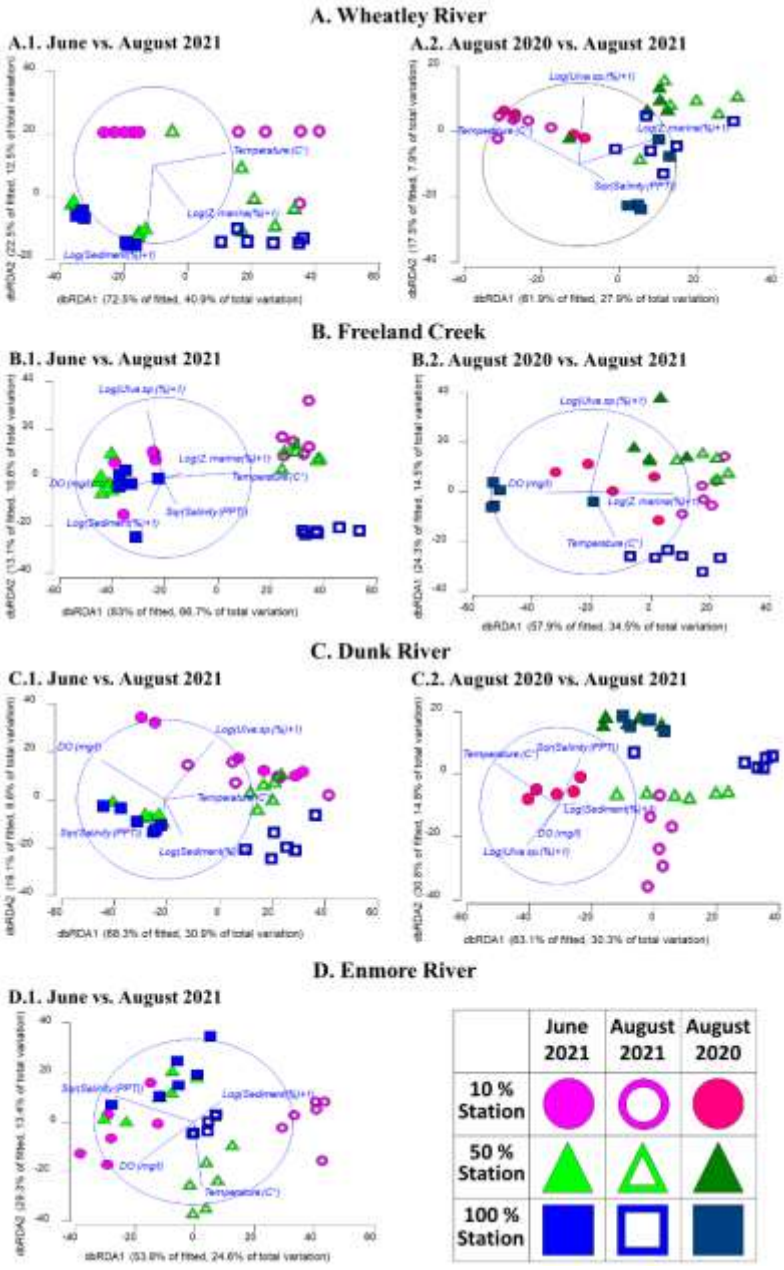

Figure 3.4: Distance-Based Redundancy Analysis (dbRDA) to visualize linear relationships between environmental variables and vegetation coverage with nearshore fish assemblages between 1) June and August 2021 and 2) August 2020 and August 2021. Fish abundance separated by adults and young-of-the-year. Selection Criteria: AIC, Selection Procedure: Best, 9999 Permutations. Note: Z. marina coverage was removed from Dunk River as it was never seen at this estuary. June and August Data. Images created using PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK).

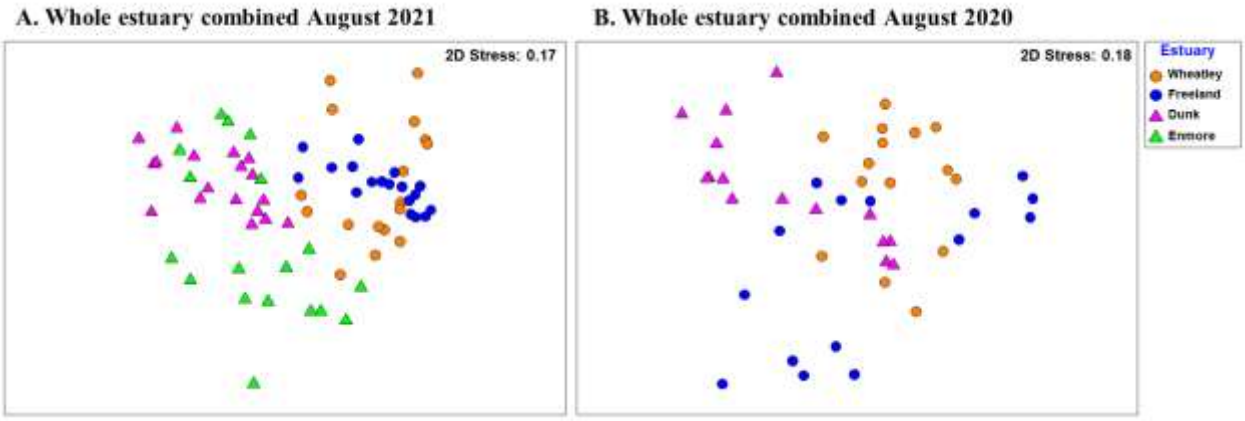

Figure 3.5: nMDS plots comparing the multivariate data cloud of A) differences between the nearshore fish assemblages of the entirety (n=16-18) of the four estuaries sampled in August 2021, and B) differences between the nearshore fish assemblages of the entirety (n=15-16) of the three estuaries sampled in August 2020 with fish abundance separated by adults and young-of-the-year. Circles represented estuaries collected on the north shore, while triangles represented estuaries collected on the south shore. Stress was found to be at or below 0.18, indicating most images offer a moderate representation of each data cloud's shape. Square root transformations, Bray-Curtis similarity, 100 restarts. Images created using PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK).

August 2020 had a lower degree of significant difference ($0.233<R<0.437$) than in August 2021 ($0.323<R<0.922$; Figure 3.5B). Interestingly, FC was found to be similar to both WR ($R=0.233$) and DR ($R=0.244$). Thus, these results suggest that while each estuary's fish assemblage is unique, similarities between shoreline exist. The SIMPER analysis showed that higher *Fundulus* sp. abundance in north shore estuaries (WR and FC) than south shore estuaries (DR and ER), and higher Atlantic silverside abundance at south shore estuaries than north shore estuaries contributed to dissimilarities between the shorelines (Table 3.4).

3.4.4 *Fundulus* Species Abundance

Finally, we examined whether the *Fundulus* sp. abundance reflected the levels of relative nutrient impact of an estuary and could distinguish the 10 % station from the 50 % station and 100 % station. No pattern of difference in *Fundulus* sp. numbers was observed between high, mid, or low nutrient impacted estuaries when examining all samples collected within an estuary in the GAM (Figure 3.6A). Instead, there was a noticeable difference between estuaries discharging to the north shore of PEI directly into the sGSL (WR and FR - higher *Fundulus* sp. abundances) versus the south shore into the Northumberland Strait (DR and ER – lower *Fundulus* sp. abundances; $p<0.05$; Figure 3.6A).

WR had a significantly higher mean *Fundulus* sp. abundance per beach seine haul than all other estuaries in August 2021 when examining only the 10 % stations ($p<0.05$; Figure 3.7A). The pattern of the 10 % stations' mean *Fundulus* sp. abundance appeared to almost mirror the eutrophic times of the of the four estuaries, with the mean abundances and eutrophic times descending in the order of WR, FC, DR, to ER (Figure 3.7C-D). There appeared to be interannual variability in this pattern, as the mean abundance at WR's 10 % station was found to be statistically similar to FC in August 2020 ($p>0.05$; Figure 3.7B). However, no DO data from 2020 existed to further examine if the eutrophic times of WR and FC were more similar to aid in possible explanations for these similarities.

In addition to the above patterns, the 10 % station had a higher *Fundulus* sp. abundance than either the 50 % or 100 % stations in each of all four estuaries ($p<0.05$; Figure 3.6B). It was also found that *Fundulus* sp. abundances had a strong, positive linear relationship with increased sea lettuce coverage ($p<0.05$; Figure 3.6C). Finally, a significant

Table 3.4: One-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across the nearshore fish assemblages of the entirety of the A) four sample in August 2021 and B) three estuaries sampled in August 2020 and Prince Edward Island. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

A. Whole estuary combined August 2021

A.1. Wheatley River and Freeland Creek

Av. dissimilarity = 52.66 %

Species	Wh. Av.Abun.	Fr. Av.Abun.	Av.Diss	Diss/ SD	Cont %
Fundulus sp.(YOY)	17.69	9.95	9.93	1.27	18.86
F. hetero-clitus (A)	14.60	5.43	9.69	1.06	18.40
A. quadracus (YOY)	5.94	10.92	6.92	1.47	13.15
Gasterosteus sp.(YOY)	3.36	6.29	6.13	1.26	11.64
					71.92%

A.2. Freeland Creek and Enmore River

Av. dissimilarity = 72.90 %

Species	En. Av.Abun.	Fr. Av.Abun.	Av.Diss	Diss/ SD	Cont %
A. quadracus (YOY)	0.37	10.92	15.04	3.17	20.63
M. menidia (YOY)	7.89	3.11	9.69	1.17	13.29
Fundulus sp.(YOY)	3.43	9.95	9.49	1.72	13.02
Gasterosteus sp.(YOY)	1.62	6.29	7.54	1.44	10.35
Syn. fuscus (YOY)	0.00	4.58	6.78	1.69	9.30
F. hetero-clitus (A)	2.67	5.43	5.93	1.29	8.13
					74.72%

Table 3.4 continued: One-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across the nearshore fish assemblages of the entirety of the A) four sample in August 2021 and B) three estuaries sampled in August 2020 and Prince Edward Island. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

A.3. Wheatley River and Dunk River

Av. dissimilarity = 73.06 %

Species	Wh. Av.Abun.	Du. Av.Abun.	Av.Diss	Diss/ SD	Cont %
Fundulus sp.(YOY)	17.69	3.11	14.23	1.49	19.47
M. menidia (YOY)	5.89	14.03	12.96	1.76	17.73
F. hetero-clitus (A)	14.60	3.62	11.30	1.08	15.47
Alosa sp . (YOY)	0.00	8.10	8.79	0.91	12.03
A. quadracus (YOY)	5.94	0.00	6.00	1.30	8.21
					72.91 %

A.4. Dunk River and Enmore River

Av. dissimilarity = 59.77 %

Species	Du. Av.Abun.	En. Av.Abun.	Av.Diss	Diss/ SD	Cont %
M. menidia (YOY)	14.03	7.89	8.09	1.63	30.27
Alosa sp. (YOY)	8.10	1.63	11.94	0.97	19.98
M. menidia (A)	4.14	1.48	5.77	1.32	9.66
Fundulus sp.(YOY)	3.11	3.43	5.61	1.15	9.39
F. hetero-clitus (A)	3.62	2.67	5.40	1.37	9.03
					78.32 %

A.5. Wheatley River and Enmore River

Av. dissimilarity = 71.40 %

Species	Wh. Av.Abun.	En. Av.Abun.	Av.Diss	Diss/ SD	Cont %
Fundulus sp.(YOY)	17.69	3.43	17.03	1.57	23.85
F. hetero-clitus (A)	14.60	2.67	15.25	1.21	21.35
M. menidia (YOY)	5.89	7.89	10.95	1.10	15.33
A. quadracus (YOY)	5.94	0.37	7.29	1.28	10.20
					70.74 %

A.6. Dunk River and Freeland Creek

Av. dissimilarity = 73.42 %

Species	Du. Av.Abun.	Fr. Av.Abun.	Av.Diss	Diss/ SD	Cont %
M. menidia (YOY)	14.03	3.11	12.27	1.95	16.71
A. quadracus (YOY)	0.00	10.92	12.24	3.69	16.68
Alosa sp. (YOY)	8.10	0.00	8.73	0.95	11.89
Fundulus sp.(YOY)	3.11	9.95	8.00	1.67	10.90
Gasterosteus sp.(YOY)	2.07	6.29	5.88	1.53	8.01
Syn. fuscus (YOY)	0.00	4.58	5.31	1.75	7.24
					71.43 %

Table 3.4 continued: One-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities across the nearshore fish assemblages of the entirety of the A) four sample in August 2021 and B) three estuaries sampled in August 2020 and Prince Edward Island. Fish counts were separated into adults and young-of-the-year. SIMPER was performed with square root transformed data using Bray Curtis similarity index and a cut-off set at 70 %.

B. Whole estuary combined August 2020

B.1. Wheatley River and Freeland Creek

Av. dissimilarity = 55.66 %

Species	Wh. Av.Abun.	Fr. Av.Abun.	Av.Diss	Diss/ SD	Cont %
Fundulus sp.(YOY)	31.92	11.21	18.07	1.38	32.46
F. hetero-clitus (A)	16.01	23.76	11.74	1.06	21.10
M. menidia (YOY)	8.26	6.47	5.84	1.18	10.50
A. quadracus (YOY)	4.19	2.83	3.66	1.03	6.58
					70.63 %

B.2. Wheatley River and Dunk River

Av. dissimilarity = 54.99 %

Species	Wh. Av.Abun.	Du. Av.Abun.	Av.Diss	Diss/ SD	Cont %
Fundulus sp.(YOY)	31.92	8.12	19.23	1.74	34.97
M. menidia (YOY)	8.26	18.64	11.69	1.17	21.25
F. hetero-clitus (A)	16.01	12.75	6.50	1.23	11.82
M. menidia (A)	2.74	5.19	3.76	1.28	6.84
					74.88 %

Dunk River and Freeland Creek

Av. dissimilarity = 59.13 %

Species	Du.. Av.Abun.	Fr. Av.Abun.	Av.Diss	Diss/ SD	Cont %
M. menidia (YOY)	18.64	6.47	15.28	1.23	25.85
F. hetero-clitus (A)	12.75	23.76	13.70	1.04	23.16
Fundulus sp.(YOY)	8.12	11.21	9.89	1.20	16.72
M. menidia (A)	5.19	1.92	4.97	1.16	8.41
					74.14 %

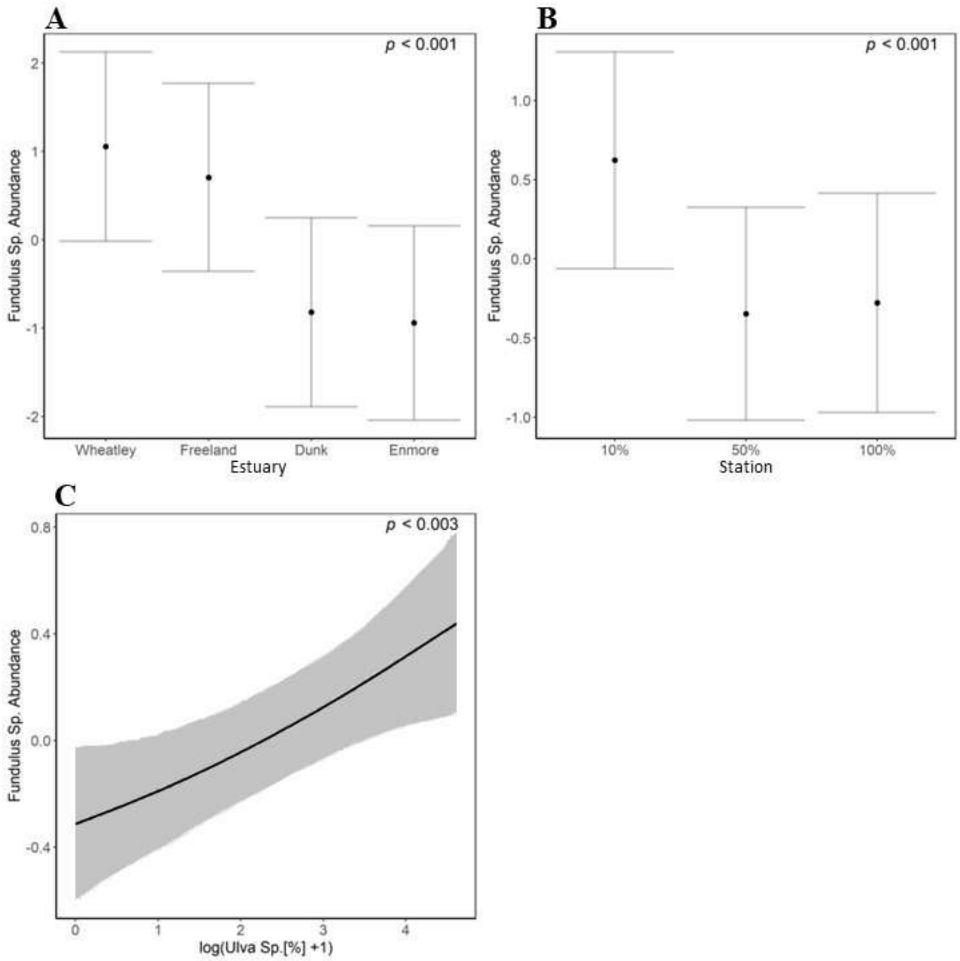

Figure 3.6: Modelled marginal smooth effects of estuary, station, and measured environmental parameters (i.e., temperature, salinity, dissolved oxygen (mg/L), *Ulva* species % coverage, *Z. marina* % coverage, sediment coverage) on the abundance of *Fundulus* species (adult and YOY combined). The generalized additive model explained 65.8% of the deviance in estuary, station, environmental parameters on Fundulus abundance, with significant effects of (top to bottom) A) Estuary's effect on the relative mean abundance of *Fundulus* species caught through June and August in 2021 (n=36-51). B) Stations' relative effect on the relative mean abundance of *Fundulus* species caught through June and August 2021 (n=12-18). C) Relationship between relative *Fundulus* species abundance and *Ulva* species % coverage at all estuaries sampled through August 2020, and June and August in 2021 (n=186). Solid, black dots or lines indicate mean effect, while light grey or bars denotes 95 % credible range. Image created in R (version 4.0.5) using ggplot2 (v. 3.3.5), wplot (v. 1.1.1), gratia (v. 0.7.0; Wickham 2009; Wood et al. 2016).

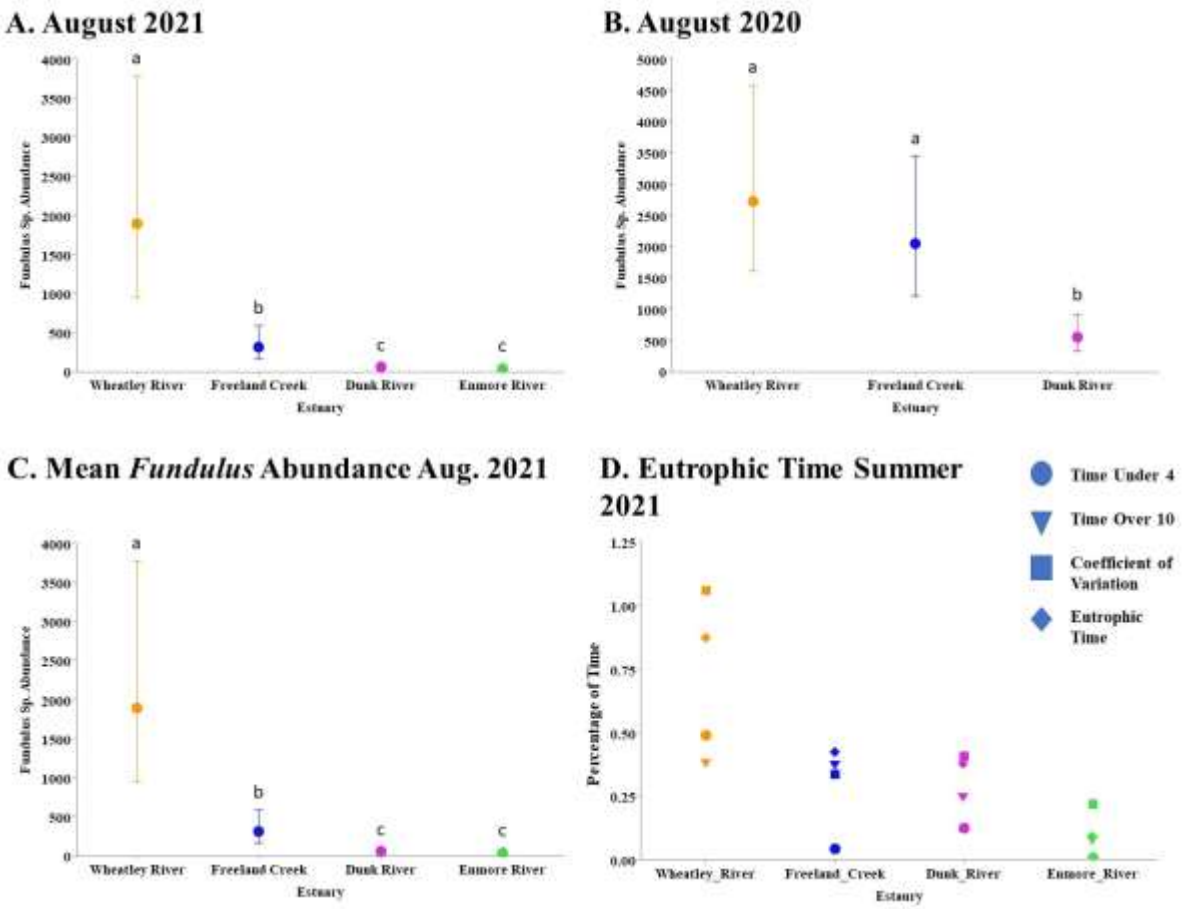

Figure 3.7: Mean *Fundulus* species abundances per beach seine haul (n=5-6) at only the 10% station within each estuary in A) August 2021 and B) August 2020. Error bars represent upper and lower 95% confidence intervals. All means and error bars were back transformed from their natural logarithm. Significance was determined with separate one-factor ANOVAs for each year followed by Tukey Post-hoc comparisons (a,b,c). The mean abundance of *Fundulus* species collected in the 10 % station during C) August 2021 and D) eutrophic time throughout the summer of 2021 are placed side-by-side to highlight similar patterns. Images created using MiniTab® Statistical Software, V 21.1 (2021 Minitab, LLC, 64-Bits).

difference between month and year was also detected ($p<0.05$; Appendix A.11).

3.5 Discussion

3.5.1 Nearshore Fish Assemblages in the Inner Estuarine Region

The inner region has been increasingly shown to be important for evaluating the health of temperate estuaries as riverine inputs, like nutrients, are often more concentrated here than in the middle or outer regions closer to the ocean (Schein et al. 2012; Niu et al. 2021; Turner et al. 2021). In the southern Gulf of Saint Lawrence (sGSL), the effects of eutrophication, like dense sea lettuce mats and chronic hypoxia, are restricted to the inner region of many estuaries (van den Heuvel et al. 2019; Coffin et al. 2021a). However, little data exist to determine if there are longitudinally distinct nearshore fish assemblages in the sGSL's estuaries, and whether eutrophication has any effect on these assemblages.

This study of four estuaries in Prince Edward Island (PEI) suggests that the inner estuarine region, represented by 10 % of the estuary's surface area, contained significantly distinct nearshore fish assemblages relative to the middle (50 % of surface area) and outer (100 % of surface area) estuarine regions. The inner regions were distinct regardless of relative level of nutrient impact (high, mid, low), season (June vs August), year (August 2020 vs August 2021), or shoreline (north shore vs south shore). These findings are consistent with results reported in other estuaries, as the changing abiotic (namely temperature and salinity) and biotic (e.g., aquatic vegetation coverage, prey availability or predation/competition) conditions create physiological and ecological 'barriers' that limit how far most fishes can venture into the freshwater or saltwater regions of an estuary (Whitfield and Elliott 2002; Foubert et al. 2018; Whitfield 2021).

Temperature and salinity are routinely identified as the main factors driving the structure of estuarine fish assemblages worldwide due to varying thermal and salinity tolerances among different fishes (Marshall and Elliott 1998; Snigirov et al. 2012; Whitfield 2021). However, PEI's estuaries receive little freshwater input due to their small watersheds, as a result, often lack the strong vertical and horizontal haloclines and thermoclines present in many other estuaries (Telesh and Khlebovich 2010; Schein et al. 2012; Coffin et al. 2017).

Herein, salinity appeared to be a factor in differentiating our stations at some estuaries (e.g., Dunk River (DR) likely due to larger watershed relative to the other estuaries sampled) and differences due to temperature mainly resulted from either sampling when water was cooler in June or when it was warmer in August.

Schein et al. (2012) suggested that due to the subtler changes in salinity and temperature along the length of PEI's estuaries, coupled with the high nitrate loading these small watersheds receive, that aquatic vegetation coverage likely plays a more important role in structuring the nearshore fish assemblages. Herein, eelgrass (*Z. marina*) and sea lettuce (*Ulva* sp.) coverage, or their absence, helps predict the station's nearshore fish assemblage. However, eelgrass was only widely present in the north shore estuaries, Wheatley River (WR) and Freeland Creek (FC), and was seldom observed in the south shore estuaries, DR and Enmore River (ER). Eelgrass is present in DR and ER (van den Heuvel et al. 2019), but it is likely this habitat was systematically missed due to the sampling design. To be precise, eelgrass beds present in DR's outer region may have been missed due to beach seining at high tide, as the eelgrass beds at this station are most accessible at low tide. Regardless, this study supports past observations that aquatic vegetation plays an important role in structuring the longitudinal fish assemblages in PEI's estuaries.

A statistical similarity between the nearshore fish assemblages in the inner region and the middle region was found in DR during August 2021, which was the only instance where the inner region was similar to one of the lower regions. The similarities may have resulted from heavy rainfall the day prior to beach seining and abnormally high sea lettuce coverage creating salinity and vegetation conditions at DR's middle station that were more comparable to the inner region than the outer region in August 2021. Regardless of the similarity that was observed between the inner and middle regions during August 2021 in DR, it was neither seasonal nor annually consistent. This leads us to speculate that longitudinal variations in the nearshore fish assemblages are likely the norm in DR.

In each of all four estuaries, higher young-of-the-year (YOY) abundances of many fishes (namely *Fundulus* sp., Atlantic silversides, fourspine stickleback, and *Gasterosteus* sp.) were sampled in the inner region compared to the middle and outer regions in August of 2020 and 2021. There was also an increased abundance of YOY in August relative to June, which is likely explained by the fact most of the fishes in PEI's estuaries spawn in either June or July (Schein et al. 2012). Many fishes prefer the inner region as a nursery, even in degraded eutrophic estuaries, as it still possesses desirable conditions like warmer temperatures, lower risk of predation, and higher productivity (Meng et al. 2002; Brady and Targett 2013; Whitfield 2020). Therefore, past studies' avoidance of the inner estuarine region may have excluded valuable information on key nursey habitat within the sGSL.

3.5.2 Mummichogs as Indicators of Eutrophication

This study provided some evidence that high abundances of mummichogs (by proxy of *Fundulus* sp.) could indicate estuarine eutrophication. Abundances of mummichogs were consistently higher in the inner region than either the middle or outer regions of all estuaries. A strong positive linear relationship between high mummichog abundance and high sea lettuce coverage was also found. These results are consistent with past studies (Schein et al. 2012; Lockfield et al. 2013). Finley et al. (2009) also found that there was generally higher mummichog abundance in the sea lettuce-infested inner region of Stanley River, PEI, than in the middle and outer regions with less sea lettuce and more eelgrass. However, the present study found that mummichog were noticeably more abundant in the inner regions of all estuaries, even ER which had the relatively lowest nutrient impact. Thus, this leads us to believe there are other factors besides sea lettuce coverage that contribute to the high mummichog abundance observed in the inner estuarine region.

Mummichogs may prefer the salinity and temperature conditions found at the inner region of the estuaries surveyed in the present study. As previously mentioned, salinity and temperature are often the driving structural forces in estuarine fish assemblages.

Mummichogs, despite being highly euryhaline, appear to be associated with warmer water temperatures and salinities around 20 PSU (Fritz and Garside 1974; Garside and Morrison 1977), which often corresponded to the inner regions of the estuaries. However, this study's GAM did not find any significant linear or non-linear relationships between salinity and temperature and mummichog abundance (Appendix A.11). Regardless, future studies may wish to investigate the influence of temperature and salinity on mummichog's longitudinal distribution if mummichogs are to be used to assess eutrophication within an estuary.

A pattern between the relative abundance of mummichogs and the level of nutrient impact was found when comparing the inner regions of the estuaries surveyed. WR's inner region, the site with the highest eutrophic time, had the highest abundance of mummichogs compared to the other three estuaries in August 2021. Of particular note is how the mean abundance of mummichogs appeared to descend with the estuaries' eutrophic time. From highest abundance and eutrophic time, the estuaries went WR, FC, DR, and ER. Unfortunately, we lacked DO data from August 2020 to see if there was interannual consistency with this pattern. In addition, this pattern in mummichog abundance and nutrient impact was obscured when examining all samples collected (inner, middle, and outer pooled together) within an estuary.

Mummichogs are well adapted to the inner region of eutrophic estuaries: being unperturbed by chronic hypoxia occurring in this region, and are often found at high abundances at sites with high sea lettuce coverage, or other fast-growing macroalgae (Finley et al. 2013; Lockfield et al. 2013; Dixon et al. 2017). Therefore, the relative abundance of mummichogs found in the inner region may offer insight into the level of nutrient impact within an estuary or between estuaries. However, developing a fixed metric of a certain abundance of *Fundulus* sp. caught per net to classify an estuaries' level of nutrient impact is unlikely due to high levels of observed variability in the number of mummichogs per beach seine haul.

3.5.3 Nearshore Fish Assemblages as Indicators of Eutrophication

This study suggested that shoreline (north vs south) may be more influential in structuring the overall nearshore fish assemblages in PEI than our classifications of an estuary's eutrophication status (high vs mid vs low nutrient impact), at least in August. Even the mummichog abundance across all regions (inner, middle, and outer all pooled together) within an estuary seems to be strongly determined by shoreline over nutrient impact. Multiple explanations exist for these observations. For one, past studies have demonstrated that the geomorphology and tidal force of an estuary often appear to be more important in determining the structure and composition of the nearshore fish assemblage than anthropogenic impacts like eutrophication (Whitfield and Elliott 2002; Harrison and Whitfield 2012). Tweedley et al. (2017) noted that the effect of an estuary's geomorphology on its nearshore fish assemblage may be so strong that it prevents the detection of any anthropogenic impact signal.

This study suggested that mummichogs are far more abundant in north shore estuaries than south shore estuaries, while Atlantic silversides appear more abundant on the south shore. Harrison and Whitfield (2012) found that temperate estuaries with similar tidal regimes and geomorphology often had similar trophic structures in their fish assemblages, with higher water residence time systems favouring detritivores and higher flushing systems favouring zooplanktivores in South Africa. The findings of this study appear to support their observation, as higher mummichog abundances (benthic feeders) were associated with the lower tidal amplitude (0.9-0.1 m) lagoon-like estuaries on PEI's north shore (WR and FC) and higher Atlantic silversides abundances (pelagic feeder) were associated with the higher amplitude (1.7-0.1 m) embayment estuaries on the south shore (DR and ER; Schein et al. 2013).

The observation of mummichogs being more abundant on PEI's north shore than south shore is not universal. Finley et al. (2009) found that Wilmot River, a eutrophic south shore estuary near DR, had higher mummichog abundance in August than Stanley River, a

eutrophic north shore estuary near WR. A likely explanation for this incongruity may relate to vegetation coverage. The sampling location selected by Finley et al. (2009) in the Stanley River contained very little coverage of sea lettuce (0.5 %) compared to Wilmott River's station (93 % sea lettuce coverage). We speculate that if sites with similar levels of sea lettuce coverage from Wilmot River and Stanley River were compared, Stanley River would likely have more mummichogs than Wilmot River based on our study's findings.

Another factor one should consider is many fishes generally make poor indicators of estuarine eutrophication (Whitfield and Elliott 2002). Firstly, estuarine fish populations often display high degrees of interannual variability which may also obscure any patterns produced by eutrophication (McGowan et al. 2022). Secondly, as Joseph et al. (2006) noted, few species in the sGSL appear to be strictly associated with either eelgrass, sediment, or sea lettuce. This study's findings support this observation, as no fishes were exclusive to either sea lettuce dominated regions in the inner region, or the eelgrass or bare sediment dominated regions in the middle and outer regions.

Perhaps most importantly, DO concentrations are routinely found to exert smaller influences on estuarine fish assemblages than temperature or salinity (Snigirov et al. 2012; Whitfield 2021). This may result from the fact many estuarine fishes have adapted to cope with temporary periods of hypoxia (Shimps et al. 2005). Dixon et al. (2017) even found oxygen-sensitive fishes, like Atlantic silversides, can persist for several hours in hypoxic (~1.31 mg/L) conditions in laboratory settings. However, certain estuarine fishes have demonstrated the ability to detect and avoid hypoxic conditions, offering some indication of nutrient impact (Wannamaker and Rice 2000). For example, juvenile weakfish (*Cynoscion regalis*) and spot (*Leiostomus xanthurus*) in eastern North American, have the ability to detect and avoid hypoxia in the inner region and vacate using the ebb and flow tides (Brady and Targett 2013). Thus, some species may offer some insight into the DO status of the estuary (Marshall and Elliott 1998; Wannamaker and Rice 2000).

Alternatively, the chosen classification system may have been too simplistic to capture the true spectrum of eutrophication present on PEI. FC's DO profile displayed eutrophic characteristics that exceeded *a-priori* assumptions, while DR's DO profile and eutrophic time were more like FC's than WR's. These observations may be explained by the fact estuaries with low tidal exchange and high riverine inputs, which leads to high nitrate inputs, are more at risk of eutrophication than estuaries with stronger tidal flushing (Coffin et al. 2018b; Kelly et al. 2021). Therefore, we can speculate that eutrophication may be exacerbated by longer residence time (2.13 days) despite low nitrate loading in FC and mitigated by shorter residence time (0.46 days) despite high nitrate loading in DR.

Non-agricultural industries in the estuaries may have added confounding variables that the current design did not account for directly or indirectly. A bivalve processing plant is found at FC's 50 % station (middle estuary) and may introduce some organic waste into this estuary. Thériault et al. (2006). found that seafood processing plants may increase an estuary's ichthyofaunal abundances as fish may be attracted to the plant's organic waste. The effects of the bivalve processing plant are unclear in FC, as there was no noticeable increase in any fishes at the 50 % station relative to the other two stations. Bivalve aquaculture present in middle and outer regions of FC, ER, and WR also introduces another variable that would be absent in pristine estuaries and thus further complicates the use of FC, ER, or the lower regions of these estuaries as reference sites. A growing body of literature suggests that the bivalve aquaculture equipment (e.g., cages, buoys, and anchoring ropes) provides habitat structure and foraging grounds for many animals, including fishes, resulting in increased abundances (see Theuerkauf et al. 2022). Future studies may need to account for the total anthropogenic impacts on the nearshore fish assemblage or the abundances of mummichogs within the watershed and estuarine area.

3.5.4 Implications for CAMP

The Community Aquatic Monitoring Programs (CAMP)'s current sampling regime excludes the nearshore fish assemblages found in the inner region of most estuaries. Results herein suggest that the inner 10 % of surface area should be included to help capture longitudinal variations in ichthyofauna that appear to be present in the sGSL's estuaries, at least in PEI. It was also found that high mummichog abundance in the inner region could potentially be used to indicate impacts of eutrophication within and between estuaries. However, some issues should be addressed about this study before adopting the inner region into CAMP.

This study did not use CAMP's exact sampling stations within each estuary. As such, the findings of this study may not be directly replicable through CAMP. However, these findings support past observations and ecological theories on factors structuring nearshore fish assemblages in estuaries and thus are likely valid and directly applicable to CAMP's design. Another important consideration is all sampled estuaries come from central and western PEI and would not capture or be representative of estuaries in New Brunswick and Nova Scotia. Therefore, future studies may need to investigate how these provinces' unique geomorphological features and anthropogenic impacts influence the nearshore fish assemblage of their estuaries' inner regions.

Another concern is that many estuaries' inner regions are challenging to beach seine. For one, the sampling stations in WR's and FC's inner region were not directly accessible by road vehicles, which is a requirement for CAMP, allowing volunteers to easily access sample sites (DFO 2011). As such, the accessibility of the inner region will have to be assessed for other CAMP estuaries if they are to be included. Another challenge presented by the inner region is the dense mats of sea lettuce that form in August, which complicate beach seining and prolong sampling (DFO 2011). DFO may consider utilizing molecular sampling techniques, like environmental DNA metabarcoding, reducing the need for extensive

physical sampling in the region. Regardless, it is apparent that CAMP is currently missing valuable information on the most impacted region in PEI's estuaries.

3.6 Conclusion

The findings of this present study suggest that the nearshore fish assemblages in the inner region of most estuaries are distinct from the middle and outer regions in estuaries in PEI, Canada. The inner region was associated with high abundances of mummichog and high abundances of the YOY of many of the fishes sampled. Secondly, the findings of this study support the existing literature which suggests the estuary's geomorphology and tidal dynamics are likely more important in structing the overall fish assemblage than anthropogenic impacts, like eutrophication. Finally, these findings appear to affirm Finley et al. (2009) observation that mummichog abundances can provide some indication of eutrophication status. Not only was a strong, positive, linear correlation with mummichog abundance and sea lettuce coverage found, but inner regions that experience higher eutrophic times also appear to have higher abundances of mummichogs relative to estuaries with lower eutrophic times in their inner region.

Chapter 4: Evaluating the Use of Environmental DNA Metabarcoding to Characterize Fish Assemblages in Temperate Estuaries

4.1 Overview

Developments in environmental DNA (eDNA) metabarcoding have demonstrated that it can complement traditional sampling methods. Beach seining, commonly used in nearshore nekton surveys, offers an incomplete insight into the entire estuarine nekton community by generally excluding non-nearshore species and can be impeded by dense macroalgae growth. The goal of this study was to evaluate whether eDNA metabarcoding could: 1) Detect seasonal, annual, and spatial shifts in fish assemblages within Prince Edward Island estuaries; 2) Identify additional fish taxa missed by beach seining, including non-nearshore taxa; and 3) Provide quantitative data (i.e., proportions) on the estuary's fish assemblage comparable to beach seining. Three stations were sampled (inner, middle, and outer estuary) in each of three estuaries in August 2020, and four estuaries in June and August 2021 across Prince Edward Island, Canada. eDNA metabarcoding detected seasonal (June to August) and interannual (August 2020 to 2021) shifts in fish assemblages and distinguished stations between 0.4-3 km apart. eDNA metabarcoding detected fish species distinct from beach seining, including the endangered winter skate (*Leucoraja ocellata*). The most abundant taxa detected by eDNA metabarcoding and beach seining often constituted similar percentages of the total composition. These findings suggest eDNA metabarcoding may serve as a complement to beach seining, identifying species commonly missed by beach seines, or even act as a replacement by providing similar quantitative data on proportional abundances.

4.2 Introduction

Developments in molecular sampling techniques, such as environmental DNA (eDNA) metabarcoding, are revolutionizing the assessment and monitoring of biodiversity (Ruppert et al. 2019). Increasingly used in ichthyofaunal studies, eDNA metabarcoding uses DNA collected from environmental media, like water or sediment, to identify and potentially quantify fishes inhabiting a particular habitat (Shelton et al. 2016; Shu et al. 2020). eDNA metabarcoding has been shown to outperform traditional sampling methods by being more time- and labour-efficient during field sampling, including in difficult-to-monitor habitats (P.F. Thomsen et al. 2012; Thomsen and Willerslev 2015; Thomsen et al. 2016; Fujii et al. 2019; Afzali et al. 2021; Andruszkiewicz Allan et al. 2021). Metabarcoding's use of reference DNA barcodes may also reduce misidentification of juvenile life stages (Maggia et al. 2017; Garcia-Vazquez et al. 2021). All these factors, coupled with standardizable collection methods, allow for eDNA metabarcoding's usage in large comprehensive studies of marine and coastal environments (Djurhuus et al. 2020).

Beach seining is a commonly used and effective sampling method in many studies and monitoring programs of estuaries, including in Canada's southern Gulf of Saint Lawrence (sGSL; Weldon et al. 2005; Steele et al. 2006; Baker et al. 2016; Kidd et al. 2021; McGowan et al. 2022). However, there are some concerns with beach seining. Firstly, beach seining offers an incomplete assessment of estuarine nekton as it is biased towards nearshore nekton, excluding deeper water species and large fish that can avoid the net (Weldon et al. 2005; Steele et al. 2006). Secondly, beach seines are impeded by dense macroalgal growth, thus limiting beach seining effectiveness in eutrophic estuaries (Finley et al. 2009; DFO 2011; Schein et al. 2012). Thirdly and finally, misidentifying the juvenile stages of similar species based on morphology is a concern, especially in the cases where citizen scientists aided in the data collection (Thériault et al. 2008; DFO 2011).

Growing evidence shows that eDNA metabarcoding could complement beach seining (P.F. Thomsen et al. 2012; Andres et al. 2022). A recent study by He et al. (2022) demonstrated eDNA metabarcoding detected greater fish diversity than beach seining and found a positive linear relationship with proportional abundances of fishes captured in beach seining in eelgrass

beds along the Atlantic coast of Nova Scotia. Thus, eDNA metabarcoding could complement beach seining by identifying missed taxa and could potentially replace beach seining in difficult-to-access habitats, like the inner region of many estuaries, if congruities in abundance data between these methods can be reliably demonstrated.

The overall goal of our study was to investigate eDNA metabarcoding's potential to complement or replace beach seining in estuaries. The specific goals of this study were to evaluate whether eDNA metabarcoding could: 1) Detect seasonal, annual, and spatial shifts in fish assemblages detected by beach seining, 2) Identify additional fish taxa from beach seining; and 3) Provide quantitative data (i.e., proportions) on the estuary's fish assemblage comparable to beach seining. To accomplish this, three estuaries were sampled in August 2020, and again, along with one additional estuary, in June and August 2021, of varying levels of eutrophication in Prince Edward Island, Canada. Each estuary was broken into three sampling stations at the inner, middle and outer regions.

4.3 Materials and Methods

4.3.1 Site Selection

Estuaries of Wheatley River, Freeland Creek, and Dunk River were sampled in August 2020, and again, along with Enmore River, in June and August 2021 in Prince Edward Island (PEI), Canada (Figure 4.1A-B). The upper estuarine boundary was defined by 0.5 PSU, and the lower limit with complete mixing and geographic features (Coffin et al. 2018b). Each estuary was divided into three sampling stations based on the estuaries' surface area: 10% station (inner estuary/ closest to the river), 50% station (middle) and 100% station (outer estuary/ closest to the ocean). The 10 % station at most estuaries, especially Wheatley River and Dunk River, was dominated by sea lettuce (*Ulva lactuca* and *U. intestinalis*), while the 50 % and 100 % stations tended to be dominated by eelgrass (*Zostera marina*) or bare sediment (van den Heuvel et al. 2019). All sampling occurred over a 250-300 m long region at the seaward boundary of each station (Figure 4.1C-F).

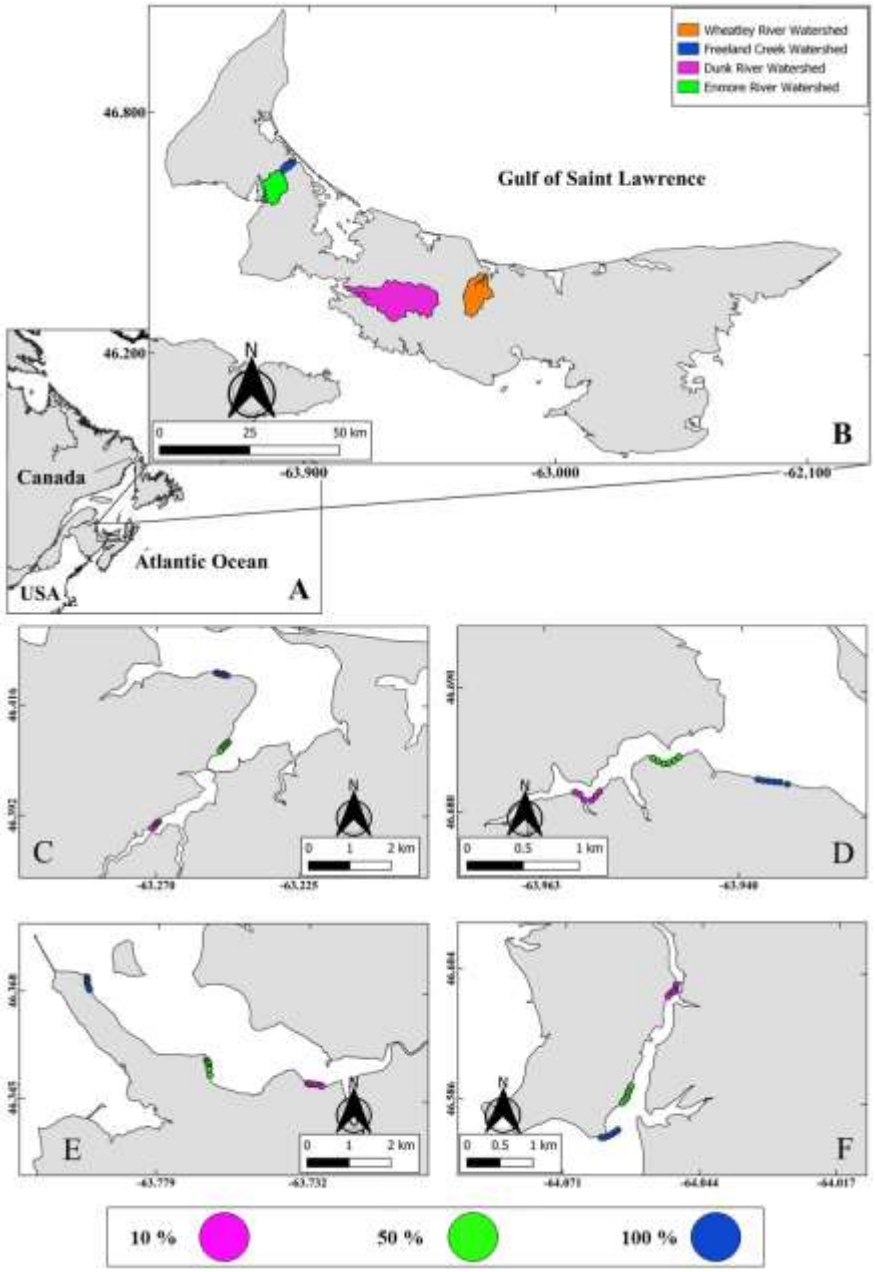

Figure 4.1: A) Study area in the context of north-eastern North America. B) Map of the sampled estuaries' watersheds in Prince Edward Island, Canada, during the summer of 2020 and 2021. Enmore River (lime green) was only sampled in 2021. The estuaries were Wheatley River (C), Freeland Creek (D), Dunk River (E), and Enmore River (F). Dissolved oxygen and salinity loggers were moored at the seaward boundary of the upstream 10% station in each estuary. Five or six beach seine nets were collected at the seaward boundary of each station. Image created using QGIS (64 Bit, Version: 3.16.11 Hannover).

4.3.2 eDNA Field Collection and Filtration

Water samples were collected prior to beach seining (24-48 h) to minimize contamination and disturbances caused by the net. Each estuary was sampled on separate days to minimize cross-estuary contamination (Thomsen and Willerslev 2015). All our field equipment (i.e., Nalgene HDPE bottles, telescopic pole, bottle holder, and boots) was cleaned with 2 X dilution of commercial bleach then rinsed with distilled water before and between water sample collection to minimize contamination. Five 1 L water samples were collected in Nalgene HDPE bottles (Thermo Fisher Scientific) 5-10 m from the shore and 20-30 cm below water's surface using a telescopic pole and a custom bottle holder, approximately 50 m apart within the 250-300 m long station. Each water bottle was rinsed three times with estuary water to remove any residual bleach before collecting 1 L. As a field blank, 1 L of distilled water was transported in Nalgene HDPE bottles into the field, submerged in each estuaries' water, placed on ice and transported back to the laboratory to be processed alongside other samples (see Thomsen and Willerslev 2015). After collection, water samples were held on ice and transported back to the lab to be filtered within 8 h of collection.

Filtration occurred in a designated room, physically separated from other laboratory stages (i.e., extraction, amplification, Polymerase Chain Reaction (PCR) cleaning up and pooling), at the University of Prince Edward Island's Biology Department. All filtration equipment was cleaned with 2 X dilution of commercial bleach for at least 15 min between samples to minimize the risk of cross-sample contamination (see Kemp and Smith 2005), after which it was rinsed with distilled water. Glass-fibre filters (Whatman Grade GF/C Glass Microfiber Filters, pore size 1.2 µm) were selected due to their predominant use in fish eDNA studies and resistance to clogging with particulates (Shu et al. 2020). A volume of 1 L of distilled water was filtered for filtration negative controls at the beginning and end of the filtration session (Goldberg et al. 2016). Filters were transferred into 1.5 mL cryovials (Thermo Fisher Scientific: N5000-1020) and 200 µL of 98 % ethanol was added to aid preservation and filters were stored at -20 ℃ until DNA extraction.

4.3.3 Beach Seining

Following the beach seining protocol specified in Schein et al. (2012), five or six non-overlapping beach seine hauls were conducted at each station, generally coinciding with the same locations eDNA samples were collected. In short, the beach seine was extended (30 m × 1.5 m seine, 3 mm mesh and 1.2 m bag) 15 m into the water perpendicular to the shoreline, then turned and walked 15 m parallel to the shore, then finally circled back to the shoreline, encompassing a 225 m^2 area. Captured fishes were sorted into age classes of either adult or young-of-the-year (YOY), and generally identified to species. However, the YOY of similar species (i.e., mummichog (*Fundulus heteroclitus macrolepidotus*) and banded killifish (*F. diaphanous*), blackspotted stickleback (*Gasterosteus aculeatus*) and threespine stickleback (*G. wheatlandi*), gaspereau (*Alosa pseudoharengus*) and American shad (*A. sapidissima*)) were identified to genus. Fish were numerated and released either downstream of the immediate sampling area or released after the proceeding beach seining haul commenced to minimize risk of recapture.

4.3.4 Laboratory Contamination Control

Procedures of eDNA extraction, RT-qPCR (real-time quantitative PCR) amplification, RT-qPCR product cleaning and pooling were conducted in physically separated rooms to reduce the risk of cross-stage contamination (Thomsen and Willerslev 2015; Goldberg et al. 2016). Workstation countertops were cleaned with 2 X dilution of commercial bleach and wiped down with 70 % ethanol to remove residual bleach. Pipettes were cleaned by exposure to UV lighting and 10 X dilution of commercial bleach between and before usage.

4.3.5 eDNA Extraction

DNA was extracted from filters using MN Nucleospin Tissue kits (Machery-Nagel), following a modification to the manufactures tissue protocol to suit glass filters (DFO-GULF environmental DNA (eDNA) extraction protocol Version 1.02). An extraction blank was included during each extraction batch. Samples from each estuary were extracted by month and year in separate batches to minimize the risk of cross-estuary and cross-month/year contamination during this process.

4.3.6 RT-qPCR Amplification

Two primer sets that amplified an overlapping region of the mitochondrial 12S rRNA gene, the novel 12S-160 (Steeves unpublished), and the recently published 12S-248F (He et al. 2022) to target actinopterygians (Table 4.1) were selected. RT-qPCR preparation was performed in an AirClean Systems PCR workstation (Model: AC632DBC) that was sterilized using UV light and 2 X dilution of commercial bleach. The eDNA extracts were run undiluted as there were no signs of PCR inhibitors after a serial dilution tests of 0 X, 10 X, and 100 X on randomly selected samples (Appendix B.1). A positive control was generated by filtering 1 L of aquarium water containing tropical fishes of the genus *Chrysiptera*, which are not present in PEI. The metabarcoding library preparation required two separate rounds of RT-qPCR. RT-qPCR was conducted on a BIORAD CFX Connect Real-Time System qPCR (Model: CFX Connect TM Optics Module).

The first round of RT-qPCR was carried out in 25 µL total reaction volume per sample comprised of 12.5 µL of QIAGEN Multiplex PCR Master Mix (Qiagen), 4.45 µL of DNA-free water, 2.4 µL (1.2 µL each of 20 µM forward and reverse primer) of the respective primer with a Nextera adaptor tail, 0.6 µL (0.3 µL each of 20 µM forward and reverse primer) of the untailed primers, 1 µL of 1 % BSA, 1.25 µL of EvaGreen® (Biotium Inc.), and 3 µL of DNA extract. All primers are listed in Table 4.1 including tailed primers with added stagger bases between the Nextera tails and locus-specific sequence that are used to increase base diversity of amplicon sequencing products as this is known to help with read quality on Illumina platforms. Initiation was 95 °C for 15 min, after which the two primers had different cycling parameters: the 12S-160F had 35 cycles of 94 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s, while the 12S-248F had 38 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s.

The second round of RT-qPCR was carried out in 25 µL total reaction volume per sample, 14.12 µL of DNA-free water, 0.25 µL of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 5.0 µL of Q5 reaction buffer, 0.5 µL of dNTP (10 µM), 1.88 µL of Illumina unique dual index primer (set A for 12S-160 and set B for 12S-248F for all cases, diluted 2X with DNA-free water), 1.0 µL of 1 % BSA, 1.25 µL of EvaGreen®, and 1.0 µL of diluted round 1 product (1:1 round 1 product to DNA free water) and had the same cycle parameters for both primers: 98 °C for 30 s for initiation followed by 98 °C for 15 s, 66 °C for

Table 4.1: Primers selected for study. Both target an overlapping region of the mitochondrial 12S rRNA gene targeting actinopterygians. Note both forward primers used the same reverse primer. Tailed sequences in bold are Illumina Oligonucleotide sequences

Primer Name	Orientation	Sequence (5'→3')	Amplicon Length (bp)	Reference
12S-160	Forward	HCGGCGTAAAG VGTGGTTA	160	Steeves unpublished
12S_NGS_160bp_RADS_For_Nextera	Forward (**Tailed**)	**TCGTCGGCAGC GTCAGATGTGT ATAAGAGACAG** HCGGCGTAAAG VGTGGTTA	320	-
12S-248F	Forward	CGTGCCAGCCAC CGCGGTT	205	He at al. 2022
12S_NGS_RADS[1]_for_Nextera (50%)	Forward (**Tailed**)	**TCGTCGGCAGC GTCAGATGTGT ATAAGAGACAG** CGTGCCAGCCAC CGCGGTT	380	-
12S_NGS_RADS[1]_for_Nex_StagN (50%)	Forward (**Tailed**)	**TCGTCGGCAGC GTCAGATGTGT ATAAGAGACAG N**CGTGCCAGCCA CCGCGGTT	380	-
MiFish-U-186 R	Reverse	CATAGTGGGGTA TCTAATCCCAGT TTG	-	Miya et al. 2015
12S_Mifish_UR_Miya_Nextera	Reverse (**Tailed**)	**GTCTCGTGGGC TCGGAGATGTG TATAAGAGACA G**CATAGTGGGGT ATCTAATCCCAG TTTG	-	-

1. 0.6 µL of each of these tailed forward primers were used to reach 1.2 µL as to stagger bases.

20 s, and 72 °C for 30 for 9 cycles. RT-qPCR products from both rounds were verified on 1.5 % agarose gels with GelGreen® Nucleic Acid Stain (Sigma-Aldrich®) to ensure the proper amplicon size.

PCR replicates (triplicates) were performed using Freeland Creek 10 % station field sample 4, and 100 % station field sample 1, collected in August 2020 and June 2021 to see if there was consistency in the RT-qPCR products. When the PCR triplicates were examined, the read proportions were found to be qualitatively similar (Appendix B.2). This suggested that the RT-qPCR and sequencing results are reproducible and reliable.

The previously mentioned samples were also used to investigate whether the number of RT-qPCR cycles influenced the fish assemblage revealed by eDNA by reducing the maximum number of RT-qPCR cycles for 12S-160 (two separate cycle thresholds: 30 cycles and 32 cycles instead of 35 cycles) and 12S-248F (two separate cycle thresholds: 32 cycles and 35 cycles instead of 38 cycles). Reducing RT-qPCR cycles appears to result in little change from the full cycle (Appendix B.2). This indicated that cycle number minimally influenced the results.

Final RT-qPCR products were pooled together according to library index set/locus by constant volume (~ 5 µL) with some poorly amplifying libraries receiving an additional volume to help normalize the amount of DNA added. Afterwards, the pooled products were purified by adding 1.5 X the volume of HighPrep™ PCR Clean-up System (Sigma-Aldrich®) to exclude fragments smaller than 200 bp following the manufacture's protocol, then resuspended in 100 µL of MN Elution Buffer BE (Machery-Nagel). Purified product was evaluated on a 1.5% gel dyed with GelGreen® Nucleic Acid Stain and excised the target size range using a sterile blade and purified using a QIAGEN QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol. The final concentration was quantified using a Qubit™ dsDNA BR Assay kit on a Qubit™ 4 Fluorometer (Thermo Fisher Scientific).

4.3.7 Sequencing and Bioinformatics

Once the libraries were prepared, each pooled and purified index was sent for high-throughput sequencing on a Novaseq 6000 (2 X 150bp) with 10 % PhiX spike-in for base diversity at the McGill Genome Centre in Montréal, Québec (sent March 3, 2021) and the Aquatic and Crop Research Development, National Research Council Canada in Saskatoon,

Saskatchewan (sent January 10, 2022). A total of 45 fields samples from August 2020 and120 fields samples from Summer 2021 were sent to be sequenced for both the 12S-160 and 12S-248F primer sets. In addition, 12 replicates (4/4 from August 2020, 8/8 from August 2021), and 7 negative (2/2 from 2020, 5/5 from 2021) and 3 positive controls (1/1 from 2020, 2/2 from 2021) were also sent for each 12S primer set.

The SCVUC pipeline (Version 2.0) was used for bioinformatic analysis (Porter and Hajibabaei 2018), identified sequences output by the pipeline with the RDP classifier and a custom 12S rRNA training set. Custom R scripts were used to filter taxonomic observations from RDP output based on bootstrap values and, to rarefy reads and generate OTU tables. Two samples with low read counts were omitted from both the 12S-160 and 12S-248F read sets to allow rarefaction to a higher read number (12S-160: 186,000; 12S-248F: 304,000). Reads were then filtered down to 1×10^{-5} to remove taxa with low proportional read contributions to a given sample, helping to reduce the risk of false positives, index hopping, minor contamination, or inconsistent or incorrect classifications (Thomsen and Willerslev 2015).

4.3.8 Multivariate Analyses

Analyses were conducted at species level, except in the genera *Fundulus* (*Fundulus heteroclitus macrolepidotus* and *F. diaphanous*) and *Alosa* (*A. pseudoharengus* and *A. sapidissima*) due to difficulties distinguishing their YOY during beach seining surveys and inconsistent classifications during bioinformatic processing. *Gasterosteus* species (*G. aculeatus* and *G. wheatlandi*) were consistently differentiated during bioinformatic processing, but not in beach seining surveys, thus were also analysed at genus level. *Gadus* (*Gad. morhua* and *Gad. ogac*) and *Scomber* (*S. scombrus* and *S. colias*). were analysed at genus-level due to concerns of possible incorrect classifications during bioinformatic processing. Adults and YOY of the fishes collected by beach seining were combined by species or genera into a single variable as eDNA cannot distinguish between age classes. Multivariate analysis was conducted using PRIMER-E V7 software (Clarke et al. 2014) and data were standardized to give the precent contribution of each taxon by sample and square root transformed. Bray-Curtis similarity index was used for resemblance measures.

Two series of two-factor crossed permutational multivariate analysis of variance (PERMANOVA)s were conducted for each estuary. The first two-factor PERMANOVA series had Month (levels: June, August) or Year (levels: August 2020 and August 2021) and Method (12S-160, 12S-248F, CAMP) as factors to examine temporal shifts detected by the three sampling methods (see Appendices B.6-B.7). The second two-factor PERMANOVA series examined each month-year combination (August 2020, June 2021, and August 2021) separately and had Station (levels: 10%, 50%, and 100%) and Method (12S-160, 12S-248F, CAMP) as factors (see Appendices B.10-B.11). Type III sum of squares with fixed effects sum to zero for mixed terms, and a maximum of 9999 permutations were used in all cases. Permutation-based pairwise t-tests were conducted on factors or factor interactions when statistical significances were detected (see Appendices B.8-B.9, B12-B.15). Two-factor similarity percentages (SIMPER) were analysed based on the Bray-Curtis index to estimate the average dissimilarity between factors (Method, Station) within each estuary and the fishes that accounted for them.

4.4 Results

4.4.1 Beach Seining and eDNA Metabarcoding Sequencing Summaries

Beach seining caught 131,824 fishes from 186 seines across August 2020, and June and August 2021, with *Alosa* sp., fourspine stickleback (*Apeltes quadracus*), *Fundulus* sp., *Gasterosteus* sp., and Atlantic silverside (*Menidia menidia*) accounting for 98% of the total. 12S-160 yielded a total of 234,628,043 reads that were spread across 164 field samples. 12S-248F's read yield was similar to 12S-160's yield, with a total of 214,963,875 reads that were spread across 162 samples. The five most abundant taxa by read abundance were also *Alosa* sp., fourspine stickleback, *Fundulus* sp, *Gasterosteus* sp., Atlantic silverside, together accounting for 84 % and 87 % of 12S-160's and 12S-1248F's total reads respectively.

4.4.2 RT-qPCR Quality Control

We experienced some level of cross-contamination in the laboratory, which is expected and unpreventable (Thomsen and Willerslev 2015). Negative controls had C_t values that were generally 4-7 cycles higher than average field samples, and therefore contain over 10- to 100-fold less fish DNA, indicating that they contained comparatively less initial concentration of

DNA than field samples (Appendix B.3). Thus, contamination was likely caused by very small amounts of DNA. Positive controls mainly contained tropical fishes (e.g., *Chrysiptera* sp.) and showed little signs of cross-contamination from our field samples and vice-versa (Appendix B.2). Very low frequency (<0.004 %) of tropical fish reads were found from the positive control in twelve estuary samples for both 12S primer sets, indicating that these reads were likely a result of index hopping rather than physical contamination (Illumina 2017). Therefore, due to the high C_t values, relatively low recovered read numbers (Appendix B.4), low diversity of fish taxa, and prevalence of non-native fish taxa in negative controls (Appendix B.2), we conclude that contamination minimally influenced our results, and effects could be ignored.

4.4.3 Detection of Species

Both 12S primer sets used were highly specific to bony fishes, with 99.7 % of 12S-160's and 98.5% of 12S-248F's total reads belonging to actinopterygians (Appendix B.5). When looking across all months and years surveyed, 12S-160 detected 37 species (excluding unknown taxa) from 26 families and 16 orders, 12S-248F detected 30 species from 22 families and 14 orders, and beach seining detected 15 species from 13 families and 11 orders (Figure 4.2). All fishes detected by beach seining in each estuary were also detected with at least one of the 12S primer sets (Figure 4.2). However, there were few detections at specific station-month-year combinations where beach seining detected certain species that both 12S primer sets failed to detect, such as cunner (*Tautogolabrus adspersus*) at Wheatley River's 10 % station during August 2020 (Figure 4.2).

The 12S-160 more frequently detected fishes that were not typical of PEI's estuaries than the 12S-248F, such as tropical fishes (i.e., *Chrysiptera* sp.), likely due to minor amounts of contamination or index hoping, and freshwater fishes (e.g., Goldfish (*Carassius auratus*), *Etheostoma* sp., *Ictalurus* sp., fathead minnow (*Pimephales promelas*)), which may be artefacts of sequencing or bioinformatics (Figure 4.2). *Ictalurus* sp. and fathead minnow tissues were used in past studies within the laboratory and may have thus contaminated some samples. Goldfish DNA was present in the lab during this study and may have also contaminated some of the field samples.

Both 12S primers sets detected larger estuarine fishes know to inhabit deeper water that

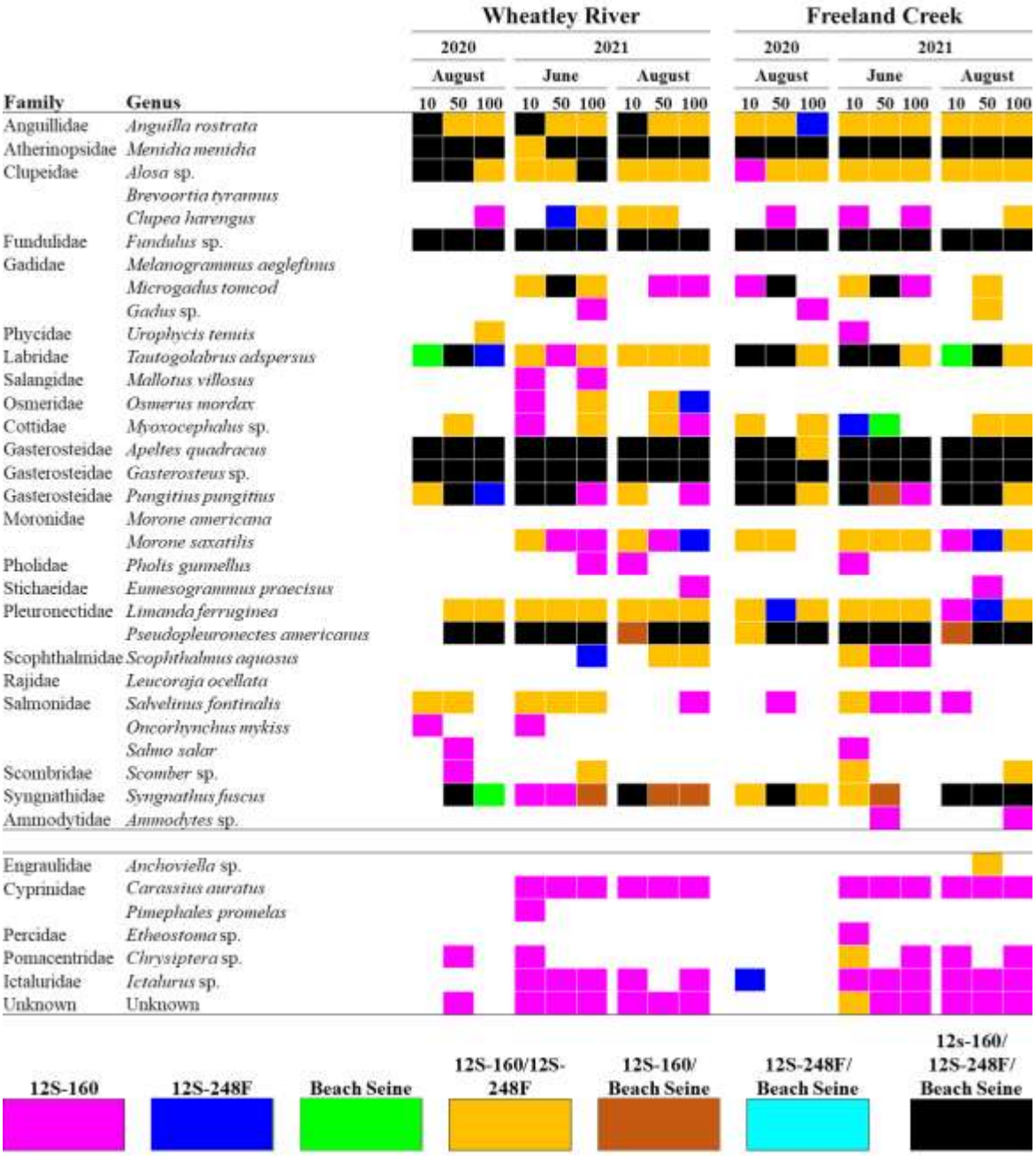

Figure 4.2: Shade plot displaying shared fish composition for all samples (n=3-6) collected at sampling station (10 %, 50 %, 100 %) by two 12S eDNA metabarcoding primer sets for the mitochondrial 12S gene (12S-160 and 12S-248F), and beach seines at two estuaries in Prince Edward Island, Canada. No colour means no detection. Computed in PRIMER-e (V7), Image made in Microsoft® Excel® for Microsoft 365 MSO (Version 2207, Build 16.0.15427.20182).

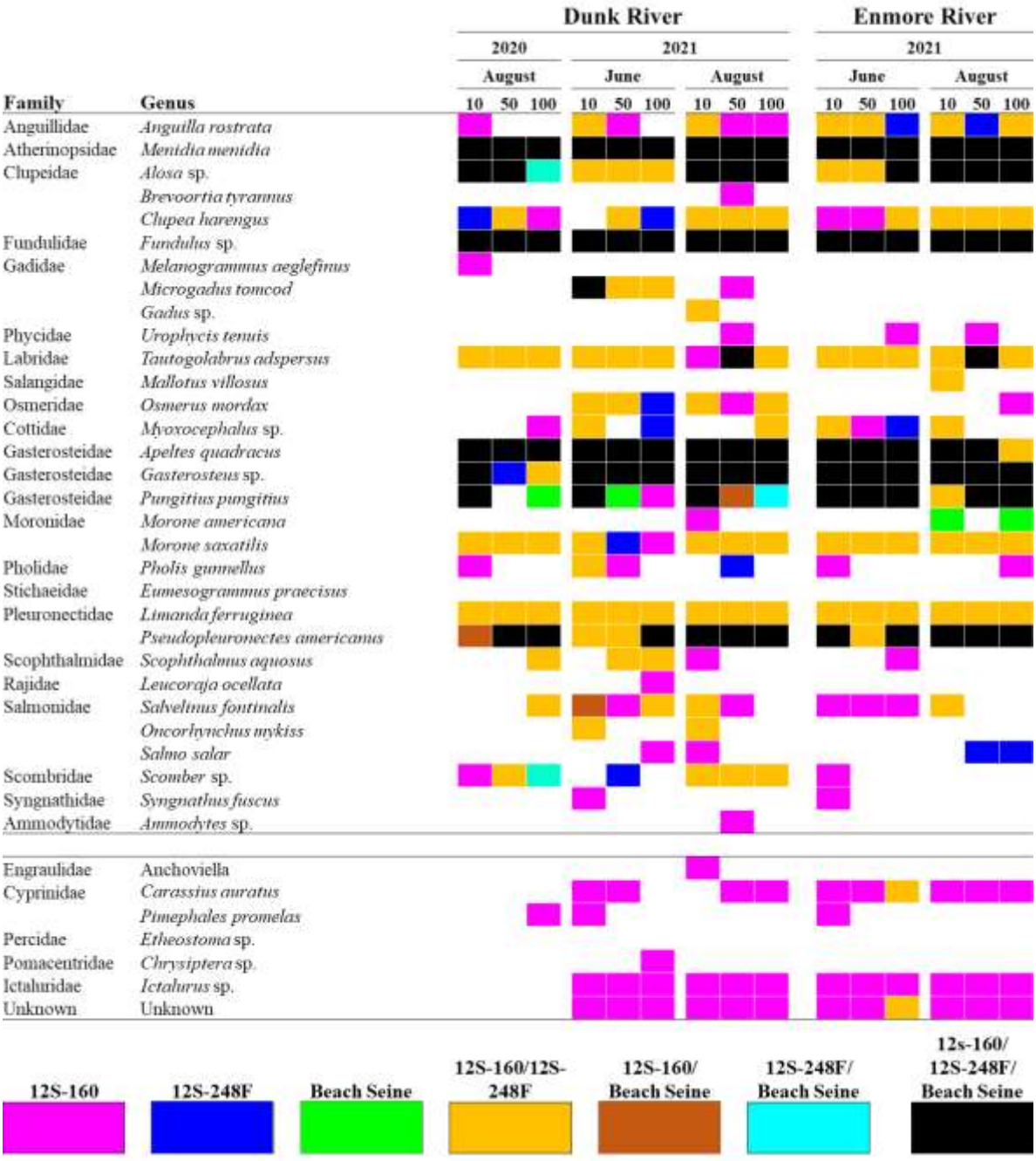

Figure 4.2 continued: Shade plot displaying shared fish composition for all samples (n=3-6) collected at sampling station (10 %, 50 %, 100 %) by two 12S eDNA metabarcoding primer sets for the mitochondrial 12S gene (12S-160 and 12S-248F), and beach seines at two estuaries in Prince Edward Island, Canada. No colour means no detection. Computed in PRIMER-e (V7), Image made in Microsoft® Excel® for Microsoft 365 MSO (Version 2207, Build 16.0.15427.2018.

beach seining failed to detect. These fishes included yellowtail flounder (*Limanda ferruginea*), striped bass (*Morone saxatilis*), brook trout (*Salvelinus fontinalis*), and *Scomber* sp. (Figure 4.2). 12S-160 also detected a chondrichthyan, the winter skate (*Leucoraja ocellata*), at Dunk River's 100 % station in June 2021 (Figure 4.2) which is an endangered species in the sGSL (Kelly and Hanson 2013).

4.4.4 Seasonal, Annual, and Spatial Variation in Fish Assemblage

The two 12S primer sets detected the seasonal and interannual shifts in the fish assemblages suggested by beach seining. Both 12S primer sets and beach seining consistently detected significant differences between June's and August's average fish assemblage in all four estuaries (P(perm)<0.05 for all tests; Appendix B.8). Both 12S primer sets and beach seining also suggested that there was significant interannual variation in the average assemblage detected at all three stations across all estuaries between August 2020 and August 2021 (P(perm)<0.05 for all tests; Appendix B.9).

Both the 12S primer sets and beach seining generally suggested that each station had a unique average assemblage compared to other stations (P(MC)<0.05 for most tests; Figure 4.3). There were several instances where either the two 12S primer sets, beach seining, or all three methods, suggest that there were similarities between certain stations. For example, in Wheatley River during June 2021, both 12S primer sets and beach seining suggested similarities between the 50 % and 100 % stations' fish assemblages (P(MC)> 0.05; Figure 4.3A.1). There were also instances were only beach seining (e.g., the 10 % and 50 % stations in Dunk River during August 2021; P(MC)>0.05; Figure 4.3C.2) or the 12S primer sets (e.g., the 10 % and 50 % stations in Freeland Creek during June 2021; P(MC)>0.05; Figure 4.3B.1) suggested similarities between certain stations. However, all similarities between stations detected by any methods were neither seasonally nor annually consistent, suggesting high temporal variability in the fish assemblages at each station.

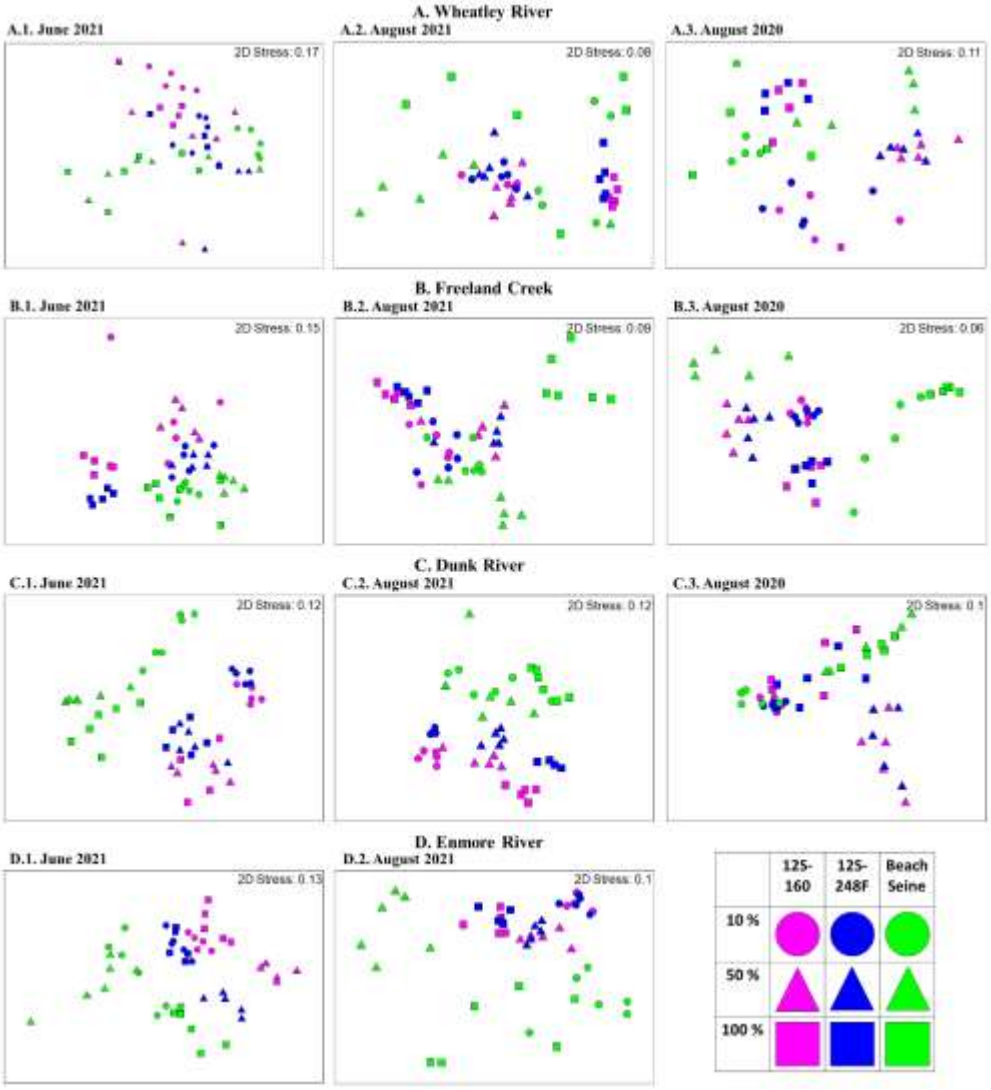

Figure 4.3: nMDS plots comparing the fish assemblages (n=3-6) of three sampling methods (12S-160 (magenta), 12S-248F (blue), beach seining (lime green)) across three stations (10 % surface area (circle), 50 % of surface area (triangle), 100 % of surface area(square)). Stress was generally found to be at or below 0.15, indicating most images offer a decent representation of each data cloud's shape. Square root transformations, Bray-Curtis similarity, 100 restarts. Images created using PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK).

4.4.5 Statistical Comparisons between the Fish Assemblages Revealed by eDNA Metabarcoding and Beach Seining

The two 12S primer sets and beach seining generally detected statistically distinct fish assemblages from one another. The average fish assemblage revealed by the two 12S primer sets were often statistically different to one another at all three stations of most estuaries across months and years ($P(MC)>0.05$ for most tests; Figure 4.3). However, there were several cases where the two primers were found to be statistically similar ($P(MC)>0.05$ for most tests; Figure 4.3 e.g., A.1-2, B.1-2., D.2). Beach seining's composition was generally statistically distinct from either 12S primer sets at all three stations across all estuaries regardless of month or year ($P(MC)<0.05$ for all tests; Figure 4.3). There were two exceptions to this trend at Wheatley River's 50 % station, where no statistical differences were found between beach seining and the 12S-248F in June and August 2021 ($P(MC)>0.05$ for both tests; Figure 4.3A.1, A.2).

Dissimilarities between the three methods were often explained by differences in the average abundances of shared species, with either the 12S primers sets or beach seining suggesting a higher average abundance than the other methods (Table 4.2). Other sources for the observed dissimilarities likely originated from the 12S primer sets detecting fishes never detected by beach seining (Table 4.2). Interestingly, the average dissimilarities between the two 12S primer sets and beach seining were generally low at Wheatley River, Freeland Creek, and Enmore River ranging between 23.69-45.35 % (Table 4.2). At the same time, Dunk River experienced higher average dissimilarity values during June and August 2021, ranging 41.56-64.81 % (Table 4.2).

4.4.6 Proportional Comparisons between the Fish Assemblages Revealed by eDNA Metabarcoding and Beach Seining

The proportions of all samples pooled by station for the two 12S primer sets and beach seining were often qualitatively similar for any given estuary, month, and year and are visualized in Figure 4.4. Generally, the most abundant taxa detected at a station were found at similar proportions between the 12S primer sets and beach seining. *Fundulus* sp., fourspine stickleback, Atlantic silverside, *Gasterosteus* sp., and winter flounder (*Pseudopleuronectes americanus*) often accounted for over 90 % of the composition per station, whether it was with the 12S primer sets

Table 4.2: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, first three shown for brevity.

Wheatley River June 2021

12S-160 and 12S-248F					
Av. Dissimilarity: 20.69%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Menidia menidia	2.34	1.87	3.34	0.83	16.16
Apeltes quadracus	2.38	1.69	2.37	1.22	11.48
Undef_ Cyprinidae	0.78	0.00	2.13	2.55	10.31
				37.95%	

12S-160 and CAMP					
Av. Dissimilarity: 30.65%					
Species	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Gasterosteus sp.	2.10	3.51	7.19	1.73	23.47
Menidia menidia	2.34	1.39	3.97	1.12	12.97
Fundulus sp.	8.74	8.47	3.43	1.38	11.18
				47.61%	

12S-248F and CAMP					
Av. Dissimilarity: 26.06%					
Species	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Gasterosteus sp.	1.69	3.51	8.50	1.58	32.63
Menidia menidia	1.87	1.39	4.16	1.10	15.95
Fundulus sp.	9.25	8.47	3.65	1.11	14.01
				62.59%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, first three shown for brevity.

Wheatley River August 2021

12S-160 and 12S-248F

Av. Dissimilarity: 12.39%

Species	160 Av. Abu	248F Av. Abu	Av. Dis	Dis/ SD	Cont%
Menidia menidia	1.13	1.47	2.19	1.03	17.66
Apeltes quadracus	3.10	3.42	1.90	1.11	15.35
Fundulus sp.	9.08	8.86	1.35	1.25	10.91
				43.92%	

12S-160 and CAMP

Av. Dissimilarity: 31.50%

Species	160 Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont%
Apeltes quadracus	3.10	3.36	7.92	1.16	25.13
Fundulus sp.	9.08	7.97	5.37	1.50	17.06
Menidia menidia	1.13	1.96	4.90	0.94	15.57
				57.76%	

12S-248F and CAMP

Av. Dissimilarity: 29.30%

Species	248F Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont %
Apeltes quadracus	3.42	3.36	7.92	1.25	27.03
Menidia menidia	1.47	1.96	4.84	1.00	16.51
Fundulus sp.	8.86	7.97	4.71	1.35	16.08
				59.62%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Wheatley River August 2020

12S-160 and 12S-248F					
Av. Dissimilarity: 12.47%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Apeltes quadracus	2.52	2.37	2.26	0.76	18.16
Gasterosteus sp.	1.95	1.59	1.69	1.37	13.56
Menidia menidia	1.58	1.53	1.42	1.21	11.41
				43.13%	

12S-160 and CAMP					
Av. Dissimilarity: 24.20%					
Species	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Menidia menidia	1.58	2.00	4.88	1.33	20.17
Gasterosteus sp.	1.95	0.83	4.27	1.97	17.64
Apeltes quadracus	2.52	1.78	3.29	1.08	13.59
				51.40%	

12S-248F and CAMP					
Av. Dissimilarity: 22.40%					
Species	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Menidia menidia	1.53	2.00	5.68	1.44	25.37
Gasterosteus sp.	1.59	0.83	3.61	1.99	16.11
Apeltes quadracus	2.37	1.78	3.18	1.01	14.18
				55.67%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Freeland Creek June 2021

12S-160 and 12S-248F

Av. Dissimilarity: 22.87%

Species	160 Av. Abu	248F Av. Abu	Av. Dis	Dis/ SD	Cont%
Undef_Cyprinidae	1.73	0.00	4.24	1.33	18.52
Carassius auratus	0.90	0.00	2.20	1.69	9.63
Fundulus sp.	7.68	8.36	2.13	1.39	9.33
				37.49%	

12S-160 and CAMP

Av. Dissimilarity: 29.97%

Species	160 Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont%
Gasterosteus sp.	2.82	4.38	4.41	1.13	14.71
Undef_Cyprinidae	1.73	0.00	4.13	1.29	13.79
Menidia menidia	3.38	2.14	3.48	1.16	11.62
				40.12%	

12S-248F and CAMP

Av. Dissimilarity: 23.69%

Species	248F Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont %
Gasterosteus sp.	2.48	4.38	5.54	1.28	23.37
Apeltes quadracus	2.20	3.12	4.01	1.49	16.92
Menidia menidia	3.10	2.14	3.47	1.08	14.63
				54.92%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Freeland Creek August 2021

12S-160 and 12S-248F

Av. Dissimilarity: 17.00%

Species	160 Av. Abu	248F Av. Abu	Av. Dis	Dis/ SD	Cont%
Apeltes quadracus	4.52	4.49	2.03	1.28	11.93
Menidia menidia	1.16	1.73	1.95	1.54	11.46
Gasteros teus sp.	1.84	2.18	1.94	1.11	11.43
				34.82%	

12S-160 and CAMP

Av. Dissimilarity: 35.57%

Species	160 Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont%
Fundulus sp.	8.15	5.40	7.89	1.23	22.17
Menidia menidia	1.16	2.06	5.97	1.32	16.78
Syngnathus fuscus	0.82	2.80	5.29	1.10	14.86
				53.81%	

12S-248F and CAMP

Av. Dissimilarity: 33.04%

Species	248F Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont %
Fundulus sp.	7.98	5.40	7.45	1.19	22.55
Menidia menidia	1.73	2.06	5.41	1.27	16.37
Syngnathus fuscus	0.76	2.80	5.41	1.14	16.36
				55.29%	

Freeland Creek August 2020

12S-160 and 12S-248F

Av. Dissimilarity: 10.01%

Species	160 Av. Abu	248F Av. Abu	Av. Dis	Dis/ SD	Cont%
Menidia menidia	3.53	3.41	1.45	1.01	14.46
Gasteros teus sp.	3.27	3.07	1.20	1.38	12.02
Apeltes quadracus	2.73	2.67	1.13	1.36	11.33
				37.81%	

12S-160 and CAMP

Av. Dissimilarity: 30.13%

Species	160 Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont%
Menidia menidia	3.53	1.96	7.59	1.26	25.20
Gasteros teus sp.	3.27	1.51	6.12	2.04	20.32
Apeltes quadracus	2.73	1.28	4.50	1.91	14.94
				60.46%	

12S-248F and CAMP

Av. Dissimilarity: 29.77%

Species	248F Av. Abu	CAMP Av. Abu	Av. Dis	Dis/ SD	Cont %
Menidia menidia	3.41	1.96	7.23	1.30	24.27
Gasteros teus sp.	3.07	1.51	5.83	2.14	19.59
Apeltes quadracus	2.67	1.28	4.42	1.75	14.85
				58.71%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Dunk River June 2021

12S-160 and 12S-248F						**12S-160 and CAMP**						**12S-248F and CAMP**					
Av. Dissimilarity: 29.50%						Av. Dissimilarity: 64.81%						Av. Dissimilarity: 58.86%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Undef_Cyprinidae	3.08	0.00	5.46	1.84	18.50	*Alosa* sp.	3.99	0.00	9.83	1.23	15.17	*Alosa* sp.	3.83	0.00	10.25	1.20	17.42
Carassius auratus	1.59	0.00	2.82	1.64	9.54	*Menidia menidia*	3.38	7.14	8.76	1.77	13.52	*Menidia menidia*	3.79	7.14	8.86	1.80	15.05
Fundulus sp.	4.09	4.65	2.32	1.36	7.86	*Fundulus* sp.	4.09	4.23	7.14	1.73	11.02	*Fundulus* sp.	4.65	4.23	7.83	1.86	13.31
				35.90%						39.71%						45.77%	

Dunk River August 2021

12S-160 and 12S-248F						**12S-160 and CAMP**						**12S-248F and CAMP**					
Av. Dissimilarity: 25.30%						Av. Dissimilarity: 49.63%						Av. Dissimilarity: 41.56%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Undef_Cyprinidae	2.23	0.00	4.67	2.99	18.45	*Alosa* sp.	1.81	3.04	5.41	1.21	10.89	*Alosa* sp.	1.34	3.04	6.35	1.10	15.28
Carassius auratus	1.17	0.00	2.43	2.21	9.59	Undef_Cyprinidae	2.23	0.00	5.17	2.85	10.41	*Fundulus* sp.	4.78	2.59	5.90	1.25	14.20
Fundulus sp.	4.54	4.78	2.01	1.59	7.94	*Fundulus* sp.	4.54	2.59	4.69	1.38	9.46	*Clupea harengus*	2.05	0.00	5.70	1.28	13.72
				35.98%						30.76%						43.19%	

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Dunk River August 2020

12S-160 and 12S-248F						**12S-160 and CAMP**						**12S-248F and CAMP**					
Av. Dissimilarity: 19.60%						Av. Dissimilarity: 33.75%						Av. Dissimilarity: 32.25%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Menidia menidia	4.72	5.04	3.52	1.09	17.96	*Menidia menidia*	4.72	6.51	8.22	1.41	24.36	*Menidia menidia*	5.04	6.51	7.54	1.43	23.37
Fundulus sp.	7.05	6.96	2.97	0.91	15.15	*Morone saxatilis*	2.13	0.00	6.22	0.68	18.45	*Morone saxatilis*	2.13	0.00	6.35	0.70	19.69
Morone saxatilis	2.13	2.13	2.38	0.68	12.16	*Fundulus* sp.	7.05	6.15	5.94	1.03	17.61	*Fundulus* sp.	6.96	6.15	5.81	1.11	18.02
				45.28%						60.41%						61.09%	

Enmore River June 2021

12S-160 and 12S-248F						**12S-160 and CAMP**						**12S-248F and CAMP**					
Av. Dissimilarity: 21.49%						Av. Dissimilarity: 40.70%						Av. Dissimilarity: 34.51%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**	**Species**	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Undef_Cyprinidae	2.08	0.00	4.23	2.03	19.69	*Gasterosteus* sp.	2.16	4.46	5.32	1.32	13.08	*Gasterosteus* sp.	1.87	4.46	6.64	1.44	19.24
Carassius auratus	1.08	0.03	2.13	1.64	9.93	*Fundulus* sp.	5.71	5.34	5.27	1.97	12.94	*Fundulus* sp.	6.35	5.34	6.36	1.76	18.44
Fundulus sp.	5.71	6.35	1.86	1.50	8.65	*Menidia menidia*	6.13	5.67	5.06	1.67	12.43	*Menidia menidia*	6.47	5.67	5.79	1.64	16.77
				38.26%						38.45%						54.46%	

1

Table 4.2 continued: Two-factor Similarity Percentage analysis (SIMPER) examining the dissimilarities between methods (12S-160, 12S-248F, Beach Seine) across all stations (10 %, 50 %, 100 %) within estuaries sampled in Prince Edward Island, Canada during August 2020 and summer 2021. Performed with a square root transformed data using Bray Curtis similarity index, the first three species were shown to for brevity.

Enmore River August 2021

12S-160 and 12S-248F					
Av. Dissimilarity: 14.49%					
Species	**160 Av. Abu**	**248F Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Morone saxatilis	1.88	1.82	1.54	1.33	10.65
Gasteros teus sp.	1.22	1.59	1.42	1.32	9.82
Clupea harengus	0.50	0.42	1.37	1.06	9.43
				29.90%	

12S-160 and CAMP					
Av. Dissimilarity: 45.35%					
Species	**160 Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont%**
Fundulus sp.	7.27	5.09	8.94	1.24	19.70
Menidia menidia	4.97	5.38	7.71	1.50	17.00
Morone saxatilis	1.88	0.00	4.82	1.60	10.64
				47.34%	

12S-248F and CAMP					
Av. Dissimilarity: 44.18%					
Species	**248F Av. Abu**	**CAMP Av. Abu**	**Av. Dis**	**Dis/ SD**	**Cont %**
Fundulus sp.	7.14	5.09	8.80	1.26	19.91
Menidia menidia	5.16	5.38	7.42	1.63	16.81
Apeltes quadracus	2.44	0.55	4.97	2.15	11.24
				47.96%	

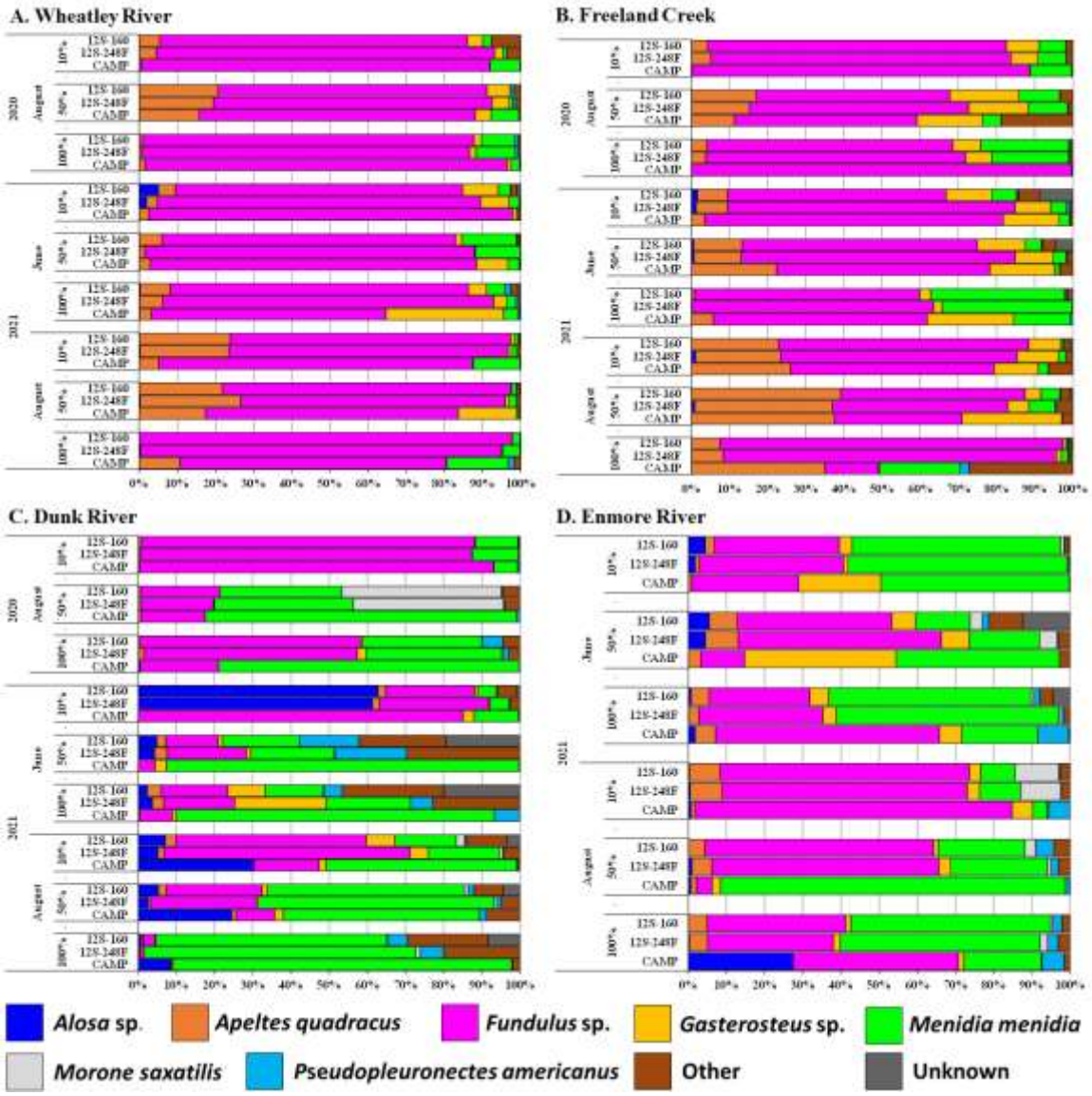

Figure 4.4: Relative proportions of fish genera for the rarefied sequencing reads for 12S-160 and 12S-248F eDNA metabarcoding primer sets (n=3-5) and relative proportions of fish counts (abundance) from beach seining (n=5-6) at three stations across four estuaries in Prince Edward Island, Canada, during August 2020, June 2021, and August 2021. Other species include *Anguilla rostrata*, *Clupea harengus*, *Microgadus tomcod*, *Limanda ferruginea*, *Osmerus mordax*, *Pungitius pungitius*, *Tautogolabrus adspersus*, *Salvelinus fontinalis*¸ and 26 other taxa. Unknown were fishes or reads unidentified down to genus level. Image made in Microsoft® Excel® for Microsoft 365 MSO (Version 2207, Build 16.0.15427.20182).

or beach seining. There were several instances where certain genera were better represented by either the 12S primer sets or beach seining. For example, striped bass was frequently detected by the 12S primer sets in Dunk River and Enmore River, while never being detected with beach seines.

The 12S primer sets displayed highly divergent results from beach seining at all stations in Dunk River throughout summer 2021, especially in June (Figure 4.4C). For example, *Alosa* sp. made over 60 % of the 12S primer sets rarefied read proportions while never being detected with beach seining in Dunk River's 10 % station during June 2021. The 12S primer sets also more frequently detected other species, namely yellowtail flounder, at the 50 % and 100 % stations where beach seining was mainly being dominated by Atlantic silversides.

4.5 Discussion

This study examined eDNA metabarcoding's potential to complement or replace beach seining in four estuaries across PEI, Canada. The results suggested that eDNA metabarcoding was generally comparable to beach seining, while also offering broader insight into the estuarine fish assemblage. eDNA metabarcoding is being increasingly seen as a complementary technique to use alongside other sampling methods (Cheang et al. 2020; Cole et al. 2022). Although, in certain circumstances where traditional methods are impractical in terms of labour and time, or there is potential concern about disturbances to the aquatic environment, eDNA metabarcoding could become a replacement (Fujii et al. 2019; García-Machado et al. 2022; He et al. 2022).

4.5.1 Seasonal, Interannual, and Spatial Resolution of eDNA Metabarcoding

eDNA metabarcoding was found to be as effective as beach seining in resolving different fish assemblages across different seasons (June vs August 2021) and years (August 2020 and 2021). These findings are in line with the growing number of studies in estuaries and coastal systems demonstrating that eDNA metabarcoding can detect seasonal shifts in fish assemblages (Sigsgaard et al. 2017; Stoeckle et al. 2021). eDNA's persistence in marine and coastal environments appears to be no longer than 20 days in the water column (Harrison et al. 2019), which suggests there was a complete turnover in eDNA between the end of June and the beginning of August.

The present study demonstrates that eDNA metabarcoding can discriminate spatially separated fish assemblages in estuaries and coastal environments. In our study, eDNA metabarcoding generally distinguished fish assemblages between stations ~1-3 km apart. These included the 50 % and 100 % stations in Enmore River in June and August in 2021, which were 400 m apart, and the 10 % from the 50 % station in Freeland Creek in August 2020 and 2021, which were 580 m apart. Kelly et al. (2018) found that at three sampling stations' (~1 km apart) eDNA communities remained distinct from one another despite incoming and outgoing tides in a fjord in Washington, USA. Oka et al. (2021) found that in tropical lagoons, with strong tide and currents, that eDNA metabarcoding from surface water could differentiate fish assemblages ~ 300 m apart. Thus, the results of the current study further demonstrate the spatial resolution of eDNA metabarcoding.

4.5.2 Comparing the Fish Assemblages of eDNA Metabarcoding and Beach Seining

Past studies have shown that eDNA metabarcoding generally identifies greater numbers of species than traditional sampling methods such as beach seining, trawling, and remote camera traps in estuaries and coastal systems (P.F. Thomsen et al. 2012; Afzali et al. 2021; Cole et al. 2022). In the current study, eDNA metabarcoding's fish assemblages routinely showed greater species richness than beach seining. However, not all studies have supported this conclusion, as Hallam et al. (2021) recently noted that their eDNA metabarcoding survey in estuaries performed worse than traditional sampling, including beach seining, which they attributed to their filters clogging with sediment preventing them from filtering their full 1 L sample.

The success of eDNA techniques herein to detect all species captured in seine nets could be attributed to excellent reference sequence coverage for PEI's estuarine fishes and selected primers sets being very specific to actinopterygians. However, it came to our attention that no sequences exist for grubby (*Myoxocephalus aenaeus*) in National Center for Biotechnology Information database. The results that no fishes were unique to beach seining deviates from many other studies that frequently find traditional methods are capable of detecting species/genera that eDNA metabarcoding failed to detect (e.g., Afzali et al. 2021; Stoeckle et al. 2021; Cole et al. 2022). Even He et al. (2022), using the 12S-248F primer set also used in the

present study, found beach seining was capable of detecting fishes eDNA metabarcoding could not in southern Nova Scotia. In most of these listed cases, eDNA metabarcoding's failed detections appear to be due to gaps in sequences in databases (see Afzali et al. 2021; Stoeckle et al. 2021).

However, beach seining was able to detect certain species generally found at low abundances at particular stations and times that eDNA metabarcoding failed to detect at the same station but did detect at other stations in the same estuary. These localized failed detections likely resulted from the fact that low-abundance taxa produce fewer molecular signals than highly abundant taxa (Di Muri et al. 2020; Afzali et al. 2021). For example, in this study cunner was only detected while beach seining Wheatley River's inner station during August 2020. eDNA metabarcoding may have failed to detect the cunner at this specific station as only one cunner was captured, compared to 14,334 *Fundulus* sp., indicating a low overall abundance of cunner in that region. The incongruities may have also stemmed from the fact that fish are mobile and there was a 24 h separation between eDNA collection and beach seining.

We were surprised by how qualitatively similar the proportions of most of the common species were between eDNA metabarcoding and beach seining. A growing number of studies show that there is often agreement with the relative abundances of fish detected between eDNA metabarcoding and traditional methods (M.Y. Stoeckle et al. 2017; van Bleijswijk et al. 2019; He et al. 2022). Afzali et al. (2021) found agreement with only the most abundant genus between trawling and eDNA metabarcoding in the northern GSL, while the selected 12S primer sets herein found agreement with at least five taxa including *Fundulus* sp., fourspine stickleback, Atlantic silverside, *Gasterosteus* sp., and winter flounder.

Beach seining may be more comparable to eDNA metabarcoding than bottom trawls as trawls are dragged over 1 km, passing tens of millions of litres of water, yet only around 1-2 L of water for eDNA were collected for these trawl-eDNA comparison studies (Thomsen et al. 2016; Afzali et al. 2021; Stoeckle et al. 2021). On the other hand, beach seining covers a smaller area and volume (225 m^2 and ~450, 000 L) from the same area water samples were collected. We speculate that this smaller area is better reflected by eDNA metabarcoding. In any case, it is apparent that eDNA metabarcoding can provide qualitatively and quantitatively (i.e.,

proportionally) similar information to beach seining while reducing the area disturbed when sampling.

Incongruities between eDNA metabarcoding and traditional sampling methods are frequently observed across studies and likely stem from biases inherent to all sampling methods skewing the fish assemblages they reveal (Stat et al. 2019; Afzali et al. 2021; Cole et al. 2022). For example, striped bass were never captured during our beach seining surveys, while eDNA metabarcoding indicates that this species may be found at higher abundances than what beach seining suggests. Many studies have noted that bottom trawling is biased against pelagic, large, or highly mobile fishes, as they can easily avoid the net but can be detected readily with eDNA, while comparatively sluggish, benthic fishes may be over represented in trawling relative to eDNA metabarcoding (Thomsen et al. 2016; Afzali et al. 2021). Similar phenomena likely occur with beach seining.

eDNA metabarcoding often excels at detecting rare or cryptic taxa that are often missed by nets (Shelton et al. 2016; Djurhuus et al. 2020). For example, Hallam et al. (2021) found that eDNA metabarcoding detected the sea lamprey (*Petromyzon marinus*), which is rare in Thames River UK, and has never been detected in a 20 year history of sampling the area with nets. In this study, winter skate, which is endangered in the sGSL (Kelly and Hanson 2013), was detected with eDNA metabarcoding at Dunk River's 50 % station in August 2021 (12S-160 only). Winter skates have never been recorded in Dunk River by CAMP since sampling began in 2007 (M. Boudreau, DFO Moncton, personal communication). Therefore, it is worth acknowledging that beach seining, and other traditional sampling methods, may not always provide the most comprehensive representation of the ichthyofaunae present in an estuary or other coastal system.

In many studies, it has been suggested that primer amplification bias plays a significant role in the taxa that any given metabarcoding primers will detect, as the primers can preferentially amplify certain taxa over others (Kelly et al. 2017; Zhang et al. 2020; Shu et al. 2021). Both of our primer sets were highly specific to actinopterygians, which may reduce our ability to detect chondrichthyans in the region but resulted in highly focused amplification and did not waste much sequencing effort on non-target classes. However, it is still possible that the

primers preferentially bind to certain genera or species more than others, so this factor cannot be entirely discounted.

Other sources of bias inherent to eDNA metabarcoding stem from differential production of eDNA by different species. Different species of fish shed eDNA at different rates depend on the species' physiology and behaviour (Thalinger et al. 2021) or the environmental conditions, like the water's temperature (Caza-Allard et al. 2022). The size of a fish also appears to influence eDNA production, as few large fish appear to produce less DNA than many small fish of equivalent biomass (Spear et al. 2021). Thus, differential shedding rates between fishes would introduce another source of variability contributing to some of eDNA metabarcoding's incongruities with beach seining.

eDNA metabarcoding is also susceptible to exogenous DNA from surrounding industries within an estuary's catchment. For example, our results showed the proportional consistency in the fish assemblages between eDNA metabarcoding and beach seining seen at Dunk River in August 2020 deteriorated in summer 2021. One possible explanation is that reduced commercial fishing activity (mainly oysters) due to the onset of the COVID-19 pandemic throughout 2020 may have resulted in Dunk River being abnormally undisturbed by seafood harvesting and processing (Yarr 2020). In summer 2021, commercial fisheries resumed, and no doubt introduced exogenous DNA. In particular, boxes of lobster bait (*Alosa* sp.) were dumped at the 10 % station in June 2021. eDNA metabarcoding cannot distinguish DNA deriving from living or dead tissue. Over 50 % of the reads of eDNA samples collected at this station were attributed to *Alosa* sp., that were never physically identified in beach seine samples but are also present during migrations in spring and early summer. Municipal sewage, fish markets, seafood processing plants (mainly for lobster), and harbour-front restaurants (closed in 2020 but re-opened in 2021) from the City of Summerside all possibly introduced exogenous DNA that contributed to the high degree of incongruities between eDNA metabarcoding and beach seining observed at Dunk River (Yamamoto et al. 2016; M.Y. Stoeckle et al. 2017; Fujii et al. 2019). Therefore, surrounding industries will need to be considered as sources of exogenous contaminating DNA if monitoring programs utilize eDNA metabarcoding.

The largest factor for the incongruities between fish proportions indicated by eDNA metabarcoding and beach seining that we observed may have resulted from our sampling design. Due to concerns over cross-contamination from beach seining, water samples for eDNA metabarcoding were collected 24 h (maximum 48 h due to rainfall events in Dunk River in August 2021) before beach seining. Kelly et al. (2018) found that the communities revealed by eDNA metabarcoding remained fairly stable at stations with incoming and outgoing tides over a 28 h period, which was our assumption going into the project in 2020. However, a recent study by Jensen et al. (2022) demonstrated eDNA reflects the diel cycles of fish assemblage in estuaries and coastal environments, changing hourly throughout the day. Fortunately, it is unlikely that the overall assemblage changes much between days in estuaries in the sGSL (Landry et al. 2007 unpublished). Nevertheless, the temporal separation between eDNA collection and beach seining potentially introduced stochasticity that may have reduced the quality of our comparisons between the two methods. Regardless of all possible sources of variation, the selected eDNA metabarcoding primer sets appear to provide similar proportional abundances of the most common species found at a station as beach seining.

4.5.3 eDNA Metabarcoding and Inconsistent Classifications

The two 12S primer sets used, while capable of identifying most fish down to species, may have difficulties reliably distinguishing *Fundulus* sp. (mummichog (*F. heteroclitus macrolepidotus*) and banded killifish (*F. diaphanous*)), and *Alosa* sp. (gaspereau (*A. pseudoharengus*) and American shad (*A. sapidissima*)) from one another. One minor concern frequently raised with beach seining surveys was misidentifying similar species' YOY(Weldon et al. 2007; DFO 2011). Thus, the eDNA metabarcoding analysis herein may not completely resolve this issue for *Fundulus* sp. and *Alosa* sp., but did do so for *Gasterosteus* sp., as threespine stickleback (*G. aculeatus*) and blackspotted stickleback (*G. wheatlandi*) appear to be reliably distinguished from one another with both 12S-primer sets. There may have been minor issues distinguishing species in the genera *Gadus* (Atlantic cod (*Gad. morhua*) and arctic cod (*Gad. ogac*)) and *Scomber* (Atlantic mackerel (*S. scombrus*) and Atlantic chub (*S. colias*)). However, Atlantic chub mackerel are not known to inhabit the sGSL (Nozères et al. 2022), so these detections may have resulted from either incorrect classification of Atlantic mackerel reads during bioinformatics or may have resulted from bait used in commercial fishing.

To be of broader use in monitoring, eDNA metabarcoding needs to be able to consistently identify the taxonomic levels of genus, and preferably species (Hleap et al. 2021). However, many universal metabarcoding primers and bioinformatic classifiers result in lower accuracy at the species or genus level than at higher taxonomic levels (Hleap et al. 2021; Xiong et al. 2022). Increasing references for the 12S mitochondrial rRNA region in sequence databases may mitigate this issue with time, as the ribosomal gene allows for higher specificity (i.e., species- or genus-level) than protein coding genes like the commonly used cytochrome oxidase subunit 1 (COI; Collins et al. 2019). However, current databases are often taxa deficient for the 12S region (Collins et al. 2019; Schenekar et al. 2020; Xiong et al. 2022). Regardless, it may be nearly impossible to achieve complete accuracy at species-level with metabarcoding due either to populations constantly evolving (i.e., hybridization and incomplete lineage sorting), and/or frequent taxonomic revisions (Schenekar et al. 2020; Hleap et al. 2021). For example, mummichog and banded killifish are known to hybridize in the GSL, which could make genetic and morphological differentiation difficult (Sargent et al. 2020) particularly in the case of mitochondrial DNA introgression from one species to another. In addition, a taxonomic revision was noticed in this study. The DNA library used in this study classified yellowtail flounder (*Limanda ferruginea*) as *Myzopsetta ferruginea*, an outdated binomial name, which caused minor confusion. Thus, monitoring programs utilizing eDNA metabarcoding may be faced with genus-level identification for certain taxa for the foreseeable future.

4.6 Conclusion

The selected 12S eDNA metabarcoding primer sets could detect seasonal and interannual shifts in the fish assemblages, and could also distinguish three spatially distinct stations (~0.4-3 km apart) that spanned the inner, middle, and outer regions of estuaries across PEI, Canada. eDNA metabarcoding was found to provide quantitative data, as the proportions of the most common species detected by eDNA metabarcoding were qualitatively similar to beach seining. We believe our findings suggest that eDNA metabarcoding may complement beach seining, allowing broader insight into the ichthyofauna that beach seining is biased against, such as striped bass, and the detection of rare species like winter skate. However, in situations with limited labour, time, difficult-to-sample-habitats, or fragile habitats, eDNA metabarcoding could

serve as a suitable replacement, offering reliable insight into the proportional abundances of fishes present within an estuary.

Chapter 5: General Discussion and Conclusion

The inner region of estuaries, closest to riverine inputs, has been increasingly recognized for its importance for evaluating estuarine health, including in Canada's southern Gulf of Saint Lawrence (sGSL; Coffin et al. 2021b). The inner region is the first to receive land-derived pollutants, including nitrogen-based agricultural fertilizers, and experiences longer water residence times compared to middle and outer regions (Schein et al. 2012; Niu et al. 2021; Turner et al. 2021). As a result, the pollutants' effects are often more pronounced in the estuary's inner region, including macroalgae over-proliferation and subsequent hypoxia in the case of nitrogen-induced eutrophication (van den Heuvel et al. 2019; Coffin et al. 2021a; Niu et al. 2021).

Fisheries and Oceans Canada (DFO) has been developing a series of indicators for a Marine Environmental Quality Guideline (MEQ) to foster efforts that will address declines in estuarine health, due to eutrophication and other anthropogenic stressors, across the sGSL (Coffin et al. 2021b). So far, dissolved oxygen (DO) variability and eelgrass coverage within the inner region are being used as indicators to assess extent of eutrophication of estuaries (Coffin et al. 2021b). DFO is currently investigating if fish assemblages could be a potential bioindicator of eutrophication, which could then be monitored using their stewardship program, the Community Aquatic Monitoring Program (DFO 2011). However, some challenges may hamper CAMP's ability to assess fish assemblages. The inner estuarine region is often excluded from CAMP's surveys due to dense mats of sea lettuce clogging the beach seines used for sampling and issues with volunteers accessing the inner region (DFO 2011). As a result, the inner estuarine region's fish assemblages have remained largely understudied across the sGSL.

This dissertation investigated the inner region's fish assemblage to see if it differed from those found at the middle and outer regions of four estuaries of varying levels of nutrient impact in Prince Edward Island (PEI), Canada. The primary objective was to investigate if environmental DNA (eDNA) metabarcoding could simplify sampling efforts by complementing or replacing beach seining in the hard-to-sample inner region of eutrophic

estuaries. In addition, the abundance of northern mummichog (*Fundulus heteroclitus macrolepidotus)* was also investigated as a potential single-species indicator of eutrophication to simplify sampling efforts for volunteers and assessment by managers.

5.1 Thesis Summary

The following summarizes the principal findings of **Chapter 3**. The inner region (defined as the innermost 10 % of estuarine surface area) of the four small, well-mixed, saltwater-dominated estuaries sampled in PEI were found to contain fish assemblages that were significantly distinct from both the middle (50 % of estuarine surface area) and outer (100 % of estuarine surface area) regions. These results were consistent regardless of the relative level of nutrient impact (high-to-low based on eutrophic time (*sensu* Coffin et al. 2018b)), shoreline (north vs south shore), season (June vs August 2021) or year (August 2020 vs August 2021). The inner region's fish assemblage appears to be generally characterized by high abundances of mummichog and the young-of-the-year (YOY) of many species (including mummichogs, Atlantic silversides (*Menidia menidia*), fourspine stickleback (*Apeltes quadracus*), and *Gasterosteus* sp.). Aquatic vegetation coverage (or lack thereof) appeared to be important in structuring the longitudinal fish assemblages across most estuaries. Temperature and salinity also appeared to contribute to structuring these assemblages. Mummichogs displayed a strong, positive linear relationship with sea lettuce coverage and, as such, may provide a simple bioindicator for eutrophication. All four estuaries sampled were found to have unique fish assemblages. However, fish assemblages in estuaries on the same shoreline (north or south shore) were found to be more like one another than those of similar levels of relative nutrient impact. The average abundance of mummichogs also mirrored these results when examining samples collected from the inner, middle, and outer regions together. Specifically, the two north shore estuaries, Wheatley River (high nutrient impact) and Freeland Creek (mid nutrient impact) had higher average abundances of mummichogs than either of the two south shore estuaries, Dunk River (mid nutrient impact) or Enmore River (low nutrient impact). However, when only the inner

region was examined, the abundance of mummichogs appear to reflect the relative level of nutrient impact, with estuaries with higher eutrophic times (i.e., Wheatley River) having higher mummichog abundance relative to estuaries with lower eutrophic times (i.e., Freeland Creek, Dunk River, and Enmore River).

The following summarizes the principal findings of **Chapter 4**. eDNA metabarcoding was generally able to detect the longitudinal shifts in the fish assemblages between the inner, middle, and outer estuarine regions that were 0.4 to 3.0 km apart, as well as the seasonal and interannual variations of these assemblages. eDNA metabarcoding detected a greater richness of genera than beach seining, while requiring less fieldwork in terms of time and labour. eDNA metabarcoding could also detect large mobile, deeper water, and potentially rare fishes that the beach seine often failed to detect. eDNA metabarcoding also indicated that certain species (i.e., striped bass (*Morone saxatilis*)) might be found at higher proportional abundances in some stations than beach seining suggested. Finally, eDNA metabarcoding and beach seining, despite having statistically different fish assemblages, often qualitatively reflected one another in terms of the percent contribution of the most abundant fishes (*Fundulus* sp., fourspine stickleback, Atlantic silverside, *Gasterosteus* sp., and winter flounder (*Pseudopleuronectes americanus*)) found at a station. Thus, eDNA metabarcoding could complement or potentially replace beach seines for monitoring fish assemblages in estuaries of the sGSL and provide quantitative data on the relative proportions of different fish in estuaries.

5.2 Implications and Recommendations for Future Research

5.2.1 Assemblages in the Inner Region of Estuaries in the Southern Gulf of Saint Lawrence

In my opinion future research is required to understand the longitudinal use of estuaries by fishes in the sGSL, and eDNA metabarcoding could complement beach seining, or most other traditional sampling methods implemented in these studies. This dissertation

provided evidence that past ichthyofauna and nekton studies across the sGSL excluded distinct fish assemblages found in the inner region from their surveys (e.g., Joseph et al. 2006; DFO 2011; Schein et al. 2012). As such, how fishes utilize specific areas within an estuary remains understudied, if not unnoticed across the sGSL. The main observations that the YOY of many species are more abundant in the inner region (and the middle region with some species) of estuaries, and how estuaries' geomorphology and hydrology may obscure patterns created by anthropogenic activities were already discussed in **Chapter 3** in detail. eDNA metabarcoding suggested that past beach seine surveys likely underestimated the prevalence of larger, mobile fishes, especially striped bass. eDNA metabarcoding also helped detect the endangered winter skate (*Leucoraja ocellata*), which has never been recorded in Dunk River since CAMP started surveying the estuary in 2007 (M. Boudreau, DFO Moncton, personal communication). Thus, combining eDNA metabarcoding with traditional sampling methods used in future studies may provide a more holistic view of the fish assemblages found at each region within an estuary.

5.2.2 Abundance of Northern Mummichogs as Indicators of Eutrophication.

Finley et al. (2009) suggested that total mummichog abundance (adult and YOY) could be used as a single species indicator to simplify the assessment of eutrophication in the sGSL. This dissertation supports their claim, as mummichogs displayed a strong positive, linear relationship with increased coverage of sea lettuce and mummichog abundance. We also found that the relative abundance of mummichogs per beach seine haul ascended with increasing eutrophic time. This suggested that mummichog abundances in the inner regions of estuaries could be compared to gauge level of nutrient impact relative to one another. However, the results also suggested that estuaries from similar shorelines (north or south shore) often had comparable abundances of mummichogs, regardless of relative levels of nutrient impact, when analysing the inner, middle, and outer regions together. Previous studies have noted that the geomorphology and hydrology of the estuaries may obscure any potential patterns produced by anthropogenic disturbances in fish assemblages (Tweedley et

al. 2017). Therefore, future studies may need to explore how estuarine geomorphology influences the distribution and abundance of mummichogs at a regional (i.e., across the sGSL or across PEI) and local (i.e., within an estuary) scale.

It is my opinion that future studies may need to investigate what other factors, besides increased productivity, contribute to the high abundance of mummichog in the inner estuarine region. This dissertation found evidence that mummichog abundance was higher in the inner region, closest to riverine inputs, than in regions closer to the open ocean. These findings are consistent with previous studies, which have attributed higher abundances to the inner region's increased productivity (Halpin 1997; Finley et al. 2009; Lockfield et al. 2013). This dissertation found that mummichog have a strong positive association with sea lettuce, which in turn supports the productivity-based attraction theory. However, temperature and salinity are routinely identified as the primary factors structuring fish distribution within estuaries (Whitfield 2021). Northern mummichog, despite being highly euryhaline, appear to be associated with warmer temperatures and salinities around 20 PSU (Fritz and Garside 1974; Garside and Morrison 1977), which often corresponds with the inner region of the selected estuaries. Thus, the influences of temperature and salinity on mummichog distribution should not be ignored.

5.2.3 Considerations and Recommendations for Using eDNA in Monitoring Programs

This dissertation demonstrated that eDNA metabarcoding could potentially complement (i.e., identify additional taxa) or replace beach seining (i.e., providing quantitative data on the composition) for monitoring estuaries across PEI and potentially the rest of the sGSL. This dissertation also demonstrated that the eDNA extraction and qPCR protocols developed by DFO could be adopted and implemented at a separate institution. However, after completing the research, I have some suggestions for managers to consider.

5.2.3.1 eDNA Metabarcoding as a Means to Reduce Time and Labour Required for Field Work

One of eDNA metabarcoding's most appealing qualities for monitoring programs is that it requires less time and labour for field collection than most traditional sampling methods, including beach seining. In my experience, eDNA often took half to a fifth of the time required for beach seining when field sampling a comparable area. I found field sampling for eDNA (collecting fifteen 1 L water samples across three stations) often took 2.5-3.5 h, including time travelling between and within stations at each estuary. It could be accomplished with two technicians comfortably. eDNA's short collection time contrasted starkly against beach seining (collecting fifteen to eighteen beach seine hauls across three stations), which took 7 h at its shortest (e.g., Dunk August 2020, Enmore August 2021) to nearly 15 h at its longest (Wheatley River August 2021). In almost all cases, the time spent beach seining had to be broken up across two days and required three or more people to be comfortably performed. Thus, I believe eDNA would be ideal for situations with short field seasons, difficult-to-sample or fragile habitats, or limited field workers.

However, much of the time saved in the field was exchanged for time spent in the laboratory. I found that filtration often took over 4 h from beginning to end. Therefore, collection and filtration often cumulatively comprised a similar amount of time as beach seining the simpler-to-sample estuaries (e.g., Enmore River). My filtration time was prolonged by having to clean equipment during filtration sessions, as I had a limited number of glass filtration units for the vacuum manifold. I believe that the time required for filtration could be reduced by having more filtration equipment for the laboratory.

Alternatively, managers may choose to develop disposable field filtration protocols (e.g., enclosed Sterivex filter units) or utilizing eDNA backpack samplers which could reduce field work (i.e., no longer carrying water samples out of field sites) and the need for laboratory filtration (e.g., Thomas et al. 2018; Miya et al. 2022). Field filtration may also reduce risk of further eDNA degradation during transport and reduce risk of contamination

by limiting times samples are handled (Majaneva et al. 2018). However, previous studies have shown that alterations to any stage of the eDNA metabarcoding workflow, including collection, often changes the communities revealed by eDNA metabarcoding (Goldberg et al. 2016; Djurhuus et al. 2017; Hermans et al. 2018; Majaneva et al. 2018). Interestingly, recent studies have found evidence that the fish assemblage compositions revealed by Sterivex filter and laboratory filtration were similar between water samples collected at stocked artificial pounds (Li et al. 2018; Di Muri et al. 2020). Regardless, future studies will be required to validate whether field filtration techniques produce similar results to laboratory filtration in natural settings of estuaries due to conflicting evidence.

The time required to complete the entire eDNA workflow (from collecting water samples to receiving the sequences) can often take several months. M.Y. Stoeckle et al. (2017) found that their workflow took around three months to complete. My complete workflow took much longer to complete, nearly seven months. I faced recurring delays due to supply chain issues throughout 2020-2021 due to the ongoing COVID-19 pandemic, which prolonged my timeline. I believe eDNA samples could be collected and ready for sequencing within a workweek if trained personnel had sufficient materials. However, even if all the samples can be filtered, extracted and amplified in under a week, a remaining bottleneck with eDNA metabarcoding is the need to accumulate enough samples to justify the costs of high-throughput sequencing (M.Y. Stoeckle et al. 2017). Therefore, there will always be additional days or weeks of laboratory work associated with eDNA collection, and eDNA should be best imagined as a means to reduce time and labour spent in the field.

5.2.3.2 eDNA Metabarcoding and Proportional Data

Estimating wildlife abundance is critical to managing and monitoring ecosystems (Spear et al. 2021). As such, eDNA's ability to provide abundance (number of individual or biomass)-related data will likely be essential for its adoption into many monitoring programs (Knudsen et al. 2019; Afzali et al. 2021; Spear et al. 2021; He et al. 2022). This dissertation

showed that eDNA metabarcoding might provide quantitative data concerning the relative proportions of species within an estuary. However, one should avoid mistaking the number of DNA sequences as a direct representation of the abundance of fish or biomass. DNA reads produced by high-throughput sequencing will be influenced by the initial concentration of DNA (which is often unknowable), the efficiency of PCR (primer binding biases, inhibitors, number of cycles), DNA captured and retained during filtration and extraction processes, and the reliability of the sequencer or bioinformatic pipeline and library quality (Shelton et al. 2016).

However, despite of all the confounding variables which may prevent a relationship, eDNA, single species and metabarcoding alike, have increasingly demonstrated the ability to estimate both fish population and biomass (Afzali et al. 2021; Spear et al. 2021; He et al. 2022). Future studies may wish to employ the RT-qPCR protocol I implemented to explore its ability to provide quantitative data on biomass. Standard curves (i.e., serial dilutions used to create regression curves) could be created to estimate the initial concentration of DNA in the samples, which could then be used to estimate abundance and/or biomass at a study site.

Perhaps of greater interest would be to develop a single species assay for mummichogs. Spear et al. (2021) demonstrated that their species-specific eDNA assay could be used to estimate walleye (*Sander vitreus*) abundances for potential fisheries management. I believe developing a similar single-species assay to quantify mummichogs should be explored in future research. A mummichog-specific assay could be utilized in MEQ guidelines to quantify the abundance of mummichog in difficult-to-sample eutrophic estuaries across the sGSL.

5.2.4 eDNA Metabarcoding's Application for CAMP

Field collection is the only stage of the eDNA workflow that does not require specialized training, as such volunteers could only be effectively utilized at this initial stage (Thomsen and Willerslev 2015; Larson et al. 2020; Meyer et al. 2021; Agersnap et al. 2022).

Developing a simple, standardizable collection methodology that minimizes the risk of contamination will be the main task of monitoring organizations wishing to utilize volunteers (Djurhuus et al. 2020; Larson et al. 2020; Meyer et al. 2021; Agersnap et al. 2022). In my opinion, the most challenging aspect of eDNA's field collection was transporting the water bottles collected at a station. I believe that supplying sterile water bottles, coolers, collection poles, and filtration equipment would be too cumbersome and costly for most volunteer-based programs.

Instead, I suggest developing disposable collection kits. Miya et al. (2022) and Agersnap et al. (2022) found that disposable eDNA field collection kits containing instructions, nitrile gloves, masks, and Sterivex filters with syringes allowed for volunteers to be easily incorporated into the field collection portion. Sterivex filters connected to syringes were simple for volunteers to use, minimized the risk of contamination, and allowed up to 1 L of water to be filtered (Agersnap et al. 2022). These kits could be easily assembled and distributed within the pre-existing CAMP network without needing to sterilize or carry heavy equipment (e.g., coolers full of water samples). However, Li et al. (2018) and Di Muri et al. (2020) note that while Sterivex filters with syringes are convenient for field filtration, they also require longer filtration times (average 18.00 ± 6.48 min (Mean ± SD)) than laboratory filtration when using similar pore size, are far costlier than standard filters (~ 15X), and generate higher amounts of plastic waste. Therefore, as previously mentioned, this field filtration technique, or other novel methods, needs to be validated by future studies within the sGSL before adoption into CAMP.

A system for retrieving the samples from the volunteer groups will have to be developed alongside field collection methods. Due to the vast geographic area covered, CAMP may choose to develop a mail-in collection method like the CALeDNA program, which collects sediment samples for eDNA analysis across California (Meyer et al. 2021). CAMP could potentially send within their collection kits a small, insulated box (e.g., styrofoam box), an ice pack, and pre-paid postage to allow the semi-refrigerated retrieval of

samples. Collected samples could be then archived in freezers for ongoing and future projects.

5.3 Conclusion

In conclusion, it was found that the inner region of PEI estuaries contains distinct fish assemblages from the middle and outer regions, which can be detected by both beach seining and our selected 12S eDNA metabarcoding primer sets. The abundance of northern mummichogs (adults and YOY) was found to display a strong positive relationship with sea lettuce coverage and was highest in the inner estuarine region. Estuaries with higher eutrophic times found in their inner region were also found to have higher mummichog abundance than the inner regions with relatively lower eutrophic times. As a result, I believe that densities of northern mummichogs could indicate eutrophication within and between estuaries. However, future studies may be needed to investigate the influences of temperature, salinity, geomorphology, and hydrology on the distribution of mummichogs within an estuary. It was also found that the fish assemblage composition revealed by beach seining and the 12S eDNA metabarcoding primer sets were often qualitatively similar. These results provide evidence that eDNA metabarcoding could potentially allow for the quantitative assessment of an estuary's fish assemblages composition.

Combining eDNA metabarcoding with other traditional sampling methods may be a relatively time- and labour-effective manner to complement existing sampling procedures used in monitoring programs, by offering greater insight into the broader faunal community being studied. eDNA metabarcoding also possesses the potential to replace traditional sampling methods, especially in situations with short field seasons, limited field workers, or difficult-to-sample habitats for existing monitoring programs. However, I believe that a simplified eDNA field collection method will need to be developed for broad usage in monitoring programs, like CAMP, that utilize volunteers.

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Appendices

Appendix A

Appendix A.1:Table of results for a two-factor crossed PERMANOVA for fish counts separated into Adults and Young-of-the-Year collected in four estuaries in Prince Edward Island, Canada during summer of 2021. Months had two levels (June, August) and Stations had three levels (10 %/inner estuary, 50 %/middle estuary, 100 %/outer estuary). Estuaries were analysed independently. Statistically significant differences in factor centroids were found between the Months, the Stations, and their interactions. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	22870	22870	35.808	**0.0001**	9938
Station	2	8553.4	4276.7	6.696	**0.0001**	9931
Mo X St	2	5650.4	2825.2	4.4234	**0.0011**	9939
Residuals	28	17883	638.69			
Total	33	55573				

B. Freeland Creek

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	43385	43385	178.68	**0.0001**	9937
Station	2	8575.1	4287.6	17.658	**0.0001**	9927
Mo X St	2	4817.5	2408.8	9.9206	**0.0001**	9943
Residuals	29	7041.4	242.81			
Total	34	64376				

C. Dunk River

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	37447	37447	48.616	**0.0001**	9946
Station	2	8523.9	4261.9	5.5331	**0.0001**	9945
Mo X St	2	8676.5	4338.2	5.6322	**0.0001**	9945
Residuals	30	23108	770.26			
Total	35	77756				

D. Enmore River

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	15110	15110	23.892	**0.0001**	9949
Station	2	17560	8779.8	13.882	**0.0001**	9936
Mo X St	2	15160	7580.1	11.985	**0.0001**	9943
Residuals	30	18973	632.44			
Total	35	66803				

Appendix A.2: Table of results for a two-factor crossed PERMANOVA for fish counts separated into Adults and Young-of-the-Year collected in three estuaries in Prince Edward Island, Canada during the August 2020 and August 2021. Year had two levels (August 2020, August 2021) and Stations had three levels (10 %/inner estuary, 50 %/middle estuary, 100 %/outer estuary). Estuaries were analysed independently. Statistically significant differences in factor centroids were found between the Year, the Stations, and their interactions. Square root transformations, Bray Curtis, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	4198.3	4198.3	6.2531	**0.0003**	9957
Station	2	12920	6460.1	9.622	**0.0001**	9937
Ye X St	2	3000.4	1500.2	2.2345	**0.0184**	9944
Residuals	26	17456	671.39			
Total	31	38108				

B. Freeland Creek

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	16257	16257	37.876	**0.0001**	9942
Station	2	16235	8117.6	18.913	**0.0001**	9951
Ye X St	2	12154	6077	14.158	**0.0001**	9916
Residuals	27	11589	429.22			
Total	32	55467				

C. Dunk River

Source	DF	Sums of Squares	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	10851	10851	16.78	**0.0001**	9942
Station	2	11092	5546.1	8.5765	**0.0001**	9937
Ye X St	2	5039.6	2519.8	3.8966	**0.0001**	9937
Residuals	27	17460	646.67			
Total	32	43836				

Appendix A.3: Pairwise comparisons of the MonthxStation interactions across the stations' average fish assemblage. Fish counts were separated into Adults and Young-of-the-Year from beach seine samples collected in June and August in Prince Edward Island, Canada during 2021. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June	10%, 50%	9	2.543	0.019	461	**0.0101**
	10%, 100%	9	3.1729	0.0015	462	**0.0011**
	50%, 100%	10	1.4407	0.1084	461	0.1313
August	10%, 50%	9	2.5013	0.0075	461	**0.0049**
	10%, 100%	9	2.9451	0.002	462	**0.0012**
	50%, 100%	10	1.7097	0.0063	462	**0.0369**

B. Freeland Creek

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June	10%, 50%	9	3.0715	0.0023	462	**0.0014**
	10%, 100%	9	2.948	0.0027	462	**0.0013**
	50%, 100%	10	3.833	0.0016	462	**0.0002**
August	10%, 50%	10	2.4389	0.0027	462	**0.004**
	10%, 100%	10	4.1771	0.0017	462	**0.0002**
	50%, 100%	10	4.4744	0.0025	461	**0.0001**

C. Dunk River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June	10%, 50%	10	3.8898	0.0018	461	**0.0003**
	10%, 100%	10	3.1349	0.0027	462	**0.0008**
	50%, 100%	10	2.1297	0.0105	462	**0.0249**
August	10%, 50%	10	1.1533	0.2726	462	0.2652
	10%, 100%	10	1.8118	0.0099	462	**0.0259**
	50%, 100%	10	1.7192	0.019	462	**0.0435**

D. Enmore River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June	10%, 50%	10	4.4693	0.0021	462	**0.0001**
	10%, 100%	10	4.3215	0.0025	462	**0.0001**
	50%, 100%	10	2.9945	0.0018	462	**0.0008**
August	10%, 50%	10	3.4302	0.0035	461	**0.0005**
	10%, 100%	10	4.7219	0.0036	462	**0.0001**
	50%, 100%	10	3.8405	0.0026	461	**0.0002**

Appendix A.4: Pairwise comparisons of the YearXStation interaction across the stations' average fish assemblage. Fish counts were separated into Adults and Young-of-the-Year from beach seine samples collected in August in Prince Edward Island, Canada during 2020 and 2021. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020	10%, 50%	8	3.3584	0.0082	126	**0.0007**
	10%, 100%	8	2.5347	0.0075	126	**0.0052**
	50%, 100%	8	2.3522	0.0162	126	**0.0097**
August 2021	10%, 50%	9	2.5013	0.0074	461	**0.0061**
	10%, 100%	9	2.9451	0.0022	462	**0.0012**
	50%, 100%	10	1.7097	0.0071	461	**0.0406**

B. Freeland Creek

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020	10%, 50%	8	4.1553	0.0099	126	**0.0001**
	10%, 100%	8	3.0338	0.007	126	**0.0021**
	50%, 100%	8	4.6923	0.0081	126	**0.0001**
August 2021	10%, 50%	10	2.4389	0.002	462	**0.0051**
	10%, 100%	10	4.1771	0.0024	462	**0.0001**
	50%, 100%	10	4.4744	0.0026	461	**0.0002**

C. Dunk River

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020	10%, 50%	8	3.57	0.009	126	**0.0011**
	10%, 100%	8	7.0119	0.0065	126	**0.0001**
	50%, 100%	8	2.2825	0.009	126	**0.0164**
August 2021	10%, 50%	10	1.1533	0.2638	462	0.2685
	10%, 100%	10	1.8118	0.009	462	**0.0256**
	50%, 100%	10	1.7192	0.0168	461	**0.0398**

Appendix A.5: Pairwise comparisons of the MonthxStation interactions between months' average nearshore fish assemblage. Fish counts were separated into Adults and Young-of-the-Year from beach seine samples collected in June and August in Prince Edward Island, Canada during 2021. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June, August	10%	8	4.4361	0.0074	126	**0.0002**
	50%	10	3.6181	0.0017	462	**0.0003**
	100%	10	4.0825	0.0024	462	**0.0001**

B. Freeland Creek

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June, August	10%	9	7.4775	0.003	462	**0.0002**
	50%	10	9.096	0.002	462	**0.0001**
	100%	10	7.9651	0.0031	462	**0.0001**

C. Dunk River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June, August	10%	10	4.1464	0.0029	462	**0.0002**
	50%	10	5.0085	0.0025	462	**0.0001**
	100%	10	4.2356	0.0025	462	**0.0001**

D. Enmore River

Month	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
June, August	10%	10	4.4693	0.0021	462	**0.0001**
	50%	10	4.3215	0.0025	462	**0.0001**
	100%	10	2.9945	0.0018	462	**0.0008**

Appendix A.6: Pairwise comparison of the YearXStation interaction between years' average fish assemblage. Fish counts were separated into Adults and Young-of-the-Year from beach seine samples collected in August in Prince Edward Island, Canada during 2020 and 2021. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). *S*ignificant values (P(MC)<0.05*)* are bolded.

A. Wheatley River

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020, August 2021	10%	8	2.132	0.0144	126	**0.016**
	50%	9	2.1859	0.0105	462	**0.0127**
	100%	9	1.6055	0.0675	462	0.0702

B. Freeland Creek

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020, August 2021	10%	9	4.4844	0.0027	462	**0.0004**
	50%	9	3.4856	0.0022	462	**0.0004**
	100%	9	5.2112	0.0025	462	**0.0001**

C. Dunk River

Year	Station	DF	t-score	P(perm)	Unique Perms	P(MC)
August 2020, August 2021	10%	9	4.2141	0.002	462	**0.0001**
	50%	9	1.5798	0.0195	462	0.0601
	100%	9	3.2466	0.0024	462	**0.0005**

Appendix A.7: Results from Distance-Based Linear Models (DISTLM) finding relationships between measured environmental variables and fish assemblages from beach seine samples collected in June and August in Prince Edward Island, Canada during 2021. Fish counts were separated into Adults and Young-of-the-Year. Selection Criteria: AIC, Selection Procedure: Best, 9999 Permutations. Note: Z. marina coverage was removed from Dunk River as it was never seen at this estuary. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Marginal Test

Total SS(trace)= 55573 Residual.df:32

Variable	**SS(trace)**	**Pseudo-F**	**P(perm)**	**Proportion**
1.Temperature °C	19784	17.69	**0.0001**	0.356
2.Sqr(Salinity (PPT))	5370.5	3.4232	**0.0138**	0.096638
3.DO (mg/l)	5436.8	3.4701	**0.0128**	0.097831
4.Log(Ulva sp.(%)+1)	4478.6	2.8049	**0.0321**	0.08059
5.Log(Z. marina(%)+1)	7366.8	4.8902	**0.0038**	0.13256
6.Log(Sediment(%)+1)	6971.6	4.5902	**0.0053**	0.12545

Overall Best Solution

AIC	**R^2**	**RSS**	**No.Var**	**Selection**
231.32	0.56438	24209	3	1,5,6

B. Freeland Creek

Total SS(trace)= 64376 Residual.df:33

Variable	**SS(trace)**	**Pseudo-F**	**P(perm)**	**Proportion**
1.Temperature °C	37553	46.199	**0.0001**	0.58333
2.Sqr(Salinity (PPT))	3742.5	2.0369	0.1241	0.058135
3.DO (mg/l)	15774	10.71	**0.0001**	0.24502
4.Log(Ulva sp.(%)+1)	8694.3	5.1527	**0.0117**	0.13505
5.Log(Z. marina(%)+1)	8463.2	4.995	**0.0086**	0.13146
6.Log(Sediment(%)+1)	6618.8	3.7817	**0.0223**	0.10281

AIC	**R^2**	**RSS**	**No.Var**	**Selection**
220.04	0.80411	12611	6	All

Appendix A.7 continued: Results from Distance-Based Linear Models (DISTLM) finding relationships between measured environmental variables and fish assemblages from beach seine samples collected in June and August in Prince Edward Island, Canada during 2021. Fish counts were separated into Adults and Young-of-the-Year. Selection Criteria: AIC, Selection Procedure: Best, 9999 Permutations. Note: Z. marina coverage was removed from Dunk River as it was never seen at this estuary. Significant values (P(perm)<0.05) are bolded.

C. Dunk River

Marginal Test

Total SS(trace)= 77756 Residual.df:34

Variable	**SS(trace)**	**Pseudo-F**	**P(perm)**	**Proportion**
1.Temperature °C	4702	2.1883	0.0827	0.060471
2.Sqr(Salinity (PPT))	5850.7	2.7665	**0.04**	0.075245
3.DO (mg/l)	13875	7.3848	**0.0005**	0.17844
4.Log(Ulva sp.(%)+1)	10922	5.5562	**0.0023**	0.14046
5.Log(Z. marina(%)+1)				
6.Log(Sediment(%)+1)	3779.8	1.7373	0.1385	0.048612

Overall Best Solution

AIC	**R^2**	**RSS**	**No.Var**	**Selection**
266.74	0.45207	42605	5	1-4,6

D. Enmore River

Total SS(trace)=66803 Residual.df:34

Variable	**SS(trace)**	**Pseudo-F**	**P(perm)**	**Proportion**
1.Temperature °C	8694.3	5.0871	**0.0008**	0.13015
2.Sqr(Salinity (PPT))	13259	8.4193	**0.0001**	0.19848
3.DO (mg/l)	14598	9.5075	**0.0001**	0.21853
4.Log(Ulva sp.(%)+1)	2968.9	1.5813	0.1572	0.044443
5.Log(Z. marina(%)+1)	4128	2.2393	0.0559	0.061793
6.Log(Sediment(%)+1)	6230.7	3.4974	**0.0089**	0.093269

AIC	**R^2**	**RSS**	**No.Var**	**Selection**
258.95	0.45709	36268	4	1-3,6

Appendix A.8: Results from Distance-Based Linear Models (DISTLM) finding relationships between measured environmental variables and fish assemblages from beach seine samples collected in August in Prince Edward Island, Canada during 2020 and 2021. Fish counts were separated into Adults and Young-of-the-Year. Selection Criteria: AIC, Selection Procedure: Best, 9999 Permutations. Note: Z. marina coverage was removed from Dunk River as it was never seen at this estuary. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Marginal Test

Total SS(trace)= 38108 Residual.df:30

Variable	SS(trace)	Pseudo-F	P(perm)	Proportion
1.Temperature °C	7735.5	7.6408	**0.0001**	0.20299
2.Sqr(Salinity (PPT))	6705	6.4055	**0.0002**	0.17595
3.DO (mg/l)	3296.5	2.8409	**0.0238**	0.086505
4.Log(Ulva sp.(%)+1)	4230	3.7458	**0.006**	0.111
5.Log(Z. marina(%)+1)	8565.5	8.6983	**0.0001**	0.22477
6.Log(Sediment(%)+1)	6047.9	5.6593	**0.0005**	0.15871

Overall Best Solution

AIC	R^2	RSS	No.Var	Selection
217.44	0.45121	20913	4	1,2,4,5

B. Freeland Creek

Total SS(trace)=55467 Residual.df:31

Variable	SS(trace)	Pseudo-F	P(perm)	Proportion
1.Temperature °C	7819.1	5.0872	**0.0024**	0.14097
2.Sqr(Salinity (PPT))	4891.2	2.998	**0.0255**	0.088183
3.DO (mg/l)	9971.7	6.7947	**0.0004**	0.17978
4.Log(Ulva sp.(%)+1)	8588.2	5.6793	**0.0013**	0.15484
5.Log(Z. marina(%)+1)	18054	14.959	**0.0001**	0.32549
6.Log(Sediment(%)+1)	6087.7	3.8218	**0.0105**	0.10975

AIC	R^2	RSS	No.Var	Selection
225.16	0.59627	22394	4	1, 3-5

Appendix A.8 continued: Results from Distance-Based Linear Models (DISTLM) finding relationships between measured environmental variables and fish assemblages from beach seine samples collected in August in Prince Edward Island, Canada during 2020 and 2021. Fish counts were separated into Adults and Young-of-the-Year. Selection Criteria: AIC, Selection Procedure: Best, 9999 Permutations. Note: Z. marina coverage was removed from Dunk River as it was never seen at this estuary. Significant values (P(perm)<0.05) are bolded.

A. Dunk River

Marginal Test				
Total SS(trace)=43836		Residual.df:31		
Variable	**SS(trace)**	**Pseudo-F**	**P(perm)**	**Proportion**
1.Temperature °C	8736.6	7.7163	**0.0001**	0.1993
2.Sqr(Salinity (PPT))	6277.3	5.1811	**0.0013**	0.1432
3.DO (mg/l)	1681	1.2362	0.2862	0.038349
4.Log(Ulva sp.(%)+1)	5386.5	4.3429	**0.0038**	0.12288
5.Log(Z. marina(%)+1)				
6.Log(Sediment(%)+1)	2408.2	1.802	0.1117	0.054936

Overall Best Solution				
AIC	**R^2**	**RSS**	**No.Var**	**Selection**
225.33	0.48085	22757	5	1-4,6

Appendix A.9: One-factor ANOSIM results for comparison of differences between the nearshore fish assemblages of the entirety of the three estuaries sampled in August 2020 and four estuaries sampled in August 2021 in Prince Edward Island, Canada. Estuary Levels; Wheatley River, Freeland Creek, Dunk River, Enmore River, unordered. Correlation method: Spearman Rank. 9999 permutations if possible. Significant values (P(perm)<0.05) are bolded.

A. Whole estuary combined August 2021

Global Test

Sig. level of sample statistic: **0.0001**	Sample statistic (R): 0.59	Number of permutations: 9999	Number of permuted statistics greater than or equal to R: 0

Pairwise Tests

	R Statistic	**Sig. Level (P(perm))**	**Possible Perms**	**Actual Perms**	**# > observed**
Dunk, Enmore	0.264	**0.0003**	Very large	9999	2
Dunk, Wheatley	0.81	**0.0001**	Very large	9999	0
Dunk, Freeland	0.922	**0.0001**	Very large	9999	0
Enmore, Wheatley	0.532	**0.0001**	Very large	9999	0
Enmore, Freeland	0.68	**0.0001**	Very large	9999	0
Wheatley, Freeland	0.323	**0.0001**	Very large	9999	0

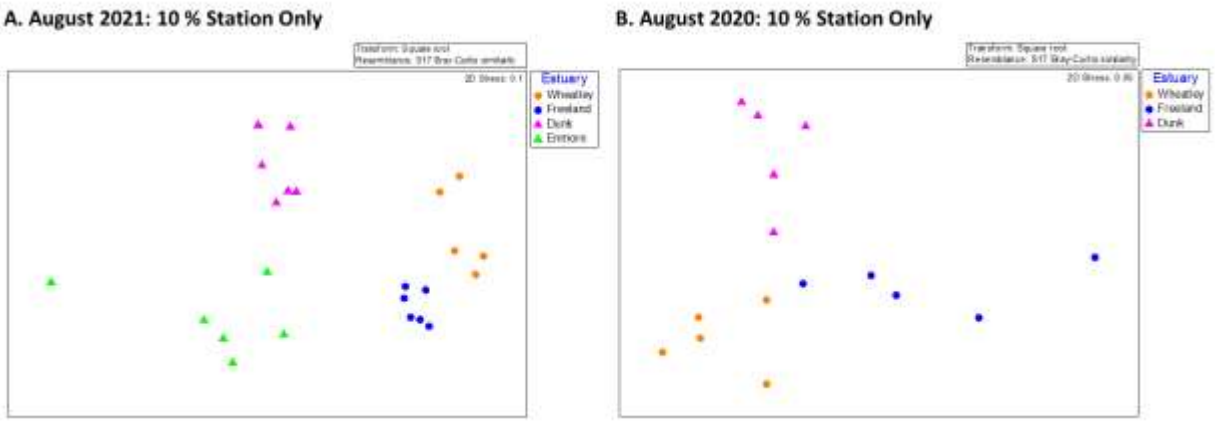
B. Whole estuary combined August 2020

Global Test

Sig. level of sample statistic: **0.0001**	Sample statistic (R): 0.308	Number of permutations: 9999	Number of permuted statistics greater than or equal to R: 0

Pairwise Tests

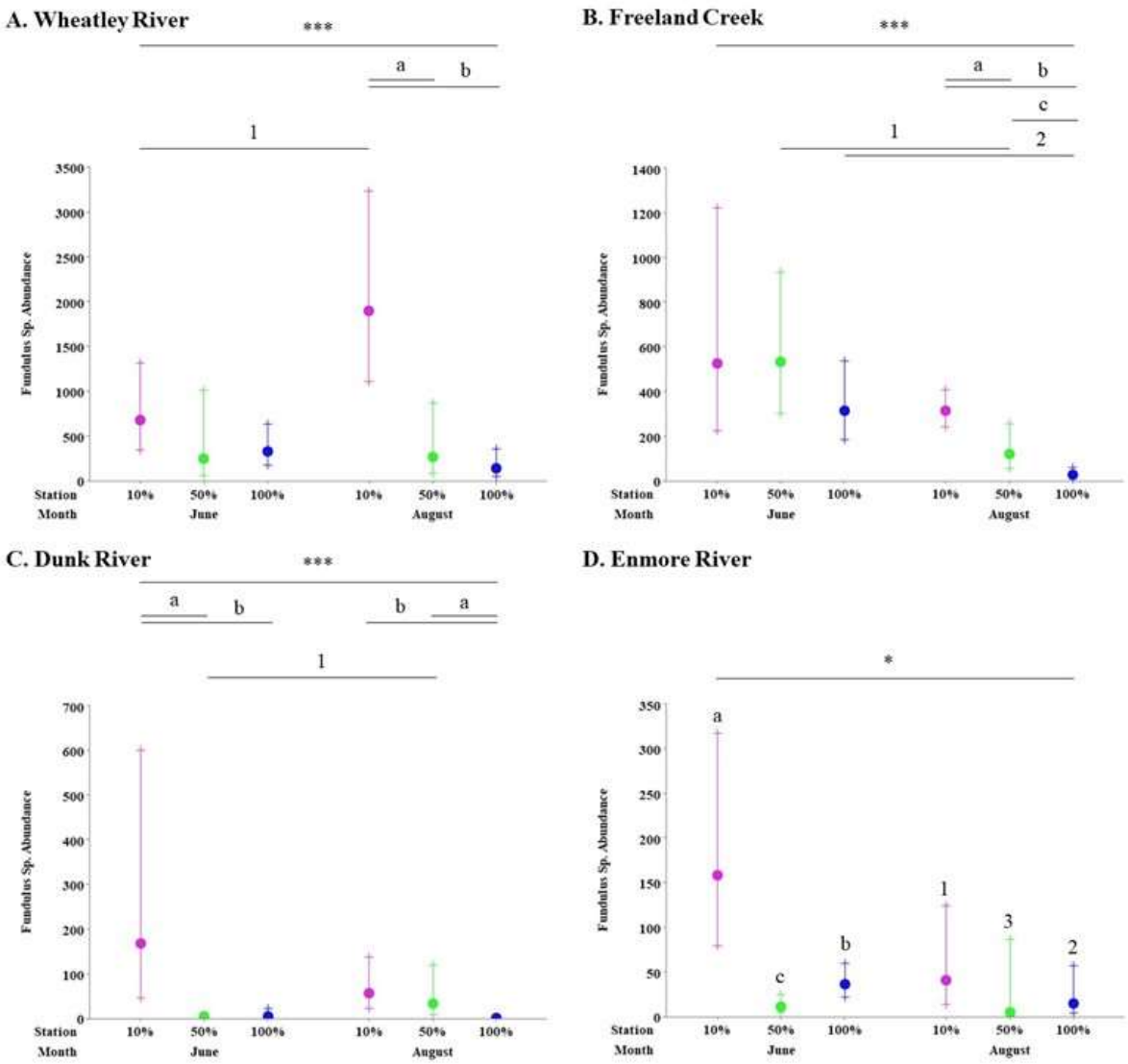
	R Statistic	**Sig. Level (P(perm))**	**Possible Perms**	**Actual Perms**	**# > observed**
Wheatley, Freeland	0.233	**0.001**	77558760	9999	10
Wheatley, Dunk	0.437	**0.0001**	77558760	9999	0
Freeland, Dunk	0.244	**0.0009**	77558760	9999	8



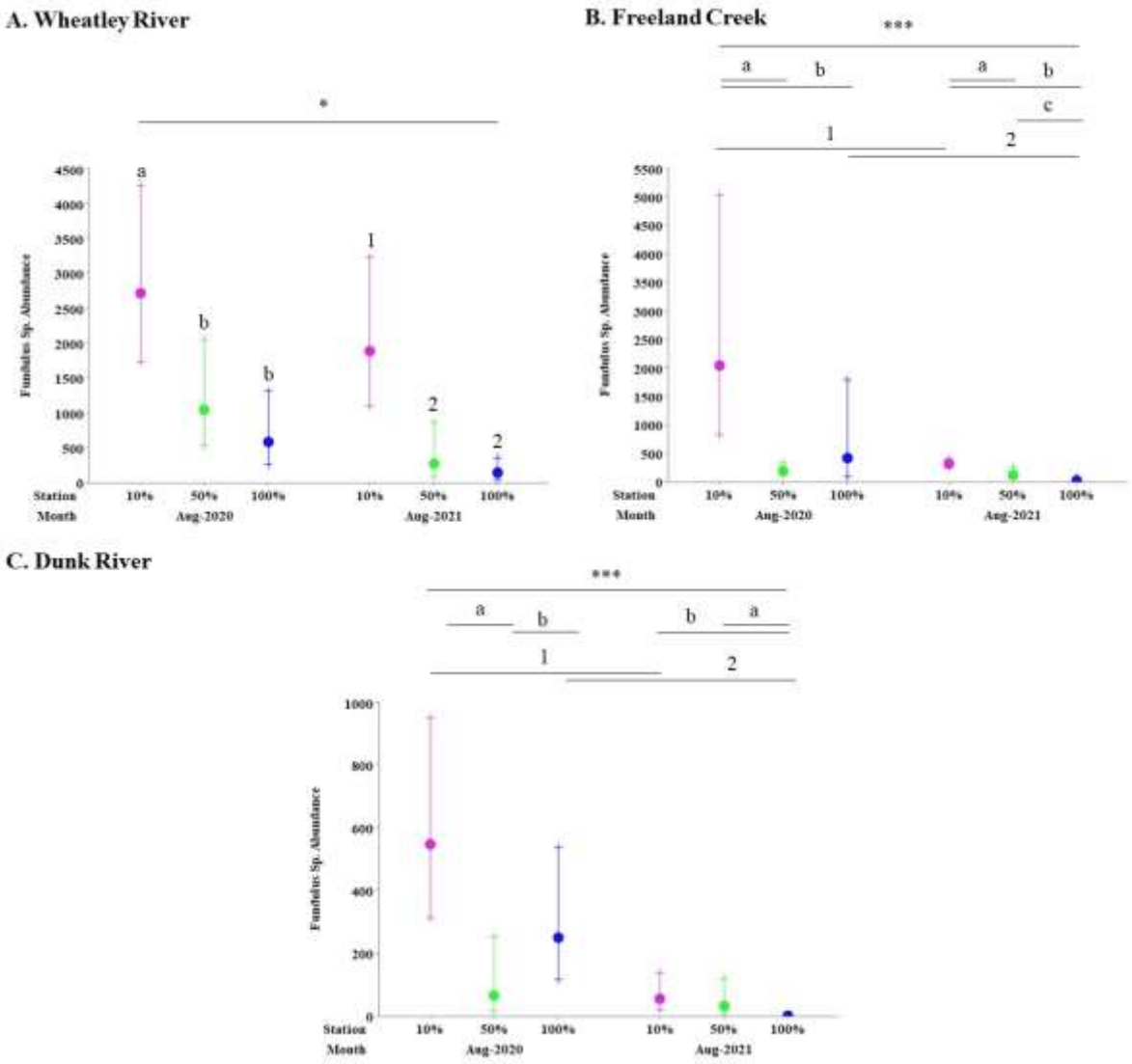
Appendix A.10: nMDS plots comparing the multivariate data cloud of A) differences between the nearshore fish assemblages of the 10 % station (inner region) of the four estuaries sampled in August 2021, and B) differences between the nearshore fish assemblages of the 10 % station of the three estuaries sampled in August 2020 with fish abundance separated by adults and young-of-the-year (Adult-vs-YOY). Circles represented estuaries collected on the north shore, while triangles represented estuaries collected on the south shore. Stress was found to be at or below 0.1, indicating most images offer a good representation of each data cloud's shape. Image was not included in **Chapter 3** as these nMDS plots showed similar patterns when all three stations (10 % , 50 % , and 100 %) were pooled together, indicating a separation between north shore estuaries and south shore estuaries at all three stations. Square root transformations, Bray-Curtis similarity, 100 restarts. Images created using PRIMER-E V7 multivariate statistical program (2021 PRIMER-E ltd, Plymouth, UK).

```
Family: Tweedie(p=1.709)
Link function: log

Formula:
Fundulus.sp. ~ s(Stationnum, bs = "re") + s(log_ulva, bs = "tp",
    k = 9) + s(Temperature, bs = "tp", k = 9) + s(sqrt_sal,
    bs = "tp", k = 9) + s(log_sed, bs = "tp", k = 9) +
    s(DO.1, bs = "tp", k = 9) + s(log_Z, bs = "tp",
    k = 9) + s(Estuary, bs = "re") + Month + Year

Parametric coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 3056.4622   342.1011   8.934 5.37e-16 ***
Month8        -0.6172     0.1852  -3.334  0.00105 **
Year          -1.5098     0.1693  -8.919 5.90e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:
                   edf Ref.df      F p-value
s(Stationnum)  1.8787829      2 24.826 < 2e-16 ***
s(log_ulva)    1.1666277      8 18.954 0.00325 **
s(Temperature) 0.2687853      8  0.044 0.36048
s(sqrt_sal)    0.0011391      8  0.000 0.61470
s(log_sed)     0.0016975      8  0.000 0.28826
s(DO.1)        0.5576475      8  0.408 0.13810
s(log_Z)       0.0003006      8  0.000 0.58096
s(Estuary)     2.9273912      3 52.809 < 2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

R-sq.(adj) =  0.474   Deviance explained = 65.8%
-REML = 1180.2  Scale est. = 3.9685    n = 186
```

Appendix A.11: Output from the generalized additive model (Wood et al. 2016) that examined differences in the abundances of Fundulus species abundances between months and estuaries and whether measured environmental parameters (i.e., temperature, dissolved oxygen, salinity, % coverage of Ulva species, % coverage of Z. marina, and % coverage of sediment) had a linear of non-linear relationship with Fundulus species abundances, from August 2020, and June and August 2021. The basis size, dispersion of residuals, homogeneity of variance, and the relationship between the observed and predicted response was assessed to verify that model assumptions were not violated. To help achieve a near normal distribution, the following parameters were transformed: Ulva, Z. marina, and sediment % coverage were natural logarithm (log (x+1)) transformed; Salinity was square root transformed; DO (mg/L), and temperature had no transformations. Fundulus species abundances were assumed to be tweedie distributed (poisson-like, discrete distribution with zeroes). The model was run in R (version 4.0.5)(R Core Team 2021) using the mgcv (v. 1.8.39)(see), and model outputs were visualized using ggplot2 (v. 3.3.5), wplot (v. 1.1.1), gratia (v. 0.7.0) (Wickham 2009; Wood et al. 2016).



Appendix A.12: Mean *Fundulus* species abundances per seine net (n=5-6) for the four estuaries sampled in June and August in summer 2021. Error bars represent upper and lower 95% confidence intervals. All means and error bars were back transformed from their natural logarithm. Statistical significances for Wheatley River, Freeland Creek, and Dunk River are shown as follows: Two-factor ANOVA significant MonthXStation interactions (***), Tukey test results from one-factor ANOVAs performed on each month for estuaries with significant MonthXStation interactions (a,b,c), and two-sample t-tests comparing changes between months at the same station (1,2). Enmore River had no significant MonthXStation interaction. Tukey tests suggest Enmore River significantly differed between months (*) and stations (June: a,b,c; August 1,2,3). Image was not included in **Chapter 3** as the general trend, that the 10 % station had the highest mean *Fundulus* species abundance, was captured in the generalized additive model. Images created using MiniTab® Statistical Software, V 21.1 (2021 Minitab, LLC, 64-Bits).



Appendix A.13: Mean *Fundulus* species abundances per seine net (n=5-6) for the three estuaries sampled in August 2020 and August 2021. Error bars represent upper and lower 95% confidence intervals. All means and error bars were back transformed from their natural logarithm. Statistical significances for Freeland Creek and Dunk River are shown as follows: Two-factor ANOVA significant YearXStation interactions (***), Tukey test results from one-factor ANOVAs performed on each year for estuaries with significant YearXStation interactions (a,b,c), and two-sample t-tests comparing changes between years at the same station (1,2). Wheatley River had no significant YearXStation interaction. Tukey tests suggest Wheatley River significantly differed between years (*) and stations (June: a,b,c; August 1,2,3). Image was not included in **Chapter 3** as the general trend, that the 10 % station had the highest mean *Fundulus* species abundance, was captured in the generalized additive model. Images created using MiniTab® Statistical Software, V 21.1 (2021 Minitab, LLC, 64-Bits).

Appendix A.14: One-factor ANOVAs comparing the natural logarithm transformed mean abundance of Fundulus per net caught throughout the estuaries sampled in August 2021 (A) and August 2020 (B). In August 2021, four were sampled (Wheatley River, Freeland Creek, Dunk River, and Enmore River), while in August 2020, three estuaries were sampled (Wheatley River, Freeland Creek, and Dunk River),. Performed in Minitab® 21.1.

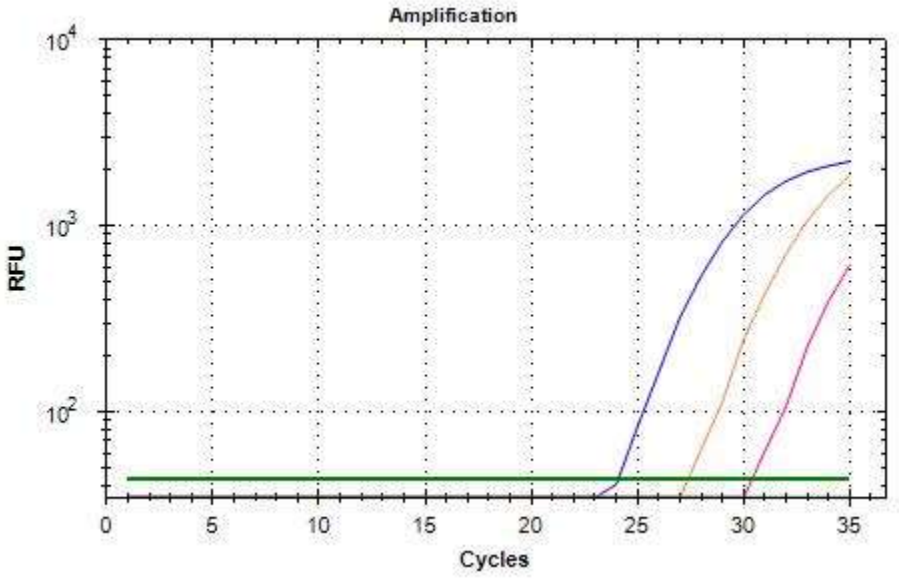
A. One-factor ANOVA: August 2021

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Estuary	3	50.89	16.9647	31.21	**0.000**
Error	19	10.33	0.5436		
Total	22	61.22			
R^2 % 83.13%			**R^2(Adjusted)** 80.47%		

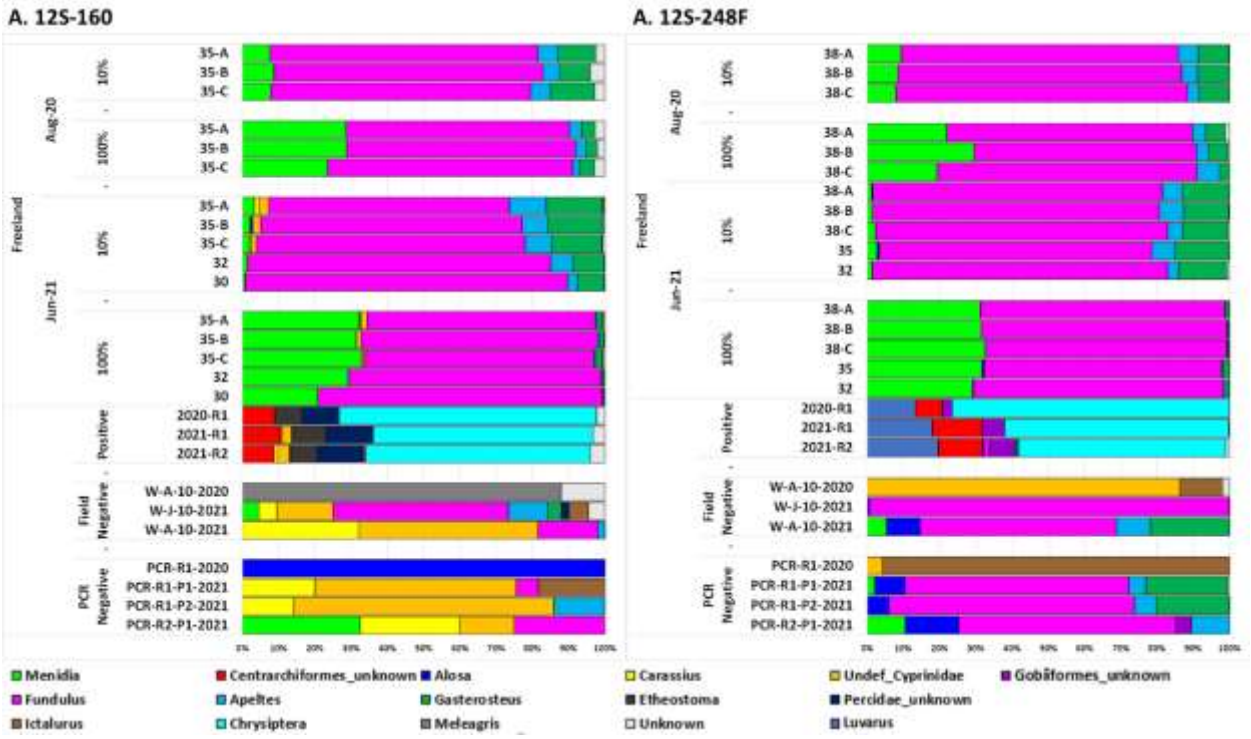
B. One-factor ANOVA: August 2020

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Estuary	2	7.308	3.6542	12.79	**0.001**
Error	12	3.428	0.2857		
Total	14	10.736			
R^2 68.07%			**R^2(Adjusted)** 62.75%		

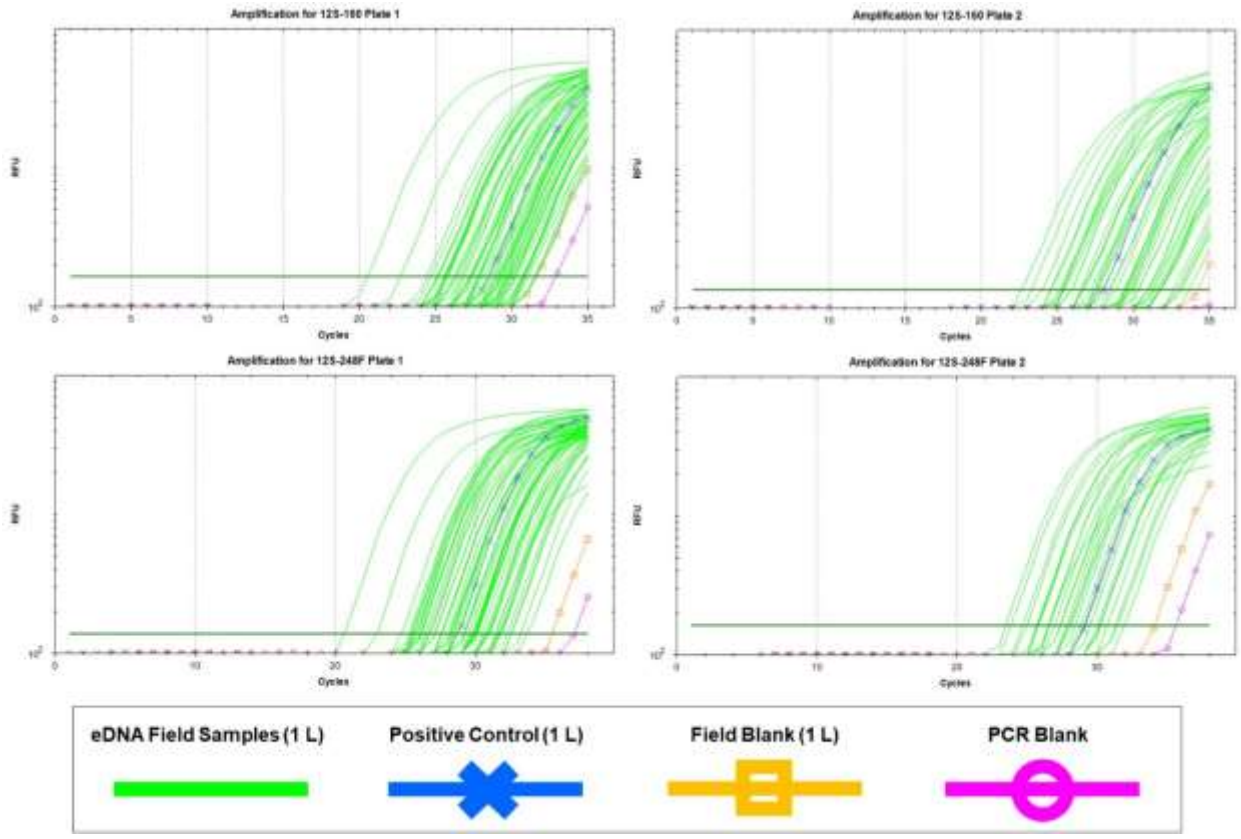
Appendix B



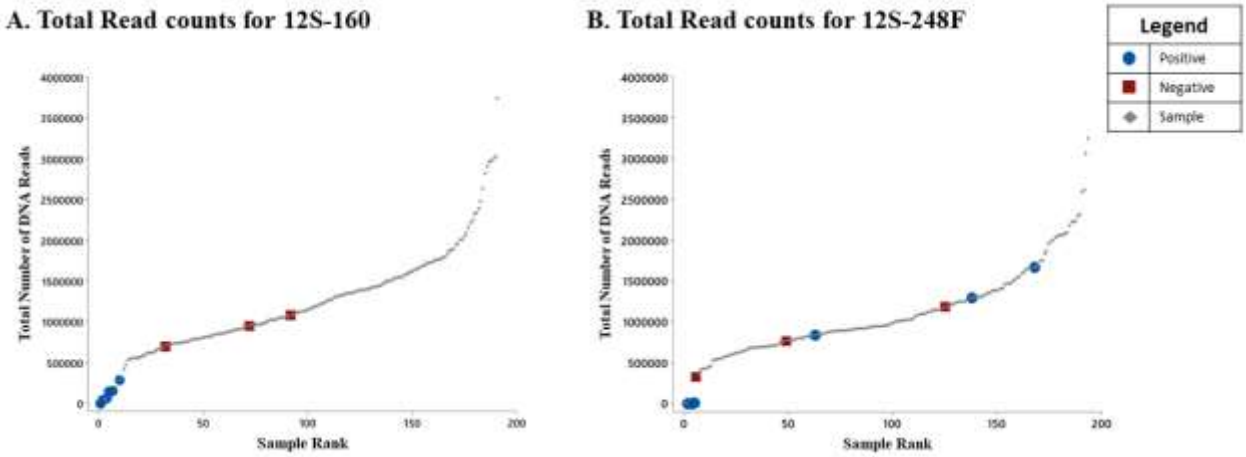
Appendix B.1: 2020 dilution test. Blue represents undiluted sample (DJ101), orange represents 10X dilution, magenta represents 100X, green represents negative control. CT values shift about 3.3 cycles for every 10-fold dilution indicates there is no inhibition present. Other dilutions showed similar results.



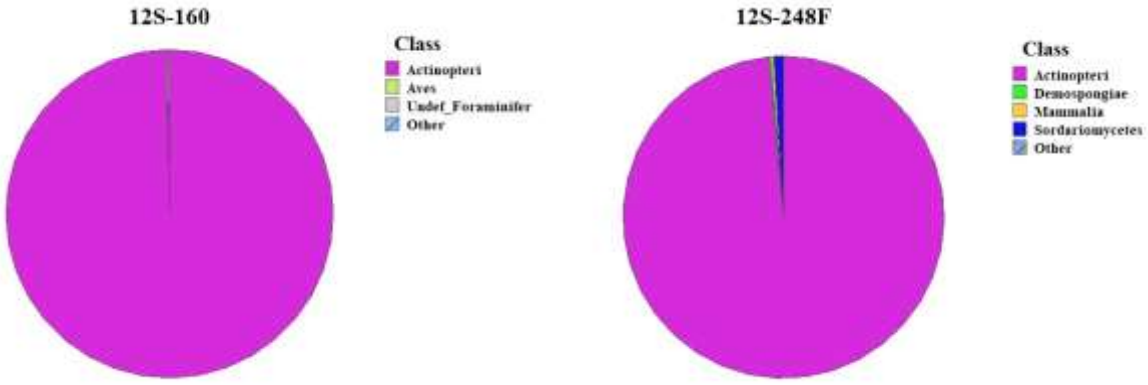
Appendix B.2: Proportion plots for PCR replicate (A,B,C), replicates from reduced PCR cycles (12S-160: 30, 32; 12S-248F: 32, 35), positive controls, and negative controls (Field Blanks and PCR blanks) to visualize read composition of raw read sequences (i.e. unrarefied and not sorted to 10^{-5}) of the 12S-160 and 12S-248F eDNA metabarcoding primer sets. PCR replicates indicate that PCR samples are consistently reproducible for both 12S metabarcoding primer sets. Reducing PCR cycles for 12S-248F (35 and 32 cycles) showed little change from the full cycle (38 cycles), indicating that cycle number minimally influenced the results. When examining the 12S-160, reducing PCR cycles (32 and 30 cycles) resulted in the failure to detect Cyprinidae (likely Goldfish (*Carassius auratus*)), which the full 35 cycles frequently detected. Possible explanations include cycle reduction reduces the chance rare taxa are amplified, higher cycle thresholds result in higher abundances of contaminates or artefactual sequences, or the higher cycle samples could have been contaminated while the lower cycle rounds were not, as the PCR reactions occurred at separate times.



Appendix B.3: RT-qPCR amplification curves (log scale) for Round 1 products for the 12S-160 and 12S-248F eDNA metabarcoding primer sets. Field samples, from collecting 1 L of water at an estuary, amplified before both types of negative control, which came out later than field samples, providing evidence that there were initially very low DNA concentrations present in the negatives, especially in 12S-248F's case. Lower Ct experienced in 12S-160 is likely a result of Goldfish DNA contaminating the negatives and some field samples. Positive controls were created from 1 L from a tropical aquarium, containing exotic fish not known to be present in PEI estuaries.



Appendix B.4: Total number of DNA sequence reads per sample ranked (from left to right) lowest to highest for all samples collected between August 2020, and June and August 2021 across four estuaries in Prince Edward Island, Canada. Two 12S metabarcoding primer sets were sequenced: A) the 12S-160 primer set (field samples and replicates n=181, positive controls n=3, negative controls n=7) and the B) 12S-248F primer set (field samples and replicates n=184, positive controls n=3, negative controls n=7). The samples were sequenced on a Novaseq 6000 (2 X 150bp) with 10 % PhiX spike-in for base diversity. The negatives from the 12S-160 primer set are relatively low compared to all field samples and positive controls, indicating low contamination. Three negatives from the 12S-248F primer set (PCR negative: PCR-R1-P2-2021=1,668,032; Field Controls: W-A-10-2021=1,294,070, W-J-10-2021=836,627) have a relatively high number of DNA reads in comparison to the field and positive controls, indicating a higher level of contamination present across these samples.



Appendix B.5: Read proportions of all samples and all reads without any taxon filtering from four estuaries (Wheatley River, Freeland Creek, Dunk River, Enmore River) in Prince Edward Island, Canada, during August 2020, June 2021, and August 2021. Negative controls, positive controls, and replicates were included. Actinopterygii comprised 99.7 % of 12S-160's reads and 98.5 % of 12S-248F's reads. Image created in Minitab® 21.1 (64-bit).

Appendix B.6: Table of results for a two-factor crossed PERMANOVA for comparing the fish assemblage composition detected by eDNA metabarcoding and beach seining in each of four estuaries in Prince Edward Island, Canada between June and August during the summer of 2021. Month had two levels (June, August), and Methods had three levels (12S-160, 12S-248F, Beach Seine). Estuaries were analysed independently. Statistically significant differences in factor centroids were found between Month, Methods, and their interactions, except at Wheatley River, where significance was found only between Moth and Method. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	5094.3	5094.3	14.16	**0.0001**	9937
Method	2	4920.2	2460.1	6.8382	**0.0001**	9947
MoxMe	2	1295.4	647.72	1.8004	0.1	9947
Residuals	85	30580	359.76			
Total	90	42021				

B. Freeland Creek

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	9650.6	9650.6	25.718	**0.0001**	9956
Method	2	10088	5043.9	13.442	**0.0001**	9942
MoxMe	2	3083.5	1541.8	4.1087	**0.0009**	9939
Residuals	89	33397	375.24			
Total	94	56685				

C. Dunk River

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	10835	10835	13.069	**0.0001**	9966
Method	2	34607	17303	20.87	**0.0001**	9921
MoxMe	2	10008	5004.2	6.0357	**0.0001**	9940
Residuals	87	72132	829.1			
Total	92	1.2655E+05				

D Enmore River

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Month	1	4943.5	4943.5	9.6735	**0.0001**	9950
Method	2	12416	6208.2	12.148	**0.0001**	9943
MoxMe	2	5723.5	2861.7	5.5999	**0.0002**	9936
Residuals	90	45993	511.04			
Total	95	69168				

Appendix B.7: Table of results for a two-factor crossed PERMANOVA for comparing the fish assemblage composition detected by eDNA metabarcoding and beach seining in each of three estuaries in Prince Edward Island, Canada between August 2020 and August 2021. Year had two levels (August 2020, August 2021), and Methods had three levels (12S-160, 12S-248F, Beach Seine). Estuaries were analysed independently. Statistically significant differences in factor centroids were found between Year, Methods, and their interactions. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	2607.9	2607.9	7.1881	**0.0013**	9940
Method	2	2445.7	1222.8	3.3705	**0.0061**	9951
YexMe	2	2362.3	1181.1	3.2556	**0.0104**	9954
Residuals	83	30113	362.81			
Total	88	37492				

B. Freeland Creek

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	10888	10888	28.415	**0.0001**	9957
Method	2	2615.4	1307.7	3.4128	**0.0028**	9930
YexMe	2	8364	4182	10.914	**0.0001**	9951
Residuals	87	33337	383.18			
Total	92	55983				

C. Dunk River

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Year	1	18655	18655	26.04	**0.0001**	9958
Method	2	15436	7718.1	10.773	**0.0001**	9939
YexMe	2	4657	2328.5	3.2503	**0.0057**	9936
Residuals	85	60895	716.41			
Total	90	1.0058E+05				

Appendix B.8: A) Pairwise comparisons between Month for Wheatley River (no significant MonthxMethod interaction), and B-D) pairwise comparisons of the MonthxMethod interactions between Month (June, August) across Methods (12S-160, 12S-248F, Beach Seine) at Freeland Creek, Dunk River, and Enmore River in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
All	85	June, August	3.763	**0.0001**	9944	0.0001

B. Freeland Creek

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	28	June, August	3.2655	**0.0001**	9950	0.0001
12S-248F	28	June, August	2.5595	**0.0001**	9952	0.0011
Beach Seine	33	June, August	4.0829	**0.0001**	9942	0.0001

C. Dunk River

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	27	June, August	2.7906	**0.0002**	9942	0.0005
12S-248F	26	June, August	3.3499	**0.0001**	9956	0.0001
Beach Seine	34	June, August	2.3132	**0.005**	9950	0.0065

D. Enmore River

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	28	June, August	3.7056	**0.0001**	9957	0.0001
12S-248F	28	June, August	2.2371	**0.0048**	9945	0.0061
Beach Seine	34	June, August	2.4143	**0.0022**	9965	0.0026

Appendix B.9: Pairwise comparisons of the YearxMethod interactions between Year (August 2020, August 2021) across Methods (12S-160, 12S-248F, Beach Seine) at three estuaries in Prince Edward Island, Canada. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	26	2020, 2021	2.654	**0.001**	9965	0.0009
12S-248F	27	2020, 2021	2.0493	**0.0163**	9955	0.0181
Beach Seine	30	2020, 2021	1.8313	**0.0421**	9952	0.0412

B. Freeland Creek

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	28	2020, 2021	3.665	**0.0001**	9941	0.0001
12S-248F	28	2020, 2021	3.1644	**0.0001**	9946	0.0001
Beach Seine	31	2020, 2021	4.6636	**0.0001**	9957	0.0001

C. Dunk River

Method	DF	Month	t-score	P(perm)	Unique Perms	P(MC)
12S-160	28	2020, 2021	3.7415	**0.0001**	9946	0.0001
12S-248F	26	2020, 2021	2.8499	**0.0004**	9949	0.0005
Beach Seine	31	2020, 2021	3.2532	**0.0001**	9957	0.0001

Appendix B.10: Table of results for a two-factor crossed PERMANOVA for comparing the fish assemblage composition detected by eDNA metabarcoding and beach seining in each of four estuaries in Prince Edward Island, Canada during summer of 2021. Stations had three levels (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary), and Methods had three levels (12S-160, 12S-248F, Beach Seine). Estuaries were analysed independently by Month. Statistically significant differences in factor centroids were found between Stations, Methods, and their interactions. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River

A.1. June 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	2861.7	1430.9	7.4626	**0.0001**	9944
Method	2	3755.3	1877.7	9.7928	**0.0001**	9940
StxMe	4	3187.6	796.9	4.1562	**0.0001**	9923
Residuals	36	6902.6	191.74			
Total	44	16712				

A.2. August 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	5294.2	2647.1	11.52	**0.0001**	9956
Method	2	2236.2	1118.1	4.866	**0.0001**	9941
StxMe	4	3994.3	998.58	4.3458	**0.0002**	9934
Residuals	37	8501.8	229.78			
Total	45	20108				

B. Freeland Creek

B.1. June 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	7514.9	3757.5	23.082	**0.0001**	9937
Method	2	5967.7	2983.9	18.33	**0.0001**	9934
StxMe	4	1448.8	362.2	2.225	**0.0031**	9915
Residuals	38	6186	162.79			
Total	46	20986				

B.2. August 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	6349.7	3174.8	22.243	**0.0001**	9951
Method	2	7180	3590	25.152	**0.0001**	9942
StxMe	4	6386.1	1596.5	11.185	**0.0001**	9947
Residuals	39	5566.6	142.73			
Total	47	25632				

Appendix B.10 continued: Table of results for a two-factor crossed PERMANOVA for comparing the fish assemblage composition detected by eDNA metabarcoding and beach seining in each of four estuaries in Prince Edward Island, Canada during summer of 2021. Stations had three levels (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary), and Methods had three levels (12S-160, 12S-248F, Beach Seine). Estuaries were analysed independently by Month. Statistically significant differences in factor centroids were found between Stations, Methods, and their interactions. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

C. Dunk River

C.1. June 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	19722	9860.9	35.175	**0.0001**	9939
Method	2	28758	14379	51.292	**0.0001**	9946
StxMe	4	11855	2963.7	10.572	**0.0001**	9929
Residuals	38	10653	280.34			
Total	46	71080				

C.2. August 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	15250	7625	23.525	**0.0001**	9957
Method	2	15941	7970.6	24.591	**0.0001**	9950
StxMe	4	3609.8	902.44	2.7842	**0.0007**	9939
Residuals	37	11993	324.13			
Total	45	45787				

D. Enmore River

D.1. June 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
St	2	5005.5	2502.8	17.319	**0.0001**	9953
Me	2	9522.7	4761.4	32.948	**0.0001**	9947
StxMe	4	6363.8	1591	11.009	**0.0001**	9950
Res	39	5636	144.51			
Total	47	26416				

D.2. August 2021

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
St	2	9007.4	4503.7	19.864	**0.0001**	9954
Me	2	8617.2	4308.6	19.003	**0.0001**	9933
StxMe	4	10436	2609	11.507	**0.0001**	9927
Res	39	8842.6	226.73			
Total	47	37717				

Appendix B.11: Table of results for a two-factor crossed PERMANOVA for comparing the fish assemblage composition detected by eDNA metabarcoding and beach seining in each of three estuaries in Prince Edward Island, Canada during August 2020. Stations had three levels (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary), and Methods had three levels (12S-160, 12S-248F, Beach Seine). Estuaries were analysed independently by Month. Statistically significant differences in factor centroids were found between Stations, Methods, and their interactions. Square root transformations, Bray Curtis Similarity, 9999 Permutations, Type III (partial) Sums of Squares, fixed effects sum to zero for mixed terms. Significant values (P(perm)<0.05) are bolded.

A. Wheatley River August 2020

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	7015.5	3507.7	33.379	**0.0001**	9951
Method	2	2163.2	1081.6	10.292	**0.0001**	9947
StxMe	4	1961.6	490.41	4.6666	**0.0001**	9937
Residuals	34	3573	105.09			
Total	42	14805				

B. Freeland Creek August 2020

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	7669.5	3834.7	52.223	**0.0001**	9955
Method	2	3945.1	1972.5	26.863	**0.0001**	9956
StxMe	4	4572	1143	15.566	**0.0001**	9934
Residuals	36	2643.5	73.43			
Total	44	18830				

C. Dunk River August 2020

Source	DF	Sums of Sq.	Mean Sum	Pseudo-F	P(perm)	Unique Perms
Station	2	16834	8416.8	34.852	**0.0001**	9956
Method	2	4560.1	2280	9.4412	**0.0001**	9946
StxMe	4	5484.2	1371	5.6772	**0.0001**	9936
Residuals	36	8693.9	241.5			
Total	44	35572				

Appendix B.12: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) across Methods (12S-160, 12S-248F, Beach Seine) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River

A.1. June 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	2.4483	0.0063	126	**0.0056**
	8	10%, 100%	2.4126	0.0077	126	**0.0017**
	8	50%, 100%	1.6174	0.0335	126	0.0818
12S-248F	7	10%, 50%	2.8626	0.0067	126	**0.0042**
	7	10%, 100%	1.9217	0.0079	126	**0.0297**
	6	50%, 100%	1.9577	0.0281	35	0.0532
Beach Seine	9	10%, 50%	2.6424	0.0219	462	**0.0144**
	9	10%, 100%	3.8525	0.0034	462	**0.0013**
	10	50%, 100%	0.88427	0.4328	462	0.4368

A.2. August 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	7	10%, 50%	1.4547	0.0657	126	0.1193
	7	10%, 100%	6.4589	0.0077	126	**0.0001**
	8	50%, 100%	6.4484	0.0093	126	**0.0001**
12S-248F	8	10%, 50%	0.9829	0.4255	126	0.4252
	8	10%, 100%	5.5771	0.0079	126	**0.0001**
	8	50%, 100%	5.2518	0.0075	126	**0.0002**
Beach Seine	9	10%, 50%	2.3485	0.0258	462	**0.0221**
	9	10%, 100%	1.5034	0.1354	462	0.1283
	10	50%, 100%	1.8775	0.0495	462	**0.0433**

Appendix B.12 continued: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) across Methods (12S-160, 12S-248F, Beach Seine) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

B. Freeland Creek

B.1. June 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	0.49015	0.9529	126	0.8852
	8	10%, 100%	2.2812	0.0078	126	**0.0074**
	8	50%, 100%	4.4699	0.0086	126	**0.0001**
12S-248F	8	10%, 50%	1.0932	0.3386	126	0.3287
	8	10%, 100%	4.5593	0.0089	126	**0.0002**
	8	50%, 100%	5.5778	0.0079	126	**0.0002**
Beach Seine	9	10%, 50%	3.5731	0.002	462	**0.0008**
	9	10%, 100%	2.4433	0.0072	462	**0.0124**
	10	50%, 100%	4.2617	0.0022	462	**0.0002**

B.2. August 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	2.0183	0.0246	126	**0.0248**
	8	10%, 100%	3.6451	0.0069	126	**0.0007**
	8	50%, 100%	4.0208	0.007	126	**0.0008**
12S-248F	8	10%, 50%	2.0905	0.0395	126	**0.0238**
	8	10%, 100%	3.7753	0.0087	126	**0.0007**
	8	50%, 100%	4.4564	0.0079	126	**0.0004**
Beach Seine	10	10%, 50%	2.7293	0.0043	461	**0.005**
	10	10%, 100%	4.9735	0.0022	462	**0.0001**
	10	50%, 100%	5.4214	0.0022	462	**0.0001**

Appendix B.12 continued: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) across Methods (12S-160, 12S-248F, Beach Seine) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

C. Dunk River

C.1. June 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	5.033	0.009	126	**0.0001**
	7	10%, 100%	4.7819	0.0071	126	**0.0001**
	7	50%, 100%	1.4257	0.0547	126	0.1024
12S-248F	8	10%, 50%	4.8551	0.0075	126	**0.0001**
	8	10%, 100%	5.7401	0.0072	126	**0.0001**
	8	50%, 100%	1.987	0.0078	126	0.0086
Beach Seine	10	10%, 50%	6.265	0.0025	462	**0.0001**
	10	10%, 100%	5.3801	0.0026	462	**0.0003**
	10	50%, 100%	2.3269	0.0031	462	**0.0122**

C.2. August 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	2.7548	0.0091	126	**0.0034**
	8	10%, 100%	7.0105	0.0079	126	**0.0001**
	8	50%, 100%	2.7694	0.0077	126	**0.0029**
12S-248F	7	10%, 50%	4.8659	0.007	126	**0.0002**
	6	10%, 100%	8.7425	0.027	35	**0.0001**
	7	50%, 100%	4.4893	0.0079	126	**0.0003**
Beach Seine	10	10%, 50%	1.0554	0.3804	462	0.3463
	10	10%, 100%	2.7344	0.0148	462	**0.0081**
	10	50%, 100%	2.1054	0.0053	462	**0.0145**

Appendix B.12 continued: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) across Methods (12S-160, 12S-248F, Beach Seine) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

D. Enmore River

D.1. June 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	4.6109	0.008	126	**0.0001**
	8	10%, 100%	3.415	0.0068	126	**0.0002**
	8	50%, 100%	3.4947	0.0084	126	**0.0004**
12S-248F	8	10%, 50%	4.6207	0.0063	126	**0.0003**
	8	10%, 100%	2.7011	0.01	126	**0.0033**
	8	50%, 100%	3.9815	0.0059	126	**0.0006**
Beach Seine	10	10%, 50%	2.0421	0.0057	462	**0.0174**
	10	10%, 100%	3.3261	0.0022	462	**0.0011**
	10	50%, 100%	4.5553	0.0026	462	**0.0002**

D.2. August 2021

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	2.8525	0.0079	126	**0.0007**
	8	10%, 100%	5.1654	0.0062	126	**0.0003**
	8	50%, 100%	2.6547	0.009	126	**0.0034**
12S-248F	8	10%, 50%	3.6073	0.0079	126	**0.0005**
	8	10%, 100%	5.0928	0.0078	126	**0.0003**
	8	50%, 100%	3.0979	0.0065	126	**0.0014**
Beach Seine	10	10%, 50%	5.4612	0.0022	462	**0.0001**
	10	10%, 100%	2.6178	0.005	462	**0.0058**
	10	50%, 100%	4.2587	0.0016	462	**0.0001**

Appendix B.13: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) Methods (12S-160, 12S-248F, Beach Seine) at three estuaries in Prince Edward Island, Canada during August 2020. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River August 2020

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	4.3995	0.0072	126	**0.0003**
	7	10%, 100%	3.5916	0.0088	126	**0.0011**
	7	50%, 100%	4.8797	0.0061	126	**0.0002**
12S-248F	8	10%, 50%	4.1987	0.0067	126	**0.0004**
	7	10%, 100%	3.5758	0.0085	126	**0.0006**
	7	50%, 100%	4.9797	0.0081	126	**0.0001**
Beach Seine	8	10%, 50%	3.62	0.0077	126	**0.0024**
	8	10%, 100%	1.4905	0.095	91	0.1231
	8	50%, 100%	3.4425	0.0074	90	**0.0043**

B. Freeland Creek August 2020

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	3.9882	0.0091	126	**0.0003**
	8	10%, 100%	3.9739	0.0087	126	**0.001**
	8	50%, 100%	4.6649	0.0088	126	**0.0002**
12S-248F	8	10%, 50%	3.3861	0.0078	126	**0.0006**
	8	10%, 100%	3.1595	0.0073	126	**0.0006**
	8	50%, 100%	4.1248	0.0086	126	**0.0001**
Beach Seine	8	10%, 50%	5.4055	0.0088	126	**0.0002**
	8	10%, 100%	3.4156	0.0074	126	**0.0055**
	8	50%, 100%	9.3755	0.0076	126	**0.0001**

Appendix B.13 continued: Pairwise comparisons of the StationxMethod interactions between Stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) Methods (12S-160, 12S-248F, Beach Seine) at three estuaries in Prince Edward Island, Canada during August 2020. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

C. Dunk River August 2020

Method	DF	Station	t-score	P(perm)	Unique Perms	P(MC)
12S-160	8	10%, 50%	5.6524	0.0089	126	**0.0001**
	8	10%, 100%	2.2887	0.0083	126	**0.022**
	8	50%, 100%	3.1434	0.008	126	**0.0012**
12S-248F	8	10%, 50%	5.2783	0.0082	126	**0.0001**
	8	10%, 100%	2.6592	0.0081	126	**0.0097**
	8	50%, 100%	2.9177	0.0074	126	**0.0037**
Beach Seine	8	10%, 50%	5.1798	0.0086	126	**0.0005**
	8	10%, 100%	7.7998	0.009	126	**0.0001**
	8	50%, 100%	1.2375	0.2544	126	0.2466

Appendix B.14: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River

A.1. June 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	2.2945	0.008	126	**0.0065**
	8	12S-160, B. Seine	4.9121	0.0077	126	**0.0002**
	8	12S-248F, B. Seine	3.5653	0.0088	126	**0.0007**
50%	7	12S-160, 12S-248F	1.7295	0.0913	126	0.086
	9	12S-160, B. Seine	1.8535	0.0446	462	**0.0462**
	8	12S-248F, B. Seine	1.9503	0.0698	210	0.0544
100%	9	12S-160, 12S-248F	1.847	0.0639	126	0.0528
	9	12S-160, B. Seine	2.8162	0.0035	462	**0.0027**
	10	12S-248F, B. Seine	2.275	0.0092	210	**0.0164**

A.2. August 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	7	12S-160, 12S-248F	0.9182	0.4843	126	0.4529
	7	12S-160, B. Seine	2.4546	0.0165	126	**0.015**
	8	12S-248F, B. Seine	2.6298	0.0094	126	**0.0073**
50%	8	12S-160, 12S-248F	1.8075	0.0334	126	**0.0264**
	8	12S-160, B. Seine	1.923	0.0463	462	**0.0481**
	9	12S-248F, B. Seine	1.551	0.0995	462	**0.1122**
100%	8	12S-160, 12S-248F	2.1422	0.033	126	**0.0288**
	9	12S-160, B. Seine	2.622	0.0064	462	**0.0104**
	9	12S-248F, B. Seine	2.3211	0.0207	462	**0.0207**

Appendix B.14 continued: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

B. Freeland Creek

B.1. June 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.5733	0.0161	126	0.0794
	8	12S-160, B. Seine	1.8773	0.0078	126	**0.0341**
	8	12S-248F, B. Seine	1.7532	0.0244	126	**0.0405**
50%	8	12S-160, 12S-248F	2.3315	0.0089	126	**0.0073**
	9	12S-160, B. Seine	3.9487	0.0026	462	**0.0001**
	9	12S-248F, B. Seine	3.3829	0.0017	462	**0.0006**
100%	8	12S-160, 12S-248F	2.481	0.0077	126	**0.0075**
	9	12S-160, B. Seine	4.8784	0.0022	462	**0.0002**
	9	12S-248F, B. Seine	4.7208	0.0018	462	**0.0006**

B.2. August 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.5654	0.0722	126	0.0847
	9	12S-160, B. Seine	2.3857	0.0061	462	**0.0075**
	9	12S-248F, B. Seine	2.0117	0.0121	461	**0.0175**
50%	8	12S-160, 12S-248F	1.4262	0.1057	126	0.1361
	9	12S-160, B. Seine	3.1473	0.002	462	**0.001**
	9	12S-248F, B. Seine	3.4178	0.0019	462	**0.0002**
100%	8	12S-160, 12S-248F	1.7879	0.0145	126	**0.0303**
	9	12S-160, B. Seine	7.0633	0.0028	461	**0.0001**
	9	12S-248F, B. Seine	6.5175	0.0025	462	**0.0003**

Appendix B.14 continued: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

C. Dunk River

C.1. June 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.8367	0.0306	126	**0.0318**
	9	12S-160, B. Seine	6.3471	0.0021	462	**0.0001**
	9	12S-248F, B. Seine	5.732	0.003	462	**0.0001**
50%	8	12S-160, 12S-248F	2.2574	0.0076	126	**0.0052**
	9	12S-160, B. Seine	7.1114	0.0027	462	**0.0001**
	9	12S-248F, B. Seine	6.3766	0.0016	462	**0.0001**
100%	7	12S-160, 12S-248F	2.2186	0.0066	126	**0.0092**
	8	12S-160, B. Seine	4.5223	0.0049	210	**0.0004**
	9	12S-248F, B. Seine	4.6066	0.0028	462	**0.0001**

C.2. August 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	2.7902	0.0071	126	**0.0045**
	9	12S-160, B. Seine	4.1316	0.0024	462	**0.0002**
	8	12S-248F, B. Seine	3.2179	0.0031	210	**0.0019**
50%	8	12S-160, 12S-248F	1.9422	0.0089	126	**0.0171**
	9	12S-160, B. Seine	2.1204	0.0023	462	**0.0092**
	9	12S-248F, B. Seine	1.9351	0.006	462	**0.0239**
100%	7	12S-160, 12S-248F	3.4202	0.0072	126	**0.0014**
	9	12S-160, B. Seine	5.719	0.0023	462	**0.0001**
	8	12S-248F, B. Seine	4.267	0.0051	210	**0.0002**

Appendix B.14 continued: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at four estuaries in Prince Edward Island, Canada during the summer of 2021. Estuaries were analysed independently by Month (June and August 2021). Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

D. Enmore River

D.1. June 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	3.2486	0.0093	126	**0.0004**
	9	12S-160, B. Seine	3.9272	0.0018	461	**0.0003**
	9	12S-248F, B. Seine	3.5115	0.0024	462	**0.0005**
50%	8	12S-160, 12S-248F	2.8979	0.0081	126	**0.0009**
	9	12S-160, B. Seine	6.1522	0.0029	462	**0.0001**
	9	12S-248F, B. Seine	4.9447	0.0017	462	**0.0001**
100%	8	12S-160, 12S-248F	2.8044	0.0071	126	**0.002**
	9	12S-160, B. Seine	4.1963	0.002	462	**0.0002**
	9	12S-248F, B. Seine	3.5246	0.002	462	**0.0006**

D.2. August 2021

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	0.66596	0.7336	126	0.7304
	9	12S-160, B. Seine	3.0983	0.0022	462	**0.0009**
	9	12S-248F, B. Seine	3.1938	0.0028	462	**0.0012**
50%	8	12S-160, 12S-248F	1.6359	0.0245	126	0.0537
	9	12S-160, B. Seine	5.6756	0.002	462	**0.0001**
	9	12S-248F, B. Seine	5.7911	0.0016	462	**0.0001**
100%	8	12S-160, 12S-248F	1.4156	0.0721	126	0.1189
	9	12S-160, B. Seine	3.3984	0.0029	462	**0.0005**
	8	12S-248F, B. Seine	3.4926	0.0023	462	**0.0006**

Appendix B.15: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at three estuaries in Prince Edward Island, Canada during August 2020. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

A. Wheatley River August 2020

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.52	0.124	126	0.1206
	8	12S-160, B. Seine	4.0691	0.0077	126	**0.0005**
	8	12S-248F, B. Seine	3.3757	0.008	126	**0.0011**
50%	8	12S-160, 12S-248F	0.8112	0.6229	126	0.5797
	8	12S-160, B. Seine	2.5113	0.0076	126	**0.0081**
	8	12S-248F, B. Seine	2.382	0.0074	126	**0.0065**
100%	6	12S-160, 12S-248F	0.68226	0.858	35	0.7048
	7	12S-160, B. Seine	2.5032	0.0149	91	**0.0127**
	7	12S-248F, B. Seine	2.65	0.0074	91	**0.0089**

B. Freeland Creek August 2020

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.013	0.4357	126	0.405
	8	12S-160, B. Seine	3.8127	0.0082	126	**0.0006**
	8	12S-248F, B. Seine	3.56	0.0092	126	**0.0003**
50%	8	12S-160, 12S-248F	1.0857	0.3736	126	0.3307
	8	12S-160, B. Seine	2.7335	0.0083	126	**0.0016**
	8	12S-248F, B. Seine	2.9625	0.0079	126	**0.0009**
100%	8	12S-160, 12S-248F	1.0207	0.4391	126	0.3977
	8	12S-160, B. Seine	9.6656	0.007	126	**0.0001**
	8	12S-248F, B. Seine	10.183	0.0087	126	**0.0001**

Appendix B.15 continued: Pairwise comparisons of the StationxMethod interactions between the Methods (12S-160, 12S-248F, Beach Seine) across the stations (10 %-inner estuary, 50 %-middle estuary, 100 %-outer estuary) at three estuaries in Prince Edward Island, Canada during August 2020. Estuaries were analysed independently. Monte Carlo (MC) tests were preferred if there was an insufficient number of permutations (<1000). Significant values (P(MC)<0.05) are bolded.

C. Dunk River August 2020

Station	DF	Method	t-score	P(perm)	Unique Perms	P(MC)
10%	8	12S-160, 12S-248F	1.0765	0.3927	126	0.3439
	8	12S-160, B. Seine	1.8254	0.0397	126	**0.0472**
	8	12S-248F, B. Seine	2.1655	0.0172	126	**0.02**
50%	8	12S-160, 12S-248F	1.0414	0.3279	126	0.3429
	8	12S-160, B. Seine	3.7413	0.0076	126	**0.0007**
	8	12S-248F, B. Seine	3.4234	0.0096	126	**0.0014**
100%	8	12S-160, 12S-248F	0.49102	0.9239	126	0.8831
	8	12S-160, B. Seine	3.1217	0.0089	126	**0.002**
	8	12S-248F, B. Seine	3.339	0.0089	126	**0.0021**