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Trophic relationships, distribution and interactions among invasive and native Laurentian Great Lakes biota assessed using metabarcoding of stomach content DNA (scDNA) and environmental DNA (eDNA)

by

Justin Glenn Mychek-Londer

A Dissertation

Submitted to the Faculty of Graduate Studies

Through the Great Lakes Institute for Environmental Research

In Partial Fulfilment of the Requirements for

The Degree of Doctor of Philosophy

At The University of Windsor

Windsor, Ontario, Canada

2018

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Trophic relationships, distribution and interactions among invasive and native Laurentian Great Lakes biota assessed using metabarcoding of stomach content DNA (scDNA) and environmental DNA (eDNA)

by

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I.Co-Authorship Declaration

I hereby declare that this dissertation incorporates material resultant from joint research, as follows: This dissertation also incorporates collaboration under the supervision of my supervisor Dr. Daniel D. Heath and co-collaborators. Collaborations were in Chapter 3 with co-collaborator Subba Rao Chaganti, and Chapter 5 with co-collaborator Katherine D. Balasingham at the time of this submission. In all cases, key ideas, primary contributions, experimental designs, and data analysis and interpretation were performed by me with additional contributions to field and data samples, data analysis and interpretation and written discussion by co-authors.

I am aware of the University of Windsor Senate Policy on Authorship and certify I have properly acknowledged contributions of other researchers to my dissertation and have obtained written permission from each of the co-author(s) to include the above material(s) in my dissertation.
I certify that, with above qualifications, this dissertation and research to which it refers, is the product of my own work.

II. Declaration of Previous Publication Submission

This thesis includes material from an original paper from Chapter Two submitted for publication to a peer reviewed journal. Although rejected it is likely to be submitted again after recommendations of reviewers have been fully addressed and is as follows: Publication title: "Mychek-Londer, J.G., MacIsaac, H.J. and D.D. Heath., 2017, "Metabarcoding primers for invasive invertebrates in the Laurentian Great Lakes." Submitted to *The Journal of Great Lakes Research*, August 20, 2017.

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ABSTRACT

As aquatic invasive species (AIS) proliferate, they pose serious threats to native taxa, foodwebs and ecosystems. Massively-parallel, high-throughput, next-generation sequencing (NGS) and metabarcoding of environmental DNA (eDNA) and predator stomach content DNA (scDNA) are new approaches to detect AIS and can facilitate detailed analyses of AIS impacts. Using such technology, degraded and digested samples, and cryptic taxa can be identified with high sensitivity. The Laurentian Great Lakes (Great Lakes) are highly invaded and to determine occurrence patterns of AIS and native species inhabiting them I used a shortened portion of Cytochrome Oxidase One (CO1) and NGS metabarcoding. I developed, optimized and validated novel target-species CO1 PCR primer sets for AIS invertebrates: *Bythotrephes longimanus*, *Cercopagis pengoi*, *Dreissena rostriformis bugensis, Dreissena polymorpha,* and *Hemimysis anomala*. I tested primer set sensitivities, specificities, and multiplexing and used these with universal primer sets to analyze field-sampled scDNA and eDNA. I analyzed influences of abiotic (spatiotemporal) and biotic (predator species and size) variables in relation to prey occurrences in scDNA. The lowest AIS DNA in $ng \cdot \mu L^{-1}$ detected with novel primer sets in PCRs was: *B. longimanus* = 2.07; *C. pengoi* = 0.0002; *D. r. bugensis* = 0.0009; *D. polymorpha* = 0.103; and *H. anomala* = 0.127 (Chapter 2). Detection limits within mixes of interfering DNA (as percentage of total DNA) were: *B. longimanus* = 3.90 %, *C. pengoi* = 0.003 %, *D. r. bugensis* = 0.020 %, *D. polymorpha* = 0.170 % and *H. anomala* = 0.019 %. To determine invertebrate AIS roles in Lake Michigan, I metabarcoded scDNA from alewife (*Alosa pseudoharengus*), bloater (*Coregonus hoyi*), ninespine stickleback (*Pungitius pungitius*), rainbow smelt (*Osmerus mordax*) and slimy sculpin (*Cottus cognatus*) sampled Spring 2009 and 2010 at 73-128 m depths from three offshore sites. I detected multiple occurrences of target AIS and three native prey

(*Leptodiaptomus sicilis*, *Limnocalanus macrurus*, and *Mysis diluviana*; Chapter 3). Driving variation in occurrences of the AIS and native taxa in scDNA was sample site, highlighting localized ecological and foodweb differences in ongoing AIS roles and impacts. Predator species effects likely reflected realized ecological feeding niches. To metabarcode scDNA from Lake Erie commercially fished predator species walleye (*Sander vitreus*), white bass (*Morone chrysops*), white perch (*Morone americana*) and yellow perch (*Perca flavescens*), I used novel AIS primer sets (Chapter Two) and a universal primer set to target fishes. Three invertebrate AIS including *C. pengoi, B. longimanus,* and *D. r. bugensis,* and AIS prey fishes gizzard shad (*Dorosoma cepedianum*), rainbow smelt and round goby (*Neogobius melanostomus*), and native prey fishes channel catfish (*Ictalurus punctatus*) and emerald shiner (*Notropis athernoides*) were detected, and all were relatively common components of scDNA. Occurrence patterns varied significantly with season, predator species, year, and predator size; however, the significances of factors also varied with prey species. Prey occurrences revealed dynamics among native and AIS prey and predators, potential competition, or prey selectivity which varied by way of these factors (Chapter 4). In 2013, 43 sites each in the Sydenham and Grand Rivers were sampled and metabarcoded for invertebrate eDNA using a universal primer set. Data revealed spatial patterns of AIS at sites within and among each river. Key AIS identified were: *D. r. bugensis, Branchiura sowerbyi, Potamothrix moldaviensis, Craspedacusta sowerbyi* and *Skistodiaptomus pallidus*. Further, I identified rare native mollusks including *Quadrula quadrula* and *Villosa fabalis* - threatened and endangered species in lower and middle reaches of the Sydenham. Novel results and information will provide important guidance to those tasked with ongoing challenges of managing, slowing spread of and eradicating AIS, and conserving native species.

DEDICATION

I dedicate this thesis to first and foremost to God and Jesus as my saviors, followed most importantly by dedication to my mother Victoria Evelyn Mychek-Londer, my father Glen Londer, brother Jonathan Gerald Londer, sister Abigail Evelyn Londer, Mark Londer, Lisa Londer, Harold Mychek, Evelyn Mychek, Jeanie Londer, Michelle LaFleur and other supportive family members, friends and mentors still with me and whom have passed on. Lastly, I dedicate this dissertation to everyone who believed in me, to doubters who did not, to the truth in science, to people that work hard for what they want in life, and to anyone passionately dedicated to preserving ecosystems and native species across the world.

ACKNOWLEDGEMENTS

I thank the staff at the University of Windsor Great Lakes Institute for Environmental Research (GLIER) and members of the Heath Environmental Genomics Lab. Funding was supplied by the Trillium Scholarship for International Graduate Students in Canada, the Canadian Aquatic Invasive Species Network (CAISN), and the Natural Sciences and Engineering Research Council (NSERC) Discovery Grant program. I thank my adviser Daniel D. Heath, who gave invaluable assistance and mentorship during all phases of this endeavor as well as thank his family for all the time he takes away from them to help his students. I also thank Hugh MacIsaac, Aaron Fisk and Timothy Johnson for support as graduate committee members during this research, their mentorship and guidance has been invaluable. I thank my family members, especially my mother and father for providing a youth with great deal of exposure to the beauty of natural environments, wildlife and the freedom and support to choose my own path in life. I thank my brother Jon Londer who has always been supportive in invaluable ways in the effort to complete this goal and who I admire for his toughness, courage, even temperament and smarts in all phases of life. I thank Katherine Balasingham, Kyle Wellband, Ryan Walters, Razagh Hashemi Shahraki, Javad Sadeghi, Clare Venney, Subba Rao Chaganti, Samir Quereshi, and Shelby Toews amongst numerous others from the Heath Lab for Environmental Genomics for technical laboratory support. I owe a great many thanks to many enthusiastic undergraduate volunteers who assisted during sample processing during dissection phases, including in particular Valerie Sokolowski, Marcus Leung, Alex Pardy, and Daniel Salehi. I especially thank Dr. Mika Tomac who was in my corner through difficult moments and who displayed tremendous effort and compassion in teaching me how to further grow spiritually, personally and professionally. I thank Mr. Ryan Flanagan who has given honest

positive encouragement and tangible assistance which has been very helpful in completing this endeavor. I also thank Dr. Chris Houser from the Department of Biological Sciences for guidance in completion of this dissertation. I thank Mary Lou Scratch and Christine Weisener for invaluable administrative assistance at GLIER. I thank Dr. Abdulkadir Hussein from the Department of Mathematics and Statistics at The University of Windsor for critical and informative assistance in developing the correct statistical tests to meet related research objectives. I thank Alison Samson and the Office of Graduate Studies for assistance, and positive interactions from almost the day I arrived on campus. Collaborators including Dr. James S. Diana, Dr. David B. Bunnell and the Ontario Ministry of Natural Resources and Forestry helped to provide samples for used for scientific analyses. I thank Mr. Todd Loop and the multi-generational family-run Loop Commercial Fisheries in Wheatley, Ontario who as individuals and which as a company were very helpful in obtaining field-samples for scientific research and whose continued passion for sustaining the Great Lakes and first-hand knowledge of Lake Erie and its fisheries is admirable.

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Figure 5.1: Map of 43 sampling sites for the Grand River. The first most site nearest to Lake Erie is in the lower right Southeast corner of the map while the uppermost site furthest from Lake Erie is at the uppermost left corner in this figure. Each little flag represents a sampling site. 151

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Figure 5.3: Proportion of matched sequences reads presented at the genus level across all eDNA samples for the Grand River and the two branches of the Sydenham River. The charts only include the sequence match data from all sites combined (all unmatched sequence data is not shown). While these charts show only at the genus level, data was identified to species level first and then organized at the genus level. 162

LIST OF ABBREVIATIONS

° **C**: degrees Celsius

AIS: Aquatic invasive species

BLAST: Basic Local Alignment Search Tool

BOLD: Barcode of Life database

bp: Base pairs

BSA: Bovine serum albumin

CO1: Cytochrome *c* oxidase subunit one

CTAB: Cetyl Trimethylammonium Bromide

ddH$_2$O: Double distilled water

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide Triphosphate

eDNA: Environmental deoxyribonucleic acid

GLIER: Great Lakes Institute for Environmental Research

L: Litre

MgCl$_2$: Magnesium chloride

m: Meter

mg: milligrams

MgSO$_4$: Magnesium Sulfate

mL: Millilitre

mm: Millimetre

NaCL: Sodium Chloride

ng: Nanograms

nm: Nanometer

NGS: Next-generation sequencing

PCR: polymerase chain reaction

pg: Picogram

pmol: Picomoles

RNase A: Ribonuclease A

rDNA: Ribosomal deoxyribonucleic acid

scDNA: Stomach content DNA

SD: Standard deviation

Taq: *Thermus aquaticus*

TE: Tris-EDTA (EthyleneDiamineTetraAcetic Acid)

TL: Total length, nearest millimeter

µL: Microlitre

UV-B: Ultraviolet

CHAPTER 1 - GENERAL INTRODUCTION

Aquatic invasive species (AIS) pose the second largest threat to aquatic biodiversity after habitat loss and they disrupt foodwebs by: causing species extinctions (Ricciardi & MacIsaac 2000; Clavereo & Garcia-Berthou 2005), changing energy flows in foodwebs (Johnson et al., 2005; Rushet al., 2012), increasing homogenization of biological communities (Rahel 2002; Villéger et al., 2011) decreasing biotic resistance to further invasion or disruption (Simberloff & Von Holle 1999; Scott & Helfman 2001), outcompeting native species for limited food/resources (Ricciardi et al., 1998; Paolucci et al., 2013), consuming egg or larval stages of native aquatic species impacting recruitment (Tyus et al., 2000; Vredenburg 2004), and inducing changes in population-specific gene expression patterns in native species (Mooney & Cleland 2001; He et al., 2017). Thus, resource managers must understand the role of AIS in foodwebs and ecosystems where important native species reside and are subject to potential ongoing impacts of AIS in order to manage and mitigate such effects (Lodge et al., 2006; Ricciardi & MacIsaac 2011).

Conventional detection methods for AIS, roles in foodwebs, ecosystems

Traditionally, visual quantification and counting or proportional estimates of target prey from predator stomach contents and water samples or advanced techniques such as stable isotopes to capture non-visible, long- term diet patterns in tissue composition have been used to determine AIS and native species roles in foodwebs and ecosystems (i.e., Brush et al., 2012; Huo et al., 2014; Mumby et al., 2017; Landry et al., 2018). However, low resolution of prey identification by isotope-based methods even when used in conjunction with visual methods can result in the failure to include rare species not abundant in diets and in samples which contain hard to identify or highly digested prey. Although perhaps not significant in terms of energetics, rare prey or taxa such as early stage AIS or at-risk native taxa occurrences may be important.

For example, some of the first detections of the AIS of spiny waterflea *Bythotrephes longimanus* in the Laurentian Great Lakes (hereafter Great Lakes) occurred when researchers were examining fish stomach contents (Bur et al., 1986). Similarly, the most reliable method thus far to detect the AIS *Hemimysis anomala* in populations that are expanding in Lake Erie has been deemed to be through the examination of fish diets (Forage Task Group 2015; Kipp et al., 2018).

Many prey are rapidly digested in gut contents (Schooley et al., 2008; Legler et al., 2010), but even when undigested, they can still be morphologically ambiguous, such as noted for many fish eggs, resting eggs of invasive invertebrates, or invasive freshwater hydroids (Auer 1982; Briski et al., 2010; Folino-Rorem et al., 2009). Inaccuracies in foodweb and ecological assessments of AIS and of rare species will limit the efficacy of ecosystem-based resource management approaches and restoration of native species (Link et al., 2008; Zimmerman & Krueger 2009). The use of DNA metabarcoding to identify potential AIS and rare species of prey in predator stomach content diet sample DNA (scDNA) and in aquatic environmental DNA (eDNA) is becoming an important approach to help alleviate some of these issues.

Environmental DNA (eDNA) and stomach content DNA (scDNA)

Instead of targeted physical specimen capture, eDNA relies on the passive capture of free DNA in water samples taken from oceans, rivers or lakes. The DNA in eDNA can occur in multiple forms, including hematological excretions, digestive remains, sloughing of epidermis, bacteria and shreckstoff, among other potential sources (Deiner & Aftermatt 2014; Deiner et al., 2016). Less passively sampled, but conceptually similar, is to analyze scDNA of predator species, as predators act as efficient samplers of their environments. DNA in scDNA might originate as whole prey, prey with varied levels of digestion, result from tertiary or non-selectively consumed prey taxa, parasites, and gut-biota, and from the predator itself. scDNA

and eDNA can be extracted and used in traditional polymerase chain reaction (PCR) followed by visualization of the resulting PCR products on agarose gels for confirmation of presence through appropriately-sized amplicons. Newly emerging suites of molecular genetic methodologies including massively parallel high-throughput next-generation sequencing (NGS), coupled with metabarcoding of eDNA and scDNA have been applied to accurately detect AIS and rare species, and define their interactions, roles in foodwebs and ecological attributes (e.g., Pompanon et al., 2012; Leray and Knowlton 2015; Balasingham et al., 2018).

Metabarcoding, high-throughput next-generation sequencing

Metabarcoding relies on every species having a unique 658 base pair (bp) segment of the mitochondrial cytochrome c oxidase one (CO1) gene marker region that differentiates it from heterospecifics (Hebert, et al., 2003a; Hebert et al., 2003b; Hebert & Gregory 2005). Combined with NGS, metabarcoding can be used to detect multiple species sequences in multiple mixed samples rapidly, cost effectively and accurately (see: Darling & Mahon 2011; Ji et al., 2013). Ideally, DNA metabarcoding PCR primer set markers for such applications would capture multiple species by being variable among species within the identification barcode, invariable at annealing sites among species expected in samples to maximize amplification of all species in a sample, and robust under experimental conditions.

Not surprisingly, these types of "universal" PCR primers have proven difficult to develop, even sometimes for species grouped at the genus or higher taxonomic levels. However, some universal primer sets have been widely used with success to detect AIS and rare native species to describe ecological interactions in foodwebs, and to examine what abiotic or biotic factors influence species roles in ecosystems through metabarcoding (e.g., Pompanon et al., 2012; Leray et al., 2013; Ley et al., 2014). One powerful approach is to use a universal primer

set coupled with highly target-specific primer sets developed using reference DNA sequences for species of interest in individual PCRs. The amplicons generated from individual samples are then combined within each sample and are uniquely tagged before submission for NGS of sample-specific, tagged DNA sequences. Sensitivity to detect target prey species or groups of species using such "universal" primer sets coupled with target-specific primer sets can be high. For example, Pochon et al. (2013) recovered DNA/PCR products of individual species present at and at greater than 0.64 % abundance from tested contrived species communities. Zhan et al. (2013) detected indicator species with biomass percentage as low as approximately 2.3×10^{-5} % when diluted amongst a mixture of other species using similar approaches. Furthermore, the sequence data from metabarcoding itself permits accurate identification of organisms or prey species in eDNA and scDNA samples, if the species-specific sequences are archived in reference databases. This step is crucial because a truly target-specific primer set, for which sequences need not be confirmed by sequencing, requires great effort of cross testing of closely related congeners for competitive- or co-amplification during PCRs (i.e., Jerde et al., 2013; Mahon et al., 2013).

In the Great Lakes region, recent analyses have used increasingly complex and informative molecular genetics-based approaches to test ecological, foodweb and AIS related hypotheses. For example, Mychek-Londer et al. (2013) used PCRs and interpreted differently sized prey species specific amplified DNA fragments visually to identify multiple species of well-preserved morphologically ambiguous fish eggs in individual diets of three benthic fish species. Carreon-Martinez et al. (2011) used DNA barcoding of fish diets to identify piscine prey of Lake Erie yellow perch (*Perca flavescens*), and Carreon-Martinez et al. (2014) presented microsatellite data suggesting larval cannibalism by yellow perch occurred at high levels in fiel

Table 1.1: Strengths and weaknesses of environmental DNA (eDNA) and stomach content DNA (scDNA) metabarcoding, and traditional-based methods for study of environments, environmental samples, and predator stomach content samples.

Metabarcoding analysis of environmental DNA (eDNA) and predator stomach content DNA (scDNA)		Traditional visual approaches for analysis of environments, environmental samples, predator stomach content	
Strength	Weakness	Strength	Weakness
1. High resolution of organism and prey species occurrences	1. Need confirmed species sequences, not all available	1. High resolution of prey species	1. Requires possible high effort to sample rare species, AIS
2. Cryptic, degraded, digested, morphologically ambiguous components in eDNA and scDNA can be identifiable	2. Life stage not accounted for	2. Life stage often determinable	2. Requires specialized training
3. Sensitive, low detection limits	3. May require subsampling	3. Some prey-species-specific body parts are relatively undigestible, can last in samples and stomach contents long periods, and be used to identify remains to species	3. Cryptic species not identified
4. Possible non-invasive sampling of environments, predator guts (lavage, fecal matter), or opportunistic, i.e., angler harvested discards	4. Quantitative aspects still emerging; challenging, need stringent controls	4. Well established approaches for attempting quantification beyond presence-absence, i.e., measure weights of organisms in samples, length to weight regressions for prey in stomach contents	4. May require subsampling
5. Lab and field controls can strengthen results and limit Type I and II errors	5. High potential for false positives, false negatives, amplification bias, inhibitors	5. Mostly non-specialized equipment can be used to sample and analyze samples, i.e. nets, scalpels, microscopes	5. Unexpected taxa easily passed over, i.e. newly arriving AIS
6. Stringent quality controls in Bioinformatics stages can limit Type I and II errors	6. Highly trained taxonomists are needed to confirm species for barcodes for databases; are becoming less common	6. Many taxonomic keys have already been developed for species	6. Highly trained traditional taxonomists becoming rare.
7. May be lower overall cost (time, effort) per sample than traditional visual methods	7. Origins of species DNA traces in eDNA and scDNA may be confounded; i.e. downstream diffusion in rivers; predators & prey in predator guts might have been sampled far from initial interactions	7. May, through and at time of sampling be able to reveal if organisms in environmental samples were likely alive or dead, or based upon relative digestive state of prey in stomach contents if the prey was likely alive when ingested (still relatively in-tact), and make a determination of time since ingestion if gastric evacuation rates and bioenergetics for the predator and prey type are known	7. Life stages can be morphologically ambiguous among multiple prey species, i.e., fish eggs, *Dreissena*spp. veligers
8. Many universal and target-species-specific PCR primer sets are already available	8. Requires some specialized training, and equipment, i.e. DNA-sequencers, though both aspects increasingly accessible and affordable		8. Degraded organisms and prey in samples may not be identifiable to species level
9. Predators (scDNA) good samplers of environments	9. Predators (scDNA) may contaminate total DNA in sample, can limit applicability of universal primer sets aimed at identifying targets in groups of species that predators also belong to; may require blocking primers; cannibalism requires non-CO1 markers not as well defined across as many species		9. Traditional sampling efforts, i.e. benthic trawling in offshore Great Lakes, sampling with waders among rivers can promote unwanted AIS spread
10. Good for samples containing many smaller sized organisms, i.e. microzooplankton	10. Advanced programming and coding (Bioinformatics steps) may be required		10. Physical sampling may have negative impacts, i.e. rare species in rare habitats they inhabit could both be negatively impacted
11. Some elements very basic, i.e. water sampling or capturing of fishes or other predators	11. Lack of matched sequences to databases may require taxonomic classification of eDNA or scDNA to genus or family level		11. May take significant time to process whole samples such as individual zooplanktivore diets containing thousands of microzooplankton
12. Training and methodologies for eDNA and scDNA applicable/translatable across different ecosystems w/potentially very different species, i.e. sampling water, lab processing steps, Bioinformatics steps can be the same or similar	12. Sample protocols still need wide definitions of and adherence to standardizations, i.e. replication levels, controls		12. Taxonomic keys can be limited in developing regions and for groups of smaller organisms i.e. rotifers compared to fishes; limits potential for community-based analyses
13. Rapid screening for target taxa, i.e. AIS, rare and at-risk native taxa possible, i.e. bulk sample processing	13. scDNA of very small sized predators i.e. invertebrates may have to be processed whole contributing large amounts of predator DNA		13. May take long periods to scan samples for single target organisms when compared to eDNA and scDNA bulk sample processing
14. Shotgun approach using universal primer sets may reveal unexpected species	14. Sensitivity and lower detection limits usually not well defined for available primer sets		14. Organisms or prey can be broken up into many pieces limiting counting of individuals
15. Approach (eDNA) may reduce unintentional transfers of AIS among sampling sites vs. traditionally styled sampling	15. Observances of taxa in eDNA and prey in scDNA may be highly localized, non-static: need spatial and temporal components with data		15. Not all information available for length-weight regressions to get proportional or other quantitative data for prey in diets
16. Reference databases for species barcodes increasing in breadth	16. Determinations of if eDNA was from live or dead organisms complicated, may require RNA-based detections		
17. DNA traces would be localized			
18. Scale of sampling effort and identified clusters of occurrences can help determine exact originations of locations of detected taxa			
19. For some species: availability of multiple genetic markers, increasing detection possibilities in eDNA and scDNA			
20. Sequence reads may be indicative of relative abundance of organisms or prey in eDNA			

samples after spawning season, even though no visual remains of these prey were identified. More recently, complex analysis of samples through metabarcoding was undertaken in which multiple species of invasive and rare native fishes were detected in aquatic eDNA from major tributaries to the Great Lakes (Balasingham et al., 2018). In another study, invertebrate invasive bivalve and gastropod species were identified in field samples using a similar eDNA approach (Klymus et al., 2017). Dynamic spatiotemporal complexities of aquatic ecosystems make high resolution quantitative data describing foodwebs, trophic relationships between predators and prey and rare native taxa ecology a critical need, and this need can be met through metabarcoding analyses. However, as eDNA and scDNA based results may influence management actions, and thus could be costly if results are inaccurate, it is imperative to employ various controls throughout metabarcoding to minimize Type I (false positives) and Type II (false negatives) errors.

Type I and Type II errors in metabarcoding of eDNA and scDNA

A concern when using metabarcoding of eDNA and scDNA with universal or target-specific PCR primer sets is that Type I and Type II errors need to be addressed (Mahon et al., 2013). Additionally, one must also rely upon the availability of reliable reference sequence data in databases such as GenBank or Barcode of Life, especially if using a universal primer. Lack of a match at the species level may require higher levels of taxonomic identification, such as to the genus or family level, reducing chances to identify important taxa (Borisenko et al., 2009; Deagle et al., 2014). Additional factors including: stochastic PCR error, unexpected haplotypes, inadequate replication and low overall or relative DNA concentrations in mixed samples (Darling & Mahon 2011; Ficetola et al., 2015) can impact DNA metabarcoding results, and all such factors should be carefully planned and accounted for during all metabarcoding research.

DNA contamination of samples among sites, among samples, or in the lab are major potential sources of error but can be limited by equipment sterilization and inclusion of blank samples or additional controls during field sampling, water filtering, DNA extraction, and PCR amplification (Bohmann et al., 2014; Jerde et al., 2012). Humic or fulvic acids (Matheson et al., 2010; McKee et al., 2015) and other substances that modify DNA, can inhibit procedures used to produce metabarcoding data. However, designing custom created digestion and extraction buffers, using appropriate specialized DNA digestion and extraction kits, use of controls, use of sample replicates and robustly-designed molecular markers with high PCR efficiency (Bohmann et al., 2014) can reduce such error.

Dissertation objectives

This dissertation uses NGS and metabarcoding to investigate the occurrences and roles of AIS and important native species within samples from predator scDNA and river water eDNA, and biotic and abiotic factors related to patterns of occurrence.

In Chapter Two, I develop and test novel AIS target-specific PCR primer sets in controlled lab experiments to help ensure that these PCR primer sets would amplify the target AIS in field samples in later chapters. These primer sets displayed high sensitivity in detecting the target, including among a mix of other species DNA, and they were robust and could perform under varied PCR conditions.

In Chapter Three, I use the novel target AIS primer sets from Chapter Two and a universal primer set to screen scDNA originating from both native and non-native predators sampled in hard to access and understudied offshore deepwater sites in Lake Michigan to describe variation in prey occurrences across space and time. I also used universal primer set data to examine its comparative amplification vs. target specific primer sets for AIS within the

same field samples. The universal primer set also permitted testing of sequencing confirmed amplification and presence-absence of three native prey species. These taxa are important Great Lakes role players, and are both invasive and endangered species outside Great Lakes regions (Spikkeland et al., 2016; Вежновец 2017; Devlin et al., 2017; Hyatt et al., 2018).

In Chapter Four, I use metabarcoding using AIS primer sets and a universal primer set that amplifies DNA from invasive fishes to analyze scDNA of economically-valuable, commercially-fished, native and non-native piscivore fishes from Lake Erie. In Chapters Three and Four I also sought to determine if abiotic and biotic factors affected presence-absence of taxa, those factors include season, year, depth, predator species, and predator total length (when available).

In Chapter Five, my objective was to use a universal primer set and metabarcoding to determine occurrences of AIS and rare and native at-risk species in waterborne captured eDNA sampled from multiple sites in the Grand River and Sydenham River, both located in southwestern Ontario, Canada. The aims were to determine if taxa were different among the rivers and different among the sites within rivers, if AIS overlapped with rare and at-risk native species, and to determine if there were patterns of AIS and rare and at-risk native species distributions at sites in each river related to distances from river mouths, and lastly determine how close sites were to one another within each river system where AIS and rare native taxa occurred, i.e., was there clustering or independent occurrences of taxa in each river.

In Chapter Six, I provide an overview of the outcomes of my dissertation, briefly identify and summarize the most important findings and results from each chapter, indicate what these findings mean in the larger context of foodwebs and ecosystems of Great Lakes and beyond, how impactful AIS appeared to be, and offer directions for future research.

Conclusion

In conclusion, the goals of my dissertation focused upon the use of state-of-the-art molecular genetic approaches to solve long-standing problems in traditionally based studies of AIS and important native species. Specifically, I describe occurrences of these taxa in eDNA and scDNA and make inferences about the ecological roles of these taxa in understudied and hard to access large scaled ecosystems making up tributaries of and within the Great Lakes proper. As native species fluctuate between ecological stability and imbalance and between high abundances and near absences because of impacts from AIS and other human induced ecological change (i.e., Madenjian et al., 2015; Paterson et al., 2014) it is important to understand if predator AIS and native species, and AIS and native species prey and other ecologically important taxa could be important factors in these observances. Inferences, methodologies and data from my novel NGS metabarcoding approaches to analyze scDNA and eDNA may be used to help answer such questions in ecosystem based models and related efforts. This will help to: manage AIS, understand AIS impacts, determine fisheries harvest and stocking levels (i.e., Bunnell et al., 2014; Rogers et al., 2014), inform efforts behind reintroduction of extirpated native species (Stewart 2010; Zimmerman & Krueger 2009), form early response strategies to newly arriving and expanding AIS (Anderson 2005), and to characterize the ecology of spatiotemporally complex and little understood large scaled Great Lakes foodwebs affected by AIS across multiple trophic levels.

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CHAPTER 2 - METABARCODING PRIMER SETS FOR INVASIVE INVERTEBRATES IN THE LAURENTIAN GREAT LAKES*

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Introduction

Aquatic invasive species (AIS) pose significant threats to ecosystems through extinction of native species (Kaufman 1992; Ricciardi & MacIsaac 2000), increasing homogenization of biological communities (Rahel 2010), changing energy dynamics in foodwebs (Johnson et al., 2005; Brush et al., 2012) decreasing biotic resistance (Scott & Helfman 2001), and outcompeting (Ricciardi et al., 1998) or preying on native species (Tyus et al., 2000). Determination of AIS establishment is often based on visual identification in environmental samples such as predator gut contents or physical samples (Garrison & Link 2000; Counihan & Bollens 2017). This can be inaccurate and time consuming as organisms may be degraded, morphologically ambiguous and challenging to identify without specialized training (Legler et al., 2010; Mychek-Londer et al., 2013). Additionally, the probability of detection is directly related to effort, making routine high-precision scanning logistically difficult (Counihan & Bollens 2017).

Metabarcoding of multiple taxa and AIS detection from extracted environmental DNA (eDNA) and predator stomach content DNA (scDNA) can help address such shortcomings (Comtet et al., 2015; Taberlet et al., 2012). Essentially, most animal species have a unique 658 base pair (bp) segment of the mitochondrial cytochrome c oxidase one (CO1) gene marker region differentiating it from heterospecifics (Hebert et al., 2003a; Hebert & Gregory 2005) which can be amplified using polymerase chain reaction (PCR) and species-specific primer sets. Sensitivity of such primer sets can be high (Pochon et al., 2013; Zhan et al., 2013), however, stochastic PCR error, unexpected haplotypes, inadequate replication, or low overall or relative DNA concentrations in mixed samples can result in false negatives for targeted species (Darling & Mahon 2011; Ficetola et al., 2008; Ficetola et al., 2014). Further, false positives can result from

eDNA and scDNA analysis as non-target sequences of closely related overlapping species may co-amplify or competitively interfere during PCR (Hebert et al., 2003b; Mahon et al., 2013; Deagle et al., 2015).

Comparing actual sequence data from amplified CO1 DNA fragments against confirmed species specific CO1 sequences published in databases such as the Barcode of Life (BOLD), can help alleviate such issues (Hebert et al., 2003a; Ratnasingham & Hebert 2007). Bench-top next-generation sequencing (NGS) platforms can provide such data; however, amplified DNA sequence read length outputs for such systems are smaller than the full 658 bp CO1 species barcode. Additionally, many platforms require adapters and tags (Loman et al., 2012) that, when including primer sequences, may result in 120-140 bp of uninformative sequence, and thus limit the availability of sequence variation otherwise available within CO1 amplified fragments (Clare 2014). Mixed eDNA and scDNA samples may contain degraded fragments and incomplete portions of the full 658 bp CO1 marker, further necessitating shorter barcode sequences (Deagle et al., 2006; Pompanon et al., 2012). Thus, metabarcoding primer sets with short read lengths and high specificity for target species are becoming more desirable for eDNA and scDNA metabarcoding analyses (Comtet et al., 2015; Deagle et al., 2015).

In the Laurentian Great Lakes (hereafter Great Lakes) around 185 non-native species have established. Five especially problematic invertebrate non-native AIS include: *Bythotrephes longimanus*, *Cercopagis pengoi*, *Hemimysis anomala*, *Dreissena rostriformis bugensis* and *Dreissena polymorpha* and each has had its own suite of negative impacts in the Great Lakes (Bunnell et al., 2011; Ricciardi & MacIsaac 2011). Identification of AIS when initially at low abundances during early invasion stages, or when in transport, can be a critical step in the prevention of further spread (Lodge et al., 2006; Simberloff 2001). Species-specific PCR primer

sets have been developed and tested for some of these AIS; however, often sensitivity or DNA concentrations used in analyses are unreported or the target primer sets amplify excessively long fragments not suitable for eDNA metabarcoding (i.e., Bronnenhuber & Wilson 2013; Marescaux & Van Doninck 2013). Thus, I sought to design and optimize metabarcoding primer sets for the five AIS above targeting a shortened region of the CO1 barcoding fragment suitable for eDNA and scDNA NGS analyses. I performed: 1) annealing temperature and DNA concentration sensitivity gradients; 2) mixed PCR DNA interference tests; and 3) primer set multiplexing experiments to better understand primer set dynamics.

Methods

DNA Extraction and dilution

Benchmark DNA for the five target AIS was collected from taxonomically validated specimens from the Great Lakes that were preserved in 95 % ethanol. Prior to DNA extraction, I rinsed a few to several individuals of a target species, or a shell free subsample of *Dreissena rostriformis bugensis* or *Dreissena polymorpha* with Milli-Q water to remove contaminants and ethanol, patted samples dry with a kimwipe, and then placed each sample into individual, sterile 1.7 mL centrifuge tubes containing Wizard Genomic DNA Purification Kit Nucleic Lysis Solution (Promega©). Tissue samples were cut using sterilized scissors to assist DNA digestion. I followed manufacturer "quick protocols" for procedures, volumes, digestion and isolation of genomic DNA, and froze samples in light-proof containers at -20 °C.

I measured starting DNA concentrations ($ng \cdot \mu L^{-1}$) using a General Electric NanoVue spectrophotometer. I used 1X Tris-EDTA as a starting blank and 3 µL of homogenously mixed DNA extract for DNA concentration determinations, replicated three to six times for each

sample. Protein and buffer contamination was inferred from corresponding 260/280 and 260/230 ratios (nm). From undiluted extractions I serially diluted targets at 1:10 sample with Milli-Q water (total volume each dilution = 100 µL) for use in PCRs. I serially diluted to 10^{-6} for each AIS DNA extract. Starting DNA concentrations in undiluted samples for each AIS differed, thus DNA concentration at each dilution level differed somewhat among species. I limited heat and light exposure during dilutions and froze samples at -20 °C in light-proof containers until PCR.

Primer development

I targeted a sub-region of the 658 bp COI barcode for AIS PCR primer set designs. From BOLD (http://www.boldsystems.org/) I downloaded and aligned all available CO1 sequences for *Bythotrephes longimanus* (N = 11), *Cercopagis pengoi* (N = 6), *Dreissena rostriformis bugensis* (N = 11)*, Dreissena polymorpha* (N = 59), and *Hemimysis anomala* (N = 9). Alignment and primer design was done in Geneious Pro 4.8.5 and Primer3 with default settings. In preliminary tests a primer set designed for *Dreissena rostriformis bugensis* also amplified *Dreissena polymorpha* seemingly as well as one initially developed just for *Dreissena polymorpha*. Thus, the primer set designed for *Dreissena rostriformis bugensis* was also used for *Dreissena polymorpha*. I expected that each taxa diverged sufficiently so each could be unambiguously identified after metabarcoding NGS, but not when only using species-specific PCR applications.

All primer sets lacked degeneracy. Suggested annealing temperatures (°C) for forward and reverse primers were: *Bythotrephes longimanus* JBTHF = 59.4 °C, JBTHR = 59.7; *Cercopagis pengoi* JCPGF = 59.0, JCPGR = 58.2; *Dreissena rostriformis bugensis* and *Dreissena polymorpha* JDBPF = 57.0, JDBPR = 60.1; and *Hemimysis anomala* JHMYF = 57.3, JHMYR = 59.8. Primers were designed for eDNA and NGS, thus I added a short 5'-end tail sequence to each forward and reverse primer to simulate common NGS adapters (Table 2.1).

Table 2.1: AIS metabarcoding (CO1) primer details. Starting DNA concentrations ($\mathbf{ng \cdot \mu L^{-1}}$) for undiluted samples and ± standard deviation are given above 10^{-1} dilutions. PCR results: N = no band at any imager setting; F = faint and very low brightness, may require manually increasing exposure to see; M = moderately bright band clearly visible at default imager settings, cleaner than next category; H = very bright band compared to and noticeably thicker than other categories under any imager settings. Amplified fragment sizes include the COI target sequence, forward and reverse primers and hypothetical procedural adapters (forward adapter 5'-end→3'-end = ACCTGCCTGCCG; reverse adapter 5'-end→3'-end = ACGCCACCGAGC). Means and standard deviations for undiluted DNA concentrations and 260/280 and 230/260 ratios are from 3-6 NanoVue spectrophotometer replicates.

Target species DNA extract	Forward primer 5'-end→3'-end sequence Reverse primer 5'-end→3'-end sequence	# base pairs in amplified fragment	Mean 260/280 ratio ± SD	Mean 230/260 ratio ± SD	PCR results at this annealing temperature (°C)					
					50.0	52.5	55.0	57.5	60.0	62.5
B. longimanus	**ACCTGCCTGCCGGCTGAGTTGGGACAGGCAGGG**	**279**								
207.3 ± 0.3	**ACGCCACCGAGCTGCTCCACTCTCTACGGCCCC**		**2.1 ± 0.02**	**2.1 ± 0.06**	**H**	**H**	**H**	**H**	**H**	**H**
10^{-1}					**M**	**M**	**M**	**M**	**M**	**H**
10^{-2}					**F**	**F**	**F**	**F**	**F**	**F**
10^{-3}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-4}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-5}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-6}					**N**	**N**	**N**	**N**	**N**	**N**
C. pengoi	**ACCTGCCTGCCGGGGCCCCTGATATGGCTTTCCC**	**338**								
24.3 ± 2.4	**ACGCCACCGAGCGGCTGTGATTCCAACAGCTCAAACA**		**2.5 ± 0.23**	**8.2 ± 0.57**	**H**	**H**	**H**	**H**	**H**	**H**
10^{-1}					**H**	**H**	**H**	**H**	**H**	**H**
10^{-2}					**H**	**H**	**H**	**H**	**H**	**H**
10^{-3}					**M**	**M**	**M**	**M**	**M**	**H**
10^{-4}					**F**	**F**	**F**	**F**	**F**	**F**
10^{-5}					**N**	**N**	**F**	**F**	**F**	**N**
10^{-6}					**N**	**N**	**N**	**N**	**N**	**N**
D. r. bugensis	**ACCTGCCTGCCGAGCATTGTTAAGGCACCGGCT**	**295**								
9.4 ± 1.4	**ACGCCACCGAGCAGGGCGGATTTGGTGGGGGT**		**1.2 ± 0.02**	**0.6 ± 0.14**	**H**	**H**	**H**	**H**	**H**	**H**
10^{-1}					**H**	**H**	**H**	**H**	**H**	**H**
10^{-2}					**M**	**M**	**M**	**M**	**M**	**H**
10^{-3}					**F**	**N**	**F**	**F**	**F**	**M**
10^{-4}					**F**	**F**	**F**	**F**	**F**	**F**
10^{-5}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-6}					**N**	**N**	**N**	**N**	**N**	**N**
D. polymorpha	**ACCTGCCTGCCGAGCATTGTTAAGGCACCGGCT**	**295**								
1026.0 ± 5.9	**ACGCCACCGAGCAGGGCGGATTTGGTGGGGGT**		**1.9 ± 0.01**	**2.3 ± 0.02**	**M**	**M**	**H**	**H**	**M**	**H**
10^{-1}					**M**	**M**	**H**	**H**	**M**	**H**
10^{-2}					**F**	**F**	**H**	**H**	**M**	**H**
10^{-3}					**F**	**F**	**M**	**M**	**F**	**M**
10^{-4}					**N**	**N**	**F**	**F**	**N**	**N**
10^{-5}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-6}					**N**	**N**	**N**	**N**	**N**	**N**
H. anomala	**ACCTGCCTGCCGTTGGGTCAGCCCGGTAGGTT**	**283**								
127.2 ± 0.3	**ACGCCACCGAGCTCCACCCCGTACCAACCCCC**		**2.0 ± 0.03**	**2.2 ± 0.06**	**H**	**H**	**H**	**H**	**H**	**H**
10^{-1}					**M**	**M**	**M**	**M**	**M**	**M**
10^{-2}					**F**	**F**	**F**	**F**	**F**	**F**
10^{-3}					**N**	**N**	**F**	**F**	**F**	**F**
10^{-4}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-5}					**N**	**N**	**N**	**N**	**N**	**N**
10^{-6}					**N**	**N**	**N**	**N**	**N**	**N**

Polymerase Chain Reaction, Serial Dilutions

PCR conditions were identical among the AIS primer sets for annealing temperature and DNA concentration sensitivity gradients. Total volume in a single PCR was 25 µL consisting of: 2.5 µL of 10X Taq reaction buffer (Bio Basic, Cat. #37A), 0.5 µL each of 10 µM forward and reverse primer, 0.1 of µL Taq polymerase at 5 $\text{units} \cdot \mu L^{-1}$ (Bio Basic, Cat. #HTD0078), 1.0 µL of 10 µM dNTPs, 3.5 µL of 20 mM $MgSO_4$ (Bio Basic Cat. #37B), 0.2 µL of 20 $\mu g \cdot \mu L^{-1}$ bovine serum albumin (BSA), 1.0 µL of sample DNA from one of the undiluted through 10^{-6} diluted stocks, and remaining total volume from nuclease free Milli-Q water. PCR thermal cycling consisted of a single initial denaturation cycle at 94 °C for 2 minutes; followed by 35 cycles of: a) denaturation at 94 °C for 45 seconds; b) annealing at a given gradient temperature for 30 seconds (details below); and c) extension at 72 °C for 45 seconds. After the 35 cycles there was a final single cycle of extension at 72 °C for 10 minutes and samples were held at 4-8 °C until removal. Six annealing temperatures were used for every experimental sample: 50.0, 52.5, 55.0, 57.5, 60.0, and 62.5 °C to test for the effects of annealing temperature on sensitivity. I included one positive control for each AIS using subsampled mastermix and either undiluted or 10^{-1} serially diluted target DNA, essentially replicating a test sample. For each target I used two negative controls: a 24 µL aliquot of mastermix that was also used for test-PCRs, plus 1 µL Milli-Q water added after loading all samples; and a 24 µL aliquot of the same mastermix which was placed in a PCR well alongside all sample preparations but to which no other components were added. One annealing temperature of 60.0 °C was used for controls.

To determine amplification success, I combined 10 µL of PCR product with 5 µL of 1X loading dye from which 6.5 µL was used in 2 % agarose gel electrophoresis. I examined fully resolved gels under UV-B light on a GelDoc™ XR Molecular Imager using Quantity One 1-D

Image Analysis Software (BioRad Laboratories Inc.). I used a quaternary scoring system for visual determinations of sensitivity: 1) N = no bands at any imager settings; 2) F = faint band, very low brightness, may require manually increasing exposure to reveal; 3) M = moderately bright band visible at default imager settings, thinner than next category; and 4) H = very bright band noticeably thicker than any other categorical bands at any imager settings. From amplified band presence on gels and serial dilution estimates I could later determine sensitivity. I also determined quantitative band intensity values for amplicons on gels using automatic image software settings and some manual adjustments to estimate combined measurements of two-dimensional amplified band area and band brightness. Band intensity trends at annealing temperatures were plotted against DNA concentration gradients for each target species to develop sensitivity curves. Because of several magnitudes of differences among the lowest and highest DNA concentrations, I used default settings and a $\log_{10}$ function in SPSS© (Version 20, IBM©, Chicago, Illinois) to normalize values across the x axis. The $\log_{10}$ transformation was modified to handle zero, low and negative values and is formulated as: sign(x) * log(1 + abs(x)). With an axis value of -99, the result of the transformation would be: sign(-99) * log(1 + abs(-99)) = -1 * log(1 + 99) = -1 * 2 = -2. “Sign” in SPSS is used to determine the sign of number (x). This operation returns -1, 0, or 1 if (x) is an integer. If (x) is a real number, sign (x) in SPPS returns -1.0, 0.0, or 1.0, depending on whether (x) is negative, zero, or positive.

DNA interference experiments

I also wanted to determine primer set sensitivities in mixed PCRs containing target and non-target DNA in a DNA interference experiment. PCRs were performed as described with some adjustments. Non-target DNA mixes for *Bythotrephes longimanus, Cercopagis pengoi* and *Hemimysis anomala* were composed of DNA from the four other species (Table 2.2). The fourth

Table 2.2: DNA concentrations ($ng \cdot \mu L^{-1}$) used in DNA interference and primer multiplexing experiments. Where a target row and column meet four bolded values are DNA concentrations used in individual mixed PCRs containing non-target DNA in amounts listed across respective rows. The "Amplification success in mix" column indicates visual levels of sensitivity for mixed PCRs. The last two columns are results for multiplexing trials that used high and low concentrations of all four primer sets in a PCR and apply only to values in bold text for respective rows indicating the target DNA concentration used. For amplification success after electrophoresis on agarose gels visualized under UV light: N = no band at any imager setting; F = faint and very low brightness, may require manually increasing exposure to see; M = moderately bright band clearly visible at default imager settings, cleaner than next category; H = very bright band compared to and noticeably thicker than other categories under any imager settings.

Target	*B. longimanus*	*C. pengoi*	*D. r. bugensis*	*D. polymorpha*	*H. anomala*	% target DNA mix	Amplification success in mix	Mplex High	Mplex Low
B. longimanus	**207.300**	6.030	3.540	9.280	31.750	80.35	H	M	M
	20.730	6.030	3.540	9.280	31.750	29.03	M	M	M
	2.073	6.030	3.540	9.280	31.750	3.93	F	F	F
	0.207	6.030	3.540	9.280	31.750	0.41	N	N	N
	0.021	6.030	3.540	9.280	31.750	0.04	N	N	N
C. pengoi	25.900	**24.300**	5.890	9.280	15.900	29.90	H	M	M
	25.900	**2.430**	5.890	9.280	15.900	4.09	H	M	M
	25.900	**0.240**	5.890	9.280	15.900	0.42	H	M	M
	25.900	**0.020**	5.890	9.280	15.900	0.03	M	F	F
	25.900	**0.002**	5.890	9.280	15.900	0.003	F	N	N
D. r. bugensis	34.500	4.050	**9.400**	0.000	21.170	13.6	H	F	F
	34.500	4.050	**0.940**	0.000	21.170	1.55	H	N	N
	34.500	4.050	**0.090**	0.000	21.170	0.15	H	N	N
	34.500	4.050	**0.009**	0.000	21.170	0.02	M	N	N
	34.500	4.050	**0.001**	0.000	21.170	0.002	N	N	N
D. polymorpha	34.500	4.050	0.000	**102.600**	21.170	63.21	H	N	N
	34.500	4.050	0.000	**10.260**	21.170	14.66	H	N	N
	34.500	4.050	0.000	**1.026**	21.170	1.69	M	N	N
	34.500	4.050	0.000	**0.103**	21.170	0.17	M	N	N
	34.500	4.050	0.000	**0.010**	21.170	0.017	N	N	N
H. anomala	20.700	21.870	2.830	22.260	**127.200**	65.28	H	N	N
	20.700	21.870	2.830	22.260	**12.720**	15.82	H	N	N
	20.700	21.870	2.830	22.260	**1.270**	1.84	M	N	N
	20.700	21.870	2.830	22.260	**0.127**	0.19	M	N	N
	20.700	21.870	2.830	22.260	**0.013**	0.019	N	N	N

non-target mix was equally composed for *Dreissena rostriformis bugensis* and *Dreissena polymorpha* and excluded each as non-targets because the single target primer set amplified both of these target species. Non-target DNA sum concentrations ranged from 48-62 $ng \cdot \mu L^{-1}$ (Table 2.2) of which 1 μL was added to 24 μL of target mastermix composed as previously described for gradient tests. I then spiked 1 μL of sample from one of the undiluted through 10^{-4} target DNA serial dilutions into an individual PCR with respective mastermix and already added non-target DNA (Table 2.2) for a total reaction volume of 26 μL. PCR thermal cycling was as described previously for gradient tests except only a single annealing temperature of 62.5 °C was used. For each target, a positive control consisted of target mastermix and 1 μL of undiluted or 10^{-1} target DNA without additions of non-targets, expecting clean amplification. Two negative controls for each target were conducted as described above, one with Milli-Q water, one lacking additions. Thus, there were five samples and three controls for each AIS in mixed PCR DNA interference experiments (Table 2.2). Limitation of heat and light exposure, electrophoresis, and UV-B imaging were completed as previously described.

Primer set multiplexing experiments

I multiplexed all four primer sets in PCRs containing DNA only from single targets. I performed primer multiplexing as described for gradient tests with minor changes. In a single PCR for multiplexing trial one I used an equal amount of each forward and reverse primer from each primer set (0.5 μL for each forward and reverse primer at of 10 μM concentration) as used in gradient tests and in trial two I used half the amount of each forward and reverse primer as used in individual gradient test PCRs (0.25 μL each of 10 μM forward and reverse primer), but unlike gradient tests, single PCRs in both multiplexing trials contained all four primer sets. In both multiplexing trials I used 1.0 μL of undiluted through the 10^{-5} diluted DNA from serial

dilutions in individual PCRs. Thus, for each target I performed 12 primer multiplexed PCRs, six at each primer concentration. For each target DNA test I also used two negative controls and a thermal cycling profile as previously described for gradient tests but only used a single annealing temperature of 62.5 °C, chosen to increase PCR primer annealing stringencies. I combined all individual PCR product with 10 µL of loading dye, mixed and subsampled 10 µL for agarose gel electrophoresis. Limitation of heat and light exposure, electrophoresis, and UV-B imaging conditions in multiplexing trials were completed as described for gradient tests.

Data Analyses

Lastly, I performed a Primer Basic Local Alignment Search Tool (BLAST) search against my primer sequences on GenBank to determine if non-target species DNA might co-amplify. Under Primer Pair Specificity Checking Parameters I set search mode to automatic, database to "nr" to expand the search for more potential matches, and the organism option was filled in at multiple entries to include all available derivations of the target species and genus and included the target order as an upper taxonomic cut-off for comparisons. I set annealing temperature ranges from 50-70 °C with a maximum difference of 10 °C between the forward and reverse primers, and removed exon junction default values. Primer BLAST stringency settings determine the required and allowed numbers and locations of sequence mismatches at annealing sites of non-targets sequences compared to my primer sequences. Lowering stringency permits greater mismatches increasing results while the highest stringency settings permit no such mismatches and respective results should essentially be target-specific. I set three parameters to apply moderate to high stringency: 1) maximum sum of mismatches at any nucleotides in either or both of the forward and reverse primer annealing sites of non-target sequences = two; 2) maximum number of the mismatches from number #1 located in non-target sequence annealing

sites at nucleotides respective to and in the last five bps of the 3'-ends of either one or both of the forward or reverse primers = one; and lastly 3) the allowed maximum sum of all mismatches at nucleotides at any positions in forward and reverse primer annealing sites of non-target sequences including those required to meet both conditions 1 and 2 above = three.

Results and Discussion

My aim was to develop and characterize metabarcoding PCR primer sets targeting a highly specific shortened portion of the COI barcoding region for five AIS that are present in, and negatively impacting, the Great Lakes. Some of the target AIS are listed among the world's worst 100 invaders (Lowe et al., 2000). Results detailed below reflect that my primers lacked degeneracies, were sensitive to low DNA concentrations, appeared robust to annealing temperature gradients, performed well when targets were diluted among highly concentrated mixes of non-target DNA, and exhibited some potential for multiplexing.

Undiluted and serially diluted single species DNA detection limits

The lowest positive detections of DNA for primer sets were: *Bythotrephes longimanus* JBTHF/JBTHR = 2.07 $\mathrm{ng \cdot \mu L^{-1}}$; *Cercopagis pengoi* JCPGF/JCPGR = 0.000243 $\mathrm{ng \cdot \mu L^{-1}}$; JDBPF/JDBPR = 0.000940 $\mathrm{ng \cdot \mu L^{-1}}$ (*Dreissena rostriformis bugensis*) and = 0.103 $\mathrm{ng \cdot \mu L^{-1}}$ (*Dreissena polymorpha*); and *Hemimysis anomala* JHMYF/JHMYR = 0.127 $\mathrm{ng \cdot \mu L^{-1}}$ (Table 2.1). Actual sensitivity likely falls between lowest detections and values at subsequent dilutions that gave negative results and applies to target DNA diluted into low volumes of purified water. Except *Hemimysis anomala,* targets are often found in pelagic offshore waters of large deep oligotrophic lakes and DNA concentrations at my sensitivity levels are expected in eDNA from composited zooplankton tows potentially containing these taxa originating from similarly

oligotrophic conditions. In lab tests, Ardura et al. (2015) developed species-specific primers targeting a 200 bp fragment of 16s rDNA for the AIS bivalve *Rangia cuneata* and spiked a single serially diluted target into only distilled water and also spiked diluted target DNA into highly concentrated non-target DNA in mixed PCRs. Those authors found similar relative sensitivities in both experiments and detection limits (0.40 $ng \cdot \mu L^{-1}$) to those from my gradient and interference tests (Table 2.1 vs. Table 2.2). My results were supported by contamination-free negative controls (blanks) and all amplified bands were clear, without stutter, smearing or notable primer-dimer.

All primer sets amplified undiluted target DNA at all PCR annealing temperatures, although medium and high annealing temperatures generally resulted in higher band intensity compared to low annealing temperatures (Table 2.1, Figure 2.1). Band intensity at annealing temperatures across all DNA concentrations showed little variation for *Hemimysis anomala* whereas *Bythotrephes longimanus* showed the most such variation among target DNA concentrations (Figure 2.1). Relative band intensities in gradient tests were similar to trends for visual based determinations of amplification (Table 2.1, Figure 2.1). For example, especially at lower DNA concentrations, *Cercopagis pengoi* (JCPGF/JCPGR) primer set band intensities exceeded band intensities for all other primer sets at all annealing temperatures (Figure 2.1) and was the most sensitive set based on visual observations (Table 2.1). Variation in sensitivity likely resulted from minor procedural variation among PCRs and quantitative gel imaging protocols.

DNA Interference Experiments

My mixed PCR DNA interference experiments used only a single (optimal) annealing temperature, but detected target DNA even at very low concentrations relative to the non-target

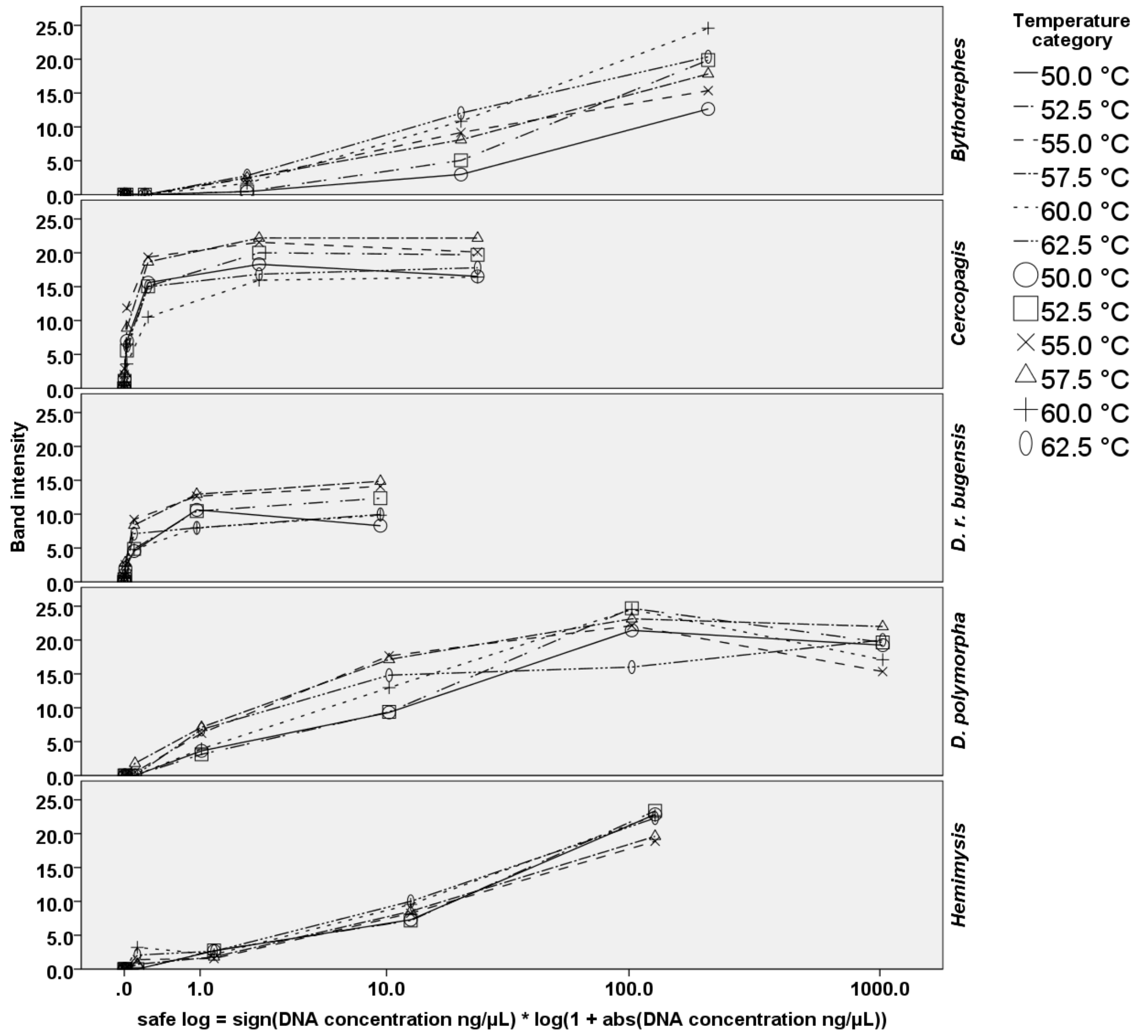

Figure 2.1: Extinction curves for target AIS annealing temperature and DNA concentration gradient tests. Band intensities account for combined quantitative measurements of band area and band brightness determined through software-based imaging of PCR amplicons on agarose gels under UV light. DNA concentrations ($\mathbf{ng \cdot \mu L^{-1}}$) for targets were made from serial dilutions of the highest starting concentrations of target DNA. Because of several orders of magnitudes of differences among the lowest and highest DNA concentrations I used a "safe" base 10 logarithmic function to normalize values across the x axis.

DNA (Table 2.2, Figure 2.2). Because target fragment sizes differed, and through my use of positive controls I am confident that non-target DNAs did not amplify. As a percentage of total DNA in a mixed PCR, the lowest spiked target DNA concentration which amplified were for *Bythotrephes longimanus* = 3.9 %, *Cercopagis pengoi* = 0.0030 %, *Dreissena rostriformis*

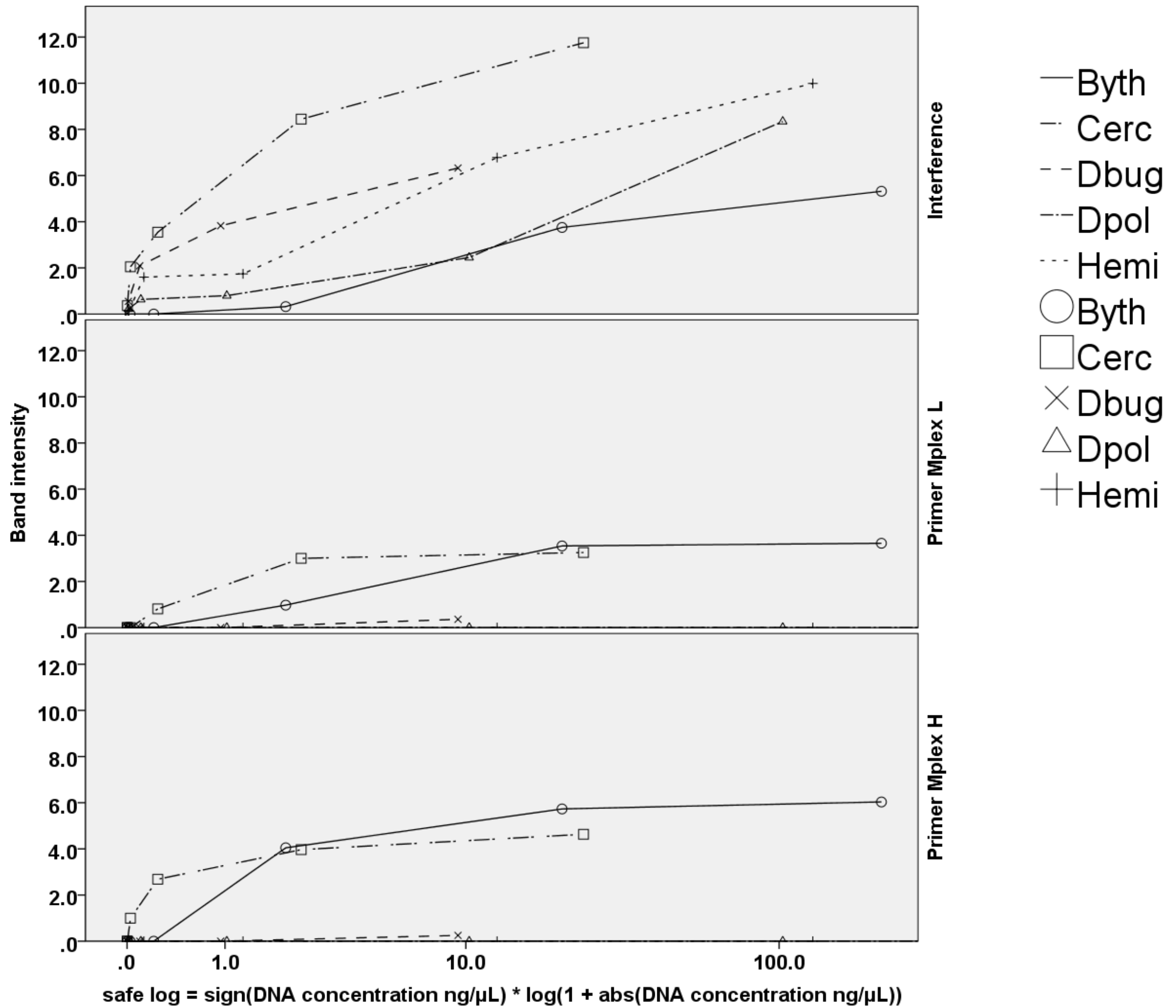

Figure 2.2: Results from DNA interference (top panel) and primer multiplexing experiments (lower two panels). Band intensities account for combined quantitative measurements of band area and band brightness determined through software-based imaging of PCR amplicons on agarose gels under UV-B light. DNA concentrations ($\mathbf{ng \cdot \mu L^{-1}}$) for targets were made from serial dilutions of the highest starting concentrations of target DNA. Because of magnitudes of orders of differences among the lowest and highest DNA concentrations I used a "safe" base 10 logarithmic function to normalize values across the x axis. Legend abbreviations for targets: Byth = *Bythotrephes longimanus*, Cerc = *Cercopagis pengoi*, Dbug = *Dreissena rostriformis bugensis*, Dpol = *Dreissena polymorpha*, and Hemi = *Hemimysis anomala*.

bugensis = 0.020 %, *Dreissena polymorpha* = 0.17 %, and *Hemimysis anomala* = 0.019 % (Table 2.2). Visual based determinations of sensitivity in mixed PCR DNA interference experiments were equal to the sensitivities determined in the non-mixed concentration gradient tests for *Bythotrephes longimanus*, *Dreissena polymorpha,* and *Hemimysis anomala*, were

similar for *Cercopagis pengoi,* and were slightly reduced for *Dreissena rostriformis bugensis* (Table 2.1 vs. Table 2.2) despite additions of 48-62 ng of non-target interfering DNA. Low detection limits in mixed interference tests are similar to Zhan et al. (2013) who identified a single target spiked in a mixed PCR at as low as $2.3 \cdot 10^{-5}$ % by concentration and were likewise similar to results obtained by others (e.g. Pochon et al., 2013; Ardura et al., 2015). Mixed PCR DNA interference results suggest primer sets can work well when applied to eDNA and scDNA based samples perhaps containing target DNA at very low concentrations relative to other taxa.

Primer Set Multiplexing

My primer multiplexing trials were met with some successes (Table 2.2, Figure 2.2). Of sixty multiplexed PCRs that each contained all four primer sets and a DNA sample from one target, *Bythotrephes longimanus* accounted for six positive results, *Cercopagis pengoi* for six, and *Dreissena rostriformis bugensis* for two positive results (Table 2.2, Figure 2.2). Visual determinations of amplicon presence and quantitative band intensities for each of these targets in both multiplexing trials were nearly identical to one another despite differences in PCR primer concentrations and Milli-Q water (Table 2.2, Figure 2.2). *Bythotrephes longimanus* amplified in multiplexed PCRs to the same visual levels using serial dilutions as for gradient and interference tests (Table 2.1, Table 2.2). *Cercopagis pengoi* amplified in multiplexed PCRs, but was relatively less sensitive by one order of magnitude compared to results for mixed PCR DNA interference tests (Table 2.2). Thus, these primer sets showed multiplexing potential; however, band intensities at DNA concentrations that amplified were reduced relative to gradient and interference tests (Figure 2.1, Figure 2.2). Undiluted *Dreissena rostriformis bugensis* DNA amplified equally in both multiplexing trials but with only very faint trace visual bands, and also exhibited relatively lower band intensity than in gradient and interference tests. Thus, the

Dreissena rostriformis bugensis and *Dreissena polymorpha* (JDBPF/JDBPR) primer set appears least suited of the primer sets that amplified in multiplexing for such applications. All multiplexing results observed for *Dreissena polymorpha* and *Hemimysis anomala* primer sets were negative. Multiplexing all my primer sets is appealing to screen for these AIS but would require further research as the exact causes of reduced sensitivities are unknown, but likely related to primer interference. However, as the single *Dreissena rostriformis bugensis* and *Dreissena polymorpha* (JDBPF/JDBPR) primer set amplifies both targets, and *Bythotrephes longimanus* (JBTHF/JBTHR) and *Cercopagis pengoi* (JCPGF/JCPGR) primer sets appear to have high potential for joint multiplexing there may be some potential for reducing workload through fewer PCRs.

Primer Set backBLASTing

Primer backBLAST searches against the entire GenBank databases returned only the expected target species sequences for primer sets: *Bythotrephes longimanus* (JBTHF/JBTHR), *Cercopagis pengoi* (JCPGF/JCPGR) and *Hemimysis anomala* (JHMYF/JHMYR). Non-target sequences for the *Dreissena rostriformis bugensis* and *Dreissena polymorpha* (JDBPF/JDBPR) primer set were returned for *Dreissena caputlacus*, noted as both endangered and invasive in Europe (Gelembiuk et al., 2006; Smith et al., 2014). Because my primer sets lack degeneracies, I believe they are highly specific to intended targets; however, NGS is a required step post-PCR for unambiguous species confirmations. As NGS is increasingly accessible and affordable but requires well designed primer sets, my novel designs and characterizations of primer set dynamics facilitate the transition to steps for their use in NGS. Although my primer sets may lack true species specificity, to develop such tools can be a complex undertaking and include rigorous in-silico, in-vitro, and/or in-situ based PCR testing of confirmed non-target voucher

sequences and samples (Cho et al., 2016). Such testing can be narrowed relative to specific study systems and through means such as BLAST searches, but approaches often rely upon queried results from databases such as GenBank which may lack reliability or potentially amplifiable reference sequences for yet to be catalogued non-targets (Cristescu 2014). Developing truly species-specific primer sets can likewise be complicated for globally distributed taxa, such as the AIS in my study, because of possible co-occurrences among potential regional ecosystem specific non-targets in eDNA or scDNA based samples which might co-amplify or competitively interfere in PCRs (Comtet et al., 2015). Hypothetically, to overcome non-target amplification confirmed by NGS, my mixed DNA interference test methodologies could be followed to determine if a target was present but unsuccessfully amplified because of the known non-target presence. This approach might also permit estimations of DNA concentrations at which these interactions may occur, if they occur differentially across concentration gradients, and ultimately would require less effort than designing and testing truly species specific primer sets.

Conclusion

Metabarcoding primer sets can have different taxonomic levels of specificity from being oriented towards amplification of single species (such as those presented here) through more “universal” PCR primers designed to amplify and capture entire communities from eDNA samples (e.g., Leray & Knowlton 2015). Metabarcoding is becoming the norm for eDNA analyses due to increasingly accessible and affordable NGS platforms. The metabarcoding primer sets I designed and characterized provide researchers studying these invertebrate AIS with valuable tools for their identification even at very low abundance. This is especially valuable when target AIS are entrained in transport vectors, present as difficult-to-identify life

stages (e.g. eggs), or when sample DNA is degraded (e.g. such as digested in predator stomach contents). The highly sensitive nature and the relative unambiguity of amplified sequence determinations from my primer sets when used with downstream NGS will make them useful as management tools to help limit the further spread and establishment of five of the world's and Great Lake's worst AIS.

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CHAPTER 3 - THE ROLE OF INVASIVE AND NATIVE ZOOPLANKTON IN FISH DIETS FROM OFFSHORE LAKE MICHIGAN: CO1 METABARCODING OF STOMACH CONTENT DNA (scDNA).

Introduction

Analyses of aquatic foodwebs have widespread applications in conservation biology, ecosystem-based resource management and in quantifying the roles and impacts of aquatic invasive species (AIS; Winemiller 1989; Link et al., 2008; Ricciardi & MacIsaac 2011; Hussey et al., 2011; Brush et al., 2012). AIS generally pose the second largest threat to aquatic biodiversity after habitat loss and disrupt foodwebs by causing species extinctions (Barel et al., 1985; Kaufman 1992; Ricciardi & MacIsaac 2011), inducing bottom-up or top-down trophic cascades in energy flow (Johnson et al., 2005; Bunnell et al., 2009; Rush et al., 2012), increasing homogenization of biological communities (Rahel 2002), decreasing biotic resistance to further invasion or disruption (Scott & Helfman 2001), outcompeting native species (Ricciardi et al., 1998), consuming eggs and larvae of native species and impacting recruitment (Tyus et al., 2000), disrupting nutrient dynamics (Matsuzaki et al., 2009) and acting as vectors for diseases and parasites (Vitule et al., 2009). Thus, resource managers need quantitative estimates of AIS roles in aquatic foodwebs such as in the above considerations to manage and mitigate these and other impacts; however, at present, ongoing range expansions and complex ecological dynamics of AIS are relatively poorly understood, especially for hard-to-access areas of large ecosystems.

The Laurentian Great Lakes (hereafter Great Lakes) have experienced numerous species introductions and invasions post-European settlement and are also hard to sample due to their size and complexity. At least 185 established non-native species are present in the Great Lakes (Wells & McClain 1973; Mills et al., 1993; Ricciardi & MacIsaac 2000). Since invading the upper Great Lakes in the early 20th century, the alewife (*Alosa pseudoharengus*) has limited the natural recruitment of lake trout (*Salvelinus namayacush*) through its predation on lake trout larvae and through its contributions as a prey fish to thiamine deficiency in adult predatory lake

trout (Krueger et al., 1995; Brown et al., 2005; Honeyfield et al., 2005; Madenjian et al., 2008). Rainbow smelt (*Osmerus mordax*), while an important prey species in the Great Lakes, also can negatively impact recruitment of native pelagic fishes (Loftus & Hulsman 1986; Myers et al., 2009). Resource competition among these fishes is difficult to demonstrate without measures of prey abundance; however, diet overlap among rainbow smelt, alewife and native fishes has been noted as moderate to high in offshore Great Lakes regions (Bunnell et al., 2015). Competition for some microcrustacean prey may also occur among alewife, rainbow smelt, and native fishes (Pothoven et al., 2009). With a rapid increase in established AIS in the Great Lakes over past decades (Lodge et al., 2006; Simberloff 2006), many AIS occupy a wide diversity of trophic levels and are now important as predators and as prey (Storch et al., 2007; Paterson et al., 2014; Keeler et al., 2015; Bunnell et al., 2015). For example, native invertebrates commonly consumed by the invasive alewife and rainbow smelt and the native fishes ninespine stickleback (*Pungitius pungitius)*, slimy sculpin (*Cottus cognatus*) and bloater (*Coregonus hoyi*) became extremely rare following introduction of the macroinvertebrate AIS *Bythotrephes longimanus* (Lehman 1991; Lehman & Cáceres, 1993; Barbiero & Tuchman, 2004; Bunnell et al., 2011). However, *Bythotrephes longimanus* is itself now a significant seasonal dietary component for some of the same predator species (Keeler et al., 2015; Staton et al., 2014). Additional key AIS that have substantially impacted Great Lakes foodwebs include the zebra mussel (*Dreissena polymorpha*), quagga mussel (*Dreissena rostriformis bugensis)*, a predatory waterflea *Cercopagis pengoi*, and the bloody red shrimp *Hemimysis anomala* (Pothoven & Madenjian, 2008; Bunnell et al., 2011; Ricciardi & MacIsaac 2011; Madenjian et al., 2015). Quantifying the roles of these AIS in foodwebs can help determine longer-term management strategies for mitigating their impact on the Great Lakes basin ecosystems and native inhabitant taxa.

Characterization of the roles of AIS in foodwebs has been traditionally performed through visual diet assessment (i.e., Garrison & Link 2000; Mychek-Londer et al., 2013; Landry et al., 2017). However, such methods can be biased by differential digestion rates, plus they are time consuming (Sutela & Huusko 2000; Schooley et al., 2008; Legler et al., 2010). Cryptic species in diets are difficult to identify even for well-trained taxonomists, especially when prey are partially digested (Briski et al., 2010; Jackson et al., 2014), reducing the identification potential of AIS and limiting potential early management responses (Puth & Post 2005; Folino-Rorem et al., 2009; Vander Zanden et al., 2010). Mitochondrial cytochrome oxidase subunit 1 (CO1; Hebert, et al., 2003a; Hebert et al., 2003b; Hebert & Gregory 2005) metabarcoding of multiple taxa or targeted species such as AIS from stomach content DNA (scDNA) can be helpful in addressing such diet determination shortcomings (Taberlet et al., 2012; Comtet et al., 2015).

There has been a recent increase in the use of metabarcoding to describe scDNA diet samples for wide ranging applications. For example, Fayle et al. (2015) found that scDNA extracted from 15 ant species sampled in the rainforests of Gabon had termite prey DNA confirmed to the family level or better. Leray et al. (2015) used a metabarcoding approach to describe diets of coral reef fishes and demonstrated that highly complex interactions in the foodweb occurred and that levels of trophic partitioning among spatiotemporally overlapping fishes were high. Sensitivity to detect target prey species or groups of species using such "universal" primer sets coupled with metabarcoding can be high (Pochon et al., 2013; Zhan et al., 2013). Furthermore, the sequence data itself permits accurate identification of the prey species if the prey barcode sequence is archived. While metabarcoding is powerful, false negatives can result from factors including: stochastic PCR error, PCR inhibitors, amplification

bias, within-species sequence variation, inadequate replication, and low overall or relative DNA concentration in mixed samples (Darling & Mahon 2011; Ficetola et al., 2008; Ficetola et al., 2014).

While published studies have examined diet using metabarcoding of scDNA to detect AIS, the Great Lakes, and especially offshore deepwater regions are particularly under-studied in these contexts. A metabarcoding approach to analyze diets of fishes from these systems could prove useful as a tool to determine AIS presence and thus apply as a methodology for predatory congeners across similarly already invaded and at-risk ecosystems. Likewise, the high sensitivity of metabarcoding could provide data not otherwise obtainable through traditional, well-established methods for describing diets and can thus better inform ongoing restoration and reestablishment of these deepwater ecosystems and native inhabitants in the Great Lakes (Zimmerman & Krueger 2009).

The goal of this study is to provide quantitative information on the presence-absence and roles of AIS in the foodweb of Lake Michigan. Specifically, I test whether metabarcoding is effective in determining AIS occurrences in diets of fishes in large offshore aquatic ecosystems. My three main objectives in this study were thus to use CO1 metabarcoding of scDNA to: 1) determine presence-absence of target AIS, and abundant or ecologically important native prey species in diets; 2) determine if variation in prey presence in predator diets existed across space and time; and 3) to assess whether specific abiotic and biotic factors affected AIS occurrences in scDNA. Metabarcoding scDNA will help managers in their understanding of the roles of non-native and native predators and of AIS as prey in the offshore Lake Michigan foodweb and ecosystem. Also, my methodological approach has applicability as a tool for similar analyses of

zooplanktivorous fish diets and thus for resource managers tasked with prevention and slowing expansions of new populations of these and other AIS beyond the Great Lakes.

Methods

Collections

Alewife, bloater, ninespine stickleback, rainbow smelt, and slimy sculpin were collected April 1 to April 15, in one or both years of 2009 and 2010 using bottom trawls for 5 to 10 minutes at three offshore sites in Lake Michigan (Figure 3.1; Table 3.1). Offshore sites at Frankfort, MI (44° 30′39″N, 86 20′18″W) and Sturgeon Bay, WI (44° 42′1″N, 87 21′26″W) were sampled using a 13 m Yankee trawl. Offshore of Two Rivers, WI the site (44° 17′57″N, 87 21′26″W) was sampled using a 31 m otter trawl. Trawled depths included 73, 82, 91, 99, 110, and 128 m (Table 3.1). All fishes collected in a trawl, or a subsample of the catch if catches were very large were immediately sorted by species, and up to 60 samples per species, per trawl, were subsampled from the catch and immediately frozen on board at -20 °C for later subsampling and stomach content recovery.

Table 3.1: Collection details for zooplanktivorous fishes. Predator fish TL = total length (nearest millimeter). All samples were taken in Julian Days of the Year 101-112 (April 1-15). N = 376 total samples. Because of limited sample availability, fishes at Frankfort were subsampled from three to five depth strata (including 73, 82, 91, 110 and 128 m). Sturgeon Bay and Two Rivers fishes were sampled from single depths of 82 and 99 m respectively. Full fish predator species names: Alewife = *Alosa pseudoharengus*, Bloater = *Coregonus hoyi*, Ninespine stickleback = *Pungitius pungitius*, Rainbow smelt = *Osmerus mordax*, and Slimy sculpin = *Cottus cognatus*.

Predator	Site	Year	Sample size	Mean TL (mm) ± SD
Alewife				
	Frankfort	2009	19	112.6 ± 12.8
	Frankfort	2010	20	103.3 ± 32.6
	Sturgeon Bay	2010	20	80.2 ± 8.2
	Two Rivers	2010	20	124.9 ± 28.9
Bloater				
	Frankfort	2009	19	146.2 ± 50.4
	Frankfort	2010	20	162.6 ± 30.9
	Sturgeon Bay	2010	20	120.1 ± 15.8
	Two Rivers	2010	20	135.2 ± 24.5
Ninespine stickleback				
	Frankfort	2009	19	68.7 ± 6.2
	Frankfort	2010	20	68.2 ± 5.7
	Sturgeon Bay	2010	20	69.7 ± 6.5
	Two Rivers	2010	20	66.3 ± 5.3
Rainbow smelt				
	Frankfort	2009	20	72.6 ± 31.0
	Frankfort	2010	20	104.8 ± 16.1
	Sturgeon Bay	2010	20	117.0 ± 15.0
	Two Rivers	2010	20	101.3 ± 4.5
Slimy sculpin				
	Frankfort	2009	19	72.0 ± 10.7
	Sturgeon Bay	2009	20	67.2 ± 13.6
	Two Rivers	2009	20	75.1 ± 11.0

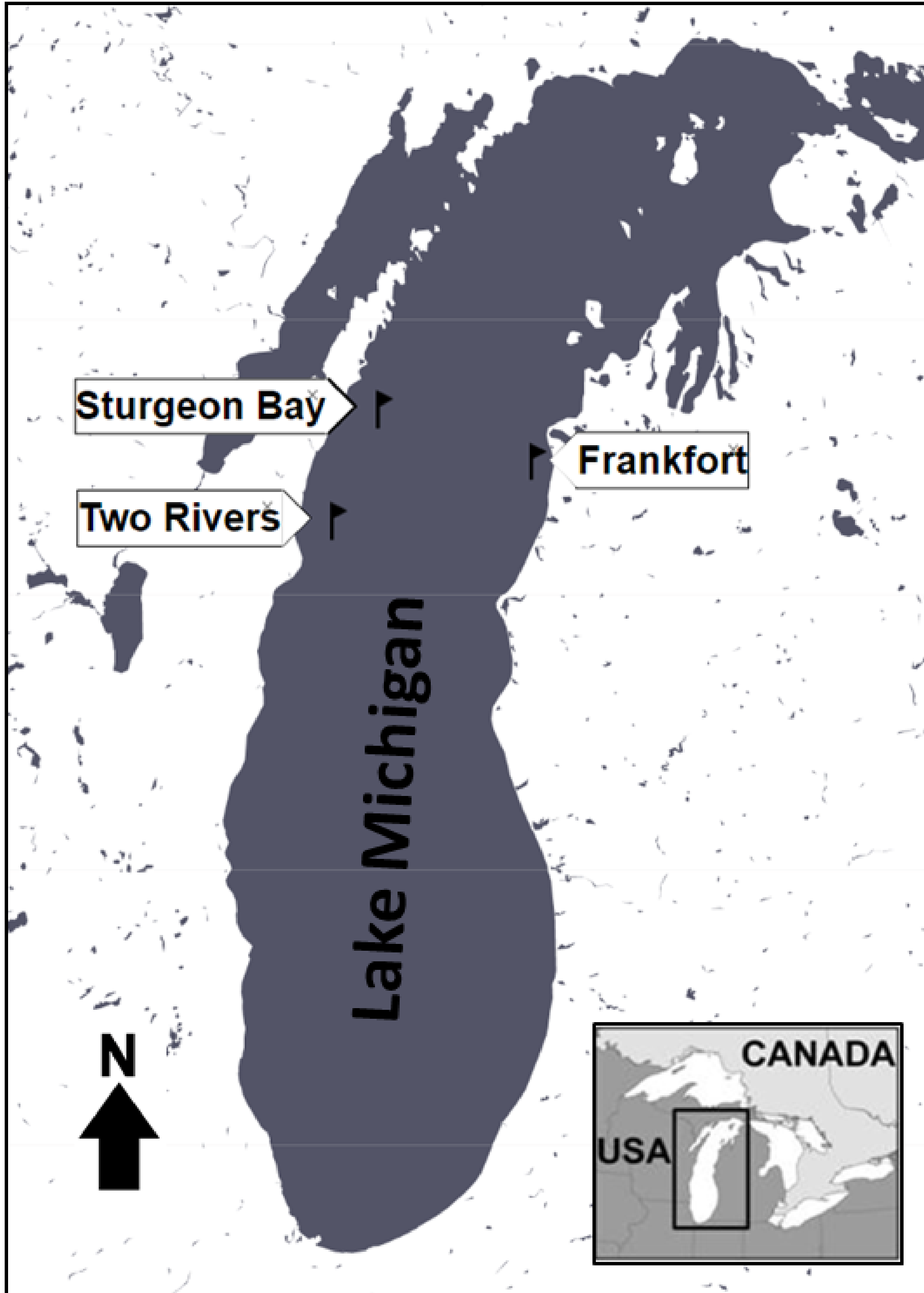

Figure 3.1: Sampling sites for the six predator fish species collected for mitochondrial cytochrome oxidase one (CO1) metabarcoding of stomach content DNA (scDNA) to determine occurrences of and the role of AIS in the Lake Michigan foodweb. Single indicators of geolocated sampling sites are given however, multiple local sites alongside these points were sampled to account different depth strata including at depths of 73, 82, 91, 99, 110, and 128 m.

Stomach content sampling

Fishes were thawed, weighed (0.1 g) and measured for total length (TL, 1.0 mm). Whole stomachs (esophagus to just below pyloric valve) were excised and individually preserved in 95 % ethanol until further processed. To achieve desired sample sizes for each species, I selected fish samples at each site and year based on stratified random sampling for body size and depth. Overall, I aimed for a balanced design and representation of diets from all sizes of a fish species.

At medium to high magnification using fine forceps, all prey materials, conspicuous prey in bolus form, or prey otherwise easily distinguishable were removed from each stomach and placed in 1.7 mL microcentrifuge tubes for DNA digestion. Next, I used small soft-tipped paintbrushes to loosen potential remaining prey tissue, while minimizing predator DNA release and added any remaining recovered prey tissue to the 1.7 mL tube. I used the entire prey contents from individual diet samples, except for the largest individual samples of alewife and bloater. These large-sized samples first required mixing of prey contents to a homogeneous level in a sanitized Petri dish. Sub samples were then obtained from a portion of the homogenous prey mix into respective 1.7 mL tubes for DNA digestion. Sub sampling was necessary as wet prey content for these large samples exceeded 0.60 mL, the target maximum starting volume for DNA extraction due to technical constraints. After transferring prey tissue, I added 95 % ethanol and vortexed contents for 30 seconds at high speed.

Extraction of scDNA

I centrifuged the tubes with the stomach contents at 13,000 x g for 15 minutes at 4 °C, discarded supernatant ethanol from each tube and used an Eppendorf 5301 VacuFuge Centrifugal Vacuum Concentrator to dry the stomach contents in each tube. Immediately after drying the stomach contents I added 200-500 µL of 1.0 mm BioSpec Products glass mill beads and added

600-900 μL of digestion buffer (5.84 g $\mathrm{NaCl \cdot L^{-1}}$, Tris-HCL pH 8.0 final concentration 50 mM, EDTA pH 8.0 final concentration 10 mM, SDS to final concentration of 0.5 %, diluted in ddH_2O). I used a Mini-Beadbeater-24 (Fisher Scientific LTD., BioSpec.) set at 50 strokes per second breaking cycles to homogenize prey followed by 60 seconds of cooling tubes on ice, repeated 3-8 times based on the volume of starting materials and visual inspections.

After homogenizing stomach content samples, I added 8 μL Proteinase-K (20 $\mathrm{mg \cdot mL^{-1}}$), digested at 38 °C with gentle rocking for 8-12 hours. I extracted scDNA from 150 μL of the supernatant using a Tecan Freedom EVO 150 Liquid Handling System and a carboxylate magnetic bead-based protocol, three ethanol washes and a final elution of extracted scDNA into 150 μL 1X TE, lastly sealing and freezing scDNA diet samples in plates at -20 °C.

Polymerase chain reactions

I used five polymerase chain reaction (PCR) primer sets targeting the mitochondrial gene cytochrome oxidase one (CO1) barcoding region to amplify prey DNA sequences. Four sets were used to target five specific AIS including: 1) *Bythotrephes longimanus*; 2) *Cercopagis pengoi;* 3) *Hemimysis anomala*; and 4) *Dreissena rostriformis bugensis* and *Dreissena polymorpha* (See Mychek-Londer Ph.D. Dissertation Chapter 2, 2018). The fifth was a universal CO1 primer set designed for aquatic microinvertebrates (Leray et al., 2013) used previously for scDNA diet samples from nearshore species of Lake Erie fishes (Shortridge 2016).

I used a three-step PCR approach for the target-specific AIS primer sets and a two-step approach for the universal primer where round-one PCRs included a 5'-end extended sequence tag on each primer. Second-round PCRs for the target AIS primer sets used the same protocols as did first-round PCRs, but used cleaned and concentrated DNA from PCR products produced in the first-round reactions to increase detection sensitivity. This second-round of PCRs was not

needed for universal primer set PCR amplicons as most scDNA diet samples successfully amplified after first-round PCRs. Third-round PCRs for the target specific AIS primer sets and second-round PCRs for the universal primer set were the same, and made up of a short-cycle PCR designed to ligate the sample identification sequence barcode and next-generation sequencer (NGS) adaptor sequences for NGS library preparation.

Total reaction volume for a single PCR for each of the five primer sets in first-round PCRs was 25 µL and consisted of: 2.5 µL of 10X Taq reaction buffer (Bio Basic, Cat. #37A); 0.5 µL each of 10 µM forward and reverse primers; 0.1 µL of Taq polymerase at 5 $units \cdot \mu L^{-1}$ (Bio Basic, Cat. #HTD0078); 1.0 µL of 10 µM dNTPs; 3.5 µL of 20 mM $MgSO_4$ (Bio Basic Cat. #37B); 0.2 µL of 20 $\mu g \cdot \mu L^{-1}$ bovine serum albumin (BSA); 1.0 µL of extracted scDNA; and the remaining total volume was nuclease free Milli-Q water. Thermal cycling protocols for all first-round PCRs consisted of: an initial denaturation cycle at 94 °C for 2 minutes; followed by 35 cycles of: a) denaturation at 94 °C for 45 seconds; b) annealing at 59 °C for 30 seconds; and c) extension at 72 °C for 45 seconds. After the 35 cycles there was a final single cycle of extension at 72 °C for 10 minutes followed by a 4 °C hold. I used two positive and negative (blank) controls for all PCRs. Positive controls used DNA extracted from whole and known species samples for the five target AIS. PCR amplification was visually assessed by agarose gel electrophoresis.

To maximize detections of AIS, I used PCR products from all first-round PCRs in additional individual second-round PCRS for the target AIS PCR primer sets and results. These second round PCRs followed the same protocol for the first round PCRs (see above) but used DNA contributions from first-round PCR products. Only one round of PCRs were needed for the universal primer set as many scDNA diet samples amplified successfully based on band

detection on agarose gels. The final second-round PCR products from target AIS primer sets and the final first-round PCR products from the universal primer set were combined for individual scDNA samples based on relative amplification strength to help ensure even sequencing depth. I cleaned each scDNA sample amplicon mix using a magnetic bead protocol which removed small amplified fragments and primer dimers less than 100 base pairs. I used Sera-Mag Speed Beads (GE Healthcare Life Sciences) following the protocol for the Agencourt AMPure XP PCR Purification Beads. Subsamples of cleaned PCR products were run on agarose gels to confirm reduction or removal of dimers and amplicons below 100 bp and the retention of amplified bands of larger size, and products were stored at -20 °C.

PCRs for sequencing library preparation, adding sequencing tags and barcodes

The final short-cycle PCR was designed to ligate a unique sequencing barcode and the NGS adaptor sequences to the PCR amplicons for each sample. These PCRs consisted of 2.5 µL of 10X Taq reaction buffer, 25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM forward primer + Uni-B adaptor, 0.5 µM reverse primer plus Uni-A adaptor, 0.1 units Taq polymerase, 10 µL of cleaned PCR product and remainder ddH_2O for a total reaction volume of 25.0 µL. Short-cycle PCR began with a 2-minute denaturation at 95 °C followed by 6 cycles of 95 °C denaturation for 30 seconds, 60 °C annealing temperature for 30 seconds, 72 °C extension for 30 seconds and a final single extension at 72 °C for 5 minutes. Subsamples from each primer set were checked using gel-electrophoresis and UV imaging to confirm small increases in fragment sizes indicative of successful ligation of the barcode and adaptor sequences.

NGS library preparation

I combined all the ligated PCR products in equal proportions and gel extracted the expected sized band from the mixture. I gel extracted in duplicate using a GenCatch™

Advanced Gel Extraction Kit (Epoch Life Science Inc.) and eluting into final volume of 20 µL. The inclusion of the target species primer set amplicons through these steps was only to verify that the agarose bands present after first or second-round PCRs were actually the intended target AIS. Measures of presence-absence from target AIS primer sets were used in favor of quantitative measures using sequence read numbers as I had not validated the target AIS primer sets as being truly species-specific.

The gel-extracted barcoded amplicons were analyzed in duplicate using an Agilent High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) to determine final DNA concentration and check that distributions of fragments at high abundances were in the size ranges expected. I next diluted each sample replicate to a final concentration of 60.0 $\mathrm{pmol} \cdot \mu\mathrm{L}^{-1}$, combined the two replicated samples in equal proportions, and sequenced the final barcoded metasample on a 318-chip on the Ion Torrent System (Life Technologies, USA).

Sequence filtering, customized database creation, and sequence querying

I used Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010) to remove sequenced amplicons with average quality scores < 19.0 and remove amplified fragments smaller than 100/150 bp for target AIS primer and universal sets, respectively. All sequences with more than three primer-template mismatches were also removed. To verify amplification of the target AIS from target species primer set PCRs, I compared the sequences resulting from my target AIS primer sets to files containing all variations of AIS specific reference CO1 sequences downloaded from GenBank and Barcode of Life (Ratnasingham and Hebert 2007; Clark et al., 2015). Universal primer set sequences were BLASTed against a custom reference sequence database containing sequences for: 1) the five selected target AIS; and; 2) sequences for three important native invertebrate prey species (*Leptodiaptomus sicilis,*

Limnocalanus macrurus, and *Mysis diluviana*). I chose the three native species as I expected them to be common diet items in both native and invasive predator scDNA samples (Mychek-Londer et al., 2013; Pothoven 2018) and they are likely to interact with the target AIS directly or indirectly (Bourdeau et al., 2011; Mumby et al., 2017). Additionally, these three native invertebrate species are reported as invasive or as posing invasion risks outside the Great Lakes (Devlin et al., 2017; Hyatt et al., 2018). My BLAST analyses against the custom databases were performed using the *map reads to reference* function available in QIIME with default parameters, except I set a minimum match value of 98 % for reads from the universal primer set, and set this value to 96 % for reads from any of the target AIS primer sets.

Presence-absence analyses of target AIS and selected native prey

Statistical analyses were based on occurrences of specific prey species in each predator scDNA sample. Presence data for the target AIS primer sets was based on positive gel image results for individual scDNA diet samples; however, I required that all presence scoring be confirmed by a match with the NGS sequence data for each such sample. Additionally, I required at least three sequences per predator scDNA sample of the given prey species to count the prey species as present to avoid sequencing artifacts driving up positive presence scoring.

For the universal primer set data, I required a minimum of 250 high quality sequences per sample for the sample to be included in my analyses. I also set the minimum sequence number threshold to three matched sequences for AIS to be identified as present in individual predator scDNA samples. If the threshold number of sequences was not met, the prey species was scored as absent for the individual scDNA sample. The binary presence-absence data for AIS prey were used in further analyses, described below, for testing for biotic and abiotic effects on the prevalence of the target AIS in the Lake Michigan planktivore diets.

I also used sequence data from the universal primer set PCRs to characterize the role of the three selected native crustacean macroinvertebrate species in the diet and scDNA of predator fishes. For the analysis of the three native prey, I required that for a prey to be counted as present: 1) at least three sequences of the native species were present per sample and; 2) the native prey sequences had a relative read abundance (RRA) greater than or equal to 0.10 %, relative to the total number of sequences recovered from that sample (see Deagle et al., 2018). For information purposes, I individually summed each individual predator's RRA value for each of these three prey (RRA for each prey as a percentage of all filtered sequences produced by the universal primer set for each scDNA diet sample). Lastly, I averaged the sum RRA percentage across the entire data set of 376 scDNA diet samples.

Statistical analyses

I tested for the influence of factors upon occurrences of the target AIS and the three selected native species using binary logistic Generalized Linear Models (GLMs). I combined the presence data from the two types of detection methodologies (target species and universal primers). If a predator was identified as having a prey species present in one or both assays, it was coded as present. Only if both assays were negative was a prey identified as absent from a predator's stomach contents. I tested the main effects of predator species, year, and sampling site as fixed effect categorical independent variables, and included depth and predator TL as continuous covariates. My subjects were the individual fish predators (coded as one or zero for prey present or absent, respectively) and I ran each statistical model for each prey species separately, one at a time. I increased the number of maximum iterations to 1000 in the modeling procedure and used all other default settings in SPSS for each GLM.

Results

Target AIS primer sets

Second-round PCR gel imaging revealed 26, 47, and 10 individual scDNA samples with positive results from the *Cercopagis pengoi*, *Dreissena spp.*, and *Hemimysis anomala* target primer sets, respectively (N = 376 total samples). No positive results resulting from the *Bythotrephes longimanus* target primer set were observed after first or second-round PCRs. Out of the 376 samples, the target species primer set sequence data confirmed the presence of *Cercopagis pengoi* presence in the diets of 19 predators (sequence read number in positive scDNA diet samples ranged from 3 to 585; Table 3.2; Figure 3.2) and confirmed *Dreissena rostriformis bugensis* in 41 scDNA diet samples (sequence read number in positive scDNA diet samples ranged from 1 to 3440; Table 3.2; Figure 3.2). No matches for *Dreissena polymorpha* resulted from the *Dreissena* spp.-based, target-specific primer set sequence data and no matches resulted from the target-specific primer set sequence data for target AIS *Hemimysis anomala*.

Universal primer set results

Across the 376 scDNA samples, the average number of reads per sample using the universal primer set was 6,914 (min = 0, max = 140,500). Of the 376 scDNA diet samples, 71 had fewer than 100 sequence reads, 114 had less than 250, 139 had less than 500, and 183 scDNA samples had less than 1000 sequence reads per sample after filtering for sequence length and quality, leaving 193 which had more than 1000 reads after filtering. Although no positive hits resulted for *Bythotrephes longimanus* using the target-specific primer set (see above), 27 scDNA diet samples were positive for *Bythotrephes longimanus* using the universal primer set with a mean sequence read number per positive sample of 317 (min = 3, max = 1,687; Table 3.2; Figure 3.2). Of the predator species sampled, alewife sampled at Frankfort and had the highest

percent frequency of occurrences of *Bythotrephes longimanus* in diet scDNA, consumed *Bythotrephes longimanus* in years 2009 and 2010, and consumed this AIS prey at four of the five depth strata alewife predators were sampled from. The second highest occurrence of *Bythotrephes longimanus* sequences from combinations of predator species and sites was from alewife scDNA at Sturgeon Bay

Table 3.2: Percent frequency of occurrence of target AIS in predator diets (N = 376) at each site. Frankfort data are for both years and all depths combined. TSPS = results from target AIS specific primer sets. UPS = results from universal primer set. Results for TSPS were based upon positive results from PCRs and gel imaging as confirmed with sequencing results. All UPS results were based upon sequencing data, and for *Dreissena rostriformis bugensis* none of the positive results from this primer set were in addition to any positive results from the target primer set but occurred within the same positive samples respective to target primer set positive results. I did not include *Dreissena polymorpha* or *Hemimysis anomala* as all results for target AIS specific and universal primer sets were negative for these two AIS. Full fish predator species names: Alewife = *Alosa pseudoharengus*, Bloater = *Coregonus hoyi*, Ninespine stickleback = *Pungitius pungitius*, Rainbow smelt = *Osmerus mordax*, and Slimy sculpin = *Cottus cognatus.*

		Predator sample size (N)	*Bythotrephes longimanus*		*Cercopagis pengoi*		*Dreissena rostriformis bugensis*	
Site	Predator		TSPS	UPS	TSPS	UPS	TSPS	UPS
Frankfort	Alewife	39	0.0	17.9	5.1	0.0	7.7	0.0
	Bloater	39	0.0	2.6	7.7	0.0	5.1	0.0
	Stickleback	39	0.0	2.6	10.3	0.0	15.4	2.5
	Smelt	40	0.0	7.5	0.0	0.0	5.0	2.5
	Sculpin	19	0.0	5.3	5.3	0.0	5.0	0.0
Sturgeon								
	Alewife	20	0.0	0.0	0.0	0.0	5.0	0.0
	Bloater	20	0.0	10.0	5.0	0.0	0.0	0.0
	Stickleback	20	0.0	0.0	20.0	0.0	5.0	0.0
	Smelt	20	0.0	0.0	0.0	0.0	5.0	0.0
	Sculpin	20	0.0	10.0	0.0	0.0	50.0	5.0
Two Rivers								
	Alewife	20	0.0	15.0	0.0	0.0	0.0	0.0
	Bloater	20	0.0	10.0	0.0	0.0	10.0	0.0
	Stickleback	20	0.0	10.0	5.0	0.0	5.0	5.0
	Smelt	20	0.0	5.0	10.0	0.0	15.0	5.0
	Sculpin	20	0.0	10.0	5.0	0.0	40.0	0.0

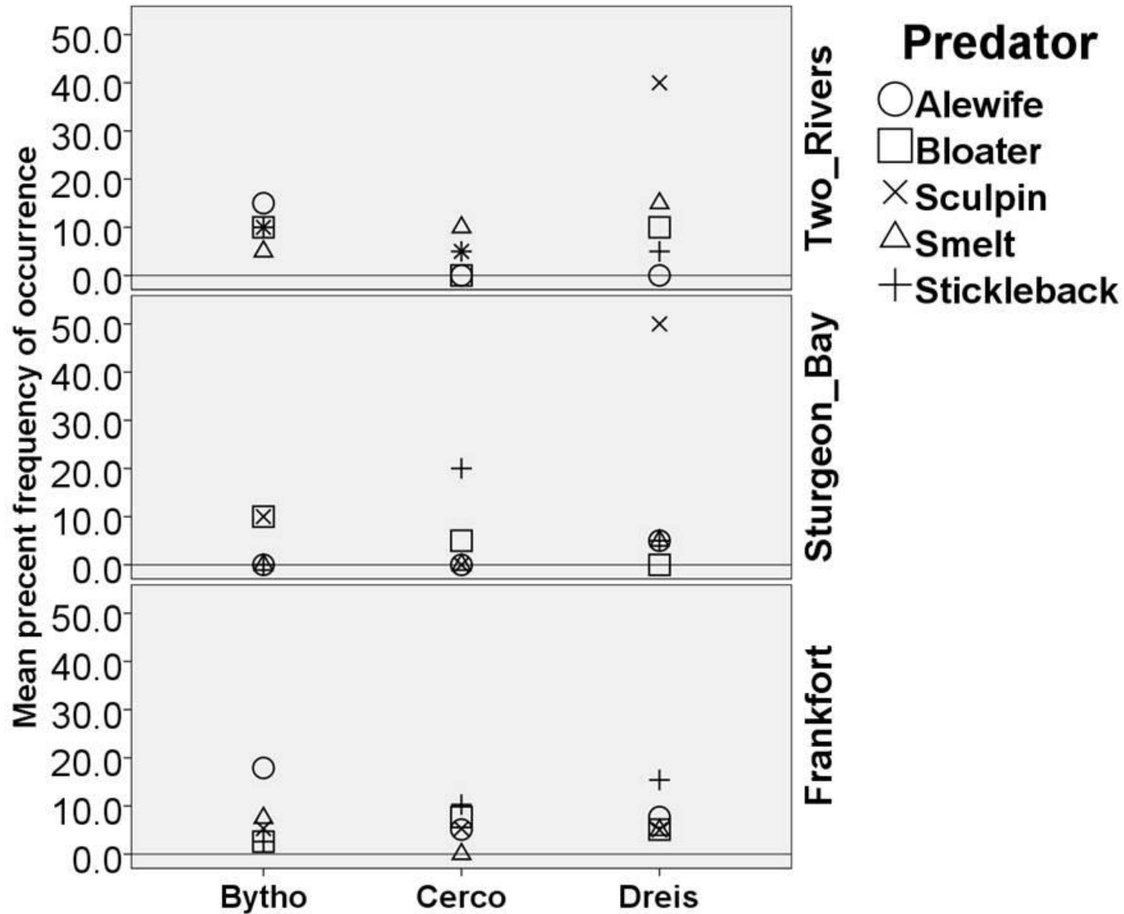

Figure 3.2: Mean percent frequency of occurrence of target AIS in predator scDNA diet samples across all predator fish samples, including from multiple years and depths at each site. The three prey on the x-axis, are named fully as *Bythotrephes longimanus*, *Cercopagis pengoi* and for Dreissenid only includes *Dreissena rostriformis bugensis.* No positive results were observed in my data set for *Dreissena polymorpha*, or *Hemimysis anomala*, thus I excluded these prey in results for this figure. Full fish predator species names: Alewife = *Alosa pseudoharengus*, bloater = *Coregonus hoyi*, ninespine stickleback = *pungitius pungitius*, rainbow smelt = *Osmerus mordax*, and slimy sculpin = *Cottus cognatus*.

The universal primer set amplified *Dreissena rostriformis bugensis* sequences, but comparatively, the *Dreissena* spp. target-species primer set had much greater sensitivity (see results above). In total, the universal primer set produced 16 individual quality-controlled *Dreissena rostriformis bugensis* sequences which occurred in 5 scDNA diet samples (Table 3.2). No target AIS sequences were produced from the universal primer set for *Cercopagis pengoi*, *Dreissena polymorpha,* or *Hemimysis anomala*. Thus, as the latter two of these three prey

respectively were also not identified using respective target primer sets, I concluded both *Dreissena polymorpha* and *Hemimysis anomala* were absent as prey in scDNA samples.

Frequencies of occurrences for the three native prey were substantially higher than AIS in scDNA samples. For example, four of five predator species contained a greater than 35 % occurrence of *Leptodiaptomus sicilis*, all five predator species contained a greater than 50 % occurrence of *Mysis diluviana* in scDNA for fishes sampled at the site Frankfort, and for four of five predator fish species sampled at Two Rivers *Limnocalanus macrurus* occurrences were between 30-60 % (Table 3.2, Figure 3.3 vs. 3.2, Figure 3.4). Positive hits for sequences for each of these three native prey types respectively occurred in 48.4, 25.0, and 53.5 % of al1 scDNA samples in the data set. An overall mean RRA of 35.1 % of all reads produced by the universal primer set per scDNA diet sample was accounted for by the three native prey species.

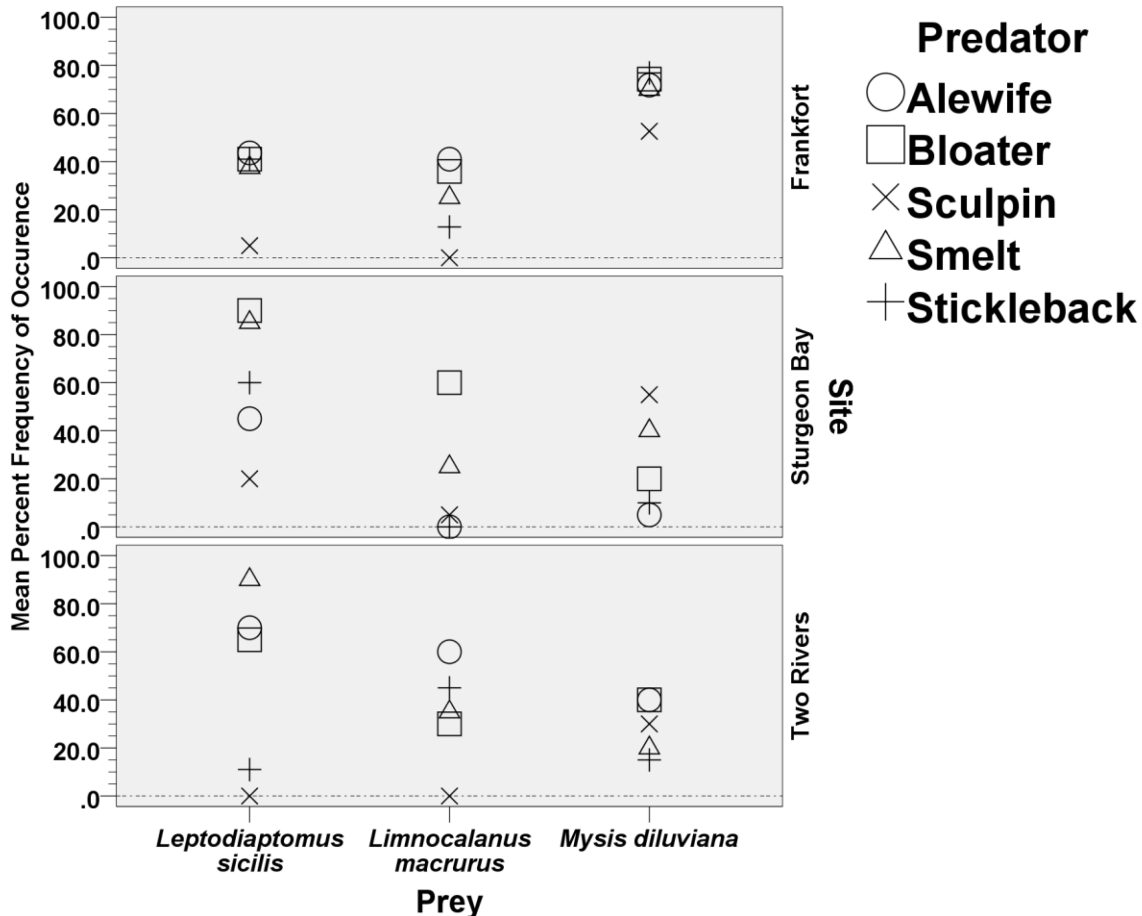

Figure 3.3: Mean percent frequency of occurrence of three native prey species in predator scDNA diet samples. All sequences in this data were produced by the universal primer set. Full fish predator species names: alewife = *Alosa pseudoharengus*, bloater = *Coregonus hoyi*, ninespine stickleback = *Pungitius pungitius*, rainbow smelt = *Osmerus mordax* and slimy sculpin = *Cottus cognatus.*

Presence-absence modeling

The presence-absence GLM for the AIS *Bythotrephes longimanus* showed significant effects of predator species total length (Table 3.3). The GLM for *Cercopagis pengoi* yielded no significant effects. The *Dreissena rostriformis bugensis* GLM resulted in a significant predator species effect, but not for any other variable (Table 3.3). GLMs for the three native prey revealed significant effects in *Leptodiaptomus sicilis* for predator species, site sampled, and year sampled (Table 3.4), *Limnocalanus macrurus* showed significant effects for predator species and site sampled, and for *Mysis diluviana* only site sampled was significant (Table 3.4).

Table 3.3: Results from the binary logistic generalized linear model occurrence analyses for the three target AIS prey I detected. I tested for the effects of fish predator species, sampling site, depth, year of sample and predator total length (TL, nearest millimeter) using filtered quality controlled sequences. My subjects were each individual fish predator scDNA sample, I tested each model one prey type at a time and the dependent variable was the assigned value of zero or one for absence or presence of the prey of interest. I tested the main effects of predator species, year, and sampling site as fixed effect categorical independent variables, and included depth and predator TL as continuous covariates in each model for each prey type. Significant values are in bold in the rightmost column.

AIS prey, model term	Wald Chi-Square	Degrees freedom	Significance
Bythotrephes longimanus			
Intercept	11.452	1	**0.001**
Predator	3.092	4	0.543
Site	0.118	2	0.943
Year	0.610	1	0.435
Depth	0.004	1	0.953
Predator total length	6.881	1	**0.009**
Cercopagis pengoi			
Intercept	3.114	1	0.078
Predator	8.857	4	0.065
Site	5.237	2	0.073
Year	0.563	1	0.453
Depth	1.183	1	0.292
Predator total length	1.111	1	0.277
Dreissena rostriformis bugensis			
Intercept	0.598	1	0.439
Predator	15.797	4	**0.003**
Site	0.802	2	0.669
Year	0.915	1	0.339
Depth	1.624	1	0.135
Predator total length	2.239	1	0.202

Table 3.4: Binary logistic generalized linear model presence-absence analyses for three native prey. I tested effects of predator, sampling site, depth, year of sample and predator length using filtered sequences amplified by the universal primer set. Subjects were each individual fish predator, I tested each model one prey type at a time, the dependent variable was the assigned value of zero or one for absence or presence of the prey of interest. I tested the main effects of predator species, year, and sampling site as fixed effect categorical independent variables, and included depth and predator TL as continuous covariates in each model for each prey type. Significant values are in bold in the rightmost column.

Native prey, model term	Wald Chi-Square	Degrees freedom	Significance
Leptodiaptomus sicilis			
Intercept	0.283	1	0.595
Predator	22.313	4	**0.000**
Site	12.524	2	**0.002**
Year	3.935	1	**0.047**
Depth	0.095	1	0.758
Predator total length	0.025	1	0.874
Limnocalanus macrurus			
Intercept	3.063	1	0.080
Predator	12.782	4	**0.012**
Site	6.585	2	**0.037**
Year	1.239	1	0.266
Depth	0.000	1	0.992
Predator total length	0.006	1	0.939
Mysis diluviana			
Intercept	1.851	1	0.174
Predator	1.322	4	0.858
Site	38.513	2	**0.000**
Year	1.888	1	0.169
Depth	2.872	1	0.090
Predator total length	0.269	1	0.604

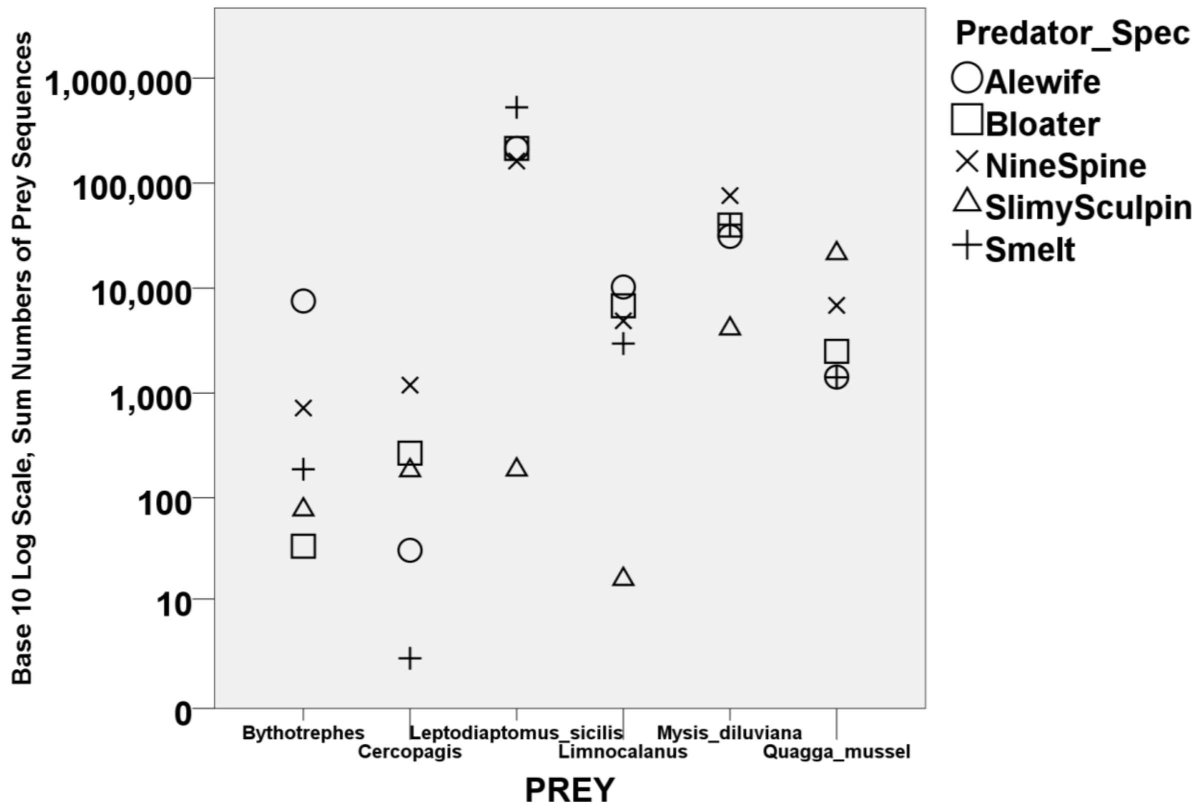

Figure 3.4: Total mean number of sequence reads per scDNA diet sample for each predator fish species at each site sampled (combining years and depths for Frankfort) using only filtered and quality controlled sequences. The Y-axis was adjusted to a base 10 log scale because some of the mean values differed so dramatically as to prevent a proper interpretation of data and in particular sequence numbers for *Leptodiaptomus sicilis* were high and a main factor in this. Full prey species names on the x-axis are: *Bythotrephes longimanus*, *Leptodiaptomus sicilis*, *Cercopagis pengoi*, *Mysis diluviana*, and *Dreissena rostriformis bugensis (*Quagga mussel). Full fish predator species names: alewife = *Alosa pseudoharengus*, bloater = *Coregonus hoyi*, ninespine stickleback = *Pungitius pungitius*, rainbow smelt = *Osmerus mordax*, and slimy sculpin = *Cottus cognatus.*

Discussion

I used CO1 metabarcoding of native and non-native zooplanktivore predator fish scDNA to identify AIS prey which have established and impacted Great Lakes foodwebs (Zimmerman & Krueger 2009; Ricciardi & MacIsaac 2000) and are classified as some of the world's worst 100 invasive species (Lowe et al., 2000). Specifically, I detected *Bythotrephes longimanus, Cercopagis pengoi,* and *Dreissena rostriformis bugensis* in multiple predator

samples. I am the first researcher to use predator scDNA metabarcoding diet analysis for planktivorous fishes from offshore regions of the Great Lakes (for other regional Great Lakes eDNA studies and one inland scDNA study see: Shortridge 2016; Gingera et al., 2017; Klymus et al., 2017; Lavigne 2017). The AIS *Dreissena polymorpha* and *Hemimysis anomala* were not detected in scDNA diet samples, despite the use of two genetic detection markers, and despite the high sensitivity of both markers for these species. My results for the three native prey contribute to the understanding of the Lake Michigan offshore foodweb (Zimmerman & Krueger 2009). The native prey exhibited frequencies of occurrences using scDNA similar to published traditional diet analyses, except for comparatively lower than expected values in this study for *Leptodiaptomus sicilis* and *Limnocalanus macrurus* prey in slimy sculpin (see: Mychek-Londer et al., 2013; Bunnell et al., 2015; Pothoven 2018). The three native prey I identified in scDNA are also important because they are themselves invasive, potentially invasive, rare or endangered outside the Great Lakes (Spikkeland et al., 2016; Вежновец 2017; Devlin et al., 2017; Hyatt et al., 2018). Site sampled, fish predator species, and fish predator TL were significant in influencing AIS and native prey occurrence patterns. Thus, metabarcoding used to target AIS or important native prey in scDNA diet samples can be used to better understand foodwebs, and is especially valuable when applied across large and difficult to sample ecosystems such as offshore Lake Michigan, where dynamic, localized and potentially interacting influences from AIS impacts and where changes in native species foodwebs are critical to understand.

Occurrences of the AIS *Bythotrephes longimanus* may reflect aspects of its adaptability, life history and reproductive strategy. For example, their cyclical parthenogenesis and lack of over-winter adult survival coupled with over-wintering resting eggs which can survive passage in gut-tracts of predator fishes (Kerfoot et al., 2011) can result in strong seasonal patterns of

availability as prey. Furthermore, the spring hatch of neonates may be delayed in the cold, deepwater benthic habitats I sampled (Yurista 1992; Keeler et al., 2015), making the pattern of consumption of this AIS by predator fishes in such offshore zones highly unpredictable. The planktonic nature of *Bythotrephes longimanus* resting eggs and prey body parts potentially suspended into the water column or unexpected hatching of resting eggs during spring turnover could have increased availability of *Bythotrephes longimanus* to more pelagic fishes in my study (Kerfoot et al., 2011). A population of *Bythotrephes longimanus* in Lake Maggiore, the second largest lake in Italy experienced a 3-month earlier onset of population growth, a 3-month earlier peak density and a nearly 6-month increase in the duration of occurrences in the water column over an ~20-year period, partially due to climate change and lake warming, exemplifying the adaptability of this AIS (Manca et al., 2007). Similarly, earlier than expected onsets of population growth for *Bythotrephes longimanus* have been observed for other lakes (i.e., Yan and Pawson, 1998; Jarnagin *et al.*, 2004). In general, shifts in timing of the early hatching of some individual *Bythotrephes longimanus* might occur variably from year to year due to the complex nature and degree of turnover in spring in offshore Lake Michigan, at which time some resupended resting eggs may possibly hatch. Typically, in large temperate systems like the Great Lakes, parthenogenetic females of *Bythotrephes longimanus* do not overwinter, or survive overwinter (Lehman, 1988; Yurista, 1997), and major swarms from hatching diapausing eggs usually begin to appear mid-summer, (i.e., Kerfoot et al., 2011; Keeler et al., 2015). However, unexpected observations of surviving overwintering female *Bythotrephes longimanus* in Lake Maggiore (Manca et al., 2007) and the potential refuge zones of the offshore waters in the Great Lakes, makes it possible that some *Bythotrephes longimanus* survived over winter in Lake Michigan. This would result in higher than expected occurrences of *Bythotrephes longimanus*

during the spring sampling period in my study. Visual confirmation of the prey or its parts at the time of stomach content removals could help to clarify the occurrence data. *Bythotrephes longimanus* presence in the scDNA of some predators was significantly affected by predator body size (fish TL) may be due to the defensive distal tail-spine which limits susceptibility to predation until fishes reach a minimal gape size (Yurista 1992; Barnhisel & Harvey 1995; Branstrator 2005; Pothoven 2012; Miehls et al., 2014). This would also serve to explain how fish predator TL was a significant factor but fish predator species was not.

None of biotic or abiotic factors included in my analyses were significantly associated with the presence of the AIS prey *Cercopagis pengoi* in predator gut contents. Life history patterns and reproductive strategies for *Cercopagis pengoi* are similar to those of *Bythotrephes longimanus*, and likely played some role in influencing predation patterns (Krylov & Panov 1998; MacIsaac et al., 1999). The lack of significance of predator TL on the pattern of presence of *Cercopagis pengoi* in scDNA may reflect that this invertebrate prey is smaller than *Bythotrephes longimanus* despite the smaller defensive spine in *Cercopagis pengoi*, which has also been cited as a factor limiting its consumption as prey in smaller fishes (Bushnoe et al., 2003). The absence of a predator body size effect suggests that the mean TL of the predators sampled may have been large enough to have escaped gape limitation for this AIS. Increases in densities of *Cercopagis pengoi* have been reported with increasing distances from shore in large aquatic ecosystems such as the Great Lakes (IUCN 2010), but I found no significant effect of location or depth on presence in the predator's diet. Recorded occurrences of *Cercopagis pengoi* in field diet samples have been noted in the literature in the spring, including in deep, offshore areas of Lakes Michigan and Huron similar to my sites (USGS, 2018), supporting my positive detections of this AIS in predator scDNA samples. While *Cercopagis pengoi* was not a major

diet component, this is the first report to use metabarcoding to identify AIS *Cercopagis pengoi* in scDNA diet samples and higher occurrences revealed herein than in traditional studies furthers what can be quantified in regards to, and what is known about the potential impacts of this AIS. Knowing the distribution of this AIS as prey will facilitate the evaluation of its potential impacts upon ecosystems, native predator fish species, non-native predator fish species, and the foodweb. This data will also promote a better understanding of levels of spatiotemporal overlap of this AIS and its predators, especially important for the hard-to-sample deepwater offshore foodweb of Lake Michigan.

The lack of *Dreissena polymorpha* in scDNA diet samples accords with its absence in the deep offshore benthic zones of the Great Lakes, likely due to ecophysiological constraints and recent ecosystem-level niche replacement by its closely related congener AIS *Dreissena rostriformis bugensis* (Mills et al., 1996; Stoeckmann 2003; Bunnell et al., 2009; Kemp and Aldridge 2018). Using the target AIS *Dreissena* spp. PCR primer set, *Dreissena rostriformis bugensis* was identified in scDNA diet samples for every combination of each of the five fish predator species and three sites sampled except in two cases, hence the predator species were likely consuming veliger or recently settled juvenile life stages of this AIS. Microscopic pelagic larval veligers remain planktonic for three to four weeks, during which time mortality rates, including from predation, can exceed 99 % (Bially & MacIsaac 2000). Traditional visual diet studies targeting alewife and rainbow smelt (Mills et al., 1995; Creque & Czesny 2012) have also documented veligers of *Dreissena rostriformis bugensis* as prey in diet samples. Slimy sculpin taken from the same trawl hauls used in this study had very few occurrences of *Dreissena rostriformis bugensis* (<1 %) and no detection of *Dreissena* spp. veligers in diet descriptions derived from traditional visual based approaches (Mychek-Londer et al., 2013).

This suggests that the high occurrences of *Dreissena rostriformis bugensis* in slimy sculpin in this study using metabarcoding reflected greater sensitivity of scDNA approaches and perhaps non-recognition or advanced digestion of the prey even when viewed under high magnification using traditional methods (Mychek-Londer et al., 2013). The high frequency of occurrence of *Dreissena rostriformis bugensis* in some slimy sculpin samples was likely a factor in the significance of fish predator species as an important predictor of its occurrence, although predator prey preference may also play a role. Additionally, the high occurrence of *Dreissena rostriformis bugensis* in slimy sculpin may relate to their lack of a swim bladder coupled with their highly benthic nature, resulting in a higher predator-prey co-occurrence and spatial overlap. For example, slimy sculpin could selectively consume recently settled veligers before the establishment of thick shells and byssal threads. However, the predator fishes I sampled may also be non-selectively consuming *Dreissena rostriformis bugensis* as a secondary byproduct of consuming preferred nearby benthic macroinvertebrates such as *Diporeia hoyi* or chironomids (e.g., Mychek-Londer et al., 2013). The high presence of *Dreissena rostriformis bugensis* in scDNA diet samples in my study may also be reflective of the high sensitivity of the target PCR primer set and may have resulted from tertiary consumption as the common prey *Mysis diluviana* has been documented to consume *Dreissena* spp., veligers (O'Malley & Bunnell 2014; O'Malley et al., 2017). *Mysis diluviana* is a commonly consumed prey (i.e., Gamble et al., 2011; Bunnell et al., 2015; this study) for all fishes in my study and stomach contents of *Mysis diluviana* could have been imparted to fish scDNA samples during scDNA digestion and extraction steps. Thus, although metabarcoding of scDNA is highly sensitive to the AIS *Dreissena rostriformis bugensis*, I caution that consideration of the life stages consumed may impact determination of prey selectivity and that scDNA metabarcoding cannot provide life stage data for prey. Hence,

the application of additional novel methodologies is necessary and can and will serve to increasingly accurately characterize specific roles of the AIS *Dreissena rostriformis bugensis* in the foodweb as actively selected prey. Regardless, the high sensitivity of my methods offers the unique advantage in determining occurrences during critical early-establishment phases when abundance of *Dreissena rostriformis bugensis* could be much lower.

The lack of *Hemimysis anomala* in my scDNA diet samples may reflect its preference for nearshore, shallow, littoral, rocky bottom habitats (de Lafontaine et al., 2012; Sun et al., 2013; Barrios-O'Neill et al., 2014; Frossard & Fontvieille 2018). Several positive PCRs for the *Hemimysis anomala* target AIS specific primer set did not generate sequence matches for this AIS, or a few closely-related congeners included in my BLAST databases. I suggest non-specific binding of my target species primer set designed for *Hemimysis anomala* occurred during PCRs, likely for the taxonomically closely related and commonly consumed native species, *Mysis diluviana.* I did detect *Mysis diluviana* sequences in my NGS libraries as a common prey when using the universal primer set. Although I did not identify *Hemimysis anomala* in scDNA of any predators, its absence helps to further define its role, or lack thereof in deep offshore ecosystems versus near-shore Lake Michigan foodwebs and in relation to other similar Great Lakes ecosystems (i.e., Pothoven et al., 2007b).

The high mean overall relative sequence read abundance (RRA) of 35.1 % across my data set for the selected native species *Leptodiaptomus sicilis, Limnocalanus macrurus,* and *Mysis diluviana* in scDNA samples demonstrates that a few important native prey taxa can account for a relatively high proportion of the diet of a diverse set of predators, despite taxonomically diverse stomach contents. Sampling site was a highly significant factor for predation on all three native invertebrate prey species, consistent with previous diet studies of

offshore predator fish species in the Great Lakes (i.e., Mychek-Londer et al., 2013; Bunnell et al., 2015; Pothoven, 2018). For example, *Mysis diluviana* had the highest frequency of occurrence at Frankfort, compared to other sites in my study, which agrees with the very high visual based occurrences of this prey at that location including in samples from the same individual trawl-hauls as samples came from in this study (i.e., Mychek-Londer et al., 2013; Bunnell et al., 2015). An increased reliance on *Mysis diluviana* in the foodweb of Lake Michigan and similar Great Lakes offshore foodwebs by the predator fish species I sampled has occurred in recent decades as a result of the disappearance of a preferred native prey, *Diporeia hoyi* (i.e., Owens & Dittman 2003; Hondorp et al., 2005; Stewart et al., 2009). *Diporeia hoyi* was shown to be abundant and eaten in large quantities at Sturgeon Bay and Two Rivers, but was determined to be absent in diets of fishes caught at Frankfort, including for samples from the same benthic trawls as fishes were sampled from for this study (see: Mychek-Londer et al., 2013; Bunnell et al., 2015). Thus, my finding that site was an important predictor in the prey presence of *Mysis diluviana* is not surprising, especially given my observation of a very high frequency of occurrence of *Mysis diluviana* at Frankfort across all predator fish species where the once preferred prey *Diporeia hoyi* was seemingly extirpated. Thus, metabarcoding scDNA diet analyses can reflect ongoing AIS-induced changes in ecological processes (e.g., prey preference) in the foodweb. Additionally, I found *Mysis diluviana* occurred in scDNA in slimy sculpin at Frankfort, Sturgeon Bay, and Two Rivers at mean frequencies of 53 %, 55 %, and 30 %, respectively, whereas traditional diet studies for slimy sculpin from the same trawl hauls reported equivalent frequency of occurrences of 72 %, 37 % and 36 % (Mychek-Londer et al., 2013). Thus foodweb dynamics captured by traditional diet studies can be comparable with scDNA CO1 metabarcoding.

All three native invertebrate prey species I examined using scDNA were common prey for all fish predator species. Analyses found significant differences among the predator species in their exploitation of the two native copepod prey species, *Leptodiaptomus sicilis* and *Limnocalanus macrurus*. This effect is likely related to site-based differences in prey availability and predator-prey demand resulting from AIS induced foodweb changes such as the patchy distribution of the preferred prey *Diporeia hoyi* (i.e., not present at Frankfort, but remained at sites Sturgeon Bay and Two Rivers). For example, increased rates of consumption of the two native copepods *Leptodiaptomus sicilis* and *Limnocalanus macrurus* seemed apparent at Frankfort compared to the other two sites as possibly seen in the occurrence data, likely contributing to the significant effects on predator species for each of these prey. This fits with trends between 1995-2005 for the fishes in my study indicating increased reliance on these copepods as *Diporeia hoyi* declined differentially at local scales across sampling sites and into my sampling period (Bunnell et al., 2015). Non-native rainbow smelt consistently exhibited the highest occurrences of *Leptodiaptomus sicilis* in my study but this prey was also consumed very frequently by bloater and alewife. Slimy sculpin seemingly lacked or had unexpectedly low occurrences of *Leptodiaptomus sicilis* and *Limnocalanus macrurus*, which is surprising given that slimy sculpin sampled from the same trawl hauls had higher occurrences of these two prey in visual-based analyses (Mychek-Londer et al., 2013). It is possible that co-occurring prey, such as chironomids, acted during PCRs to significantly reduce the ability of the universal primer set to perform as well in amplifying the two native copepod prey species. For example, it may be possible that chironomid species or another prey unique to or highly abundant in slimy sculpin scDNA have CO1 DNA fragments that more easily and preferentially anneal to the universal primers, and through preferential competitive exclusion during PCRs would have

limited the amplification of co-occurring DNA fragments of *Leptodiaptomus sicilis* and *Limnocalanus macrurus* species in slimy sculpin diets. The predator species effects for *Leptodiaptomus sicilis* and *Limnocalanus macrurus* in my study may have in fact partly resulted from the very low occurrences I observed for these prey in slimy sculpin. However, the other predators varied in levels of occurrences of *Leptodiaptomus sicilis* and *Limnocalanus macrurus* as well, except at Frankfort where occurrences of these prey among predator species seemed more similar. The importance of sampling site and fish predator species in determining prey occurrences in a study such as this is perhaps not surprising, as native and AIS predator species are able to respond and adapt to dynamic foodweb conditions (i.e., Mychek-Londer et al., 2013; Bunnell et al., 2015; Leray et al., 2015).

Whether or not non-native fish predators should consume more AIS prey than native predator species only seemed to hold for one of the AIS predators. Lake Michigan alewife have some of the highest proportions of the AIS *Bythotrephes longimanus* as prey in summer and fall, when this prey is abundant (Bunnell et al., 2015; Keeler et al., 2015). In my study, alewife had the first and second highest mean occurrences of *Bythotrephes longimanus* at each sampling site and had the overall highest mean percent occurrence of this AIS prey in Lake Michigan of the predator fish species I sampled. In contrast, native ninespine stickleback had the highest observed mean percent frequency of occurrences of AIS *Cercopagis pengoi* sequences in scDNA diet samples at two of three sites vs. every other combination of fish predator species and site sampled. Further, as many as one in five stickleback sampled from Sturgeon Bay contained *Cercopagis pengoi* prey, while all remaining non-native fish predator species sampled at this site had none. Thus, the determinations of whether or not AIS or native predator species are eating AIS prey more often appear inconsistent. While the literature has identified these *Bythotrephes*

longimanus and *Cercopagis pengoi* using traditional diet studies (Bushnoe et al., 2003; Pothoven et al., 2007a; Bunnell et al., 2015), less research has characterized them more generally for offshore Great Lakes fishes, especially for ninespine stickleback (see Gamble et al., 2011; Bunnell et al., 2015). Ninespine stickleback may consume *Cercopagis pengoi* prey at high occurrences relative to other fishes I sampled as it may act as a replacement prey for ninespine stickleback as their once preferred prey including many native cladocerans are now generally extirpated or greatly reduced in abundance, perhaps due to *Bythotrephes longimanus* and *Cercopagis pengoi* both consuming high amounts of and limiting availability of those native taxa (i.e., Barbiero & Tuchman, 2004; Bunnell et al., 2011; Bunnell et al, 2015). *Cercopagis pengoi* habitat selection in avoidance of its predator *Bythotrephes longimanus* may have also increased its availability to ninespine stickleback (Witt & Cáceres 2004), possibly across diurnal scales (Armenio et al., 2017). Thus, predator species is an important consideration in design and analyses of metabarcoding scDNA studies to detect and characterize AIS within foodwebs. However, it appears specializations of individual predators or localized foodweb conditions were more important in influencing prey occurrence patterns than was invasive status of predator species.

In summary, I used metabarcoding to identify AIS and native invertebrate prey species in scDNA diet samples from five zooplanktivorous predator fishes (two AIS and three native fish species) sampled in the spring from three offshore Lake Michigan sites and various depths ranging from between 73-128 m. I targeted five AIS prey and detected three of the AIS in scDNA diet samples using a novel CO1 metabarcoding of scDNA approach. Results of examinations of biotic and abiotic factors on the patterns of AIS and native prey presence in scDNA diet samples indicated that site based ecological variation was the most important factor

examined, followed by predator species, and predator species total length which were less important predictors of prey occurrences in scDNA GLM analyses. Variability in these predictors seemed to reflect the overall status of the hard to access deep offshore Lake Michigan benthopelagic foodweb in that native prey species occurrences still dominated in predator scDNA, but also in that AIS are also possibly becoming more common as prey, likely as the ecosystem has become more invaded over time and predators become more habituated to consuming these AIS. Additionally, prey occurrences in predator scDNA seemed to reflect localized conditions of foodwebs at the sampled sites which have been differentially impacted by AIS. Metabarcoding data also likely reflected predator-species-specific abilities to respond to ecological change, predator specializations, and prey selection as in some cases at sites where *Diporeia hoyi* had become absent due to AIS effects, there may have increased predation pressure on remaining native prey among both native and non-native predators such that diet overlap may have been increased. In conclusion, my methodologies provide a useful guideline for other researchers to follow who wish to use a metabarcoding approach to detect selected AIS and native prey species in scDNA of these and similar predator congeners. Further, such data will be useful to inform efforts to prevent the further spread of the target AIS and to help manage their effects on ecosystems and foodwebs where they have established.

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CHAPTER 4 - METABARCODING OF NATIVE AND INVASIVE PREY IN STOMACH CONTENT DNA (scDNA) OF COMMERCIALLY HARVESTED LAKE ERIE FISHES

Introduction

Aquatic invasive species (AIS) are well known to affect the structure and function of ecosystems and their inhabitants. For example, species extinction and loss of biodiversity (Barel et al., 1985; Kaufman 1992; Rahel 2002; Ricciardi & MacIsaac 2011), induction of trophic cascades or changes in energy flows in foodwebs (Johnson et al., 2005; Bunnell et al., 2009; Brush et al., 2012), reduced biotic resistance (Scott & Helfman 2001), competition for limited prey resources with native taxa (Ricciardi et al., 1998), consumption of susceptible life stages of native species causing recruitment limitation (Tyus et al., 2000), disruption of nutrient dynamics (Matsuzaki et al., 2009) and disease and parasite vector transmission (Vitule et al., 2009) are impacts attributed to invasion and proliferation by AIS. However, quantifying AIS roles in foodwebs can assist in managing and mitigating such impacts (David et al., 2017; Ricciardi & MacIsaac 2011).

The Laurentian Great Lakes (hereafter Great Lakes) have at least 186 established non-native species (Wells & McClain 1973; Mills et al., 1993; Ricciardi & MacIsaac 2000; Simberloff 2006). At least 25 non-native fishes have established in the Great Lakes and at least 32 native Lake Erie fishes are considered rare (Noble 2002) with AIS identified as one of the leading causes of their decline (Dextrase & Mandrak 2005). The AIS round goby (*Neogobius melanostomus*) has spread widely throughout the Great Lakes (Hayden and Miner 2009) and has impacted foodwebs where it occurs in high numbers. Specifically, round gobies have contributed to localized extinction of native fishes through competitive exclusion of habitats needed for reproduction (Janssen & Jude 2001), reduced native invertebrate biota through selective predation (Barton et al., 2005), and consumed eggs and young of the year native fish

species (French & Jude 2001; Mychek-Londer et al., 2013). AIS fishes such as rainbow smelt (*Osmerus mordax*) and gizzard shad (*Dorosoma cepedianum*) also negatively impact foodwebs and native species in the Great Lakes through consumption of eggs and larvae of native fishes and or potential competition for native prey (i.e., Loftus 1980; Evans & Loftus 1987; Hartman et al., 1992). Those two species have themselves been recorded as important, and sometimes primary prey consumed by native and non-native piscivores (i.e., Knight et al., 1984; Diana 1990; Madenjian et al., 1998; Madenjian et al., 2006; Pothoven et al., 2017). Five invertebrate AIS have been shown to have especially high impacts in Great Lakes foodwebs including: *Bythotrephes longimanus*, zebra mussels (*Dreissena polymorpha*), quagga mussels (*Dreissena rostriformis bugensis)*, *Cercopagis pengoi* and *Hemimysis anomala*. Those invertebrate AIS have been shown to be prey for many fishes in the Great Lakes (MacIsaac et al., 1999; Pothoven & Madenjian, 2008; Bunnell et al., 2011; Ricciardi & MacIsaac 2011; Madenjian et al., 2015). Understanding the roles AIS play in Great Lakes foodwebs can help predict future impacts as they establish and increase in numbers or fluctuate in abundances after establishment. Such predictions are critical for effective management efforts aiming to mitigate AIS impacts.

Analysis of the AIS listed above as well as others in foodwebs has traditionally been based on visual identification of field sampled predator diets including prey and prey species counting and volumetric- or weight-based prey species proportional determinations (i.e., Garrison & Link 2000; Mychek-Londer et al., 2013; Bunnell et al., 2015; Landry et al., 2017). However, biases and inaccuracies can affect outcomes as prey are often too digested, broken into many pieces, or so numerous as to require many hours of counting for an individual sample (Sutela & Huusko 2000; Schooley et al., 2008; Legler et al., 2010). Additionally, specialized taxonomic training may be needed to properly identify cryptic species (i.e., Gamble et al., 2011;

Briski et al., 2010; Jackson et al., 2014). Such limitations may be especially important during the early stages of invasion and expansion, when appearance of the AIS in stomachs may be infrequent or limited in quantity (Puth & Post 2005; Hulme 2006; Lodge et al., 2006; Folino-Rorem et al., 2009; Harvey et al., 2009; Vander Zanden et al., 2010). The emerging molecular genetic technology of metabarcoding of environmental DNA (eDNA) including predator stomach content DNA (scDNA) has applicability in helping to address such issues (Taberlet et al., 2012; Comtet et al., 2015).

Recent metabarcoding studies describing the composition of scDNA have been undertaken in aquatic foodwebs. For example, Aguilar et al. (2017) identified digested prey DNA sequences in scDNA from three sympatric predatory species of native and non-native catfishes and found dramatically increased proportions of prey species identification at the species level (92 %) when compared to traditional visual diet analyses (10 %) due to a high prevalence of digested items. Leray et al. (2015) used a metabarcoding approach and universal primer set to describe diets of spatiotemporally overlapping coral reef fishes and determined highly complex interactions in the foodweb, reflecting trophic partitioning. While universal metabarcoding primers provide broad taxonomic coverage, such primers may have amplification biases and should be supplemented with species-targeted primers with known high efficiency for specific species (i.e., Pochon et al., 2013; Zhan et al., 2013). In such cases, the metabarcoding sequencing confirms the identification of the target species. While powerful, such a metabarcoding approach can be affected by false negatives resulting from PCR error, unexpected haplotypes, inadequate replication, low overall or relative DNA concentrations in mixed samples, polymerase inhibitors in the extracted eDNA, or by limited taxonomic coverage in the reference sequence database (Darling & Mahon 2011; Ficetola et al., 2008). Despite such

limitations, metabarcoding of scDNA overall can be a powerful and effective method to generate detailed and sensitive frequency of occurrence data for use in scDNA diet analyses.

To determine presence-absence of AIS and selected common native prey in the Western Basin of Lake Erie foodweb, I analyzed Lake Erie scDNA from four predator fish species using a CO1 (see: Hebert, et al., 2003a; Hebert et al., 2003b; Hebert & Gregory 2005) metabarcoding approach with main objectives to:1) determine occurrences of five invertebrate AIS in predator scDNA; 2) determine occurrences of three AIS and two native piscine prey in predator diet scDNA; and 3) determine if variation in prey occurrences from predator diet scDNA existed in relation to selected measured abiotic (year, season) and biotic (predator species, predator total length (TL)) variables. Determination of allowable fisheries harvest levels for the predator species included in this study could be improved with a better understanding of factors influencing prey occurrences. In order of importance, I expect that the factors of predator species > season > predator TL > year of sampling will explain the most to least variation in the occurrences of AIS and native prey species in predator scDNA in this study. The predator species effect is expected because predators can partition resources in ways that may help reduce diet overlap and competition for prey. I predict an important seasonal effect because of expected ontogeny and behavioral changes in predator and prey species over my sampling periods (for example, those related to changes from spring spawning events through fall declines in prey resource composition and availability). Predator size effects should be driven by prey defense mechanisms such as dorsal and pectoral spines that serve to limit their consumption until the predators grow past sized-based gape limitations. Because all fishes in this study have had at least some time to behaviorally adapt to AIS and native prey foodweb dynamics, I expect only minimally different levels of occurrences of native versus AIS prey based upon the predator

species origin (i.e., native and AIS). I expect that because trophic interactions are based on community composition, which is not likely to change dramatically from year to year, that few year-based effects on occurrences of prey will be observed.

Methods

Collections

Fishes were sampled summer (August 9) and fall (November 27) in 2014 and spring (April 22), summer (August 24) and fall (November 2) in 2015 in the Western Basin of Lake Erie. Commercial bottom-set gill nets were set at 10 m depth, and retrieved after 18-24 hours. After collection, fish were transferred to shore, collected by researchers and stored on ice in coolers during transport to the Great Lakes Institute for Environmental Research (GLIER) at The University of Windsor, Ontario, Canada. At GLIER whole fishes were frozen at -20 °C. Later, the fish were thawed and up to 15 fish per species per sample date were weighed (1.0 g) and measured for TL (mm; Table 4.1). Whole stomachs (esophagus to just below pyloric valve junction) were excised and individually preserved in 95 % ethanol in sealed 500 mL jars.

Stomach dissections, prey removal

I removed each preserved stomach from ethanol, rinsed it with Milli-Q water to remove excess ethanol, patted it dry, and placed it into a sanitary dissection dish. I dissected each stomach and transferred conspicuous prey to another separate dish. Next, I used water and soft tipped paint brushes to separate prey tissue from stomach tissue. I attempted to minimize predator DNA release at all stages of stomach content recovery. Many samples had prey volumes greater than my target of 0.70 mL for scDNA extraction. For the target volume, I first manually subsampled small amounts of tissue from the largest prey in diets, such as from

relatively undigested fish remains, and continued subsampling through to smaller-sized partial remains of bones, spinal columns, and other conspicuous relatively macroscopic diet items. If something was in bolus form, it was manually separated and components subsampled. I mixed subsamples with the slurry of smaller constituents of partial and unidentifiable prey remains also subsampled from the whole diet sample if needed, and included a relatively small volume of chyme as a portion of the subsample. I homogenously mixed everything within a sanitary Petri dish using manual shearing with scissors if needed. I then selected three to four randomized portions of the mix until achieving the 0.70 mL subsample for each sample. Some predator stomachs had little or no observable prey tissue; for these I sampled a small amount of chyme (~ 0.20 mL) from the stomach and combined it with ddH_2O (~0.50 mL) to reach 0.70 mL. I then centrifuged the mix for each such sample for later DNA extraction from pelleted material. Subsamples of subsampled mixes of prey from non-empty stomachs were similarly each transferred to a 1.7 mL tube for scDNA extraction. I next filled each predators sample tube with representative scDNA with 95 % ethanol to a sum wet volume of 1.6 mL and vortexed each tube for 30 seconds at high speed.

Table 4.1: Numbers of predator species sampled in this diet metabarcoding study with season, year of sampling, sample size and mean TL. Total sample size was 252 fish. TL = total length in millimeters. SD = standard deviation. No yellow perch were sampled Fall, 2014.

Predator	Summer 2014	Fall 2014	Spring 2015	Summer 2015	Fall 2015
Walleye	14	15	14	14	14
TL ± SD	447.0 ± 48.7	481.2 ± 78.4	475.9 ± 16.9	326.4 ± 56.3	457.9 ± 12.1
White Bass	14	9	14	6	14
TL ± SD	318.6 ± 34.1	296.7 ± 31.2	358.1 ± 10.5	304.0 ± 33.9	320.8 ± 11.1
White Perch	14	14	13	15	14
TL ± SD	246.5 ± 29.3	227.6 ± 65.3	267.5 ± 17.5	226.5 ± 14.9	272.1 ± 11.7
Yellow Perch	13	n/a	14	13	14
TL ± SD	211.2 ± 17.7	n/a	235.9 ± 10.6	239.5 ± 24.5	236.6 ± 22.5

Digestion and extraction of scDNA

I extracted scDNA following the protocol described in Chapter Three (Mychek-Londer, Ph.D. Dissertation, 2018). Briefly, I centrifuged the prey tissue tubes and removed the supernatant. I used an Eppendorf 5301 VacuFuge Centrifugal Vacuum Concentrator to evaporate remaining ethanol and used 1.0 mm diameter glass beads and 600-900 µL of digestion buffer ($5.84\ \mathrm{g\ NaCL \cdot L^{-1}}$, Tris-HCL pH 8.0 final concentration 50 mM, EDTA pH 8.0 final concentration 10 mM, SDS to final concentration of 0.5 %, diluted in ddH2O) to homogenize the tissue samples. Subsequently, I added 12 µL Proteinase-K ($20\ \mathrm{mg \cdot mL^{-1}}$) and incubated the reactions with gentle rocking at 37.5 °C for 18-24 hours. I then centrifuged the tubes, removed 150 µL of supernatant and transferred the entire volume to wells on 96 well DNA extraction plates. Using a carboxylate magnetic bead-based protocol on a Tecan Freedom EVO 150 Liquid

Handling System, I extracted scDNA samples and eluted into 150 µL 1X TE and froze DNA at -20 °C.

Polymerase chain reactions

I used five primer sets targeting the mitochondrial gene cytochrome oxidase I (COI) barcoding region to amplify target invertebrate AIS prey sequences, four were target specific for five AIS in the Great Lakes including: 1) *Bythotrephes longimanus*; 2) *Cercopagis pengoi;* 3) *Hemimysis anomala*; and 4) *Dreissena rostriformis bugensis* and *Dreissena polymorpha* (Mychek-Londer Ph.D. Dissertation Chapter Two, 2018). The fifth primer set was a "universal" primer set designed to amplify a shortened fragment of COI for native and non-native fishes present in the Great Lakes (Balasingham et al., 2018). I followed PCR protocols described in Chapter 2 and in Balasingham et al., (2018). Briefly, I used a two-step PCR approach where the first PCR amplified the target CO1 fragment from the scDNA and the second PCR was to ligate the sample identification barcode and adaptor sequences for the next-generation sequencing library preparation. For all first-round PCRs, I used two negative and two positive controls, without and with DNA. Positive control benchmark DNA was collected from established Great Lakes sub-populations of each target AIS or for each predator fish species and preserved in 95 % ethanol.

NGS library preparation

I initially determined observed band presence-absence on gels for each PCR. The absence of a visible band on an agarose gel does not indicate a lack of PCR product, rather it may indicate low concentration. To address this variation in amplicon concentration, I combined the five PCRs (four targeted and one universal primer sets) for each scDNA sample based on the presence or absence of a visible agarose gel band such that 1 µL or 5 µL were used for positive

or negative band results respectively when combining the PCRs. Each combination of PCR products were cleaned using magnetic beads using the process already described (see above). I then ligated a unique sequencing barcode to the amplicons using the second-round short-cycle PCRs. Short-cycle PCRs consisted of 2.5 µL of 10X Taq reaction buffer, 25 mm $MgCL_2$, 0.2 mm of each dNTP, 0.5 µm forward primer + Uni-B and 0.5 µm A + barcode + key + Uni-A adaptor, 0.1 units Taq polymerase, 10 µL of cleaned PCR product and remainder Milli-Q water to total volume of 25.0 µL. Short-cycle PCR cycling began with 2-minute denaturation at 95 °C followed by 6 cycles of 95 °C denaturation for 30 seconds, 60 °C annealing temperature for 30 seconds, 72 °C extension for 30 seconds and a final single extension at 72 °C for 5 minutes. A selection of second-round PCR samples for each primer set was checked using gel-electrophoresis to confirm increases in fragment sizes after tagging and barcoding.

I combined all second-round PCRs prior to amplicon purification. PCRs were combined in equal amounts. I gel extracted four replicates of the metasample (combined PCRs) in separate enlarged gel wells. Visible bands of the amplified gel fragments were manually excised and processed using a GenCatch™ Advanced Gel Extraction Kit (Epoch Life Science Inc.), following manufacturer protocols, and eluted into a final volume of 20 µL. Amplicon size and concentration was assessed using an Agilent High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). I next diluted each sample to a final concentration of 60.0 $\mathrm{pmol} \cdot \mu L^{-1}$, combined all samples in equal proportions, and sequenced this final barcoded metasample from this mixture on a 318-chip on the Ion Torrent High-Throughput DNA Sequencing System (Life Technologies, USA).

Ion Torrent sequences, filtering

I used Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010) to filter sequences in the Ion Torrent produced FASTQ file. I filtered out sequenced amplicons with: minimum average quality scores less than 19.0, fragment sizes smaller than 200 bp (see Mychek-Londer Ph.D. Dissertation Chapter Two, 2018; Balasingham et al., 2018 for expected sizes), and removed amplicons which had greater than three primer-template mismatches and used all other default settings for the split_libraries.py function in QIIME.

Customized database creation and sequence querying for five target invertebrate AIS

Filtered sequences resulting from the four target invertebrate AIS primer sets targeting five AIS were compared to species-specific reference databases composed of COI sequences downloaded from GenBank and Barcode of Life for those five target invertebrate AIS (Ratnasingham & Hebert 2007; Clark et al., 2015). I excluded any sequences which had reference database identity match values below 99.0 % and next excluded results from any individual sample which had fewer than three matched sequences (i.e., excluded "singletons" and "doubletons"). If these quality thresholds were not met, the prey species would be counted as absent in all subsequent analyses. I thus generated prey occurrence data for the two years, three seasons and four predators for each target invertebrate AIS.

Customized databases, sequence querying for invasive and native fish prey species

I used similar sequence filtering and BLASTing steps as above to match fish prey species scDNA sequences produced from the universal primer set. I included three non-native fish prey known to be prevalent in the Great Lakes ecosystems, specifically round goby, gizzard shad, and rainbow smelt. I also included two native fish prey species, channel catfish (*Ictalurus punctatus*) and emerald shiner (*Notropis athernoides*). Specific prey species were chosen because of the

focus on AIS in my study, coupled with the potential for the selected predator species competing for prey taxa including these two native species. To address the expected prevalence of predator tissue DNA PCR amplification with the universal fish PCR primers, I first matched all fish prey sequences to the four predator species examined in this project. For predator species matches, I required a 99 % match identity to the reference CO1 sequences. All metabarcoded sequences that matched the predator from which the scDNA diet samples were derived (i.e., walleye stomach sample and walleye sequences) were removed from further analyses as they were likely due to predator DNA contamination in the scDNA.

I then BLASTED remaining sequences against species level reference sequences for my five selected AIS and native fish prey. I summed identified prey sequences in each scDNA sample for fish prey and added remaining, yet unmatched, sequences after excluding the "host" species sequences. I required that each predator scDNA metabarcode sequence library have at least 1000 sequences, and any individual samples with fewer sequences were deemed to result from "empty" stomachs and the prey numbers were set to zero. Lastly, I required at least three sequences of a prey species in a scDNA sample for that prey to be counted. If the sequence data for any scDNA sample did not meet the set thresholds for prey species, that prey species was scored as "absent" from that a predator stomach.

Presence-absence analyses of universal primer and target AIS primer set sequences

I used each target invertebrate AIS prey species with positive hits in a presence-absence logistic binary Generalized Linear Models (GLMs in SPSS) to test for the effects of predator species, year, season, and predator TL (nearest mm) as a continuous covariate. My dependent variable was presence-absence for each prey species for each individual predator stomach content sample. I tested each target AIS invertebrate prey one at a time in each GLM with all

predictor variables in the model. I increased the maximum number of iterations to 10,000 to ensure for model convergence, but used default settings for all other options in SPSS. I followed the same presence-absence model when using occurrence data from the universal primer set for selected native and non-native fish prey species.

Results

Based upon my criteria, 22 (out of a total of 252 filtered samples) predator stomachs scored positive for *Bythotrephes longimanus* with a mean sequence read number of 46.2 (±75.1 standard deviation (SD)). *Cercopagis pengoi* was present in 45 samples with a mean sequence read number of 17.7 (± 23.6 SD). *Dreissena rostriformis bugensis* was present in 248 scDNA samples with a mean sequence read number of 167.3 (± 427.6 SD).

Bythotrephes longimanus occurred only in summer samples and in three of four predator species I sampled, while *Cercopagis pengoi* occurred in at least one of each of the four predator species sampled in each season in each year (Figure 4.1; Table 4.2).

Dreissena rostriformis bugensis occurred in almost all samples (Figure 4.1; Table 4.2). Neither *Dreissena polymorpha* nor *Hemimysis anomala* were detected in any of the predator scDNA. There was considerable variation in target fish prey species occurrences across predator species, year of capture and season of capture based on sequencing results (Table 4.3).

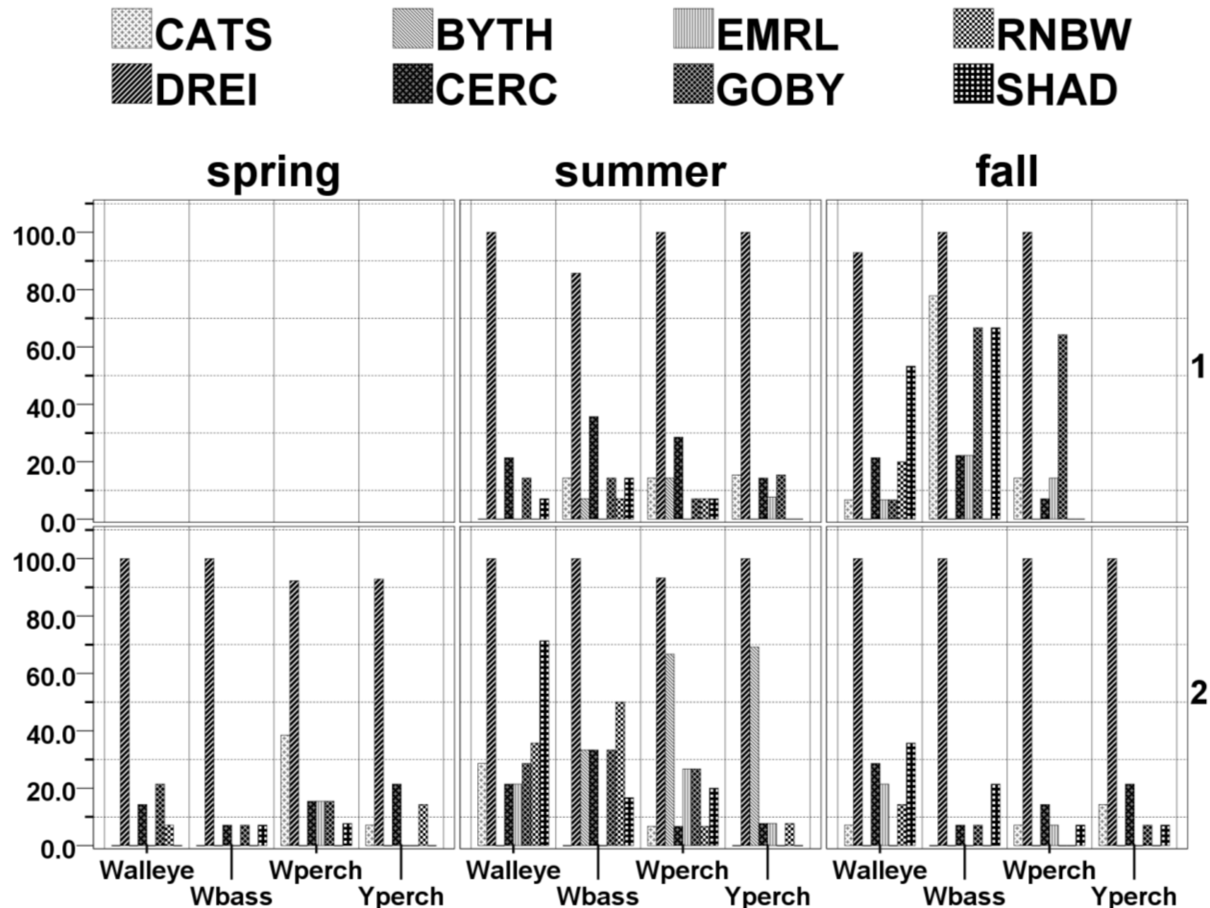

Figure 4.1: Percent frequency of occurrence (% on y-axis) for each of the eight AIS and native prey in predator diets. One can follow grid lines upward such that a set of two gridlines will encapsulate all potential prey for a predator species on the x-axis they correspond to. Additionally, the bar graphs can be further identified because channel catfish = CATS is always the first bar at the left most part of a series of prey for a predator species in a season and year, followed always in order by DREI = *Dreissena rostriformis bugensis,* BYTH = *Bythotrephes longimanus,* CERC = *Cercopagis pengoi,* EMRL = emerald shiner, GOBY = round goby, RNBW = rainbow smelt and with SHAD = gizzard shad always as the last potential bar for a series of spots for eight bars moving left to right. Further, DREI is always the second bar in each series and always had 100 % or otherwise very similarly high occurrences. Year 1 = 2014, Year 2 = 2015. No samples were taken Spring, 2014. No yellow perch were sampled Fall, 2014. An absence of a bar indicates that I did not detect this prey for the given predator species.

Table 4.2: Frequency of occurrence (%) of target invertebrate AIS in scDNA of all four sampled predator species determined by CO1 metabarcoding. An AIS was scored as "present" in a stomach if the sequences first passed filtering requirements in QIIME including for minimum sequence quality scores, minimum sequence lengths and minimum match identities for local BLASTs. Cases noted as 0.0 % had no occurrences of the AIS for the combination of predator and season. Note I report results for only three of five targeted AIS invertebrate species as *Dreissena polymorpha* and *Hemimysis anomala* were not detected in any samples.

Season	Predator(↓) Prey(→)	*Bythotrephes longimanus*	*Cercopagis Pengoi*	*Dreissena rostriformis bugensis*
Summer 2014	Walleye	0.0	21.4	100.0
	White bass	7.1	35.7	85.7
	White perch	14.3	28.6	100.0
	Yellow perch	0.0	14.3	100.0
Fall 2014	Walleye	0.0	21.4	92.9
	White bass	0.0	22.2	100.0
	White perch	0.0	7.1	100.0
Spring 2015	Walleye	0.0	14.3	100.0
	White bass	0.0	7.1	100.0
	White perch	0.0	15.4	92.3
	Yellow perch	0.0	21.4	92.9
Summer 2015	Walleye	0.0	21.4	100.0
	White bass	33.3	33.3	100.0
	White perch	66.7	6.7	93.3
	Yellow perch	69.2	7.7	100.0
Fall 2015	Walleye	0.0	28.6	100.0
	White bass	0.0	7.1	100.0
	White perch	0.0	14.3	100.0
	Yellow perch	0.0	21.4	100.0

Table 4.3: Frequency of occurrence (%) of target fish prey in scDNA of four sampled predator species determined by CO1 metabarcoding. Prey was scored as "present" in a stomach if the sequences first passed filtering requirements in QIIME including for minimum sequence quality scores, minimum sequence lengths, minimum match identities for local BLASTs, minimum numbers of sequences produced in each sample and number of sequences of a prey type that were produced in each sample (see methods). Cases noted as 0.0 % had no occurrences of the AIS for the combination of predator and season.

Season	Predator	Channel catfish	Gizzard Shad	Emerald shiner	Rainbow Smelt	Round goby
Summer 2014	Walleye	0.0	7.1	0.0	0.0	14.3
	White bass	14.3	14.3	0.0	7.1	14.3
	White perch	14.3	7.1	0.0	7.1	7.1
	Yellow perch	15.4	0.0	7.7	0.0	15.4
Fall 2014	Walleye	6.7	53.3	6.7	20.0	6.7
	White bass	77.8	66.7	22.2	0.0	66.7
	White perch	14.3	0.0	14.3	0.0	64.3
Spring 2015	Walleye	0.0	0.0	0.0	7.1	21.4
	White bass	0.0	7.1	0.0	0.0	7.1
	White perch	38.5	7.7	15.4	0.0	15.4
	Yellow perch	7.1	0.0	0.0	14.3	0.0
Summer 2015	Walleye	28.6	71.4	21.4	35.7	28.6
	White bass	0.0	16.7	0.0	50.0	33.3
	White perch	6.7	20.0	26.7	6.7	26.7
	Yellow perch	0.0	0.0	7.7	7.7	0.0
Fall 2015	Walleye	7.1	35.7	21.4	14.3	0.0
	White bass	0.0	21.4	0.0	0.0	7.1
	White perch	7.1	7.1	7.1	0.0	0.0
	Yellow perch	14.3	7.1	0.0	0.0	7.1

I found significant effects of predator species, predator total length, season, and year on patterns of occurrences among statistical models for various prey GLMs in scDNA samples across my data set (Table 4.4; Figures: 4.2, 4.3, 4.4). I found that occurrences for channel catfish, gizzard shad, and round goby prey were significantly related to the predator species consuming the prey (Table 4.4: P = 0.011, P = 0.004, P = 0.017, respectively). Also, I found that

channel catfish and round goby prey occurrences in their GLMs were significantly related to predator species total length (Table 4.4: P ≤ 0.001, and P = 0.006 respectively). Season was significant in the GLMs for channel catfish and gizzard shad prey (Table 4.4: P = 0.050, and P = 0.008). Year was significant in GLMs for *Bythotrephes longimanus,* channel catfish and round goby prey species sequences in scDNA diet samples (Table 4.4: P = 0.000, P = 0.044, and P = 0.046 respectively).

Table 4.4: Results of binary logistic GLM analyses (P-values) of occurrences for prey species identified using metabarcoding of stomach content DNA across four predator species, sampled in 3 seasons over 2 years. Significant results are in bold. Wald Chi-square estimates are in parentheses below significance values.

Prey from scDNA	Predator species	Year	Season	Predator TL
Bythotrephes longimanus	0.877 (0.68)	**0.000** (17.64)	**0.049** (6.85)	0.792 (0.07)
Cercopagis pengoi	0.947 (0.37)	0.471 (0.52)	0.785 (0.48)	0.757 (1.00)
Dreissena rostriformis bugensis	0.887 (0.64)	0.376 (0.78)	0.476 (1.49)	0.858 (0.03)
Ictalurus punctatus	**0.011** (11.15)	**0.044** (4.04)	**0.050** (5.95)	**0.000** (15.05)
Notropis athernoides	0.165 (5.10)	0.206 (1.60)	0.377 (1.95)	0.684 (0.17)
Dorosoma cepedianum	**0.004** (13.29)	0.254 (1.30)	**0.008** (9.71)	0.537 (0.38)
Osmerus mordax	0.077 (6.86)	0.201 (1.64)	0.505 (1.37)	0.223 (1.49)
Neogobius melanostomus	**0.017** (10.17)	**0.046** (3.99)	0.613 (0.98)	**0.006** (7.46)

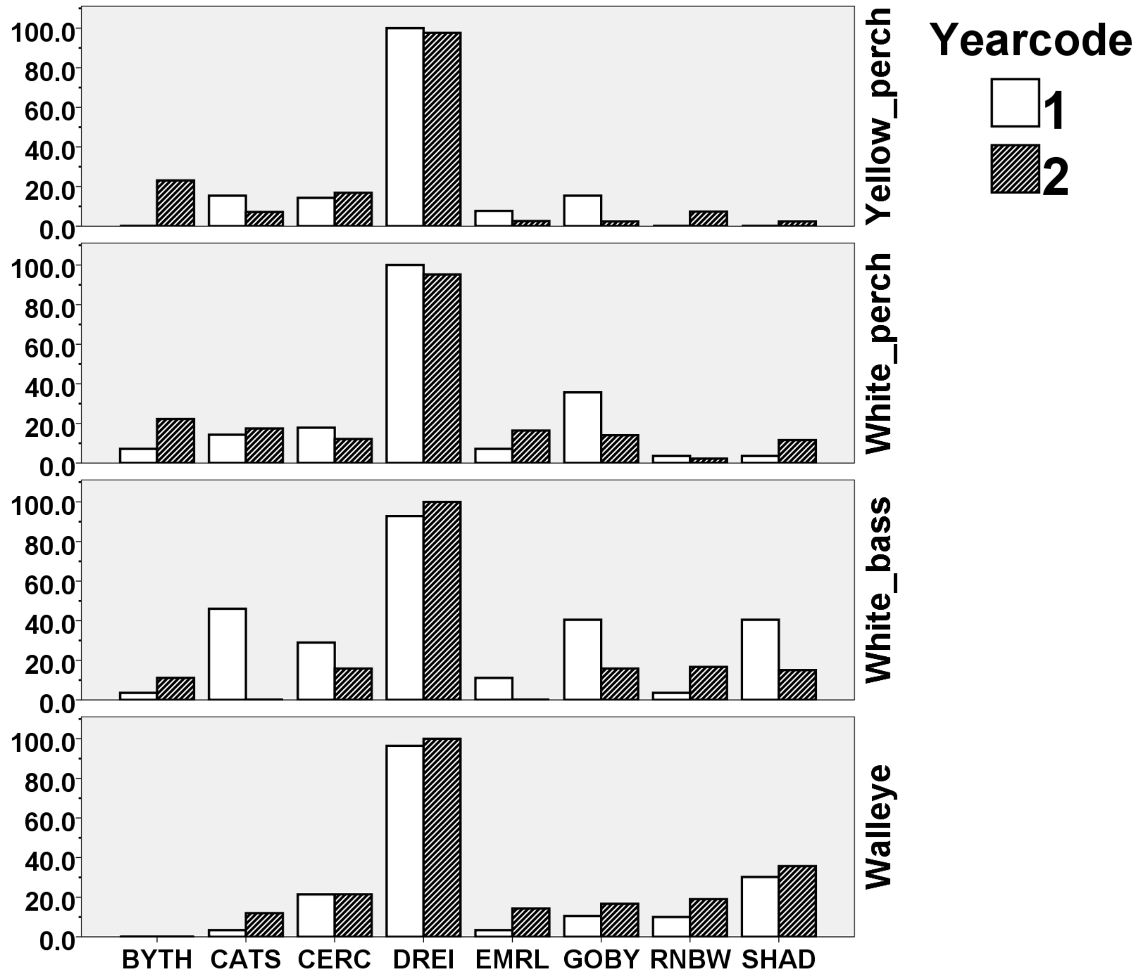

Figure 4.2: Mean percent frequency of occurrence (%) for the eight prey types and four predator species according to the year sampled. BYTH = *Bythotrephes longimanus,* CATS = channel catfish, CERC = *Cercopagis pengoi,* DREI = *Dreissena rostriformis bugensis,* EMRL = emerald shiner, GOBY = round goby, RNBW = rainbow smelt and SHAD = gizzard shad. Year 1 = 2014, Year 2 = 2015. No samples were taken in Spring, 2014 and no yellow perch were sampled Fall, 2014.

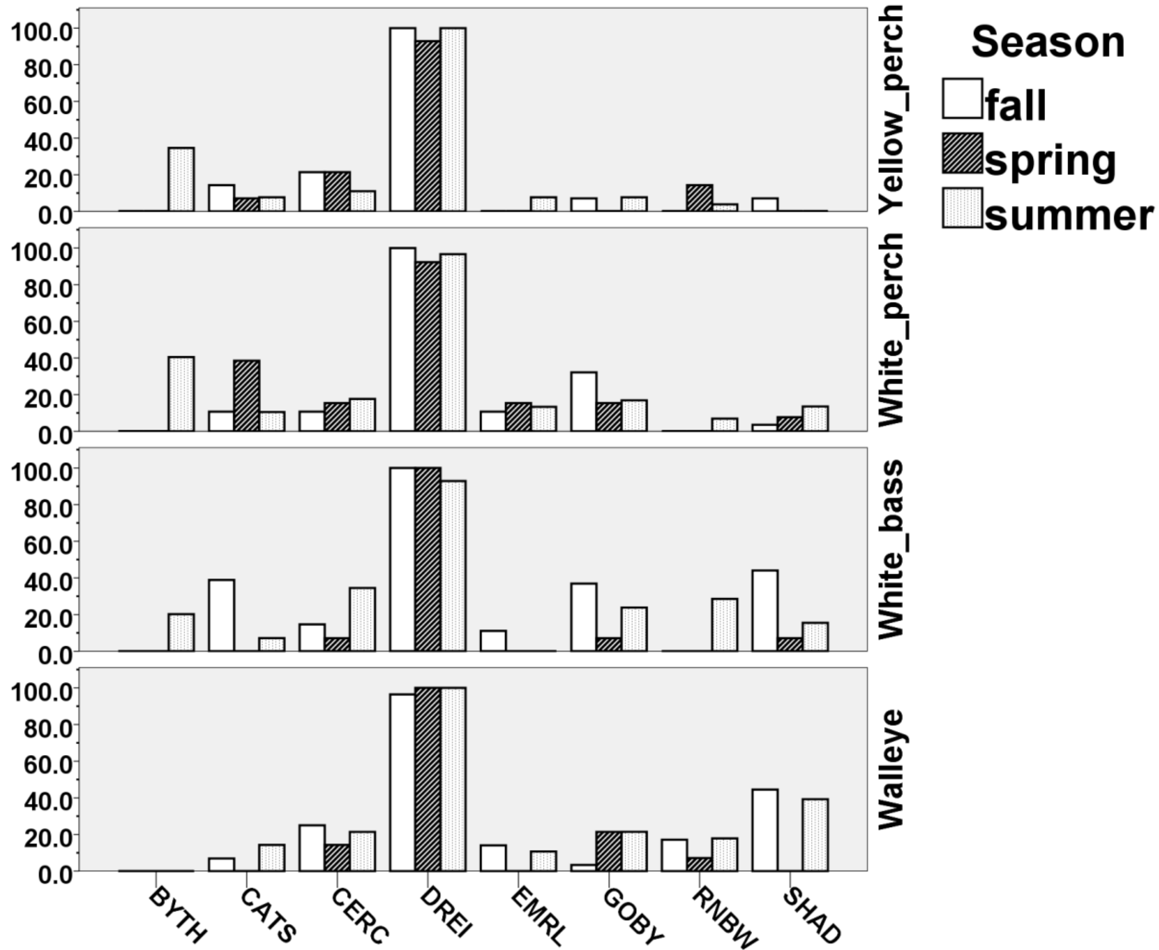

Figure 4.3: Percent frequency of occurrence (%) for each of four predator species and eight prey types according to the season sampled. BYTH = *Bythotrephes longimanus,* CATS = channel catfish, CERC = *Cercopagis pengoi,* DREI = *Dreissena rostriformis bugensis,* EMRL = emerald shiner, GOBY = round goby, RNBW = rainbow smelt, and SHAD = gizzard shad.

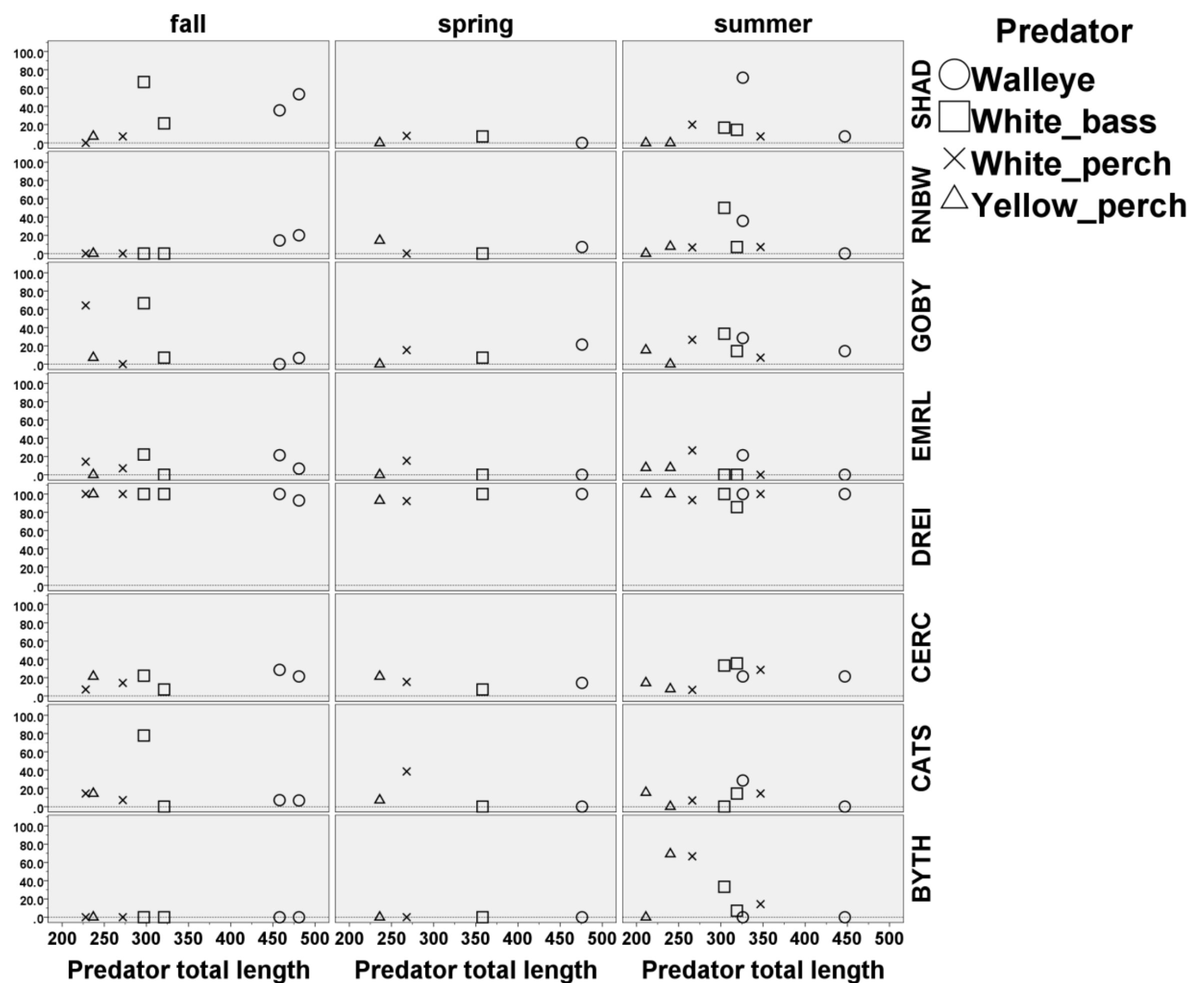

Figure 4.4: Mean percent (y-axis) frequency of occurrence of each of the eight prey types in my study according to season and total length (nearest mm, x axis) of each predator. There are two symbols for each predator species in each panel except for spring panels, reflective of the two different years sampled in my study. BYTH = *Bythotrephes longimanus,* CATS = channel catfish, CERC = *Cercopagis pengoi,* DREI = *Dreissena rostriformis bugensis,* EMRL = emerald shiner, GOBY = round goby, RNBW = rainbow smelt and SHAD = gizzard shad.

Discussion

I detected three of five target invertebrate AIS prey, three of three non-native target fish prey, and both target native fish prey in Lake Erie predator scDNA samples using CO1 metabarcoding. Some of the detected AIS are included in a list of the world's 100 worst invasive species (Lowe et al., 2000). Results show that the western basin of Lake Erie is a highly invaded

ecosystem in which AIS prey are commonly found in predator diets, resulting in a foodweb designated as “unstable” owing to ongoing impacts from AIS (Markham & Knight 2017), or reflective of a new and novel foodweb and dynamics. For example, *Dreissena rostriformis bugensis* occurred in nearly every predator scDNA sample across my entire data set, on average 18.4 % of all samples contained the AIS invertebrate prey *Cercopagis pengoi*, and the mean Summer, 2015 occurrences of *Bythotrephes longimanus* for white and yellow perch were respectively 66.7 % and 69.2 %. Invasive preyfishes in scDNA were mostly as, or more, common than native preyfishes, i.e., across all samples the mean frequency of occurrence of gizzard shad was 18.0 %, 17.7 % for round goby, and 8.9 % for rainbow smelt and for native prey channel catfish and emerald shiner occurrences were 13.3 % and 7.9 %, respectively. I hypothesize that variability in occurrences of preyfishes in scDNA in this component of the foodweb reflects: 1) relative abundances of the prey species in the field, 2) prey consumption variation due to reproductive patterns of the predators and prey, 3) specific prey selection strategies of predators, 4) ability of predators to adapt to short term seasonal and longer term dynamics of prey in ways that reduce competition and diet overlap, and 5) recent ongoing ecological changes induced from AIS (Markham & Knight 2017; Forage Task Group 2018).

My results suggest that native predator fishes may have been slightly better overall at consuming AIS invertebrates. For example, white bass and white perch were the only predators which consumed any of the AIS *Bythotrephes longimanus* in summer, 2014 (7.1 and 14.3 % respectively). In summer, 2015 yellow perch also consumed *Bythotrephes longimanus* and nearly tied with white perch for the highest frequency in their scDNA at 69.2 %, 66.7 %, respectively. Essentially, in every season and year sampled, a native predator had the highest mean occurrence of the AIS *Cercopagis pengoi*. However, *Cercopagis pengoi* also was very

common in scDNA of the AIS predator white perch. Patterns of occurrences of *Bythotrephes longimanus* and *Cercopagis pengoi* in the diets of all sampled predator species across seasons were very different; however, with equivalent non-significant outcomes for predator species as a factor driving those patterns. As virtually all predators had *Dreissena rostriformis bugensis* in their scDNA, no obvious distinction between native and non-native predators could be made in the occurrences of *Dreissena rostriformis bugensis* in their diets.

CO1 metabarcoding appeared to provide better detection of *Bythotrephes longimanus* in stomach contents than traditional visual-based diet analysis for some seasons. For example, LEBS et al. (2015) and Scarbro et al. (2016) found Western Basin Lake Erie yellow and white perch diets sampled in spring and summer respectively had occurrences of *Bythotrephes longimanus* of 3.6 % and 17.0 %, and of 5.2 % and 20.0 %. In my data set, I did not find this AIS prey in spring, but summer, 2015 occurrences were 66.7 % and 69.2 % respectively for yellow and white perch. The absence of *Bythotrephes longimanus* in spring scDNA compared to Scarbro et al. (2016) spring results may simply reflect that my samples were taken before resting eggs deposited the prior winter began to hatch in large numbers. Seasonal abundances of *Bythotrephes longimanus* tend to have the highest densities at the conclusion of summer (Keeler et al., 2015), concurrent with all positive hits in my study. Indeed, I found a significant seasonal effect on the occurrence of *Bythotrephes longimanus* in scDNA. This may reflect: 1) diet shifts from spring to summer in which predators begin to consume this prey more often as it rapidly increases in abundance, followed by decreased consumption into fall as remaining adult *Bythotrephes longimanus* likely experienced high mortality from cumulative predation and increasingly colder temperatures (Kerfoot et al., 2016); 2) availability of alternative prey or; 3) predator shifts in gape towards larger preferred prey types as the season progresses. My findings

of high occurrences of *Bythotrephes longimanus* in summer, 2015 are consistent with previous central basin Lake Erie yellow perch diet estimates for summers, 2004-2008 where the diet content was "dominated" by *Bythotrephes longimanus* and emerald shiner (Markham & Knight 2017). Using traditional diet analyses, Panos et al. (2018) found that yellow perch sampled in June in the western basin of Lake Erie consumed *Bythotrephes longimanus* at a frequency of occurrence of 16 % and in September at 10 %, both levels more similar to the lower levels of occurrence that I observed in predator scDNA in summer, 2014.

A significant year effect for the occurrence of *Bythotrephes longimanus* prey (2014 and 2015), seems attributable to the large differences in occurrence in the two summers for predators that consumed this prey. This is interesting as the lower values in one year in my study were closer to values published in other studies. This could reflect the timing of sampling in each year, or differing levels of spatial overlap of predators and prey among sampling bouts in each year, whereby larger swarms of the AIS may have occurred at local scales during sampling in given years. The finding of an interannual effect for *Bythotrephes longimanus* occurrences is supported by survey-based findings from the Lake Erie Forage Task Group Report (2018), which reported that western basin Lake Erie cladoceran biomass in 2015 was the highest ever recorded in the annual time series (from: 1999-2017). Though not specified as particular to *Bythotrephes longimanus* as it is also a cladoceran, it would suggest a large prey base was available for *Bythotrephes longimanus* in 2015 versus 2014 as native cladocerans are a main prey for this AIS (Barbiero & Tuchman 2004). Increased availability of prey for *Bythotrephes longimanus* could have influenced the much higher occurrences of *Bythotrephes longimanus* in scDNA in 2015 I observed, compared to 2014 through a stronger year-class. However, given the inclusion of only two years, yearly variation may be due to many other potential biotic or biotic variables that

could have influenced the outcome. Regardless, *Bythotrephes longimanus* can contribute substantially to foodwebs, not only as prey but also as predators by selectively consuming smaller zooplankton and as possible competitors for these and other zooplankton prey (i.e., Barbiero & Tuchman 2004; Kerfoot et al., 2016). Additionally, *Bythotrephes longimanus* can non-lethally influence the distribution of its potential prey taxa as they will actively avoid them and occupy different areas of the water column or become locally absent (Lehman & Cáceres 1993; Pangle et al., 2007; Bordeau et al., 2011; Bunnell et al., 2011). These changes can in turn affect the availability of the native prey to other native predators including young of the year fish. Additionally, potential negative influences on predators that consume large amounts of *Bythotrephes longimanus* prey can result from the low energy density of this AIS relative to native prey it might displace (Kerfoot et al., 2016; Staples et al., 2017). Further, *Bythotrephes longimanus* defensive spines work to limit their consumption by small sized gape limited fishes that would otherwise be eating the native prey displaced by *Bythotrephes longimanus*. Thus, the roles of the AIS *Bythotrephes longimanus* in predator diets, and foodwebs in general, characterized through CO1 metabarcoding of scDNA, helps us to understand the complexity of ongoing impacts of this AIS in the western basin of Lake Erie.

I identified *Cercopagis pengoi* in scDNA consistently across all seasons in both sampling years in predator scDNA. Ecological data for *Cercopagis pengoi* as a prey item within Lake Erie for the predator species I sampled is not well described in literature. The Forage Task Group of Lake Erie (2018) determined that in September 2017, yellow perch in the western basin of Lake Erie consumed *Cercopagis pengoi* at a frequency of occurrence of 9.0 %. Creque and Czesny (2012) described diets of summer and fall collected Lake Michigan yellow perch and found an average percent frequency of occurrence of *Cercopagis pengoi* in diets of only 3.5 %, whereas

my Fall, 2015 yellow perch had a mean frequency of occurrence of 21.7 %. However, making comparisons between Lakes Michigan and Erie is problematic due to fundamental differences in the Lake Michigan and Lake Erie foodwebs and respective yellow perch population ecologies. Perhaps the consistent occurrences of *Cercopagis pengoi* in all Lake Erie predator scDNA relates to increased habitat suitability for this AIS due to the warmer and generally shallower waters in the Lake Erie Western Basin, compared to more offshore regions of the Great Lakes (i.e., Forage Task Group 2018; Ptáčníková et al., 2015). Seasonal or decreased abundances of *Bythotrephes longimanus* which can prey upon *Cercopagis pengoi* to the point of changing its distribution, may have resulted in increases in both the population size and availability of *Cercopagis pengoi* to predators as well as increasing the potential spatiotemporal overlap of this prey with predators (Witt & Cáceres 2004; Ptáčníková et al., 2015). However, if *Bythotrephes longimanus* was reduced to low enough levels, one might expect to see higher predation of *Cercopagis pengoi*, possibly explaining apparently consistent occurrences of this AIS across seasons (i.e., MacIsaac et al., 1999; Pothoven et al., 2007; Cavaletto et al., 2010; Forage Task Group, 2018). *Cercopagis pengoi* may have also occurred frequently in predator scDNA as a result of a large prey base for *Cercopagis pengoi* in the more productive ecosystem of the Western Basin of Lake Erie compared to Lake Michigan results, a form of a "bottom up" effect (Vanderpoleg et al., 2002; Benoit et al., 2002; Laxson et al., 2003; Forage Task Group, 2018). This is important because *Cercopagis pengoi* can compete with young of the year fishes or other native taxa for common preferred prey (i.e., MacIsaac et al., 1999; Laxson et al., 2003; Bushnoe et al., 2003). Interestingly, I found no factors (including predator species, season sampled, and year sampled) to have affected the patterns of occurrences of *Cercopagis pengoi* as prey in my study, reflecting the consistency of this prey in the diets of my selected predators. This suggests that possible

large-scale regional and longer-term temporal influences beyond single seasons and two years may be driving the ecology and potential impacts of *Cercopagis pengoi* in the Lake Erie foodweb. Although the effect may be minor, one hypothesis is that *Cercopagis pengoi* could benefit planktivorous fishes by transferring energy from zooplankton prey that are difficult for fish to capture to the predators of *Cercopagis pengoi*, but would require acceptance of several other assumptions (Vanderploeg et al., 2002). Potential consequences for the predators of this AIS and the invaded ecosystem include ongoing behaviorally-induced "top down" trophic cascade effects or "bottom up" competition effects and are notable considerations for resource managers.

The invertebrate AIS *Dreissena rostriformis bugensis* was present in most of all of the scDNA samples in this study, i.e., > 85 % for all predators in all seasons. The occurrences of the target AIS prey *Dreissena rostriformis bugensis* in yellow and white perch from the Western basin of Lake Erie were much higher than those reported by Scarbro et al. (2016). In fact, my frequency of occurrence for this prey species was substantially higher than any reported in the literature for all predator species in my study. This raises the possibility that results could be contamination; however, all my controls indicated no field or lab-based contamination. Hence, I instead suggest metabarcoding using CO1 and the primer set specific to *Dreissena rostriformis bugensis* was so sensitive that results reflect actual ecological conditions in the foodweb. For example, because *Dreissena rostriformis bugensis* existed at the time of sampling at very high abundances in essentially all of Lake Erie (GLANSIS 2018; LEBS et al., 2015), it is possible that some level of selective predation occurred. Specifically, perhaps small, recently established benthic veligers with relatively undeveloped bivalve shells or larger sized shelled juveniles or adults were intentionally targeted as prey (Mills et al., 1995). While published data on my fish

predator species in this regard is limited, at least adult yellow perch from the Western Basin have been found to eat dreissenids at high levels through traditional diet studies, and these AIS are sometimes noted as main prey components in their diets (Knight et al., 1984; LEBS et al., 2018).

Negative environmental effects are well documented resulting from juvenile and adult *Dreissena rostriformis bugensis* benthic establishment. Those effects include competition-induced microplankton prey reduction, decreased phytoplankton abundance, or spatial competition for limited habitat structure with native benthic invertebrates (i.e., GLANSIS 2018; Benson et al., 2018). Few descriptions of ecological or bioenergetics impacts from *Dreissena rostriformis bugensis* resulting from consumption by fish predators are available. However, omnivorous Lake Erie lake whitefish (*Coregonus clupeaformis*) began to consume shelled adult dreissenids at high levels after they became locally abundant and in the absence of preferred native benthic invertebrate prey excluded by *Dreissena* spp. (Madenjian et al., 2010; Pothoven & Madenjian 2008). This diet shift resulted in less energy available to lake whitefish and is thought to have played a role in corresponding reducing growth rates (Madenjian et al., 2010; Pothoven & Madenjian 2008). The loss of energy is likely due in part to the shell of this AIS lacking nutritional content, but being highly indigestible and causing a false sense of fullness when consumed in large quantities (Madenjian et al., 2010; Pothoven & Madenjian 2008). For example, Lake Erie yellow perch have exhibited very low measures of body condition possibly in relation to consumption of *Dreissena* spp. (Markham & Knight 2017).

Another possible explanation for the very high occurrences of *Dreissena rostriformis bugensis* in my diet study is secondary and non-selective feeding on *Dreissena rostriformis bugensis* occurring as predators target other benthic prey such as round gobies (i.e., Madenjian et al., 2011). Additionally, unintentional consumption of *Dreissena rostriformis bugensis*

planktonic eggs, larvae, and veligers may occur, and CO1 metabarcoding would be sensitive to the presence of such incidental prey. This could easily happen as *Dreissena rostriformis bugensis* are likely present in the environment at very high abundances in their juvenile planktonic life stages over the entire sampling period of this study, as they can reproduce year-round. Information regarding the consumption of early life stages of *Dreissena* spp. for any Great Lakes fish predator species is generally limited and this ecological aspect of *Dreissena rostriformis bugensis* in the foodwebs of the Great Lakes appears not generally well understood. However, such incidental consumption of early life stages of *Dreissena rostriformis bugensis* has recently been proposed as a potential important component of "lost biomass" in ecosystem based models for invaded lakes (see Campbell et al., 2009; Bowen et al., 2018). The lack of data on the role of the early life stages of this AIS may be due to the fact that the planktonic life forms of *Dreissena rostriformis bugensis* are very small, hard to identify and may begin to digest quickly upon consumption, becoming unrecognizable despite leaving potential trace DNA that would have been picked up in my study. This highlights the importance of metabarcoding to help untangle complex Great Lakes foodweb interactions. However, the very high sensitivity for *Dreissena rostriformis bugensis* in scDNA was in part due to my development of target-species PCR primers with exceptional sensitivity (0.002 % by molecular weight in a mixed species DNA sample; Mychek-Londer Ph.D. Dissertation Chapter Two, 2018). A lower level of occurrence of *Dreissena rostriformis bugensis* using metabarcoding was found for Lake Michigan planktivorous fishes (Mychek-Londer Ph.D. Dissertation Chapter Three, 2018), which also supports the robustness of the results of this study and would indicate that contamination effects are even less likely. Predators from the Western Basin of Lake Erie may simply more commonly encounter *Dreissena rostriformis bugensis* in the comparatively shallower sampling area versus

offshore environments of Lake Michigan. In any case, it is clear that some Lake Erie AIS such as *Dreissena rostriformis bugensis* are more integrated into the foodweb than previously thought, perhaps due to predator habituation to eat *Dreissena rostriformis bugensis* as prey or because of its abundances at some life stage in this ecosystem.

I did not identify any *Hemimysis anomala* in the scDNA samples, similar to Scarbro et al. (2016) who only found an average occurrence of only 0.125 % in Western Basin Lake Erie yellow and white perch diet samples. These results may reflect the strong diurnal activity cycles of *Hemimysis anomala* that are dominated by daytime hiding in interstitial spaces to avoid fish predation and nighttime hunting for prey in the water column (Kipp et al., 2007; Boscarino et al., 2012). Lantry et al. (2012) found very low frequencies (< 0.10 %) of *Hemimysis anomala* in diets of a nearshore sampled population of Lake Ontario yellow perch taken in June, July, August, and September. That work indicated that *Hemimysis anomala* prey may be only present in fish predators that feed in the water column at or near dark, and have the ability to capture swift moving prey (Lantry et al., 2012). However, those conditions should not necessarily exclude the predators I sampled. Fishes I sampled in gill nets set in 10 m depth strata were likely relatively far away from suitable nearshore littoral habitat areas for *Hemimysis anomala* prey. The lack of *Hemimysis anomala* in my metabarcoding study provides some support of previous studies showing this AIS is not yet well incorporated into native or introduced fish diets in the Western Basin of Lake Erie.

Recent research has reported patterns of AIS and native prey fishes in diets which appear generally similar to my own results. The Forage Task Group (2018), which reported diets for the predator species I sampled, noted similar levels of occurrences of prey fishes in diets for samples from the western basin of Lake Erie. For example, they reported walleye having a high reliance

on gizzard shad (63 % frequency of occurrence), and also noted that round goby only occurred in 10 % of walleye diets in that study, levels similar to observances I recorded. Pothoven et al. (2017) found that the frequency occurrence of fish prey from April to November in walleye diets sampled in a shallow region of Saginaw Bay of Lake Huron was on average: 1) 12.5 % for rainbow smelt prey; 2) 9.0 % for *Notropis* spp. prey; and 3) 14.0 % for round goby prey. I found very similar overall mean percent occurrence of these prey in all walleye scDNA samples, i.e., 1) 14.1 %; 2) 12.3 %; and 3) 15.4 % respectively. Thus, CO1 metabarcoding of piscine prey, even with high contributions of the predator species DNA, can provide high resolution diet data, and likely improved the diet assessment by identifying what could be potentially otherwise unidentifiable digested fish prey remains.

In my study, three of five prey fish species (channel catfish, gizzard shad, round goby) had a significant predator species effect related to their occurrences in scDNA. For channel catfish, the significant predator species effect may have been influenced by the very high variation in the occurrences of this prey species in the four different predator species diets. It seems likely that ontogenic and seasonally-based reproductive periods of spawning in predators and channel catfish prey may have driven the variation behind the predator species effect for this prey. Neither invasive nor native predator species seemed to show any clear and consistent trend in their consumption of channel catfish.

Gizzard shad was consumed at a high rate of occurrence by walleye, especially in summer, 2015 (71.4 %) and fall of both years (53.3 %, 35.7 %) and this fits well with previous descriptions of western basin Lake Erie walleye diet studies indicating a preference for this prey. Gizzard shad also showed a lower but still high rate of occurrence in scDNA of the native predator white bass in these same years and seasons, including the highest mean occurrence of

this AIS prey in scDNA of this predator from all fishes sampled Fall, 2014 (66.7 %), in agreement with Hartman (1998) who demonstrated that gizzard shad made up 56-97 % of white bass diets sampled between July and October from the western basin Lake Erie. It may have been these high occurrences, especially for walleye, in combination with the more consistent absences or generally much lower levels of gizzard shad for yellow perch and white perch which drove the significance of the predator species effect in the GLM for this AIS prey. In the literature gizzard shad is consistently identified as a preferred prey for walleye and white bass when available and in this study these two native predators appeared better at consuming gizzard shad than the AIS predator white perch and native predator yellow perch (). Thus, as has been described historically for the role of gizzard shad in the foodweb of the Western Basin of Lake Erie, the role of this AIS today still appears to be as "a cornerstone for piscivore production" especially for walleye and perhaps white bass, but appear to remain a less important prey for white and yellow perch production (Hartman 1998; Hartman and Margraf 1992).

For the AIS prey round goby, no distinction between native or non-native predator consumption was apparent. Essentially all species consumed round goby at low to moderate levels, although yellow perch generally lacked this prey, consistent with traditional diet data from the Forage Task Group (2018). All predators I studied have been shown to consume round goby in the Great Lakes, consistent with my results and thus round goby is now clearly an important component of the Western Basin of Lake Erie foodweb. This is especially true in that round goby acts as a conduit in transferring energy into higher trophic levels because it is a specialized predator capable of high levels *Dreissena* spp. consumption (Johnson et al., 2005; Bunnell et al., 2009; Madenjian et al., 2010).

For the fish prey species emerald shiner and rainbow smelt, predator species was not a significant factor affecting their occurrences in sampled predator scDNA. Native predators were generally better at consuming these two prey fishes than the AIS predator white perch, but it may relate to white perch having a lower tendency for piscivory than walleye and white bass. Additionally, the relatively weak age-zero and age-one year classes of emerald shiner in 2013 and 2014 in the Western Basin of Lake Erie may have contributed to a general lack of availability of this prey and a related lack of a significant effect for predator species upon this prey (Forage Task Group 2018). Rainbow smelt year classes also were at low abundances at the time of sampling possibly explaining relatively low levels of predation across all predator species and limited power to detect a predator species effect (Forage Task Group 2018).

Predator size (TL) had a significant effect on the occurrence of channel catfish in scDNA diet samples. Channel catfish belong to a genus of fishes that have evolved novel predation defense mechanisms, including locking dorsal and pectoral spines, which might affect the ability of predators to successfully consume this prey. A logical explanation of the significant effect on channel catfish occurrences in scDNA would relate to predator gape limitation with the expectation that larger fishes would be better at consuming this prey (i.e., Diana 1995; Fine et al., 1997). For example, in examination of mean TLs between individuals of each predator species with and without channel catfish as prey, the differences overall were very small (with channel catfish mean TL ± SD = 248.2 ± 38.6; without channel catfish TL = 243.8 ± 37.6). Likewise, differences in mean TLs were also very small for yellow perch with channel catfish prey (TL = 231.6 ± 2.8 vs. without prey = 224.8 ± 20.5) but were largest for walleye (mean TL with prey = 446.2 ± 70.2; without prey = 353.0 ± 82.8), and moderate for white bass (mean TL with prey = 328.3 ± 29.7; without prey = 300.1 ± 35.7). It thus seems likely that gape limitation

or similar effect contributed to variation in predation on channel catfish in Lake Erie based on predator size. Perhaps as predators gain size they can surpass defensive mechanisms and can consume the prey with greater efficiency and lower prey handling costs.

Unlike channel catfish, round goby are not typically believed to have defensive anti-predation mechanisms and mainly rely upon grouping together, hiding in benthos and shelters, and cryptic patterning to avoid predators; however, predator TL was also significant factor for its occurrence patterns in scDNA. Though information on gape limited predation of round goby by Great Lakes predator fishes is limited, Truemper and Lauer (2005) hypothesized that that yellow perch in Southern Lake Michigan chose either round goby or alewife (*Alosa pseudoharengus*) as prey when both were available depending upon their total length. Specifically, yellow perch ate round goby at smaller sizes and once they attained the minimum gape size required, they switched from round goby to alewife. My data supports this hypothesis; for example, predator fishes that did not, and which did eat round goby had mean TLs (mm, ± SD) as follows: walleye that did not consume goby = 445.2 ± 70.8, walleye that did = 396.4 ± 91.5; white bass not consuming goby = 325.5 ± 31.9, white bass that did = 315.6 ± 33.7; white perch not consuming goby = 250.7 ± 29.1, white perch that did = 236.6 ± 56.8; and yellow perch not consuming goby = 232.1 ± 21.4 and yellow perch that did consume round goby = 212.7 ± 22.0. Thus, predators from all assayed species had a lower mean TL for individuals that consumed round gobies versus those that did not. Thus, round goby may be an important prey for fishes in the foodweb of the Western Basin Lake Erie prior to attaining sizes great enough to switch to larger or other relatively better-defended preyfishes. There was no significant effect for predator TL on the consumption of gizzard shad, emerald shiner, or rainbow smelt prey fishes. Thus, including not only various predator species, but also a substantial size range within each predator species

sampled when using a metabarcoding approach of scDNA is an important step to allow in-depth and meaningful interpretation of such detailed diet data.

My analyses showed strong seasonal effects on the patterns of fish prey detected for channel catfish and gizzard shad. Pothoven et al., (2017) found that for Lake Huron walleye and yellow perch, diets were dominated by rainbow smelt in the spring-summer months while the prey composition shifted towards gizzard shad in the summer to fall. I observed a similar trend, in that gizzard shad exhibited a significant seasonal effect. The lack of a seasonal affect for rainbow smelt in my study may relate to the lower abundance of rainbow smelt (average fish · ha^{-1}, 2014-2015 = 1.5) in comparison to much higher abundances of gizzard shad (average fish · ha^{-1}, 2014-2015 = 29.1) in the western basin of Lake Erie (Forage Task Group, 2018). This effect seemed greatest in walleye as they consumed more gizzard shad when transitioning from spring to summer months and generally maintained or increased consumption of gizzard shad into fall. This likely in part reflects walleye spawning in the spring and thus reduced feeding at that time such as when I sampled in late April. I found no seasonal effect for rainbow smelt and emerald shiner prey occurrences, perhaps because both of these fishes were at relatively low abundances in the Western Basin of Lake Erie (rainbow smelt see above; emerald shiner average fish · ha^{-1}, 2014-2015 = 1.05; Forage Task Group, 2018) and consequently there were many instances of low and non-occurrences which may have limited the power of my analysis to detect seasonal variation. For channel catfish prey, the seasonal effect appeared more due to each predator species individually and independently having what appeared to be somewhat different occurrences of this prey in different seasons. Seasonal influences on prey consumption are often related to changing interactions of predators and prey caused by a variety of factors. For example, during predator spawning periods often prey consumption is reduced, dynamic

temporal and spatial aspects of prey densities exist related to their own ecologies, prey year class abundances can vary, and growth patterns of predators and prey variably progress across seasons – all of these affect availability and abundances of the prey. Due to these influences, and based on my results, findings suggest that temporal dynamics on the scale of season, at a minimum, are important considerations for any diet study, but especially for scDNA metabarcoding studies targeting community oriented foodweb dynamics as the method is sensitive to biotic and abiotic environmental factors due to the high resolution of prey detection that is possible.

Sample year effects were found for channel catfish and round goby prey. For example, although 77.8 % of predator white bass in Fall, 2014 contained channel catfish as prey, in Fall, 2015 I did not identify this prey in white bass scDNA. In Summer, 2014 walleye had no occurrence of channel catfish, but in Summer, 2015 channel catfish prey occurred at a mean frequency of occurrence of 28.6 % in walleye scDNA. Sample year effects are hard to characterize and may be due to possible shifting year classes of predators, or slightly different times I sampled in each year. More years of data are needed to effectively address year effects. However, channel catfish are There was also an interannual effect in the round goby GLM which may have resulted because mean proportions of round goby in scDNA in predators in 2014 consuming round goby appeared to have been higher than compared to in 2015. Although the effect is difficult to fully explain, apparent round goby abundances in Lake Erie can vary dramatically in only a short time span. For example, in 1999 the abundance per hectare of round goby in the Central Basin of Lake Erie was estimated to be 2,789 individuals per hectare, and was estimated to have dropped nearly 50 % to 1,461 individuals per hectare in the years from 2000 and 2002 (Johnson et al., 2005), and the 2014. Thus, it is entirely possible that the year affect for round goby was a true reflection of a possible reduction in the availability of this prey

to predators in 2015 compared to 2014 but potential reasons as to why would need additional explanation. There were no year based effects in GLMs for emerald shiner or rainbow smelt prey, and congruent with the lack of effects of other factors on occurrences of these prey taxa might reflect low abundances of them in the foodweb at the time of sampling (Forage Task Group 2018). My data show that at minimum, multiple years should be sampled if using metabarcoding to determine diets, particularly for large, dynamic, complex ecosystems such as the Western Basin of Lake Erie.

In summary, I was successful in detecting invertebrate and fish AIS and native prey using metabarcoding of scDNA of Western Lake Erie sampled native and AIS predator fishes. I found that AIS, as both predators and prey, are major integrated components of the Western Basin of Lake Erie foodweb. Despite substantial variation in the frequency of occurrence among the prey species in my study, likely in part related to the high proportion of predator DNA sequences in scDNA, my metabarcoding approach was sensitive to key target AIS and native prey in predator scDNA. The methods can be implemented further and elsewhere to complement well established foodweb and diet study methodologies. The results of this study will also better inform conservation and management decisions of public interest such as in determining AIS extermination strategies, limiting the spread of these AIS beyond the Great Lakes, and can help to better parameterize models for improving commercial fish harvest levels. My work also highlights the complex nature of large lake foodwebs with punctuated seasons, diverse species, potential competition, and AIS potentially participating in aspects of both “bottom up” and “top down” induced trophic cascade type effects such as in the Western Basin of Lake Erie.

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CHAPTER 5 - MAPPING INVASIVE AND NATIVE INVERTEBRATES IN TWO GREAT LAKES TRIBUTARIES USING ENVIRONMENTAL DNA (eDNA) METABARCODING

Introduction

Aquatic invasive species (AIS) have detrimental impacts on ecosystems, economies, and native species and can directly or indirectly contribute to species extinctions and extirpations (Gibbons et al., 2000; Dextrase & Mandrak 2006; Ricciardi & MacIsaac 2011; Simberloff & Rejmánek 2011). The role of AIS within ecosystems is critical information needed to assess their potential impact (Ricciardi et al., 1998; Johnson et al., 2005), limit further spread (Lodge et al., 2006; Vander Zanden & Olden 2008) and generate remediation plans for impacted ecosystems and species (Zimmerman & Krueger 2009; Ricciardi et al., 2017). AIS detection and monitoring has been traditionally accomplished through field sampling, visual counting, proportional based estimates, and frequency of occurrence (FOO) estimates (i.e., Gamble et al., 2011; Vasek et al., 2014; Landry et al., 2017). However, such methods may be biased by several factors, including: identification skills of the observer, resources available to sample multiple field sites and sort through numerous samples to detect rare species, degraded materials, immature or resting stages, and cryptic species that make accurate visual taxonomic identification difficult or impossible (Metcalfe-Smith et al., 2000a; Folino-Rorem et al., 2009; Legler et al., 2010, Briski et al., 2011; Nannini et al., 2016).

To overcome such limitations, AIS and rare species occurrences can be detected through metabarcoding of environmental DNA (eDNA) samples. Such eDNA metabarcoding approaches have been applied in lotic systems to determine the makeup of aquatic invertebrate, amphibian and fish communities (Deiner et al., 2016; Elbrecht et al., 2017; Li et al., 2018) and to detect newly arriving AIS (Goldberg et al., 2013; De Ventura 2017; Xia et al., 2018). However, much of the published literature using eDNA metabarcoding for invertebrates has assigned

sequences to the genus, family, or even higher taxonomic classifications, potentially limiting opportunities to identify AIS and rare native species (Taberlet et al., 2012; Deagle et al., 2014; Lacoursière-Roussel et al., 2018).

As the Great Lakes have experienced ongoing AIS introductions and expansions (i.e., MacIsaac et al., 1999; Simberloff 2006; Bates 2018; Cangelosi 2018), eDNA metabarcoding studies have been undertaken to determine the presence of such taxa. For example, Klymus et al. (2017) and Balasingham et al. (2018) each used novel PCR primer sets and metabarcoding to target AIS and rare native aquatic species in the Great Lakes and their tributaries. The Grand and Sydenham Rivers in southwestern Ontario eDNA sampled by Balasingham et al. (2018) were assessed for fish taxa. However, those tributaries are also good candidates for invertebrate based eDNA metabarcoding studies as they harbor uniquely diverse biological communities such as a variety of mollusks endemic to these areas (Metcalfe-Smith et al., 2003; Metcalfe-Smith et al., 2000b; Gillis et al., 2017). Additionally, they are prime candidates for ongoing introductions and range expansions of AIS, including those moving upstream from Lakes Erie and St. Clair into which they empty, or through such means as jump dispersal or downstream diffusion (Levri et al., 2007; Levri & Jacoby, 2008; Levri & Clark 2015; Krebs et al., 2018). Balasingham et al. (2018) proposed that fish AIS are moving upstream from the Great Lakes into the two rivers they sampled, and found eDNA from the AIS fish round goby (*Neogobius melanostomus*) at many sites spanning nearly the entire river. However, metabarcoding of aquatic eDNA to determine the occurrences of rare invertebrate native species and AIS has not been undertaken for these tributaries (but see Currier et al., 2018), yet such an approach can provide valuable data needed to better understand the distributions and potential impacts of the AIS and where they may co-occur with rare native taxa.

In this study I used a universal PCR CO1 primer set designed to target a broad range of aquatic invertebrates (Leray et al., 2013) expected in eDNA sampled from sites in both the Grand and Sydenham Rivers. I used a broad approach to search eDNA sample CO1 sequences for species-level sequence matches for target taxa and taxonomic classes of interest. Information from such an analysis will inform our understanding of how AIS may be impacting Great Lakes tributaries, and importantly, how they may be positively or negatively impacting at-risk native species. My approach highlights the emerging analytical power of eDNA metabarcoding in biologically diverse ecosystems such as the Grand and Sydenham Rivers, and is applicable to other comparable large Great Lakes tributaries. I hypothesize that: 1) AIS will be more common in the lower reaches of each stream, consistent with diffusion invasion from the lakes; 2) I will detect DNA sequences of rare, native, at-risk mollusk species throughout my sampled region, consistent with drift dispersal of the early life stages; 3) the occurrences of AIS and rare taxa will be different between the two river systems; but 4) common native rotifer and chironomid species (chosen to target as major biological components of the benthic and pelagic zones of the rivers) will show similar occurrence patterns in both river systems. Data from eDNA metabarcoding will help to inform the status and current distribution of invertebrate taxa, including documented AIS, and rare native species, as well as potentially yet undocumented species. The distributions of these taxa within each stream can inform local-scaled management efforts to understand AIS ecology, prevent further AIS expansion up- or downstream, and promote an assessment of local habitat factors that may be associated with desirable and rare native species.

Methods

Field sites

Samples in this study originated from, and were initially utilized according to details in Balasingham et al. (2018). Methods therein describe much of the sample processing used in this study and I cite Balasingham et al. (2018) and provide summaries for protocol changes as noted. Both the Grand and Sydenham Rivers were sampled for waterborne eDNA (Balasingham et al., 2018) and are in Southern Ontario, Canada (Table 5.1, Figures 5.1, 5.2). The north branch of the Sydenham River is approximately 70 km in length with mean annual discharge of 17 $m^3 \cdot s^{-1}$. The east branch is approximately 100 km in length with mean annual discharge of 34 $m^3 \cdot s^{-1}$ and the combined drainage area is 2,725 km^2; this branch empties into Lake St. Clair. The Grand River is approximately 300 km in length and drains the largest watershed in southern Ontario (6,800 km^2) emptying into Lake Erie (Table 5.1, Figures 5.1, 5.2). Sites were numbered starting at number 1 at sites proximal to the lake and the highest site numbers of 43 applied to the most upstream sampling site in each river system (Figures 5.1, 5.2).

Field sampling

Details for related field sampling, field- and lab-based controls, and additional methods are outlined in Balasingham et al. (2018). Water samples were taken at 86 sites, 43 in each river following a modified field sampling protocol from Jerde et al. (2011) meant to reduce field sampling error. Fisheries and Oceans Canada (DFO) conducted sampling efforts in September to early October 2013 in the Grand River, and in mid- to late October 2013 in the Sydenham River. Ten to 15 replicated river surface samples and two to three replicated near river-bottom-samples were taken at most sites, and subsamples were later filtered to recover solids for eDNA extraction (see below).

Table 5.1: Sites where water samples for eDNA were taken. Up to 10-15 replicates from the surface and near-bottom were taken, subsampled and filtered to capture eDNA. Fisheries and Oceans Canada (DFO) conducted sampling efforts in September to early October 2013 in the Grand River, and in mid- to late October 2013 in the Sydenham River.

Waterbody	Latitude	Longitude	Site	Waterbody	Latitude	Longitude	Site
Grand	42.91373	-79.80906	**1G**	East Sydenham	42.58202	-82.39388	**1S**
Grand	42.91330	-79.82272	**2G**	East Sydenham	42.58783	-82.39364	**2S**
Grand	42.91936	-79.83070	**3G**	East Sydenham	42.59768	-82.35619	**3S**
Grand	42.93319	-79.84689	**4G**	East Sydenham	42.59572	-82.29549	**4S**
Grand	42.94561	-79.86110	**5G**	East Sydenham	42.59948	-82.28603	**5S**
Grand	42.95892	-79.87048	**6G**	East Sydenham	42.59303	-82.26957	**6S**
Grand	43.02789	-79.89435	**7G**	East Sydenham	42.58983	-82.18858	**7S**
Grand	43.03932	-79.90611	**8G**	East Sydenham	42.59189	-82.17706	**8S**
Grand	43.05627	-79.91694	**9G**	East Sydenham	42.59572	-82.15779	**9S**
Grand	43.06345	-79.93033	**10G**	East Sydenham	42.59261	-82.14767	**10S**
Grand	43.07413	-79.95948	**11G**	East Sydenham	42.58908	-82.12897	**11S**
Grand	43.07931	-79.97327	**12G**	East Sydenham	42.60624	-82.04412	**12S**
Grand	43.08146	-79.97858	**13G**	East Sydenham	42.65044	-82.00870	**13S**
Grand	43.09219	-80.03560	**14G**	East Sydenham	42.70536	-81.98021	**14S**
Grand	43.08931	-80.03954	**15G**	East Sydenham	42.75710	-81.92388	**15S**
Grand	43.09391	-80.06511	**16G**	East Sydenham	42.76695	-81.84780	**16S**
Grand	43.09741	-80.07076	**17G**	East Sydenham	42.83095	-81.85126	**17S**
Grand	43.09909	-80.08580	**18G**	East Sydenham	42.84698	-81.82445	**18S**
Grand	43.11189	-80.12534	**19G**	East Sydenham	42.85545	-81.81327	**19S**
Grand	43.10515	-80.12981	**20G**	East Sydenham	42.85950	-81.78986	**20S**
Grand	43.10353	-80.13687	**21G**	East Sydenham	42.87008	-81.76925	**21S**
Grand	43.10189	-80.14156	**22G**	East Sydenham	42.86702	-81.74447	**22S**
Grand	43.09151	-80.16015	**23G**	East Sydenham	42.88176	-81.74226	**23S**
Grand	43.09219	-80.18549	**24G**	East Sydenham	42.89152	-81.68716	**24S**
Grand	43.09272	-80.19473	**25G**	East Sydenham	42.93166	-81.66692	**25S**
Grand	43.09798	-80.21476	**26G**	East Sydenham	42.94012	-81.65647	**26S**
Grand	43.09563	-80.21755	**27G**	East Sydenham	42.95665	-81.63218	**27S**
Grand	43.09224	-80.23489	**28G**	East Sydenham	42.95962	-81.62516	**28S**
Grand	43.10027	-80.24047	**29G**	East Sydenham	42.97577	-81.59409	**29S**
Grand	43.10721	-80.22956	**30G**	East Sydenham	42.98474	-81.56042	**30S**
Grand	43.11749	-80.20948	**31G**	East Sydenham	43.00267	-81.50877	**31S**
Grand	43.12784	-80.19896	**32G**	East Sydenham	43.01409	-81.50308	**32S**
Grand	43.14402	-80.20522	**33G**	North Sydenham	42.60316	-82.38595	**1N**
Grand	43.12929	-80.21369	**34G**	North Sydenham	42.61902	-82.38555	**2N**
Grand	43.11983	-80.21988	**35G**	North Sydenham	42.63224	-82.37353	**3N**
Grand	43.11790	-80.24152	**36G**	North Sydenham	42.64116	-82.37782	**4N**
Grand	43.11039	-80.24418	**37G**	North Sydenham	42.65658	-82.37445	**5N**
Grand	43.10748	-80.26532	**38G**	North Sydenham	42.65704	-82.38836	**6N**
Grand	43.11577	-80.26820	**39G**	North Sydenham	42.66848	-82.40307	**7N**
Grand	43.12109	-80.26335	**40G**	North Sydenham	42.69466	-82.40025	**8N**
Grand	43.12880	-80.26395	**41G**	North Sydenham	42.69556	-82.38743	**9N**
Grand	43.13933	-80.27583	**42G**	North Sydenham	42.70698	-82.39045	**10N**
Grand	43.14898	-80.29637	**43G**	North Sydenham	42.72166	-82.36298	**11N**

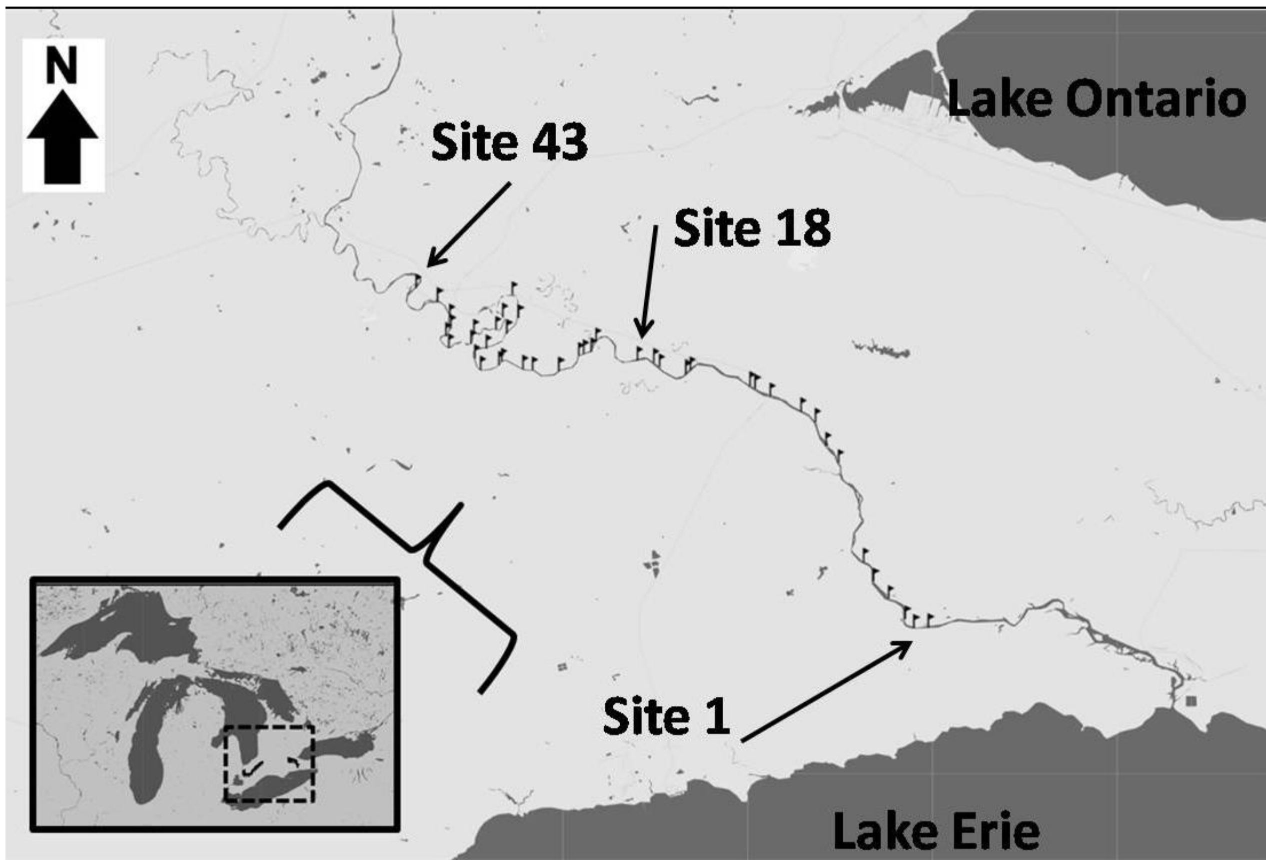

Figure 5.1: Map of 43 sampling sites for the Grand River. The first site is nearest to Lake Erie near the lower right corner of the map while the uppermost site farthest from Lake Erie. Each little flag represents a sampling site.

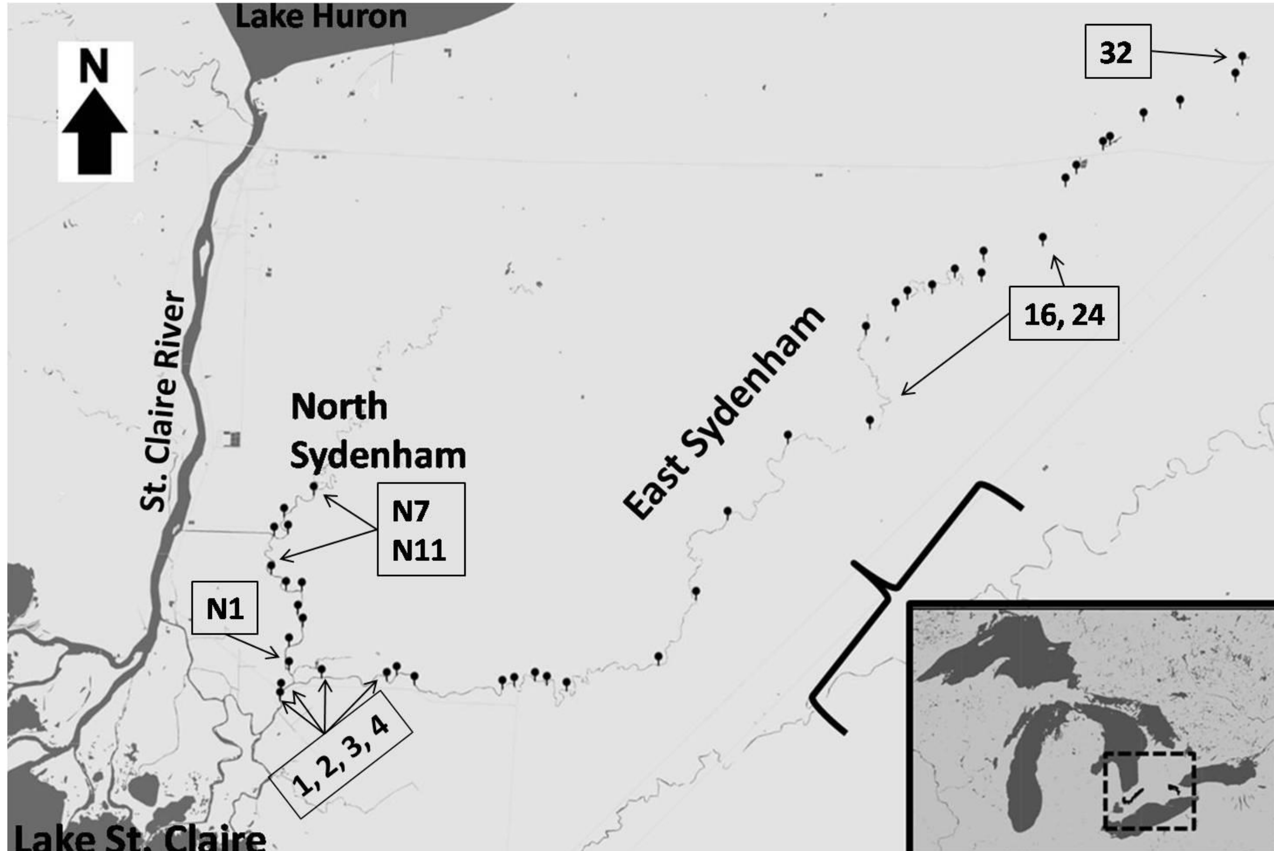

Figure 5.2: North and east channel sampling sites on the Sydenham River. The system drains east to west into Lake St. Claire (lower left). North channel sites 1-11 (N = 11) begin just north of the channel junction. East channel sites 1-32 include two sites just below the junction.

Filtering, DNA digestion, eDNA extraction

A subset of 2-5 field samples per individual sampling site from all 43 sites in each river system were randomly chosen for eDNA analysis. Within 24 hours of collection, all water samples were filtered using glass microfiber filter paper (47 mm diameter, 1.2 µm pore size; Whatman, Maidstone, UK). Prior to filtering each set of river samples for each site, 500 mL of ddH2O was filtered on a separate filter to act as a laboratory control, followed by the filtration of river samples on a single, new filter or through up to four new filters if they contained high organic content and suspended solids. If multiple filters were used for river samples from a

single site, those filters were each individually and equally subsampled and then combined for individual site-based DNA extractions. If the laboratory control created for each site or any other control(s) tested positive for target DNA during PCR amplification, the corresponding sample representing a field site was excluded due to potential contamination (i.e., false positives). Each filter or the individual groups of subsamples (for sites that required multiple filters) were placed in a 15-ml Falcon tube and stored at -20 °C until DNA extraction.

DNA digestion and extraction methods for each eDNA sample are outlined in full detail in Balasingham et al. (2018). In brief, DNA was extracted using glass beads and a bead-breaker (BioSpec Cat. No. 11079110) at 3,000 $\text{strokes} \cdot \text{minute}^{-1}$ using a Mini-Beadbeater-24 (Fisher Scientific LTD, BioSpec.) and cetyltrimethylammonium bromide (CTAB) digestion buffer with phenol–chloroform–isoamyl. An isopropanol and sodium acetate precipitation-concentration step was also performed before eventual resuspension of DNA in 30 µL of 10 mM TE buffer and 1.0 mm of 20 $\text{mg} \cdot \mu\text{L}^{-1}$ RNase. The resulting eDNA was used for both the Balasingham et al.'s (2018) fish eDNA identification study and this invertebrate eDNA study.

First-round PCRs

I used a two-step PCR approach where the first-round PCRs amplified eDNA using a universal PCR primer set (Leray et al., 2013) and the second-round of PCRs was a short-cycle to ligate the barcodes and adaptor sequences for next-generation sequencing to the first-round PCR amplicons. Full-cycle, first-round PCRs for each extracted sample of eDNA consisted of 2.5 µL of 10X Taq reaction buffer, 25 mM $MgCl_2$, 0.5 µM of each universal forward and reverse primer from Leray et al. (2013), 0.2 mM of each dNTP, 0.2 $\text{mg} \cdot \text{mL}^{-1}$ BSA, 0.1 units Taq polymerase, 1.0 µL of eDNA sample and ddH2O for a total reaction volume of 25 µL. PCRs used an initial denaturation of 95 °C for 2 minutes, 40 cycles of 30 seconds at 94 °C, 30 seconds annealing

temperature at 52 °C, 30 seconds at 72 °C for extension, a single cycle extension at 72 °C for 10 minutes, and a hold at 4 °C. Blank controls were included for all PCRs as well as the field blank controls (i.e., clean water samples handled in the field as for actual river water samples; n = 7) and field blank PCR products were included in the next-generation sequencing (NGS) library.

Amplicon processing, second-round PCRs

To remove dimers and fragments less than 100 bp after eDNA first-round PCR amplifications, PCR products were cleaned using magnetic beads (Agencourt AMPure XP, Beckman Coulter, Mississauga, ON, Canada). I next ligated unique barcode identifiers (as well as adaptor sequences for the NGS) to amplicons for each eDNA sample using a short-cycle second-round PCR for NGS library preparation. Second-round PCRs consisted of 2.5 µL of 10X Taq reaction buffer, 25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM forward primer + Uni-B adaptor, 0.5 µM reverse primer plus Uni-A adaptor (See: Balasingham et al., 2018; Mychek-Londer Ph.D. Dissertation Chapter Two 2018 for relevant adapter details), 0.1 units Taq polymerase, 10 µL of cleaned PCR product and remainder ddH_2O for a total reaction volume of 25.0 µL. Short-cycle second-round PCRs used denaturation for 2 minutes at 95 °C followed by 6 cycles of 95 °C denaturation for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension for 5 minutes at 72 °C.

NGS library preparation

Second-round PCR products with attached adaptors were purified again with magnetic beads but this time after first combining 10 µL of each cleaned barcoded PCR product from up to 72 samples into a 1.5 mL microcentrifuge tube. Products were left to resuspend at 20 °C overnight. I added equal volumes together from these tubes, then added equal volumes of isopropanol and one-tenth volume of 3 M sodium acetate (pH 5.2) to precipitate the amplicons.

Combined PCR products (i.e., NGS library) were centrifuged, following which I discarded the isopropanol and washed the pellet once with 70 % ice-cold ethanol. The tubes were centrifuged again, ethanol was discarded, and the library was eluted in 50 µL of ddH_2O. Next, 20 µL of the library was gel-extracted and bioanalysed to determine final DNA concentration using an Agilent High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Finally, the library was diluted to a final concentration of 60.0 $\mathrm{pmol \cdot \mu L^{-1}}$ and sequenced on a 318-chip using the Ion Torrent Sequencer (Life Technologies, USA).

NGS sequence data processing

NGS data were filtered and processed using Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso et al., 2010). I removed amplified sequences smaller than 200 bp, removed sequences with more than three primer-template mismatches, and those with average quality scores lower than 19.0. This provided the final sequence reads for each sample, from which I only used results for samples with greater than 750 sequences. I excluded all samples where this threshold was not met.

BLAST analyses

The filtered NGS sequences were compared against custom reference database files. I generated reference files composed of CO1 species-level sequences that were available from Genbank and Barcode of Life databases for mollusks, chironomids, rotifers, calanoid copepods, amphipods, mysids, bosminids, and daphnids (i.e., most major plankton and key benthic groups in freshwater). Additional known or suspected invertebrate AIS which are listed on Great Lakes Invasive Species Watchlists (i.e., US EPA, 2008: Baker et al., 2011; Sturtevant 2016; Alsip et al., 2017; GLANSIS 2018) but were not already included in the taxonomic groupings listed above, were also included. The selection of the invertebrate species included in databases was

informed by the lists of AIS present in the Great Lakes, those which could invade, and species including native rotifers and chironomids that play important community ecological roles such as competitors for algal prey or as prey with and for the AIS and rare native mollusks in these ecosystems (McMahon 1991; Yeager et al., 1994). An abbreviated list of taxa included in my reference database is given in Table 5.2. The entire list of all species in my database is available as a digital appendix upon request.

For any species to be included as “present” based on my eDNA metabarcoding, I required that NGS sequences matched to the database sequences with minimum match values of 98.0 %. I only classified a species as “present” in a sample and at a site if I recovered a minimum of two species-level sequence matches (i.e., I excluded all “singletons” but accepted doubletons). To confirm my BLAST matches, I back-BLASTed my lowest match score sequences to the full database on NCBI. The broader species coverage in the NCBI database would allow me to show the distribution of NGS sequence read counts for each river (two sections of the Sydenham) for all detected taxa and allow for a more community-oriented view. Though sequences in the resultant figure are organized at the genus level for display purposes, the sequences that constitute this data were originally first identified to the species level for all 56 taxa (except for the four mollusks previously only identified to genus level).

Table 5.2: Selected species and species groupings used for BLAST identification of CO1 metabarcoded sequences of aquatic eDNA from the Grand and Sydenham Rivers. "Number" in the right hand column is the number of species with COI sequences at species level included in the BLAST database. Some taxa listed for posterity as part of larger groupings (e.g., *Dreissena* spp. are included in the number of species for mollusks, but two are also individually listed). Species groupings are roughly organized into taxonomic categories which included species belonged to. Established AIS and AIS with high likelihood to expand into the Great Lakes are included. A list of all 11,565 species that made up the database is available upon request.

Taxonomy	Groupings for species sequences	Number species
Chironomidae	*Chironomidae*, all COI sequences	1019
Rotifera	Rotifera, all COI species sequences	160
Crustacea	*Calanoida*, all resultant COI species	372
	Amphipoda, all COI species sequences	955
	Mysidae, all COI species sequences	28
	Bosminidae, all COI species sequences	19
	Chydoridae, all COI species sequences	35
	Cherax, all COI species	19
	Bythotrephes longimanus, target AIS	1
	Cercopagis pengoi, AIS	1
	Daphnids, all COI sequences	76
Mollusca	All species sequences on GBANK	8735
	Rare mollusk spp. poss. in Sydenham Riv	62
	Dreissena rostriformis bugensis, AIS	1
	Dreissena polymorpha, AIS	1
Gastropod	*Bellamya*, AIS Watchlist, COI all species	26
	Cipangopaludina chinensis, AIS Watchlist	1
	Pomacea, Watchlist all COI species seqs	17
	Potamopyrgus, Watchlist all COI species	4
	Radix auricularia, AIS	1
	Valvata piscinalis, AIS	1
	Viviparus georgianus, AIS	1
Bivalvia, Class	*Corbicula,* Watchlist, all COI species seqs	13
	Corbula gibba, AIS Watchlist	1
	Lasmigona subviridis, AIS Watchlist	1
	Limnoperna, Watchlist, all species COI	5
	Pisidium henslowanum, AIS Watchlist	1
	Pisidium supinum, AIS Watchlist	1
Oligochaeta,	*Branchiura sowerbyi,* AIS Watchlist	1
	Potamothrix bedoti, AIS Watchlist	1
	Potamothrix heuscheri, AIS Watchlist	1
	Potamothrix moldaviensis, AIS Watchlist	1
	Potamothrix vejdovskyi, AIS Watchlist	1
	Ripistes parasite, AIS Watchlist	1
Hydrozoa	*Cordylophora caspia,* AIS Watchlist	1
	Craspedacusta sowerbyi, AIS Watchlist	1

Results

A total of 184 Sydenham River samples (109 surface, 72 bottom, and 3 field-controls) and 170 Grand River samples (108 surface, 58 bottom, and 4 field-controls) were taken from the 43 sites in each river and subsampled for filtering and processing of eDNA. The Grand and Sydenham River NGS sequencing files produced 2,741,900 and 831,886 sequence reads, respectively, after quality filtering. In total, reference databases contained 11,565 species; however, a large proportion of these were Mollusca species (N = 8,735; See Table 5.2 for abbreviated list; Complete digital Appendix upon request). The mean number of quality filtered sequence reads per site was 10,118 (min = 1, max = 54,571) for the Grand River and 4,333 (min = 2, max = 11,924) for the Sydenham River; however, I eliminated all samples with less than 750 sequence reads (N = 13). I identified exactly 52 invertebrate taxa to the species level using BLAST to my custom reference databases. I also identified four mollusks that could only be identified at the genus level, but as these may be rare and could have high value in the Grand and Sydenham Rivers, I included them for a grand total of 56 taxa used for analyses. Each of the 52 species and 4 mollusk genera are listed according to site (43 sites per river) within each of the Grand (Table 5.3) and Sydenham Rivers (Table 5.4). Site numbers for sampling in each river listed in Tables 5.3 and 5.4 for taxa occurrences can be referenced to Table 5.1 for coordinate-based geographical locations. Each consecutive site number is listed in contiguous upstream order (Figures 5.1, 5.2).

Known AIS recovered in my eDNA metabarcoding analyses were: *Dreissena rostriformis bugensis*, *Craspedacusta sowerbyi, Branchiura sowerbyi*, *Potamothrix moldaviensis* and *Skistodiaptomus pallidus*. I detected the AIS *Potamothrix moldaviensis* and detected the AIS copepod *Skistodiaptomus pallidus* only in the Grand River and not the Sydenham River, and

detected both *Craspedacusta sowerbyi* and *Branchiura sowerbyi* in both the Grand and Sydenham rivers. Identified species that are not well described in the literature, which may be non-native and/or rare included: *Amphinaias refulgens, Cardiida spp., Chydoridae brevilbaris, Cricotopus triannulatus, Cricotopus tricinctus, Eulimella ventricosa, Gammarus crinicaudatus, Microtendipes pedellus, Mysella bidentata, Physella acuta, Pleuroxus denticulataus, Radix swinhoei, Rheotanytarsus pellucidus, Rheotanytarsus spp., Stempellina spp., Stylommatophora spp., Tanytarsus glabrescens, Tanytarsus guerlus,* and *Thienemanniella xena.*

Native mollusk species present in eDNA samples included some rare and at-risk (including threatened and endangered) taxa, specifically *Lasmigona complanata, Ligumia recta, Musculium transversum, Pyganodon grandis, Quadrula quadrula, Sphaerium fabale, Sphaerium striatimum, Villosa fabalis, Physella ancillaria,* and *Zonitoides nitidus.* Of particular importance among my finds were *Quadrula quadrula* which is a threatened species in Ontario, and *Villosa fabalis* which is an endangered species in Ontario. Fewer occurrences of AIS and rare native species were found when compared to more common native taxa, including some for some chironomids and rotifers (Tables 5.3, 5.4).

Table 5.3: Occurrences of species in the Grand River, Southwestern Ontario Canada. Sampling site numbers 1-43 in top row start from site nearest Lake Erie to sites upstream. Filled black squares indicate taxa presence for native and possible invasive taxa not well described in literature. Invasive species occurrences are shown in gray boxes. Native rare and at-risk species shown with an X.

		Site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Taxa	Genus	Species																																											
Bivalve	*Amphinaias*	*refulgens*	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Cardiida*	spp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Dreissena*	*b_rostriformis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Lasmigona*	*complanata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Ligumia*	*recta*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Musculium*	*transversum*	.	.	.	.	■	■	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Mysella*	*bidentata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Pyganodon*	*grandis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Quadrula*	*quadrula*	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaeridae*	spp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaerium*	*fabale*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaerium*	*striatinum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Villosa*	*fabalis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*sylvestris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*triannulatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.
Chironomid	*Cricotopus*	*tricinctus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*trifascia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	■	.	.
Chironomid	*Cricotopus*	*trifasciatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Microtendipes*	*pedellus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	■	.	.
Chironomid	*Orthocladius*	*dubitatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.
Chironomid	*Orthocladius*	*oliveri*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Polypedilum*	*convictum*	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Rheotanytarsus*	*pellucidus*	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	■	.	.	.	.	■	.	.	.	.	.	.
Chironomid	*Tanytarsus*	*glabrescens*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Tanytarsus*	*guerlus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Thienemanniella*	*xena*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.
Cladoceran	*Bosmina*	*longirostris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	■	.	.	.	.	.	.	■	.	.	.	.	.
Cladoceran	*Pleuroxus*	*denticulatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Copepod	*Acanthocyclops*	*robustus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Copepod	*Skistodiaptomus*	*pallidus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	▒	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	▒	.	.	.	.	.	.	.	.	.	.
Jellyfish	*Craspedacusta*	*sowerbyii*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	▒	.	.	.	▒	.	▒	▒	▒	.	.	▒	.	.	.	.	▒	.
Gammarid	*Chydoridae*	*breviilabris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.
Gammarid	*Gammarus*	*crinicaudatus*	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Eulimella*	*ventricosa*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	■	.	.	.	■	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	*acuta*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	*ancillaria*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	spp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Radix*	*swinhoei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Stylommatophora*	spp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Zonitoides*	*nitidus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mollusc	*Deroceras*	*reticulatum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.
Olgochaete	*Branchiura*	*sowerbyi*	.	.	.	.	▒	▒	.	.	.	.	.	.	▒	.	▒	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	▒	▒	.	.	.	.	.
Olgochaete	*Potamothrix*	*moldaviensis*	.	.	.	.	.	.	.	.	.	.	.	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Brachionus*	*bidentatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Brachionus*	*calyciflorus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Brachionus*	*q._brevispinus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Brachionus*	*urceolaris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Keratella*	*cochlearis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Keratella*	*hiemalis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Lepadella*	*patella*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Polyarthra*	*dolichoptera*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Polyarthra*	*vulgaris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*kitina*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*pectinata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*tremula*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*t._oblonga*	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■

Table 5.4: Sydenham River, Southwestern Ontario, Canada. Occurrences of species. Moving upstream sample sites 1-32 are for the east channel and N1-N11 for the north channel. Filled black squares indicate taxa presence for native and possible invasive taxa not well described in literature. Invasive species occurrences are shown in gray boxes. Native, rare and at-risk species shown with an X. Dark line on right for display purposes: marks end of the east channel, begin display for north channel, i.e., N1 geographically nearer to the east channel site 1 than site 32.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	N 1	N 2	N 3	N 4	N 5	N 6	N 7	N 8	N 9	N 10	N 11
Taxa	Genus	Species	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Amphinaias*	*refulgens*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Cardiida*	*spp.*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X	X	.	.	.	.	.	.	.
Bivalve	*Dreissena*	*b_rostriformis*	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Lasmigona*	*complanata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Ligumia*	*recta*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Musculium*	*transversum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■
Bivalve	*Mysella*	*bidentata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.
Bivalve	*Pyganodon*	*grandis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	■	.	.	.	.	.	.	.	.	■	■	■	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Quadrula*	*quadrula*	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaeridae*	*spp.*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaerium*	*fabale*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	X	X	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Sphaerium*	*striatinum*	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	■	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bivalve	*Villosa*	*fabalis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*sylvestris*	.	.	■	.	.	■	.	■	.	.	.	.	.	.	.	■	.	.	.	.	.	.	■	.	■	.	.	.	.	.	.	.	.	.	.	.	■	■	.	■	.	.	.
Chironomid	*Cricotopus*	*triannulatus*	.	.	.	.	.	■	.	.	■	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*tricinctus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.
Chironomid	*Cricotopus*	*trifascia*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Cricotopus*	*trifasciatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	■	.	■	■	.	.
Chironomid	*Microtendipes*	*pedellus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Orthocladius*	*dubitatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Orthocladius*	*oliveri*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Polypedilum*	*convictum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■
Chironomid	*Rheotanytarsus*	*pellucidus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Tanytarsus*	*glabrescens*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chironomid	*Tanytarsus*	*guerlus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■
Chironomid	*Thienemanniella*	*xena*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	■	■	■	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.
Cladoceran	*Bosmina*	*longirostris*	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cladoceran	*Pleuroxus*	*denticulatus*	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Copepod	*Acanthocyclops*	*robustus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.
Copepod	*Skistodiaptomus*	*pallidus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Jellyfish	*Craspedacusta*	*sowerbyii*	.	.	.	.	.	.	.	.	.	▒	.	.	.	.	.	.	.	.	.	▒	.	.	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gammarid	*Chydoridae*	*breviilabris*	■	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.
Gammarid	*Gammarus*	*crinicaudatus*	.	■	.	■	■	■	.	■	.	■	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	■	.	.	.	.	.	.	.
Gastropod	*Eulimella*	*ventricosa*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	*acuta*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	*ancillaria*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Physella*	*spp.*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Radix*	*swinhoei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Stylommatophora*	*spp.*	.	.	.	.	.	■	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gastropod	*Zonitoides*	*nitidus*	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mollusc	*Deroceras*	*reticulatum*	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Olgochaete	*Branchiura*	*sowerbyi*	.	.	.	.	▒	.	.	▒	.	▒	.	.	.	.	.	▒	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Olgochaete	*Potamothrix*	*moldaviensis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Brachionus*	*bidentatus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	■
Rotifer	*Brachionus*	*calyciflorus*	■	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	■	.	.	■	■	■	■	■	■	■	.	■		■
Rotifer	*Brachionus*	*q._brevispinus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	■	.
Rotifer	*Brachionus*	*urceolaris*	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	■
Rotifer	*Keratella*	*cochlearis*	■	■	■	■	■	■	■	■	.	■	.	■	.	.	.	■	.	■	.	.	.	.	.	.	.	■	■	■	■	■		■	■	■	■	■	■	■	■	■	.	■	.
Rotifer	*Keratella*	*hiemalis*	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Lepadella*	*patella*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.
Rotifer	*Polyarthra*	*dolichoptera*	.	.	.	.	.	.	.	■	.	■	.	.	.	.	.	■	.	■	.	.	.	.	.	.	.	.	■	■	■	.	.	.	.	■	■	■	■	■	■	■	.	.	.
Rotifer	*Polyarthra*	*vulgaris*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	■	■	■		■	■	.	.	.
Rotifer	*Synchaeta*	*kitina*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	■	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*pectinata*	.	■	.	.	■	.	.	.	■	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	■	■	■	■	■	■	■	■	■	■	■	.
Rotifer	*Synchaeta*	*tremula*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	■	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rotifer	*Synchaeta*	*t._oblonga*	■	■	■	■	■	■	■	■	■	■	■	■	.	.	.	■	.	.	.	■	.	.	.	.	.	.	.	.	.	.	■	■	■	■	.	■	.	■	■	■	■	■	■

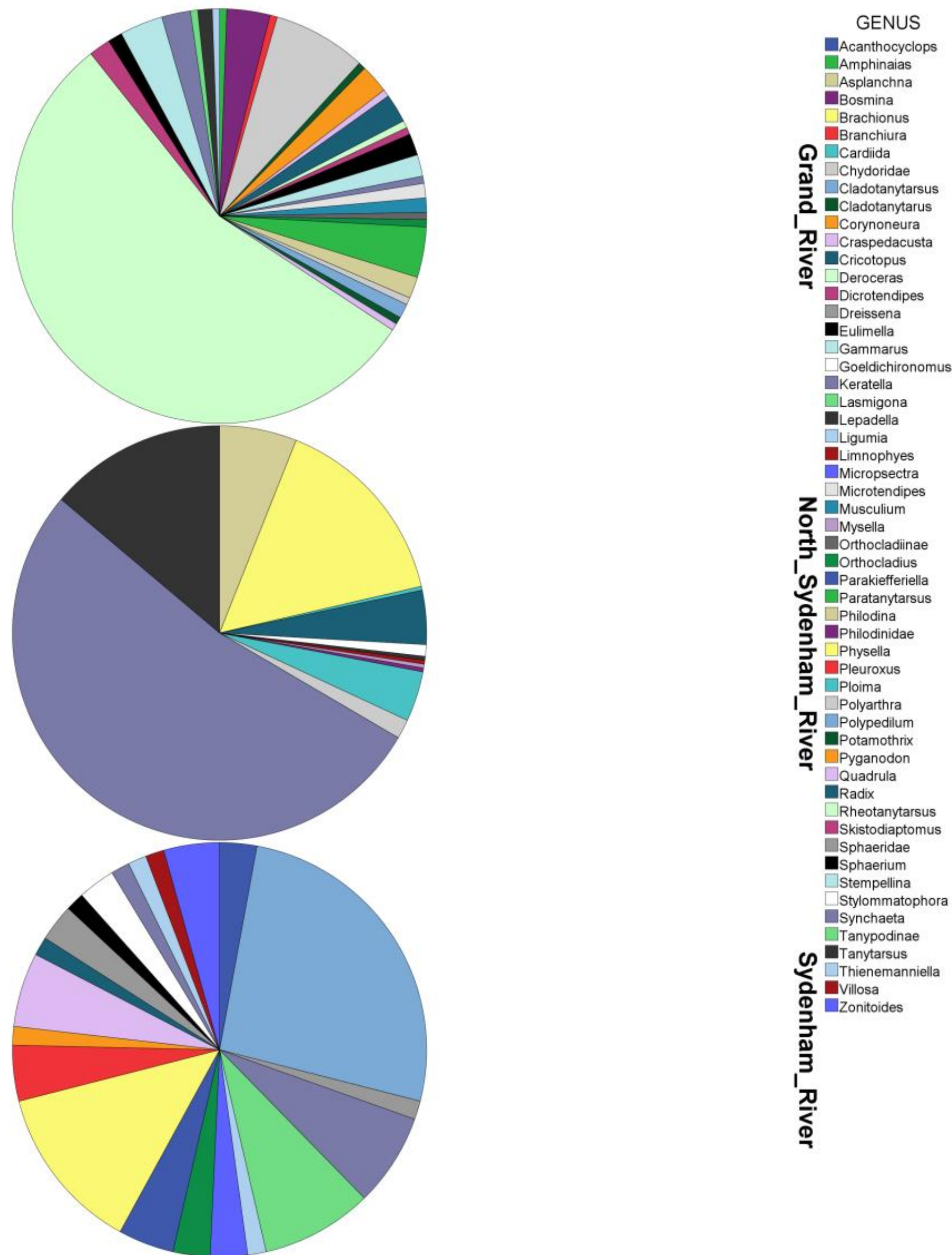

Figure 5.3: Proportion of matched sequence reads presented at the genus level across all eDNA samples for the Grand River and the two branches of the Sydenham River. The charts only include the sequence match data from all sites combined (all unmatched sequence data is not shown). While these charts show only at the genus level, data was identified to species level first and then organized at the genus level.

Discussion

I found possible early establishing AIS and rare, at-risk, and endangered native species in two major tributaries of the Great Lakes using eDNA metabarcoding. It is clear the Grand River

and east and north channels of the Sydenham River have different compositions of taxa, and indeed there is considerable variation among sites sampled within each river. For example, the 43 sites sampled in the Grand River had only 23 of the 56 total species identified in this study, whereas I identified 33 of these 56 taxa in the east channel of the Sydenham River (despite ten fewer sample sites than for the Grand) and identified 21 of these 56 taxa in the north channel of the Sydenham River sites despite sampling 31 fewer sites here than in the Grand River. Interestingly, neither AIS nor rare native mollusks were identified in the north branch of the Sydenham River, but this portion of the Sydenham did have nine native taxa which did not occur in the Sydenham East branch. Although absolute differences were small, the east branch of the Sydenham River had comparatively more AIS, more cumulative species of native taxa, and more rare native mollusk species present across its sites than did Grand River sites. Generally, AIS did not occur in a decreasing linear fashion moving upstream from the lake towards upper stream reaches in either the Grand or Sydenham Rivers. However, some AIS did appear to have distributions suggestive of some form of human mediated or other jump dispersal mechanism followed by potential downstream diffusion, such as from drift of larvae or veligers which may have contributed to their movement and patterns of occurrences. It is not surprising that these very different Great Lakes tributaries exhibited different spatial patterns of AIS, rare mollusks and other native species occurrences and my data highlights the need to treat each tributary and the sites within them as potentially unique ecosystems for conservation of rare native species and AIS management.

The AIS I identified have potential negative impacts on species and in ecosystems. For example, *Dreissena* spp. can extirpate native mollusks through competition for space or food, fast growth rates, or other factors impacting local foodweb dynamics (Ten Winkel & Davids

1983; Sprung & Rose 1988; MacIsaac et al., 1992; Dermott & Munawar 1993; Ricciardi et al., 1998; Baldwin et al., 2002). *Dreissena* spp. might also facilitate round goby expansion as it is a preferred prey (i.e., Jude et al., 1992; Ghedotti et al., 1995; Mychek-Londer et al., 2013), but could also limit *Dreissena rostriformis bugensis* spread - such as into upper river reaches - through predation. Balasingham et al. (2018) found round goby eDNA at most of the same sites in both the Sydenham and Grand River as in this study, suggesting its occurrences could be limiting expansions of *Dreissena rostriformis bugensis* into upper river reaches. As *Dreissena* spp. may be able to survive in round goby gut-tracts (i.e., Gatlin et al., 2013), and round gobies occur across most of the Grand and Sydenham Rivers (Poos et al., 2010; Balasingham et al., 2018; Bronnenhuber et al., 2011; Raab et al., 2018), the species could act as a vector to further spread *Dreissena* spp. throughout the rivers. This is especially concerning because of a noted "reservoir effect" above lowhead dams which appear to increase both round goby and *Dreissena* spp. recruitment (Smith et al., 2015; Raab et al., 2018). Thus, in addition to monitoring *Dreissena* spp., and secondarily, round goby using an eDNA approach, monitoring *Dreissena* spp. in round goby diet composition using metabarcoding of scDNA would be valuable.

Occurrences of *Dreissena* spp. in eDNA were perhaps limited by my use of a universal PCR primer set (Leray et al., 2013) not specifically created to target *Dreissena* spp. Mychek-Londer Chapters Two and Three Ph.D. Dissertation (2018) tested a highly sensitive PCR primer set designed for *Dreissena rostriformis bugensis* and *Dreissena polymorpha* which amplified the former taxa in field sampled scDNA better than the universal PCR primer set. Thus, *Dreissena* spp. may have been detected at additional locations had the more sensitive species-specific PCR primer sets been included in this study. Although the location where I detected *Dreissena* spp. eDNA was near the river mouth, it was also adjacent to the junction of the north and east

channels of the Sydenham River, and thus while downstream diffusion may not pose much concern, upstream transport could result in impacts on rare mollusks. *Dreissena* spp. eggs and veligers could be transported upstream by attachment to waterfowl, as plankton in small vessel ballast tanks or live wells, through bait bucket introductions, counter currents, among many other vectors, and could then diffuse downstream during planktonic reproductive stages (i.e., Johnson et al., 2001; Rup et al., 2010; Kharchenko 1995). Because of such opportunities for dispersal, coupled with potential harmful consequences, traditional monitoring approaches for *Dreissena* spp. in the Grand and Sydenham Rivers should continue but can greatly benefit from complementary inclusion of eDNA metabarcoding by: 1) reducing physical impact on environments via non-invasive sampling techniques; 2) decreasing sampling effort due to high sensitivities possible in detection; and 3) by reducing specialized training needed to properly identify *Dreissena* spp., including for egg or veliger life stages.

I found the freshwater jellyfish AIS *Craspedacusta sowerbyi* at multiple locations in each river. *Craspedacusta sowerbyi* is native to China, occupies diverse habitats (DeVries, 1992; Peard, 2002), was discovered in 1933 in Lake Erie, and it exists in Lake St. Clair. It also exists in regional inland water bodies, was found in Quebec in 1955 and only as recently as 2002 was discovered in the Muskoka Lakes region (Mills et al., 1993; Peard 2002; GLANSIS 2018; McKercher et al., 2018). To my knowledge, I am the first to report *Craspedacusta sowerbyi* in the Grand River and Sydenham River. In the Grand River, most occurrences of *Craspedacusta sowerbyi* were at adjacent or clustered sampling sites along river reaches, and only in the uppermost river sections sampled. In the east channel of the Sydenham River, two of the three sites where *Craspedacusta sowerbyi* occurred were in the middle-upper section relatively far upstream from the rivermouth. The detection sites were clustered, similar to the pattern of

occurrences in the Grand River. However, the third occurrence of *Craspedacusta sowerbyi* in the east channel of the Sydenham River was much further downstream from the other two sites. Thus, it is possible that upstream populations of *Craspedacusta sowerbyi* in the Grand River may have expanded by diffusion (leading to the clumped distribution). In the Sydenham River, on the other hand, the pattern of distribution is different, perhaps resulting from independent localized introduction events with little evidence for large scale secondary dispersal. However, *Craspedacusta sowerbyi* has a complicated life cycle and is responsive to environmental conditions that may have factored into its distribution in Great Lakes tributaries; these factors include 1) microscopic podocyst resting bodies; 2) frustular planktonic larvae from asexual polyp budding; 3) sexually produced planktonic planular larvae from adult hydromedusae; 4) sessile individual polyps which can attach to stable surfaces; and 5) polyp colonies with multiple attached individuals that can fully or singly detach and newly establish elsewhere (Angradi, 1998; Acker & Muscat, 1976; Pennak, 1989; Peard, 2002). Adult hydromedusae are most easily recognized but are also the least common of the life stages in the environment. Other life stages are inconspicuous and adults are produced only sporadically and may go several years between bloom events characterized by localized high adult abundances (Kato & Hirabayashi, 1991; Angradi 1998; Peard 2002). Additional visual-based research at locations where *Craspedacusta sowerbyi* occurred would help to reveal life stages present and if these varied among sites.

Coexistence of *Craspedacusta sowerbyi* and rare native mollusks in my study are of potential concern. For example, *Craspedacusta sowerbyi* overlapped with *Sphaerium fabale* at a single sampling site in each river system. *Sphaerium fabale* is a mollusk species of special concern in the State of Michigan, considered imperiled in Ontario and has dramatically declined in the Great Lakes with expansion of *Dreissena* spp. ranges (Lozano et al., 2000, Nalepa et al.,

1998). *Craspedacusta sowerbyi* could have similar impacts on *Sphaerium fabale* (see below). Notably, at or at very close to sites in the Sydenham River where *Craspedacusta sowerbyi* and *Sphaerium fabale* distribution overlapped, several other important rare and endangered mollusks are also present, raising the possibility that *Craspedacusta sowerbyi* could affect a variety of at-risk or rare mollusk species. Dodson and Cooper (1983) suggested that *Craspedacusta sowerbyi* is an opportunistic predator that prefers rotifer prey similar to the rotifer taxa recovered in my study (i.e., Jiffry 1984; Nichols & Garling 2000). Another potential impact resulting from *Craspedacusta sowerbyi* invasion includes predation of such taxa as calanoid copepods (Spadinger and Maier 1999), which might include those I identified as unique to each river system. Dumont (1994) speculated that *Craspedacusta sowerbyi* may even consume fish eggs and even young-of-year fish, although Spadinger and Maier (1999) note that expected levels would be minimal. Jankowski et al. (2005) described possible trophic cascades that could result from *Craspedacusta sowerbyi* predation effects whereby algal species two trophic levels below their main invertebrate prey could dramatically increase. Such a trophic cascade may benefit some rare native mussel species if availability and quality of algal foods increased (Cabana & Rasmussen 1996; Raikow & Hamilton 2001). Whether or not *Craspedacusta sowerbyi* could directly or indirectly impact native fishes that native mussels rely upon for reproduction, or if could affect at-risk native mussel species recruitment unknown. However, *Craspedacusta sowerbyi* could be a critical AIS to consider in future decision making and applications of ongoing restoration and preservation efforts of these ecosystems and rare native taxa such as the mollusks they appear to overlap with.

Two oligochaete AIS were identified in this study including *Branchiura sowerbyi* which is native to Southeast Asia, but introduced and common on every continent except Antarctica

(Carroll & Dorris 1972). It was first noted in Ohio in 1930 and in Lakes St. Clair and Erie in by the 1960's (Mills et al., 1993; U.S. Geological Survey 2018a). I detected *Branchiura sowerbyi* at seven sites in the lower-middle and upper sections of the Grand River, and at three sites in the lower-middle and middle reaches of the east channel of the Sydenham River; these are likely the first reported observances for this AIS in these systems. As a large filter feeder, *Branchiura sowerbyi* can burrow into sediment deeper than its congeners (up to 20 cm) and can transport large quantities of benthic materials to the sediment-water interface. This may impact native mollusks in the Grand and Sydenham Rivers due to increases in local turbidity or changes in bacterial biofilm components (Matisoff et al., 1999). Further, *Branchiura sowerbyi* could disperse in search of better food resources (Wang & Matisoff 1997) and serve as a host for numerous myxosporean parasites that could negatively affect the health of susceptible fishes (i.e., Liyanage et al., 2003; Wahab et al., 1989).

The AIS *Potamothrix moldaviensis* was the second oligochaete detected in this study but was rare and only found at a single site in the lower-middle Grand River. It is of Ponto-Caspian origin and has been reported in Lakes Erie and St. Clair since the late 1960's (U.S. Geological Survey 2018b). Both *Branchiura sowerbyi* and *Potamothrix moldaviensis* have the potential to rapidly spread, since they are characterized by high adaptability to local conditions (Carroll & Dorris 1972; Matisoff et al., 1999; Milbrink 1999; Milbrink & Timm 2001; et al., Paunovic et al., 2005). Thus, developing a better understanding of the distribution and ecology of these AIS in each river may assist in containing further spread. The ecology of most oligochaete species in the Great Lakes is poorly described, and as many of the native and AIS oligochaetes are cryptic in appearance (Gigorovich et al., 2003; Spencer & Hudson 2003) metabarcoding approaches could be particularly useful to achieving such management goals.

I did not identify the AIS copepod *Skistodiaptomus pallidus* in the Sydenham River but did identify it in the Grand River at two contiguous sites in its lower-section and at a single site in the upper section. These are the first reports of *Skistodiaptomus pallidus* in the Grand River, and to my knowledge, are the first such records in any inland waterbody in the Great Lakes region (Kipp et al., 2018). It seems likely that the clustered *Skistodiaptomus pallidus* detections in the Grand River resulted from diffusion; however, the third location appeared far enough away from the other two sites such that is likely the results of independent localized (jump) dispersal. The reproductive cycle of *Skistodiaptomus pallidus* may have influenced its occurrences and distribution and may play a role in its ability to sustain localized populations or expand its range. *Skistodiaptomus pallidus* breeds March to November, producing up to five generations. Females can produce up to 20 eggs in a brood in late summer, depositing eggs into sediments at densities of up to 105· m^{-2}. Eggs can diapause, allowing for downstream drift, and can begin hatching as early as December into June of the following year (Kipp et al., 2018). *Skistodiaptomus pallidus* is also an efficient omnivorous predator that could impact native species by inducing cascade-type effects through selective consumption of algae and rotifers (Geiling & Campbell 1972; Williamson & Butler 1986). There have been no recent reports of *Skistodiaptomus pallidus* in the Great Lakes, and it is also thought that it might generally occur only sporadically as a result of main populations in coastal wetlands or tributaries being washed into the lakes during times of flood events. *Skistodiaptomus pallidus* has potential for impacting rare native mollusks or other important native taxa in the Grand River and further study of its ecology as well as monitoring for possible expansions should be explored.

The rare native mollusk species differed in patterns of occurrence not only among river systems, but in important ways among samples sites within each river; many species seemed to

occur independently from where other rare native mollusk species were detected. However, one important area where the rare native mollusks seemed to co-occur and cluster was in the middle reaches of the east channel of the Sydenham River. That some areas seem to host clustering rare mollusks, while other mollusks seemed to occur far from these sites suggests that some type of diffusive downstream or fish-mediated upstream dispersal may be occurring, or reflects taxa-specific preferred habitat criteria or remnant populations that have surpassed survival bottlenecks in recent decades. Many of the rare native mussel species endemic to these river systems can in fact live 10-30 years or longer (i.e., Clark 1980; Neves and Moyer 1988; Heller 1991; McMahon 1991; Carney 2003a; Carney 2003b), thus partitioning exact means of dispersal is confounded by this fact and in that I only detected eDNA and made no measurements of size- or year- classes. However, my highly sensitive methods help to better define the areas where intensive sampling efforts can be undertaken and should become especially useful as guidelines such as for areas like the middle section of the east channel of the Sydenham River where it appeared that multiple occurrences of multiple rare native species of mollusks occurred in clusters. Additionally, at these same sites that the rare native mollusks occurred there were co-occurrences of AIS which are of utmost concern, including *Craspedacusta sowerbyi.* I identified the endangered rare native species *Villosa fabalis* in the same middle reaches of the east branch of the Sydenham, and at a site adjacent to where this AIS of freshwater jellyfish occurred. *Villosa fabalis* was also recently noted in literature and monitoring reports having used traditional sampling means as occurring in the middle reaches of the east channel of The Sydenham and these populations were suggested as some of the last, or perhaps *the* last existent with natural recruitment still occurring in the entire world (West et al., 2000; Metcalfe-Smith et al., 2003). Thus, its potential for

restoration in areas in this river may depend at minimum, upon the preservation of their genetic diversity and naturally reproducing populations.

If habitat-based factors related to the occurrences of the rare mollusk taxa that I documented are better understood, factors beneficial to these species can be implemented elsewhere within these river systems. The most likely place to focus future sampling and research efforts is the middle sections of the east channel of the Sydenham, as there were important native taxa and AIS that co-occurred here. Using traditional methods to find these mussels with consistent detection is challenging, especially without prior knowledge of expected distributions because mussels are spatially clustered, can be very small (i.e., *Villosa fabalis*), cryptic in appearance, and because many species are rare. Further, because there is the potential to disturb sensitive habitats in which these mussels occur, metabarcoding can help to safely determine where rare taxa are distributed (Wisniewski et al., 2013).

The occurrence data based on eDNA metabarcoding analyses in my study focused on AIS and rare and at-risk native species can inform management strategies for both AIS eradication efforts and for conservation of native taxa. AIS appeared to occur mainly in the mid- to upper reaches of the Grand River and the lower to mid-reaches in the east channel of the Sydenham River. Spatial patterns in occurrence data suggested that factors related to both diffusive invasion (either upstream to downstream) and other factors, such as jump type dispersal, are at play in determining the distributions of the native and AIS, and over potentially variable time scales. Key locations were identified where AIS and rare native taxa (and other co-occurring species of interest) co-occurred which require further monitoring and research efforts that should not be delayed. Using traditional based methods to map species occurrences would have been much costlier and time intensive, and possibly more impactful to the habitat and rare

native species (i.e., Metcalfe-Smith et al., 2000a; Gillis et al., 2017). CO1 metabarcoding showed that each river contains different biological communities, different species of AIS, and different rare native species of ecological importance. Interestingly, the distribution of my target species also varied within each river. Further examinations of patterns of taxonomic diversity in each river and among sites within those rivers would help to possibly reveal important habitat characteristics which may have influenced the occurrences of the rare native taxa and AIS. Such information can possibly help our understanding of factors influencing spread and establishment of AIS and the capability of rare natives to persist, recover and stabilize in the face of environmental stressors coupled with AIS.

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CHAPTER 6 – GENERAL CONCLUSIONS

Introduction

Some of the many impacts of aquatic invasive species (AIS) include native extinction (Ricciardi et al., 1998; Ricciardi & MacIsaac 2011), changes in energy flow in foodwebs (Johnson et al., 2005; Brush et al., 2012), and changes in native species gene expression profiles in the presence of AIS (Mooney & Cleland 2001; He et al., 2017). The Laurentian Great Lakes (hereafter Great Lakes) have experienced numerous species invasions and AIS are now prevalent components in foodwebs. For example, the AIS fish round goby (*Neogobius melanostomus*) may cause extirpations of populations of native benthic fishes through resource competition, consumption of the eggs of native fishes and alterations of the benthic biological community composition (i.e., Dubs & Corkum 1996; Janssen & Jude 2001; Mychek-Londer et al., 2013; Kipp & Ricciardi 2012). Expansion of the AIS round goby was likely in part driven by availability of two established and abundant invertebrate AIS prey, *Dreissena* spp. bivalves, which round goby consume at high levels (i.e., Simberloff 2006; Mychek-Londer et al., 2013). These and other invertebrate AIS have had direct wide-ranging negative impacts upon ecosystems, foodwebs and native Great Lakes biota (Nalepa et al., 2009; Bunnell et al., 2009).

Traditionally, AIS have been documented as prey and predators and their trophic roles defined through field observation and various forms of visual detection and identification, counting procedures, proportional estimates, and by quantifying frequencies of occurrences. Specifically, zooplankton tows, water samples, and stomach contents of predators are methods traditionally used for invertebrate AIS detection (i.e., Madenjian et al., 2006; Rothlisberger et al., 2010; Keeler et al., 2015). Stable isotope methodologies, especially when used in combination with traditional diet studies, are powerful for trophic interaction characterization and can reveal long term and real-time patterns in diet (i.e., Brush et al., 2012; Mumby et al., 2017). However,

pitfalls still remain for not commonly consumed and/or highly digested prey (Schooley et al., 2008; Legler et al., 2010) or cryptic prey taxa (Belyaeva & Taylor 2009). Metabarcoding of predator stomach content DNA (scDNA) and of environmental DNA (eDNA) are two emerging genetic approaches that can help resolve such issues and define some aspects of trophic interactions with greater resolution such as for organism and prey occurrence data, especially for studies with an AIS focus. For example, metabarcoding was successfully used in the Great Lakes and their tributaries to determine the distributions of rare native and invasive AIS from waterborne eDNA for fishes (Balasingham et al., 2018) and invertebrate AIS (Klymus et al., 2017), and each showed high sensitivity in detecting targeted species.

In my dissertation, I also used metabarcoding of eDNA and scDNA to address hypotheses on the roles and interactions of AIS and important native species in Great Lakes foodwebs and ecosystems. In Chapter Two (first data chapter) I developed and optimized novel PCR primer sets to ensure high reliability of AIS detection when using CO1 metabarcoding in later chapters. The main objective in both Chapters Three and Four was to use metabarcoding and the AIS PCR primer sets developed in Chapter Two coupled with use of distinct "universal" PCR primer sets for the analyses of field sampled scDNA taken from native and AIS of predator fishes in Lakes Michigan and Erie. A goal in each of these chapters was to determine which factors influenced the occurrences of AIS and native prey in diet scDNA of both native and AIS predators. For the last data chapter, Chapter Five, my main objective was to use metabarcoding of water sampled eDNA to identify invertebrate AIS and to determine distributions of these taxa and of rare at-risk native mollusks and additional native species at sites in two major Great Lakes tributaries. An additional objective was to determine where potentially rare and at-risk species co-occurred with AIS at sites in these tributaries and what possible impacts could result.

Chapter Two

The target-species primer sets developed in Chapter Two were shown to be robust and sensitive. These were also used in my Chapter Three Lake Michigan planktivore and Chapter Four Lake Erie predator-fish scDNA metabarcoding diet studies. While my use of a widely cited universal invertebrate primer set was successful in detecting AIS in scDNA samples in Chapter Three the concurrent use of Chapter Two target primer sets increased both the power and specificity of results. For example, the target primer set for *Dreissena rostriformis bugensis* I developed in Chapter Two had a better overall detection rate for this AIS than did the universal invertebrate primer used for metabarcoding of field-sampled offshore Lake Michigan planktivore scDNA. Further, the universal primer set did not amplify *Cercopagis pengoi* in any scDNA in Chapter Three whereas the novel target primer set I developed to target this AIS consistently revealed its presence in Chapter Three scDNA samples.

Additionally, the novel target AIS primer sets I developed in Chapter Two were used in Chapter Four analyses of Lake Erie predator fish scDNA. Although a different universal primer set was used in Chapter Four than in Chapter Three (instead, a universal primer set targeting Great Lakes fish species was used in Chapter Four), the outcomes from the target vs. the universal primer sets from Chapter Three helped validate results for the target AIS primer sets used in Chapter Four. The results in Chapter Four suggested that the novel target AIS primer sets were very sensitive such as to detect AIS in scDNA at very low levels even in complex scDNA sample mixtures. This success with scDNA and high sensitivity was despite the fact that commercially caught fishes from Chapter Four may have sat in gill nets for up to 24 hours prior to being frozen for preservation. During this time, stomach contents (and their DNA) would have become increasingly digested. As in Chapter Three, the use of the novel primer sets from

Chapter Two in Chapter Four scDNA analyses thus provided more information about AIS occurrences than if only a universal primer set had been used. In fact, my approach and novel results using the primer sets from Chapter Two provided a level of resolution in AIS frequency of occurrence data in the western basin of Lake Erie in predator diets which has thus far not been possible through traditional diet studies. The high sensitivities of the Chapter Two target species primer sets may have detected even the smallest life stages and digested remains of the target AIS in field samples, though admittedly, the energetic contributions of these prey, such as *Dreissena* spp. veligers may be minor. Thus, such novel PCR primer sets as I developed can be and may become especially valuable to researchers as monitoring tools for AIS expected at possible low abundances and can be used to analyze samples with potentially degraded eDNA or scDNA. Thus my approach is especially useful for foodweb and ecosystem studies when the target AIS primer sets are used in conjunction with universal primer sets to determine AIS co-occurrences with important native or other taxa and to resolve potential species interactions.

Chapter Three

In Chapter Three, I achieved the main objectives and built upon outcomes from Chapter Two by using the novel primer sets for metabarcoding of field sampled scDNA from two AIS predators (alewife and rainbow smelt) and three native fish predators (bloater, ninespine stickleback, and slimy sculpin). I identified two of five target invertebrate AIS, including *Cercopagis pengoi* and *Dreissena rostriformis bugensis* using the novel primer sets developed in Chapter Two. I also identified the AIS *Bythotrephes longimanus* and three commonly consumed native invertebrate prey *Leptodiaptomus sicilis, Limnocalanus macrurus* and *Mysis diluviana* in Lake Michigan predator scDNA samples using a universal primer set. The AIS and some of the native prey taxa I identified are themselves invasive outside the Great Lakes. I explicitly tested

for the effects of sampling site, depth, year, and predator species size on the AIS and native prey taxa occurrences in scDNA in Chapter Three. A predator size effect for the AIS prey *Bythotrephes longimanus* suggested some predators may have been gape limited in their ability to consume this taxa, reflecting unique predator defensive adaptations which have helped this AIS to persist and spread into new ecosystems. However, sample site was the most important factor driving the occurrence of the target prey taxa, followed by predator species, which supports previously published traditional diet studies. Site-based variation in prey occurrences likely reflects localized bottom up and top-down effects and the resultant local-scaled differences in foodweb structure and realized predator feeding niches or prey selection strategies resulting as *Dreissena* spp. expanded into offshore waters and preferred native prey of predator fishes studied in this chapter *Diporeia hoyi* concomitantly disappeared or declined at sampling sites. Such predator behaviors likely helped to reduce diet overlap and potential competition for food in the face of changes in availability and abundances of preferred native prey in these offshore regions. The important findings in Chapter Three would not have been possible without inclusion of both the target species primer sets from Chapter Two and the combined use of the universal primer set. For example, occurrences of native invertebrate prey species I targeted in scDNA seemed more variable among predators at two sites (Sturgeon Bay, Two Rivers) where another native preferred prey (*Diporeia hoyi*) was still available to predators. However, occurrences of the native invertebrate prey I targeted and identified in scDNA with the universal primer set at the third site where the other native preferred prey, *Diporeia hoyi*, was extirpated, were much more similar among predator species and were found at higher occurrences in scDNA. Increased predation pressure on these native invertebrate taxa is likely occurring at the site of Frankfort and, to a lesser degree, at the other sites that were not as yet impacted by *Diporeia hoyi*

extirpations and losses. Such variation and AIS induced changes in predation pressure by the fish species I studied could be of concern if they reach levels that impact the abundances of the targeted native taxa, especially if it limited *Mysis diluviana* availability as it is a key preferred native invertebrate prey for not only the fishes I studied, but essentially all fishes in the offshore ecosystems of Lake Michigan. Additionally, the prevalence of some of the invertebrate AIS I identified in offshore Lake Michigan predator scDNA is concerning as it may reflect growing populations that could provide propagules for further range expansions into tributaries of Lake Michigan and to other sites in the Great Lakes – a possibility explored in Chapter Five. Additionally, Chapter Three results complement Chapter Four results as these very different ecosystems sampled for scDNA provide a broader comparative view of the role of AIS in Great Lakes foodwebs.

Chapter Four

The deep and vast open offshore ecosystems sampled in Chapter Three had generally lower or much lower target AIS occurrences in scDNA compared to the scDNA from the relatively shallower, warmer more eutrophic nearshore waters of the relatively enclosed western basin of Lake Erie sampled for Chapter Four. In Chapter Four, I met my main objectives including to: determine occurrences of up to five AIS invertebrates using the novel PCR metabarcoding primer sets I developed in Chapter Two; determine occurrences in scDNA of piscine prey (AIS: round goby, gizzard shad and rainbow smelt; native species: emerald shiner and channel catfish) using a universal fish PCR primer set; and determine variation in prey occurrences related to abiotic (year, season) and biotic (predator species, predator total length (TL)) variables. Metabarcoding of predator scDNA from AIS white perch and native white bass, walleye and yellow perch reflected, as expected, the high resolution and sensitivity possible in

the approach. For example, I detected the AIS *Dreissena rostriformis bugensis* at occurrence levels previously unreported as high in the literature for this AIS in predator diets, likely in large in part due to detection of mostly unexplored multiple life stages and non-selective consumption of this AIS by predator fishes. This likely includes secondary predation when targeting nearby benthic preferred prey, or incidental consumption of microscopic planktonic life stages. My data indicating high sensitivity for the target AIS primer set for *Dreissena rostriformis bugensis* in Chapter Two, lends support to my findings in Chapter Four as *Dreissena rostriformis bugensis* yielded positive hits even when its DNA was highly diluted among a mix of other species DNA. Also, my inclusion of multiple negative controls in sample preparations and during lab procedures helped to ensure that the high occurrences of *Dreissena rostriformis bugensis* in Chapter Four were unlikely to be related to contamination. While the metabarcoding approach I used for this AIS would clearly be advantageous in situations where early detection and early warning strategies are needed, partitioning exact levels of predator selectivity for this AIS and determining which of the varied life stages of *Dreissena rostriformis bugensis* occurred in scDNA will require additional research steps. While it did not occur with as high of frequencies in Chapter Four scDNA samples, *Cercopagis pengoi* was still a relatively common prey item in Lake Erie predator diets and had higher occurrences than in Chapter Three and typically reported in traditional studies of Great Lakes prey consumption. This highlights the power and sensitivity of the target AIS primer sets I developed and used and differences in the foodwebs of the different ecosystems in each chapter. Interpreting the presence-absence patterns and factors affecting invertebrate prey in stomach contents of essentially piscivorous predators is complex; however, my goals in as much for this Chapter were achieved and such data provides an important understanding of a little explored dimension of the Lake Erie foodweb.

All five targeted fish prey species in Chapter Four were amplified using a universal primer set designed to amplify native and AIS fishes of the Great Lakes. My study was the first use of this recently designed and published universal primer set to test its applicability in scDNA, as in previous research it has only been used for eDNA analyses. Occurrences of some of the fish prey in scDNA depended on predator species and predator size, consistent with prey-selection and prey-specific predation defenses. Such patterns of predation highlight the power of my approach to define trophic interactions. i.e., predator size affected channel catfish occurrences and this prey species has evolved defensive mechanisms such as locking spines which can limit their susceptibility to predation across varied life stages of both the predator and prey. In an opposite way, it appeared that the lack of similar defensive mechanisms in round goby resulted in a predator size effect such that predators may have consumed round goby as a highly abundant and easy to capture prey until predators became large enough to target larger-sized and or better defended and preferred prey species. In summary, novel data from metabarcoding scDNA increased what is known about AIS and native species predator-prey relationships across multiple trophic levels in the foodweb of the Western Basin of Lake Erie in Chapter Four. The data will help resource managers to better predict how to best respond to the ongoing dynamic AIS and native predator and prey relationships in the foodweb of the western basin of Lake Erie. Notably, the high occurrences of AIS in the western basin presents a danger in that many tributaries to Lake Erie and the western basin are presently uninvaded by these AIS in that the main lakes could serve as a large secondary source for invasion of the tributaries or other inland water bodies. This is compounded by the fact that many of the tributaries have dams that are thought to block the upstream movement of some AIS since this would limit the hope that one day such dams can be removed. Thus, metabarcoding using scDNA of predators

could be used for sensitive monitoring of AIS distribution and changes in abundance and potentially could be used to minimize the effects of these invaders on ecosystems such as Lake Erie and its Western Basin.

Chapter Five

Despite using similar methods and approaches, the goals of Chapter Five were complementary to, but different from, those of Chapters Three and Four. In this chapter I used aquatic environmental DNA (eDNA) rather than stomach content DNA to characterize the presence of and possible ecological interactions among important AIS and native invertebrate species from tributary river water samples. My previous work on scDNA showed that native and AIS prey were both found commonly in the diets of introduced and native predators, and thus it is likely that AIS are also becoming normalized in other foodwebs of the Great Lakes and potentially in the two tributaries I sampled in southwestern, Ontario, creating basis for study and comparison. Additionally, I greatly expanded my reference sequence databases used in this chapter to determine species compositions rather than the more targeted species approach. Over 11,000 species with multiple species level sequences were included in the BLAST database for reference. This included all rare and at-risk native mollusks of Ontario, the Great Lakes and in the region but also included all available sequences for all Mollusca species that were available in Barcode of Life (BOLD) databases for download for comparison to sequences from metabarcoding of eDNA. In order to determine where AIS might overlap spatially with (and hence potentially impact) rare native taxa, I also included all species of AIS presently in the Great Lakes and all species of AIS which have a high potential to arrive and spread in the Great Lakes in the reference database, based on published lists available characterizing and predicting the potential arrivals of such species. I also included all chironomid species sequences and all

rotifer species sequences in the reference database. The chironomids and rotifers are taxa that can play important roles as potential prey for AIS taxa I identified in these rivers, as potential prey at early life stages for the rare native mussel species also identified, or as important competitors with those native mussel species for algal-based foods. My work shows that the tributaries I sampled can be expected to show broad similarities in terms of a few relatively common invertebrates observed, but generally that tributaries seemed to show substantial differences in the diversity and abundance of AIS and native invertebrate species in each of the communities. Native rare bivalve mollusks I identified include species officially designated as threatened and endangered and are of high concern to managers, as well as are protected by governmental environmental agencies in Ontario and Canada. These rare native taxa, like the AIS, had occurrences that differed among tributaries and among the sites sampled within each river system. Some locations where I identified the rare native mollusks were also locations where AIS were noted to occur, and possible impacts from the AIS upon these rare native species are unknown, but feasible potential impacts were identified. The main objective of identifying where AIS and the rare native taxa occurred in each river was achieved and will permit researchers to examine interactions among the native and non-native species and is highly informative for narrowing the spatial scale for such detailed ecological analyses. Besides decreased effort in sampling, sites identified with important rare native species in each river and with potentially impactful AIS should be examined further for any particular habitat characteristics which are critical for species occurrences, as well as can be identified more efficiently for such tasks through the data provided in this chapter. The reference database did include two of the same target AIS as in Chapters Three and Four, as well as included the three native prey from Chapter Three, all which amplified by the use of the same primer set as was

used in this chapter, but outside of a single occurrence of *Dreissena rostriformis bugensis* in this chapter, none of these other prey was identified. Another objective was to assess if AIS may be moving upstream from the rivermouth areas into upper reaches in each tributary. Some AIS were identified in middle reaches of each river, some in lower reaches, and some in upper reaches, and there did not seem to be a consistent pattern to where AIS were distributed. Drift of eDNA from populations from upstream to downstream may have played a role in the spatial patterns observed for native and AIS invertebrates I identified, but are complicated by diverse and complex life history characteristics of the AIS and rare native mollusks. For example, birds and other biota can initially mediate dispersals of the AIS which may have previously occurred and would explain the scattered distribution throughout the tributaries, but, some of the rare native mussels can distribute upstream via fishes and live on scales of decades. The introduction of AIS through diverse vectors remains as an ongoing concern for additional, tertiary sources of the spread of these AIS from the locations I identified into other areas of the tributaries, and to other nearby regional inland water bodies. In summary, the data I provided in Chapter Five helps to better understand AIS and native species dynamics at local and at regional scales across all the sites within each tributary and among tributaries that were sampled, furthers the understanding of the roles of these taxa in foodwebs, and helps future efforts to limit the spread of AIS into areas where rare native species including the rare mollusks I detected can be further protected.

Future directions

There are many possible future directions for research from this stage forward. For example, the novel PCR metabarcoding primer sets I developed in Chapter Two can be examined for use in additional field settings and used to assist in analysis of additional predator

species scDNA. They could also be used to analyze the DNA from sediments in areas where predator diets were sampled from to examine for the presence of important taxa of interest and in relation to developing historical comparisons of what prey communities may have previously been composed like before invasions. These primer sets could also be tested for co-amplification of closely related target taxa and used on additional media such as fish fecal matter and in ballast water analysis from vessels.

While I was able to generate occurrence data in Chapters Three, Four and Five, additional metabarcoding loci (e.g. microsatellite DNA) could be used to determine quantitative measures of prey number in scDNA and of organism counts in eDNA. Management strategies for sustaining high levels of harvest of commercially caught fishes sampled in Chapter Four could benefit from the creation of a dedicated scDNA monitoring program, and stomachs and their contents are frequent discarded from recreationally harvested fishes and could be consistently obtained without great effort for such determinations. Although metabarcoding is an efficient and cost effective means to gather a lot of information about the ecosystem, more importantly, a dedicated monitoring program could reveal important localized AIS introductions and AIS establishing phases, allowing for initial research baselines to be established. To accomplish this, many more target species-specific primer sets need to be developed, and where possible, opportunities to create group-level primer sets, such as a primer set that would amplify all Great Lakes copepods should be pursued. As such a suite of molecular genetic tools will eventually emerge, the implementation of such a style of monitoring tasks will also likely slowly gain wide acceptance and use in efforts to manage AIS and rare native species, such as from building upon the work I report in this dissertation. One could hypothetically use DNA metabarcoding to describe diets of the AIS and native prey themselves (secondary predation detection, i.e., diets of

Bythotrephes longimanus) to better define the predator-prey dynamics in the foodwebs studied, or one could attempt to capture the AIS in the field that were found to occur in eDNA for similar uses. Lastly, it would be ideal to also use already or yet to be developed highly target specific primer sets which may have higher sensitivity than universal primer sets to identify species of special interest, such as for the rare mollusks in my study to increasingly reveal possible co-occurrences of these taxa and AIS.

In conclusion, I successfully identified multiple AIS and native prey in scDNA of multiple predator species sampled in the Great Lakes using metabarcoding, and identified AIS and rare native taxa in eDNA from water samples taken from two major Great Lakes tributaries. Laboratory and field based experiments confirmed the usefulness of the metabarcoding approach using target-species primer sets in combination with universal primer sets in determining occurrences and distributions of AIS and native taxa in samples. The results were and will continue to be useful in understanding the roles of AIS and native species as they interact in the complex ecosystems and foodwebs of the Laurentian Great Lakes.

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