A Dissertation
entitled
Environmental DNA Detection and Population Genetic Patterns of Native and Invasive Great Lakes Fishes
by
Matthew R. Snyder
Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in
Biology

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An Abstract of

Environmental DNA Detection and Population Genetic Patterns of Native and Invasive Great Lakes Fishes

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Invasive species are one of the top threats to native biodiversity. Their population genetics and genomics can be useful in control and management of invasive species and can be regarded as accidental evolutionary experiments. Here, in a temporal study of the high impact invasive round goby *Neogobius melanostomus* in the Laurentian Great Lakes, the ability of invasion genetics to track sources and temporal changes in population structure was demonstrated. We tested for three possible alternative temporal patterns in population genetic diversity over time – termed the 'genetic stasis', 'supplementation', and 'replacement' hypotheses. 'Genetic stasis' or no change in allelic composition over time could be caused by a large number of introduced propagules that possibly possess all (or most) of the diversity present in the native source, or by a density dependent process circumventing the establishment of later arrivals. Alternatively, there may be 'genetic supplementation' in which populations that experienced an initial founder effect then gain diversity over time. Finally, 'replacement' of all or some of the initial founding genetic diversity could result when the early arrivals are the best dispersers, followed by those that are better competitors. Results showed that near the site of initial establishment (the invasion core), high genetic diversity due to a large number of introduced individuals precluded significant changes in allelic

composition over time. Further from the invasion core, some slight changes in genetic diversity occurred soon after population establishment. Results supported 'genetic stasis' and the founder takes all hypothesis. Due to the territoriality of adult round gobies, it is possible that a density dependent process circumvented establishment of later arrivals. Additional introductions from separate native sources were implicated in some areas of the invasion.

Detection of newly introduced species before they can become established and characterizing native community composition are top concerns of management agencies. Metabarcode environmental (e)DNA assays are non-invasive sampling tools for detecting species. Targeted and general metabarcode assays and an associated custom library preparation and bioinformatic pipeline that reduce error were designed and tested. This protocol discerned 100% of species present in electrofishing surveys in the Maumee River from just six water samples. Four 1L water samples in the Maumee River were sufficient to identify 88% of species present in concomitant electrofishing surveys and 73% of those in a much larger effort (44 sampling events in 22 sites). Proportions of species-specific high-throughput sequencing reads were weakly correlated with taxa assessed using morphological surveys. Our method identified more invasive species in more samples than did morphological sampling. Haplotypic diversity discerned with metabarcode assays significantly differed from that determined with traditional population genetic data collection. The protocol increased confidence in metabarcode surveys by removing cross-contamination, index-hops (sequence to sample mis-assignment, leading to false positives), and sequencing error and achieved a high detection efficiency.

To evaluate the utility of this approach, the protocol was applied to potential retail sources of invasive species in the Great Lakes, including bait and pond stores. Metabarcode assays found a much greater number of stores with illegal native and invasive species

compared to morphological sampling. These included juveniles of valuable fishery species, such as walleye *Sander vitreus* and yellow perch *Perca flavescens,* and unestablished, potentially high impact invasive species, including the Eurasian ruffe *Gymnocephalus cernua*, Eurasian ide *Leuciscus idus*, and silver *Hypophthalmichthys molitrix* and bighead *H. nobilis* carps. Presence of illegal species was unrelated to retailer supply chains. Surveys showed that bait dumping is common among anglers in the Great Lakes. There appears to be serious risk of introduction of non-native species via this vector.

For Jen and Dahlia. You both inspire me to always do the next right thing.
I love you.

Acknowledgements

This dissertation would not be possible without the love and support of my family. My parents inspired a love of the natural world in me which carried over into my academic life. I will be forever grateful. My wife, Jen, and daughter, Dahlia, inspire me to be the best possible scientist, academic, and citizen I can be. Jen's no-nonsense attitude is well balanced by her creative side. I have spent over a decade trying to impress you. Dahlia, your smile makes it possible for me to get through every day. I can't wait to see the person you become. My sister continues to show me that it is possible to make a difference. You gave me a copy of A Sand County Almanac, by Aldo Leopold, in high school. I had not fully come to realize the impact of your doing so until this very moment. Without this formative work that helped inspire the environmental movement in America, I would not be where I am today.

I would like to thank my advisor, Carol Stepien, for your guidance and mentorship. You helped me tremendously with my development as a scientist and writer. I also thank my dissertation committee, Drs. Jonathan Bossenbroek, Kerry Naish, Matthew Neilson, and William Von Sigler. Your input was indispensable in making this work what it is. I also honor my former committee member and leader of the Conservation Genetics and Genomics Laboratory USGS Leetown Science Center, Dr. Timothy King. Dr. King tragically passed away during the second year of my PhD dissertation, after helping with my qualifying exam. His work on conservation genetics and communication skills were inspiring, and he will continue to be missed by the whole community.

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Preface

This dissertation develops and applies cutting edge molecular diagnostic tools for the detection and investigation of native and invasive species. Chapter 2 is nearly identical to its published form. Chapters 3 and 4 are anticipated to be submitted for review and subsequent publication in scientific journals. Chapter 2 was published in *Molecular Ecology* two years ago with only slight rewording/reformatting for the dissertation:

Snyder MR, Stepien CA (2017) Genetic patterns across an invasion's history: a test of change versus stasis for the Eurasian round goby in North America. *Molecular Ecology*, 26, 1075–1090.

Chapter 3 is planned for submission to *Metabarcoding and Metagenomics* and Chapter 4 to *Biological Conservation*, both in the coming two months.

None of this work would be possible without funding procured from various research grants by Carol Stepien. For a list of grants, see the Acknowledgements section in each chapter. Additionally, many collaborators and colleagues helped with sample collection (see Acknowledgements in each chapter). I also thank past and present members of the Genetics and Genomics Group at NOAA Pacific Marine Environmental Lab (formerly Great Lakes Genetics/Genomics Lab at the University of Toledo) for their support in various forms – K. Andrews, D. Eddins, A. Elz, K. Klymus, E. Kramer, N. Marshall, M. Niner, C. Paight, C. Prichard, H. Scheppler, E.. Slikas, and S. Yerga-Woolwine.

Chapter 1

Introduction and Background

1.1 Rationale

The USGS spent >$17 million on invasive species research and >$20 million on fisheries assessment in 2018 (USGS 2019a). Identification and management of invasive species are top environmental concerns (Lodge and Finnoff 2008; Estévez et al. 2015). Knowledge of the distribution of native species is important for managing fisheries (Cooke et al. 2016) and conservation of rare taxa (Dobson et al. 1997; Margules et al. 2002). Introductions of exotic species comprise accidental experiments, offering the opportunity to study evolutionary and ecological processes that unfold in real-time with their relative successes and spread into new habitats (Huey et al. 2005). Molecular genetics provide tools to interpret and understand these scenarios (Allendorf et al. 2013; Bourne et al. 2018). Recent methods, such as analyses of environmental (e)DNA, offer the means to accurately and quickly identify species in the environment from water, larvae, or egg samples, and thus comprise a new opportunity to eradicate invasive species before they gain a foothold towards establishment (Zaiko et al. 2018), or to conserve rare native taxa. Genetic tools also can be used to gauge the relative success of invasions throughout their various stages of introduction, establishment, spread, and persistence, and add to our knowledge of the

fundamental ecological story governing new populations (Allendorf and Lundquist 2003). ***The central rationale underlying this dissertation is that invasion genetics is a valuable tool for understanding biological invasions and eDNA can be used to detect introduced and native species community diversity, thereby aiding in conservation and management.***

1.2 Invasive species in the Great Lakes

Non-native species are documented on every continent, representing appreciable proportions of the biota on all except Antarctica, and comprises one of the greatest threats to extant native biodiversity (Ricciardi 2013). The Laurentian Great Lakes are among the most invaded aquatic habitats on the earth, harboring >186 non-native species that have reproducing populations, including 43 fish species (~19% of total fish diversity), many of which have exerted large ecological and economic consequences (Ricciardi 2006; NOAA 2019). Prevention of exotic introductions has become a major management concern in the Great Lakes (Kelly et al. 2014; US EPA 2015; NOAA 2019). Aquatic invasive species in the Great Lakes region cost industries and households >$200 million annually (Lodge and Finnoff 2008; USFWS 2012). Most invasive species have been introduced into the Great Lakes via ballast water exchange (Ricciardi 2006), however, the pond and aquarium trades also are recognized as a potential vector (Kolar and Lodge 2002; Vander Zanden and Olden 2008; Lockwood et al. 2013a).

Managers are concerned that invasive carps, particularly the silver *Hypophthalmichthys molitrix* and bighead *H. nobilis* carps that already are high impact invaders in the Mississippi River basin, will enter and become established in the Great Lakes (Kolar et al. 2005). Silver

and bighead carps are projected to have severe effects on Great Lakes' food chains and fisheries (Kolar et al. 2005). Electric barriers between the Great Lakes and the Illinois River originally were installed in the Chicago shipping canal to prevent the round goby from escaping into the latter, but were too late, and now are the primary "prevention" to invasive carp species entering the Great Lakes from the south. Even after a voltage increase in 2011, the Illinois River electric "barriers" were shown to regularly be breached by fishes swimming alongside metal boats, including silver and bighead carps (Parker et al. 2015). The original barrier was decommissioned, and three new ones were completed in 2013 (US ACE 2019). A permanent physical separation between the Illinois River and Lake Michigan is projected to cost $9.5 billion (Wittmann et al. 2014). Silver and bighead carps are known bait contaminants in their established range in the Mississippi River basin (Kolar et al. 2005). If present in Great Lakes bait stocks, they may be introduced via this vector.

1.3 Invasion biology and genetics

The genetic composition and diversity of invasions are believed to play significant roles in their relative success and persistence (Stepien et al. 2005; Allendorf et al. 2013; Forsman 2014). The classic scenario is that the introduction of just a few propagules into a new region usually leads to a founder effect followed by genetic bottlenecks that may lower potential for adaptation and ecological fitness of a population (Baker and Stebbins 1965). In reality, many exotics appear to be as successful in their invasive range, or even more so, as in their native range (Williamson and Garvey 2005; Sokołowska and Fey 2011). Introduced populations with high genetic diversity are predicted to be more likely to persist and spread (Roman and Darling 2007; Allendorf et al. 2013), which may result from repeated introductions from one

or a number of sources and/or by a large number of initial introduced propagules (Lockwood et al. 2013b). Several recent studies in invasion genetics appear to support this hypothesis, indicating that successful introductions often possess high levels of genetic diversity (Roman and Darling 2007; Brown and Stepien 2009; Stepien et al. 2013, 2019).

It is highly unlikely that any individual species will be introduced into a new environment. When they are, those having low propagule pressure and/or low genetic diversity are unlikely to become established and may become extirpated (Allendorf and Lundquist 2003; Figure 1–1). Sustained propagule pressure would facilitate establishment and eventual spread of the invasion, after an initial lag time. Just a small percentage of introduced species are successful (i.e., those that reach and progress quickly through the second stage of an invasion). Those that do, often show a lag time between establishment and rapid population growth, expansion, and ecological effects. Further propagule pressure may augment establishment and spread into additional areas. As the invasion spreads, newly invaded areas then undergo the same process. In traditional invasion ecology theory, the "tens rule" states that only about 10% introduced species usually become established (Lockwood et al. 2013c). Less than ~10% of those usually go on to spread and become invasive (i.e., cause ecological or economic effects). There often is a 10-year lag time for a species to progress through these stages.

Early detection may facilitate eradication of non-native individuals before they become an established population or aid more effective control at later stages. The response to a new non-native species is effectively the same whether or not there is evidence that they will become invasive – managers should attempt eradication if possible (Simberloff 2003). For this reason, the terms invasive, non-native, alien, etc. are used interchangeably throughout much of this dissertation.

A thorough and complete understanding of biological invasions is recognized as essential to develop effective management and control strategies (Allendorf et al. 2013). It is possible for invasion genetics/genomics studies to aid prediction of which introduced species may become invasive by calculating propagule pressure, genetic diversity, and the adaptational responses of introduced populations (Stepien et al. 2005; Rius et al. 2015a; Bourne et al. 2018). Additionally, it is possible that with eDNA sampling, and diagnostic assays, which appear more efficient and effective than traditional sampling methods, managers may be able to detect invasions earlier (Darling and Mahon 2011; Zaiko et al. 2018). Overall, prevention is recognized as more effective and economical than control (Leung et al. 2002) and identifying an invasive species early after introduction makes eradication more likely (Figure 1–1).

1.4 New tools for identifying invasive species and community composition

Identification and control of invasive species is critical for the protection of native biodiversity (Allendorf and Lundquist 2003; Ricciardi 2013). Identifying native community composition is a high priority for conservation, management, and discerning anthropogenic impacts on the environment (Attrill and Depledge 1997; Myers et al. 2000; Margules et al. 2002). Morphological surveys require a high degree of taxonomic expertise and are time consuming, expensive, and prone to false negatives, especially with rare native or newly introduced potentially invasive species (Balmford and Gaston 1999; Reynolds et al. 2011; Darling and Mahon 2011). It often is not possible for a non-native species to be detected with traditional methods until its density is appreciably high (Figure 1–2A). Species

identification often is very difficult, especially with eggs or larvae, for which morphological characters frequently are lacking and closely related species appear identical, necessitating highly skilled and trained personnel for processing samples.

Individuals continually shed DNA into the environment in the forms of mucus, urine, feces, and skin cells (Ficetola et al. 2008; Barnes and Turner 2016). eDNA methods involve the collection of genetic material from the environment, often in water or sediment/soil. Through filtering and/or centrifugation, genetic material is concentrated, and DNA is extracted and purified using standard laboratory methods. One approach is to identify the presence or absence of a single targeted taxon, using traditional polymerase chain reactions (PCR), quantitative PCR (qPCR), or digital droplet PCR (ddPCR), of which the latter two approaches can be used to quantify the relative amount of DNA (Rees et al. 2014). The approach of metabarcoding utilizes high throughput sequencing (HTS) and DNA amplification to simultaneously sequence millions of individual amplicons, which then are compared to one or more reference databases to identify the taxa (Shokralla et al. 2012; Deiner et al. 2017). Additionally, samples can be indexed (tagged) during PCR to facilitate the pooling of multiple samples for a single HTS run, offering significant reductions in cost per sample (Illumina 2018). Theoretically, the species density required for detection is considerably lower for eDNA than for traditional sampling (Figure 1–2B; Darling and Mahon 2011; Wilcox et al. 2016), potentially facilitating the initiation of management actions before the invasion has passed beyond the first stage of establishment when eradication is still feasible (Figure 1–1; Allendorf and Lundquist 2003).

eDNA assays recently have been used to detect the presence/absence of single invasive species – including invasive carps belonging to *Hypopthalmichthys* spp. (Jerde et al. 2011; Erickson et al. 2016), Eurasian ruffe *Gymnocephalus cernua* (Tucker et al. 2016), New Zealand

mudsnail *Potamopyrgus antipodarum* (Goldberg et al. 2013), sea lamprey *Petromyzon marinus* (Gingera et al. 2016), and some Ponto-Caspian gobies (Adrian-Kalchhauser and Burkhardt-Holm 2016) – as well as rare native species – including giant salamanders *Andrias* spp. (Fukumoto et al. 2015) and Mekong giant catfish *Pangasianodon gigas* (Eva et al. 2016). Metabarcode assays are becoming more widely used (Deiner et al. 2017) and have been conducted in various types of aquatic habitats as *de novo* assessments of community composition (Miya et al. 2015; Bista et al. 2017; Kelly et al. 2018), in comparison to traditional morphological sampling (Civade et al. 2016; Lawson Handley et al. 2019; Fujii et al. 2019), and also to identify invasive species and their populations (Brown et al. 2016; Marshall and Stepien 2019a; Stepien et al. 2019).

1.5 The dissertation research

This dissertation research contains three parts. **Part I** is a temporal population genetics investigation of the round goby in the Great Lakes (previously published as Snyder & Stepien 2017 in *Molecular Ecology*), which analyzed patterns of population genetics over the timespan of a successful high impact invasion. **Part II** is the development and testing of new eDNA metabarcode high-throughput sequencing assays, a sequence library preparation protocol, and bioinformatic pipeline for the detection of native and invasive species and community structure in the Great Lakes. **Part III** is the application of the targeted eDNA metabarcode high-throughput sequence metabarcode approach developed in **Part II** to identify invasive species in the bait and pond store trades in the Great lakes. This research adds to the scientific understanding of successful aquatic invasive species. In addition, it provides an improved methodological framework for assessment of community composition

using targeted metabarcode high-throughput sequencing and bioinformatic methodology and applies this protocol to detect invasive and illegal native species in an understudied yet important vector.

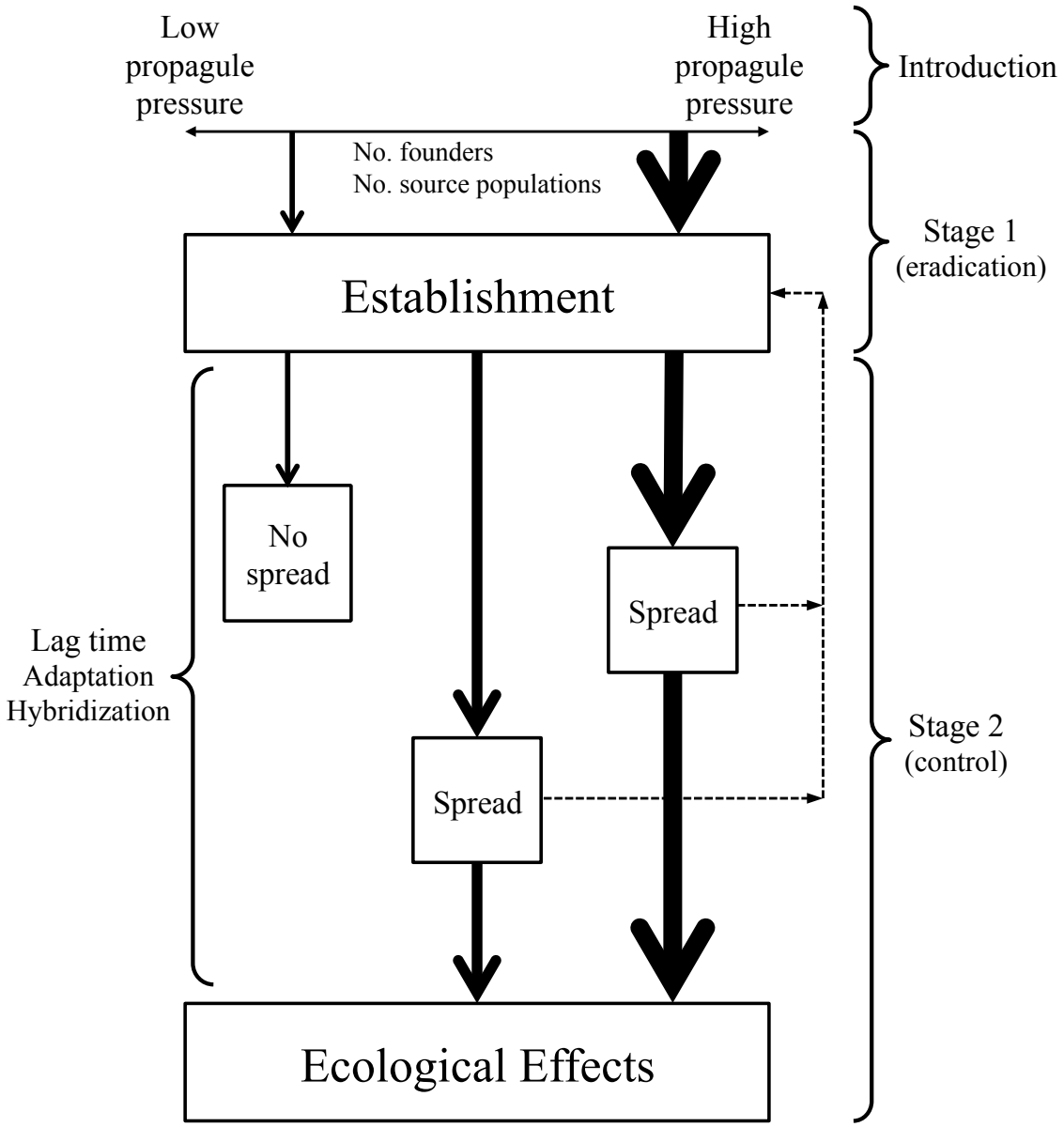

Figure 1–1 The effect of propagule pressure on the process of biological invasions. Adapted from Allendorf and Lundquist (2003).

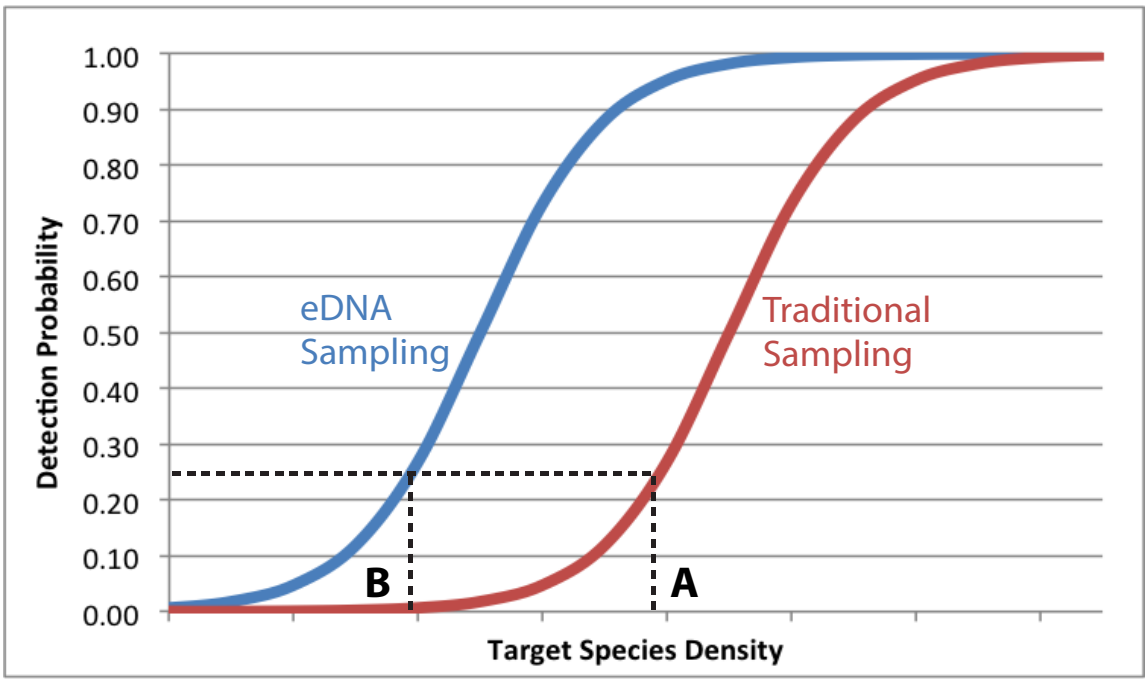

Figure 1–2 Hypothetical comparison of eDNA vs. traditional sampling methods for detecting species at varying densities. Adapted from Darling and Mahon (2011).

Chapter 2

Genetic patterns across an invasion's history: a test of change versus stasis for the Eurasian round goby in North America

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2.1 Abstract

Biological invasions comprise accidental evolutionary experiments, whose genetic compositions underlie relative success, spread, and persistence in new habitats. However, little is known about whether, or how, their population genetic patterns change temporally and/or spatially across the invasion's history. Theory predicts that most would undergo founder effect, exhibit low genetic divergence across the new range, and gain variation over time via new arriving propagules. To test these predictions, we analyze population genetic diversity and divergence patterns of the Eurasian round goby *Neogobius melanostomus* across the two decades of its North American invasion in the Laurentian Great Lakes, comparing

results from 13 nuclear DNA microsatellite loci and mitochondrial DNA cytochrome *b* sequences. We test whether "genetic stasis", "genetic replacement", and/or "genetic supplement" scenarios have occurred at the invasion's core and expansion sites, in comparison to its primary native source population in the Dnieper River, Black Sea. Results reveal pronounced genetic divergence across the exotic range, with population areas remaining genetically distinct and statistically consistent across two decades, supporting "genetic stasis" and "founder takes most". The original genotypes continue to predominate, whose high population growth likely outpaced the relative success of later arrivals. The original invasion core has stayed the most similar to the native source. Secondary expansion sites indicate slight allelic composition convergence towards the core population over time, attributable to some early "genetic supplementation". The geographic and temporal coverage of this investigation offers a rare opportunity to discern population dynamics over time and space in context of invasion genetic theory versus reality.

2.2 Introduction

Introductions of non-native species into new locales comprise accidental experiments, which can be used to test evolutionary theory (Huey et al. 2005), since genetic diversity and composition underlie their relative success, spread, and persistence (Wares et al. 2005; Stepien et al. 2005; Wilson et al. 2009; Forsman 2014). Understanding the evolutionary scenarios of exotic invasions has become increasingly important with their growing prevalence in most ecosystems worldwide and their serious impacts on native biodiversity (Williamson 1999; Allendorf et al. 2013; Ricciardi 2013). Traditional ecological theory predicts that most exotic populations would undergo founder effects that limit their relative

adaptive success in new areas (Baker and Stebbins 1965; Allendorf and Lundquist 2003; Lockwood et al. 2013b, d). However, recent studies have shown that some exotic populations appear as successful (or even more so) in their introduced locations as across their native ranges (Williamson and Garvey 2005; Brown and Stepien 2009, 2010; Sokołowska and Fey 2011). Relatively high levels of genetic variability in these invasions may stem from repeated introductions from one or more source populations and/or by large numbers of introduced propagules, termed "propagule pressure" (Williamson 1999; Lockwood et al. 2013b). Such very diverse populations are presumed to be better able to survive, reproduce, and adapt in a variety of new habitats (Lewontin 1974; Wares et al. 2005; Lockwood et al. 2013d). However, studies of invasions across their temporal trajectories are relatively rare, often because samples are unavailable from their early stages or across a time continuum (Brown and Stepien 2010; Kekkonen 2016).

It is difficult (and unlikely) for new arrivals to successfully survive, reproduce, spread, and adapt in new habitats (Elton 1958; Williamson and Fitter 1996). Moreover, the selective forces acting on a population are predicted to vary over the invasion's time course, as well as across the species' life history stages and may alter genetic diversity and divergence patterns (Hedrick 1995; Weinig 2000; Stepien et al. 2013). Initial colonists entering a new environment might be better dispersers, and later arrivals that are better competitors may replace the earlier ones (Wilson et al. 2009; Phillips et al. 2010; Allendorf et al. 2013). The gene pool of the new population thus would change over time, with the later-arriving genotypes gaining increased representation and the earlier ones remaining most prevalent along the invasion front, which has been termed the "leading edge" hypothesis (Hewitt 1996). However, if the original colonists reproduce rapidly enough to greatly outnumber later arrivals (and their genotypes) and there is little to no adaptive advantage between them,

then the population would stay relatively consistent; Waters *et al.* (2013) termed this the "founder takes all" hypothesis. Another alternative would be that new populations might accumulate genetic variation over time, either from new arrivals or via spread from neighboring populations. Such admixture might enhance genetic diversity and adaptive potential in a variety of habitats across the new range (Lewontin 1974; Wilson et al. 2009).

The present study evaluates these alternative genetic scenarios across nearly 25 years of a successful introduction, using the introduction of the Eurasian round goby *Neogobius melanostomus* (Pallas 1814) in the Laurentian Great Lakes of North America as a model. The round goby first was discovered in the St. Clair River and Lake St. Clair in 1990, where it was accidentally introduced via ballast water transport from commercial trans-oceanic ships (Jude et al. 1992; Hensler and Jude 2007). Ballast water has been a common vector for many introductions of Ponto-Caspian species into the Great Lakes over the past 30 years, including the dreissenid mussels – zebra *Dreissena polymorpha* (Hebert et al. 1989) and quagga *D. rostriformis* (May and Marsden 1992) – and spiny water flea *Bythotrephes longimanus* (Johannsson et al. 1991). By 1995 the round goby had spread throughout the Great Lakes region (Jude et al. 1995), enhanced by shipping transport (Hensler and Jude 2007). In many shallow water areas it now is one of the most common benthic species (USGS 2016) and has exerted ecological and economic impacts, notably as an egg predator on important sport and commercial fishes (Steinhart et al. 2004; Kornis et al. 2012).

Our collections of round goby samples over the two and a half decades following the round goby's discovery offer a rare opportunity to evaluate whether population genetic changes or relative stasis has characterized the temporal and spatial range of this successful invasion. Earlier genetic analyses by our laboratory (Brown and Stepien 2009) identified the port population area of the Dnieper River Delta in the Black Sea near Kherson, Ukraine as

the likely primary source for the Great Lakes introduction. Across the Great Lakes, the invasive round goby's range is characterized by high genetic diversity and pronounced population genetic structure; both indicate a large number of introduced propagules and lack of founder effect (Stepien and Tumeo 2006; Brown and Stepien 2009). This high genetic variability, as well as its ecological and physiological flexibility (Moskal'kova 1996; MacInnis and Corkum 2000; Karsiotis et al. 2012), prior establishment of its native dreissenid mussel prey (Dreissena polymorpha and D. rostriformis; Ricciardi 2001; DeVanna et al. 2011), and release from native predators and parasites (Muzzall et al. 1995; Kvach and Stepien 2008), are hypothesized to have facilitated the round goby's success.

The present study tests the history of genetic variability of the round goby introduction using 13 unlinked nuclear DNA microsatellite loci and mitochondrial (mt) DNA cytochrome (cyt) *b* gene sequences (Appendix A.1) at five invasive locations in the Great Lakes (including the invasion core and peripheral range expansion sites) in relation to the founding source population. We test the following genetic scenarios:

1. **Genetic stasis**. Genotypes of the initially established colonists have persisted over time in population areas. This might stem from all (or most) of the introduction's genetic diversity becoming established early on and maintained. The overall genetic composition would remain representative of the invasion population source(s). Genetic variability of the new population likely would be lower than the source population(s) due to founder effect, particularly with loss of rare alleles, which might further decline over time due to drift, but no gain of significant numbers or proportions of alleles would occur over time. "Genetic stasis" also is predicted by the "founder takes all" hypothesis (Waters et al. 2013), in which the original established genotypes crowd out contributions by later arrivals, due to rapid population increase by the former. Genetic stasis in given areas

across the new range also might be accompanied by considerable genetic divergence among population areas, when more than one source differentially contribute to several simultaneous foundings across the invasive range (Kolbe et al. 2004; Darling et al. 2008; Stepien et al. 2013). In that case, regional populations might retain their original genetic signatures over time despite the respective growth and expansion of neighboring ones.

2. **Genetic supplement**. Genotypes of the initial established colonists persist and are supplemented by later-arriving genotypes from single or multiple sources. Allelic richness thus increases over time and the genetic composition of the population would change. For example, the *B. longimanus* invasion in the Great Lakes appeared to have been genetically supplemented from repeated European introductions (Berg et al. 2002).
3. **Genetic replacement**. Genotypes of the initially established colonists are replaced by later arrivals from single or multiple sources. These might be better competitors or more fit than the original colonists. Overall genetic diversity of the population might remain the same or decline, but its allelic composition would change. The change in predominant alleles would be distinguishable from random drift in scenario 1. For example, the original invasive population of *D. polymorpha* in the Hudson River died out and then was genetically replaced by new arrivals (Brown and Stepien 2010; Strayer et al. 2011; Stepien et al. 2013).

2.3 Methods

2.3.1 Sampling design and collections

We analyzed 1096 round goby individuals from the early, mid, and late invasion stages at five key population areas in the Great Lakes. Two of these sites were located where

the species was initially discovered – termed the invasion core (the St. Clair River and Lake St. Clair) – and three comprised later expansion sites (one in Lake Michigan and two in Lake Erie; Figure 2–1). Each was sampled near the round goby's approximate time of establishment at that location and again in 2007, 2011, and 2013, with additional temporal replicates in the Lake Erie locations. We relate these to samples collected in 2002, 2007, and 2013 at their primary native genetic source region – previously identified by our laboratory in Brown and Stepien (2009) – located in the Dnieper River Delta at the port of Kherson, Ukraine in the northwestern Black Sea (Figure 2–1; Table 2.1). Date of initial establishment by the round goby per location was obtained from the U.S.G.S. Nonindigenous Aquatic Species Database factsheet (Clapp et al. 2001; Fuller et al. 2019). Generations since initial establishment of that population area were calculated using the minimum age at maturity (two years) for round goby females in the Great Lakes (MacInnis and Corkum 2000).

Collections were made with a two-meter seine or minnow traps in shallow areas, or bottom trawls in deeper waters by federal or state agencies and members of our laboratory. Whole samples or fin clips were preserved in 95% ethanol in individually labeled vials and then archived at room temperature prior to DNA extraction. Collections followed the University of Toledo's International Animal Care and Use Committee protocol #105400 and were obtained under Ohio Department of Natural Resources permit #17-159 and Michigan Department of Natural Resources permits.

2.3.2 Genetic data collection

Genomic DNA was extracted using QIAGEN DNeasy kits (Qiagen Inc., Valencia, CA), with quality and quantity assessed on 1% agarose mini-gels stained with ethidium bromide and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific™ Inc.,

Waltham, MA). DNA was stored at 4°C until amplification via Polymerase Chain Reaction (PCR).

Amplification and alignment of the mtDNA cyt *b* gene followed Brown and Stepien (2008, 2009). Primer sets for nuclear μsat loci were from Feldheim et al. (2009; Ame 10, 17, 129, and 133) and Dufour et al. (2007; Nme 1-5 and 7-10). PCR amplifications totaled 10 μL of 0.035 units *Taq* DNA polymerase, 100 μM dNTPs, 60mM KCl, 2.8 mM $MgCl_2$ (or DMSO for Ame 194), 12 mM Tris–HCl, 0.5 μM of each primer, and ≥80 ng template. Positive (known genotype) and negative (no template) controls were included in each run. PCR comprised initial denaturation at 94°C (3 min), followed by 40 cycles of 94°C (30 sec), annealing at primer specific temperatures (1 min; Appendix A.1B), and extension at 72°C (1 min), capped by final 3 min extension. PCR products were diluted 1:50 or 1:100, of which 1 μL was added to 13 μL formamide and Applied Biosystems Gene Scan 500 size standard (ABI, Fullerton, CA) in 96-well plates, denatured at 95°C (2 min), and analyzed on an ABI 3130xl Genetic Analyzer with GENEMAPPER v3.7. Electropherograms were checked manually to verify allele sizes.

2.3.3 Genetic data analyses

To establish whether samples represented true populations and statistical assumptions were met, μsat loci were tested for conformance to Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) assumptions, as well as possible scoring errors, null alleles, and large allele dropout following our laboratory protocols (see Haponski et al. 2014). LOSITAN (Antao et al. 2008) and BAYESCAN (Foll and Gaggiotti 2008) evaluated whether loci conformed to expectations of neutrality or were under possible

selection. Colony v2.0.6.1 (Jones and Wang 2010) tested for the presence of full siblings in the data set.

We analyzed spatial and temporal patterns among round goby population sites, including relative genetic diversity and composition, to weigh the evidence for and against three scenarios. Significant changes in genetic composition over time might support either "genetic replacement**"** or "genetic supplement", whereas negligible alteration involving just rare alleles/haplotypes likely reflects sampling error, suggesting "genetic stasis". We determined numbers of haplotypes/alleles (N_H/N_A), numbers and proportions of private haplotypes/alleles (N_{PH} and P_{PH}/N_{PA} and P_{PA}), observed and expected heterozygosities (H_O/H_E), and haplotype diversity (h) in ARLEQUIN v3.5 (Excoffier and Lischer 2010) and allelic richness (A_R) in FSTAT v2.9.3.2 (Goudet 1995). Friedman rank sum and post-hoc tests in R v3.0.1 (R Core Team 2015) identified significant differences in allelic richness and observed heterozygosity. Percentages of each haplotype present are illustrated with stacked bar graphs (per sample) and pie charts (per population; Microsoft EXCEL; Microsoft, Redmond, WA).

To discern whether samples significantly diverged over time and space, we calculated pairwise comparison θ_{ST} values (an F_{ST} analogue) in ARLEQUIN (cyt *b*) or FSTAT (μsats) and performed exact tests in GENEPOP (Rousset 2008). Significance was determined after sequential Bonferroni correction (Rice 1989). The relationships between genetic divergence and geographic distance (km; nearest waterway) and time (years) at sampling sites were evaluated using separate Mantel (1967) tests in ARLEQUIN. Nearest waterway geographic distances (km) between all Great Lakes sites were measured using the Path tool in GOOGLE EARTH (Google, Mountain View, CA). Analysis of Covariance (ANCOVA) compared the regression slopes of genetic divergence versus temporal separation in R.

Analysis of Molecular Variance (AMOVA) in ARLEQUIN with locations as population groups, and temporal samples as populations, evaluated the relative spatial and temporal partitioning of genetic variation. Additionally, three-dimensional Factorial Correspondence Analysis in GENETIX v4.05 (Belkhir et al. 2004) was used for non-parametric exploratory visualization of spatial and temporal trends in population structure.

The frequency-based methods described analyzed the population as the unit of comparison, whereas Bayesian model-based approaches in STRUCTURE v2.3.3 (Evanno et al. 2005) and GENECLASS2 (Piry et al. 2004) were based on assignments of individual fish. STRUCTURE assigned a likelihood for each individual belonging to one (or more) *K* number(s) of genetic demes. We evaluated *K*s from one to the total number of samples plus one ($N_{Sites\ by\ Year}$+1=27) and conducted three hierarchical analyses of population structure, with and without the Location Priors function. Each *K* was evaluated in 10 independent runs, with burn-ins of 50,000 and 100,000 replicates, and Δ*K* likelihood (Evanno et al. 2005) in STRUCTURE HARVESTER (Earl and vonHoldt 2012). Bayesian assignment tests in GENECLASS2 used the compute likelihood function, 100,000 simulated individuals, the method of criterion by Rannala and Mountain (1997), and the simulation algorithm from Paetkau et al. (2004).

Sequence data are available in NCBI GenBank, whose accession numbers are in Appendix A.2. Arlequin input files with the genetic and geographic coordinate data are deposited in Dryad (DOI: http://dx.doi.org/10.5061/dryad.p5kk8).

2.4 Results

2.4.1 MtDNA diversity and composition

We discerned 13 cyt *b* sequence haplotypes among the round goby samples, including 10 in the Great Lakes and three exclusive to the native Dnieper River Delta (Appendix A.2). Haplotypes per sampling location numbered from three (Lake Michigan, a later expansion site) to eight (Dnieper River and the Central Lake Erie expansion site). Haplotypes collected at a location in a single year numbered from just one (Lake Michigan 1999) to six in the Great Lakes (Central Lake Erie 2013) and seven for the native population (Dnieper River 2013). Seven haplotypes were private to a single population location and year (Appendix A.3).

Haplotypes identified in Lake Michigan, which had the lowest diversity and was the more remote expansion area, increased from one in 1999 to three in later sampling years (θ_{ST}=0.157, p<0.05; Appendix A.4). Its sample size was lower in 1999 (N=19), since the goby then was very rare (we obtained and analyzed all those obtained in a federal survey). If its haplotypic distributions in 1999 and 2007 had been identical, the likelihood of the earlier sample comprising just that one haplotype (Ame1) would have been very low given the multiplicative probability rule (p=0.004, e.g., 0.745^{19}; Appendix A–1).

Over time most of the other population temporal samplings varied only by singleton haplotypes, attributable to sampling stochasticity. Notably, in 2013, three new unique singletons were detected in the Dnieper River and three others in Central Lake Erie. Moreover, both locations in 2013 also had a shared new haplotype that was not previously obtained (Nme 89). Electropherograms for novel haplotypes were rechecked manually to verify that they were not errors. If haplotypic composition at the time when earlier samples

were taken in Central Lake Erie and the Dnieper Rive were actually identical to those in 2013, probabilities of obtaining samples without these rare haplotypes would be very low and in some cases significantly so. Given the multiplicative probability rule, the likelihood of earlier samples in Central Lake Erie not containing any of the rare haplotypes if they were actually present in the population would have been 0.107 (1998; N=24), 0.067 (2002; N=29), 0.01 (2007; N=50), and 0.011 (2011; N=48). In the Dnieper River the same likelihoods would have been 0.057 (2002, 2005; N=25 for both). One additional individual possessed haplotype Ame88 in the 2013 St. Clair River sample, in comparison to its occurrence in a single individual in 1993; none were obtained in 2007 or 2011 (Appendix A–1).

2.4.2 Nuclear DNA microsatellite genetic diversity

All μsat loci and population samples conformed to Hardy Weinberg equilibrium and linkage disequilibrium expectations, lacked null alleles, and showed no evidence of selection. The sole samplings with full siblings occurred in Lake Michigan, with two groups in 1999 (one comprising three individuals and one with two, of N=19) and one in 2011 (two individuals, N=44). Analyses thus were run with and without the full sibling multiples (the latter with a single representative per group). Mean differences between the two analysis sets were slight: 0.0005±0.0001 for spatial θ_{ST} and 0.02±0.003 for the individual assignments. Mean differences for temporal θ_{ST} were 0.0012±0.0002 and 0.03±0.01 for individual assignments. Since these variations were negligible, results from the complete data set are presented here.

We recovered 246 μsat alleles (mean per locus=18.9±2.69), of which 77 occurred only at a single population site and 63 of those just in one year (Table 2.1). The Dnieper River source population overall possessed the greatest number of alleles (N_A=190) and the

greatest proportion of private alleles (P_{PA}=0.25), which were absent in the Great Lakes. Within the Great Lakes, the most alleles occurred in the St. Clair River (143) – the original introduction site – and the fewest in Lake Michigan (118). The largest proportion of private alleles among Great Lakes' sites were found at the Lake Erie Islands location (0.06), and the lowest in the St. Clair River and Central Lake Erie (both 0.03). Allelic richness (A_R) at the original invasion core locations (St. Clair River and Lake St. Clair) did not significantly differ from the native Dnieper River source population. A_R values were significantly lower at the early expansion margins (Central Lake Erie, Lake Erie Islands, and Lake Michigan) in comparison to the native source.

2.4.3 Genetic divergence among sites

The Lake Erie Islands expansion population alone significantly diverged from the other samples with the cyt *b* sequence data (Table 2.2A), due to greater representation of haplotypes Ame8 and 57 (respectively colored light grey and blue in Figure 2–1). All round goby populations significantly differed from one another in μsat allelic composition (Table 2.2B). The original invasion core sites at the St. Clair River and Lake St. Clair were genetically more similar to each other (θ_{ST}=0.004, p<0.00004), as well as to their Dnieper River source (θ_{ST}=0.025, p<0.00001). θ_{ST} values were greatest between the Dnieper River and the Great Lakes' later expansion populations (e.g., central Lake Erie; 0.047, p<0.00001 and Lake Michigan; θ_{ST}=0.067, p<0.00001), which are located further away from the invasion core. The expansion population in the Lake Erie Islands was more similar to the core (θ_{ST}=0.007, p<0.00001). The Central Lake Erie expansion population was genetically closest to its neighboring one in the Lake Erie Islands (θ_{ST}=0.009, p<0.00001).

A Mantel test discerned that 98% (p=0.013) of the overall μsat genetic divergence was explained by geographic distance among the Great Lakes' sampling locations (R^2=0.98, F=389.8, p=4.51•10^{-8}; Appendix A–2). This relationship also was significant without the Lake Michigan population (R^2=0.87, F=34.57, p=4.2•10^{-3}).

2.4.4 Temporal trends within sampling sites

MtDNA haplotype compositions at population locations (Appendix A.4) remained consistent over time (except for Lake Michigan), but their nuclear μsat allelic frequencies varied significantly (Table 2.3). AMOVA partitioned 2.43% (p<0.00001) of the μsat variation among the populations and 2.02% (p<0.00001) across their temporal replicates. Of the temporal comparisons, 86% (θ_{ST}) and 96% (exact tests) were significant following sequential Bonferroni correction. All round goby populations stayed statistically consistent in A_R over time, except that the first sampling (1999) at the Lake Michigan expansion site was significantly lower than most others (Table 2.1).

Mantel tests depicted significant relationships between genetic divergence and time for just the Lake Michigan and Central Lake Erie expansion sites (p=0.040 and 0.031), which each underwent significant temporal alterations in allelic frequencies (Table 2.3). Lake Michigan exhibited the most change from earlier to later samplings, with net gain of 50 alleles from 1999–2007, followed by net loss of 13 in subsequent years. Re-analysis without the first (1999) sampling revealed that the relationship between genetic divergence and time was no longer significant. ANCOVA analyses indicated significant differences in slopes of the relationships between genetic divergence and time for Lake Michigan versus all other sites ($5.02•10^{-05}<p<0.004$) except the Dnieper River. The slope for the Dnieper River also

significantly differed from most sites in the Great Lakes ($0.007<p<0.017$), except Lake Michigan and Central Lake Erie.

Almost all gains or losses of μsat alleles after initial samplings of populations involved those with ≤5% representation, implicating stochastic sampling error as the source of that variation. Most pairwise θ_{ST} values denoted greater genetic distinctiveness among populations in early sampling years than later (Table 2.3). However, all populations remained significantly different from all others. Reductions in θ_{ST} between expansion edge populations and the invasion core were most pronounced between their first and second samplings (1990s and 2007), denoting changes sometime between those two temporal replicates. The close genetic relationship between the St. Clair River and the Lake St. Clair core populations stayed relatively consistent over time (Table 2.3).

2.4.5 Population structure and assignments

Three-dimensional Factorial Correspondence Analysis based on μsat loci showed that the Lake Michigan expansion population was very different from all others in the Great Lakes (Figure 2–2A) and remained so over time (Figure 2–2B). During their relative expansions, the genetic compositions of the Lake Michigan and Central Lake Erie populations each independently converged towards the invasion core, and then stayed relatively consistent (Figure 2–2C). Bayesian modeling of population structure (Figure 2–2D and 2–2E) provided greatest support for K=2 (ΔK=28.2) and K=6 population groups (ΔK=16.4). At K=2, the Dnieper River founding population and the Lake Michigan expansion site each differed from all others, and the St. Clair River core site possessed the most admixture (Figure 2–2d). At K=6, the Dnieper River, Lake Michigan, and Central Lake Erie populations were the most distinct (Figure 2–2E). Over time, the St. Clair River and

Lake St. Clair core populations both assigned more to the same hypothetical population colored yellow, which occurred earlier in the former. The Lake Erie Islands expansion population underwent just minor temporal fluctuations, showing considerable admixture. The Central Lake Erie expansion population assigned mostly to pink, which appeared to decline over time.

All round goby individuals from the Dnieper River source population correctly self-assigned (Figure 2–2E, Table 2.2C). Just the invasion core populations – St. Clair River (0.29) and Lake St. Clair (0.27) – had appreciable proportions of individuals mis-assign to the Dnieper River source (Table 2.2C). For the St. Clair River, 31% of individuals mis-assigned to Lake St. Clair, indicating similarity between the invasion core sites. Overall, most round goby individuals correctly assigned to their respective home population except for slightly greater mis-assignment of the Lake Erie Islands to the Lake St. Clair core, which was its likely source. No gobies sampled elsewhere mis-assigned to Lake Michigan, except for two individuals from the Lake Erie Islands.

Most populations showed the greatest proportion of self-assignments in the first sample year (Appendix A.5). Exceptions were the Dnieper River and Lake Michigan, with most assignments to the correct location but a different year. As time progressed, most Great Lakes populations (all except Lake Michigan) had fewer correct assignments to location and year, suggesting genetic convergence. This trend appeared more pronounced for the Lake Erie expansion populations. For example, after the first sampling in 1998, the Lake Erie Islands expansion population had more individuals mis-assign to the Lake St. Clair invasion core. Likewise, individuals sampled from the Central Lake Erie expansion population increasingly mis-assigned to the Lake St. Clair core after 1997.

2.5 Discussion

2.5.1 Genetic diversity of newly founded invasions

Invasion genetic theory predicts that most introductions undergo founder effects, which may influence their relative successes in new habitats (Baker and Stebbins 1965; Allendorf and Lundquist 2003; Lockwood et al. 2013b, d). This does not appear to be the case for the round goby. Across the round goby's North American range, Brown and Stepien (2008) described an overall pattern of substantial genetic diversity attributed to high propagule pressure with little founder effects (mean HO invasive/native range=0.49/0.46; Brown and Stepien 2008, 2009). Results of the present investigation agree with this assessment, and additionally discern the trajectory of these patterns over the history of the round goby's expansion, focusing on the Great Lakes. The present study discerned statistically similar A_R and heterozygosity values between the native source population and the invasion core, yet a greater proportion of rare (private) alleles/haplotypes in the former may indicate some loss of allelic variation.

Many other successful invasions are characterized by comparable genetic diversity in the their native and introduced ranges. Notably, Rius et al. (2015b) reported that 74% of marine introductions experienced no significant loss of genetic diversity. In the freshwater Great Lakes, populations of the zebra and quagga dreissenid mussels possessed high genetic diversity, traced to multiple founding sources, and displayed no founder effects in comparison to their native Eurasian populations (mean HO invasive/native range=0.72/0.76; Brown and Stepien 2010; Stepien et al. 2013). The Asian tiger mosquito *Aedes albopictus* displayed greater genetic diversity in its introduced Australian range than in its hypothesized Indonesian source population, implicating founding admixture (Beebe et al.

2013). Initially, the Eurasian spiny waterflea *B. longimanus* introduction in the Great Lakes displayed small founder effects, which disappeared due to repeated introductions from multiple native locations (Berg et al. 2002). Exotic populations of western corn rootworm *Diabrotica virgifera virgifera* in Croatia had fewer alleles early in the invasion than later (Lemic et al. 2013).

In contrast to those examples and to the round goby, some introductions have succeeded despite low levels of genetic diversity and/or propagule pressure. These include the miconia tree *Miconia calvescens* in tropical Pacific islands (Le Roux et al. 2008), two Indo-Pacific lionfishes *Pterois volitans* and *P. miles* that became established in the Western Atlantic and Caribbean (Betancur-R. et al. 2011), and serially introduced western mosquitofish *Gambusia affinis* in New Zealand (Purcell et al. 2012). Exotic Asian paddle crab *Charybdis japonica* populations in New Zealand are genetically homogeneous and show lower genetic diversity than in the native range (Wong et al. 2016). In an extreme example, Sacramento pikeminnow *Ptychocheilus grandis* populations were very successful in short-range invasions into coastal Californian rivers, with less than four effective founders and 49.6% reduction in A_R (Kinziger et al. 2014). Likewise, numbers of exotic moose *Alces alces* in Newfoundland greatly increased despite being founded by just three individuals (Broders et al. 1999). Thus, genetic diversity does not appear predictive of all successful invasions.

2.5.2 Spatial divergence patterns of invasions

Our results and earlier studies (Brown and Stepien 2009) demonstrate significant genetic divergence among round goby populations in the Great Lakes; there thus is pronounced structure across the invasion. Present findings support a significant pattern of genetic isolation by geographic distance among the Great Lakes' sites assayed here, with

greater divergence and geographical separation of the Lake Michigan population. In contrast, patterns analyzed across the entire North American range by Brown and Stepien (2009) implicated jump dispersal and did not correspond to overall isolation by distance. For native round goby populations within the Black and Caspian Sea basins, isolation by distance held true (Brown and Stepien 2008); the similarity to our findings here for the invasion core and Lake Erie populations, denotes a model of natural dispersal as in the native systems.

As in the round goby, pronounced population divergences characterize the ranges of some successful introductions, paralleling those across their native ranges (see Stepien et al. 2005 and those below). For example, exotic dreissenid mussel populations possessed high differentiation across the Great Lakes, as well as in their later expansions across North America and among native Eurasian populations (Brown and Stepien 2010; Stepien et al. 2013). Northern snakehead *Channa argus* displayed pronounced spatial structuring across their invasive range in the eastern U.S. due to multiple independent introductions (Wegleitner et al. 2016). Significant genetic and transcriptomic divergence between populations of the European green crab *Carcinus maenas* occurred in its North American Atlantic coastal range, but were greater in the native range (Tepolt and Palumbi 2015).

For the round goby, the closer genetic relationship between the invasion core and the Lake Erie Islands expansion population might have resulted from one-way gene flow from the former via downstream dispersal. The greater genetic difference characterizing the Central Lake Erie expansion population might stem from natural dispersal and inter-basin ballast water exchange. Finally, the Lake Michigan population likely became established via the latter vector, leading to its greater difference from the other assayed locations. Together, these explain the overall genetic isolation by geographic distance pattern among these invasive populations.

2.5.3 Temporal patterns in invasion genetics

Round goby populations have maintained genetic consistency at the introduction core in the Great Lakes, as well as in expansion locations after initial establishment and growth. Our results for the round goby support the "genetic stasis" and "founder takes most" scenarios – the latter constituting our modification of the "founder takes all" hypothesis outlined by Waters et al. (2013). An exception is that a small amount of "genetic supplementation" occurred early in the expansion populations. During initial spread, secondary founder effects temporarily characterized the population expansions in Lakes Michigan and Erie, which initially had lower A_R values; these then dissipated early. The genotypes that arrived and established first at given locations have persisted, with some convergence in spread populations attributable to slight "genetic supplementation". Overall, the Great Lakes invasive populations assayed have retained their individual genetic compositions and differences from one another.

As in the round goby, exotic bighead goby *Ponticola kessleri* populations showed no significant genetic changes over two years post-introduction in Switzerland, where they significantly diverged among different river systems (Adrian-Kalchhauser et al. 2016). The bighead goby belongs to the same family (Gobiidae) and subfamily (Benthophilinae) as the round goby and shares a common native biogeographic history in the Ponto-Caspian region (Neilson and Stepien 2009; Stepien and Neilson 2013). In another case of genetic stasis, invasive populations of red fox *Vulpes vulpes* in California showed no significant changes over several generations, except in those that were culled (Sacks et al. 2016). Also similar to the round goby, zebra and quagga mussel populations exhibited pronounced divergences across their exotic North American ranges, with some populations changing significantly over their temporal courses yet remaining distinctive (Brown and Stepien 2010; Stepien et al. 2013).

The southern bull kelp *Durvillaea antarctica* has high dispersal ability yet displayed pronounced genetic differences among even closely located populations in its native Chile implying that a density dependent process, "founder takes all", circumvented new arrivals from establishing (Fraser et al. 2010; Waters et al. 2013); this process appears to have characterized both the round goby (this study and Brown and Stepien 2009) and most of the dreissenid mussel invasion (Brown and Stepien 2010; Stepien et al. 2013) across North America.

The "leading edge" hypothesis predicts that populations at an introduction's periphery would have lower genetic variability due to founder effects and then diverge as a result of drift (Hewitt 1996; Slatkin and Excoffier 2012). This outcome has been supported for many temperate populations that originated from post-glacial refugia expansions (Hewitt 1996; Sepulveda-Villet and Stepien 2012). Similarly, genomic analysis of the bank vole *Myodes glareolus* showed decreasing genetic diversity along transects stretching ~200 km from the invasion core to the periphery in Ireland (White et al. 2013). Secondary jump dispersals frequently display lower diversity at their "leading edges", including the European green crab's transport from the North American Atlantic to Pacific coasts (Tepolt and Palumbi 2015) and trans-locations of dreissenids from the eastern Great Lakes to western North American reservoirs via overland boat trailers (Brown and Stepien 2010; Stepien et al. 2013).

The "leading edge" hypothesis might have explained some secondary expansion patterns of the round goby, if its populations retained lower diversity over their invasional time course. The Lake Michigan population at the time of our earliest sample (1999) was very small, had low genetic diversity, and contained two full sibling groups. Its secondary founder effect then dissipated with arrival of new colonizing individuals (and their alleles), supporting some "genetic supplementation" and contradicting the "leading edge" hypothesis. When the first Lake Michigan sample was removed from the analysis relating

genetic divergence to time between samplings, the regression slope for θ_{ST} became statistically similar to zero. Thus, this population has remained most like itself over time in allelic composition, supporting "founder takes most" and "genetic stasis" after initial establishment, along with some "genetic supplementation" associated with great population size increase. The population displayed some slight convergence towards the invasion core over time yet has stayed distinctive. Its overall population genetic composition, as is the case for the other round goby populations we assayed, has retained consistency. The offspring of its established genotypes have been prolific over generations, continuing to monopolize habitats and resources. "Genetic supplementation" also occurred to a lesser degree early in the Central Lake Erie expansion population, followed by "genetic stasis".

Similar to the round goby, western corn rootworm populations showed some convergence over time with divergence values being slightly lower later in the invasion than earlier yet retaining their significant genetic differences (Lemic et al. 2013). The western flower thrips insect *Frankliniella occidentalis*, which is native to North America, underwent genetic bottlenecks in seven of 14 exotic populations in China (Yang et al. 2012), some of which were genetically different, but did not vary temporally from 2009–2012 (Yang et al. 2015). This pattern of high genetic divergence and temporal stability is congruent with overall patterns of the round goby invasion.

Peripheral expansion round goby populations (Lake Michigan and Central Lake Erie) exhibited more fluctuations than the invasion core. Small population sizes in the early expansion samplings, as well as smaller sample sizes, may have influenced these results. However, samples were taken from multiple trawls at these locations, and the extra effort required to obtain relatively few individuals indicates that these likely reflected the

population status at that time. Over time, these populations tended to become more like the invasion core, yet retained significant respective divergences.

Moreover, the Central Lake Erie population may have been supplemented by additional propagules from overseas, as suggested by Brown and Stepien's (2008; 2009) analyses across Eurasia and North America and by our cyt *b* results. Notably, the latest Central Lake Erie sample possessed a wider variety of mtDNA haplotypes than any other exotic population. These haplotypes were all singletons, which are not known to occur in any other round goby population; they either may indicate supplementation by new propagules from overseas or those previously un-sampled simply due to stochastic sampling error. At present, it cannot be discerned whether the three singletons in the Central Lake Erie 2013 sample originated directly from the Dnieper River or elsewhere in the round goby's native/exotic range. The same is true for the three other private singletons sampled from the founding source Dnieper River population in 2013. That site is a major commercial shipping port whose genetic composition may have varied due to the influence of continued shipping (and propagule arrivals from other port areas) or may reflect sampling variation, as the earlier sample sizes were smaller (N=25 in 2002 and 2007, and 53 in 2013).

2.6 Conclusions

Temporal variations among round goby population samples stemmed from slight gains or losses of low-frequency alleles/haplotypes, reflecting some small "genetic supplementation" at the early expansion edges and/or sampling error. Most of the invasion expansion sites showed just slightly more allelic gains than losses over time when sample sizes were small in the earliest samples, providing evidence against large-scale "genetic

supplement". Overall findings support "genetic stasis" and "founder takes most" after initial establishment and population growth, countering the alternative "genetic replacement". Genetic replacement is the sole scenario that is completely rejected by our results. Since there were no appreciable losses coupled with gains of other alleles, genetic compositions stayed consistent except during early establishment and growth at the expansion population sites. These factors have maintained genetic diversity and population structure patterns across this very successful introduction. Congruent results would be predicted for other introduced species when the invasion is large, the population has high reproductive success, and later arriving genotypes do not have a competitive advantage over those that initially established.

The present study is the first known to the authors that analyzes population genetic patterns across the broad geographic and relatively complete temporal range of a highly successful introduction. We predict that the round goby's high genetic diversity and divergence patterns will be retained in the future. Some population genetic convergence may continue towards that of the invasion core, but pronounced genetic structure likely will continue to characterize populations across its overall range. Individuals are predicted to continue to disperse into connected waterways or be transported to other locales via ballast water or bait buckets, establishing new populations and continuing this genetic pattern. The round goby's broad temperature range and wide salinity tolerance (Cross and Rawding 2009; Karsiotis et al. 2012) likely will aid its establishment in other freshwater and estuarine systems, possibly stretching to the North American Atlantic, Pacific, and Gulf of Mexico coasts, especially if its native dreissenid mussel prey has already invaded those habitats.

2.7 Acknowledgements

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Table 2.1 Genetic diversity of round goby populations and samples from microsatellite data. Sampling site locations and collection years, with population genetic results (calculated in ARLEQUIN and GENEPOP): estimated number of generations since the population was discovered (Gen), number of individuals analyzed (N), allelic richness ± standard error (A_R), observed heterozygosity ± standard error (H_O), total number of alleles (N_A), and proportion of alleles that appear private (P_{PA}) for 13 microsatellite loci. In bold are summaries for locations (all sampling years combined).

Site (Map Label) and Discovery Year	Sample Year(s)	Gen	N	A_R	H_O	N_A	P_{PA}
L. Michigan (LM)	**All**	---	**163**	**8.3±1.3**	**0.59±0.06**	**118**	**0.04**
1997	1999	1.0	19	2.9±0.3	0.49±0.07	46	0.00
	2007	5.0	50	4.3±0.5	0.56±0.06	96	0.02
	2011	7.0	44	4.3±0.4	0.63±0.06	89	0.01
	2013	8.0	50	4.1±0.5	0.62±0.06	83	0.01
St. Clair R. (SR)	**All**	---	**179**	**10.0±1.5**	**0.60±0.05**	**143**	**0.03**
1990	1993	1.5	45	4.6±0.5	0.60±0.05	114	0.02
	2007	8.5	50	4.4±0.4	0.61±0.05	106	0.03
	2011	10.5	34	4.5±0.5	0.66±0.05	98	0.00
	2013	11.5	50	4.3±0.4	0.56±0.05	97	0.00
L. St. Clair (LC)	**All**	---	**171**	**9.7±1.5**	**0.60±0.05**	**137**	**0.05**
1994	1998	2.0	39	4.5±0.4	0.59±0.05	97	0.00
	2007	6.5	50	4.4±0.4	0.56±0.05	108	0.01
	2011	8.5	32	4.6±0.5	0.63±0.05	100	0.02
	2013	9.5	50	4.6±0.4	0.63±0.06	109	0.03
L. Erie Islands (EI)	**All**	---	**285**	**9.1±1.4**	**0.59±0.05**	**142**	**0.06**
1993	1998	2.5	51	4.2±0.5	0.53±0.06	94	0.02
	2002	4.5	50	4.3±0.4	0.58±0.05	96	0.00
	2005	6.0	49	4.3±0.4	0.60±0.04	105	0.03
	2007	7.0	40	4.4±0.4	0.63±0.05	96	0.01
	2011	9.0	45	4.3±0.4	0.61±0.05	99	0.02
	2013	10.0	50	4.3±0.5	0.62±0.05	100	0.01
Central L. Erie (CE)	**All**	---	**201**	**8.5±1.1**	**0.59±0.04**	**120**	**0.03**
1993	1998	2.5	24	4.0±0.4	0.45±0.04	71	0.03
	2002	4.5	29	3.8±0.4	0.58±0.06	79	0.00
	2007	7.0	50	4.1±0.4	0.64±0.05	91	0.01
	2011	9.0	48	4.0±0.4	0.60±0.05	90	0.00
	2013	10.0	50	4.0±0.4	0.58±0.04	91	0.01
Dnieper R. Black Sea (DR)	**All**	---	**102**	**14.5±2.4**	**0.62±0.04**	**190**	**0.25**
Native	2002	---	25	4.7±0.5	0.63±0.05	111	0.05
	2007	---	25	7.6±0.5	0.66±0.05	121	0.08
	2013	---	53	8.1±0.5	0.59±0.05	146	0.14

Table 2.2 Population comparisons. Pairwise comparisons between round goby population samples, based on A) mtDNA cytochrome *b* sequences and B) nuclear DNA microsatellites. Divergences among population samples using exact tests (from GENEPOP; above diagonal) and θ_{ST} – calculated using a) ARLEQUIN or b) FSTAT; below diagonal. *=p<0.05 but not significant following Bonferroni correction, **=remained significant (p<α) after sequential Bonferroni correction, NS=p>0.05. c) Genetic assignment test results of round goby individuals to populations using GENECLASS2 based on nuclear DNA microsatellite data. Proportion of individuals assigned to each population. Population self-assignments are underlined. * indicates assignment proportions greater than 0.25.

(A)	LM	SR	LC	EI	CE	DR
L. Michigan (LM)	~	NS	NS	**	NS	NS
St. Clair R. (SR)	0.003	~	NS	**	NS	NS
L. St. Clair (LC)	0.015	0.015	~	**	*	*
L. Erie Islands (EI)	0.040**	0.061**	0.020**	~	**	**
Central L. Erie (CE)	-0.002	-0.003	0.006*	0.044**	~	NS
Dnieper R. (DR)	0.009	-0.005	0.007	0.052**	-0.002	~
Mean	**0.013**	**0.014**	**0.013**	**0.043**	**0.009**	**0.012**

(B)	LM	SR	LC	EI	CE	DR
L. Michigan (LM)	~	**	**	**	**	**
St. Clair R. (SR)	0.042**	~	**	**	**	**
L. St. Clair (LC)	0.047**	0.004**	~	**	**	**
L. Erie Islands (EI)	0.055**	0.014**	0.007**	~	**	**
Central L. Erie (CE)	0.065**	0.020**	0.016**	0.009**	~	**
Dnieper R. (DR)	0.067**	0.025**	0.026**	0.037**	0.047**	~
Mean	**0.055**	**0.021**	**0.020**	**0.024**	**0.031**	**0.040**

(C)	Assigned to						
Location of sample	**LM**	**SR**	**LC**	**EI**	**CE**	**DR**	***N***
L. Michigan (LM)	0.72*	0.06	0.06	-	0.01	0.15	163
St. Clair R. (SR)	-	0.36*	0.31*	0.02	0.02	0.29*	179
L. St. Clair (LC)	-	0.08	0.60*	0.03	0.01	0.27*	171
L. Erie Islands (EI)	0.01	0.09	0.34*	0.32*	0.06	0.19	285
Central L. Erie (CE)	-	0.06	0.28*	0.07	0.42*	0.15	201
Dnieper R. (DR)	-	-	-	-	-	1.00*	102

Table 2.3 Pairwise sample divergence values for nuclear DNA microsatellite data. Pairwise comparisons between round goby temporal samples based on microsatellite data. Exact tests (calculated in GENEPOP; above diagonal) and θ_{ST} (FSTAT; below diagonal). *=p<0.05 but not significant following Bonferroni correction, **=remained significant (p<α) after sequential Bonferroni correction, NS=p>0.05.

	L. Michigan (LM)				St. Clair R. (SR)				L. St. Clair (LC)			
	1999	**2007**	**2011**	**2013**	**1993**	**2007**	**2011**	**2013**	**1998**	**2007**	**2011**	**2013**
LM1999	~	**	**	**	**	**	**	**	**	**	**	**
LM2007	0.081**	~	**	**	**	**	**	**	**	**	**	**
LM2011	0.077**	0.008*	~	**	**	**	**	**	**	**	**	**
LM2013	0.104**	0.023**	0.018**	~	**	**	**	**	**	**	**	**
SR1993	0.140**	0.045**	0.055**	0.065**	~	**	*	**	*	NS	*	**
SR2007	0.148**	0.046**	0.056**	0.054**	0.008*	~	**	**	**	NS	**	**
SR2011	0.141**	0.047**	0.048**	0.046**	0.028**	0.017**	~	**	**	**	NS	NS
SR2013	0.147**	0.040**	0.043**	0.037**	0.020**	0.007*	0.011*	~	**	**	**	**
LC1998	0.163**	0.052**	0.063**	0.072**	0.006*	0.012**	0.041**	0.030**	~	**	**	**
LC2007	0.156**	0.051**	0.064**	0.070**	0.005	0.005	0.025**	0.019**	0.007*	~	*	**
LC2011	0.147**	0.040**	0.040**	0.051**	0.014**	0.015**	0.006	0.015**	0.018**	0.008*	~	**
LC2013	0.153**	0.046**	0.044**	0.042**	0.030**	0.018**	0.004	0.008*	0.035**	0.022**	0.004	~
EI1998	0.184**	0.073**	0.088**	0.092**	0.027**	0.025**	0.052**	0.045**	0.020**	0.017**	0.033**	0.045**
EI2002	0.177**	0.059**	0.064**	0.071**	0.020**	0.017**	0.028**	0.030**	0.015**	0.010*	0.010*	0.019**
EI2005	0.161**	0.052**	0.059**	0.075**	0.013**	0.018**	0.034**	0.033**	0.016**	0.012**	0.011**	0.030**
EI2007	0.167**	0.051**	0.054**	0.063**	0.017**	0.019**	0.042**	0.033**	0.008*	0.014**	0.019**	0.032**
EI2011	0.152**	0.047**	0.040**	0.047**	0.036**	0.026**	0.012**	0.021**	0.046**	0.030**	0.005	0.007*
EI2013	0.168**	0.048**	0.050**	0.051**	0.020**	0.021**	0.023**	0.018**	0.020**	0.015**	0.007*	0.010**
CE1998	0.201**	0.058**	0.067**	0.070**	0.037*	0.032	0.055**	0.040*	0.030	0.042*	0.039	0.049**
CE2002	0.221**	0.093**	0.103**	0.101**	0.049**	0.037**	0.066**	0.060**	0.040**	0.045**	0.056**	0.062**
CE2007	0.177**	0.059**	0.069**	0.072**	0.021**	0.019**	0.043**	0.036**	0.015**	0.018**	0.025**	0.040**
CE2011	0.174**	0.063**	0.072**	0.067**	0.034**	0.025**	0.025**	0.031**	0.036**	0.027**	0.024**	0.025**
CE2013	0.180**	0.069**	0.067**	0.071**	0.040**	0.030**	0.016**	0.031**	0.041**	0.028**	0.011*	0.013**
DR2002	0.209**	0.091**	0.090**	0.094**	0.063**	0.072**	0.063**	0.061**	0.074**	0.075**	0.056**	0.055**
DR2007	0.170**	0.060**	0.065**	0.064**	0.027**	0.033**	0.049**	0.036**	0.030**	0.039**	0.033**	0.040**
DR2013	0.182**	0.064**	0.077**	0.085**	0.020**	0.026**	0.056**	0.032**	0.025**	0.029**	0.040**	0.046**

Table 2.3 continued

	L. Erie Islands (EI)						Central L. Erie (CE)					Dnieper R (DR)		
	1998	2002	2005	2007	2011	2013	1998	2002	2007	2011	2013	2002	2007	2013
LM1999	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LM2007	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LM2011	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LM2013	**	**	**	**	**	**	**	**	**	**	**	**	**	**
SR1993	**	**	**	**	**	**	**	**	**	**	**	**	**	**
SR2007	**	**	**	**	**	**	NS	**	**	**	**	**	**	**
SR2011	**	**	**	**	*	**	**	**	**	**	**	**	**	**
SR2013	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LC1998	**	**	**	**	**	**	NS	**	**	**	**	**	**	**
LC2007	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LC2011	**	**	**	**	NS	**	**	**	**	**	**	**	**	**
LC2013	**	**	**	**	*	**	**	**	**	**	**	**	**	**
EI1998	~	**	**	**	**	**	NS	**	**	**	**	**	**	**
EI2002	0.009*	~	**	**	**	**	NS	**	**	**	**	**	**	**
EI2005	0.008*	0.006*	~	**	**	**	NS	**	**	**	**	**	**	**
EI2007	0.008*	0.006*	0.008*	~	**	**	NS	**	**	**	**	**	**	**
EI2011	0.047**	0.015**	0.018**	0.030**	~	**	**	**	**	**	**	**	**	**
EI2013	0.023**	0.006*	0.017**	0.011**	0.017**	~	**	**	**	**	**	**	**	**
CE1998	0.031	0.028	0.032	0.027	0.045**	0.035	~	**	**	**	**	**	**	**
CE2002	0.022**	0.030**	0.035**	0.022**	0.063**	0.048**	0.026	~	**	**	**	**	**	**
CE2007	0.013**	0.010**	0.016**	0.008*	0.039**	0.024**	0.019	0.006	~	**	**	**	**	**
CE2011	0.016**	0.014**	0.021**	0.023**	0.026**	0.023**	0.021	0.014*	0.013**	~	**	**	**	**
CE2013	0.034**	0.009*	0.022**	0.030**	0.007*	0.020**	0.050**	0.050**	0.029**	0.018**	~	**	**	**
DR2002	0.105**	0.075**	0.084**	0.078**	0.069**	0.063**	0.098**	0.115**	0.082**	0.090**	0.076**	~	**	**
DR2007	0.056**	0.045**	0.044**	0.033**	0.053**	0.037**	0.049**	0.070**	0.042**	0.057**	0.060**	0.020*	~	**
DR2013	0.050**	0.041**	0.047**	0.038**	0.065**	0.035**	0.058**	0.079**	0.046**	0.063**	0.064**	0.055**	0.025**	~

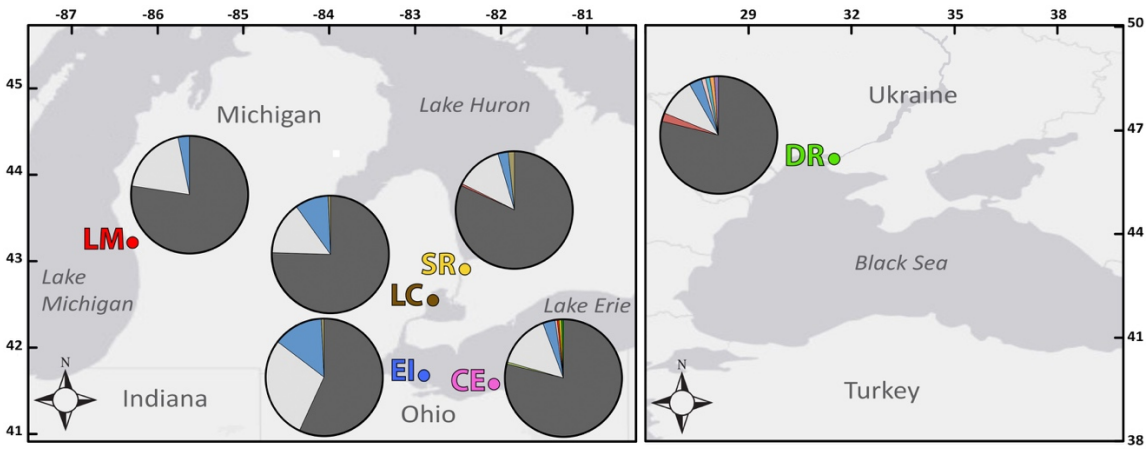

Figure 2–1 Map of locations sampled for North America (left) and Europe (right), showing mtDNA cytochrome *b* haplotype representation (See Table 2.1 for map label abbreviations).

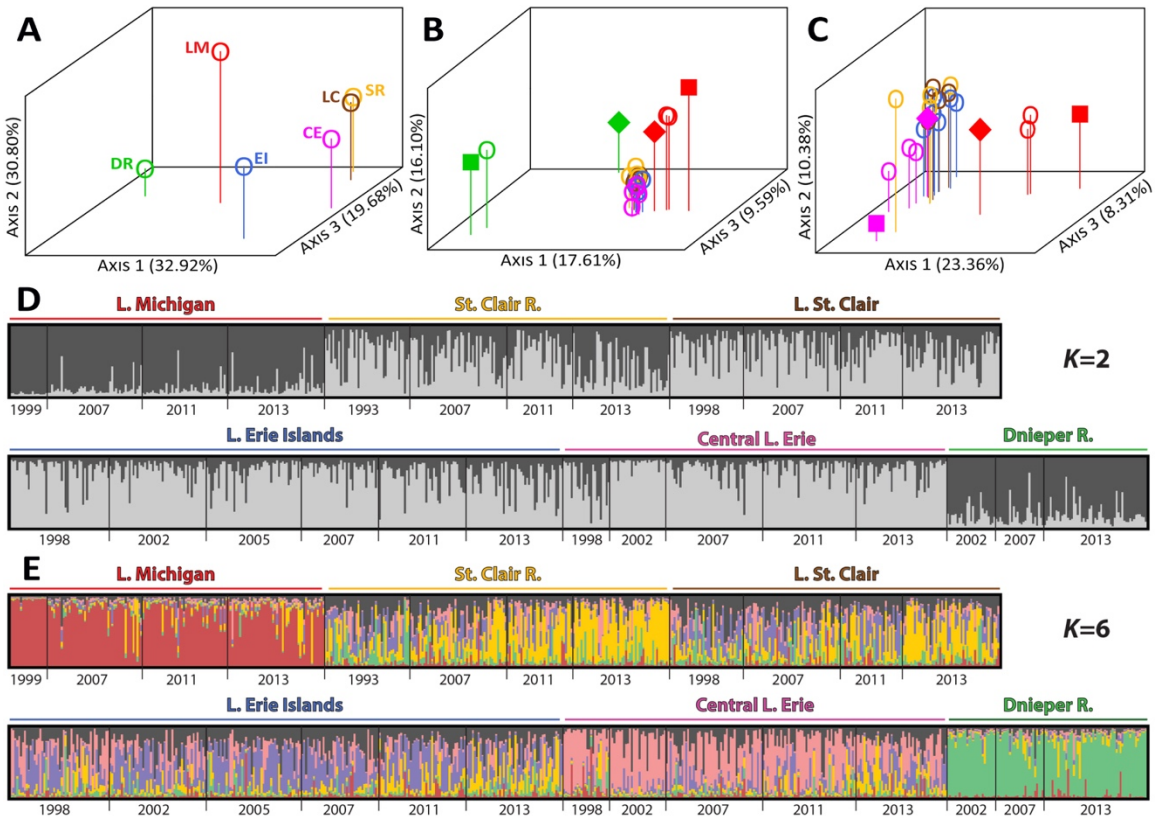

Figure 2–2 Population structure. A–C) 3-Dimensional Factorial Correspondence Analyses (GENETIX) based on nuclear microsatellite data for A) sampling locations (all years combined), B) temporal samples (years) per population, and C) without the Dnieper River samples (for improved visualization of the invasive populations; See Figure 2–1 for location color key) Squares indicate earliest temporal sample and diamonds the most recent samples for those locations with appreciable change over time. Analyses explained A) 83.4%, B) 43.3%, and C) 42.1% of the variation. D & E) Bayesian STRUCTURE analysis results for population groups. STRUCTURE HARVESTER indicated greatest support for D) K=2 (ΔK=28.21) and E) K=6 (ΔK=16.36) round goby population groups, from nuclear microsatellite data.

Chapter 3

Increasing confidence in species compositions discerned with multiple environmental DNA metabarcode high-throughput sequencing assays: community profiling of Great Lakes fishes

3.1 Abstract

Community composition data are valuable for conservation management, including identifying rare native and invasive species, along with abundant ones. Morphological surveys require considerable taxonomic expertise, are time consuming and expensive, can kill rare taxa and damage habitats, and often are prone to false negatives. Environmental DNA (eDNA) metabarcode high-throughput sequencing (HTS) assays facilitate detection of community diversity and are more sensitive, less damaging, and relatively time and cost-efficient. However, a trade off exists between stringency of bioinformatic filtering to remove false positives and the potential for false negatives. The present study design thus employs four mitochondrial (mt) DNA assays and a bioinformatic pipeline to increase confidence in species identifications by removing false positives from several potential sources. Positive controls calculate sequencing error and species hits are removed that fall below this cutoff in single unless they occurred in multiple assays. Mock communities determined a strong

relationship between observed and expected sequence reads and few false negatives. Tank experiments showed a high detection probability. Our approach identified 100% of the species in a larval fish sample (Detroit River, Lake St. Clair tributary, Laurentian Great Lakes), 100% from an eDNA water sample concomitant with electrofishing surveys in the Wabash River (Mississippi River drainage), and 88% in the Maumee River (Lake Erie tributary, Great Lakes). 73% of species were detected from just four 1L water samples in comparison to four months of electrofishing surveys in the Maumee River. Detected total fish diversity using the four assays was greater in the two rivers, identifying 35 additional species missed by electrofishing. Communities were better ecologically differentiated with the eDNA assays than with morphological sampling, which was further improved by combining results from the four different markers. HTS metabarcoding analyses for round goby and silver carp, all population haplotypes also discerned with Sanger sequencing, along with some others, meriting further investigation. Overall, the use of multiple assays and a custom bioinformatics to discern error from true positive detections can improve confidence in eDNA metabarcode HTS detection of environmental biodiversity.

3.2 Introduction

Assessment of species compositions and diversities from biological communities is fundamental for ecological and environmental comparisons (Elton 1966; Begon et al. 2006; Morin 2009), facilitating conservation efforts (Myers et al. 2000; Margules et al. 2002) and evaluation of anthropogenic impacts (Attrill and Depledge 1997). Identifying rare and/or endangered species is of particular interest to managers (Dobson et al. 1997; Margules et al. 2002). along with detection of non-native species (Allendorf and Lundquist 2003). However,

such surveys are costly to conduct, require extensive taxonomic expertise, and are prone to false negatives (Attrill and Depledge 1997; Balmford and Gaston 1999; Darling and Mahon 2011).

Environmental DNA (eDNA) methods involve sampling and analysis of genetic material from water, sediment, gut contents, etc., to identify the presence of single or multiple taxa (Ficetola et al. 2008; Rees et al. 2014). Extraction of DNA from an environmental sample can be followed by multiple approaches, including determination of taxon presence/absence with traditional polymerase chain reactions (PCR), quantitative (qPCR), or digital droplet (ddPCR) versus simultaneous analysis of multiple samples and multiple taxa using metabarcoding and high-throughput sequencing (HTS) assays (Shokralla et al. 2012; Rees et al. 2014). With metabarcode/HTS assays, PCR uses primers that often target multiple taxa and the resultant libraries are sequenced on a HTS platform such as Illumina MiSeq®, generating millions of sequence reads, which then are compared to a reference database to determine the likely species of origin (Shokralla et al. 2012; Deiner et al. 2017). In addition, multiple samples can be "tagged" and distinguished bioinformatically (Klymus et al. 2017).

Metabarcoding HTS eDNA assays have been used to evaluate community compositions and shown to be as effective or complementary (see Deiner et al. 2017) and more cost efficient than traditional sampling surveys (Smart et al. 2016). PCR inhibition is a challenge in some environmental samples, leading to amplification failure or false negatives (Civade et al. 2016; Fujii et al. 2019). Some studies that have compared biomass of taxa to the relative proportions of sequence reads returned from metabarcode HTS assays have found positive correlations (Hänfling et al. 2016; Thomsen et al. 2016a; Marshall and Stepien 2019a), while others have not (Shaw et al. 2016; Gillet et al. 2018). Primer biases for or

against certain taxa can affect these relationships and/or species detections (Xiong et al. 2016; Alberdi et al. 2017; Kelly et al. 2017). Some general markers have used less variable gene regions such as mitochondrial (mt) 12S RNA to ensure a better match between primers and possible target sequences, often to the detriment of taxonomic fidelity (limiting resolution to the species or genus level, or higher) (e.g., Miya et al. 2015; Valentini et al. 2016; Cilleros et al. 2019). eDNA with metabarcoding and HTS also have been used to evaluate population genetic information from mtDNA sequence haplotypes employing specifically designed, targeted markers (Sigsgaard et al. 2017; Parsons et al. 2018; Marshall and Stepien 2019a; Stepien et al. 2019).

Error from incorrect base calls and/or sequence to sample mis-assignments due to index-hopping (when the wrong index is incorporated into a HTS library) can result in false positives (Xiong et al. 2016; Deiner et al. 2017). Incorrect base calls can artificially inflate haplotypic diversity in population genetics (Tsuji et al. 2018). Sequencing error and index-hopping are especially problematic when using metabarcoding to discern invasive species, since the consequence of a false positive could lead to wasted effort and funds, including unnecessary response by management agencies to either verify the presence of or attempt to eradicate non-natives (Zaiko et al. 2018). More stringent bioinformatic filtering can remove some of this error but may negatively restrict detection capability, particularly of rare taxa. Better protocols are needed to alleviate issues of primer bias and error in eDNA assay data, while still identifying as many taxa as possible (Zinger et al. 2019).

3.2.1 Objectives

The objectives of the present research were to: (1) employ new eDNA metabarcode HTS assays designed by our lab, which use a robust reference database and relatively high

sequence variation in the mtDNA cytochrome (cyt) *b* gene to resolve species-level and some population-level genetic variation for freshwater fishes, including native and invasive species, (2) develop a library prep and associated bioinformatic pipeline to reduce possible sources of error and increase confidence in results with a multiple assay approach, (3) compare and combine results with a previously published mt 12S rDNA assay (MiFish; Miya et al. 2015) that has lower species-level resolution due to its slower evolutionary rate, and (4) evaluate the efficiency and accuracy of this method compared to traditional sampling and morphological identifications. These assays and the pipeline we developed were tested on: (A) laboratory mock communities (mixtures of varying concentrations of extracted DNA of known target taxa), (B) laboratory aquarium experiments, and environmental samples in comparison with traditional sampling and morphological species identifications, including: (C) eDNA water samples from two large rivers (the Wabash and Maumee), and *de novo* sampling and sequencing from Lakes Erie and St. Clair, and (D) a larval fish tow in reference to traditional sampling and morphological identification. Taxonomy and nomenclature presented follow Fishbase.org.

3.3 Methods

3.3.1 eDNA metabarcode HTS assays

New eDNA metabarcode HTS assays were designed, tested, and applied that targeted the mtDNA cyt *b* gene, to distinguish among fish species in the Great Lakes (native and introduced), along with predicted future invasive species. Cyt *b* sequences >1000bp were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) for all Great Lakes fishes (Hubbs and Lagler 2007), all invasive fish species listed in the USGS Nonindigenous Aquatic

Species database (USGS 2019b), and all predicted future invasive fish species from the NOAA Great Lakes Aquatic Nonindigenous Species Information System (NOAA 2019; Appendix B.1). Sequences for two native catostomids (*Erimyzon claviformis* and *Moxostoma lacerum*), three *Coregonus* spp. (*Coregonus hubbsi*, *C. nipigon*, and *C. reighardi*), two cyprinids (*Margariscus natchtriebi* and *Semotilus corporalis*), and troutperch *Percopsis omiscomaycus* were unavailable on GenBank (accessions listed as "No records" in Appendix B.1). The final dataset included >95% of native Great Lakes fishes and 100% of those that are current or predicted invaders. Three different cyt *b* assays were designed to either target all Great Lakes fishes (FishCyt*b*), cyprinid fishes (CarpCyt*b*; Stepien et al. 2019), or currently and potentially invasive gobies (GobyCyt*b*) (see Stepien and Neilson 2013). Conserved sequence regions adjacent to variable ones <250bp, which differentiated among all target species and encompassed as much intraspecific variation as possible, were identified by visually inspecting alignments of consensus sequences for fish families (FishCyt*b* assay) or species (CarpCyt*b* and GobyCyt*b* assays). Primers were constructed with degenerate bases that incorporated >75% of the variation in the target taxa (Table 3.1, Appendix B–1).

The marker sets FishCyt*b* amplified 154 nucleotides (NT) of the cyt *b* gene, beginning at NT 855, CarpCyt*b* 136 NT beginning at NT 114, and GobyCyt*b* 167 NT from NT 42 (Table 3.1). A published assay targeting the mt 12S RNA gene (MiFish; Miya et al. 2015) was used for comparison. All primer sets included the Illumina sequencing adapters and four unique spacer inserts, e–h, at the 5' end (Table 3.1; Klymus et al. 2017). Spacer inserts varied from 7–14NT long, offsetting sequences to increase library diversity, thereby improving the quality of HTS data on the Illumina platform (Fadrosh et al. 2014; Wu et al. 2015).

3.3.2 Experiment Series A. In vitro tests: Mock Communities

To test the sensitivity of our cyt *b* metabarcode assays and associated bioinformatic pipeline (see below), mock communities were prepared that each contained different concentrations of genomic DNA from each target taxon (Suppl. material: Tables 2A–B). Taxa (species or haplotypes) compositions were selected based on the target assay. Ten fish species (two native and eight invasive) from five families were used for the FishCyt*b* assay. Six invasive cyprinids (including three haplotypes of silver carp *Hypophthalmichthys molitrix*), invasive round goby *Neogobius melanostomus*, and the native walleye *Sander vitreus* were used for the CarpCyt*b* assay. Seven invasive gobiid species (including three haplotypes of the round goby) and walleye were used for the GobyCyt*b* assay.

To avoid contamination of haplotypes from other specimens, sterile single use razor blades were used to remove internal lateral muscle tissue from sacrificed fish. DNA was extracted using Qiagen DNeasy kits (Hilden, Germany, EU). Genomic DNA was serially diluted 1:4, until the concentration of the extraction was <0.1ng μl^{-1}. The five least concentrated dilutions from each taxon then were mixed into mock communities in a factorial design (Appendix B.2A). Concentrations of DNA from individual taxa in MCs varied from <0.01 to ~20ng μl^{-1}. These mock communities then were processed with the appropriate assay to determine limits of detection and evaluate the relative abundance of input DNA concentration to sequence reads returned from Illumina MiSeq.

3.3.3 Experiment Series B. In situ tests: Laboratory aquaria

To further investigate sensitivity of the assays to detect species, several laboratory aquarium experiments were conducted, with the first (experiment B1) containing invasive round and freshwater tubenose *Proterorhinus semilunaris* gobies, collected from the Harley

Ensign Memorial Boat Launch, Harrison Charter Township, MI (LSC in Figure 3–1). Fish were transported to the laboratory and maintained for seven days before the experiment began.

Three 40L aquaria were decontaminated with 10% bleach, thoroughly rinsed with deionized water, and filled with 30L of dechlorinated tap water. Bleach sterilized PVC tubes (to provide habitat) and bubblers were added, and aquarium water was thoroughly mixed and one liter of water was sampled in a sterile container from the surface, before the fish were added (aquarium B1, 0 hr control samples). DNA extraction and PCR amplification then were attempted (see below) on these control samples. The three aquaria were kept covered after populating them with varying proportions of round and tubenose gobies, at relative species densities similar to those found in the Great Lakes (Kornis et al. 2012). The three aquaria respectively contained 17 round gobies, 18 round gobies and one tubenose goby, and 15 round gobies and 4 tubenose gobies, which were measured to total length (mm) and weighed (g). Water was sampled 24, 48, and 72 hrs after the addition of fishes, from which eDNA was extracted immediately (see below).

After the experiment, fishes were anesthetized and sacrificed under approved IACUC protocol #205400 (see below). DNA was extracted from muscle tissue and the complete cyt *b* gene for each individual was amplified and Sanger sequenced following Snyder and Stepien (2017). The aquarium water samples then were processed with the GobyCyt*b* assay (see Results).

Experiments B2 and 3: Two additional aquaria containing higher fish species diversities were sampled and processed using the four assays. One ~2,200 L Great Lakes fishes display aquarium at the University of Toledo's Lake Erie Center, Oregon, OH (hereafter; display aquarium B2), contained six fish species belonging to five families

(Appendix B.3A). Species in that aquarium were weighed (g) and measured (mm TL) after duplicate water samples were collected at the surface, following mixing the water. Another ~72,500 L aquarium located at a commercial outdoor recreation outfitter in Rossford, OH (hereafter; Aquarium B3) contained 14 Great Lakes fish species belonging to eight families (Appendix B.3B). Water samples from both aquaria were taken as described above and processed with all four assays (see below).

3.3.4 Experiment Series C: Field surveys of eDNA water samples using our assays versus conventional sampling and morphological identifications

To assess the comparability among these assays and to results from traditional morphological sampling, several eDNA water samples were collected concomitant with conventional surveys (Figure 3–1, Appendix B.4). These included: (Experiment C1) Duplicate eDNA water samples collected before and after two electrofishing transects conducted by us in the Wabash River, IN, where bighead and silver carps are prevalent as invasive species, on September 2, 2016 (sites WAB 1 and 2). (Experiment C2) The Ohio EPA conducted 44 electrofishing surveys at 22 sites in the Maumee River, OH (a western Lake Erie tributary) in summer (June–September) 2012, from which all fish were identified to species, counted, and weighed (g) by them (MAU1–MAU4; OEPA 2014, 2015), from which concomitantly collected water samples were collected for us from the surface and just four of them then were analyzed here (MAU1–4). (Experiment C3) We sampled and tested eDNA water from Lake St. Clair sampled by us at the Harley Ensign Memorial Boat Launch, Harrison Charter Township, MI (LSC 1; June 5, 2017) and (C4) at the Franz Theodore Stone Laboratory, Put-In-Bay, Gibraltar Island, Lake Erie, OH (LEI; July 29, 2017) (Figure 3–1, Appendix B.4). Experiments C3 and C4 were *de novo* sequencing of environmental DNA

water samples without accompanying morphological survey data, designed to further test the ability of our eDNA metabarcode assays to differentiate among the fish communities from various habitats.

For C3, to investigate the applicability of the assays to population genetics of the invasive round goby, eDNA water samples were collected at the surface (LSC 2) and 10 cm above the benthos (LSC 3), immediately prior to seining 60 round gobies on November 16, 2016 (Figure 3–1, Appendix B.4; collected under a Michigan collection permit), from which complete cyt *b* sequences were obtained following Snyder and Stepien (2017). For C1, cyt *b* sequence haplotypes and population genetic diversity of the silver carp previously were assessed from 37 individuals collected from a Wabash River site (WAB 3; Stepien et al. 2019), which are further analyzed here using eDNA water samples collected at the surface, as above, on September 2, 2016.

All four eDNA assays were used to analyze the eDNA extracted from all environmental samples. The CarpCyt*b* and GobyCyt*b* assays were respectively designed to target and distinguish among silver carp or round goby haplotypes, whereas the FishCyt*b* and MiFish assays distinguished to species (or, in the case of MiFish, genus or family due to lack of resolution power). Traditional population genetics sampling results were compared to those from eDNA water samples using the CarpCyt*b* and GobyCyt*b* assays (see Data analysis below).

All eDNA water samples were collected in bleach-sterilized 1L bottles, stored on ice in brief transit from the field, and then frozen at -80°C. Field locations were mapped using STEPMAP (stepmap.com), which holds no copyright on data or layers presented (Figure 3–1).

3.3.5 Experiment Series D: Metabarcoding HTS assays versus traditional ichthyoplankton analysis

To test the ability of our metabarcode HTS approach for discerning ichthyoplankton species, a bongo net tow of larval fishes from the Detroit River was collected and morphologically identified by USGS scientists in May 2013 (DRL) and stored in EtOH, from which we extracted the DNA, and analyzed it here *en masse* using our assays (Figure 3–1, Appendix B.4).

3.3.6 Ethics statement

All native fishes except those used in the mock communities were released *in situ* and invasive fishes were anesthetized and sacrificed under the approved University of Toledo IACUC #205400, "Genetic studies for fishery management" to CAS using an overdose of 250mg/L tricaine methane sulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA). All fishes were collected by our lab under Ohio Department of Natural Resources (ODNR) permit #17-159 or Michigan Department of Natural Resources permits or by collaborators with their permits (see Acknowledgements).

3.3.7 DNA capture, extraction, and library prep

Environmental samples from turbid habitats, such as the Maumee or Wabash rivers or western Lake Erie, can be difficult to filter (Williams et al. 2017), and thus our eDNA water samples were concentrated via centrifugation. Centrifugation, DNA extraction, and sequence library preparation (prep) followed Stepien et al. (2019). DNA from the Detroit River larvae samples previously was extracted from homogenized tissues following a modified CTAB protocol in our laboratory (Turner et al. 2015).

We employed a two-step library prep protocol. First step PCR reactions were 25 or

50µl (with the latter used for inhibited samples, assessed according to the presence of primer dimer, see below) containing 1X Qiagen AllTaq PCR buffer, 0.3mM dNTPs, 0.5µM of each primer, an additional 1.5mM MgCl2, 5U AllTaq, 1–8µl template DNA, and ddH20 to total 50µl. PCR conditions were: 2min initial denaturation at 95°C, followed by 40 cycles of 95°C for 5sec, primer specific annealing temp for 15sec (Table 3.1), and 72°C for 10sec. Reactions were assessed by gel electrophoresis using 1% agarose gels stained with GelRed (Biotium, Fremont, CA, USA). Successful reactions were cleaned with MagBio HighPrep™ beads (MagBio Genomics, Gaithersburg, MD, USA) at a 0.7X ratio.

Amplifications were attempted on negative (reagents only) extractions (three times per marker), negative centrifugations (sterile ddH_2O; three times per marker), aquarium B1 0hr controls (before the addition of fishes; three times per marker), as well as on no-template PCR controls (one for every reaction). Reactions that showed no amplification in their associated no-template PCR controls from the first step were indexed for HTS. To discern any potential contamination during the clean-up step, a "clean-up negative control" was conducted using the clean-up reagents from each set of reactions, on which indexing (see below) was attempted.

Libraries were indexed using 2.5 µl of unique combinations of 5' and 3' Nextera 96 indices (Illumina®, San Diego, CA, USA), using the lowest numbers of shared forward and reverse indices per marker and spacer inserts, following the manufacturer's protocol for 12 thermalcycles. Indexed samples were visualized on 1% agarose gels stained with GelRed. Successful reactions were bead cleaned (see above) at 1X ratio, sized, and quantified on an Agilent Bioanalyzer (Santa Clara, CA, USA), and pooled in equimolar concentrations. Pools then were bead cleaned at 0.8X ratio to remove any primer dimer. Pippen Prep (Sage Science, Beverly, MA) removed any amplicons >600bp, which might be undesirable PCR

multimers potentially affecting run quality. Resulting libraries were sequenced in five separate Illumina MiSeq runs by Ohio State Wooster's Molecular and Cellular Imaging Center (https://mcic.osu.edu/home), targeting a 40% PhiX spike in.

To quantify and correct for potential errors, which might include incorrect base calls, undetectable index-hops (see below), and/or cross contamination of samples, positive controls were amplified for each marker on each run (see Deiner et al. 2017). To avoid potential contamination or index-hopping (see below) from positive controls, these samples were constructed by mixing equal mass (ng) of DNA extractions from 10 marine species (species that cannot live in freshwater environments). Each individual extraction was sequenced for the region of cyt *b* that contained our eDNA assays. The species and accession numbers for associated sequences are listed in Appendix B.5. FASTQ files for all samples sequenced will be deposited in the NCBI Sequence Read Archive upon publication of this work in a peer reviewed scientific journal.

3.3.8 Bioinformatic pipeline

Primers were trimmed from raw reads using a custom PYTHON v3.7.1 script (made available on Dryad at time of publication), which allowed for sequencing errors in 30% of the primer nucleotides, which is a standard approach in metabarcode assays (see Martin 2011). Errors in positive controls tended to occur at the NT immediately following the forward or reverse primer, presumably due to PCR, and thus the first and last NTs were trimmed from the target region. The trimming script also removed any reads having the wrong spacer insert, which might result from index-hopping, which occurs when the wrong index is incorporated into an HTS library, leading to sequence-to-sample mis-assignment or cross-contamination (Xiong et al. 2016; MacConaill et al. 2018). Since we used four sets of

spacer primers per assay, most errors introduced by those mechanisms were identified and removed by this script, along with non-informative sequences <100 NT, resulting from primer dimer (Khodakov et al. 2016). Trimmed reads were merged in DADA2 (Callahan et al. 2016), which corrected potential sequence errors and removed chimeras using a de-noising algorithm. DADA2 was run using the default parameters, with "MaxE" set to "(3, 5)". Inputs were truncated at median Q score <30, for the first 10 samples/marker/run, using DADA2's plotQualityProfile function. De-noised sequences with 100% similarity in DADA 2 are termed amplicon sequence variants (ASVs).

Unique ASVs were subjected to the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the command line, to obtain the top 500 results/ASV from custom databases. The custom database for samples in the Great Lakes consisted of all cyt *b* or 12S (MiFish) sequences on GenBank for species that have established (native or invasive) or are predicted future invaders. Although the cyt *b* reference database was robust (see above), the 12S reference sequence database on GenBank was missing 53 (23%) of the known Great Lakes' species. These included native taxa, notably 80% of *Coregonus* spp., 47% of catastomids, 22% of cyprinids, 21% of percids, and several others. Also absent were several current (e.g., round and tubenose goby) and predicted possible Great Lakes' invaders, including steelcolor shiner *Cyprinella whipplei*, starry goby *Benthophilus stellatus*, and the Black Sea *Neogobius fluviatilis* and Caspian Sea *Neogobius pallasi* monkey gobies. Samples from outside the Great Lakes (i.e., the Wabash River) were subjected to BLAST searches against a database containing all available Actinopterygii fish cyt *b* or 12S sequences. A custom PERL v5.18.2 script summarized BLAST results, which removed sequence hits having <90% query cover or identity. All species hits/ASV passing

this filter and having the lowest expectation (e)value (best match) were combined into a list of potential taxa.

Sequence error was calculated as the greatest number of sequences belonging to an unexpected ASV in a positive control, divided by the total number of reads in the sample (hereafter, calculated error). Index-hopping is a potential source of error, which has been observed for ~0.1% of sequences on a MiSeq run (MacConaill et al. 2018). Thus, calculated errors below that value were rounded up to 0.1%.

In our multiple assay approach, species incidences were scored as valid when they were greater than the calculated error cutoff in a single assay or occurred in multiple assays (hereafter termed the MetaAssay approach). This approach compensated for potential primer set biases. We compared these results to use of 0.1% as the cutoff, as well as evaluated all positive hits (without frequency based filtering). Metabarcode sequence data for replicates from the same site were combined, by applying the bioinformatic filter for each separately and then combining ASVs and read counts afterwards. Species detections per assay and for the combined assays (MetaAssay approach) were compared to results discerned via the accompanying morphological sampling and analyses.

3.3.9 Data analysis

Mock communities assessed the ability of the metabarcode HTS assays to preserve concentration of the input genetic material in the output proportions of sequence reads (see Klymus et al. 2017; Marshall and Stepien, 2019). Taxon identifications (species or haplotype) and log % proportions of observed reads (response variable) in mock communities were compared to those expected based on input ng μl^{-1} of DNA (independent variable) from each species or haplotype. Linear models were constructed and Spearman rank correlation

coefficients were calculated for all mock communities and per species across libraries, and the results were plotted in R (R Core Team 2015).

Results from eDNA metabarcode HTS assays were compared with their concomitant electrofishing transects, as well as across all of 2012 OEPA fish survey results for the Maumee River. Species appearing unique to eDNA HTS assays and/or capture-based surveys were determined for individual samples and regions (i.e., Detroit River larvae and Wabash or Maumee River). Some morphological or eDNA assay identification results were restricted to the genus or family levels (see Results). False negatives were determined using a relaxed detection criterion, which considered a species present when identified at the genus level. Species richness values were determined for morphological and eDNA assays. These values were compared among all survey methods (individual eDNA HTS assays, the MetaAssay approach, and capture based surveys) and regions using *t*-tests in R, with significance adjusted with sequential Bonferroni correction (Rice 1989). Biomass proportions of species from morphological survey data were statistically compared to the proportions of sequence reads for single eDNA HTS assays using linear models and Spearman rank correlation coefficients in R. Analysis of Covariance (ANCOVA) was used to compare the slopes for the relationships among the different markers to evaluate concordance (Zar 2010).

To investigate the ability of metabarcode assays to differentiate among habitats, Non-metric Multi-dimensional Scaling (NMDS) with Bray and Curtis dissimilarity was calculated in VEGAN (Oksanen et al. 2019) for morphological sampling, individual eDNA HTS assays, and the MetaAssay approach. Differences between samples from the Wabash and Maumee Rivers (the two regions having multiple samples for all survey methods) were statistically tested using ANOVA, with the ADONIS2 function in VEGAN, and the assumption that groups of points do not significantly differ in their distance to the centroid

was tested using BETADISPER

FASTCLUSTER in R plotted dendrograms of the eDNA HTS assays and morphological samples using binary distance and the Ward's D2 agglomeration method (Müllner 2013), which further explored habitat comparisons. PVCLUST calculated approximate unbiased (AU) percent bootstrap support for each node in the dendrogram (10,000 replications), using the same distance and agglomeration method (Suzuki and Shimodaira 2015).

Numbers and proportions of population haplotypes were calculated from HTS reads and traditional Sanger sequencing of individuals. F_{ST} and exact tests of population differentiation were assessed in Arlequin (Excoffier and Lischer 2010) to compare traditional and eDNA methods for determining haplotypic frequencies.

3.4 Results

3.4.1 High-throughput sequencing metrics

No amplifications occurred in aquarium B1 for the 0 hr control samples or negative extractions, centrifugations, no-template PCR, or indexing controls. 27,961,011 reads were obtained for all libraries (mean/sample/assay±SE=229,189±18,645; Appendix B.6). A mean of 204,320±17,738 reads/sample/assay were successfully trimmed, with most others comprising short sequences from primer dimer. DADA2 merged an average 0.80±0.01 of trimmed reads, having a mean of 75.4±11.4 ASVs/sample/assay. Of those, a mean of 23±1.7 had BLAST hits to our fish databases for identity and query cover >90% (mea query cover=99.83±0.01%, mean identity=99.14±0.02%). 2,631 ASVs had BLAST hits that passed the % identity/query cover filter, with 2,342 (89%) identified to single species and 59, 103,

43, and 75 hits to the level of genus for the FishCyt*b*, CarpCyt*b*, GobyCyt*b*, and MiFish assays, respectively. These included 14 genera, which were primarily *Carassius* (13% of the overall genus level hits), *Carpiodes* (19%), and *Ictiobus* (39%), which were detected in samples for which either morphology and/or another one of our eDNA markers resolved a congener to species level. Nine 12S MiFish hits were resolvable only to the family level, of which six were to Cyprinidae, and three to Catostomidae; all were discarded. Not all samples led to successful libraries for every marker, presumably due to primer specific inhibition (see dashes in Table 3.2). For all positive control cases, the most abundant unexpected sequence was closely related to an expected sequence. Error frequencies calculated from the positive controls ranged from 0.18–0.42% (mean=0.27±0.02%).

3.4.2 Experiment Series A: Mock Communities

Observed and expected reads for mock communities or taxa (haplotypes or species) were significantly positively correlated (mean R^2=0.88, p<0.01 for all; Appendix B.2C and B–2), with their slopes significantly differing from zero (p<0.01 for all). Slopes for just three of the 15 mock communities (two for FishCyt*b* and one for GobyCyt*b*) significantly differed from 1.0 (range=0.61–1.33, p<0.05 for all three). Two of the 30 taxa had slopes that statistically differed from 1.0, including smallmouth bass *Micropterus dolomieu* in the FishCyt*b* mock communities (slope=0.60, p<0.05) and round goby haplotype 1 for the GobyCyt*b* mock communities (slope=1.28, p<0.05). Despite proportions of reads from several taxa in mock communities that did not maintain rank abundances with respect to input ng of genomic DNA (Appendix B–2), Pearson rank coefficients for all mock communities were statistically significant (mean ϱ=0.92±0.01, p<0.05 for all; Appendix B.2C).

A small number of false negatives occurred in the mock community experiments, all

for taxa that had the lowest concentrations of genomic DNA in that sample. Among the FishCyt*b* mock communities, six false negatives occurred in the 50 total taxa occurrences, and there were three false negatives each among the 50 for the CarpCyt*b* and GobyCyt*b* respective mock communities. Those sequences all had a very low number of reads (<20) in raw FASTQ files, which the DADA2 denoising algorithm classified as error and thus were excluded from the final results. 0–2 taxa per mock community (mean=0.93±0.18) occurred in the merged reads at frequencies lower than the calculated error cutoff. The mean number of this type of false negative decreased when 0.1% was used as the cutoff (0.73±0.15).

3.4.3 Experiment Series B: Display aquaria

False negatives in the display aquarium results ranged from 0 (MiFish and MetaAssay in display aquarium B2) to 50% (CarpCyt*b* and GobyCyt*b* in display aquarium B2) of the species present (Table 3.2A). All species in display aquarium B1 were discerned by one or more of the assays. Many species that were held live in other lab tanks of the facility housing display aquarium B1, which shared common tank cleaning equipment, also were identified with our assays, including emerald shiner *Notropis atherinoides*, goldfish *Carassius auratus*, and grass carp *Ctenopharyngodon idella* (Appendix B.6). Only one species was not detected by the assays, which was present in display aquarium B3 – the longnose gar *Lepisosteus osseus*. Several species of feeder fish, including emerald shiner and golden shiner *Notemigonus crysoleucas*, along with *Alosa* spp., which is commonly used in fish meal (Frimodt and Dore 1995; Miles and Chapman 2006) additionally were detected in display aquarium B3 (Appendix B.7). Staff confirmed using fish meal and feeder fish in that aquarium. There was an apparent misidentification by the staff who provided the morphological census for the B3 aquarium. Florida largemouth bass *Micropterus floridanus* was detected by our assays, as well as

largemouth *M. salmoides* and smallmouth bass *M. dolomieu*, with just the latter two being identified by the staff.

3.4.4 Experiment Series C & D: Metabarcode HTS assays versus morphological identifications

Several false negatives (taxa identified with morphological sampling but not with the eDNA approach) using single assays with the calculated error cutoff, were positive when 0.1% was used for filtering (*N*=13) or when all ASVs were accepted (*N*=48). However, with the MetaAssay approach, which detected more species than single assays (see below), just one false negative using the calculated error cutoff occurred when all ASVs were accepted. When ASVs above 0.1% were accepted, several index-hops were apparent, including for the Black Sea sprat *Clupeonella cultriventris*, which is a possible future invader of the Great Lakes that has not been documented in North America (NOAA 2019), and silver carp outside of its known established invasive range in the Mississippi River basin (Kolar et al. 2005), presumably mis-assigned from MCs on the same run or (more likely) due to cross contamination (both had been sequenced in our lab). Cod *Gadus* spp. and rockfish *Sebastes* spp., marine taxa that were used in our positive controls here, also occurred in the results when 0.1% was used as the frequency-based filtering cutoff. Thus, our results used the calculated error cutoff due to the likelihood of false positives under conditions of less stringent filtering.

Morphological capture-based surveys and eDNA assay results did not completely overlap. 51 taxa (74%) were in common between the two identification approaches for the environmental samples (Detroit River larvae, Maumee and Wabash Rivers; Appendix B.7). Since hybrid species (e.g., hybrid striped bass *Morone chrysops* x *saxatilis*) identified morphologically possess a single mtDNA genome, the eDNA HTS assays discerned the

maternal species. Some morphological identifications that were unresolved to species (see Experiment D below) were identified by the assays. Several eDNA assay false negatives for species detected with morphological surveys were discerned at the genus level by the eDNA HTS assays. With these corrections, 93% of the species detected with morphological surveys also were identified by the eDNA HTS assays. This was 80% when all electrofishing survey data in the Maumee River collected throughout the entire four months of intensive summer 2012 collections by the OEPA were considered, compared to just four one-liter surface water samples collected from single sites for our assays (Experiment C3).

Experiment C1: 18 fish species from five families were morphologically sampled in the Wabash River. Our eDNA HTS assays identified all species (100%) that were found in electrofishing surveys, along with an additional 21 species (Figure 3–2, Table 3.2A, Appendix B.7). Experiment C2: 33 species from 11 families were detected with the electrofishing surveys conducted concomitant with eDNA water sampling. Our metabarcode approach detected 29 of these (88%), along with an additional 19 species. 59 species in 12 families were collected in all 44 morphological surveys in 22 sites across the Maumee River in four months of sampling during Summer 2012. eDNA HTS assays found 43 (73%) of those species and an additional nine species from just four 1 L water samples (corresponding to 9% of surveys in 18% of the sites).

Experiment D: Seven taxa were identified with morphology in the Detroit River larvae sample, with three not resolved to species (*Morone* spp., percid, and unknown sp.). Our metabarcoding HTS assays identified all to species, including resolution of the unknown percid larva as the yellow perch *Perca flavescens* and the *Morone* spp. as two separate species: the invasive white perch *M. americana* and the native white bass *M. chrysops* (Table 3.2A, Appendix B.7).

Just four of the taxa found in the Maumee River electrofishing surveys Maumee River conducted concomitant with our four single eDNA water samples were not detected with our eDNA HTS assays – northern hogsucker *Hypentelium nigricans*, longnose gar, stonecat *Noturus flavus*, and white crappie *Pomoxis annularis* (Figure 3–2, Appendix B.7). Thirteen additional species discerned in the Maumee River across the Ohio EPA's entire four month-long morphological electrofishing survey (Experiment C2) did not occur in our eDNA assays of the four water samples, including rock bass *Ambloplites rupestris*, five darter species (*Ammocrypta pellucida*, *Etheostoma blennioides*, *E. flabellare*, *E. nigrim,* and *Percina maculata*), central stoneroller *Campostoma anomalum*, common shiner *Luxilus cornutus*, silver chub *Macrhybopsis storeriana*, golden redhorse *Moxostoma erythrurum*, tadpole madtom *Noturus gyrinus*, brindled madtom *N. miurus*, and creek chub *Semotilus atromaculatus*.

Species results from the single assays did not completely overlap. Of the 347 individual species detections across all samples, 111 (32%) occurred in single assays. 21 (6%) of the detections were scored as positive according to the MetaAssay criteria alone, meaning that their hits in single assays fell below the cutoff values, but the taxa were identified by more than one marker. Mean proportions of false negatives from single assays in samples taken concomitant with electrofishing surveys were 0.48±0.04. When all samples from a single region were combined, this value was reduced to 0.34±0.04. In both cases, the highest proportions of false negatives appeared for the CarpCyt*b* assay in the Maumee River. The MetaAssay approach had significantly fewer false negatives after sequential Bonferroni correction than did single assays ($p<0.004$ for all). Mean proportions of false negatives using the MetaAssay approach were 0.17±0.05 for the individual sampling sites or 0.09±0.03 when all samples from each region were combined. Six of the common false negatives from the MiFish assay were attributable to species that lacked 12S sequences in GenBank (quillback

Carpiodes cyprinus, highfin carpsucker *Ca. velifer*, shorthead redhorse *Moxostoma macrolepidotum*, ghost shiner *Notropis buchanani*, and white crappie).

100% detection efficiency from eDNA assays occurred in the Maumee River 1 sample, which had the lowest morphological species richness (Table 3.2A). Comparing the four eDNA water samples to the all electrofishing surveys conducted by the Ohio EPA in the Maumee River in summer 2012, each single assay detected between 26% (CarpCyt*b*) and 48% of the species present (MiFish). The MetaAssay approach found 73% of the species overall in this watershed level community, based on just four individual site one-liter samples (corresponding to 9% of the electrofishing surveys).

A mean of 4.6±1.0 taxa in the single assays or 14.5±5.3 using the MetaAssay results were undetected in the concomitant morphological samples. The 19 species uniquely found in the eDNA HTS assays of the Maumee River, were reduced to nine when all electrofishing identification data from all samples and sites across that entire watershed were included (Table 3.2A, Figure 3–2). Two unlikely false positives occurred with the MiFish assay. One was several apparent matches to the non-native blacktip jumprock *Moxostoma cervinum* in the Wabash River, likely due to the absence of most *Moxostomar* spp. from the 12S database – six of the seven known to be present in the watershed had no 12S reference sequence (Simon 2006). In this region, other *Moxostoma* spp. were detected with cyt *b* assays and/or morphology in every sample in which the MiFish marker had a hit for blacktip jumprock. Its hits with the MiFish assay were discarded from the final dataset. The other likely false positive with the MiFish assay was the marine white croaker *Genyonemus lineatus*, which was not found in positive controls. Just one species in that family is known to occupy the sampled regions – the freshwater drum *Aplodinotus grunniens*. That species does have a single 12S reference sequence in the BLAST database to which multiple MiFish sequences had a

match. Without a reasonable explanation, hits for that species also were removed from the final dataset.

The eDNA assays found every invasive species that were collected in the morphological surveys, including silver carp, common carp *Cyprinus carpio*, flathead catfish *Pylodictis olivaris*, round goby, and white perch (Appendix B.7). Ghost shiner eDNA was not identified from two Maumee River sites where it was physically collected, but occurred in eDNA assay results from another sample in the region. Both of these false negatives occurred when the species represented <0.1% of total fish biomass. Our assays identified more samples that possessed invasive species. For example, just a single electrofishing survey in the Wabash River caught silver carp, yet every eDNA sample detected this species with at least one marker. Our assays identified invasive grass carp in the Maumee and Wabash rivers (where they are known to occur), but they were not caught in either. Tubenose goby was not sampled morphologically in the Maumee River but was present in the eDNA assay results (and is known to occur in the watershed).

3.4.5 Proportions of species assessed with morphology versus metabarcode assays

Proportions of species-specific HTS reads and biomass were left-skewed and thus here were log-transformed for linear models and plotting. The proportions of taxa in display aquarium B2 showed very low variance in both numbers of species and biomass, as did their percent sequence reads. Therefore, relationships between biomass and sequence reads for aquarium experiment B2 were disregarded. Proportions of species-specific reads from the other aquarium (B3) and environmental samples (C1, C2, and D) showed weak positive relationships with their morphological detections. Only one R^2 and Spearman rank correlation coefficient was significant for a single assay among the single samples (CarpCyt*b*

assay in display aquarium B3; Appendix B.8). Relationships increased slightly at the regional scale (Figure 3–3), with 25% showing significant R^2 and 31% a significant Spearman rank correlation coefficient. None of the slopes for display aquarium B2 or the Detroit River larvae sample (B3) significantly differed from 1.0, and 75% were significant for the Wabash (C2) and Maumee (C3) river samples. ANCOVA found that slopes per assay did not significantly differ based on all morphological surveys versus those conducted concomitant with eDNA sampling in the Maumee River. Just the FishCyt*b* marker had significant R^2 and Spearman rank correlation coefficients, shown in the Wabash (C2) and Maumee (C3) rivers. ANCOVA discerned significant differences in the relationships among the markers within samples (Figure 3–3).

3.4.6 Community comparisons

Some species were found to be unique to geographic regions. In the Wabash River, these included blue sucker *Cycleptus elongatus* and invasive silver carp *Hypophthalmichthys molitrix,* both with morphology and eDNA HTS assays, and gravel chub *Erimystax x-punctatus* and mooneye *Hiodon tergisus* discerned solely with the eDNA HTS assays. Species uniquely appearing in the Maumee River included pumpkinseed *Lepomis gibbosus*, orangespotted sunfish *Lepomis humilis*, invasive ghost shiner, spotted sucker *Minytrema melanops*, and common logperch *Percina caprodes* were detected with both the morphology and eDNA HTS assays, and black crappie *Pomoxis nigromaculatus*, spoonhead sculpin *Cottus ricei*, orangethroat darter *Etheostoma spectabile*, and invasive tubenose goby were found only with the latter. Lakes St. Clair and Erie were surveyed with the eDNA assays alone. Black bullhead *Ameiurus melas* was the sole unique species appearing in the Lake Erie Islands of Lake Erie, and invasive chum salmon *Oncorhynchus keta* appeared in Lake St. Clair alone (where it was introduced for

sport fishing).

Species richnesses discerned from single eDNA assays ranged from 6–19, with the MetaAssay approach detecting the most taxa, including 38 from Maumee River (MAU) 1 (C2, Table 3.2B). Regionally, the greatest richness from single assays was 31 taxa (MiFish in the Maumee River) and 53 using the MetaAssay approach (also the Maumee River). Richness values obtained from morphology and the eDNA assays were not significantly correlated. No significant difference in species richness was found after sequential Bonferroni correction between the approaches, based on all samples. However, variation may have been influenced by the lower species diversity in the Detroit River larval sample (C1). Richness values for samples in the Maumee and Wabash Rivers were statistically significantly greater using the MetaAssay approach than for all other methods, including single assays and morphology ($p<0.004$ for all). Notably, numbers of taxa detected with the MetaAssay approach, which were missing from morphological surveys, were always greater than the false negatives in all but two samples (MAU 2 and 3; Table 3.2A). Number of replicates and/or samples collected per region was a significant predictor of species richness in eDNA HTS assays for single assays ($R^2=0.73$, $p<0.001$) and the MetaAssay results ($R^2=0.79$, $p<0.001$).

NMDS plots discerned more discrete grouping of regional samples using the MetaAssay approach than with single eDNA assays (Figure 3–4). Significant differences in distances to the centroid were not found for the Wabash and Maumee rivers. The MetaAssay approach (df=1, $F=6.32$, $p=0.030$) as well as the FishCyt*b* (df=1, $F=3.00$, $p=0.031$), CarpCyt*b* (df=1, $F=4.73$, $p=0.030$), and MiFish assays (df=1, $F=4.18$, $p=0.028$) found significance differences between the Wabash and Maumee river communities. The GobyCyt*b* assay and morphological surveys did not. Notably, the Lake Erie samples were

very close to those from Lake St. Clair (the other lentic habitat) with all survey methods that analyzed both.

Some samples did not cluster by geographic region in the dendrograms, when single assay results were used together with morphology (Figure 3–5A). Notably, none of the Lake Erie Islands samples clustered together. Two Maumee River samples processed with the CarpCyt*b* assay clustered with Lake St. Clair samples. One Wabash River sample for which only one of the two replicate samples produced a successful library for the GobyCyt*b* assay clustered with the Lake Erie Islands and Lake St. Clair samples. When just the MetaAssay approach and morphological data were used, all samples clustered according to region, showing improved resolution and site-specific discrimination (Figure 3–5B). Most (single assays) or all (MetaAssay approach) samples from the two Great Lakes sites (Erie and St. Clair) were more similar to each other than either was to the river samples.

3.4.7 Population genetics

All of the haplotypes actually present in experiment B1 and discerned by the eDNA HTS assays fell above the calculated error cutoff in the goby aquarium experiments (Appendix B–3). The sole significant differences in their proportions based on the number of individuals, biomass (weight), or eDNA reads for any of the assays was between the 72hrs sample in the round goby-only aquarium versus all other time points and for its morphologically assessed proportions (F_{ST} $p<0.05$ for all). This was attributed to the death of an individual having haplotype 57, between the 48 and 72hrs samplings. An appreciable number of false haplotypes were present in eDNA HTS assay results. Most, but not all, fell below the calculated error from positive controls. 15 occurrences of false round goby haplotypes were above the calculated error cutoff for the GobyCyt*b* assay, four appearing in

two or more samples. For ASVs >0.1% frequency, 10 additional false haplotypes appeared. Thus, our tests of the feasibility of populations genetics from eDNA assays instead were based on the calculated error cutoff.

Traditional Sanger sequencing of tissue samples discerned three round goby haplotypes in Lake St. Clair – RG 1 (78% of individuals), 8 (12%), and 57 (10%) (Figure 3–6). All three haplotypes were found in the benthic eDNA water sample processed with the GobyCyt*b* assay. The two rare haplotypes were absent from surface water eDNA (even when accepting ASVs at any frequency). Both surface and benthic water samples contained multiple haplotypes that were above the calculated error cutoff, which were undetected by the Sanger sequencing population analysis and were not in GenBank.

Sanger sequencing discerned three silver carp haplotypes that were physically sampled in the Wabash River (designated as SC A, B, and H), constituting 49%, 48.5%, and 3% of that population, which were sampled at a separate time (Stepien et al. 2019). The CarpCyt*b* assay differentiated all three of these haplotypes in the eDNA water samples, whose read proportions were: 67%, 30%, and <0.5% in a different year (Figure 3–6). Three additional previously undiscovered haplotypes were detected – all above the calculated error cutoff and at greater frequency than the rare haplotype H. Comparisons of our eDNA assays with traditional population genetics based on Sanger sequencing of mtDNA haplotypes showed significant frequency differences after sequential Bonferroni correction using F_{ST} (mean F_{ST}=0.179, p<0.0002 for all) and exact tests (p<0.0001 for all).

3.5 Discussion

3.5.1 Error and limits of detection

Mock community results (Experiment series A) defined a detection limit for our HTS metabarcode assay pipeline. Some false negatives in MCs were attributable to the denoising algorithm, particularly for closely related taxa, since all that were absent from the merged reads were present in unmerged ones at low abundances and were closely related to another taxon that occurred in high abundance. Additionally, bioinformatic filtering criteria (e.g., read abundances and BLAST matches) can affect final results (Frimodt and Dore 1995; Bylemans et al. 2018a). We were unable to identify a specific quantity of genomic DNA below which taxa were undetectable, since that value varied among MCs and markers and likely is dependent on the overall proportions of all DNA present.

We evaluated various frequency-based filters to eliminate index-hops and/or cross-contamination from positive controls (since the likelihood that sequencing error would BLAST to a different species was low). Given the large number of samples that can be pooled on a HTS run, such sources of error could result in false positives (Xiong et al. 2016; MacConaill et al. 2018). False positives may lead to wasted time and resources since managers may respond to discovery of a new exotic species or implement conservation practices for a rare native species. In our investigation, risks of cross-contamination or index-hops were reduced using our custom spacer insert library prep protocol and a trimming script that removed error. Despite the fact that the most common error in every positive control was closely related to an expected sequence (implicating sequencing error as the origin of these unexpected ASVs), the use of the calculated error cutoff was the sole method that eliminated all apparent index-hops.

Several studies have used positive controls to apply frequency based bioinformatic filtering (Hänfling et al. 2016; Port et al. 2016; see Deiner et al. 2017). We found that this approach likely resulted in false negatives in the single assays. However, when a sufficient number of targeted and general primer sets are used and combined with the MetaAssay approach, false negatives are significantly reduced, and detections greatly improved. Future eDNA work should utilize a library prep and bioinformatic pipeline that reduces error, assesses effectiveness using positive controls or MCs, and investigates multiple values for frequency-based filtering to discern which performs best.

3.5.2 Comparability of metabarcode assays to morphological sampling

An important goal of eDNA research is to determine the degree of overlap in community diversity discerned using genetic and morphological survey methods, which was investigated here in experiment series C and D. Detection efficiency in studies of vertebrates that compared morphological survey results to metabarcoding have shown a wide range of overlap, ranging from 25% (e.g., Gillet et al. 2018; Cilleros et al. 2019) to >90% (e.g., Hänfling et al. 2016; Port et al. 2016; Stoeckle et al. 2017). Relatively few cases have been published in which 100% of the morphologically sampled species also were discerned with metabarcoding in a single site or watershed (as we found here for the Wabash River). To our knowledge, most were cases in studies that employed multiple primer sets and/or were from low-diversity environments (Shaw et al. 2016; Civade et al. 2016; Evans et al. 2017; Fujii et al. 2019). Shaw et al. (2016) achieved 100% incidence regionally but had lower success on a per site basis. The trend of a higher regional or watershed level detection level than from individual sites is common in eDNA assays (Cilleros et al. 2019; Lawson Handley et al. 2019; Fujii et al. 2019), and likely reflects the overall amount of water sampled or filtered or the

fact that spatial variability in eDNA sampling is not equivalent to morphological capture. High identification efficiency (few false negatives and large number of species uniquely discerned with genetic assays) in the present study likely was aided by our use of multiple assays, since our sampling was limited. The 100% detection efficiency we found in the Wabash River may have been aided by our samples being collected in duplicate. One replicate at each site was collected after the electrofishing transect was completed, possibly mixing the water and incorporating more eDNA into those samples. Although detection frequencies have been shown to be related to the stringency of bioinformatic filtering (Alberdi et al. 2017; Evans et al. 2017), even when all sequences were accepted regardless of frequency, our eDNA assays did not discern all species present in the Maumee River. Since our results analyzed just single 1L water samples taken at four (9% of electrofishing surveys) of those sites, a very high efficiency was shown per sampling effort. More water sampling effort and more sites or samples would be needed to discern the additional species.

Employing a similar approach to ours, Evans et al. (2017) applied three metabarcode HTS assays to 31 water samples collected in a small Michigan pond, in a much smaller system than examined here. They used varying stringencies of bioinformatic filtering based on numbers of samples and/or assays in which a species was detected, comparing eDNA HTS results to morphological sampling. Their low and moderate stringency bioinformatic methods found all 10 species that were present in capture-based surveys. Three false negatives occurred when they applied the highest stringency of bioinformatic filtering. Eleven species were detected solely with their eDNA HTS assays. The authors used rarefaction to show that ≥8 samples needed to be processed with all three assays in order for the species accumulation curve to reach an asymptote. Given our result that species richness was correlated with numbers of samples taken in a region, it is likely that more intensive

sampling schemes would increase the total diversity detected and improve the overlap between metabarcode HTS and capture-based surveys, a conclusion that has been corroborated by other investigations (Evans et al. 2017; Bylemans et al. 2018b). For example, Civade et al. (2016) achieved 90% detection of morphologically sampled species using a single metabarcode assay by filtering 45L of water per sample site, which is putatively the same as taking 45 1L samples per site. Even with a relatively small number of 1 L water samples (N=4), we discerned 73% of the diversity present across 44 electrofishing surveys conducted over four months of effort by the OEPA. Obviously, the time and effort needed to obtain community composition data with the metabarcode HTS approach demonstrated here is much less than with traditional methods. As indicated here, the joint use of several targeted and general eDNA metabarcode HTS assays can increase detection when sampling is limited.

Some traditional capture or visual surveys can be thwarted by physical or environmental conditions (Fujii et al. 2019). In our study, the lowest morphology-based species richness in the Maumee River was discerned near its mouth (Maumee River 1 at river mile 9.4), which was one of the deepest locations sampled and possessed high suspended solids that decreased visibility (OEPA 2014). It is likely that electrofishing simply could not effectively sample fishes in those conditions. In that environmental sample, our eDNA HTS identified 100% of the species present in the concomitant morphological sampling. Fuji et al. (2019) applied eDNA HTS assays to backwater lakes in Japan, where capture-based methods were deemed difficult. The two sites where eDNA assays discerned all taxa present in their morphological surveys contained just four and eight species. Different nets have different biases, selectively capturing some species while leaving others unsampled, for which capture avoidances vary with conditions and among species, and eDNA assays also are variable

(Deiner et al. 2017).

Another possible explanation for the 100% detection efficiency of our eDNA metabarcode HTS assays at our MAU 1 site is that we censused a larger spatial extent than capture based methods, especially in this lotic system (see Cilleros et al. 2019; Fremier et al. 2019 for comparison). Just three of the 13 species present in the morphological sampling surveys at that site did not also occur farther upstream. Transport of eDNA in large rivers, like the Maumee River, has been recorded up to 130km (Pont et al. 2018). Such movement of eDNA could explain the better detection here. More thorough eDNA sampling and on-site filtering of larger water quantities can improve the overlap between eDNA HTS assays and morphological results (Shaw et al. 2016; Evans et al. 2017; Cantera et al. 2019).

Invasive species detected solely with our eDNA HTS assays all were found within their known geographic ranges, except for the round goby detected in the Wabash River. This species also was detected in nearby bait shops (see Chapter 4), and may have recently expanded its range into the region. This species detection in the Wabash River occurred in single assay from a single sample, and thus there is a remote possibility that it was a false positive that was not removed by our pipeline. Fuji et al. (2019) identified two new invasive fish species from the pond and aquarium trade, the three-lips *Opsariichthys uncirostris* and pale chub *Zacco platypus*, using metabarcoding HTS assays of Japanese backwater lakes. Gillet et al. (2018) discerned invasive tilapia *Oreochromis niloticus* and common carp *Cyprinus carpio* that were not found in morphological surveys of a reservoir in the Middle Mekong Basin, Laos. Although qPCR is regarded as a more effective method for detecting single very rare species in the environment, including newly introduced ones (Zaiko et al. 2018), metabarcoding HTS assays also are very effective for their identifications and do not require prior taxon-specific primers and probes.

3.5.3 Community diversity in traditional morphological versus eDNA metabarcode HTS analyses

eDNA metabarcode HTS assays often discern greater diversity in habitats compared to morphological sampling (see Deiner et al. 2017), as was determined in our study (Experiment series C). The total number of species found is related to the number of samples or volume of water processed (Lawson Handley et al. 2019; Cantera et al. 2019). In some cases, greater diversity or unique species detected may results from an incomplete reference database, which may lead to hits for closely related taxa instead, as occurred in a survey comparing rotenone fish sampling to metabarcode detection from 39 river sites in French Guinea (Cantera et al. 2019). In our investigation, the use of a robust cyt *b* database and removal of a few improbable hits from the 12S MiFish assay avoided a similar result. Many of the species found here solely from the eDNA assays in the Maumee or Wabash rivers either had small body sizes (e.g., golden shiner, mooneye) or were benthic (bowfin *Amia calva*, spoonhead sculpin, blue catfish *Ictalurus furcatus*, and round and freshwater tubenose gobies) and are less susceptible to electrofishing capture. Port et al. (2016) found a similar result in which fish taxa found exclusively with metabarcoding were difficult to see in their visual surveys of kelp forests in Monterey Bay, CA. Hänfling et al. (2016) likewise found eels using metabarcode HTS assays in United Kingdom lakes that avoided gillnet surveys.

Our MetaAssay approach and most of our single eDNA HTS assays differentiated among the taxon compositions in geographic regions more effectively than did morphological surveys. This may be related to the greater total diversity detected with the MetaAssay approach. Cilleros et al. (2019) found that metabarcoding HTS better differentiated among river drainages than traditional morphological sampling, uncovering

more unique species.

3.5.4 Relative abundances of species in eDNA metabarcode assays

Our mock community results showed that these cyt *b* assays maintained good relationships between input concentrations of DNA and proportions of sequence reads. Environmental samples showed a weaker, but often positive relationship, depending on the marker used. Several environmental factors can affect the relationship between proportions of species present and observed sequence reads. These include eDNA transport and settling rates in water (Deiner and Altermatt 2014; Pont et al. 2018), which likely are subject to varying amounts of degradation depending on environmental conditions (Barnes et al. 2014; Jo et al. 2019) or whether the eDNA is intra or extra-cellular/organellar (Turner et al. 2014). Variation in numbers of mitochondria in different types of cells that are shed at different rates by different species likely also affects this relationship (Robin and Wong 1988; Klymus et al. 2017; Jo et al. 2019). Subsampling that occurs during collection and library prep, as well as primer bias, also can affect these results (Deiner et al. 2017).

Hänfling et al. (2016) discerned that metabarcode assay read abundances were positively correlated with findings of long-term and concomitant morphological surveys of fishes in three United Kingdom lakes. Multiple studies have found positive correlations at higher taxonomic levels (Thomsen et al. 2016a; Gillet et al. 2018), although because different taxa in families often are not ecological equivalents (e.g., benthic invertivore darters and several piscivorous species in Percidae) these results are less useful than comparisons at the species level. Gillet et al. (2018) found that their 12S marker showed a better relationship than one targeting cyt *b* to relative proportions of fish orders and families obtained from gillnetting in an Asian reservoir. Results from various studies thus should be interpreted with

caution and here indicate that the marker used likely will affect the relationships.

3.5.5 Population genetic patterns from eDNA metabarcode HTS results

Our goby aquarium experiments (B1) showed the promise of metabarcode assays as useful tools for collecting population genetic information. Although some apparent false haplotypes likely were among the sequences, the relative proportions of the higher frequency true haplotypes were not statistically different than found with conventional sequencing of individuals. Aquarium experiments using invasive Eurasian zebra *Dreissena polymorpha* and quagga *D. rostriformis* mussels revealed similar haplotype compositions and frequencies to those actually present, using similar targeted metabarcode HTS assays (Marshall and Stepien 2019a). In our study here, tests conducted in the field showed that traditional data collection methods may yield different results than found with eDNA HTS assays of water samples. Although employing a targeted marker and sampling from the appropriate location in the water column detected all of the haplotypic diversity discerned with traditional methods, the proportions of those haplotypes differed. The factors that can affect eDNA proportions shed by various taxa in comparison to physical sampling of the organisms (see above) could influence results as well. Additionally, the haplotypic diversities of the realm of populations targeted for metabarcode assays should be known in order to design primers that are able to best differentiate them, yet assays surveying eDNA likely would have ability to detect new variation and/or changes in their frequencies, possibly enhanced by the greater numbers screened.

Tsuji et al. (2018) identified eight of nine ayu *Plecoglossus altivelis altivelis* mtDNA control region haplotypes from eDNA aquarium samples using a single metabarcode assay. Despite use of DADA2's denoising algorithm (albeit, without any frequency based

bioinformatic filtering), they found 31 false haplotypes, seven of which occurred across all 15 replicates. This likely was due to the fact that error on the Illumina platform is non-random, posing challenges for gathering population genetics data with HTS metabarcode assays, even under stringent bioinformatic filtering (Nakamura et al. 2011; Schirmer et al. 2016). We likewise here discerned additional previously undiscovered haplotypes, which might be the result of similar errors.

To our knowledge, few studies have examined whether haplotype identities and their frequencies from traditional PCR and Sanger sequencing of tissue samples match those found with eDNA metabarcode HTS assays in the environment. Parsons et al. (2018) compared 88 harbor porpoise *Phocoena phocoena* tissue extractions that had been Sanger sequenced for the mtDNA control region, using metabarcoding of that gene in HTS analysis of 36 eDNA water samples collected in the fluke prints of diving aggregations. Five of the 28 haplotypes also were recovered in the eDNA HTS results, along with three additional haplotypes (two of which were previously unknown and might constitute false haplotypes). Notably, a whale shark *Rhincodon typus* aggregation was sampled offshore of Qatar in the Arabian Gulf with biopsy spears and sequenced for the complete mtDNA control region by Sigsgaard et al. (2017). Eight sites where whale sharks were observed then were sampled in triplicate, and the extractions pooled and sequenced using two HTS metabarcode assays targeting portions of the same gene. One of the markers yielded very similar haplotype frequencies to those determined from tissue sampling, and the other did not, with complete recovery of all haplotypes likely due to the large number of water samples (N=24) collected (Sigsgaard et al. 2017).

3.6 Conclusions

A high-throughput sequencing metabarcode assay pipeline featuring high discrimination of species and low false positive probability was demonstrated (MetaAssay approach). This approach found nearly all the diversity sampled over much more extensive traditional electrofishing surveys, and yielded an appreciable number of additional species not identified morphologically. The MetaAssay approach and single assays better differentiated among communities from ecological regions than did the traditional sampling. Regionally, the metabarcode method detected all or a considerable amount of diversity present in the electrofishing surveys and many species that were not. Per site, traditional electrofishing surveys combined with eDNA metabarcode HTS assays performed better than either method alone; however, more intensive eDNA sampling at each location would likely improve the performance of the eDNA HTS method presented here. eDNA HTS reads can sometimes be used as a proxy for proportional abundance of taxa within the system, but results are marker dependent. Current technological limitations render population genetic analyses using metabarcode HTS data more problematic, but as error incidences decline and longer read lengths become feasible, this line of research has considerable potential merit.

3.7 Acknowledgements

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development, Tom Blomquist, Carson Prichard, and James Willey for early consultation and work on primer development, USGS scientist Edward Roseman for collecting and identifying the species in the larval fish sample, Mariusz Sapota, Yuriy Kvach, and Matthew Neilson for collecting unestablished potential invaders for use in mock communities, Mark Pyron for conducting electrofishing surveys in the Wabash River, Dave Altfater and the entire OEPA stream sampling crew for conducting the electrofishing surveys and collecting water samples in the Maumee River, Keith Wernert for obtaining permission for us to sample display aquarium 3 and providing the census of fishes within, and Scott McBride, Fred Averick, and David Butterfield for logistical support. This work represents partial fulfillment of the requirements for a PhD for MRS at the University of Toledo.

Table 3.1 Primers used for HTS eDNA assays in this study. Table indicates primer element function, primer name, direction (Dir; F=forward, R=reverse), and sequences for each primer element. Annealing temperatures (T_A) are given only for the target specific primers. Primer topology was 5`–Illumina sequencing adapter, spacer insert, target specific primer–3`. Spacer inserts were from Klymus et al. (2017). Previously published assays: CarpCyt*b* (Stepien et al. 2019) and MiFish (Miya et al. 2015).

Function	Name	Dir	Sequence 5`–3`	T_A
Target specific	FishCyt*b*	F	GCCTACGCYATYCTHCGMTCHATYCC	50° C
		R	GGGTGTTCNACNGGYATNCCNCCAATTCA	
	CarpCytb	F	KRTGAAAYTTYGGMTCYCTHCTAGG	54° C
		R	AARAAGAATGATGCYCCRTTRGC	
	GobyCytb	F	AACVCAYCCVCTVCTWAAAATYGC	50° C
		R	AGTCANCCRAARTTWACRTCWCGRC	
	MiFish	F	GTCGGTAAAACTCGTGCCAGC	65° C
		R	CATAGTGGGGTATCTAATCCCAGTTTG	
Adapter	Illumina seq	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	
Spacer inserts	e	F	TCCTATG	
		R	CGTACTAGATGTACGA	
	f	F	ATGCTACAGT	
		R	TCACTAGCTGACGC	
	g	F	CGAGGCTACAACTC	
		R	GAGTAGCTGA	
	h	F	GATACGATCTCGCACTC	
		R	ATCGGCT	

Table 3.2. Morphological versus eDNA sampling diversity. (A) Number of species discerned with morphology and species unique to eDNA. Proportion of false negatives in eDNA results (in parentheses). Regional samples were combined in the Maumee (1–4) and Wabash (1–2) River. Maumee R. all shows values using species detected in all summer 2012 electrofishing surveys in the region regardless of whether concomitant eDNA data was processed. (B) Species richness for morphology and eDNA assay methods. Samples taken in the same year in the same watershed were combined regionally (e.g., Maumee R. all).

A						
Location	**Morphology**	**MetaAssay**	**FishCytb**	**CarpCytb**	**GobyCytb**	**MiFish**
Display tank 1	6	5 (0.00)	0 (0.17)	2 (0.50)	0 (0.50)	0 (0.17)
Display tank 2	14	12 (0.06)	7 (0.25)	3 (0.38)	7 (0.25)	4 (0.19)
Maumee R. 1	13	20 (0.00)	1 (0.23)	1 (0.38)	3 (0.23)	7 (0.31)
Maumee R. 2	22	6 (0.41)	3 (0.45)	3 (0.73)	1 (0.73)	1 (0.77)
Maumee R. 3	23	4 (0.26)	0 (0.57)	1 (0.70)	0 (0.78)	3 (0.52)
Maumee R. 4	23	10 (0.30)	1 (0.61)	1 (0.83)	4 (0.65)	6 (0.57)
Maumee R. 1–4	33	18 (0.12)	2 (0.36)	4 (0.67)	6 (0.58)	9 (0.39)
Maumee R. all	59	9 (0.29)	0 (0.56)	2 (0.76)	3 (0.64)	6 (0.54)
Wabash R. 1	13	16 (0.23)	0 (0.54)	5 (0.54)	–	13 (0.46)
Wabash R. 2	12	14 (0.08)	7 (0.67)	1 (0.50)	2 (0.83)	7 (0.17)
Wabash R. 1–2	18	21 (0.00)	9 (0.33)	5 (0.39)	2 (0.83)	14 (0.22)
Detroit River larvae	7	0 (0.00)	0 (0.14)	0 (0.57)	0 (0.00)	0 (0.29)
B						
Display tank 1	6	11	5	5	3	5
Display tank 2	14	27	19	12	19	17
Maumee R. 1	13	38	19	14	18	18
Maumee R. 2	22	25	15	12	9	7
Maumee R. 3	23	26	18	10	6	16
Maumee R. 4	23	28	17	6	15	19
Maumee R. 1–4	33	42	26	20	24	31
Lake St. Clair 1	–	16	6	7	8	8
Lake St. Clair 2	–	16	–	10	12	12
Lake St. Clair 3	–	16	9	11	9	–
Lake St. Clair all	–	23	6	16	16	8
Lake Erie Islands	–	14	7	8	–	9
Wabash R. 1	13	30	8	14	–	21
Wabash R. 2	12	29	14	10	5	16
Wabash R. 3	–	27	17	11	9	11
Wabash R. 1–2	18	37	22	20	10	30
Detroit River larvae	7	9	6	4	8	6

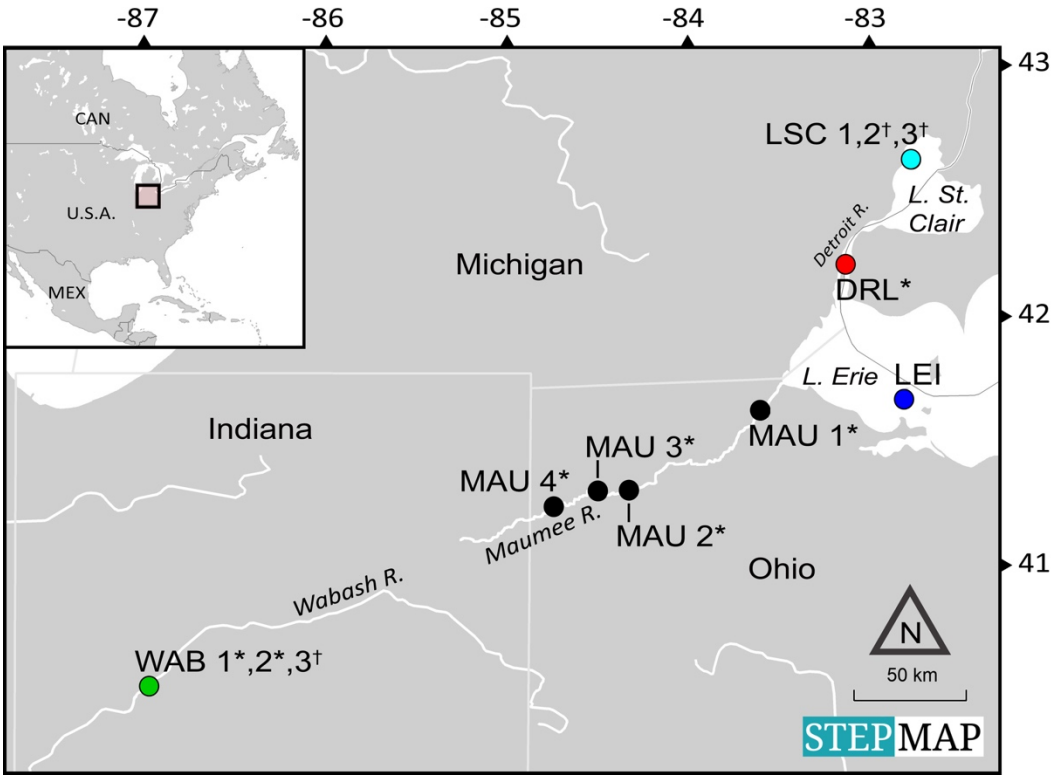

Figure 3–1 Map of sample sites in the Wabash River (WAB), the Maumee River (MAU), Lake St. Clair (LSC), and Lake Erie Islands (LEI). At some sites, electrofishing surveys (*) or traditional population genetics sampling and data collection (†) were conducted and compared to eDNA metabarcode HTS assay results. Sites in the Wabash River (WAB) and Lake St. Clair (LSC) were in too close proximity to be depicted separately (see Appendix B.4 for geographic coordinates).

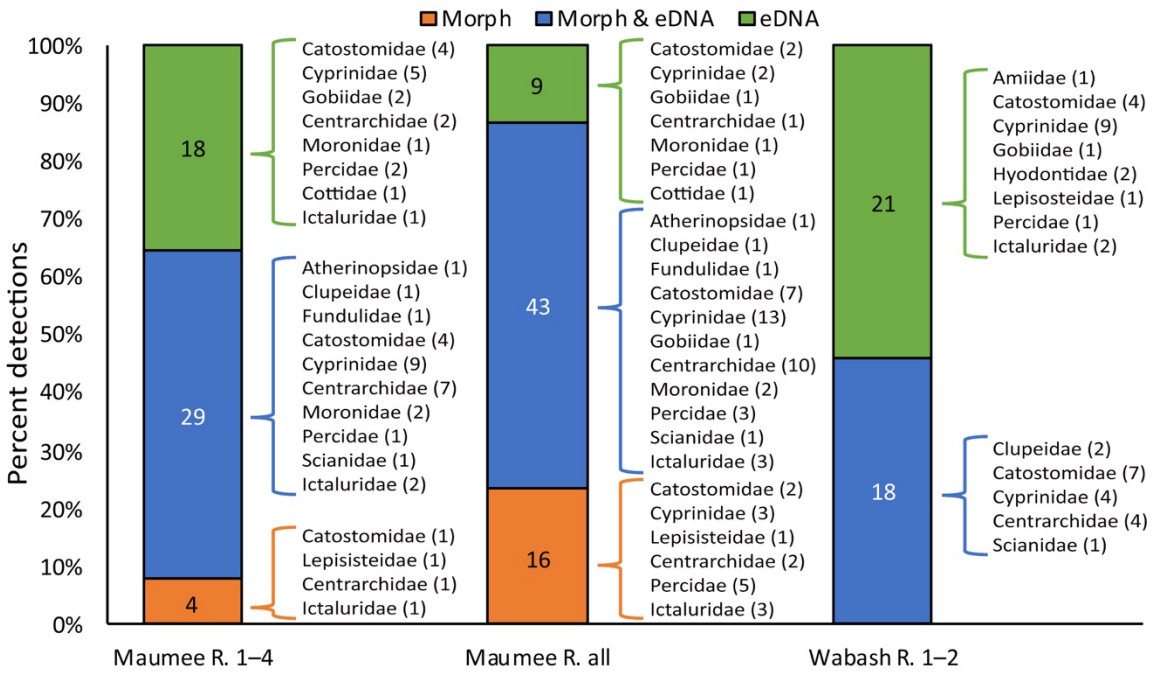

Figure 3–2 Families (number of species) detected with morphology (Morph), eDNA HTS assays, or both methods. Samples taken concomitant with electrofishing surveys were combined (Maumee R. 1–4, Wabash R. 1–2). Maumee R. all: comparison of four eDNA water samples to 44 electrofishing transects from 22 sites in the Maumee River, June–September 2012.

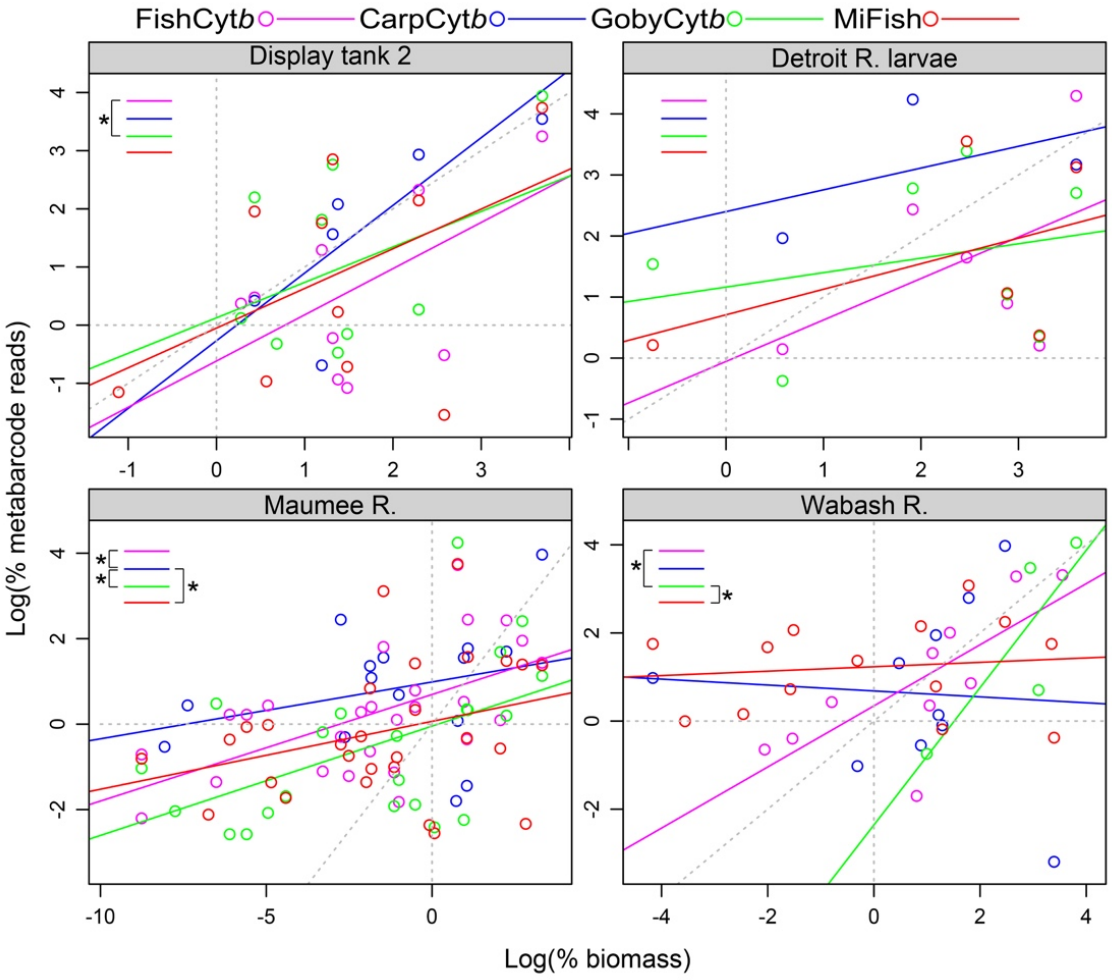

Figure 3–3 Proportions of species discerned with morphology versus high-throughput sequencing reads. Solid lines are fitted regression models for each assay. Dotted lines show x and y = 0 and a 1:1 relationship. Top left corner of each panel: significant differences between slopes (ANCOVA) indicated with brackets.

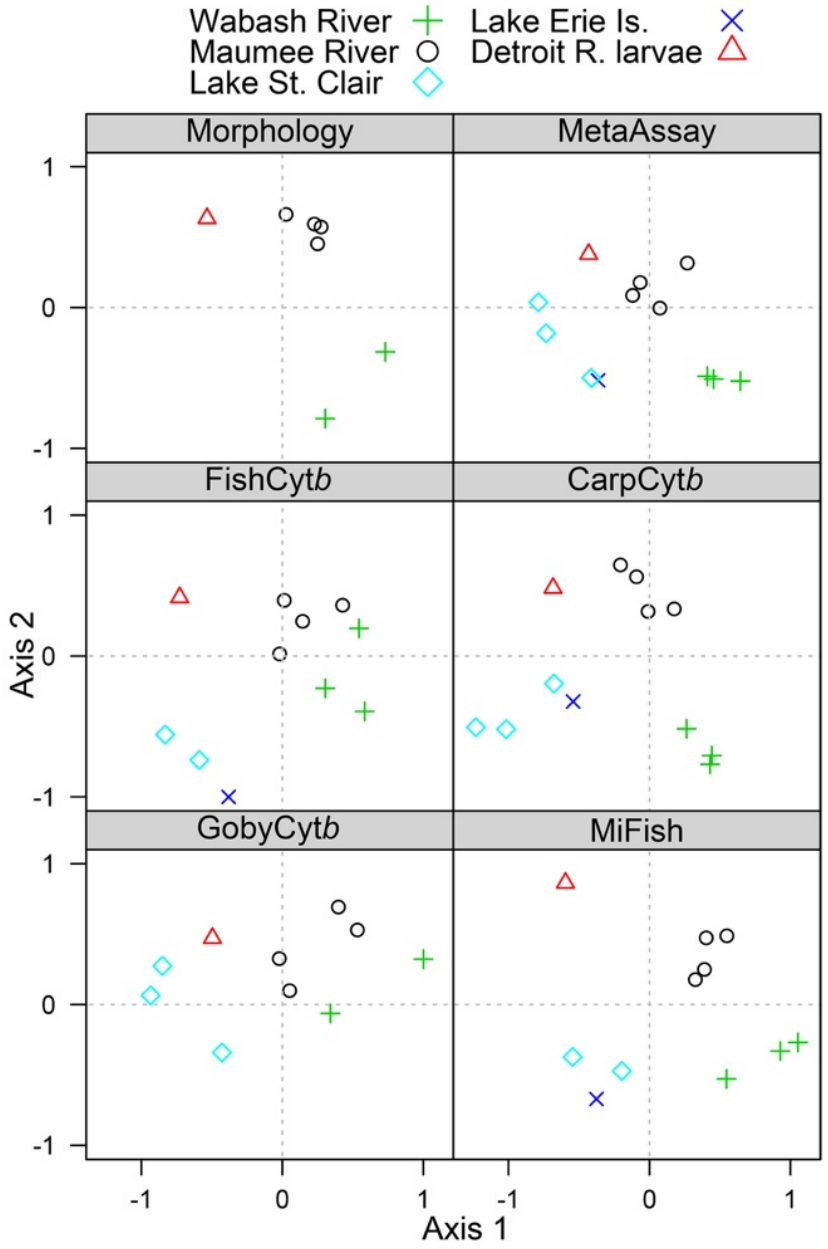

Figure 3–4 Non-metric multi-dimensional scaling plot based on Bray and Curtis dissimilarity of environmental samples with eDNA metabarcode assays and morphological capture based methods (where both were conducted concomitantly).

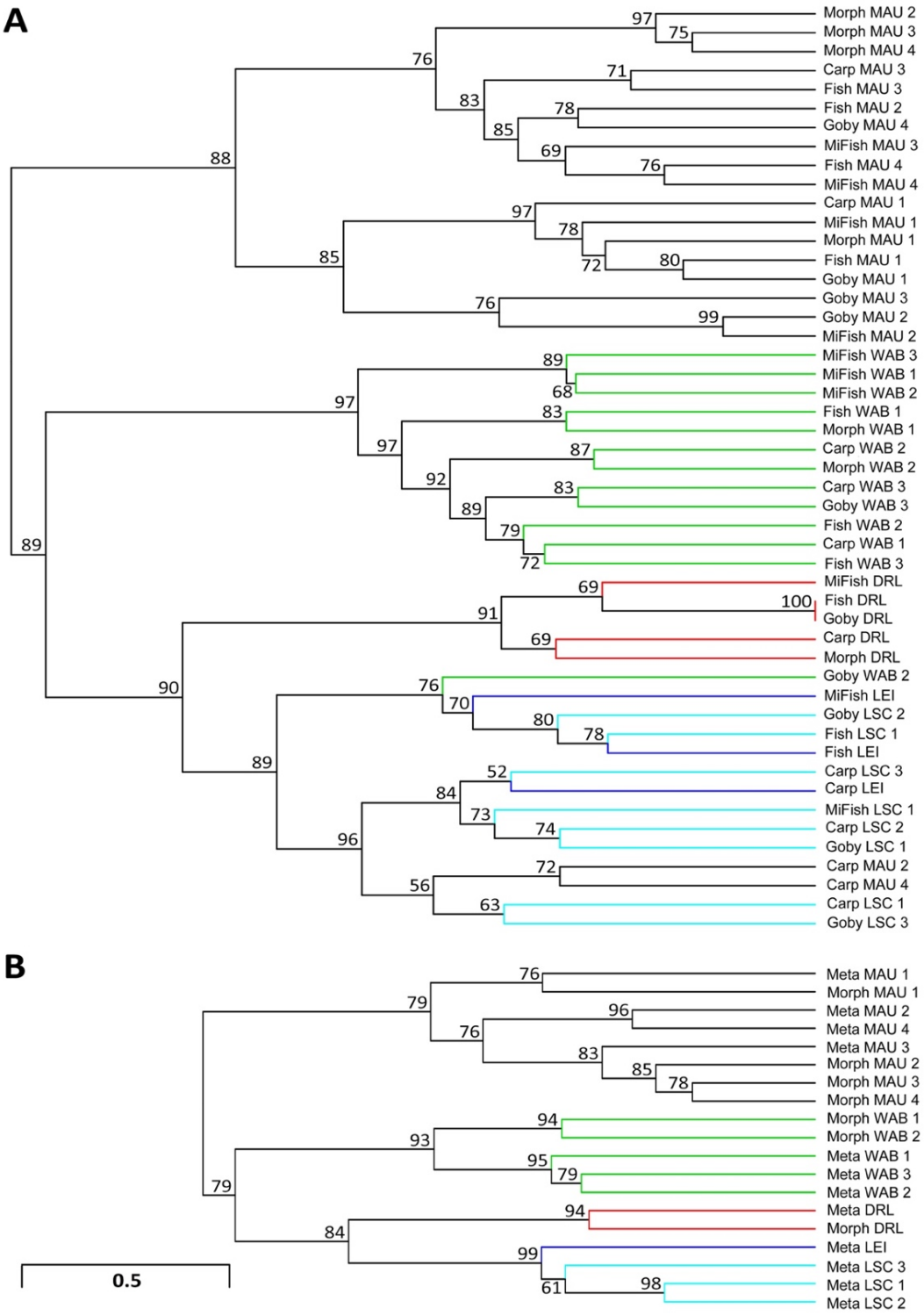

Figure 3–5 Dendrogram of eDNA and morphological samples using binary distance and Ward's D2 agglomeration method. (A) Samples processed with individual eDNA assays and morphological data. Fish=FishCyt*b*, Carp=CarpCyt*b*, Goby=GobyCyt*b*, Morph=morphological sampling. (B) Samples processed with the MetaAssay approach (Meta) and morphological data. See Figure 3–1 for color key and site abbreviation.

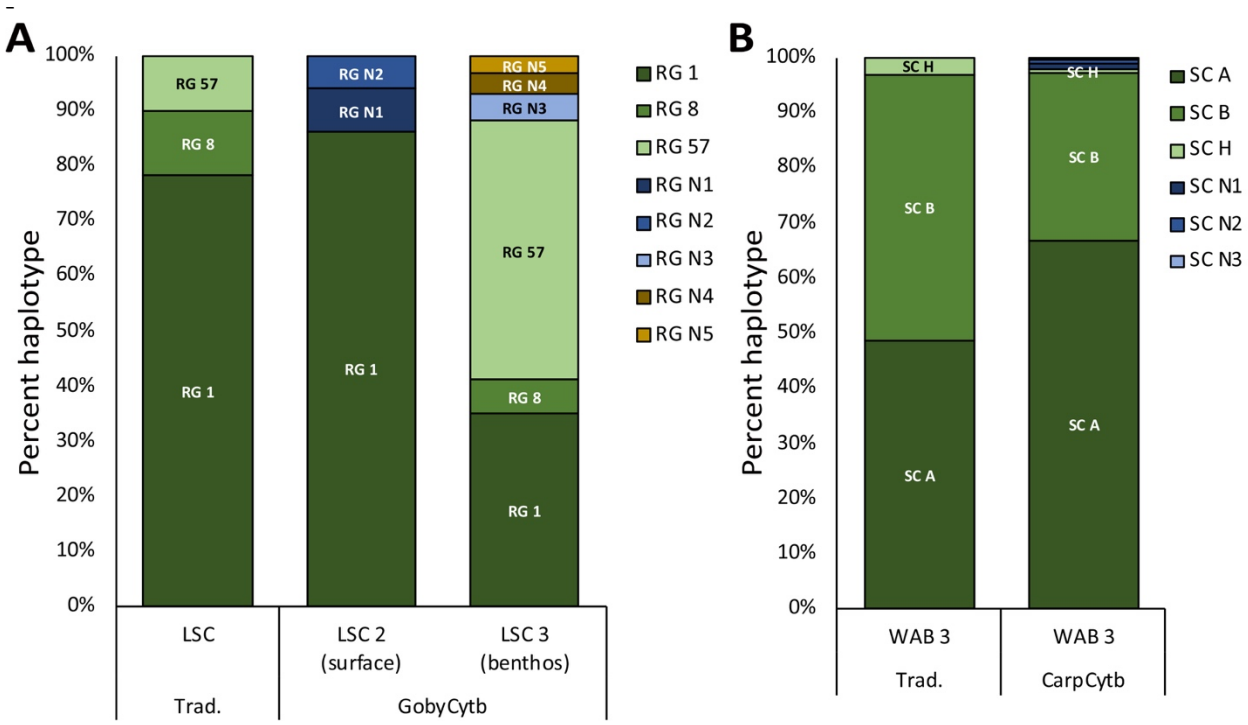

Figure 3–6 Haplotypic diversity assessed with eDNA. Round goby (RG) in Lake St. Clair (LSC2: surface, LSC3: benthos) and silver carp (SC) haplotypes in the Wabash River (WAB) assessed with traditional population genetic sampling (Trad) and the GobyCyt*b* and CarpCyt*b* eDNA assays. New haplotypes (N) not within the known cytochrome *b* diversity of either species with a frequency less than 1% of sequence reads are not labeled on the plot for clarity.

Chapter 4

Detecting aquatic invasive species in bait and pond stores using targeted environmental DNA high-throughput sequencing metabarcode assays: angler, retailer, and manager implications

4.1 Abstract

Bait and pond supply stores comprise potential yet little-studied vectors for aquatic invasive species (AIS) spread. We tested for AIS and illegal native species in 51 live bait and 21 pond stores from three watersheds: western-central Lake Erie (Ohio), Lake St. Clair (Michigan), and Wabash River (Indiana) using targeted environmental DNA (eDNA) metabarcode assays of water samples, coupled with morphological sampling and identifications. Retailers were questioned about their supply chains and anglers were surveyed about live baitfish use and disposal. Assays revealed unadvertised species in 100% of the bait stores, with 61% having illegal native non-bait and 88% with AIS. The 13 native non-bait species included juvenile walleye, yellow perch, and white sucker. Eleven AIS

encompassed Eurasian ruffe in seven stores (all watersheds), silver carp in five (all watersheds, including a Lake Erie store in two separate years), and bighead carp in two Lake Erie stores that also had silver carp. Among pond stores, two in Lake St. Clair had bighead carp eDNA, one also containing silver carp, and a Wabash River location showed European ide. Unadvertised invasive snails were discerned in 55% of pond stores. Four contained zebra mussel eDNA and two had invasive bryozoans. Illegal native species and AIS thus were widespread, showing no relationship to their variable and extensive supply chain sources. Live baitfish dumping was widely reported in Lakes St. Clair (35%) and Erie (50%). Consumer behavior and AIS prevalence in the bait and pond trades thus appear to pose serious risks for AIS introductions and spread.

4.2 Introduction

4.2.1 Invasive species in the retail bait and pond trades

The retail trade in live animals, including bait and pond stores, has been regarded as a potential vector for introduction of aquatic invasive species (AIS) (Litvak and Mandrak 1993; Ludwig and Leitch 1996; Kolar and Lodge 2002; Vander Zanden and Olden 2008) but has been little investigated to date, likely due to limited resources and lack of cost-effective and efficient identification techniques. Pond store and aquarium retailers have introduced >150 AIS in the USA, accounting for 1/3 of the species on the list of the world's 100 worst AIS (Padilla and Williams 2004). Overall, AIS in the Laurentian Great Lakes have exerted severe economic and ecological effects, with 188 listed in the NOAA Great Lakes Aquatic Nonindigenous Species Information System (GLANSIS; NOAA 2019). AIS cost taxpayers and businesses in the Great Lakes' region >$200 million annually (Lodge and Finnoff 2008;

USFWS 2012), and threaten a $7 billion commercial and recreational fishing industry (GLFC 2019). Currently, there is considerable concern that the AIS bighead (*Hypophthalmichthys nobilis*) and silver (*H. molitrix*) carps, which are now at the gateways to the Great Lakes, might become established (Kolar et al. 2005; Stern et al. 2014; GLFC 2018). They are projected to significantly impact Great Lakes fisheries due to their filter feeding low on the food web (Irons et al. 2007; Sass et al. 2014), high fecundity, rapid growth, and large sizes (Kolar et al. 2005; Cuddington et al. 2014). These carps may be present in bait sold by retailers, as they readily resemble a wide range of other "minnows", including their native cyprinid relatives, as well as gizzard shad (*Dorosoma cepedianum*) (Kolar et al. 2005).

The USA Lacey Act of 1900 (16 U.S.C. §§ 3371-3378) and the Alien Species Prevention and Enforcement Act of 1992 (P.L. 102-393) include regulation of the retail bait and pond trades, banning the importation, shipment, or sale of "injurious" species, and rendering violation of state wildlife regulations a federal crime (US 102nd Congress 1992; USFWS 2006; Nathan et al. 2014). At the state and regional levels, surveillance of bait and pond stores may include random and/or yearly inspections (AMFGLEO 2016; LED MDNR 2017). Morphological sampling rarely identifies AIS, likely because these are relatively rare in store tanks and because many young fishes at the "minnow" stage look alike (Keller and Lodge 2007; AMFGLEO 2016; LED MDNR 2017; Kevin Kale, ODNR Fisheries Biologist, pers. comm.). The aim of the present investigation was to develop and ground-truth diagnostic metabarcode high-throughput sequencing (HTS) assays and an associated bioinformatic pipeline to serve as potential tools for managers and researchers.

4.2.2 Environmental DNA detection

Organisms regularly shed environmental (e)DNA in their mucus, skin cells, and

waste products, which may persist for hours to days in aquatic systems (Ficetola et al. 2008; Barnes and Turner 2016). This material can be genetically analyzed to determine presence/absence of single or multiple species (Rees et al. 2014). eDNA assays have been shown to be more sensitive than traditional morphological sampling for identifying rare AIS and other taxa, with most studies relying on single species detection using PCR or quantitative (q)PCR (Adrian-Kalchhauser and Burkhardt-Holm 2016; Erickson et al. 2016; Zaiko et al. 2018). A more recent approach involves metabarcoding, which involves PCR amplification of DNA sequences from multiple species in a sample, from which an amplicon library is created, then sequenced *en masse* on a high-throughput sequencing (HTS) platform such as Illumina MiSeq®, generating millions of reads that are compared to a reference database to identify taxa whose genetic material is present (Shokralla et al. 2012). Multiple indexed samples (here up to 96) can be run in a single lane (Illumina 2018).

4.2.3 Objectives

Our present research objectives were to: (1) determine whether and to what degree illegal native species and AIS are present in bait and pond stores in Great Lakes watersheds, (2) identify which species are involved, and whether they differ among the three focus watersheds, (3) elucidate whether and to what degree their morphological and eDNA HTS assay identifications match, and (4) statistically compare illegal native species and AIS incidence using the two approaches. In addition, anglers and retailers were surveyed to evaluate awareness, possibility of AIS release, and relationships to supply chains. This protocol is designed to be adaptable for management and conservation use.

4.3 Methods

4.3.1 Bait & pond store sampling

Bait and pond retailers in northeast Indiana (IN), southeast Michigan (MI), and the Lake Erie watershed of Ohio (OH) were identified from Google Maps™ searches (e.g., "bait shop", "bait store", "bait fish", "emerald shiners", "pond shop", "pond store", "pond fish", "koi", "pond snails"), conversations with anglers, and roadside observations. Retailers were telephoned to confirm that live baitfish or pond species were for sale. We sampled 51 bait stores (48 in 2016 and 49 in 2017, with 46 in common for both years; Figure 4–1, Appendix C.1) during June–August. According to local anglers, diversity of bait fishes sold increases later in the season. Thus 19 stores previously sampled were re-sampled in September–November 2017. One bait store that had genetic evidence of silver carp in 2016 (see Results) was re-sampled twice. In addition, 21 pond supply stores were sampled in May–June 2017 (Figure 4–1, Appendix C.1).

Collections were made under the University of Toledo Institutional Animal Care and Use Committee (IACUC) protocol #205400, issued to CAS and KPC and covering the research team. All sampling equipment first was decontaminated for >10 min in 10% bleach and thoroughly rinsed in ddH2O between uses. We purchased >24 baitfish or one pond fish and/or 3 snails from each bait or pond store tank unless a flow-through (common) water system was used, from which ≥2 samples were obtained. Advertised species were recorded. In the parking lots, specimens immediately were filtered from water using sterile colanders, moved to a clean bucket of dechlorinated water, immediately sacrificed with an overdose of 250mg/mL tricaine methane sulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) following our IACUC protocol, placed in labeled plastic bags, and stored in a

designated cooler on ice for transport to the lab. Water (containing eDNA) was stored in labeled sterile 700ml plastic jars on ice in a separate cooler, and frozen at -80°C. In the lab, fish were identified to species using taxonomic keys (Trautman 1981; Hubbs and Lagler 2007), pat dried, weighed (g), and measured to total length (mm). Taxonomy and nomenclature for all species followed Fishbase.org for fishes or the World Register of Marine Species (marinespecies.org) for invertebrates.

4.3.2 Genetic detection of species

4.3.2.1 eDNA assay design

MtDNA cytochrome (cyt)*b* sequences >1000bp were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) for all Great Lakes fishes (Hubbs and Lagler 2007), all established invasive species listed in the USGS Nonindigenous Aquatic Species database (USGS 2019b), and all predicted future AIS from the NOAA Great Lakes Aquatic Nonindigenous Species Information System (NOAA 2019; Appendix B.2). Sequences for two native catostomid fishes (*Erimyzon claviformis* and *Moxostoma lacerum*), three extant *Coregonus* spp. (*Coregonus hubbsi*, *Coregonus nipigon*, *Coregonus reighardi*) and one that is extinct (*Corregonus johannae*), two cyprinids (*Margariscus natchtriebi*, *Semotilus corporalis*) and troutperch (*Percopsis omiscomaycus*) were unavailable on GenBank (accessions listed as "No records" in Appendix C.2). The final reference dataset included >95% of Great Lakes fishes and 100% of the AIS.

Three metabarcode assays were designed to target all Great Lakes fishes (FishCyt*b*), invasive cyprinid fishes (CarpCyt*b*) (Stepien et al. 2019), and invasive goby fishes (GobyCyt*b*). Conserved regions adjacent to variable areas <250bp that differentiated among all targeted species and discerned as much intraspecific variation as possible were identified

by visually inspecting alignments of consensus sequences of families (FishCty*b* assay) or species (CarpCyt*b* and GobyCyt*b*). Primers were constructed with degenerate bases that incorporated most of the variation in the targeted taxa (Appendix C.3).

The GobyCyt*b* and CarpCyt*b* assays respectively amplified 167 and 136 nucleotides (NTs), beginning at bases 42 and 114 in cyt *b*. An original shorter FishCty*b* (-S) assay targeted a 55NT region beginning at base 954 that was designed to be less susceptible to DNA degradation (Table 4.1). To increase taxonomic fidelity and alleviate library preparation issues associated with sequencing very short amplicons on the Illumina HTS platform (when pooling multiple markers; see below), we designed a longer 154NT FishCty*b* (-L) assay beginning at base 855 (Table 4.1) that shared the same reverse primer. This longer assay was used for the 2017 bait and pond store samples, and all re-runs (see below).

An assay targeting the mitochondrial 16S rRNA gene (Mollusk16S) designed for mollusks and other invertebrates by the CAS lab (Klymus et al. 2017) was used for pond stores selling snails. All primer sets included the Illumina® sequencing primer and four unique spacer inserts, labeled e–h (7-14 NT), at the 5' end (Appendix C.3) to increase library diversity and improve HTS data quality (Fadrosh et al. 2014; Wu et al. 2015).

4.3.2.2 DNA processing, library prep, and HTS

Genetic material was centrifuged, extracted, and subjected to a two-step library preparation method for sequencing using positive controls (see Deiner et al. 2017) to calculate sequencing error following Stepien et al. (2019). Positive controls were constructed by mixing equal mass (ug) of genomic DNA from 10 marine fish species that are absent from the Great Lakes ecosystem. Each was Sanger sequenced for the cyt *b* region of our eDNA assays (protocols in Appendix C1–B2; GenBank accessions in Appendix C.4;

FASTQ files in NCBI Sequence Read Archive Bioproject # PRJNA548536).

4.3.3 Retailer and angler surveys

Sampled stores were asked to complete a survey about their supply chains over the phone or at a later visit (so as not to influence the species sold during sampling). Angler surveys were distributed through an online list serve at the International Association for Great Lakes Research conference in Detroit, MI (May 2017), through the SeaGrant network, and at local boat launches and marinas. Questions included fishing experience, bait use and disposal, and AIS awareness (Appendix C4).

4.3.4 Data analysis

4.3.4.1 Categorizing detections: legal bait and pond species

Laws regulating bait species for sale in MI (MDNR 2016) and IN (IDNR 2019a) are more restrictive than OH (Ohio 2016), with MI banning all AIS (Appendix C.5). Three AIS are legal bait in OH – common carp (*Cyprinus carpio*), rainbow smelt (*Osmerus mordax*), and skipjack herring (*Alosa chrysochloris*). Invasive alewife (*Alosa pseudoharengus*) and threadfin shad (*Dorosoma petenense*) are legal IN bait when caught and used in the same water body. Morphological and eDNA detections in bait stores were categorized as advertised or unadvertised legal bait, native non-bait, or AIS based on the respective state laws.

No laws govern private pond stockings in the three states, provided that they are obtained in-state and there is no connection to a larger waterbody or stream. Regulations specify what species can be reared and sold by aquaculture facilities. IN has 36 legal species (Indiana 2014), MI has 55 (Michigan 1996), and OH has 94 (ODNR 2019a), with endangered taxa and most AIS being prohibited. However, some AIS are legal in aquaculture

in all three states, including all ornamental pond species (see Results; Appendix C.5). Pond retailers either sell ornamental species or those intended for establishment in small private lakes for recreational fishing purposes. Pond store detections were categorized based on the state, with non-ornamental species classified as either being native non-pond or unadvertised AIS. Native non-pond classification was based on whether the store was advertising ornamental or recreational species.

To our knowledge, no laws exist in any of the sampled states against rearing or selling native invertebrates. Most invertebrates sold by ornamental pond stores were mystery snails (*Cipangopaulidina* spp.), which often were advertised as trapdoor snails (see Results). Although invasive in the Great Lakes, no laws prohibit them from being stocked in private ponds. Thus, invertebrates discerned with the Mollusk16S assay were categorized as advertised species or unadvertised AIS.

4.3.4.2 HTS eDNA assay bioinformatic pipeline

HTS data analyses were modified from Stepien et al. (2019) (Appendix C3). In brief, primers were trimmed from raw sequence reads and detectable index-hops (Xiong et al. 2016; MacConaill et al. 2018) were removed based on the correct spacer insert and primer set using a custom PYTHON v3.7.1 script. Reads from assays targeting cyt *b* were subjected to a BLAST against the custom database used for primer design, with addition of positive control sequences, and for the Mollusk16S assay against the entirety of GenBank. Sequencing error was calculated as the frequency of the most common unexpected amplicon sequence variants (ASV) in a positive control. Error frequencies <0.1% were rounded up to this value, which is the observed rate of index-hopping on Illumina MiSeq (MacConaill et al. 2018).

Species detections were ranked as valid if they occurred at a frequency that was above the error determined from positive controls for that marker in that run, or if they were present in multiple markers, recognizing that individual primer sets might have some bias (Evans et al. 2017; Bylemans et al. 2018a). Assay results were combined and compared to morphological survey data using a custom PYTHON script. Samples having AIS detections not known to occur within the sampling area (see Results) at any read frequency were re-run for the GobyCytb, CarpCytb, FishCtyb-L, and Mollusk16S assays (the latter for pond stores only using a fish-blocking primer; Klymus et al., 2017) to confirm presence. We avoided shared 5' or 3' indices for single spacer and primer combinations. All custom scripts were deposited in Dryad (to be submitted upon publication).

4.3.4.3 Relationships among morphological, eDNA assay, and survey results

Generalized linear models in R related potential predictor and response variables. Response variables included the presence of advertised or unadvertised bait or pond species, native non-bait or pond species, AIS, or non-native species found with eDNA assays or morphological identifications (see Results). Predictor variables were species advertised for sale, region, supplier, and day/month of sampling. Linear models compared reported bait dumping to possible predictive factors, including years of fishing experience, areas most often fished, AIS awareness, and bait type used (live fish, live non-fish, or artificial). Separate models were constructed for all anglers and for those using live fish bait. AIS awareness and eDNA results, supply chains, and angler survey data were mapped in ESRI ArcGIS v10.7 (Redmond, CA).

4.4 Results

4.4.1 Morphological sampling

Five legal bait fish species (all cyprinids) were advertised during visits to bait stores (Appendix C.6), and 41% did not advertise any of the same species during multiple visits. Some advertised ambiguously (e.g., "minnows" or "shiners"; 14% of sampling events). Two MI stores advertised mudminnows (Umbridae), yet none were sampled or detected with our eDNA assays (see below). 71% of stores sold one or more unadvertised species during at least one sampling event (Table 4.1A), and 67% of them sold an unadvertised legal bait species. Five stores in 2016 and two stores in 2017 sold only unadvertised species (Appendix C.6). In 2016, all unadvertised morphological samples were legal bait. In 2017, native non-bait and AIS were both sold by four stores (8%). Brook stickleback (*Culaea inconstans*), an unadvertised legal bait sold by several OH stores, was found in one MI store where it is here classified as native non-bait (Appendix C.6). Yellow perch (*Perca flavescens*), a native non-bait species in all states, occurred in two OH stores, and AIS western mosquitofish (*Gambusia affinis*) was sold by four OH stores. Four stores advertised and sold goldfish (*Carassius auratus*) for use as bait, though it is not legal to be sold as bait in any of the sampled states (Appendix C.5). Neither state nor supplier were significant predictors of unadvertised bait, native non-bait, or AIS, and year was not a predictor for any type of unadvertised bait.

Most stores that supply recreational fishing species deliver the fish directly to the customer's pond, and thus 19 of the pond fish retailers solely sold ornamental species. The two that sold recreational fishes also sold ornamentals. Among retailers with ornamentals, 95% sold koi (aka, common carp), 76% sold goldfish, 71% mystery snails, and 24% mosquitofish. All native species sold by recreational pond fish retailers were legal except for

striped bass (*Morone saxatilis*), an AIS sold in OH. All morphological samples collected in pond stores were the advertised species.

4.4.2 Genetic detection of species

4.4.2.1 High-throughput sequencing metrics

49,275,676 sequence reads were obtained for bait and pond store libraries (mean/sample /assay±SE=113,801±3,624; Appendix C.7). DADA2 merged an average proportion of 0.79±0.01 trimmed reads/sample/assay (mean=64,385±1,400), averaging18±0.6 ASVs/sample/assay. Frequencies of error in positive controls ranged from 0.07–0.29% (mean=0.19±0.02%). Amplification was not observed in any negative centrifugation, extraction, or clean up controls. At least one sample from every bait store was successfully sequenced with at least one assay and 81% were sequenced with three assays (Appendix C.8). Twenty (of *N*=21) pond fish retailer samples were successfully sequenced with all of the fish assays. One sample did not yield any successful libraries, presumably due to inhibition. All 13 pond stores that sold mollusks were successfully sequenced with the Mollusk16S assay. 94% of the 5569 BLAST hits that passed the filter were identified to single species. Of hits that could not be resolved to species, only the two subclass level hits (mollusk Hygrophila) contained taxa of different classifications (advertised or unadvertised AIS), which were regarded as the advertised species.

4.4.2.2 Detection efficiencies of eDNA assays versus morphological sampling

Fifteen (32.6%) of the 46 species discerned with the eDNA HTS assays also were morphologically identified as present in the bait stores (Appendix C.9). Of the 215 total morphological bait detections (every species in every store), eDNA HTS assays found 89%.

Creek chub (*Semotilus atromaculatus*) was not identified with eDNA HTS assays in samples where it had been identified by the student researcher and often was advertised. Instead, our assays found large proportions of reads that BLASTed to white sucker (*Catostomus commersonii*). This was considered to be a morphological misidentification. With this correction, 91% of the 215 morphological findings were confirmed with our eDNA HTS assays. All but one of the 23 eDNA false negatives were unadvertised legal bait species. The exception was a goldfish false negative in a sample for which only the FishCytb-L assay successfully amplified. eDNA HTS assays of bait store samples yielded many more individual species detections (totaling 619), of which 65% were not identified from morphological examinations. eDNA HTS assays discerned 31% more bait stores containing unadvertised species (legal bait, native non-bait, and AIS; $p<0.001$ for each) than were found morphologically. All of the 23 unadvertised species identified using the eDNA assays in the pond stores were undetected in the morphological examinations.

4.4.2.3 Unadvertised species in bait stores

Since no significant differences occurred in our eDNA assay results among sampling years or events at given locations, the results were combined for further analyses. Results did not statistically differ among the three states. One or more unadvertised species were detected in 100% of the bait stores on at least one visit (Table 4.1A), which always included one or more legal-bait species (Appendix C.10). Just five samples (5%) solely contained bait species that were all advertised for sale. Twenty-four (23%) had unadvertised legal bait alone (no native non-bait or AIS). Five stores (10%) contained just unadvertised legal bait (no native non-bait or AIS), according to eDNA HTS assay results (Figure 4–1, Appendix C.10).

Native non-bait was found in 61% of stores (Figure 4–1, Table 4.1A), most commonly containing (22% of stores) walleye (*Sander vitreus*) or yellow perch (33%) (Figure 4–2A). Year was not a significant predictor of native non-bait eDNA detections. AIS commonly used in fish food (e.g., *Alosa* spp.) were disregarded (Frimodt and Dore 1995; Miles and Chapman 2006). The eDNA HTS assays discerned unadvertised AIS in 88% of stores (Table 4.1A), which were most commonly goldfish (25%), mosquitofish (31%), or round goby *Neogobius melanostomus* (55%) (Figure 4–2A). Three species not established in the lower Great Lakes were identified and confirmed in re-runs (Figure 4–2A). These included Eurasian ruffe (*Gymnocephalus cernua*) in seven (14%), bighead carp in two (4%), and silver carp in five stores (10%), including twice in one store in both sampling years (Figure 4–1, Table 4.1A, Appendix C.8). Bighead carp hits occurred in stores that also had silver carp. In 2017, AIS detections showed a 25% increase, although these did not significantly differ between years. Native non-bait species was the sole significant predictor of AIS eDNA hits ($p<0.001$).

4.4.2.4 Unadvertised species in pond store retailers

15% of the pond stores contained eDNA of unadvertised legal fishes, goldfish, or mosquitofish (Figure 4–2B). Native non-pond fishes (excluding those advertised for sale by recreational pond fish suppliers) were found with the fish assays in 25% of stores (Figure 4–1, Table 4.1B), including single store occurrences of gizzard shad (*Dorosoma cepedianum*), largemouth bass (*Micropterus salmoides*), walleye, white sucker, or yellow perch (Figure 4–2B). State or supplier did not significantly predict the presence of native non-pond species.

Pond store eDNA assays also identified fish food species, which were disregarded as for the bait stores. Grass carp (*Ctenopharyngodon idella*) was identified in one (5%) ornamental

pond fish supplier. Round goby was detected in four stores (19%) (Figure 4–2B, Appendix C.11). Three fish species not established in the Great Lakes were detected and confirmed with re-runs, included the Eurasian ide (also called orfe) (*Leuciscus idus*) in one store and bighead carp in two stores, one of which also had silver carp (Figure 4–1, 2B, Appendix C.11).

11 pond stores amplified with the Mollusk16S primer had hits for snails, totaling eight different snail species across all. Four stores solely contained *Cipangopaludina chinensis*, four had *Ci. Japonica* alone, and three possessed both. All advertised snails were AIS, and 55% of the pond stores selling mollusks had genetic evidence of unadvertised AIS snails (Table 4.1C). These included *Planorbarius corneus, Physa acuta, Helisoma trivolvis, Gyraulus parvus,* and *Melanoides tuberculata*, each in single stores (Figure 4–2B). The Mollusk16S assay identified zebra mussel *Dreissena polymorpha* in four stores and invasive Bryozoa in two: *Fredericella indica* and *Lophopodella carteri* (Figure 4–2B, Appendix C.12). No variables (state, supplier, species sold, native non-ornamental detections) were significant predictors of these AIS.

4.4.3 Retailer and angler surveys

Among bait stores, 50% in 2016 and 61% in 2017 divulged their suppliers, reporting sourcing from a variety of chains to ensure fish were in stock all season. A maximum of 10 bait stores named the same supplier and 11 specified the same state (OH) (Appendix C–1). Just two of the pond stores had the same supplier (Appendix C–2). Supplier was not significantly related to the presence of unadvertised legal bait or AIS discerned with morphology or eDNA assays. Bait stores that sourced from a single supplier located along the southern shore of Lake Erie's western basin (OH) were 43% more likely to have native

non-bait in our eDNA HTS assay results (p=0.043). This supplier refused to divulge their bait source(s). No significant relationship was found between unadvertised species of any type and supply chains for the pond stores.

We received 179 complete responses to the angler surveys (Appendix C.13), who reported fishing in all five Great Lakes and inland lakes and streams in all adjacent US states, except Illinois. Anglers reported having 0–70 years of fishing experience, with 173 (96.7%) aware of at least one AIS, some familiar with all 10 species listed, and several naming others (e.g., dreissenid mussels, alewife, Atlantic salmon; Appendix C.13). 53% of the anglers reported using live fish bait (Figure 4–3), which varied by region and was significantly greater for those fishing in Lake Erie (40, 61.6%, of the 61 reports, p<0.001). 23% of all anglers and 44% of those using live fish bait reported dumping it into waterways (Appendix C.13). Frequency of bait dumping varied by region (Figure 4–3), with significant frequencies reported into the Lake St. Clair region (the Lakes Huron-Erie Corridor of MI) (80% of those using live fish bait, p=0.016), OH inland lakes and streams (60%, p=0.037), and Lake Erie (50%, p=0.042).

4.5 Discussion

4.5.1 Morphological sampling versus eDNA HTS analyses

Our eDNA HTS assays revealed relatively few false negatives and more detections of unadvertised species than did morphological sampling. This result is expected as retailer tanks typically contain dozens (pond stores) or hundreds to thousands (bait) of individuals, and thus are unlikely to be accurately represented in a relatively small sample. Confidence levels in genetic results are much higher than for morphological identifications (Imtiaz et al.

2017), which corresponds to our findings. Morphological sampling provides counts of species in tanks but results in many more false negatives. Our assays are restricted to relative abundances of eDNA reads, but sampled many more individuals and thus found many more unadvertised species.

Most studies comparing eDNA HTS methods to morphological sampling of fishes in the environment showed that it was at least as good or provided a valuable complement to traditional methods (Deiner et al. 2017). Studies that relied on just a single eDNA HTS assay for identifications more often have experienced false negatives due to primer bias (Kelly et al. 2014; Miya et al. 2015). This trend can be alleviated with use of multiple primer sets (Thomsen et al. 2012; Evans et al. 2017), as done here. Our study, which used three assays, found that use of multiple eDNA HTS assays improved resolution over morphological surveys for distinguishing species in retail tanks.

Most unadvertised species that were for sale in bait and pond stores, as detected with our eDNA HTS assays or from morphological examinations, were legal taxa. It is unlikely that every bait shipment to a store contains the same species. Previously stocked species may be less numerous in the tank, but their DNA may remain (Zaiko et al. 2018). Bait stores may be incapable or unmotivated to determine exactly what species they have for sale. Identification of AIS and illegal native species is necessary for their removal from sale (Litvak and Mandrak 1993; Nathan et al. 2015).

4.5.2 Sources of eDNA in tanks

Cells and free DNA can adhere to nets, traps, or other fish during collection or might be accidentally introduced to tanks via filleting of game fish in the store. If either were the case here, high numbers of eDNA hits for native non-bait species would be expected in

stores that filleted game fish, and/or for native non-bait and established AIS in stores that sourced bait from the environment. None of those trends were observed here.

Persistence of eDNA in the store tanks may be influenced by water chemistry, temperature, microbial communities, total biomass present, and/or UV radiation (Barnes et al. 2014). Studies of eDNA degradation in tanks or mesocosms have estimated that eDNA is undetectable after <14 days (Barnes et al. 2014; Strickler et al. 2015; Jo et al. 2019). Moreover, almost every bait and pond store we sampled had filters that actively removed waste from tanks, and the tanks contained very large numbers of live individuals. Thus, it is most likely that our eDNA assay results reflected the actual present-day or very recent physical compositions of the species in tanks. The addition of eRNA (see method in Pochon et al. 2017) could be undertaken to differentiate live metabolizing species in the tanks, if applied to our study design.

4.5.3 Unadvertised species detection implications

Most of our morphological and eDNA assay detections of unadvertised species were of taxa that are legal for sale either as bait or by aquaculture facilities. The release of a legal species that originated from aquaculture or came from a different geographic region could transmit parasites or diseases into wild populations (Meyer 1991; Goodwin et al. 2004; Walker and Winton 2010). For example, a non-native tapeworm parasite (*Bothriocephalus acheilognathi*) was introduced into Lake Mead, NV, through bait releases of infected invasive grass carp (Heckman et al. 1993). Viral hemorrhagic septicemia virus (VHS) is a swiftly evolving finfish disease known to occur in aquaculture operations, which has caused large fish kills in the Great Lakes, and is known to be present in aquaculture operations (Walker and Winton 2010; Pierce and Stepien 2012; Stepien et al. 2015).

Although not statistically significant, the increase in native non-bait and AIS observed in our 2017 results (both eDNA assays and morphology) could reflect a decline in availability of local bait species (Egan 2017; Copper 2017) or was due to variation in suppliers. Ecological projections predict additional declines in bait fish availability in the face of silver and bighead carp invasions (Zhang et al. 2016). It may become more difficult to collect legal bait in Lake Erie and other locations, necessitating that retailers source from out of state, further increasing the risk of AIS introductions. The two most commonly detected AIS with our metabarcode assay (round goby and western mosquitofish) are species that occupy the same habitats as legal bait species, implicating collection from the environment as the source of these non-native taxa.

Bait dumping is a known vector for the introduction or secondary spread of many AIS, including Eurasian ruffe (Winfield et al. 1996), round goby (Janssen and Jude 2001), and smallmouth bass (*Micropterus dolomieu*) (Jackson 2002). Many of the invasive fishes that we detected are well-established in the lower Great Lakes (e.g., goldfish, grass carp, mosquitofish, and round goby). However, supplementing the genetic variation of an invasive population can increase its success (Baker and Stebbins 1965), and their release from bait could lead to secondary spread into new areas. A reproducing population of grass carp recently was discovered in the Sandusky River, a tributary to Lake Erie (Embke et al. 2016), nearby where many stores and suppliers reported collecting their bait in our study.

Several AIS that currently are not known in the Lower Great Lakes were discerned in the present investigation. Predicted impacts of Eurasian ruffe in Lake Erie include competition with the economically valuable native yellow perch fishery (Ogle 1998), with its AIS populations presently solely known from the Upper Great Lakes (Stepien et al. 2018). Silver and bighead carps are predicted to significantly alter food webs and threaten native

fisheries if they become established in the Great Lakes (Kolar et al. 2005; Irons et al. 2007; Sass et al. 2014). Potential impacts of invasion by Eurasian ide are understudied but include competition with native species (USGS 2018). A cultivar of this fish, the golden orfe, whose coloration renders it a popular ornamental species, is widely sold in many areas of the world, including the USA (USGS 2018), and thus might become established.

Gastropod mollusks (snails), including cryptic AIS, often are introduced to new ecosystems via the pond store trade (Keller and Lodge 2007). Species in the families Physidae, Thiaridae, Planorbidae, and Lymnaeidae have established populations globally (Pointier and Augustin 1999; Cowie and Robinson 2003) and were found in the pond stores assayed here. Duggan (2010) identified similar snail AIS in home aquaria in New Zealand. Pond and aquarium snails, including *Ph. acuta* and *Me. tuberculata,* typically live in association with aquatic plants and often appear cryptic (Cowie & Robinson 2003, Duggan 2010). Many of these snails have histories of accidental releases leading to established AIS populations, with some being long established and others having more limited distributions, raising ecological concerns (Duggan 2010). Introductions can lead to negative ecological interactions with related native species (Zukowski and Walker 2009). For example, the continued release and range expansions of invasive *Cipangopaludina* spp. (e.g., the Chinese mystery snail group) pose significant threats to native snails (Van Bocxlaer and Strong 2019). Moreover, invasive *Me. tuberculate* found here are intermediate hosts to several human pathogenic trematode flukes, constituting human health concerns (Pinto and Melo 2011). Stricter laws and policies are needed to regulate sale of potential and already established AIS (Keller and Lodge 2007), since all advertised pond snails found in our investigation are AIS.

By comparison, Litvak and Mandrake (1993) identified five native non-bait and one AIS (common carp) in their morphological survey of just four baitfish dealers in Ontario,

Canada. Nathan et al. (2015) detected bighead and silver carps, goldfish, rudd (*Scardinius erythrophthalmus*), and round and tubenose (*Proterorhinus semilunaris*) gobies in single-target species qPCR surveys of 576 bait stores across the eight US Great Lakes states. Numbers of hits for AIS were similar to ours, including three retailers in their study having silver carp along the southern shore of Lake Erie near Sandusky. Three of the five bait stores from which we identified silver carp eDNA were previously sampled in 2012–2013 by Nathan et al. (2014), who did not then find AIS in these retailers (L. Nathan pers. comm.). However, those stores were located relatively near (<120 km) three other OH stores in which Nathan et al. (2014) discerned silver carp eDNA with qPCR (L. Nathan pers. comm.). qPCR assays only detect single species, whereas metabarcoding can identify multiple species from entire communities (Shokralla et al. 2012; Rees et al. 2014). Using multiple metabarcode HTS assays, we identified six AIS that Nathan et al. (2014) did not test for or find: Eurasian ruffe, common carp, grass carp, mosquitofish, rainbow smelt (*Osmerus mordax*), and white perch (*Morone americana*). Nathan et al. (2014) also did not attempt to detect illegal native taxa with any qPCR assays, whereas we identified 13 illegal native species.

Mahon et al. (2014) applied a single metabarcoding assay to six bait stores in the Great Lakes and found a maximum of three unadvertised legal bait species per retailer. They also detected two unadvertised native non-bait species and invasive white perch in MI, where it was already established. In comparison, we discerned 13 native non-bait species and 11 AIS in our eDNA HTS assay analyses of 51 bait stores, in a more comprehensive investigation. The majority of sequence reads from the stores in Mahon et al. (2014) were classified as "unknown fish". It is unclear if this lower resolution was due to their marker selection or their bioinformatic pipeline. Almost all of our hits were resolved to species, and our results showed a high degree of confidence due to the bioinformatic filtering criteria

employed. Our approach, which combined multiple targeted and general metabarcoding HTS assays, identified a wide range of species, significantly extending and enhancing those prior studies of AIS and non-legal species for sale in bait stores. Moreover, we conducted a novel investigation of pond stores, discerning many AIS snails.

4.5.4 Retailers and angler behavior

Our investigation showed that retailers obtained their inventories from a wide diversity of sources, with few sharing common suppliers. This appeared especially true for pond stores. Survey response frequencies resembled those reported in other studies. One Lake Erie OH bait supplier was significantly associated with native non-bait species presence, whose proximity to Lake Erie indicated that they obtained their bait from local waters and were unwilling or unable to identify species present. The present results indicated that an appreciable number (20%) of bait stores sourced bait from out of state (similar to numbers found by Kilian et al. (2012), posing a risk of AIS introductions and spread. Additional regulations requiring stores and suppliers to list bait source(s) likely would help alleviate this potential spread of AIS and illegal sales of young native non-bait species and provide additional avenues for possible enforcement when they are found.

Just a small number of bait stores (<10) sampled here posted any information for anglers about proper disposal of unused bait or AIS. One store in the Maumee River region of Northwest OH displayed a "best practices" approach, providing pamphlets with pictures of legal bait and AIS (including bighead and silver carps), encouraging anglers to throw unused bait in the trash. In a mail survey, 49–84% of bait stores in the Great Lakes claimed to communicate information about AIS to customers either with signage, printed information, or in conversations (Connelly et al. 2018), but those results were not verified

with actual visits to retailers, and were not found to be the case in our study. Nathan et al. (2014) discerned that just 22% of 525 bait stores surveyed in the Great Lakes region displayed signage or other materials about AIS. When provided with materials and signage, only 54% of those stores continued to display them one year later (Nathan et al. 2014). There is an apparent lack of awareness of regulations among bait retailers. We found that several stores sold goldfish for use as bait, although they are not legal to use.

All three states sampled provide online resources on best practices for pond construction and management to ensure that there are no connections with other water bodies (MDNR 1999; MDEQ 2017; IDNR 2019b; ODNR 2019b). However, we observed no information posted in the pond stores about this topic or AIS. To our knowledge, ours appears to be the first study to investigate and identify unadvertised species for sale in pond stores or to examine pond store communications with customers about AIS.

Most anglers surveyed were aware of AIS, which did not necessarily result in proper bait disposal behavior. Surveys previously conducted in the Canadian Great Lakes' provinces have indicated that >50% of anglers were unfamiliar with bait fish disposal regulations and 41% dumped unused bait (Litvak and Mandrak 1993). Nearly all of their respondents reportedly did so believing they were doing a service to the environment by not throwing unused bait in the trash (Litvak and Mandrak 1993). Another study found that 65% of Maryland anglers using live fish bait reported dumping it into the water bodies (Kilian et al. 2012). Given the >13 million yearly angler fishing days in the Great Lakes (USDI et al. 2016), the frequency of dumping, and the presence of AIS in bait stocks, hundreds of introductions are likely every year due to this vector. These studies, and our findings, illustrate the importance of further AIS education, especially against bait dumping and release of pond species.

4.6 Conclusions

A large proportion of unadvertised species detected in the bait and pond store trades are illegal native or AIS, including silver and bighead carps. Their presence, particularly in bait, poses serious threat of introduction into the Great Lakes. AIS presence appeared unrelated to single supply chains, highlighting an industry-wide lack of willingness, motivation, and/or ability to properly identify species for sale and remove those that are illegal. Pond stores communicated no information about potential escape of the legal advertised and unadvertised invasive species for sale. Bait dumping is a common practice among anglers, particularly in the lower Great Lakes, and pond stores provided little to no information on proper pond construction and management. Thus, there is high risk of AIS introductions and spread via these vectors. A genetic surveillance program could help managers to accurately survey presence of illegal species in bait and pond stores.

4.7 Acknowledgements

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Table 4.1 Bait and pond store morphological and eDNA assay results. Summary of detections of species grouped by state and totals for (A) bait stores for all years and seasons combined with morphology and eDNA HTS assays and (B) pond stores from fish cyt *b* or (C) mollusk16S assays. Number of samples (and proportion) that had evidence of any unadvertised species, native non-bait/pond, aquatic invasive species (AIS), or *Hypopthalmichthys* (*Hypop.*) spp. (invasive silver or bighead carps). All morphological samples from pond fish retailers were found to be the advertised species, and thus only eDNA HTS assay results are shown (B and C).

A. Bait stores			Unadvertised				
	State	N Shops	All	Legal	Native	AIS	Hypop. spp.
Morphology	IN	4	2 (0.50)	2 (0.50)	–	–	–
	MI	14	11 (0.79)	11 (0.79)	3 (0.21)	0 (0.00)	–
	OH	33	23 (0.70)	21 (0.64)	1 (0.03)	4 (0.12)	–
	Total	51	36 (0.71)	34 (0.67)	4 (0.08)	4 (0.08)	–
eDNA	IN	4	4 (1.00)	4 (1.00)	3 (0.75)	4 (1.00)	1 (0.25)
	MI	14	14 (1.00)	14 (1.00)	11 (0.79)	14 (1.00)	1 (0.07)
	OH	33	33 (1.00)	33 (1.00)	17 (0.52)	27 (0.82)	3 (0.09)
	Total	51	51 (1.00)	51 (1.00)	31 (0.61)	45 (0.88)	5 (0.10)
B. Pond store fishes	IN	1	1 (1.00)	1 (1.00)	–	1 (1.00)	–
	MI	9	5 (0.56)	–	3 (0.33)	2 (0.33)	2 (0.22)
	OH	10	6 (0.60)	2 (0.20)	2 (0.20)	2 (0.20)	–
	Total	20	12 (0.60)	3 (0.15)	5 (0.25)	5 (0.25)	2 (0.10)
C. Pond store mollusks	MI	5	–	–	–	3 (0.60)	–
	OH	6	–	–	–	3 (0.50)	–
	Total	11	–	–	–	6 (0.55)	–

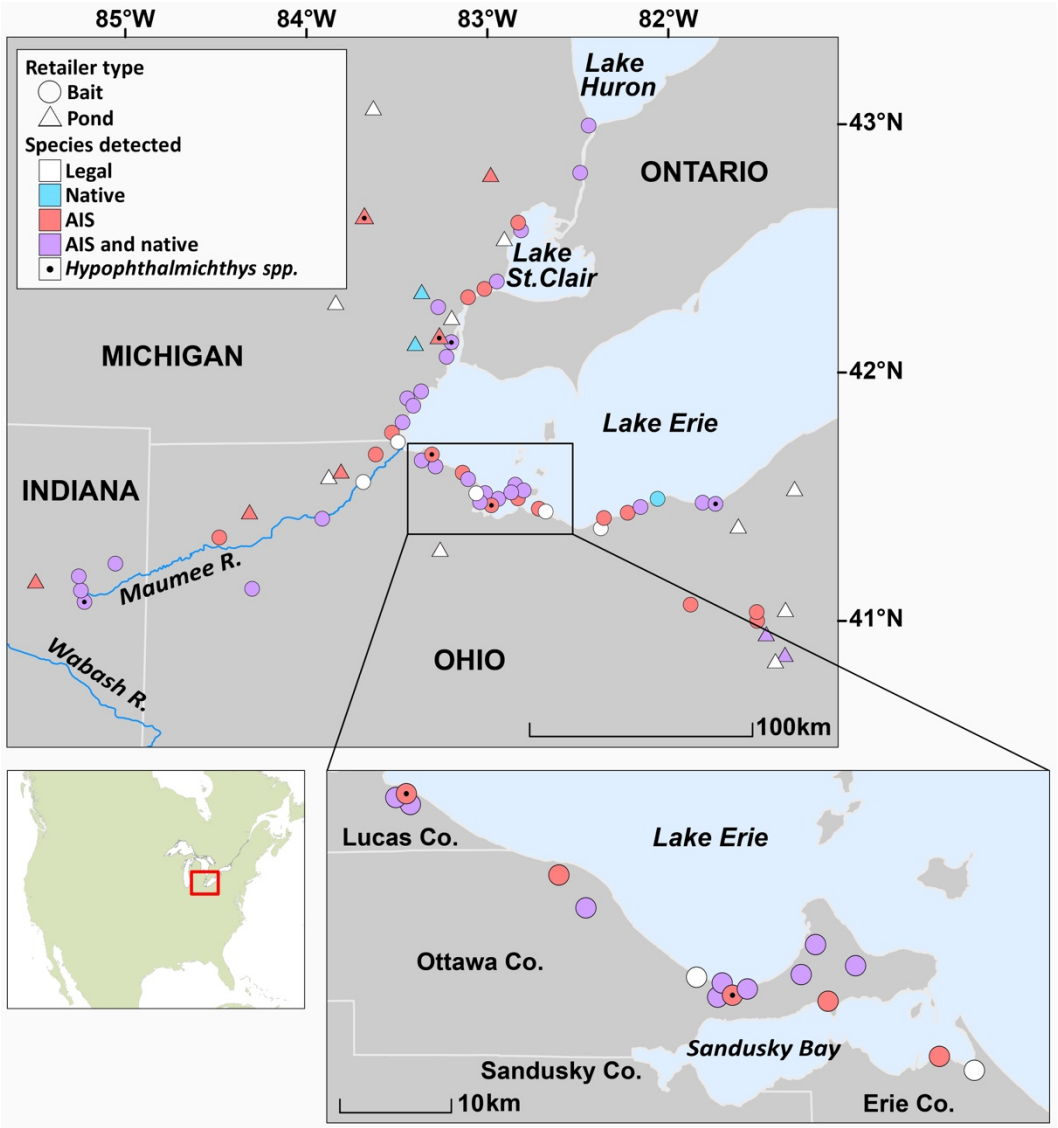


Figure 4–1 Map of bait and pond stores showing eDNA assay results.

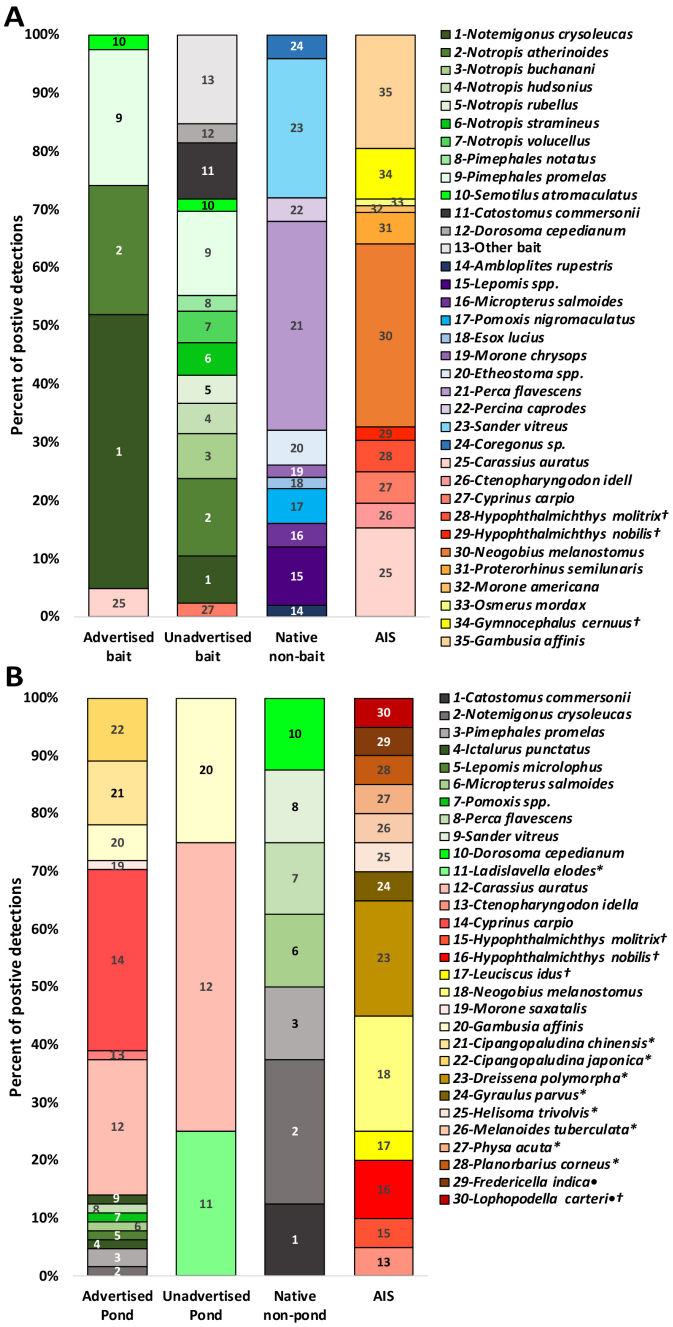


Figure 4–2 Detections of species in (A) bait and (B) pond stores using high-throughput sequencing metabarcode eDNA assays. Legal bait species detections of <2% are combined in the "Other bait" category. Taxa are ordered native to invasive (≥25 in A, ≥12 in B). Only unadvertised AIS are in the AIS column. †=unestablished aquatic invasive species on the GLANSIS watch list, *=Mollusk, •=Bryozoan.

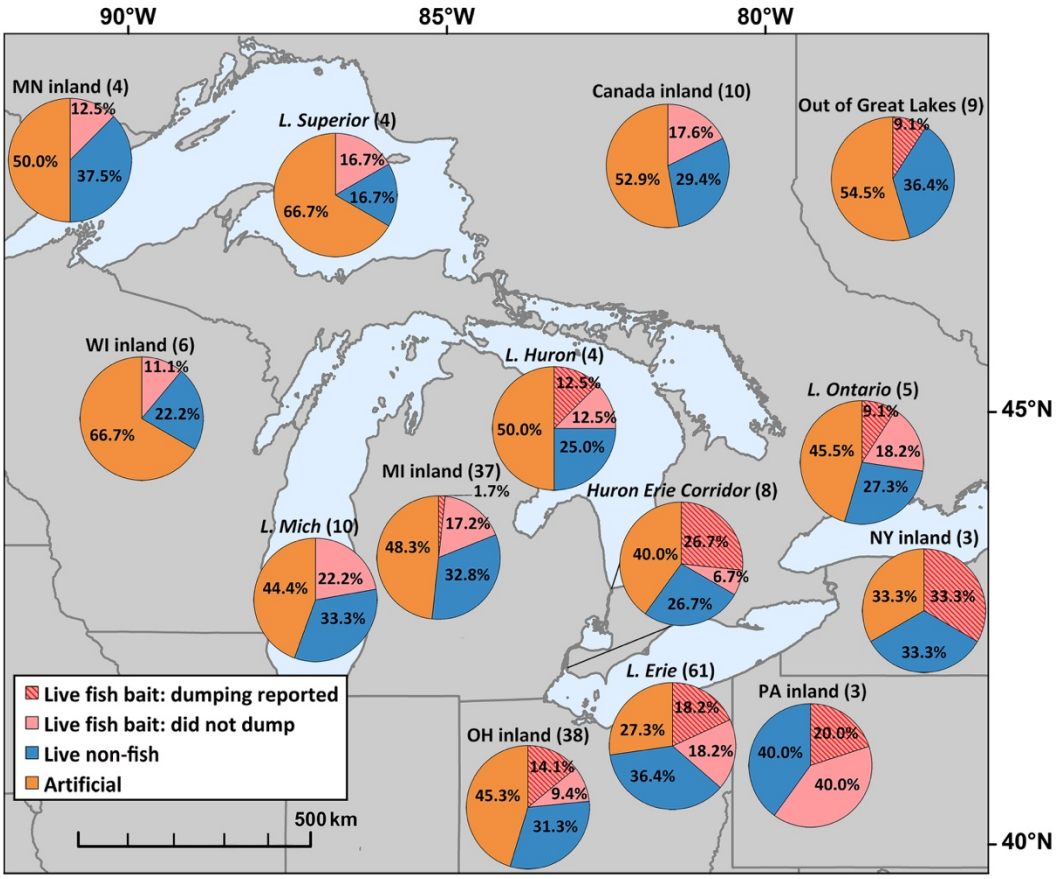


Figure 4–3 Map of angler bait use and reported dumping in the Great Lakes region. Parentheses enclose the number of anglers that reported fishing per location. Pie charts represent proportions of total reported bait uses (many anglers reported using multiple types of bait).

Chapter 5

Conclusions and Future Work

5.1 General Discussion

This dissertation research investigated temporal and spatial patterns in population structure of a high impact invasive species, developed a metabarcode high-throughput sequencing assay method with low error and high detection probability, and applied this pipeline to detect invasive species in a little studied retail vector. Chapter 2 utilized samples collected over two decades (the entire temporal range at the time of the commencement of the work) and a large spatial extent of the round goby to conduct an invasion genetics investigation. Results showed that population structure in an invasive species is dynamic over time, with 'genetic stasis' existing alongside bottlenecks and subsequent recovery of genetic diversity across the range of this species, as also discerned by some partnering studies (Stepien et al. 2013, 2019; Marshall and Stepien 2019b). It is likely that not all introductions of an invasive species originate from the same source population (Brown and Stepien 2009, 2010; Marshall and Stepien 2019b) as was found here, with Lake Erie possessing different cytochrome (cyt) *b* haplotypes indicating contribution from one or more other sources than

the original invasion. It is likely that if the round goby continues to expand its range, similar temporal and spatial patterns of population structure will continue.

Since the invention of high-throughput sequencing (HTS) using the Roche 454 platform in 2005, the number of platforms and rate of throughput have increased, while the cost per sample has declined (Shokralla et al. 2012; Rees et al. 2014). Metabarcode detection of community diversity has become popular, but there is considerable debate concerning its sensitivity and accuracy (Deiner et al. 2017; Zaiko et al. 2018; Evans and Lamberti 2018). Chapter 3 presents a metabarcode survey method and bioinformatic pipeline that discerns a greater proportion of the morphologically sampled and detected species using fewer samples than most other studies (Evans et al. 2017; Bylemans et al. 2018; Lawson Handley et al. 2019). Error was greatly reduced with a custom library preparation procedure and a bioinformatic pipeline that removes cross contamination, index-hops, and sequencing error. Although metabarcoding is not considered an ideal method for detecting single invasive species (Zaiko et al. 2018), this protocol detected more non-natives in more samples than found with traditional morphological sampling.

In Chapter 4, this pipeline was applied to invasive species detection in bait and pond stores. Many more instances of unadvertised legal and illegal native or invasive species were found with the metabarcode HTS assays versus morphological sampling. The proportion of stores with detections of these unadvertised species was similar to those found in other studies that employed genetic methods (Mahon et al. 2014; Nathan et al. 2015) and was much greater than those using morphological surveys (Litvak and Mandrak 1993; Keller and Lodge 2007). Notaly, silver carp was detected in both bait and pond retail stores, which may appear camouflaged with other "minnows" in retail store tanks, as was found in some previous eDNA assays of bait stores (Nathan et al. 2015).

5.2 Genetic variation in populations of invasive species

With the falling cost of HTS and the power of genomic approaches to detect even very fine population structure (Andrews and Luikart 2014), it is likely that the future of studies of this type will use genomic tools. Transcriptomic approaches may be used to investigate selective pressures and gene expression in introduced species (Alvarez et al. 2015). Reduced representation genomic sequencing technologies also yield sufficient data to test many invasion genetics questions at reduced cost and time (Andrews et al. 2016; Bourne et al. 2018). Genomic methods have been used to detect admixture between invasive and native subspecies of cutthroat trout *Oncorhynchus clarkii* in Northwest Montana (Hohenlohe et al. 2013). Unlike the round goby, invasive common myna *Acridotheres tristis* in Australia were shown in a study using reduced representation genomic analyses to be less diverse at the original invasion locations and more diverse where initially separate invasive populations were admixed (Ewart et al. 2019).

These data sets, which are very large in comparison to those of microsatellites, offer ability to screen thousands of loci across the genome for variability, find areas that are neutral or under selection, and use them to test evolutionary theory in non-model organisms (Alvarez et al. 2015; Andrews et al. 2016). Reference genomes can be assembled *de novo*, allowing flexibility in targeting species for investigation (De Wit et al. 2012). With non-native species there is particular potential for these approaches to identify evolutionary processes playing out over the course of an invasion and to understand the role of gene expression in invasiveness (Yu et al. 2012; Rius et al. 2015).

5.3 Metabarcode eDNA assays in the environment

5.3.1 Need to compare and standardize HTS metabarcode assay and bioinformatic protocols

If metabarcode high-throughput sequencing assays are not at least as effective in detecting species in the environment, then they should be carried out alongside traditional capture-based or visual surveys. With high variation among results versus those obtained in traditional surveys (see Chapter 3), consensus on pipelines and protocols needs to be further developed. A large degree of variation exists in DNA capture and extraction methodology and results (Rees et al. 2014) as well as subsequent metabarcode primers, assays, protocols and bioinformatic pipelines (Deiner et al. 2017). It is unlikely that all methods from sampling to library preparation will work with all types of samples, i.e., produce successful libraries and have distinguishing identification power. However, standard guidelines to experimental design including sampling, number of markers used, controls (positive and negative), and bioinformatic pipeline would greatly benefit the community (Zinger et al. 2019). Such standardization would eliminate at least some of the barriers to informative comparisons of results among studies, taxa, and ecosystems.

5.3.2 Improving detection efficiency

A relationship has been observed in many studies between the number of samples collected and the total species richness (number of taxa) detected or the overlap in community composition assessed with metabarcode and traditional surveys (Evans et al. 2017; Dickie et al. 2018; Bylemans et al. 2018; Lawson Handley et al. 2019). Whether distribution of genetic material from different taxa in the environment is random or not, it is simply unlikely that a single 1L water sample collected at a single specific location would

contain DNA from every species present. This is especially true when the ecology of organisms is considered. For example, some organisms are more sedentary (e.g., ambush predators) or occupy the benthos (e.g., darters or catfish). This uneven distribution also likely causes problems in the relationship of biomass to sequence reads. Several researchers have called for increased sampling in metabarcode assays (Goldberg et al. 2016; Balasingham et al. 2018; Lacoursière-Roussel et al. 2018; Zinger et al. 2019). Although HTS offers the depth and potential to pool large numbers of samples on a single run (Shokralla et al. 2012; Illumina 2018), there is still an added cost in resources and time associated with processing a larger number of libraries.

Future studies could lead to simplification and/or standardization of sampling and data processing schemes. A larger number of replicate samples collected at the location of each electrofishing transect in this dissertation research likely would have increased detection efficiency on a per site basis. If collected, one could perform an experiment comparing complete separate processing of individual samples to pooling DNA extractions prior to the first step PCR and/or pooling filters or pellets before extraction. In the absence of a large number of libraries to be sequenced, results from Chapter 3 showed that multiple gene markers that target a wide variation in taxa can detect an appreciable proportion of the species present in much larger traditional sampling efforts.

5.3.3 Quantitative assessment of species

Size fractionation has shown that most eDNA is in whole cells or organelles (Turner et al. 2014). Whether the exact origin of these cells is mucus, epidermis, feces, etc. is understudied, but likely differs among species (Barnes and Turner 2016). The fate of different types of cells/organelles in the environment (i.e., transport, settling, degradation)

likely also varies (Barnes and Turner 2016). Shedding rates are affected by environmental conditions (Poté et al. 2009), age of the organism (Maruyama et al. 2014), and behavior, and varies among species (Klymus et al. 2015). It seems improbable that a single water sample would contain eDNA in the same proportion as the biomass of species in the environment. This is likely why a better relationship was observed when metabarcode results were combined regionally in this dissertation research and further supports more intense sampling per site and regionally.

Several researchers have used internal standards to quantify the number of copies of target markers in environmental samples (Pierce et al., 2012 a,b; Ushio et al. 2018; Hardwick et al. 2018). A similar method was proposed by Dr. Carol Stepien in her USEPA Great Lakes Restoration Initiative Grant for use in the present study. Higher numbers of copies assessed with qPCR have been shown to be positively correlated with biomass (Doi et al. 2015; Gingera et al. 2016; Hinlo et al. 2017). Relative number of copies of a target marker among species would in theory be affected by the exact same factors as proportions of genetic material from species in the environment. These analyses may not produce data that are more informative than relative proportions of sequence reads, which have a weak relationship to biomass of species detected. However, a quantitative assessment of species in a metabarcode assay would increase the possibility of making comparisons between samples about the density of a specific species.

5.3.4 Population genetic data from metabarcode assays

Although improved sampling may result in a better match in haplotypic frequencies between eDNA HTS assays and traditional population genetic methods, technological limitations with respect to error rates currently make it difficult to accurately identify

intraspecific sequence variation using metabarcode assays (Nakamura et al. 2011; Schirmer et al. 2016; Tsuji et al. 2018). Current denoising platforms use only empirically derived errors from the user's data (Callahan et al. 2016; Edgar 2016). Several studies have investigated the observed error profiles on Illumina HTS platforms (Nakamura et al. 2011; Schirmer et al. 2016). A well-trained bioinformatician (likely in collaboration with a statistician) could conceivably use the known/common errors on these platforms to develop a denoising algorithm that can detect and correct these errors as well. This is a project that I would very much like to undertake in the future.

Short read lengths obtained from Illumina HTS platforms also make detecting population variation difficult. For both the silver carp with the CarpCyt*b* assay and round goby with the GobyCyt*b* assay, the most common haplotypes within the North American range of these invasive species were ≤2NT substitutions different from each other. The GobyCyt*b* assay detected no haplotypic variation in the closely related tubenose goby. Knowledge of the potential variation discernable within the sampling range was required in order to design these assays. It is entirely possible that individuals with new real haplotypes were sequenced in this dissertation research, but that this variation was outside the region amplified by the assays.

Longer read lengths up to >20 kilo bases (Kb) are possible using the PacBio (Menlo Park, CA) SMRT sequencing platform. Average error rates sequencing molecules ≥13Kb on this platform are 13–15% (Ardui et al. 2018). Error rates that are even better than 0.1% (average error on Illumina Miseq platform) are achieved with molecules ≤5Kb on the SMRT sequencing platform (Ardui et al. 2018). Size fractionation experiments show that most eDNA is likely present in whole organelles if not single or multiple cells (Turner et al. 2014). Deiner et al. (2017) showed that it is possible to amplify whole mitochondrial genomes of

ichthyofauna from the environment. In their approach, sonication was used to randomly sheer amplicons, and adapters were added with ligation and PCR. Libraries were sequenced on a short-read Illumina platform. They were able to build scaffolds of whole mitogenomes from species but because they may be chimeric in nature, they were not able to conduct comparisons using longer read lengths.

It would be possible to choose one or more restriction enzymes to digest whole mitogenome amplicons (~16Kb in fishes) into three of four smaller molecules or design PCR primers to amplify subsections of the mitochondria ~4–5Kb, which could then be sequenced on a PacBio SMRT sequencer. This would allow *de novo* assessment of population structure from eDNA. If primers were designed to target a small group of taxa or a single species, these data could provide valuable population genetic information, albeit with haplotypic frequencies still potentially affected by uneven distribution/incomplete mixing of genetic material from individuals. If primers that are less taxa specific were used, the length of reads would greatly expand the potential reference database for species identification. To this author's knowledge, no published studies have used PacBio platforms for eDNA analyses of eukaryotes, as has been done for bacterial metabarcoding of the entire 16S rRNA gene (Schloss et al. 2016). This seems to be an underrecognized potential avenue that could facilitate an advance in eDNA data collection and analysis.

Kimmerling et al. (2018) used sonication of genomic DNA followed by PCR and enzymatic ligation of adapters to identify species from bulk larval fish samples collected near a tropical reef in the Gulf of Aqaba in the Red Sea offshore of Jordan. It is possible that this method, which should be free of bias from PCR of initial template, could be modified to target larger molecules and yield population genetic data. It seems unlikely that enough DNA from any single target group of organisms exists in environmental samples for this method

to be successful, but it could be applied to ichthyoplankton or zooplankton samples either with or without purification of mitochondrial DNA (see Azimzadeh et al. 2016).

5.3.5 Metabarcoding in invasive species management

qPCR often is recognized as a potentially more accurate and sensitive method for identifying very rare species in the environment compared to metabarcoding (Rees et al. 2014; Zaiko et al. 2018), although its detection efficiency also is affected by the volume of water processed (Sepulveda et al. 2019). However, some studies, including this dissertation research, have shown that metabarcode assays are more efficient than traditional sampling for discerning rare invasive species (Brown et al. 2016; Marshall and Stepien 2019; Stepien et al. 2019). None of the invasive species found with metabarcode assays in the environment in this dissertation research were outside of their known established range, but often were absent from the morphological sampling data. Increased sampling could possibly result in additional metabarcode findings of non-native species. Even with the use of the pipeline developed here that reduced false positive detections, the best course of action is for managers to conduct more intensive traditional sampling to attempt to confirm the presence of a non-native species discerned with metabarcode assays outside of their known range.

Several states in the USA conduct morphological sampling of bait stores, though discoveries of invasive species are rare (see Chapter 4). To this author's knowledge, no state has a genetic monitoring program for invasive species in the bait or pond trades, though there is an effort at the Michigan DNR to initiate one (Lucas Nathan, MDNR, pers. comm.). It seems unlikely that managers could legally fine a retailer based on an eDNA detection of an invasive species, but if one occurs, more intensive sampling/inspection of the retailer's

stocks could be carried out. With the large number of potential introduction events every year via this vector (see Chapter 4), such a monitoring program may be warranted.

References

Adrian-Kalchhauser I, Burkhardt-Holm P (2016) An eDNA assay to monitor a globally invasive fish species from flowing freshwater. PLOS ONE 11: e0147558. https://doi.org/10.1371/journal.pone.0147558

Adrian-Kalchhauser I, Hirsch PE, Behrmann-Godel J, N'Guyen A, Watzlawczyk S, Gertzen S, Borcherding J, Burkhardt-Holm P (2016) The invasive bighead goby *Ponticola kessleri* displays large-scale genetic similarities and small-scale genetic differentiation in relation to shipping patterns. Molecular Ecology 25: 1925–1943. https://doi.org/10.1111/mec.13595

Alberdi A, Aizpurua O, Gilbert MTP, Bohmann K (2017) Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods in Ecology and Evolution 9: 134–147. https://doi.org/10.1111/2041-210X.12849

Allendorf FW, Luikart GH, Aitken SN (2013) Invasive Species. In: Conservation and the Genetics of Populations, 2 edition. Wiley-Blackwell (Hoboken, NJ): 421–439.

Allendorf FW, Lundquist LL (2003) Introduction: Population Biology, Evolution, and Control of Invasive Species. Conservation Biology 17: 24–30. https://doi.org/10.1046/j.1523-1739.2003.02365.x

Alvarez M, Schrey AW, Richards CL (2015) Ten years of transcriptomics in wild populations: what have we learned about their ecology and evolution? Molecular Ecology 24: 710–725. https://doi.org/10.1111/mec.13055

AMFGLEO (2016) Annual Agency Reports. In: Association of Midwest Fish and Game Law Enforcement Officers. http://static1.squarespace.com/static/53dd062ce4b0461efc568dee/t/57d811c320099e5bb1f2a9d6/1473778201251/2016+AMFGLEO+Agency+Reports.pdf. Accessed 20 Apr 2019

Andrews KR, Good JM, Miller MR, Luikart G, Hohenlohe PA (2016) Harnessing the power of RADseq for ecological and evolutionary genomics. Nature Reviews Genetics 17: 81–92. https://doi.org/10.1038/nrg.2015.28

Andrews KR, Luikart G (2014) Recent novel approaches for population genomics data analysis. Molecular Ecology 23: 1661–1667. https://doi.org/10.1111/mec.12686

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a F_{st}-outlier method. BMC Bioinformatics 9: 323. https://doi.org/10.1186/1471-2105-9-323

Ardui S, Ameur A, Vermeesch JR, Hestand MS (2018) Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. Nucleic Acids Research 46: 2159–2168. https://doi.org/10.1093/nar/gky066

Attrill MJ, Depledge MH (1997) Community and population indicators of ecosystem health: targeting links between levels of biological organisation. Aquatic Toxicology 38: 183–197. https://doi.org/10.1016/S0166-445X(96)00839-9

Azimzadeh P, Asadzadeh Aghdaei H, Tarban P, Akhondi MM, Shirazi A, Khorram Khorshid HR (2016) Comparison of three methods for mitochondria isolation from the human liver cell line (HepG2). Gastroenterology and Hepatology From Bed to Bench 9: 105–113

Baker H, Stebbins G (eds) (1965) The Genetics of Colonizing Species. Academic Press (New York, NY).

Balasingham KD, Walter RP, Mandrak NE, Heath DD (2018) Environmental DNA detection of rare and invasive fish species in two Great Lakes tributaries. Molecular Ecology 27: 112–127. https://doi.org/10.1111/mec.14395

Balmford A, Gaston KJ (1999) Why biodiversity surveys are good value. Nature 398: 204–205. https://doi.org/10.1038/18339

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

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM (2014) Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science & Technology 48: 1819–1827. https://doi.org/10.1021/es404734p

Beebe NW, Ambrose L, Hill LA, Davis JB, Hapgood G, Cooper RD, Russell RC, Ritchie SA, Reimer LJ, Lobo NF, Syafruddin D, van den Hurk AF (2013) Tracing the tiger: population genetics provides valuable insights into the *Aedes* (*Stegomyia*) *albopictus* invasion of the Australasian Region. PLoS neglected tropical diseases 7: e2361. https://doi.org/10.1371/journal.pntd.0002361

Begon M, Townsend CR, Harper JL (2006) Ecology: From Individuals to Ecosystems, 4 edition. Wiley-Blackwell (Malden, MA): 1–750.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. In: Laboratoire Génome,

Populations, Interactions, CNRS UMR 5171. Université de Montpellier II, Montpellier, France. http://kimura.univ-montp2.fr/genetix/. Accessed 25 Jun 2019

Berg DJ, Garton DW, Macisaac HJ, Panov VE, Telesh IV (2002) Changes in genetic structure of North American *Bythotrephes* populations following invasion from Lake Ladoga, Russia. Freshwater Biology 47: 275–282. https://doi.org/10.1046/j.1365-2427.2002.00805.x

Betancur-R. R, Hines A, Acero P. A, Ortí G, Wilbur AE, Freshwater DW (2011) Reconstructing the lionfish invasion: insights into Greater Caribbean biogeography: Reconstructing the lionfish invasion. Journal of Biogeography 38: 1281–1293. https://doi.org/10.1111/j.1365-2699.2011.02496.x

Bista I, Carvalho GR, Walsh K, Seymour M, Hajibabaei M, Lallias D, Christmas M, Creer S (2017) Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nature Communications 8: 14087. https://doi.org/10.1038/ncomms14087

Bourne SD, Hudson J, Holman LE, Rius M (2018) Marine Invasion Genomics: Revealing Ecological and Evolutionary Consequences of Biological Invasions: 1–36. https://doi.org/10.1007/13836_2018_21

Broders HG, Mahoney SP, Montevecchi WA, Davidson WS (1999) Population genetic structure and the effect of founder events on the genetic variability of moose, *Alces alces*, in Canada. Molecular Ecology 8: 1309–1315

Brown EA, Chain FJJ, Zhan A, MacIsaac HJ, Cristescu ME (2016) Early detection of aquatic invaders using metabarcoding reveals a high number of non-indigenous species in Canadian ports. Diversity and Distributions 22: 1045–1059. https://doi.org/10.1111/ddi.12465

Brown JE, Stepien CA (2009) Invasion genetics of the Eurasian round goby in North America: Tracing sources and spread patterns. Molecular Ecology 18: 64–79. https://doi.org/10.1111/j.1365-294X.2008.04014.x

Brown JE, Stepien CA (2010) Population genetic history of the dreissenid mussel invasions: Expansion patterns across North America. Biological Invasions 12: 3687–3710. https://doi.org/10.1007/s10530-010-9763-2

Brown JE, Stepien CA (2008) Ancient divisions, recent expansions: phylogeography and population genetics of the round goby *Apollonia melanostoma*. Molecular Ecology 17: 2598–2615. https://doi.org/10.1111/j.1365-294X.2008.03777.x

Bylemans J, Gleeson DM, Hardy CM, Furlan E (2018a) Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). Ecology and Evolution 8: 8697–8712. https://doi.org/10.1002/ece3.4387

Bylemans J, Gleeson DM, Lintermans M, Hardy CM, Beitzel M, Gilligan DM, Furlan EM (2018b) Monitoring riverine fish communities through eDNA metabarcoding: determining optimal sampling strategies along an altitudinal and biodiversity gradient. Metabarcoding and Metagenomics 2: e30457. https://doi.org/10.3897/mbmg.2.30457

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13: 581–583. https://doi.org/10.1038/nmeth.3869

Cantera I, Cilleros K, Valentini A, Cerdan A, Dejean T, Iribar A, Taberlet P, Vigouroux R, Brosse S (2019) Optimizing environmental DNA sampling effort for fish inventories in tropical streams and rivers. Scientific Reports 9: 3085. https://doi.org/10.1038/s41598-019-39399-5

Cilleros K, Valentini A, Allard L, Dejean T, Etienne R, Grenouillet G, Iribar A, Taberlet P, Vigouroux R, Brosse S (2019) Unlocking biodiversity and conservation studies in high-diversity environments using environmental DNA (eDNA): a test with Guianese freshwater fishes. Molecular Ecology Resources 19: 27–46. https://doi.org/10.1111/1755-0998.12900

Civade R, Dejean T, Valentini A, Roset N, Raymond J-C, Bonin A, Taberlet P, Pont D (2016) Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. PLOS ONE 11: e0157366. https://doi.org/10.1371/journal.pone.0157366

Clapp DF, Schneeberger PJ, Jude DJ, Madison G, Pistis C (2001) Monitoring round goby (*Neogobius melanostomus*) population expansion in eastern and northern Lake Michigan. Journal of Great Lakes Research 27: 335–341. https://doi.org/10.1016/S0380-1330(01)70649-1

Connelly NA, Lauber TB, Stedman RC, Knuth BA (2018) Bait dealers' roles in preventing the spread of aquatic invasive species and fish pathogens in the Great Lakes region. Journal of Great Lakes Research 44: 514–520. https://doi.org/10.1016/j.jglr.2018.04.005

Cooke SJ, Martins EG, Struthers DP, Gutowsky LFG, Power M, Doka SE, Dettmers JM, Crook DA, Lucas MC, Holbrook CM, Krueger CC (2016) A moving target—incorporating knowledge of the spatial ecology of fish into the assessment and management of freshwater fish populations. Environmental Monitoring and Assessment 188: 239. https://doi.org/10.1007/s10661-016-5228-0

Copper M (2017) Erie-area bait shops, anglers search for Lake Erie emerald shiners. In: GoErie.com. https://www.goerie.com/news/20170704/erie-area-bait-shops-anglers-search-for-lake-erie-emerald-shiners, https://www.goerie.com/news/20170704/erie-area-bait-shops-anglers-search-for-lake-erie-emerald-shiners. Accessed 18 Mar 2019

Cowie RH, Robinson DG (2003) Pathways of introduction of nonindigenous land and freshwater snails and slugs. In: Carlton J (ed) Invasive Species: Vectors and Management Strategies. Island Press 93–122.

Cross EE, Rawding RS (2009) Acute thermal tolerance in the round goby, *Apollonia melanostoma* (*Neogobius melanostomus*). Journal of Thermal Biology 34: 85–92. https://doi.org/10.1016/j.jtherbio.2008.10.006

Cuddington K, Currie WJS, Koops MA (2014) Could an Asian carp population establish in the Great Lakes from a small introduction? Biological Invasions 16: 903–917. https://doi.org/10.1007/s10530-013-0547-3

Darling JA, Bagley MJ, Roman J, Tepolt CK, Geller JB (2008) Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. Molecular Ecology 17: 4992–5007. https://doi.org/10.1111/j.1365-294X.2008.03978.x

Darling JA, Mahon AR (2011) From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research 111: 978–988. https://doi.org/10.1016/j.envres.2011.02.001

De Wit P, Pespeni MH, Ladner JT, Barshis DJ, Seneca F, Jaris H, Therkildsen NO, Morikawa M, Palumbi SR (2012) The simple fool's guide to population genomics via RNA-Seq: an introduction to high-throughput sequencing data analysis. Molecular Ecology Resources 12: 1058–1067. https://doi.org/10.1111/1755-0998.12003

Deiner K, Altermatt F (2014) Transport Distance of Invertebrate Environmental DNA in a Natural River. PLOS ONE 9: e88786. https://doi.org/10.1371/journal.pone.0088786

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

Deiner K, Renshaw MA, Li Y, Olds BP, Lodge DM, Pfrender ME (2017b) Long-range PCR allows sequencing of mitochondrial genomes from environmental DNA. Methods in Ecology and Evolution 8: 1888–1898. https://doi.org/10.1111/2041-210X.12836

DeVanna KM, Bodamer BL, Wellington CG, Hammer E, Mayer CM, Bossenbroek JM (2011) An alternative hypothesis to invasional meltdown in the Laurentian Great Lakes region: General facilitation by *Dreissena*. Journal of Great Lakes Research 37: 632–641. https://doi.org/10.1016/j.jglr.2011.07.005

Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE, Powell JR, Weaver L (2018) Towards robust and repeatable sampling methods in eDNA-based studies. https://doi.org/10.1111/1755-0998.12907

Dobson AP, Rodriguez JP, Roberts WM, Wilcove DS (1997) Geographic distribution of endangered species in the United States. Science 275: 550–553. https://doi.org/10.1126/science.275.5299.550

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

Dufour BA, Hogan TM, Heath DD (2007) Ten polymorphic microsatellite markers in the invasive round goby (*Neogobius melanostomus*) and cross-species amplification. Molecular Ecology Notes 7: 1205–1207. https://doi.org/10.1111/j.1471-8286.2007.01833.x

Duggan IC (2010) The freshwater aquarium trade as a vector for incidental invertebrate fauna. Biological Invasions 12: 3757–3770. https://doi.org/10.1007/s10530-010-9768-x

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359–361. https://doi.org/10.1007/s12686-011-9548-7

Edgar RC (2016) UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing: 081257. https://doi.org/10.1101/081257

Egan D (2017) Shiner shortage has bait shops all in a tangle. In: Crain's Cleveland Business. https://www.crainscleveland.com/article/20170701/news/170709996/shiner-shortage-has-bait-shops-all-tangle. Accessed 18 Mar 2019

Elton CS (1966) The Pattern of Animal Communities. Springer Netherlands (Rotterdam, Netherlands):

Elton CS (1958) The Ecology of Invasions by Animals and Plants, New edition. University of Chicago Press (Chicago): 1–222.

Embke HS, Kocovsky PM, Richter CA, Pritt JJ, Mayer CM, Qian SS (2016) First direct confirmation of grass carp spawning in a Great Lakes tributary. Journal of Great Lakes Research 42: 899–903. https://doi.org/10.1016/j.jglr.2016.05.002

Erickson RA, Rees CB, Coulter AA, Merkes CM, McCalla SG, Touzinsky KF, Walleser L, Goforth RR, Amberg JJ (2016) Detecting the movement and spawning activity of bigheaded carps with environmental DNA. Molecular Ecology Resources 16: 957–965. https://doi.org/10.1111/1755-0998.12533

Estévez RA, Anderson CB, Pizarro JC, Burgman MA (2015) Clarifying values, risk perceptions, and attitudes to resolve or avoid social conflicts in invasive species management. Conservation Biology 29: 19–30. https://doi.org/10.1111/cobi.12359

Eva B, Harmony P, Thomas G, Francois G, Alice V, Claude M, Tony D (2016) Trails of river monsters: Detecting critically endangered Mekong giant catfish *Pangasianodon gigas* using environmental DNA. Global Ecology and Conservation 7: 148–156. https://doi.org/10.1016/j.gecco.2016.06.007

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Evans NT, Lamberti GA (2018) Freshwater fisheries assessment using environmental DNA: A primer on the method, its potential, and shortcomings as a conservation tool. Fisheries Research 197: 60–66. https://doi.org/10.1016/j.fishres.2017.09.013

Evans NT, Li Y, Renshaw MA, Olds BP, Deiner K, Turner CR, Jerde CL, Lodge DM, Lamberti GA, Pfrender ME (2017) Fish community assessment with eDNA metabarcoding: effects of sampling design and bioinformatic filtering. Canadian Journal of Fisheries and Aquatic Sciences 74: 1362–1374. https://doi.org/10.1139/cjfas-2016-0306

Ewart KM, Griffin AS, Johnson RN, Kark S, Cohen TM, Lo N, Major RE (2019) Two speed invasion: assisted and intrinsic dispersal of common mynas over 150 years of colonization. Journal of Biogeography 46: 45–57. https://doi.org/10.1111/jbi.13473

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM, Ravel J (2014) An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2: 1–6. https://doi.org/10.1186/2049-2618-2-6

Feldheim KA, Willink P, Brown JE, Murphy DJ, Neilson ME, Stepien CA (2009) Microsatellite loci for Ponto-Caspian gobies: markers for assessing exotic invasions. Molecular Ecology Resources 9: 639–644. https://doi.org/10.1111/j.1755-0998.2008.02495.x

Ficetola, Claude M, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biology Letters 4: 423–425. https://doi.org/10.1098/rsbl.2008.0118

Foll M, Gaggiotti O (2008) A genome scan method to identify selected loci appropriate for both dominant and codominant markers: A Bayesian perspective. https://doi.org/10.1534/genetics.108.092221

Forsman A (2014) Effects of genotypic and phenotypic variation on establishment are important for conservation, invasion, and infection biology. Proceedings of the

National Academy of Sciences 111: 302–307. https://doi.org/10.1073/pnas.1317745111

Fraser CI, Thiel M, Spencer HG, Waters JM (2010) Contemporary habitat discontinuity and historic glacial ice drive genetic divergence in Chilean kelp. BMC evolutionary biology 10: 203. https://doi.org/10.1186/1471-2148-10-203

Fremier AK, Strickler KM, Parzych J, Powers S, Goldberg CS (2019) Stream Transport and Retention of Environmental DNA Pulse Releases in Relation to Hydrogeomorphic Scaling Factors. https://doi.org/10.1021/acs.est.8b06829

Frimodt C, Dore I (1995) Multilingual Illustrated Guide to the World's Commercial Coldwater Fish, 1 edition. Wiley-Blackwell (Farnham, Surrey): 1–264.

Fujii K, Doi H, Matsuoka S, Nagano M, Sato H, Yamanaka H (2019) Environmental DNA metabarcoding for fish community analysis in backwater lakes: a comparison of capture methods. PLOS ONE 14: e0210357. https://doi.org/10.1371/journal.pone.0210357

Fukumoto S, Ushimaru A, Minamoto T (2015) A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. Journal of Applied Ecology 52: 358–365. https://doi.org/10.1111/1365-2664.12392

Fuller P, Benson A, Maynard E, Neil;son ME, Larson J, Fusaro A (2019) *Neogobius melanostomus*. In: USGS Nonindigenous Aquatic Species Database, Gainesville, FL. http://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=713. Accessed 25 Jun 2019

Gillet B, Cottet M, Destanque T, Kue K, Descloux S, Chanudet V, Hughes S (2018) Direct fishing and eDNA metabarcoding for biomonitoring during a 3-year survey significantly improves number of fish detected around a South East Asian reservoir. PLOS ONE 13: e0208592. https://doi.org/10.1371/journal.pone.0208592

Gingera TD, Steeves TB, Boguski DA, Whyard S, Li W, Docker MF (2016) Detection and identification of lampreys in Great Lakes streams using environmental DNA. Journal of Great Lakes Research 42: 649–659. https://doi.org/10.1016/j.jglr.2016.02.017

GLFC (2019) The Great Lakes fishery: a world-class resource! In: Great Lakes Fishery Commission. http://www.glfc.org/the-fishery.php. Accessed 18 Feb 2019

GLFC (2018) Asian carp. In: Great Lakes Fishery Commission. http://www.glfc.org/asian-carp.php. Accessed 18 Feb 2019

Goldberg CS, Supulveda A, Ray A (2013) Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science 32: 792–800. https://doi.org/10.1899/13-046.1

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE, Taberlet P (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7: 1299–1307. https://doi.org/10.1111/2041-210X.12595

Goodwin AE, Peterson JE, Meyers TR, Money DJ (2004) Transmission of exotic fish viruses. Fisheries 29: 19–23. https://doi.org/10.1577/1548-8446(2004)29[19:TOEFV]2.0.CO;2

Goudet J (1995) FSTAT (Version 1.2): A Computer Program to Calculate *F*-Statistics. Journal of Heredity 86: 485–486. https://doi.org/10.1093/oxfordjournals.jhered.a111627

Hänfling B, Handley LL, Read DS, Hahn C, Li J, Nichols P, Blackman RC, Oliver A, Winfield IJ (2016) Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. Molecular Ecology 25: 3101–3119. https://doi.org/10.1111/mec.13660

Haponski AE, Dean H, Blake BE, Stepien CA (2014) Genetic history of walleyes spawning in Lake Erie's Cattaraugus Creek: a comparison of pre- and poststocking. Transactions of the American Fisheries Society 143: 1295–1307. https://doi.org/10.1080/00028487.2014.935477

Hardwick SA, Chen WY, Wong T, Kanakamedala BS, Deveson IW, Ongley SE, Santini NS, Marcellin E, Smith MA, Nielsen LK, Lovelock CE, Neilan BA, Mercer TR (2018) Synthetic microbe communities provide internal reference standards for metagenome sequencing and analysis. Nature Communications 9: 3096. https://doi.org/10.1038/s41467-018-05555-0

Hebert PDN, Muncaster BW, Mackie GL (1989) Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 46: 1587–1591. https://doi.org/10.1139/f89-202

Heckman RA, Greger PD, Furtek RC (1993) The Asian fish tapeworm, *Bothriocephalus acheilognathi*, in fishes from Nevada. Journal of the Helminthological Society of Washington 60: 127–128

Hedrick PW (1995) Genetic polymorphism in a temporally varying environment: effects of delayed germination or diapause. Heredity 75: 164. https://doi.org/10.1038/hdy.1995.119

Hensler SR, Jude DJ (2007) Diel vertical migration of round goby larvae in the Great Lakes. Journal of Great Lakes Research 33: 295–302. https://doi.org/10.3394/0380-1330(2007)33[295:DVMORG]2.0.CO;2

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58: 247–276. https://doi.org/10.1111/j.1095-8312.1996.tb01434.x

Hinlo R, Furlan E, Suitor L, Gleeson D (2017) Environmental DNA monitoring and management of invasive fish: Comparison of eDNA and fyke netting. Management of Biological Invasions 8: 89–100. https://doi.org/10.3391/mbi.2017.8.1.09

Hohenlohe PA, Day MD, Amish SJ, Miller MR, Kamps-Hughes N, Boyer MC, Muhlfeld CC, Allendorf FW, Johnson EA, Luikart G (2013) Genomic patterns of introgression in rainbow and westslope cutthroat trout illuminated by overlapping paired-end RAD sequencing. Molecular Ecology 22: 3002–3013. https://doi.org/10.1111/mec.12239

Hubbs CL, Lagler KF (2007) Fishes of the Great Lakes Region, Revised Edition. University of Michigan Press (Ann Arbor, Michigan):

Huey R, Gilchrist G, Hendry A (2005) Using invasive species to study evolution: Case studies with Drosophila and salmon. In: Sax DF, Stachowicz JJ, Gaines SD (eds) Species Invasions: Insights into Ecology, Evolution, and Biogeography. Sinauer Associates Inc. (Sunderland, MA): 139–164.

IDNR (2019a) Indiana bait dealer's license regulations. In: Indiana Department of Natural Resources. https://www.in.gov/dnr/fishwild/files/fw-Bait_Dealer_Regs.pdf. Accessed 20 Apr 2019

IDNR (2019b) Indiana fish pond management. In: Indiana Department of Natural Resources. https://www.in.gov/dnr/fishwild/files/fishmgt.pdf. Accessed 20 Apr 2019

Illumina (2018) Nextera XT DNA Library Prep Kit Reference Guide. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-03.pdf. Accessed 20 Apr 2019

Imtiaz A, Nor SAM, Naim DM (2017) Review: Progress and potential of DNA barcoding for species identification of fish species. Biodiversitas: Journal of Biological Diversity 18: 1394–1405. https://doi.org/10.13057/biodiv/d180415

Indiana (2014) Article 9. Fish and Wildlife. In: Indiana Administrative Code. http://www.in.gov/legislative/iac/T03120/A00090.PDF? Accessed 20 Apr 2019

Irons KS, Sass GG, McClelland MA, Stafford JD (2007) Reduced condition factor of two native fish species coincident with invasion of non-native Asian carps in the Illinois River, U.S.A. Is this evidence for competition and reduced fitness? Journal of Fish Biology 71: 258–273. https://doi.org/10.1111/j.1095-8649.2007.01670.x

Jackson DJ (2002) Ecological effects of *Micropterus* introductions: the dark side of black bass. In: Phillip DP, Ridgway MS (eds) Black Bass: Ecology, Conservation, and management221–232.

Janssen J, Jude DJ (2001) Recruitment failure of mottled sculpin *Cottus bairdi* in Calumet Harbor, southern Lake Michigan, induced by the newly Introdiced round goby *Neogobius melanostomus*. Journal of Great Lakes Research 27: 319–328. https://doi.org/10.1016/S0380-1330(01)70647-8

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4: 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jo T, Murakami H, Yamamoto S, Masuda R, Minamoto T (2019) Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. Ecology and Evolution 9: 1135–1146. https://doi.org/10.1002/ece3.4802

Johannsson OE, Mills EL, O'Gorman R (1991) Changes in the nearshore and offshore zooplankton communities in Lake Ontario: 1981-88. Canadian Journal of Fisheries and Aquatic Sciences 48: 1546–1557. https://doi.org/10.1139/f91-183

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources 10: 551–555. https://doi.org/10.1111/j.1755-0998.2009.02787.x

Jude DJ, Janssens J, Crawford G (1995) Ecology, distribution, and impact of the newly introduced round & tubenose gobies on the biota of the St. Clair & Detroit Rivers. In: Munawar M, Edsall T, Leach J (eds) The Lake Huron Ecosystem: Ecology, Fisheries and Management. Ecovision World Monograph Series447–460.

Jude DJ, Reider RH, Smith GR (1992) Establishment of Gobiidae in the Great Lakes Basin. Canadian Journal of Fisheries and Aquatic Sciences 49: 416–421. https://doi.org/10.1139/f92-047

Karsiotis SI, Pierce LR, Brown JE, Stepien CA (2012) Salinity tolerance of the invasive round goby: Experimental implications for seawater ballast exchange and spread to North American estuaries. Journal of Great Lakes Research 38: 121–128. https://doi.org/10.1016/j.jglr.2011.12.010

Kekkonen J (2016) Temporal genetic monitoring of declining and invasive wildlife populations: current state and future directions. In: Angelici FM (ed) Problematic Wildlife: A Cross-Disciplinary Approach. Springer International Publishing (Cham): 269–294.

Keller RP, Lodge DM (2007) Species invasions from commerce in live aquatic organisms: problems and possible solutions. BioScience 57: 428–436. https://doi.org/10.1641/B570509

Kelly RP, Closek CJ, O'Donnell JL, Kralj JE, Shelton AO, Samhouri JF (2017) Genetic and manual urvey methods yield different and complementary views of an ecosystem. Frontiers in Marine Science 3. https://doi.org/10.3389/fmars.2016.00283

Kelly RP, Gallego R, Jacobs-Palmer E (2018) The effect of tides on nearshore environmental DNA. PeerJ 6: e4521. https://doi.org/10.7717/peerj.4521

Kelly RP, Port JA, Yamahara KM, Crowder LB (2014) Using environmental DNA to census marine fishes in a large mesocosm. PLOS ONE 9: e86175. https://doi.org/10.1371/journal.pone.0086175

Khodakov D, Wang C, Zhang DY (2016) Diagnostics based on nucleic acid sequence variant profiling: PCR, hybridization, and NGS approaches. Advanced Drug Delivery Reviews 105: 3–19. https://doi.org/10.1016/j.addr.2016.04.005

Kilian JV, Klauda RJ, Widman S, Kashiwagi M, Bourquin R, Weglein S, Schuster J (2012) An assessment of a bait industry and angler behavior as a vector of invasive species. Biological Invasions 14: 1469–1481. https://doi.org/10.1007/s10530-012-0173-5

Kinziger AP, Nakamoto RJ, Harvey BC (2014) Local-scale invasion pathways and small founder numbers in introduced Sacramento pikeminnow (*Ptychocheilus grandis*). Conservation Genetics 15: 1–9. https://doi.org/10.1007/s10592-013-0516-5

Klymus KE, Marshall NT, Stepien CA (2017) Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. PLOS ONE 12: e0177643. https://doi.org/10.1371/journal.pone.0177643

Kolar C, Chapman D, Courtenay W, Housel C, Williams J, Jennings D (2005) Asian carps of the genus *Hypophthalmichthys* (Pisces, Cyprinidae) — a biological synopsis and environmental risk assessment. In: National Invasive Species Council materials. http://digitalcommons.unl.edu/natlinvasive/5

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

Kolar CS, Lodge DM (2002) Ecological predictions and risk assessment for alien fishes in North America. Science 298: 1233–1236. https://doi.org/10.1126/science.1075753

Kolbe JJ, Glor RE, Schettino LR, Lara AC, Larson A, Losos JB (2004) Genetic variation increases during biological invasion by a Cuban lizard. Nature 431: 177. https://doi.org/10.1038/nature02807

Kornis MS, Mercado-Silva N, Zanden MJV (2012) Twenty years of invasion: a review of round goby *Neogobius melanostomus* biology, spread and ecological implications. Journal of Fish Biology 80: 235–285. https://doi.org/10.1111/j.1095-8649.2011.03157.x

Kvach Y, Stepien CA (2008) Metazoan parasites of introduced round and tubenose gobies in the Great Lakes: support for the "enemy release hypothesis." Journal of Great Lakes Research 34: 23–35. https://doi.org/10.3394/0380-1330(2008)34[23:MPOIRA]2.0.CO;2

Lacoursière-Roussel A, Howland K, Normandeau E, Grey EK, Archambault P, Deiner K, Lodge DM, Hernandez C, Leduc N, Bernatchez L (2018) eDNA metabarcoding as a new surveillance approach for coastal Arctic biodiversity. Ecology and Evolution 8: 7763–7777. https://doi.org/10.1002/ece3.4213

Lawson Handley L, Read DS, Winfield IJ, Kimbell H, Johnson H, Li J, Hahn C, Blackman R, Wilcox R, Donnelly R, Szitenberg A, Hänfling B (2019) Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. Environmental DNA 1: 26–39. https://doi.org/10.1002/edn3.5

Le Roux JJ, Wieczorek AM, Meyer J-Y (2008) Genetic diversity and structure of the invasive tree *Miconia calvescens* in Pacific islands. Diversity and Distributions 14: 935–948. https://doi.org/10.1111/j.1472-4642.2008.00504.x

LED MDNR (2017) 2017 Annual Programs Report. In: Law Enforcement Division of the Michigan Department of Natural Resources. https://www.michigan.gov/documents/dnr/ledannualrprt_598700_7.pdf. Accessed 20 Apr 2019

Lemic D, Mikac KM, Bažok R (2013) Historical and contemporary population genetics of the invasive western corn rootworm (Coleoptera: Chrysomelidae) in Croatia. Environmental Entomology 42: 811–819. https://doi.org/10.1603/EN12351

Leung B, Lodge DM, Finnoff D, Shogren JF, Lewis MA, Lamberti G (2002) An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species. Proceedings of the Royal Society B: Biological Sciences 269: 2407–2413. https://doi.org/10.1098/rspb.2002.2179

Lewontin RC (1974) The Genetic Basis of Evolutionary Change, 1st US-1st Printing edition. Columbia Univ Pr (New York): 1–352.

Litvak MK, Mandrak NE (1993) Ecology of freshwater baitfish use in Canada and the United States. Fisheries 18: 6–13. https://doi.org/10.1577/1548-8446(1993)018<0006:EOFBUI>2.0.CO;2

Lockwood JL, Hoopes MF, Marchetti MP (2013a) Transport vectors and pathways. In: Invasion Ecology, 2nd Edition. Wiley-Blackwell (West Sussex, UK): 24–49.

Lockwood JL, Hoopes MF, Marchetti MP (2013b) Propagules. In: Invasion Ecology, 2nd Edition. Wiley-Blackwell (West Sussex, UK): 74–98.

Lockwood JL, Hoopes MF, Marchetti MP (2013c) An introduction to invasion ecology. In: Invasion Ecology, 2nd Edition. Wiley-Blackwell (West Sussex, UK): 1–23.

Lockwood JL, Hoopes MF, Marchetti MP (2013d) Evolution of invaders. In: Invasion Ecology, 2nd Edition. Wiley-Blackwell (West Sussex, UK): 277–298.

Lodge DM, Finnoff D (2008) Annual losses to Great Lakes region by ship-borne invasive species at least $200 million. In: Invasive Species in the Great Lakes: Costing Us Our Future. https://www.invasive.org/gist/products/library/lodge_factsheet.pdf. Accessed 20 Apr 2019

Ludwig HR, Leitch JA (1996) Interbasin transfer of aquatic biota via anglers' bait buckets. Fisheries 21: 14–18. https://doi.org/10.1577/1548-8446(1996)021<0014:ITOABV>2.0.CO;2

MacConaill LE, Burns RT, Nag A, Coleman HA, Slevin MK, Giorda K, Light M, Lai K, Jarosz M, McNeill MS, Ducar MD, Meyerson M, Thorner AR (2018) Unique, dual-indexed sequencing adapters with UMIs effectively eliminate index cross-talk and significantly improve sensitivity of massively parallel sequencing. BMC Genomics 19: 30. https://doi.org/10.1186/s12864-017-4428-5

MacInnis AJ, Corkum LD (2000) Fecundity and reproductive season of the round goby *Neogobius melanostomus* in the upper Detroit River. Transactions of the American Fisheries Society 129: 136–144. https://doi.org/10.1577/1548-8659(2000)129<0136:FARSOT>2.0.CO;2

Mahon AR, Nathan LR, Jerde CL (2014) Meta-genomic surveillance of invasive species in the bait trade. Conservation Genetics Resources 6: 563–567. https://doi.org/10.1007/s12686-014-0213-9

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209–220

Margules CR, Pressey RL, Williams PH (2002) Representing biodiversity: Data and procedures for identifying priority areas for conservation. Journal of Biosciences 27: 309–326. https://doi.org/10.1007/BF02704962

Marshall NT, Stepien CA (2019a) Invasion genetics from eDNA and thousands of larvae: A targeted metabarcoding assay that distinguishes species and population variation of zebra and quagga mussels: 1–24. https://doi.org/10.1002/ece3.4985

Marshall NT, Stepien CA (2019b) Invasion genetics from eDNA and thousands of larvae: a targeted metabarcoding assay that distinguishes species and population variation of zebra and quagga mussels. Ecology and Evolution 9: 3515–3538. https://doi.org/10.1002/ece3.4985

Martin M (2011) Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. EMBnet.journal 17: 10–12. https://doi.org/10.14806/ej.17.1.200

Maruyama A, Nakamura K, Yamanaka H, Kondoh M, Minamoto T (2014) The release rate of environmental DNA from juvenile and adult fish. PloS One 9: e114639. https://doi.org/10.1371/journal.pone.0114639

May B, Marsden JE (1992) Genetic identification and implications of another invasive species of dreissenid mussel in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 49: 1501–1506. https://doi.org/10.1139/f92-166

MDEQ (2017) Building a pond in Michigan. In: Michigan Department of Environmental Quality. https://www.michigan.gov/documents/deq/wrd-permit-pond_559023_7.pdf. Accessed 20 Apr 2019

MDNR (2016) Minnow, wiggler, and crayfish regulations for commercial purposes and waters that are closed to the personal take of minnows. In: Michigan Department of Natural Resources. https://www.michigan.gov/documents/dnr/FO-216-07_182409_7.pdf. Accessed 20 Apr 2019

MDNR (1999) Building and managing ponds. In: Michigan Department of Natural resources. https://www.michigandnr.com/publications/pdfs/huntingwildlifehabitat/landowners_guide/Habitat_Mgmt/Wetland/Building_Managing_Ponds.htm. Accessed 20 Apr 2019

Meyer FP (1991) Aquaculture disease and health management. Journal of Animal Science 69: 4201–4208. https://doi.org/10.2527/1991.69104201x

Michigan (1996) Michigan Aquaculture Development Act

Miles RD, Chapman FA (2006) The benefits of fish meal in aquaculture diets. In: University of Florida Intitute of Food and Agriculture Sciences. http://edis.ifas.ufl.edu/pdffiles/fa/fa12200.pdf. Accessed 20 Apr 2019

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

Morin PJ (2009) Community Ecology. John Wiley & Sons (Oxford, UK): 1–418.

Moskal'kova K (1996) Ecological and morphophysiological prerequisites to range extention in the round goby Neogobius melanostomus under conditions of anthropogenic pollution. Journal of Ichthyology 36: 584–590

Müllner D (2013) Fastcluster: fast hierarchical, agglomerative clustering routines for R and Python. Journal of Statistical Software 53: 1–18. https://doi.org/10.18637/jss.v053.i09

Muzzall PM, Peebles CR, Thomas MV (1995) Parasites of the round goby, *Neogobius melanostomus*, and tubenose goby, *Proterorhinus marmoratus* (Perciformes: Gobiidae), from the St. Clair River and Lake St. Clair, Michigan. Journal of the Helminthological Society of Washington 62: 226–228

Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB da, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403: 853. https://doi.org/10.1038/35002501

Nakamura K, Oshima T, Morimoto T, Ikeda S, Yoshikawa H, Shiwa Y, Ishikawa S, Linak MC, Hirai A, Takahashi H, Altaf-Ul-Amin M, Ogasawara N, Kanaya S (2011) Sequence-specific error profile of Illumina sequencers. Nucleic Acids Research 39: e90–e90. https://doi.org/10.1093/nar/gkr344

Nathan LR, Jerde CL, Budny ML, Mahon AR (2015) The use of environmental DNA in invasive species surveillance of the Great Lakes commercial bait trade. Conservation Biology 29: 430–439. https://doi.org/10.1111/cobi.12381

Nathan LR, Jerde CL, McVeigh M, Mahon AR (2014) An assessment of angler education and bait trade regulations to prevent invasive species introductions in the Laurentian Great Lakes. Management of Biological Invasions 5: 319–326

Neilson ME, Stepien CA (2009) Escape from the Ponto-Caspian: Evolution and biogeography of an endemic goby species flock (Benthophilinae: Gobiidae: Teleostei). Molecular Phylogenetics and Evolution 52: 84–102. https://doi.org/10.1016/j.ympev.2008.12.023

NOAA (2019) GLANSIS – Great Lakes Aquatic Nonindigenous Species Information System. https://www.glerl.noaa.gov/glansis/. Accessed 20 Apr 2019

ODNR (2019a) Aquaculture law digest. Ohio Department of Natural Resources Division of Wildlife

ODNR (2019b) Ohio pond management handbook, a guide to managing ponds for fishing and attracting wildlife. Ohio Department of Natural Resources Division of Wildlife

OEPA (2014) Biological and Water Quality Study of the Maumee River and Auglaize River 2012–2013. In: Ohio EPA Division of Surface Water. https://www.epa.ohio.gov/Portals/35/documents/MaumeeTSD_2014.pdf

OEPA (2015) Biological Criteria for the Protection of Aquatic Life: Volume III. Standardized Biological Field Sampling and Laboratory Methods for Assessing Fish and Macroinvertebrate Communities. In: Ohio EPA Division of Surface Water. https://epa.ohio.gov/portals/35/documents/BioCrit15_Vol3.pdf#page41

Ogle DH (1998) A synopsis of the biology and life history of ruffe. Journal of Great Lakes Research 24: 170–185. https://doi.org/10.1016/S0380-1330(98)70811-1

Ohio (2016) 1501:31-13-04 Bait and bait dealers. In: Ohio Division of Wildlife. http://codes.ohio.gov/oac/1501:31-13-04v1. Accessed 20 Apr 2019

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H (2019) Package 'vegan.' In: CRAN. https://cran.ism.ac.jp/web/packages/vegan. Accessed 20 Apr 2019

Padilla DK, Williams SL (2004) Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. Frontiers in Ecology and the Environment 2: 131–138. https://doi.org/10.1890/1540-9295(2004)002[0131:BBWAAO]2.0.CO;2

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Molecular Ecology 13: 55–65

Parker AD, Glover DC, Finney ST, Rogers PB, Stewart JG, Simmonds RL (2015) Direct observations of fish incapacitation rates at a large electrical fish barrier in the Chicago Sanitary and Ship Canal. Journal of Great Lakes Research 41: 396–404. https://doi.org/10.1016/j.jglr.2015.03.004

Parsons KM, Everett M, Dahlheim M, Park L (2018) Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. Royal Society Open Science 5: 180537. https://doi.org/10.1098/rsos.180537

Phillips BL, Brown GP, Shine R (2010) Evolutionarily accelerated invasions: the rate of dispersal evolves upwards during the range advance of cane toads. Journal of Evolutionary Biology 23: 2595–2601. https://doi.org/10.1111/j.1420-9101.2010.02118.x

Pierce LR, Stepien CA (2012) Evolution and biogeography of an emerging quasispecies: Diversity patterns of the fish Viral Hemorrhagic Septicemia virus (VHSv). Molecular Phylogenetics and Evolution 63: 327–341. https://doi.org/10.1016/j.ympev.2011.12.024

Pierce, L.R., J.C. Willey, E.L. Crawford, D.W. Leaman, V.V. Palsule, M. Faisal, R.K. Kim, B.S. Shepherd & C.A. Stepien. 2013. A new StaRT-PCR approach to detect and quantify fish Viral Hemorrhagic Septicemia virus (VHSv): Enhanced quality control with internal standards. *Journal of Virological Methods.* 189:129-142. https://doi.org/*10.1016/j.jviromet.2013.01.006*

Pierce, L.R., J.C. Willey, E.L. Crawford, V.V. Palsule, B.S. Shepherd & C.A. Stepien. 2013. Accurate detection and quantification of the fish Viral Hemorrhagic Septicemia virus (VHSv) with a two-color fluorometric real-time PCR assay. *PLoS One.* 8(8): e71851. https://doi.org/*10.1371/journal.pone.0071851*

Pinto HA, Melo ALD (2011) A checklist of trematodes (Platyhelminthes) transmitted by *Melanoides tuberculata* (Mollusca: Thiaridae). Zootaxa 2799: 15–28. https://doi.org/10.11646/zootaxa.2799.1.2

Piry S, Alapetite A, Cornuet J-M, Paetkau D, Baudouin L, Estoup A (2004) GENECLASS2: a software for genetic assignment and first-generation migrant detection. The Journal of Heredity 95: 536–539. https://doi.org/10.1093/jhered/esh074

Pochon X, Zaiko A, Fletcher LM, Laroche O, Wood SA (2017) Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. PLOS ONE 12: e0187636. https://doi.org/10.1371/journal.pone.0187636

Pointier J-P, Augustin D (1999) Biological control and invading freshwater snails.A case study. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 322: 1093–1098. https://doi.org/10.1016/S0764-4469(99)00108-0

Pont D, Rocle M, Valentini A, Civade R, Jean P, Maire A, Roset N, Schabuss M, Zornig H, Dejean T (2018) Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Scientific Reports 8: 10361. https://doi.org/10.1038/s41598-018-28424-8

Port JA, O'Donnell JL, Romero-Maraccini OC, Leary PR, Litvin SY, Nickols KJ, Yamahara KM, Kelly RP (2016) Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. Molecular Ecology 25: 527–541. https://doi.org/10.1111/mec.13481

Poté J, Mavingui P, Navarro E, Rosselli W, Wildi W, Simonet P, Vogel TM (2009) Extracellular plant DNA in Geneva groundwater and traditional artesian drinking water fountains. Chemosphere 75: 498–504. https://doi.org/10.1016/j.chemosphere.2008.12.048

Purcell KM, Ling N, Stockwell CA (2012) Evaluation of the introduction history and genetic diversity of a serially introduced fish population in New Zealand. Biological Invasions 14: 2057–2065. https://doi.org/10.1007/s10530-012-0213-1

R Core Team (2015) R: a language and environment for statistical computing

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Sciences 94: 9197–9201. https://doi.org/10.1073/pnas.94.17.9197

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) REVIEW: The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. Journal of Applied Ecology 51: 1450–1459. https://doi.org/10.1111/1365-2664.12306

Reynolds JH, Thompson WL, Russell B (2011) Planning for success: Identifying effective and efficient survey designs for monitoring. Biological Conservation 144: 1278–1284. https://doi.org/10.1016/j.biocon.2010.12.002

Ricciardi A (2013) Invasive species. In: Meyers RA (ed) Encyclopedia of Sustainability Science and Technology. Springer (New York, NY): 161–178.

Ricciardi A (2006) Patterns of invasion in the Laurentian Great Lakes in relation to changes in vector activity. Diversity and Distributions 12: 425–433. https://doi.org/10.1111/j.1366-9516.2006.00262.x

Ricciardi A (2001) Facilitative interactions among aquatic invaders: is an "invasional meltdown" occurring in the Great Lakes? Canadian Journal of Fisheries and Aquatic Sciences 58: 2513–2525. https://doi.org/10.1139/f01-178

Rice WR (1989) Analyzing Tables of Statistical Tests. Evolution 43: 223–225. https://doi.org/10.2307/2409177

Rius M, Bourne S, Hornsby HG, Chapman MA (2015a) Applications of next-generation sequencing to the study of biological invasions. Current Zoology 61: 488–504. https://doi.org/10.1093/czoolo/61.3.488

Rius M, Turon X, Bernardi G, Volckaert FAM, Viard F (2015b) Marine invasion genetics: from spatio-temporal patterns to evolutionary outcomes. Biological Invasions 17: 869–885. https://doi.org/10.1007/s10530-014-0792-0

Robin ED, Wong R (1988) Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. Journal of Cellular Physiology 136: 507–513. https://doi.org/10.1002/jcp.1041360316

Roman J, Darling JA (2007) Paradox lost: genetic diversity and the success of aquatic invasions. Trends in Ecology & Evolution 22: 454–464. https://doi.org/10.1016/j.tree.2007.07.002

Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molecular Ecology Resources 8: 103–106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

Sacks BN, Brazeal JL, Lewis JC (2016) Landscape genetics of the nonnative red fox of California. Ecology and Evolution 6: 4775–4791. https://doi.org/10.1002/ece3.2229

Sass GG, Hinz C, Erickson AC, McClelland NN, McClelland MA, Epifanio JM (2014) Invasive bighead and silver carp effects on zooplankton communities in the Illinois River, Illinois, USA. Journal of Great Lakes Research 40: 911–921. https://doi.org/10.1016/j.jglr.2014.08.010

Schirmer M, D'Amore R, Ijaz UZ, Hall N, Quince C (2016) Illumina error profiles: resolving fine-scale variation in metagenomic sequencing data. BMC Bioinformatics 17: 125. https://doi.org/10.1186/s12859-016-0976-y

Schloss PD, Jenior ML, Koumpouras CC, Westcott SL, Highlander SK (2016) Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. PeerJ 4: e1869. https://doi.org/10.7717/peerj.1869

Sepulveda AJ, Schabacker J, Smith S, Al-Chokhachy R, Luikart G, Amish SJ (2019) Improved detection of rare, endangered and invasive trout in using a new large-volume sampling method for eDNA capture. Environmental DNA 00: 1–11. https://doi.org/10.1002/edn3.23

Sepulveda-Villet OJ, Stepien CA (2012) Waterscape genetics of the yellow perch (*Perca flavescens*): patterns across large connected ecosystems and isolated relict populations. Molecular Ecology 21: 5795–5826. https://doi.org/10.1111/mec.12044

Shaw JLA, Clarke LJ, Wedderburn SD, Barnes TC, Weyrich LS, Cooper A (2016) Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. Biological Conservation 197: 131–138. https://doi.org/10.1016/j.biocon.2016.03.010

Shokralla S, Spall JL, Gibson JF, Hajibabaei M (2012) Next-generation sequencing technologies for environmental DNA research. Molecular Ecology 21: 1794–1805. https://doi.org/10.1111/j.1365-294X.2012.05538.x

Sigsgaard EE, Nielsen IB, Bach SS, Lorenzen ED, Robinson DP, Knudsen SW, Pedersen MW, Jaidah MA, Orlando L, Willerslev E, Møller PR, Thomsen PF (2017) Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. Nature Ecology & Evolution 1: 0004. https://doi.org/10.1038/s41559-016-0004

Simon T (2006) Biodiversity of fishes in the Wabash River: Status, indicators, and threats. Proceedings of the Indiana Academy of Science 115: 136–148

Slatkin M, Excoffier L (2012) Serial founder effects during range expansion: a spatial analog of genetic drift. Genetics 191: 171–181. https://doi.org/10.1534/genetics.112.139022

Smart AS, Weeks AR, Rooyen AR van, Moore A, McCarthy MA, Tingley R (2016) Assessing the cost-efficiency of environmental DNA sampling. Methods in Ecology and Evolution 7: 1291–1298. https://doi.org/10.1111/2041-210X.12598

Snyder MR, Stepien CA (2017) Genetic patterns across an invasion's history: a test of change versus stasis for the Eurasian round goby in North America. Molecular Ecology 26: 1075–1090. https://doi.org/10.1111/mec.13997

Sokołowska E, Fey DP (2011) Age and growth of the round goby Neogobius melanostomus in the Gulf of Gdańsk several years after invasion. Is the Baltic Sea a new Promised Land? Journal of Fish Biology 78: 1993–2009. https://doi.org/10.1111/j.1095-8649.2011.02986.x

Steinhart GB, Marschall EA, Stein RA (2004) Round goby predation on smallmouth bass offspring in nests during simulated catch-and-release angling. Transactions of the American Fisheries Society 133: 121–131. https://doi.org/10.1577/T03-020

Stepien CA, Brown JE, Neilson ME, Tumeo MA (2005) Genetic diversity of invasive species in the Great Lakes versus their Eurasian source populations: insights for risk analysis. Risk Analysis: An Official Publication of the Society for Risk Analysis 25: 1043–1060. https://doi.org/10.1111/j.1539-6924.2005.00655.x

Stepien CA, Eddins DJ, Snyder MR, Marshall N. T. (2018) Genetic change versus stasis over the time course of invasions: trajectories of two concurrent, allopatric introductions of the Eurasian ruffe. Aquatic Invasions 13: 537–552. https://doi.org/10.3391/ai.2018.13.4.11

Stepien CA, Grigorovich IA, Gray MA, Sullivan TJ, Yerga-Woolwine S, G. Kalayci (2013) Evolutionary, biogeographic, and population genetic relationships of dreissenid mussels, with revision of component taxa. In: Nalepa TF, Schloesser DW (eds) Quagga and Zebra Mussels: Biology, Impacts, and Control. CRC Press (New York, NY): 403–444.

Stepien CA, Neilson ME (2013) What's in a name? Taxonomy and nomenclature of invasive gobies in the Great Lakes and beyond. Journal of Great Lakes Research 39: 555–559. https://doi.org/10.1016/j.jglr.2013.09.020

Stepien CA, Pierce LR, Leaman DW, Niner MD, Shepherd BS (2015) Gene diversification of an emerging pathogen: a decade of mutation in a novel fish viral hemorrhagic septicemia (VHS) substrain since Its first appearance in the Laurentian Great Lakes. PLOS ONE 10: e0135146. https://doi.org/10.1371/journal.pone.0135146

Stepien CA, Snyder MR, Elz AE (2019) Invasion genetics of the silver carp *Hypophthalmichthys molitrix* across North America: Differentiation of fronts, introgression, and eDNA metabarcode detection. PLOS ONE 14: e0203012. https://doi.org/10.1371/journal.pone.0203012

Stepien CA, Tumeo MA (2006) Invasion genetics of Ponto-Caspian gobies in the Great Lakes: a 'cryptic' species, absence of founder effects, and comparative risk analysis. Biological Invasions 8: 61–78. https://doi.org/10.1007/s10530-005-0237-x

Stern CV, Upton HF, Cynthia B (2014) Asian carp and the Great Lakes region. Congressional Research Service

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

Strayer DL, Cid N, Malcom HM (2011) Long-term changes in a population of an invasive bivalve and its effects. Oecologia 165: 1063–1072. https://doi.org/10.1007/s00442-010-1792-0

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation 183: 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Suzuki R, Shimodaira H (2015) Package "pvclust": Hierarchical Clustering with P-Values via Multiscale Bootstrap Resampling. In: CRAN. https://cran.r-project.org/web/packages/pvclust/pvclust.pdf. Accessed 12 Jun 2019

Tepolt CK, Palumbi SR (2015) Transcriptome sequencing reveals both neutral and adaptive genome dynamics in a marine invader. Molecular Ecology 24: 4145–4158. https://doi.org/10.1111/mec.13294

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLOS ONE 7: e41732. https://doi.org/10.1371/journal.pone.0041732

Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E (2016a) Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes. PLOS ONE 11: e0165252. https://doi.org/10.1371/journal.pone.0165252

Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E (2016b) Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PLOS ONE 11: e0165252. https://doi.org/10.1371/journal.pone.0165252

Trautman MB (1981) The Fishes of Ohio, Revised Edition. Ohio State University Press (Columbus, Ohio):

Tsuji S, Miya M, Ushio M, Sato H, Minamoto T, Yamanaka H (2018) Evaluating intraspecific diversity of a fish population using environmental DNA: An approach to distinguish true haplotypes from erroneous sequences: 429993. https://doi.org/10.1101/429993

Tucker AJ, Chadderton WL, Jerde CL, Renshaw MA, Uy K, Gantz C, Mahon AR, Bowen A, Strakosh T, Bossenbroek JM, Sieracki JL, Beletsky D, Bergner J, Lodge DM (2016) A sensitive environmental DNA (eDNA) assay leads to new insights on Ruffe (*Gymnocephalus cernua*) spread in North America. Biological Invasions 18: 3205–3222. https://doi.org/10.1007/s10530-016-1209-z

Turner CR, Barnes MA, Xu CCY, Jones SE, Jerde CL, Lodge DM (2014) Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods in Ecology and Evolution 5: 676–684. https://doi.org/10.1111/2041-210X.12206

Turner CR, Uy KL, Everhart RC (2015) Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biological Conservation 183: 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

US 102nd Congress (1992) Alien Species Prevention and Enforcement Act. In: Government Publishing Office. https://www.govinfo.gov/content/pkg/STATUTE-106/pdf/STATUTE-106-Pg1729.pdf. Accessed 20 Jun 2019

US ACE (2019) Electric barriers. In: US Army Corps of Enguineers Chicago district. https://www.lrc.usace.army.mil/Missions/Civil-Works-Projects/ANS-Portal/Barrier/

US EPA R 05 (2015) Invasive Species in the Great Lakes. In: US EPA. https://www.epa.gov/greatlakes/invasive-species-great-lakes. Accessed 24 Jun 2019

USDI, USFWS, USDOC, USCB (2016) National survey of fishing, hunting, and wildlife-associated recreation. U.S. Department of the Interior, U.S. Fish and Wildlife Service, and U.S. Department of Commerce, U.S. Census Bureau.

USFWS (2012) The cost of invasive species. In: U.S. Fish and Wildlife Service. https://www.fws.gov/verobeach/pythonpdf/costofinvasivesfactsheet.pdf

USFWS (2006) Lacey Act. US Fish and Wildlife Service

USGS (2019a) Budget Justifications and Performance Information Fiscal Year 2019. The United States Department of Interior and U.S. Geological Survey

USGS (2016) Compiled reports to the Great Lakes Fishery Commission of the annual bottom trawl and acoustic surveys, 2015. United States Geological Survey Great Lakes Science Center (Ann Arbor, MI):

USGS (2019b) NAS – Nonindigenous Aquatic Species Database. https://nas.er.usgs.gov/. Accessed 20 Apr 2019

USGS (2018) Ide (*Leuciscus idus*) – species profile. In: USGS Nonindigenous Aquatic Species Database. https://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=557. Accessed 21 Feb 2019

Ushio M, Murakami H, Masuda R, Sado T, Miya M, Sakurai S, Yamanaka H, Minamoto T, Kondoh M (2018) Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. Metabarcoding and Metagenomics 2: e23297. https://doi.org/10.3897/mbmg.2.23297

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, Gaboriaud C, Jean P, Poulet N, Roset N, Copp GH, Geniez

P, Pont D, Argillier C, Baudoin J-M, Peroux T, Crivelli AJ, Olivier A, Acqueberge M, Brun ML, Møller PR, Willerslev E, Dejean T (2016) Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology 25: 929–942. https://doi.org/10.1111/mec.13428

Van Bocxlaer B, Strong EE (2019) Viviparidae Gray, 1847. In: Lydeard C, Cummings CS (eds) Freshwater Mollusks of the World. A Distribution Atlas. John Hopkins University Press (Baltimore, MD): 43–50.

Vander Zanden MJ, Olden JD (2008) A management framework for preventing the secondary spread of aquatic invasive species. Canadian Journal of Fisheries and Aquatic Sciences 65: 1512–1522. https://doi.org/10.1139/F08-099

Walker PJ, Winton JR (2010) Emerging viral diseases of fish and shrimp. Veterinary Research 41: 51. https://doi.org/10.1051/vetres/2010022

Wares JP, Hughes AR, Grosberg RK (2005) Mechanisms that drive evolutionary change: insights from species introductions and invasions. In: Sax DF, Stachowicz JJ, Gaines SD (eds) Species Invasions: Insights into Ecology, Evolution, and Biogeography. Sinauer Associates Inc. (Sunderland, MA): 229–257.

Waters JM, Fraser CI, Hewitt GM (2013) Founder takes all: density-dependent processes structure biodiversity. Trends in Ecology & Evolution 28: 78–85. https://doi.org/10.1016/j.tree.2012.08.024

Wegleitner B, Tucker A, Chadderton W, Mahon A (2016) Identifying the genetic structure of introduced populations of northern snakehead (*Channa argus*) in Eastern USA. Aquatic Invasions 11: 199–208. https://doi.org/10.3391/ai.2016.11.2.09

Weinig C (2000) Plasticity versus canalization: population differences in the timing of shade-avoidance responses. Evolution; International Journal of Organic Evolution 54: 441–451

White TA, Perkins SE, Heckel G, Searle JB (2013) Adaptive evolution during an ongoing range expansion: the invasive bank vole (*Myodes glareolus*) in Ireland. Molecular Ecology 22: 2971–2985. https://doi.org/10.1111/mec.12343

Wilcox TM, McKelvey KS, Young MK, Sepulveda AJ, Shepard BB, Jane SF, Whiteley AR, Lowe WH, Schwartz MK (2016) Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis*. Biological Conservation 194: 209–216. https://doi.org/10.1016/j.biocon.2015.12.023

Williams KE, Huyvaert KP, Piaggio AJ (2017) Clearing muddied waters: Capture of environmental DNA from turbid waters. PLOS ONE 12: e0179282. https://doi.org/10.1371/journal.pone.0179282

Williamson CJ, Garvey JE (2005) Growth, fecundity, and diets of newly established silver carp in the Middle Mississippi River. Transactions of the American Fisheries Society 134: 1423–1430. https://doi.org/10.1577/T04-106.1

Williamson M (1999) Invasions. Ecography 22: 5–12

Williamson M, Fitter A (1996) The varying success of invaders. Ecology 77: 1661–1666. https://doi.org/10.2307/2265769

Wilson JRU, Dormontt EE, Prentis PJ, Lowe AJ, Richardson DM (2009) Something in the way you move: Dispersal pathways affect invasion success. Trends in Ecology & Evolution 24: 136–144. https://doi.org/10.1016/j.tree.2008.10.007

Winfield IJ, Adams CE, Fletcher JM (1996) Recent introductions of the ruffe (*Gymnocephalus cernuus*) to three United Kingdom lakes containing *Coregonus* species. Annales Zoologici Fennici 33: 459–466

Wittmann ME, Cooke RM, Rothlisberger JD, Lodge DM (2014) Using structured expert judgment to assess invasive species prevention: asian carp and the mississippi—Great Lakes hydrologic connection. Environmental Science & Technology 48: 2150–2156. https://doi.org/10.1021/es4043098

Wong NA, Tooman LK, Sewell MA, Lavery SD (2016) The population genetics and origin of invasion of the invasive Asian paddle crab, *Charybdis japonica* (A. Milne-Edwards, 1861) (Brachyura: Portunidae) in north-eastern New Zealand. Marine Biology 163: 133. https://doi.org/10.1007/s00227-016-2906-y

Wu L, Wen C, Qin Y, Yin H, Tu Q, Nostrand JDV, Yuan T, Yuan M, Deng Y, Zhou J (2015) Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. BMC Microbiology 15: 125. https://doi.org/10.1186/s12866-015-0450-4

Xiong W, Li H, Zhan A (2016) Early detection of invasive species in marine ecosystems using high-throughput sequencing: technical challenges and possible solutions. Marine Biology 163: 139. https://doi.org/10.1007/s00227-016-2911-1

Yang X-M, Lou H, Sun J-T, Zhu Y-M, Xue X-F, Hong X-Y (2015) Temporal genetic dynamics of an invasive species, *Frankliniella occidentalis* (Pergande), in an early phase of establishment. Scientific Reports 5. https://doi.org/10.1038/srep11877

Yang X-M, Sun J-T, Xue X-F, Li J-B, Hong X-Y (2012) Invasion genetics of the western flower thrips in China: evidence for genetic bottleneck, hybridization and bridgehead effect. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0034567

Yu H, Wan FH, Guo JY (2012) Different thermal tolerance and hsp gene expression in invasive and indigenous sibling species of Bemisia tabaci

Zaiko A, Pochon X, Garcia-Vazquez E, Olenin S, Wood SA (2018) Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: management and surveillance of non-indigenous species. Frontiers in Marine Science 5. https://doi.org/10.3389/fmars.2018.00322

Zar JH (2010) Biostatistical Analysis, 5 edition. Pearson (Upper Saddle River, NJ): 1–944.

Zhang H, Rutherford ES, Mason DM, Breck JT, Wittmann ME, Cooke RM, Lodge DM, Rothlisberger JD, Zhu X, Johnson TB (2016) Forecasting the impacts of silver and bighead carp on the Lake Erie food web. Transactions of the American Fisheries Society 145: 136–162. https://doi.org/10.1080/00028487.2015.1069211

Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE, Barba MD, Dickie IA, Dumbrell AJ, Ficetola GF, Fierer N, Fumagalli L, Gilbert MTP, Jarman S, Jumpponen A, Kauserud H, Orlando L, Pansu J, Pawlowski J, Tedersoo L, Thomsen PF, Willerslev E, Taberlet P (2019) DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. Molecular Ecology 28: 1857–1862. https://doi.org/10.1111/mec.15060

Zukowski S, Walker KF (2009) Freshwater snails in competition: alien *Physa acuta* (Physidae) and native *Glyptophysa gibbosa* (Planorbidae) in the River Murray, South Australia. Marine and Freshwater Research 60: 999–1005. https://doi.org/10.1071/MF08183

Appendix A

Chapter 2 Supplementary Tables and Figures

Appendix A.1 Primers, annealing temperatures, references, and information for loci used in study. Primers for A) mtDNA cytochrome *b* gene and B) 13 nuclear DNA microsatellite loci. Primer DNA sequences, annealing temperatures (T_A, °C), reference, numbers of alleles (N_A), and their relative size ranges (R_S, base pairs, bp). Ame loci were developed prior to name change from *Apollonia melanostoma* to *Neogobius melanostomus* (Neilson & Stepien 2009; Stepien & Neilson 2013).

(A)

Primer	Sequence 5'–3'	T_A (°C)	Reference
L14724	GTGACTTGAAAAACCACCGTT	52	Brown & Stepien 2008, 2009
H5	GAATTYTRCGTTTGGGAG	52	” ”
L15066	TTGGTCGAGGCCTCTATTACG	52	” ”

(B)

Primer	Sequence 5'–3'	T_A (°C)	Reference	N_A	R_S (bp)
*Ame*10F	ATGCGAAGCCGATTTCTG	52	Feldheim *et al.* 2008	12	196–256
*Ame*10R	CCATATGTCAGGCGATATTCC	52	” ”	”	”
*Ame*17F	GGCGCAACCTCATTTTAATC	58	” ”	29	153-282
*Ame*17R	GTTTAGGCGGGGGTTAAGAG	58	” ”	”	”
*Ame*129F	TGCTCGGTCCTACTTCAAGC	56	” ”	43	99-351
*Ame*129R	GCATTCACATTCCTCCCACT	56	” ”	”	”
*Ame*133F	GCCCACCCCTTCACTCTT	56	” ”	23	181-345
*Ame*133R	GGCTATGGCATTTTCTCTCC	56	” ”	”	”
*Nme*1F	CGAGCGCTAAAATAGAAGAAAA	48	Dufour *et al.* 2007	26	212-364
*Nme*1R	TCCAGTGGCTTGAGTGATGT	48	” ”	”	”
*Nme*2F	TGTGTAATGACGTGGAATAGCC	55	” ”	12	234-276
*Nme*2R	CAATAGGCCAGGATGAATGAG	55	” ”	”	”
*Nme*3F	GCGGGAGTCAAGAATTGAAC	48	” ”	19	112-228
*Nme*3R	TTGTTAGAATGTATTATGCCATAGCC	48	” ”	”	”
*Nme*4F	TGTGCTTGGTTAAGGTGGTG	55	” ”	10	53-149
*Nme*4R	CCGGACAGAAACAACTTAAAGC	55	” ”	”	”
*Nme*5F	GTCACACCGATCTTCGACTG	48	” ”	12	77-141
*Nme*5R	GATTTACTTGATTCATCACT	48	” ”	”	”
*Nme*7F	AATGGATGGGTCAATTGCAT	48	” ”	10	122-222
*Nme*7R	AAGGTTGAGCTGCCACTGAG	48	” ”	”	”
*Nme*8F	ATGGAGTTTCTGGGCAGTTG	55	” ”	17	179-349
*Nme*8R	CTCCGTCGATTGTGTTCTGA	55	” ”	”	”

Primer	Sequence 5'–3'	T_A (°C)	Reference		N_A	R_S (bp)
*Nme*9F	GGGGTGCACTTGTTTAGCTC	59	”	”	22	121-253
*Nme*9R	AACGGACAAGTGGAAGAAGG	59	”	”	”	”
*Nme*10F	GCGATTATGAGGTTCGGAGA	48	”	”	11	259-289
*Nme*10R	ATCAGCAACCCCTGAACAGA	48	”	”	”	”

Appendix A.2 MtDNA cytochrome *b* haplotypes discerned in this study. Designation follows Brown & Stepien (2008, 2009). GenBank accession numbers (Acc #; http://www.ncbi.nlm.nih.gov). Ame haplotypes were designated prior to name change from *Apollonia melanostoma* to *Neogobius melanostomus* (Neilson & Stepien 2009; Stepien & Neilson 2013). See Table 2.1 for site abbreviations.

		Percent Representation						
Haplotype	**Acc #**	**LM**	**SR**	**LC**	**EI**	**CE**	**DR**	**Mean**
Ame 1	EU331156	77.4	81.7	75.5	56.9	78.9	78.8	*74.9*
Ame 5	EU331160	-	-	-	-	0.6	-	*0.6*
Ame 7	EU331162	-	0.6	-	-	-	2.4	*1.5*
Ame 8	EU331163	19.5	13.1	14.6	28.5	14.9	10.6	*16.9*
Ame 57	EU331207	3.1	2.9	9.3	13.9	3.4	3.5	*6.0*
Ame 88	EU564125	-	1.7	0.7	0.8	-	-	*1.1*
Nme 89	KT231987	-	-	-	-	0.6	1.2	*0.9*
Nme 91	KT231989	-	-	-	-	-	1.2	*1.2*
Nme 92	KT231990	-	-	-	-	-	1.2	*1.2*
Nme 93	KT231991	-	-	-	-	-	1.2	*1.2*
Nme 94	KT231992	-	-	-	-	0.6	-	*0.6*
Nme 95	KT231993	-	-	-	-	0.6	-	*0.6*
Nme 96	KT231994	-	-	-	-	0.6	-	*0.6*

Appendix A.3 Genetic diversity of round goby population sampling years and locations from mtDNA cytochrome *b* gene sequence haplotypes. Sample year(s), sample sizes (*N*), mtDNA sequence haplotypes, gene diversity ± standard deviation (*h*), number of haplotypes (N_H), and proportion of haplotypes that appeared private (P_{PH}). Values in bold combine results from all sampling years per population location. Calculations performed in ARLEQUIN.

Site	Sample Year(s)	*N*	Haplotypes	*h*	N_H	P_{PH}
L. Michigan	**All**	**163**	**1,8,57**	**0.32±0.05**	**3**	**0.00**
	1999	19	1	0.00±0.00	1	0.00
	2007	50	1,8,57	0.41±0.08	3	0.00
	2011	44	1,8,57	0.46±0.06	3	0.00
	2013	50	1,8,57	0.34±0.07	3	0.00
St. Clair R.	**All**	**179**	**1,7,8,57,88**	**0.36±0.05**	**5**	**0.00**
	1993	45	1,7,8,57,88	0.50±0.08	5	0.00
	2007	50	1,8,57	0.25±0.07	4	0.00
	2011	34	1,8	0.12±0.07	3	0.00
	2013	50	1,8,88	0.34±0.08	3	0.00
L. St. Clair	**All**	**171**	**1,8,57,88**	**0.41±0.05**	**4**	**0.00**
	1998	39	1,8,57,88	0.47±0.09	4	0.00
	2007	50	1,8,57	0.46±0.08	3	0.00
	2011	32	1,8,57	0.40±0.10	3	0.00
	2013	50	1,8,57	0.30±0.09	3	0.00
L. Erie Islands	**All**	**285**	**1,8,57,88**	**0.58±0.02**	**5**	**0.00**
	1998	51	1,8,57	0.58±0.03	4	0.00
	2002	50	1,8,57	0.59±0.05	3	0.00
	2005	49	1,8,57,88	0.57±0.05	4	0.00
	2007	40	1,8,57,88	0.53±0.08	4	0.00
	2011	45	1,8,57	0.59±0.06	3	0.00
	2013	50	1,8,57	0.59±0.05	3	0.00
Central L. Erie	**All**	**201**	**1,5,8,57,89,94-96**	**0.33±0.05**	**8**	**0.50**
	1998	24	1,8	0.32±0.11	2	0.00
	2002	29	1,8,57	0.42±0.12	3	0.00
	2007	50	1,5,8,57	0.32±0.08	4	0.25
	2011	48	1,8,57	0.44±0.08	3	0.00
	2013	50	1,8,89,94-96	0.32±0.09	6	0.50
Dnieper R.	**All**	**102**	**1,7,8,57,89,91-93**	**0.37±0.06**	**8**	**0.38**
	2002	25	1,7,8,57	0.42±0.12	4	0.00
	2007	24	1,7,8,57	0.47±0.11	4	0.00
	2013	53	1,8,57,89,91-93	0.35±0.10	7	0.43

Appendix A.4 Pairwise sample divergence values from mtDNA cytochrome *b* sequence data. Pairwise comparisons of mtDNA sequence data between round goby temporal samples. Exact tests (GENEPOP; above diagonal) and θ_{ST} (ARLEQUIN; below diagonal). *=p<0.05 but not significant following Bonferroni correction, **=remained significant (p<α) after sequential Bonferroni correction, NS=p>0.05.

	L. Michigan (LM)				St. Clair R. (SR)				L. St. Clair (LC)			
	1999	**2007**	**2011**	**2013**	**1993**	**2007**	**2011**	**2013**	**1998**	**2007**	**2011**	**2013**
LM1999	~	NS	*	NS	NS	NS	NS	NS	NS	*	NS	NS
LM2007	0.059	~	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LM2011	0.157*	0.003	~	NS	NS	NS	*	NS	NS	*	NS	NS
LM2013	0.070	-0.011	0.004	~	NS	NS	NS	NS	NS	NS	NS	NS
SR1993	0.048	-0.017	0.022	0.006	~	NS	NS	NS	NS	NS	NS	NS
SR2007	0.027	0.001	0.046	-0.010	0.012*	~	NS	NS	NS	NS	NS	NS
SR2011	0.007	0.038	0.112*	0.028	0.042	-0.006*	~	NS	NS	*	NS	NS
SR2013	0.065	0.008	0.026*	-0.012	0.024	-0.007	0.019*	~	NS	*	NS	NS
LC1998	0.054	-0.015	0.030	0.013	-0.025	0.018	0.049	0.032*	~	NS	NS	NS
LC2007	0.075*	0.010	0.070*	0.053*	-0.010*	0.055*	0.082*	0.079*	-0.016*	~	NS	NS
LC2011	0.083	-0.029	-0.014	-0.028	-0.018	-0.012	0.041	-0.011	-0.014*	0.018*	~	NS
LC2013	0.022	-0.013	0.038*	-0.008	-0.009	-0.017	0.004	0.002	-0.007	0.020*	-0.019*	~
EI1998	0.245**	0.068*	0.025	0.100**	0.072*	0.159**	0.224**	0.136**	0.078*	0.103*	0.058*	0.130**
EI2002	0.120**	0.018	0.044	0.061**	0.003*	0.083**	0.119**	0.093*	-0.001	-0.003	0.021	0.048*
EI2005	0.111**	-0.002	0.001	0.024	-0.003*	0.054*	0.097*	0.050	-0.002	0.015	-0.006	0.030
EI2007	0.075	-0.007	0.035	0.029	-0.021	0.040*	0.074*	0.054	-0.024	-0.020	-0.003*	0.009
EI2011	0.120**	0.009	0.032	0.051	-0.004*	0.076*	0.117*	0.083*	-0.006	-0.004	0.011	0.041
EI2013	0.124*	0.039	0.085**	0.091**	0.014*	0.105**	0.133**	0.122*	0.007	-0.008*	0.046*	0.063
CE1998	0.140	-0.013	-0.009	-0.032	0.005	-0.015	0.045	-0.027	0.013	0.053	-0.034	-0.009
CE2002	0.086	-0.036	-0.017	-0.030	-0.027	-0.014	0.044	-0.010	-0.024	0.004	-0.047*	-0.025
CE2007	0.019	-0.009	0.036*	-0.009	-0.004	-0.016	0.001	0.000	-0.001	0.028	-0.018*	-0.021*
CE2011	0.067	-0.023	0.003	-0.007	-0.021	0.007	0.048	0.015	-0.020	0.002	-0.028	-0.010
CE2013	0.026	-0.011	0.029	-0.012	-0.004	-0.017	0.004	-0.004	-0.001	0.030	-0.021*	-0.021*
DR2002	0.026	-0.011	0.033	-0.007	-0.013	-0.012	0.009	0.003	-0.006	0.019	-0.019*	-0.020
DR2007	0.047	-0.018	0.011	-0.015	-0.016	-0.009	0.024	-0.002	-0.009	0.019	-0.028*	-0.017
DR2013	-0.005	0.013	0.085**	0.022	0.007	-0.003	-0.002	0.027	0.008	0.029	0.012*	-0.013*

	L. Erie Islands (EI)						Central L. Erie (CE)					Dnieper R (DR)		
	1998	**2002**	**2005**	**2007**	**2011**	**2013**	**1998**	**2002**	**2007**	**2011**	**2013**	**2002**	**2007**	**2013**
LM1999	**	*	*	*	*	*	NS	NS	NS	*	NS	NS	NS	NS
LM2007	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LM2011	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	*
LM2013	*	*	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS
SR1993	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SR2007	**	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS
SR2011	**	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS
SR2013	**	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS	NS
LC1998	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LC2007	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
LC2011	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LC2013	*	*	*	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS
EI1998	~	NS	NS	*	NS	*	*	NS	*	*	**	*	*	**
EI2002	0.037*	~	NS	NS	NS	NS	NS	NS	*	NS	*	*	NS	*
EI2005	0.010*	-0.008*	~	NS	NS	NS	NS	NS	*	NS	*	NS	NS	*
EI2007	0.064*	-0.014*	-0.007*	~	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
EI2011	0.028	-0.023*	-0.015*	-0.017*	~	NS	NS	NS	*	NS	*	NS	NS	*
EI2013	0.079	-0.014	0.017*	-0.008*	-0.012*	~	*	NS	*	NS	*	*	NS	*
CE1998	0.082	0.055	0.018	0.028*	0.045*	0.083*	~	NS	NS	NS	NS	NS	NS	NS
CE2002	0.048	0.007	-0.016	-0.016	-0.002*	0.030*	-0.034*	~	NS	NS	NS	NS	NS	NS
CE2007	0.132	0.056	0.035*	0.016	0.048	0.074*	-0.012*	-0.023*	~	NS	NS	NS	NS	NS
CE2011	0.057	0.006	-0.009	-0.014	-0.002	0.026	-0.009*	-0.036*	-0.007*	~	NS	NS	NS	NS
CE2013	0.123*	0.054*	0.031*	0.016	0.046	0.073	-0.017*	-0.026*	-0.019*	-0.008*	~	NS	NS	NS
DR2002	0.114*	0.042	0.026	0.008	0.035	0.056	-0.010	-0.025*	-0.018*	-0.009*	-0.018	~	NS	NS
DR2007	0.085	0.032	0.011	0.003	0.023	0.051	-0.021	-0.033	-0.016*	-0.017*	-0.017	-0.039	~	NS
DR2013	0.179*	0.072*	0.063*	0.027	0.068	0.078	0.025	0.005	-0.009	0.015*	-0.005	-0.010	0.002	~

Appendix A.5 Individual round goby assignments to population samples (using GENECLASS2) based on nuclear DNA microsatellite data. Proportion of individuals assigned to each sample based on microsatellite data. Underlined values are sample self-assignments. * indicates highest proportion(s) of individuals assigned per sample. Only within population sample comparisons and those for which the highest proportion of individuals assigned are shown.

Sample	Assigned to L. Michigan (LM)				*N*
	1999	**2007**	**2011**	**2013**	
LM1999	0.21	0.16	0.63*	-	19
LM2007	-	0.38*	0.24	0.06	50
LM2011	-	0.11	0.50*	0.09	44
LM2013	-	0.10	0.10	0.36*	50

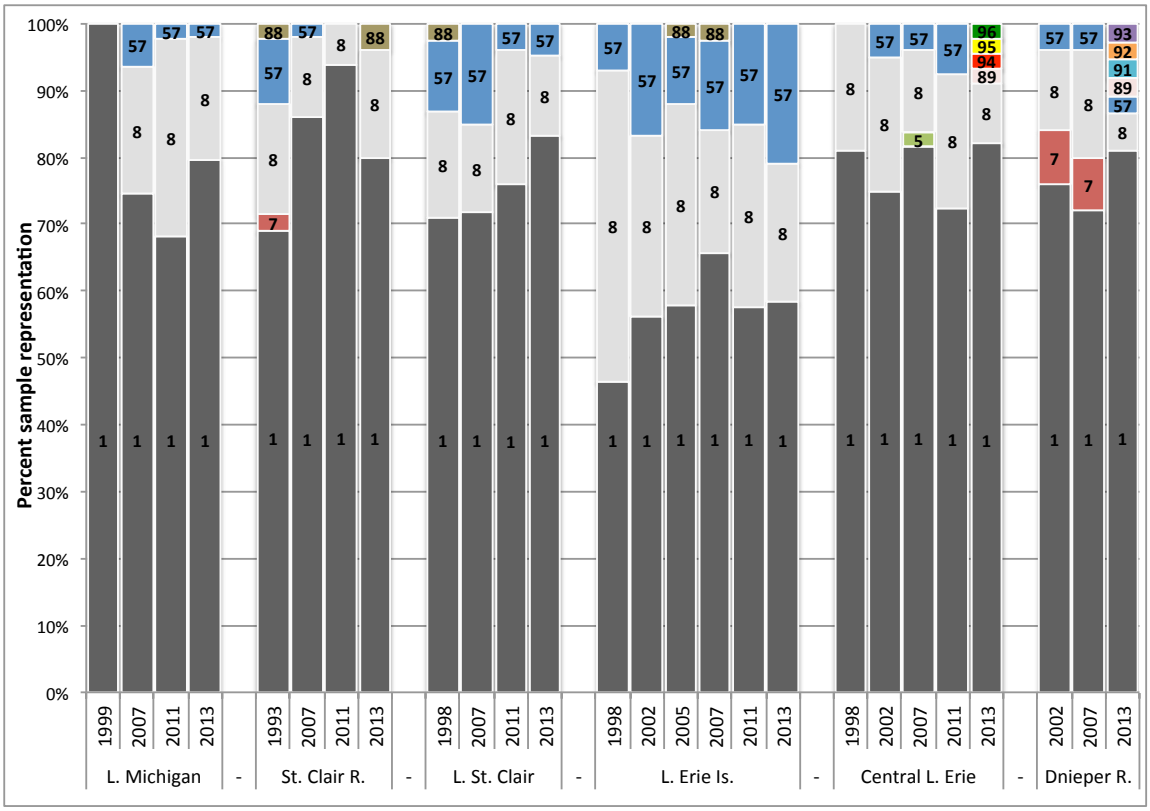
	St. Clair R. (SR)				DR	LC	*N*
	1993	**2007**	**2011**	**2013**	**2007**	**2013**	
SR1993	0.29*	0.07	0.02	-	0.18	0.07	45
SR2007	0.14	0.08	0.14	-	0.20*	0.14	50
SR2011	0.12	0.06	0.32*	-	0.09	0.12	34
SR2013	0.02	0.02	0.02	0.22	0.26	0.28*	50

	L. St. Clair (LC)				SR	*N*
	1998	**2007**	**2011**	**2013**	**1993**	
LC1998	0.21*	0.03	0.13	0.08	0.21*	39
LC2007	0.06	0.10	0.28*	0.14	0.12	50
LC2011	0.03	0.06	0.19*	0.13	0.13	32
LC2013	0.02	0.02	0.20	0.34*	0.10	50

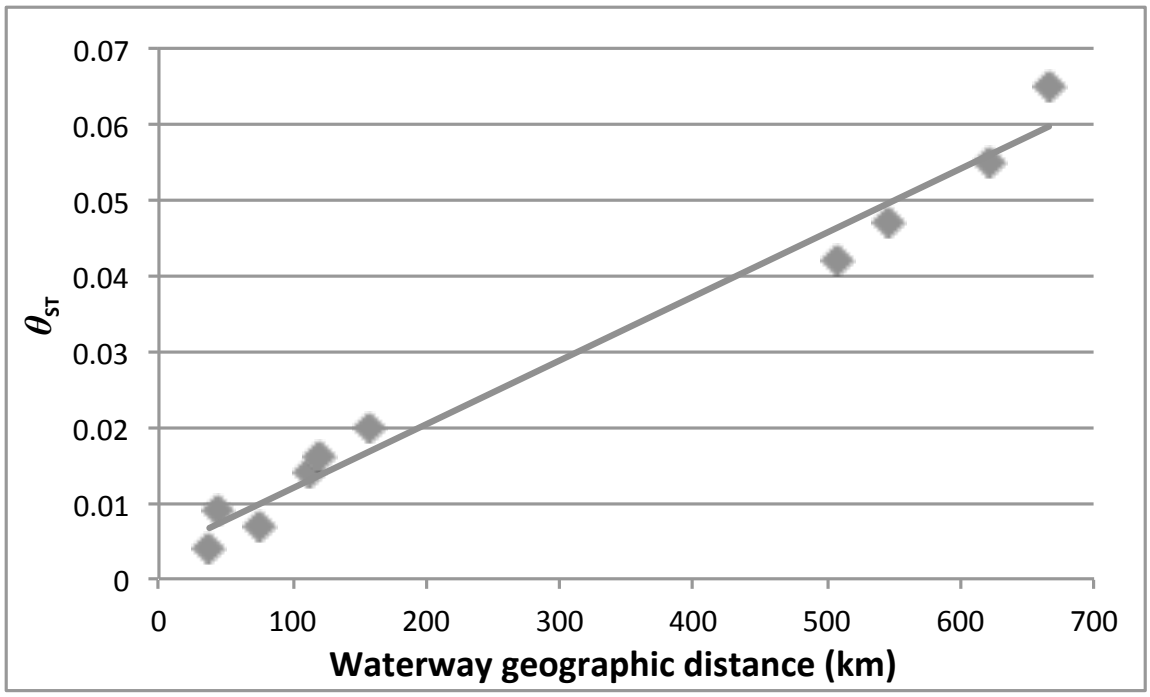
	L. Erie Islands (EI)						LC		*N*
	1998	**2002**	**2005**	**2007**	**2011**	**2013**	**2011**	**2013**	
EI1998	0.18*	0.04	0.10	0.04	0.04	-	0.04	0.14	51
EI2002	0.02	0.04	0.02	0.06	0.08	-	0.22*	0.14	50
EI2005	0.02	0.02	0.16	0.04	0.08	0.02	0.18*	0.02	49
EI2007	0.03	0.08	0.05	0.13	0.05	0.03	0.18*	0.13	40
EI2011	-	0.07	0.04	-	0.16	-	0.29*	0.20	45
EI2013	-	0.00	0.00	0.06	0.04	0.12	0.10	0.40*	50

	Central L. Erie (CE)					LC		*N*
	1998	**2002**	**2007**	**2011**	**2013**	**2011**	**2013**	
CE1998	0.58*	0.08	-	0.08	-	-	-	24
CE2002	0.38*	0.10	0.03	0.07	-	-	-	29
CE2007	0.18*	0.04	0.12	0.02	-	0.18*	0.02	50
CE2011	0.15	0.04	-	0.19*	0.02	0.10	0.19*	48
CE2013	0.02	0.02	-	0.02	0.08	0.12	0.26*	50

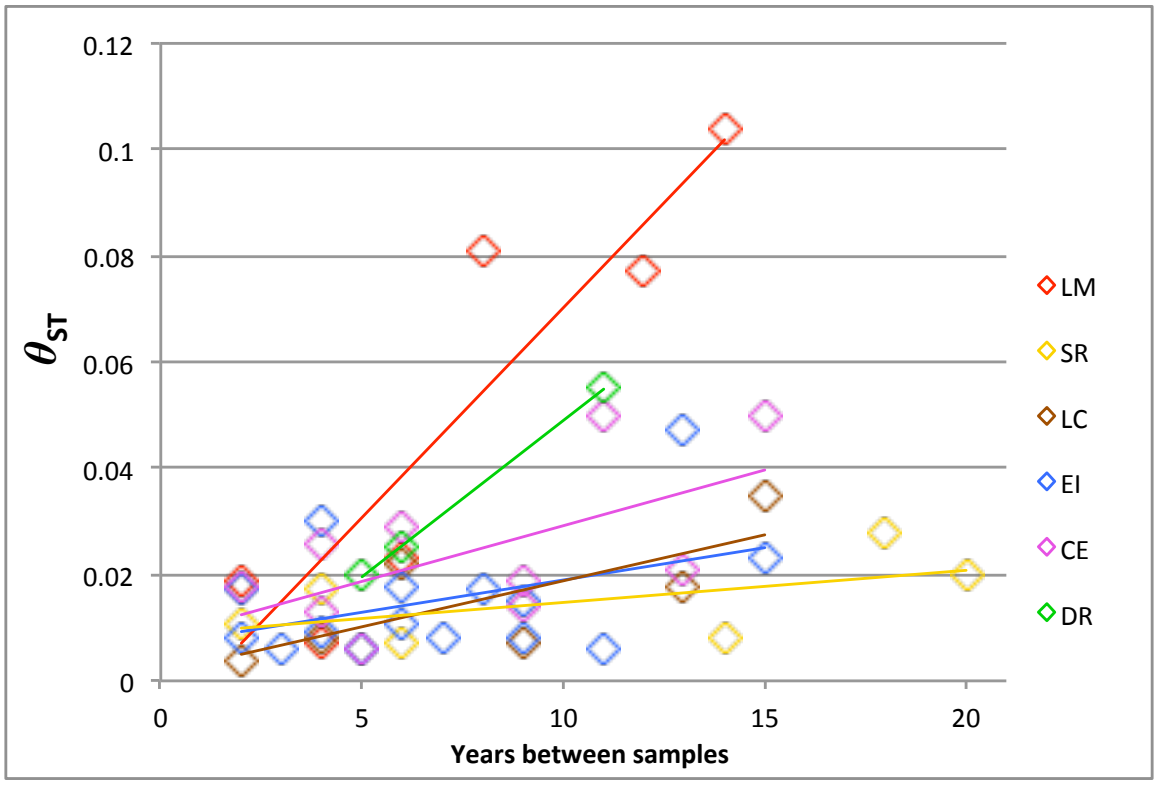
	Dnieper R. (DR)			*N*
	2002	**2007**	**2013**	
DR2002	0.40	0.56*	0.04	25
DR2007	0.08	0.79*	0.13	24
DR2013	0.08	0.30	0.57*	53



Appendix A–1 MtDNA cytochrome *b* haplotypic composition of round goby temporal samples. Numbers denote different haplotypes. See Appendix A.2.



Appendix A–2 Relationship of population genetic divergences versus geographic distances for round goby populations. A Mantel test (ARLEQUIN) determined that 98% (p=0.013) of the overall variation in θ_{ST} from microsatellite data was determined by geographic distance between populations. (Linear model fit in R: R^2=0.98, F=389.8, p=4.51•10^{-8}).



Appendix A–3 Relationships between genetic distances and sampling events for round goby populations. Mantel tests based on μsat data at individual population locations (ARLEQUIN) determined 83% (L. Michigan, p=0.040*), 32% (St. Clair River, p=0.120), 55% (Lake St. Clair, p=0.085), 17% (Lake Erie Is., p=0.097), 39% (Central Lake Erie, p=0.031*), and 99.9% (Dnieper River, p=0.169) of variation in θ_{ST} were determined by years between sampling events. Dnieper River had a low number of available samples, likely affecting significance. ANCOVA analysis (R) indicated the slope for Lake Michigan was significantly different from all others ($0.000<p<0.004$) except the Dnieper River ($p>0.289$). The slope of the Dnieper River population significantly differed from that of the St. Clair River alone ($p<0.007$).

Appendix B

Chapter 3 Supplementary Tables and Figures

Appendix B.1 Species and GenBank accession numbers used to design the eDNA assays and for the BLAST database. Common name, species name, taxonomy (order and family), and Accession numbers available on GenBank as of January 5, 2018 for species used in the design of eDNA assays in this study. *=non-native species either established or predicted future invader of the Great Lakes. In order to discern all possible regions with the potential to design eDNA assays, only sequences longer than 1000bp were included.

Common name	Species	Taxonomy	Accession
Lake sturgeon	*Acipenser fulvescens*	Order: Acipenseriformes Family: Acipenseridae	AJ245829.1, KU985070.1, KU985081.1, KU985082.2, KU985084.1, NC_030325.1
Paddlefish	*Polyodon spathula*	Order: Acipenseriformes Family: Polydontidae	AJ245841.1, AP004353.1, AY442349.1, AY510086.1, KU985085.2, KU985086.2, NC_004419.1
Bowfin	*Amia calva*	Order: Amiiformes Family: Amiidae	AB018999.1, AB042952.1, NC_004742.1
American eel	*Anguilla rostrata*	Order: Anguilliformes Family: Anguillidae	AB021767.1, AF006716.1, AF006717.1, AF485271.1-AF485276.1, AP007249.2, KJ564170.1-KJ564184.1, KJ564186.1, KJ564187.1, KJ564189.1-KJ564217.1, NC_006547.2
Bigscale sand smelt*	*Atherina boyeri*	Order: Atheriniformes Family: Atherinidae	AB848929.1, EU036421.1, EU036422.1, EU253549.1, EU253550.1

Common name	Species	Taxonomy	Accession
Brook silverside	*Labidesthes sicculus*	Order: Atheriniformes Family: Atherinopsidae	JQ282031.1, KC736409.1
Skipjack herring*	*Alosa chrysochloris*	Order: Clupeiformes Family: Clupeidae	EF653231.1, EF653232.1, KJ158092.1, MG958209.1
Alewife*	*Alosa pseudoharengus*		AP009132.1, NC_009576.1
American shad*	*Alosa sapidissima*		EU552616.1, HQ331537.1, KY769128.1, NC_014690.1
Black sea sprat*	*Clupeonella cultriventris*		AP009615.1, NC_015109.1
Gizzard shad	*Dorosoma cepedianum*		DQ536426.1, EU552584.1-EU552586.1, NC_008107.1
Banded killifish	*Fundulus diaphanus*	Order: Cyprinidontiformes Family: Fundulidae	FJ445394.1, FJ445395.1, KX359040.1, KX359041.1, NC_012361.1
Starhead topminnow	*Fundulus dispar*		GQ119707.1, GQ119708.1, L31599.1, U77119.1, U77120.1
Blackstripe topminnow	*Fundulus notatus*		KF245643.1-KF245748.1, KP013106.1, KP059009.1, NC_028293.1
Mosquitofish*	*Gambusia affinis*	Order: Cyprinidontiformes Family: Poeceliidae	AP004422.1, EF017514.1, KP059011.1, NC_004388.1
River carpsucker	*Carpiodes carpio*	Order: Cypriniformes Family: Catastomidae	AB126083.1, AF454867.1, AP006763.1, AY366087.1, JF799431.1, JN053177.1, JN053185.1, JN053187.1, JN053188.1, JN053190.1, JN053193.1, JN053194.1, JN053208.1, JN053221.1, JN053222.1, JN053237.1-JN053239.1, JN053245.1-JN053253.1, JN053255.1-JN053260.1, NC_005257.1
Quillback	*Carpiodes cyprinus*		JF799432.1, JF799433.1, JN053178.1, JN053179.1, JN053183.1, JN053191.1, JN053195.1-JN053197.1, JN053199.1, JN053203.1, JN053205.1, JN053209.1-JN053220.1, JN053228.1, JN053241.1, JN053242.1, JN053261.1, JN053263.1, JX488761.1
Highfin carpsucker	*Carpiodes velifer*		JF799434.1, JN053180.1-JN053182.1, JN053184.1, JN053186.1, JN053189.1, JN053192.1, JN053204.1, JN053262.1, JX488762.1
Longnose sucker	*Catostomus catostomus*		AF454871.1, EU676808.1, JX258854.1-JX258858.1, KJ441284.1, KP757032.1-KP757038.1, KT203373.1, KT203374.1, KU697931.1, KU761848.1, KU761849.1

Common name	Species	Taxonomy	Accession
White sucker	*Catostomus commersonii*		AB127394.1, HQ446762.1, JF799435.1-JF799437.1, JX488781.1, KP013114.1, KU697932.1, NC_008647.1
Western chubsucker	*Erimyzon claviformis*		No records
Eastern chubsucker	*Erimyzon oblongus*		AF454876.1, AP011228.1, NC_013064.1
Lake chubsucker	*Erimyzon sucetta*		AF454878.1, KU697910.1
Northern hogsucker	*Hypentelium nigricans*		AB242169.1, AF454909.1, AY253341.1-AY253405.1, AY253407.1-AY253413.1, JF799441.1, NC_008676.1
Smallmouth buffalo	*Ictiobus bubalus*		AP009316.1, FJ226281.1, FJ226285.1, FJ226287.1-FJ226290.1, FJ226299.1, FJ226300.1, FJ226302.1, FJ226333.1, FJ226335.1-FJ226364.1, JF799443.1, JX488763.1-JX488765.1, NC_013071.1
Bigmouth buffalo	*Ictiobus cyprinellus*		FJ226256.1-FJ226270.1, FJ226286.1, FJ226291.1-FJ226298.1, FJ226301.1, FJ226303.1-FJ226308.1, JF799444.1, JX488766.1, JX488767.1, KP306894.1, NC_026528.1
Black buffalo	*Ictiobus niger*		FJ226271.1-FJ226280.1, FJ226282.1-FJ226284.1, FJ226309.1, FJ226313.1-FJ226315.1, FJ226321.1, FJ226325.1-FJ226332.1, JF799446.1, JX488768.1, JX488769.1
Spotted sucker	*Minytrema melanops*		AB242166.1, AF454879.1, DQ536432.1, JF799447.1-JF799449.1, KU697909.1, NC_008113.1
Silver redhorse	*Moxostoma anisurum*		AF454880.1, AF454881.1, JF799450.1-JF799452.1
River redhorse	*Moxostoma carinatum*		AF454884.1, JF799455.1-JF799459.1, JX488820.1
Black redhorse	*Moxostoma duquesnei*		AF454894.1, AF454895.1
Golden redhorse	*Moxostoma erythrurum*		AF454886.1, AF454887.1, AY253421.1, JF799463.1-JF799470.1, KJ441285.1, KU697911.1
Copper redhorse	*Moxostoma hubbsi*		AF522289.1, JF799471.1, JF799472.1, JX488821.1
Harelip sucker	*Moxostoma lacerum*		No records
Shorthead redhorse	*Moxostoma macrolepidotum*		AF454890.1, JF799473.1-JF799476.1
Greater redhorse	*Moxostoma valenciennesi*		AF454893.1, JF799487.1

Common name	Species	Taxonomy	Accession
Oriental weatherfish*	*Misgurnus anguillicaudatus*	Order: Cypriniformes Family: Cobitidae	AB473261.1-AB473407.1, AB599977.1-AB599980.1, AB614357.1-AB614359.1, AB674743.1-AB674748.1, AB899670.1-AB899684.1, AF051868.1, AP011291.1, AY625700.1, DQ026434.1, DQ105238.1, DQ105240.1, DQ105241.1, DQ886941.1, EF088651.1, EF376188.1, EF424602.1-EF424608.1, EF508555.1-EF508559.1, EF595974.1-EF595982.1, EU131132.1-EU131140.1, EU145021.1-EU145024.1, EU670766.1, GU583669.1-GU583677.1, HM856629.1, KC509900.1, KC509901.1, KC734881.1, KC762740.1, KC823274.1, KC881110.1, KC884745.1, KF736233.1, KM186181.1, KM576227.1, KM576236.1, KM576243.1, NC_011209.1
Common bleak*	*Alburnus alburnus*	Order: Cypriniformes Family: Cyprinidae	AB239593.1, AF090743.1-AF090745.1, AY026393.1, DQ350253.1, DQ350254.1, HM560060.1-HM560062.1, JQ436541.1, KJ463863.1, NC_008659.1, Y10443.1
Central stoneroller	*Campostoma anomalum*		AF452079.1, DQ324063.1, DQ486786.1-DQ486788.1, DQ486795.1-DQ486801.1, DQ486803.1-DQ486811.1, DQ486813.1, DQ486816.1-DQ486822.1, DQ486824.1, DQ486826.1-DQ486828.1, DQ486837.1, JQ712313.1, KY398932.1
Largescale stoneroller	*Campostoma oligolepis*		DQ324064.1, DQ486793.1, DQ486794.1, DQ486802.1, DQ486812.1, DQ486814.1, DQ486815.1, DQ486823.1, DQ486825.1, DQ486829.1-DQ486836.1, DQ486838.1, HQ446741.1, JQ712314.1

Common name	Species	Taxonomy	Accession
Goldfish*	*Carassius auratus*		AB006953.1, AB111951.1, AF051858.1, AP011236.1, AP011239.1, EF055472.1, EF483931.1, GU086395.1-GU086397.1, GU135503.1-GU135601.1, GU135603.1-GU135605.1, HQ689793.1-HQ689890.1, HQ689910.1-HQ689912.1, HQ875340.1, JN105355.1, JX183534.1-JX183536.1, KF147851.1, KJ476998.1, KJ735886.1-KJ735908.1, KJ874428.1-KJ874431.1, KM657141.1-KM657143.1, KM659025.1, KT756205.1, KU146528.1, KX505165.1, MF443758.1-MF443771.1, NC_002079.1, NC_006580.1, NC_015142.1
Crucian carp*	*Carassius carassius*		AY714387.1, JQ911695.1, KX781320.1, NC_006291.1
Prussian carp*	*Carassius gibelio*		GU138989.1, HM000005.1, HM000006.1, HM000010.1, HM000019.1, HM000022.1, HM000025.1-HM000029.1, KU896991.1, KU896992.1, KX505166.1, KX611160.1
Northern redbelly dace	*Chrosomus eos*		AP009151.1, EU755056.1, NC_015364.1
Southern redbelly dace	*Chrosomus erythrogaster*		AP011276.1, AY281055.1, EU755049.1-EU755055.1, NC_031570.1
Redside dace	*Clinostomus elongatus*		AP011280.1, GU182772.1-GU182776.1, GU182820.1-GU182822.1, JN053201.1, JN053223.1, JN053254.1, NC_031572.1
Lake chub	*Couesius plumbeus*		AF452083.1, AP011274.1, AY281053.1, NC_031568.1
Grass carp*	*Ctenopharyngodon idella*		AB900162.1, AF051860.1, AF420424.1, HM237985.1-HM238043.1, JN673556.1
Satinfin shiner	*Cyprinella analostana*		GQ275236.1, GQ275237.1
Red shiner	*Cyprinella lutrensis*		AB070206.1, DQ324095.1, GQ275183.1-GQ275190.1, GQ275194.1, KR061540.1-KR061551.1, KR061553.1, KR061555.1-KR061557.1, KR061559.1, KR061561.1-KR061563.1, KR061566.1-KR061569.1, NC_008643.1
Spotfin shiner	*Cyprinella spiloptera*		DQ536422.1, GQ275218.1-GQ275223.1, NC_008103.1, U66605.1
Steelcolor shiner*	*Cyprinella whipplei*		GQ275230.1-GQ275233.1

Common name	Species	Taxonomy	Accession
Common carp*	*Cyprinus carpio*		AB126083.1, AB158803.1-AB158807.1, AF454867.1, AP006763.1, AY347276.1-AY347295.1, AY366087.1, EU676848.1, EU689059.1-EU689072.1, HQ443697.1, JF799431.1, JN053177.1, JN053185.1, JN053187.1, JN053188.1, JN053190.1, JN053193.1, JN053194.1, JN053201.1, JN053208.1, JN053221.1-JN053223.1, JN053237.1-JN053239.1, JN053245.1-JN053260.1, KF574485.1-KF574490.1, NC_005257.1
Gravel chub	*Erimystax x-punctatus*		AF117172.1, AF117173.1, AY486043.1-AY486054.1, KC763653.1
Tonguetied minnow	*Exoglossum laurae*		JF949841.1, JQ712316.1, JX442989.1, KY398933.1
Cutlips minnow	*Exoglossum maxillingua*		JX442988.1, KC763683.1
Brassy minnow	*Hybognathus hankinsoni*		AF452080.1, EU811090.1
Eastern silvery minnow	*Hybognathus regius*		EU811087.1, EU811088.1, GQ275151.1
Bigeye chub	*Hybopsis amblops*		EU917316.1-EU917406.1, EU917408.1-EU917417.1, HQ446747.1
Silver carp*	*Hypophthalmichthys molitrix*		AB198974.1, AF051866.1, EU315941.1, JQ231114.1, KJ671449.1, KJ671450.1, KJ679503.1, KJ729076.1, KJ729092.1-KJ729094.1, KJ746938.1-KJ746940.1, KJ746943.1-KJ746957.1, KJ746960.1, KJ746961.1, KJ746964.1, KJ746965.1, KP013119.1, KY126320.1, MF180230.1-MF180232.1, NC_010156.1, NC_034667.1
Bighead carp*	*Hypophthalmichthys nobilis*		AP011217.1, EU343733.1, HM162839.1, JQ346141.1, KJ679504.1, KJ679505.1, KJ710362.1, KJ710363.1, KJ729077.1-KJ729091.1, KJ729095.1-KJ729097.1, KJ746935.1-KJ746937.1, KJ746941.1, KJ746942.1, KJ746958.1, KJ746959.1, KJ746962.1, KJ746963.1, KJ746966.1, KJ756343.1, KY126320.1, MF180233.1-MF180235.1, NC_010194.1, NC_034667.1
Eurasian ide/orfe*	*Leuciscus idus*		AY026397.1, HM560098.1, HM560099.1
Common dace*	*Leuciscus leuciscus*		AY509823.1, DQ664302.1-DQ664306.1, HM560100.1, HM560101.1
Striped shiner	*Luxilus chrysocephalus*		AF117166.1, AF117167.1, AP012079.1, GQ275161.1, NC_033923.1, U66595.1, U66596.1

Common name	Species	Taxonomy	Accession
Common shiner	*Luxilus cornutus*		AP012090.1, NC_033931.1, U66597.1
Redfin shiner	*Lythrurus umbratilis*		AP012094.1, GQ275160.1, NC_033935.1, U17274.1
Silver chub	*Macrhybopsis storeriana*		KC763654.1, KX139438.1, NC_030485.1
Allegheny pearl dace	*Margariscus margarita*		AF452072.1, AP012081.1, JX443011.1
Northern pearl dace	*Margariscus natchtriebi*		No records
Black carp*	*Mylopharyngodon piceus*		AF051870.1, AP011216.1, DQ026435.1, EU979305.1, EU979307.1, MF687109.1, MF687137.1, NC_011141.1
Hornyhead chub	*Nocomis biguttatus*		AP012082.1, AY486057.1, GQ275149.1, JQ712283.1, JQ712284.1, JQ712322.1-JQ712325.1, KM281559.1-KM281563.1, KM281565.1-KM281583.1, KM281585.1, NC_033924.1
River chub	*Nocomis micropogon*		AF452077.1, GQ275148.1, JQ712294.1-JQ712297.1, JQ712344.1-JQ712348.1, JQ712356.1
Golden shiner	*Notemigonus crysoleucas*		AB127393.1, KP013116.1, MG570412.1, MG570425.1, MG570428.1, MG570438.1, NC_008646.1, U01318.1
Pugnose shiner	*Notropis anogenus*		AY140698.1
Emerald shiner	*Notropis atherinoides*		AF261220.1, AF352272.1-AF352274.1, AP012083.1, AY096008.1, AY281062.1, HM224297.1, KT834521.1, NC_033925.1
Bridle shiner	*Notropis bifrenatus*		AP012097.1, KC763658.1, NC_033938.1
River shiner	*Notropis blennius*		AF117170.1, AF117171.1
Bigeye shiner	*Notropis boops*		AF352261.1
Silverjaw minnow	*Notropis buccatus*		AF117154.1-AF117157.1, GQ275154.1, KC763688.1
Ghost shiner	*Notropis buchanani*		AY281058.1, GQ275162.1, HM179622.1-HM179630.1
Ironcolor shiner	*Notropis chalybaeus*		KC763697.1
Bigmouth shiner	*Notropis dorsalis*		AF117162.1, AF117163.1, AF117174.1, AF117175.1
Blackchin shiner	*Notropis heterodon*		AY140697.1
Blacknose shiner	*Notropis heterolepis*		AY140696.1
Spottail shiner	*Notropis hudsonius*		HQ446752.1, KT834523.1
Silver shiner	*Notropis photogenis*		AF352280.1, AF352281.1
Swallowtail shiner	*Notropis procne*		KC763670.1

Common name	Species	Taxonomy	Accession
Rosyface shiner	*Notropis rubellus*		AF117194.1, AF117195.1, AF469164.1, EU084794.1-EU084867.1
Sand shiner	*Notropis stramineus*		DQ536429.1, HM179631.1-HM179637.1, NC_008110.1
Weed shiner	*Notropis texanus*		AF352267.1
Mimic shiner	*Notropis volucellus*		AF352268.1, HM179557.1-HM179596.1
Pugnose minnow	*Opsopoeodus emiliae*		AF261221.1, AP012085.1, GQ184496.1-GQ184498.1, GQ275152.1, GQ275153.1, NC_033926.1
Suckermouth minnow	*Phenacobius mirabilis*		DQ536431.1, JF949845.1, NC_008112.1
Finescale dace	*Phoxinus neogaeus*		EU755058.1, EU755059.1
Eurasian minnow*	*Phoxinus phoxinus*		AB671170.1, AP009309.1, AP011272.1, EF094550.1, EU352213.1, EU755036.1, KC992395.1, KX265376.1-KX265402.1, NC_020358.1
Bluntnose minnow	*Pimephales notatus*		AP012101.1, GQ184499.1-GQ184518.1, GQ275155.1, HQ446759.1, KU856827.1-KU856888.1, KU856890.1-KU856949.1, NC_033941.1, U66606.1
Fathead minnow	*Pimephales promelas*		AP011279.1, GQ184519.1-GQ184522.1, GQ275158.1, GQ275159.1, KT278765.1, KT289925.1, KU856825.1, NC_028087.1
Bullhead minnow	*Pimephales vigilax*		AF117202.1, AF117203.1, AP012102.1, GQ184528.1-GQ184534.1, GQ275157.1, KU856822.1-KU856824.1
Stone moroko*	*Pseudorasbora parva*		AB366541.1, AB677449.1, AF051873.1, AY952995.1, EU934500.1-EU934504.1, HM117852.1-HM117901.1, HM224302.1, HM560155.1, JF802126.1, JX472459.1, KJ135626.1, LC098191.1-LC098196.1, NC_015614.1, Y10453.1
Eastern blacknose dace	*Rhinichthys atratulus*		AF452078.1, AP012104.1, JX442984.1, KF640094.1, KF640095.1, KY398975.1, NC_033943.1
Great lakes longnose dace	*Rhinichthys cataractae*		AP012105.1, EU811101.1, FJ744108.1, HQ446760.1, JQ712320.1, JX442982.1, KF640096.1-KF640151.1, KF640153.1-KF640157.1, NC_033944.1
Western blacknose dace	*Rhinichthys obtusus*		DQ990250.1

Common name	Species	Taxonomy	Accession
Common roach*	*Rutilus rutilus*		AF090772.1, DQ061933.1, DQ447727.1, FJ025068.1, FJ025072.1, FJ025074.1, FJ025077.1-FJ025079.1, HM156751.1-HM156759.1, HM560167.1, KC696559.1, KF784808.1, KF784810.1-KF784815.1, KF784819.1-KF784822.1, KF784831.1-KF784833.1, KF784838.1-KF784841.1, KU302643.1, KX583754.1-KX583795.1, KX583814.1-KX583817.1, KX583835.1-KX583919.1, KX588545.1-KX588552.1
Common rudd*	*Scardinius erythrophthalmus*		AP011263.1, AY509835.1-AY509848.1, EF105295.1, EU856057.1, HM560171.1, NC_031561.1
Creek chub	*Semotilus atromaculatus*		AF452082.1, AP012107.1, HM224307.1, HQ446761.1, NC_033946.1
Fallfish	*Semotilus corporalis*		No records
Grass pickerel	*Esox americanus*	Order: Esociformes Family: Esocidae	AY497427.1-AY497436.1
Northern pike	*Esox lucius*		AP004103.1, AY497445.1-AY497453.1, FJ425091.1-FJ425097.1, HM177469.1, HM177470.1, KT124232.1-KT124235.1, KT203375.1-KT203379.1, KU244688.1, KU244696.1, KU659805.1, KY399416.1-KY399442.1, NC_004593.1
Muskellunge	*Esox masquinongy*		AY497455.1, AY497456.1
Chain pickerel	*Esox niger*		AP013046.1, AY497437.1-AY497441.1
Central mudminnow	*Umbra limi*	Order: Esociformes Family: Umbridae	AY497458.1, KP013095.1, NC_028282.1
Burbot	*Lota lota*	Order: Gadiformes Family: Lotidae	AP004412.1, DQ174052.1, DQ174053.1, KC844053.1, KM201364.1, KM363244.1, KT327178.1, KU244689.1, KU244691.1, KU244692.1, NC_004379.1
Fourspine stickleback	*Apeltes quadracus*	Order: Gasterosteiformes Family: Gasterosteidae	AB445126.1, NC_011580.1
Brook stickleback	*Culaea inconstans*		AB445125.1, NC_011577.1

Common name	Species	Taxonomy	Accession
Threespine stickleback*	*Gasterosteus aculeatus*		AB094606.1-AB094627.1, AB678412.1-AB678418.1, AF356079.1, AP002944.1, AY116004.1, AY787224.1, KJ628012.1, KR912169.1-KR912173.1, KT971020.1-KT971072.1, LC108042.1, LC108074.1, LC108076.1, LC108085.1, LC108093.1, LC108094.1
Ninespine stickleback	*Pungitius pungitius*		AB094628.1, AB445130.1, AF356080.1, GU227740.1-GU227783.1, JF798872.1-JF798929.1, JQ982981.1-JQ983070.1, KJ627975.1-KJ627989.1, KR779233.1-KR779244.1, KT583722.1, KT583723.1, KT989571.1, KX384721.1-KX384725.1, LC108045.1, LC108047.1, LC108050.1, LC108053.1, LC108055.1, LC108069.1-LC108073.1, LC108075.1, LC108086.1, LC108087.1, LC108089.1, LC108090.1, LC108097.1, NC_011571.1
Racer goby*	*Babka gymnotrachelus*	Order: Gobiiformes Family: Gobiidae	EU444667.1, FJ526765.1-FJ526767.1, KC886267.1, KC886268.1, KF415509.1
Starry goby*	*Benthophilus stellatus*		FJ526780.1
Caucasian dwarf goby*	*Knipowitschia caucasica*		FJ526796.1, KF214248.1-KF214256.1, KT809447.1-KT809449.1
Black Sea Monkey goby*	*Neogobius fluviatilis*		EU444672.1, FJ526749.1-FJ526753.1, KC886273.1-KC886275.1, KF549991.1-KF549993.1, KJ605175.1-KJ605184.1
Round goby*	*Neogobius melanostomus*		EU331156.1-EU331236.1, EU564119.1-EU564125.1, KC886276.1-KC886278.1, KF549988.1-KF549990.1, KJ605185.1, KT231987.1-KT232004.1, KX619643.1
Caspian Sea Monkey goby*	*Neogobius pallasi*		GQ444372.1-GQ444434.1
Bighead goby*	*Ponticola kessleri*		EU444669.1, FJ526768.1-FJ526770.1, KC886259.1, KC886260.1, KJ605186.1-KJ605189.1, KM583832.1, NC_025638.1
Marine tubenose goby*	*Proterorhinus marmoratus*		EU444614.1-EU444617.1, EU444620.1-EU444624.1, EU444629.1, EU444635.1, EU444637.1-EU444648.1, EU444652.1-EU444657.1, EU444666.1, KF415640.1

Common name	Species	Taxonomy	Accession
Freshwater tubenose goby*	*Proterorhinus semilunaris*		EU444604.1-EU444609.1, EU444612.1, EU444613.1, EU444625.1-EU444628.1, EU444632.1-EU444634.1, EU444649.1-EU444651.1, EU444658.1-EU444665.1, KJ605190.1-KJ605212.1
Mooneye	*Hiodon tergisus*	Order: Hiodontiformes Family: Hiodontidae	AP009499.1, NC_015082.1
Spotted gar	*Lepisosteus oculatus*	Order: Lepisisteiformes Family: Lepisosteidae	AB042861.1, AY442350.1, JF912051.1-JF912053.1, NC_004744.1
Longnose gar	*Lepisosteus osseus*		DQ536423.1, JF912057.1-JF912059.1, NC_008104.1
Shortnose gar	*Lepisosteus platostomus*		JF912054.1-JF912056.1
European smelt*	*Osmerus eperlanus*	Order: Osmeriformes Family: Osmeridae	EU492295.1, EU492321.1, FJ010889.1
Rainbow smelt*	*Osmerus mordax*		AB114911.1, FJ010902.2-FJ010904.2, HM106493.1, HQ115272.1, HQ915956.1, KP257703.1-KP257780.1, NC_015246.1
Rock bass	*Ambloplites rupestris*	Order: Perciformes Family: Centrarchidae	AY115977.1, AY115978.1, AY225663.1, EU501059.1-EU501080.1, KY660677.1, NC_035659.1
Redbreast sunfish*	*Lepomis auritus*		AY115969.1, AY115970.1, AY828949.1-AY828957.1, JF742827.1, MF621723.1, NC_036385.1
Green sunfish	*Lepomis cyanellus*		AY115973.1, AY115974.1, AY828958.1, AY828959.1, JF742828.1, KC427094.1, KP013087.1, NC_020359.1
Pumpkinseed	*Lepomis gibbosus*		AY828960.1-AY828962.1, JF742829.1, KJ513207.1, KP013097.1, MF621724.1-MF621726.1, NC_028284.1
Warmouth	*Lepomis gulosus*		AY115971.1, AY115972.1, AY828963.1, JF742830.1
Orangespotted sunfish	*Lepomis humilis*		AY374293.1, AY828964.1, AY828965.1, JF742831.1
Bluegill	*Lepomis macrochirus*		AP005993.1, AY115975.1, AY115976.1, AY225667.1, AY828966.1-AY828968.1, JN389795.2, KP013118.1, MF621712.1-MF621714.1, NC_015984.2
Longear sunfish	*Lepomis megalotis*		AY828973.1-AY828977.1, JF742833.1, KF571551.1-KF571627.1
Redear sunfish	*Lepomis microlophus*		AY828978.1-AY828982.1, JF742834.1

Common name	Species	Taxonomy	Accession
Smallmouth bass	*Micropterus dolomieu*		AB378749.1, AB378750.1, AY115997.1, AY115998.1, AY225685.1-AY225694.1, HM070845.1-HM070849.1, HM070897.1, HM070903.1, HM070904.1, KC819834.1, KU171303.1-KU171330.1, MF621710.1, MF621711.1, NC_011361.1
Florida largemouth bass*	*Micropterus floridanus*		HM070866.1, HM070868.1-HM070881.1, HM070883.1, HM070887.1-HM070889.1, HM070899.1, HQ391897.1, NC_014689.1
Largemouth bass	*Micropterus salmoides*		AF479273.1, AP014537.1, AY115999.1, AY116000.1, AY225675.1-AY225684.1, DQ536425.1, HM070864.1, HM070865.1, HM070867.1, HM070882.1, HM070891.1, HM070900.1, HM070901.1, HM070910.1, HM070911.1, HQ391896.1, KC819835.1, KX588083.1-KX588092.1, L14074.1, NC_008106.1, NC_014686.1
White crappie	*Pomoxis annularis*		AY115989.1, AY115990.1, JF742839.1
Black crappie	*Pomoxis nigromaculatus*		AY115991.1, AY115992.1, JF742840.1, KP013112.1, MF621715.1, MF621719.1, NC_028298.1
Northern snakehead*	*Channa argus*	Order: Perciformes Family: Channidae	AP006041.1, GU937112.1, JN681169.1-JN681171.1, JX978723.1, KC823605.1, KM077026.1, KT358952.1, KT358953.1, NC_015191.1
White perch*	*Morone americana*	Order: Perciformes Family: Moronidae	KU641485.1, NC_030281.1
White bass	*Morone chrysops*		AF240745.1, AY374295.1, AY770838.1
Yellow bass*	*Morone mississippiensis*		AF045362.1
Striped bass	*Morone saxatilis*		AF240746.1, HM447585.1, NC_014353.1
Amur sleeper*	*Percottus glenii*	Order: Perciformes Family: Odonotobutidae	AB560893.1, AY722208.1, AY722217.1, AY722243.1, AY722244.1, EF031143.1, EF031144.1, KC292213.1, KC493693.1-KC493753.1, KF415632.1, KM657956.1, NC_020350.1
Western sand darter	*Ammocrypta clara*	Order: Perciformes Family: Percidae	AF045350.1, AF183941.1, HQ128065.1
Eastern sand darter	*Ammocrypta pellucida*		AF183943.1, AY374257.1, FJ381008.1

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Greenside darter	*Etheostoma blennioides*		AF288426.1, AF386539.1, AY374261.1, EF587846.1-EF587848.1, EU118843.1-EU118896.1, EU296656.1, EU296659.1, EU296664.1, EU296665.1, EU296667.1, EU716042.1, EU716043.1, HQ128092.1, HQ128093.1, KT880218.1
Rainbow darter	*Etheostoma caeruleum*		AY374263.1, DQ465072.1-DQ465226.1, FJ381011.1-FJ381027.1, GQ250800.1-GQ250833.1, KT880220.1, KY660678.1, NC_035660.1
Bluntnose darter	*Etheostoma chlorosomum*		HQ128105.1, HQ128106.1, JQ397531.1
Iowa darter	*Etheostoma exile*		AF386541.1
Fantail darter	*Etheostoma flabellare*		AF045342.1, AF386544.1, AF412526.1, HQ128131.1
Least darter	*Etheostoma microperca*		FJ381003.1, KM035907.1-KM035910.1, KM035913.1-KM035923.1, KM035925.1-KM035931.1, KM035933.1, KM035934.1
Johnny darter	*Etheostoma nigrum*		AF183945.1, AY374268.1, GQ183642.1-GQ183677.1, KT289926.1
Tessellated darter	*Etheostoma olmstedi*		GQ183678.1-GQ183700.1
Orangethroat darter	*Etheostoma spectabile*		AF045344.1, DQ465068.1, EU046673.1, FJ381042.1-FJ381067.1, FJ381071.1, FJ381072.1, HQ128229.1, KF377052.1-KF377118.1, KF377120.1-KF377122.1
Banded darter	*Etheostoma zonale*		AF288449.1, AP005994.1, AY964705.1, AY964706.1, EU296686.1, HQ128252.1, KF592243.1-KF592245.1, KF592249.1-KF592251.1, KF592253.1-KF592260.1, KF592268.1-KF592271.1, KF592273.1, KF592276.1-KF592279.1, KF592285.1, KF592288.1, KF592292.1-KF592294.1, KF592298.1, KF592299.1, KF592302.1-KF592304.1, KF592308.1-KF592311.1, KF592313.1-KF592316.1, KF592318.1-KF592321.1, KF592328.1, KF592331.1, KF592332.1, KF592336.1-KF592339.1, KF592341.1-KF592346.1, KF592350.1, KF592353.1-KF592355.1, KF592358.1-KF592365.1, KF592370.1, KF592371.1, KF592374.1, KF592379.1, KF592380.1, KF592384.1-KF592387.1, KF592390.1, KF592394.1, KF592398.1-KF592400.1, KF592402.1, KF592404.1, KF592411.1, KF592415.1, KF592419.1, KF592420.1

Common name	Species	Taxonomy	Accession
Eurasian ruffe*	*Gymnocephalus cernua*		AF045356.1, AF386598.1, KC819833.1, KM978956.1, NC_025785.1
Yellow perch	*Perca flavescens*		AF045357.1, AF386600.1, AF546115.1, AY374280.1, EU348833.1-EU348838.1, JX629442.1-JX629448.1, KC819830.1, MF621736.1, NC_019572.1
European perch*	*Perca fluviatilis*		AF045358.1, AF386599.1, AF546116.1, AF546117.1, AY374281.1, AY929376.1, EU348839.1-EU348846.1, FJ172663.1, FJ172664.1, FJ788389.1, FJ788391.1-FJ788393.1, FJ788400.1-FJ788411.1, KM410088.1, NC_026313.1
Logperch	*Percina caprodes*		AF045354.1, AF386550.1, AY770841.1, DQ493482.1-DQ493490.1, EF587838.1-EF587841.1, EU046670.1, EU379093.1-EU379095.1, KC211182.1, KT880217.1
Channel darter	*Percina copelandi*		AF386568.1, AY374283.1
Gilt darter	*Percina evides*		AF375938.1-AF375955.1, AY374284.1, DQ493500.1
Blackside darter	*Percina maculata*		AF045353.1, AF386557.1
Slenderhead darter	*Percina phoxocephala*		AF386563.1, AY374289.1, KM209994.1-KM210030.1
River darter	*Percina shumardi*		AF386571.1, AF386572.1
Sauger	*Sander canadensis*		KC663435.1, KC819814.1-KC819818.1, KT211477.1, KT211478.1, NC_021444.1
Zander*	*Sander lucioperca*		AF546122.1, AY374291.1, FJ788390.1, FJ788394.1-FJ788399.1, GQ214532.1-GQ214534.1, HM049965.1, JX025362.1-JX025365.1, KC819823.1-KC819826.1, KC960516.1-KC960521.1, KM410087.1, KP125333.1, NC_026533.1
Walleye	*Sander vitreus*		AF045359.1, AF386602.1, KC819819.1-KC819822.1, KP013098.1, KT211421.1-KT211476.1, NC_028285.1
Freshwater drum	*Aplodinotus grunniens*	Order: Perciformes Family: Scianidae	AY225662.1, KP722606.1, KT880216.1
Pirate perch	*Aphredoderus sayanus*	Order: Percopsiformes Family: Apherdoderidae	AP004403.1, NC_004372.1
Troutperch	*Percopsis omiscomaycus*	Order: Percopsiformes Family: Percopsidae	No records
Chestnut lamprey	*Ichthyomyzon castaneus*	Order: Petromyzontiformes Family: Petromyzontidae	GQ206168.1

Common name	Species	Taxonomy	Accession
Northern brook lamprey	*Ichthyomyzon fossor*		GQ206170.1, KM267716.1, NC_025552.1
Silver lamprey	*Ichthyomyzon unicuspis*		GQ206171.1, KM267717.1, NC_025553.1
American brook lamprey	*Lampetra appendix*		GQ206179.1, KJ684697.1-KJ684704.1, KM267719.1, NC_025583.1
Sea lamprey*	*Petromyzon marinus*		GQ206148.1, KJ684768.1, NC_001626.1, U11880.1
European Flounder* (exterpated)	*Platichthys flesus*	Order: Pleuronectiformes Family: Pleuronectidae	AB125334.1, EU224026.1, EU492120.1, EU492121.1, EU492293.1, EU492294.1, FJ515658.1
Cisco	*Coregonus artedi*	Order: Salmoniformes Family: Salmonidae	JX960771.1, JX960772.1, MF621765.1, MF621766.1, NC_036393.1
Lake whitefish	*Coregonus clupeaformis*		JQ390060.1, JQ661482.1-JQ661487.1, JX960775.1, JX960776.1, NC_020762.1
Bloater	*Coregonus hoyi*		JX960777.1, JX960778.1
Ives lake cisco	*Coregonus hubbsi*		No records
Kiyi	*Coregonus kiyi*		JX960780.1
Blackfin cisco	*Coregonus nigripinnis*		JX960788.1
Nipigon tullibee	*Coregonus nipigon*		No records
Shortnose cisco	*Coregonus reighardi*		No records
Siskiwit lake cisco	*Coregonus zenithicus*		JX960796.1
Deepwater cisco (extinct)	*Corregonus johannae*		No records
Pink salmon*	*Oncorhynchus gorbuscha*		EF455489.1, FJ435607.1, FJ435608.1, JX185439.1, JX185440.1, JX185443.1, JX185444.1, JX960805.1, JX960806.1, KU761855.1, KU872713.1, NC_010959.1
Chum salmon*	*Oncorhynchus keta*		AB039896.1, AF125212.1, AP010773.1, FJ435616.1, FJ435617.1, JX960807.1, JX960808.1, KU872716.1, KX958410.1, NC_017838.1
Coho salmon*	*Oncorhynchus kisutch*		EF126369.1, FJ435609.1, FJ435610.1, JX185441.1, JX185442.1, JX258853.1, JX960809.1, JX960810.1, KJ740755.1-KJ740761.1, KP671851.1, KU761856.1, KU761857.1, KU872712.1, MF621749.1, MF621751.1, NC_009263.1

Common name	Species	Taxonomy	Accession
Rainbow trout*	*Oncorhynchus mykiss*		AF125208.1, AF125209.1, AY032629.1-AY032632.1, AY587167.1-AY587185.1, D58401.1, DQ288268.1-DQ288271.1, FJ435586.1-FJ435602.1, HQ167694.1, JX960813.1-JX960815.1, KP013084.1, KP085590.1, KU761858.1, KU761859.1, KU872710.1, L29771.1, LC050735.1, MF621750.1, NC_001717.1
Chinook salmon*	*Oncorhynchus tshawytscha*		AF392054.1, FJ435603.1, FJ435604.1, HQ167695.1, JX960819.1, JX960820.1, KU761862.1, KU761863.1, KU872715.1, KX958411.1, NC_002980.1
Pygmy whitefish	*Prosopium coulterii*		JX960823.1, JX960824.1, KT630746.1-KT630748.1
Round whitefish	*Prosopium cylindraceum*		AP013050.1, JQ390062.1, JX960825.1, JX960826.1, KT630744.1, KU244693.1, KU244694.1, KU761864.1-KU761866.1, MF621759.1, MF621764.1, MF621767.1, MF621768.1, NC_020764.1
Atlantic salmon*	*Salmo salar*		AF053591.1, AF133701.1, AF202032.1, BT044011.1, EF584212.1, EU492280.1, EU492281.1, FJ435618.1-FJ435620.1, HQ167697.1, JQ390055.1, JQ390056.1, JX960833.1, JX960834.1, KF792729.1, KY122205.1, KY122206.1, LC012541.1, NC_001960.1, U12143.1
Brown trout*	*Salmo trutta*		AM910409.1, D58400.1, EU492108.1, EU492109.1, EU492282.1, EU492348.1, FJ435621.1-FJ435623.1, FJ655773.1, HQ167696.1, JN995186.1, JQ390057.1, JX960835.1-JX960837.1, JX960839.1, KF985666.1-KF985738.1, KT279167.1-KT279175.1, KT279178.1-KT279198.1, KT633607.1, LC011387.1, LC137015.1, LC145638.1, MF621760.1-MF621763.1, NC_010007.1, NC_024032.1
Brook trout	*Salvelinus fontinalis*		AF154850.1, D58399.1, HQ167699.1, JX960851.1, JX960852.1, KU872718.1, MF621737.1-MF621739.1, NC_000860.1
Lake trout/siscowet	*Salvelinus namaycush*		JX960857.1, JX960858.1, KT630743.1, KU761867.1-KU761869.1, MF621742.1, MF621744.1-MF621748.1, NC_036392.1

Common name	Species	Taxonomy	Accession
Arctic Grayling (exterpated)	*Thymallus arcticus*		AF319544.1, GQ452036.1, JX960861.1, JX960862.1, KJ866481.1, KT630732.1-KT630734.1, KU258419.1, KU258420.1, KU761871.1-KU761876.1, MF621752.1-MF621758.1
Mottled sculpin	*Cottus bairdii*	Order: Scorpaeniformes Family: Cottidae	AF549123.1-AF549127.1, AF549162.1-AF549167.1, AY116363.1, AY833333.1-AY833336.1, KP013090.1, NC_028277.1
Slimy sculpin	*Cottus cognatus*		AF549118.1-AF549120.1, AY116364.1, AY116365.1, AY833342.1
European bullhead*	*Cottus gobio*		AY116366.1
Spoonhead sculpin	*Cottus ricei*		AY833363.1
Deepwater sculpin	*Myoxocephalus thompsonii*		AY338275.1, AY338276.1, AY833369.1
White catfish	*Ameiurus catus*	Order: Siluriformes Family: Ictaluridae	AF484159.1, AF484163.1, AY184267.1, AY184270.1, EF491729.1, KM264126.1, KM576102.1, NC_028151.1
Black bullhead	*Ameiurus melas*		AY184263.1, AY184273.1, KT804702.1
Yellow bullhead	*Ameiurus natalis*		AF484158.1, AY184255.1, AY184265.1, AY458888.1, MF621735.1, NC_036391.1
Brown bullhead	*Ameiurus nebulosus*		AY184257.1, AY184264.1, AY184271.1, AY458889.1, MF621731.1, MF621733.1, MF621734.1, NC_036387.1
Blue catfish*	*Ictalurus furcatus*		AF484159.1, EF491729.1, KM264126.1, KM576102.1, NC_028151.1
Channel catfish	*Ictalurus punctatus*		AB045119.1, AB069646.1, AF477829.1, AF482987.1, AY184253.1, AY184254.1, AY458886.1, AY791413.1, EU490914.1, GQ396767.1, GQ396769.1-GQ396773.1, GQ396792.1, GQ396793.1, JN015529.1, MF621716.1-MF621718.1, MF621720.1-MF621722.1, NC_003489.1
Stonecat	*Noturus flavus*		AY327287.1-AY327290.1, AY458892.1, KM264121.1
Tadpole madtom	*Noturus gyrinus*		AY327295.1-AY327297.1, AY458890.1
Margined madtom	*Noturus insignis*		AY327301.1-AY327303.1, AY458891.1
Brindled madtom	*Noturus miurus*		AY327306.1-AY327308.1, DQ790738.1, DQ790739.1, KM264123.1, KM363003.1-KM363065.1
Northern madtom	*Noturus stigmosus*		AY327319.1, AY327320.1

Common name	Species	Taxonomy	Accession
Flathead catfish*	*Pylodictis olivaris*		AF484161.1, AY458887.1, DQ790748.1, GQ396768.1, MF621727.1-MF621730.1, NC_036386.1
Wels catfish*	*Silurus glanis*	Order: Siluriformes Family: Siluridae	AM398435.2, NC_014261.1
Shortsnouted pipefish*	*Syngnathus abaster*	Order: Sygnathiformes Family: Sygnathidae	AF356060.1, JX228141.1

Appendix B.2 Mock communities. (A) Hypothetical design (ng/µl genomic DNA) of mock community (MC) experiments. Each extraction was serially diluted 1:4 such that the mock community in which it was least concentrated would have <0.05ng/µl. (B) Species included in mock communities for the three cyt *b* assays. •=non-native species established in the Great Lakes. †=unestablished non-native species on the GLANSIS watch list. (C) Linear regression slope (m) and R^2 and Spearman rank coefficient (ϱ) for each mock community (MC 1–5) and values for species across libraries for each marker. Significance for slope is the difference from one. *=p<0.05, **=p<0.01, and ***=p<0.001.

A	**MC 1**	**MC 2**	**MC 3**	**MC 4**	**MC 5**	**Total taxon**
Taxon 1	312.5	62.5	12.5	2.5	0.5	*390.5*
Taxon 2	0.5	312.5	62.5	12.5	2.5	*390.5*
Taxon 3	2.5	0.5	312.5	62.5	12.5	*390.5*
Taxon 4	12.5	2.5	0.5	312.5	62.5	*390.5*
Taxon 5	62.5	12.5	2.5	0.5	312.5	*390.5*
Taxon 6	156.25	31.25	6.25	1.25	0.25	*195.25*
Taxon 7	0.25	156.25	31.25	6.25	1.25	*195.25*
Taxon 8	1.25	0.25	156.25	31.25	6.25	*195.25*
Taxon 9	6.25	1.25	0.25	156.25	31.25	*195.25*
Taxon 10	31.25	6.25	1.25	0.25	156.25	*195.25*
Total MC	*585.75*	*585.75*	*585.75*	*585.75*	*585.75*	

B

FishCyt*b*	**CarpCyt*b***	**GobyCyt*b***
Sander vitreus	*Carassius auratus*•	*Neogobius melanostomus 1*•
Perca fluviatilis†	*Ctenopharyngodon idella*•	*N. melanostomus 8*•
Gymnocephalus cernua•	*Cyprinus carpio*•	*N. melanostomus 57*•
Hypophthalmichthys molitrix†	*Hypophthalmichthys molitrix A*†	*N. fluviatilis*†
Ctenopharyngodon idella•	*H. molitrix B*†	*N. pallasi*†
Cyprinus carpio•	*H. molitrix H*†	*Proterorhinus semilunaris*•
Neogobius melanostomus•	*H. nobilis*†	*Proterorhinus marmoratus*†
Proterorhinus semilunaris•	*Mylopharyngodon piceus*†	*Babka gymnotrachelus*†
Micropterus dolomieu	*Neogobius melanostomus*•	*Ponticola kessleri*†
Clupeonella cultriventris†	*Sander vitreus*	*Sander vitreus*

C

FishCyt*b*	***m***	***R*²**	ϱ
MC 1	0.84	0.87***	0.97***
MC 2	0.61**	0.86***	0.87**
MC 3	0.67*	0.89**	0.89*
MC 4	1.03	0.93***	0.95***
MC 5	0.88	0.87***	0.90**
All FishCytb MCs	*0.82***	*0.80****	*0.90****
Sander vitreus	0.90	0.96**	1.00*
Perca fluviatilis†	0.79	0.92*	1.00
Gymnocephalus cernua•	0.73	0.89	1.00
Hypophthalmichthys molitrix†	0.57	0.93*	1.00
Ctenopharyngodon idella•	1.27	0.93*	1.00
Cyprinus carpio•	0.89	0.83	0.80
Neogobius melanostomus•	0.83	0.95**	1.00*
Proterorhinus semilunaris•	0.74	0.92**	1.00*
Micropterus dolomieu	0.60*	0.96*	1.00

Clupeonella cultriventris†	0.88	0.95**	1.00*

CarpCyt*b*	***m***	***R*²**	*ϱ*
MC 1	1.12	0.94***	0.87**
MC 2	0.88	0.94***	0.98***
MC 3	0.82	0.94***	0.95***
MC 4	0.81	0.72**	0.90**
MC 5	0.88	0.85***	0.87**
All CarpCytb MCs	*0.86**	*0.86****	*0.93****
Carassius auratus•	0.57	0.51	0.80
Ctenopharyngodon idella•	0.80	0.91*	0.90
Cyprinus carpio•	1.01	0.94**	0.90
Hypophthalmichthys molitrix A†	1.09	0.98*	1.00
H. molitrix B†	0.83	0.02	0.20
H. molitrix H†	0.88	0.97**	0.90
H. nobilis†	0.83	0.87*	0.90
Mylopharyngodon piceus†	0.92	0.98***	0.90
Neogobius melanostomus•	1.26	0.93**	1.00*
Sander vitreus	0.98	0.54	0.70

GobyCyt*b*	***m***	***R*²**	*ϱ*
MC 1	1.09	0.83***	0.90**
MC 2	1.05	0.86***	0.95***
MC 3	0.81	0.88***	0.93***
MC 4	1.04	0.90***	0.93***
MC 5	1.33*	0.94***	0.96***
All GobyCytb MCS	*1.04*	*0.81****	*0.90****
Neogobius melanostomus 1•	1.28*	0.99***	1.00*
N. melanostomus 8•	1.45	0.97*	1.00
N. melanostomus 57•	1.04	0.91*	1.00*
N. fluviatilis†	0.86	0.97**	1.00*
N. pallasi†	1.04	0.94**	1.00*
Proterorhinus semilunaris•	1.26	0.98**	1.00*
P. marmoratus†	1.22	0.95**	1.00*
Babka gymnotrachelus†	1.13	0.93**	1.00*
Ponticola kessleri†	0.81	0.97**	1.00*
Sander vitreus	1.00	0.93**	1.00*

Appendix B.3 Species present in high diversity tank experiments. Number of individuals (*N*) and grams of biomass (g) for species in display tanks (A) 1 at University of Toledo's Lake Erie Center and (B) 2 at a commercial fishing outfitter in Rossford, OH. Fishes in display tank 1 were weighed and measured. Grams of biomass in display tank 2 was estimated by the aquarist at the facility.

A					
Order	**Family**	**Common name**	**Scientific name**	***N***	**g**
Acipenseriformes	Acipenseridae	Lake sturgeon	*Acipenser fulvescens*	1	646
Amiiformes	Amiidae	Bowfin	*Amia calva*	1	351
Perciformes	Centrarchidae	Bluegill	*Lepomis macrochirus*	2	242
Perciformes	Percidae	Yellow perch	*Perca flavescens*	2	397
		Walleye	*Sander vitreus*	1	202
Siluriformes	Icataluridae	Brown bullhead	*Ameiurus nebulosus*	1	427
B					
Amiiformes	Amiidae	Bowfin	*Amia calva*	1	3629
Cypriniformes	Catostomidae	Smallmouth buffalo	*Ictiobus bubalus*	2	20410
		Bigmouth buffalo	*ictiobus cyprinellus*	2	20410
Cypriniformes	Cyprinidae	Common carp	*Cyprinus carpio*	2	8164
Lepisosteiformes	Lepisosteidae	Longnose gar	*Lepisosteus osseus*	3	9523
		Shortnose gar	*Lepisosteus platostomus*	1	680
Perciformes	Centrarchidae	Bluegill	*Lepomis macrochirus*	3	2721
		Smallmouth bass	*Micropterus dolomieu*	3	6801
		Largemouth bass	*Micropterus salmoides*	34	82555
		Hybrid striped bass	*Morone chrysops* × *saxatilis*	2	9072
		Black crappie	*Pomoxis nigromaculatus*	8	7695
Perciformes	Percidae	Walleye	*Sander vitreus*	2	3174
Perciformes	Sciaenidae	Freshwater drum	*Aplodinotus grunniens*	1	4082
Siluriformes	Icataluridae	Blue catfish	*Ictalurus furcatus*	1	27215

Appendix B.4 Field sampling for tests of environmental DNA assays. Samples collected concomitant with (*) morphological capture-based surveys (electrofishing in the Wabash and Maumee Rivers, ichthyoplankton collected with bongo nets in the Detroit River) or (†) traditional population genetics sampling and Sanger sequencing (silver carp collected via electrofishing in the Wabash River or round goby collected with minnow seines in Lake St. Clair). II=water samples collected in duplicate, for which data were combined.

Sample Name	Location	Year	Lat, Long
gWAB 1*II	Wabash River, Lafayette, IN	2016	40.4516, -86.8944
WAB 2*II	" "	" "	40.4197, -86.8972
WAB 3†II	Lagoon near Wabash River, Lafayette, IN	" "	40.4302, -86.8980
MAU 1*	Maumee River, RM09.4	2012	41.6089, -83.5794
MAU 2*	Maumee River, RM60.0	" "	41.2914, -84.2819
MAU 3*	Maumee River, RM69.2	" "	41.2842, -84.4344
MAU4*	Maumee River, RM91.5	" "	41.2219, -84.6697
LSC 1II	Lake St Clair, Clinton Township, MI	2017	42.5929, -82.7768
LSC 2†II	Lake St Clair, Clinton Township, MI	2016	42.5935, -82.7743
LSC 3†II	Lake St Clair, Clinton Township, MI	2016	42.5935, -82.7743
LEII	Lake Erie, Stone Lab, Put In Bay, OH	2017	41.6581, -82.8212
DRL*	Detroit River larvae	2013	42.1807, -83.1272

Appendix B.5. Species and GenBank accessions used in positive controls for cytochrome (Cyt) *b* and 12S RNA (to be submitted before publication) genes.

	Accession	
Species	**Cyt *b***	**12S**
Clinitrachus argentatus	MK990528	MN154385
Cristiceps argyropluera	MK990529	MN154386
Eliginus gracilis	MK990530	MN154387
Gadus macrocephalus	MK990531	MN154388
Gibbonsia montereyensis	MK990532	MN154389
Myxodes viridis	MK990533	MN154390
Gadus chalcogrammus	MK990534	MN154391
Myxodes cristatus	MK990538, MK990539	MN154392
Clinus superciliosis	MK990535	MN154393
Sebastes alutus	MK990536	MN154394
Ribeiroclinus eigenmanni	MK990537	MN154395

Appendix B.6. High-throughput sequencing metrics. Assay (Fish=FishCyt*b*, Goby=GobyCyt*b*, Carp=CarpCyt*b*, and MiFish assay from Miya et al. 2015), sample description, library name in Sequence Read Archive, number of raw reads returned from HTS, successfully trimmed (had both primers and the correct spacer insert), merged without chimeras in DADA2, proportion of trimmed that merged, unique ASVs per library, and number with BLAST results above 90% query cover and identity. All FASTQs will be deposited in the Sequence Read Archive upon publication of this chapter in a peer reviewed journal.

Run 1			**Reads**				**ASVs**	
Assay	**Sample desc.**	**Library**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**Raw**	**Filtered**
Goby	Goby MC 1	gMC1	554,359	541,414	477,504	0.88	30	23
	Goby MC 2	gMC2	538,418	525,359	460,496	0.88	40	28
	Goby MC 3	gMC3	489,904	479,820	428,559	0.89	24	20
	Goby MC 4	gMC4	542,086	527,792	453,195	0.86	31	27
	Goby MC 5	gMC5	540,631	530,058	462,261	0.87	51	36
		Total	*2,665,398*	*2,604,443*	*2,282,015*	–	–	–
		Mean	*533,080*	*520,889*	*456,403*	*0.88*	*35.2*	*26.8*
Run 2								
Fish	Goby Tanks 2	9BKC05	636,249	617,505	470,120	0.76	71	4
	Goby Tanks 3	9BKC06	583,451	567,228	452,514	0.80	67	4
	Goby Tanks 5	9BKC08	743,919	723,453	265,936	0.37	10	3
	Goby Tanks 6	9BKC09	1,037,986	1,000,940	499,093	0.50	24	5
	Goby Tanks 8	9BKC11	827,057	799,916	507,542	0.63	28	5
	Goby Tanks 9	9BKC12	1,376,503	1,332,607	941,158	0.71	37	6
	Pos. Cont.	9GFMC1	408,358	399,568	329,879	0.83	33	13
		Total	*5,613,523*	*5,441,217*	*3,466,242*	–	–	–
		Mean	*801,932*	*777,317*	*495,177*	*0.64*	*38.6*	*5.7*
Goby	Goby Tanks 1	gBKC04	466,724	284,393	141,661	0.50	14	13
	Goby Tanks 2	gBKC05	222,219	165,909	88,408	0.53	19	10
	Goby Tanks 3	gBKC06	312,292	231,563	122,992	0.53	14	8
	Goby Tanks 4	gBKC07	479,149	328,647	157,307	0.48	45	12
	Goby Tanks 5	gBKC08	389,221	321,436	163,403	0.51	22	17
	Goby Tanks 6	gBKC09	297,543	200,828	98,199	0.49	25	13
	Goby Tanks 7	gBKC10	365,237	318,929	173,317	0.54	30	25
	Goby Tanks 8	gBKC11	391,301	317,175	169,343	0.53	32	23
	Goby Tanks 9	gBKC12	322,950	278,610	146,739	0.53	54	21
	Pos. Cont.	gMC1	215,686	208,043	184,526	0.89	28	17
		Total	*3,462,322*	*2,655,533*	*1,445,895*	–	–	–
		Mean	*346,232*	*265,553*	*144,590*	*0.54*	*28.3*	*15.9*
Run 3								
Carp	DRL	cBFO01	222,762	219,576	203,664	0.93	12	10
	Disp. Tank 1	cBIW05	213,148	209,147	197,019	0.94	47	27
	Goby Tanks 1	cBKC04	207,142	202,443	164,653	0.81	39	17
	Goby Tanks 2	cBKC05	239,950	236,423	224,936	0.95	28	17
	Goby Tanks 3	cBKC06	323,476	317,682	300,649	0.95	28	21
	Goby Tanks 4	cBKC07	327,799	320,116	302,089	0.94	33	24
	Goby Tanks 5	cBKC08	323,285	311,647	291,875	0.94	26	22
	Goby Tanks 6	cBKC09	304,201	299,610	283,692	0.95	36	27
	Goby Tanks 7	cBKC10	232,791	229,152	217,574	0.95	27	23
	Goby Tanks 8	cBKC11	183,043	180,059	172,082	0.96	16	14
	Goby Tanks 9	cBKC12	241,393	237,500	228,321	0.96	25	22
		Total	*2,818,990*	*2,763,355*	*2,586,554*	–	–	–
		Mean	*256,272*	*251,214*	*235,141*	*0.94*	*28.8*	*20.4*

			Reads				ASVs	
Assay	**Sample desc.**	**Library**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**Raw**	**Filtered**
Goby	DRL	gBFO01	429,389	420,571	386,864	0.92	46	18
	Disp. Tank 1	gBIW05	753,536	530,272	450,956	0.85	125	20
	LSC 2	gBKT01	297,649	228,303	203,906	0.89	54	34
	LSC 3	gBKT03	304,576	253,515	226,883	0.89	27	25
	Pos. Cont.	gMC1	215,686	208,043	184,526	0.89	28	17
		Total	*2,000,836*	*1,640,704*	*1,453,135*	–	–	–
		Mean	*400,167*	*328,141*	*290,627*	*0.89*	*56.0*	*22.8*
MiFish	DRL	mBFO01	305,420	299,469	261,182	0.87	11	11
	Disp. Tank 1	mBIW5A	398,532	386,258	336,312	0.87	17	16
	Pos. Cont.	BLE00	240,353	235,285	204,050	0.87	13	13
		Total	*944,305*	*921,012*	*801,544*	–	–	–
		Mean	*314,768*	*307,004*	*267,181*	*0.87*	*13.7*	*13.3*
Run 4								
Fish	Display Tank 1	fBIW5A	143,512	142,335	131,411	0.92	16	13
	WAB 1	fBKW01	139,278	100,082	69,721	0.70	476	7
	WAB 2	fBKW03	401,315	331,617	236,009	0.71	792	26
	WAB 2	fBKW04	206,614	180,262	146,361	0.81	332	10
	MAU 3	fBKZ17	265,546	232,588	121,776	0.52	178	43
	MAU 4	fBKZ22	138,723	134,420	89,759	0.67	249	37
	LEI	fBLA02	46,757	43,488	25,274	0.58	98	74
	Display Tank 2	fBLA06	58,538	57,457	39,688	0.69	99	87
	Fish MC 1	fGFMC1	115,090	113,527	104,414	0.92	11	11
	Fish MC 3	fGFMC3	106,390	105,159	98,633	0.94	7	7
	Fish MC 5	fGFMC5	97,691	96,615	89,290	0.92	10	10
	Pos. Cont.	fBLE00	95,380	94,626	89,580	0.95	18	14
		Total	*1,814,834*	*1,632,176*	*1,241,916*	–	–	–
		Mean	*151,236*	*136,015*	*103,493*	*0.76*	*190.5*	*28.3*
Carp	LSC 2	cBKT02	212,330	133,838	125,105	0.93	48	23
	LSC 3	cBKT04	100,654	93,383	86,038	0.92	70	12
	WAB 1	cBKW01	48,623	18,373	16,533	0.90	28	12
	WAB 1	cBKW02	149,555	132,516	118,161	0.89	88	28
	WAB 3	cBKW05	129,549	127,175	117,333	0.92	43	27
	MAU 4	cBKZ22	204,826	154,833	139,912	0.90	72	20
	LEI	cBLA02	114,995	41,727	17,694	0.42	83	74
	LSC 1	cBLA03	112,700	75,138	61,352	0.82	23	20
	LSC 1	cBLA05	93,095	88,818	79,486	0.89	19	15
	Display Tank 2	cBLA07	254,306	237,564	220,253	0.93	57	32
	Carp MC 1	cMC1	90,648	85,241	76,053	0.89	13	13
	Carp MC 2	cMC2	121,646	114,095	102,742	0.90	14	14
	Carp MC 3	cMC3	84,354	79,336	76,019	0.96	10	10
	Carp MC 4	cMC4	78,626	73,840	69,242	0.94	11	10
	Carp MC 5	cMC5	96,733	90,655	82,984	0.92	9	9
	Pos. Cont.	cBLE00	122,687	120,887	114,661	0.95	29	28
		Total	*2,015,327*	*1,667,419*	*1,503,568*	–	–	–
		Mean	*125,958*	*104,214*	*93,973*	*0.90*	*38.6*	*21.7*
Goby	WAB 2	gBKW04	196,252	144,535	112,972	0.78	345	16
	WAB 3	gBKW05	232,453	169,314	107,817	0.64	424	16
	MAU 2	gBKZ14	266,060	124,315	88,640	0.71	475	23
	LSC 1	gBLA04	146,312	132,596	121,062	0.91	112	23
	Display Tank 2	gBLA06	157,224	138,323	120,167	0.87	79	30
	Pos. Cont.	gBLE00	116,610	115,115	106,936	0.93	22	18
		Total	*1,114,911*	*824,198*	*657,594*	–	–	–
		Mean	*185,819*	*137,366*	*109,599*	*0.80*	*242.8*	*21.0*

			Reads				ASVs	
Assay	**Sample desc.**	**Library**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**Raw**	**Filtered**
MiFish	WAB 2	mBKW03	120,177	115,842	87,560	0.76	30	23
	WAB 2	mBKW04	135,126	129,519	107,398	0.83	24	21
	WAB 3	mBKW06	91,084	89,992	64,164	0.71	22	16
	MAU 3	mBKZ17	115,892	104,948	85,606	0.82	33	31
	LEI	mBLA01	112,173	74,478	62,284	0.84	24	18
	LSC 1	mBLA04	77,634	72,392	62,304	0.86	10	9
	Display Tank 2	mBLA06	156,755	153,573	116,466	0.76	40	36
	Pos. Cont.	mBLE00	124,134	122,929	108,221	0.88	12	12
		Total	*932,975*	*863,673*	*694,003*	–	–	–
		Mean	*116,622*	*107,959*	*86,750*	*0.80*	*24.4*	*20.8*
Run 5								
Fish	DRL	fBFO01	102,242	101,029	91,315	0.90	10	10
	WAB 3	fBKW05	259,226	225,846	169,585	0.75	479	27
	WAB 3	fBKW06	67,558	62,108	46,327	0.75	126	15
	MAU 1	fBKZ03	149,862	148,386	132,395	0.89	37	34
	MAU 2	fBKZ14	277,687	226,598	155,150	0.68	498	26
	LEI	fBLA01	36,324	34,910	20,084	0.58	104	102
	LSC 1	fBLA05	77,180	74,562	54,782	0.73	120	105
	Display Tank 2	fBLA07	83,459	80,698	61,542	0.76	80	56
	Fish MC 2	fGFMC2	120,094	118,399	106,649	0.90	12	12
	Fish MC 4	fGFMC4	72,153	71,245	65,388	0.92	12	12
	Pos. Cont.	fBLE01	71,667	70,713	66,095	0.93	17	15
		Total	*1,317,452*	*1,214,494*	*969,312*	–	–	–
		Mean	*119,768*	*110,409*	*88,119*	*0.80*	*135.9*	*37.6*
Carp	LSC 3	cBKT03	177,136	168,416	148,526	0.88	43	26
	WAB 2	cBKW03	79,486	75,947	71,595	0.94	25	15
	WAB 2	cBKW04	63,038	61,528	57,792	0.94	22	10
	Wab 3	cBKW06	68,513	66,368	60,445	0.91	24	13
	MAU 1	cBKZ03	169,881	167,357	131,415	0.79	32	29
	MAU 2	cBKZ14	86,737	53,511	43,305	0.81	31	30
	MAU 3	cBKZ17	94,228	91,125	80,861	0.89	89	20
	LEI	cBLA01	159,595	65,298	25,878	0.40	120	111
	LSC 1	cBLA04	120,931	106,462	96,002	0.90	32	22
	Display Tank 2	cBLA06	70,634	64,040	58,997	0.92	31	23
	Pos. Cont.	cBLE01	106,432	104,922	99,027	0.94	26	25
		Total	*1,196,611*	*1,024,974*	*873,843*	–	–	–
		Mean	*108,783*	*93,179*	*79,440*	*0.85*	*43.2*	*29.5*
Goby	LSC 3	gBKT04	41,063	4,134	3,206	0.78	19	19
	MAU 1	gBKZ03	137,472	133,881	119,900	0.90	40	34
	MAU 3	gBKZ17	191,927	107,215	77,812	0.73	394	31
	MAU 4	gBKZ22	229,044	151,260	115,237	0.76	379	36
	LSC 1	gBLA03b	91,944	62,765	49,222	0.78	89	18
	Display Tank 2	gBLA07	196,744	176,893	155,597	0.88	92	16
	Pos. Cont.	gBLE01	109,213	107,532	97,427	0.91	22	17
		Total	*997,407*	*743,680*	*618,401*	–	–	–
		Mean	*142,487*	*106,240*	*88,343*	*0.83*	*147.9*	*24.4*

			Reads				ASVs	
Assay	**Sample desc.**	**Library**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**Raw**	**Filtered**
MiFish	WAB 1	mBKW01	73,286	64,324	43,993	0.68	37	19
	WAB 1	mBKW02	140,774	128,020	95,957	0.75	36	28
	WAB 3	mBKW05	71,311	69,654	30,993	0.44	17	11
	MAU 1	mBKZ03	84,962	83,089	64,767	0.78	31	31
	MAU 2	mBKZ14	88,189	39,234	32,604	0.83	17	15
	MAU 4	mBKZ22	147,225	119,633	82,980	0.69	43	35
	LEI	mBLA02	108,166	90,459	80,903	0.89	22	15
	LSC 1	mBLA03	58,491	52,526	40,068	0.76	15	12
	Display Tank 2	mBLA07	183,611	174,347	130,088	0.75	40	34
	Pos. Cont.	mBLE01	110,105	108,869	94,097	0.86	12	12
		Total	*1,066,120*	*930,155*	*696,450*	–	–	–
		Mean	*106,612*	*93,016*	*69,645*	*0.75*	*27.0*	*21.2*

		Reads				ASVs	
	All runs	**Raw**	**Trimmed**	**Merged**	**Prop.**	**Raw**	**Filtered**
	Total	27,961,011	24,927,033	19,290,472	–	–	–
	Mean	229,189	204,320	158,119	0.80	75.4	23.0
	±SE	±18,645	±17,738	±12,588	±0.01	±11.4	±1.7

Appendix B.7 Morphological and eDNA HTS assay survey results. Table indicates (-) negative and positive (+) detections. Some species in the Maumee River were only found in sites where eDNA water samples were not examined(++). Total detections from morphology in the Maumee River shows *N* species in samples with concomitant eDNA water samples processed/*N* total species detected in all electrofishing surveys in the watershed.

			Display tanks		Detroit R. larvae		Maumee R.		L. St. Clair	L. Erie Is.	Wabash R.	
Order	Family	Species	Morph	eDNA	Morph	eDNA	Morph	eDNA	eDNA	eDNA	Morph	eDNA
Acipenseriformes	Acipenseridae	*Acipenser fulvescens*	+	+	-	-	-	-	-	-	-	-
Amiiformes	Amiidae	*Amia calva*	+	+	-	-	-	-	-	-	-	+
Atheriniformes	Atherinopsidae	*Labidesthes sicculus*	-	-	-	-	+	+	-	-	-	-
Clupeiformes	Clupeidae	*Alosa* sp.	-	+	-	-	-	-	-	-	-	-
		Al. chrysochloris	-	+	-	-	-	-	-	-	+	+
		Al. pseudoharengus	-	+	-	-	-	-	-	-	-	-
		Dorosoma cepedianum	-	-	+	+	+	+	+	-	+	+
Cyprinidontiformes	Fundulidae	*Fundulus notatus*	-	-	-	-	+	+	-	-	-	-
Cypriniformes	Catastomidae	*Carpiodes* sp.	-	-	-	+	-	+	+	+	-	+
		Ca. carpio	-	-	-	-	-	+	-	-	+	+
		Ca. cyprinus	-	-	+	-	+	-	-	-	-	-
		Ca. velifer	-	-	-	-	-	+	-	-	+	-
		Catostomus commersonii	-	-	+	+	++	+	+	+	-	-
		Cycleptus sp.	-	-	-	-	-	-	-	-	-	+
		Cy. Elongatus	-	-	-	-	-	-	-	-	+	+
		Hypentelium nigricans	-	-	-	-	+	-	-	-	-	+
		Ictiobus sp.	-	+	-	-	-	+	-	-	-	+
		Ic. Bubalus	+	-	-	-	+	-	-	-	+	-
		Ic. Cyprinellus	+	-	-	-	+	-	-	-	-	+
		Moxostoma sp.	-	-	-	-	-	-	-	-	-	+
		Mo. Anisurum	-	-	-	-	++	+	+	-	+	+
		Mo. Breviceps	-	-	-	-	-	-	-	-	-	+
		Mo. Carinatum	-	-	-	-	-	+	+	-	-	+
		Mo. Erythrurum	-	-	-	-	++	-	-	-	+	+
		Mo macrolepidotum	-	-	-	-	+	+	+	-	+	+
Cypriniformes	Cyprinidae	*Campostoma anomalum*	-	-	-	-	++	-	-	-	-	-
		Carassius sp.	-	+	-	-	-	+	+	-	-	-
		Cr. Auratus	-	-	-	-	+	-	+	-	-	-
		Cr. Auratus x Cyprinus carpio	-	-	-	-	++	-	-	-	-	-
		Ctenopharyngodon sp.	-	-	-	-	-	-	-	-	-	+

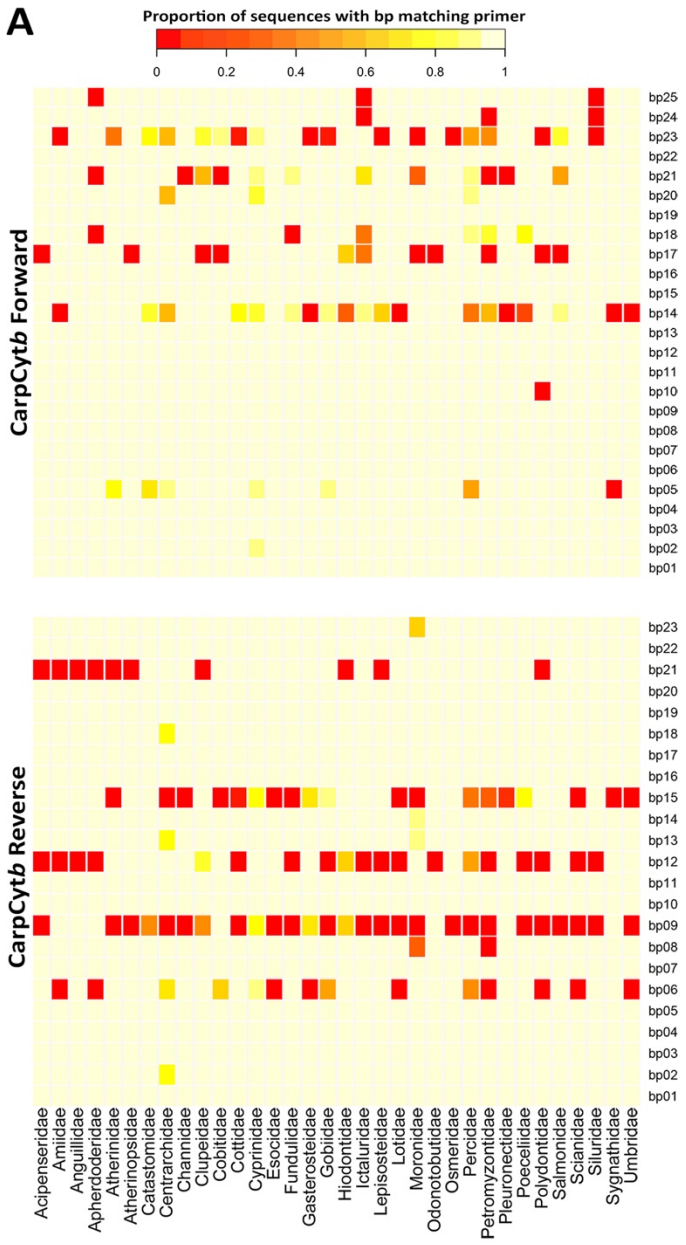
Order	Family	Species	Morph	eDNA	Morph	eDNA	Morph	eDNA	eDNA	eDNA	Morph	eDNA
		Ct. idella	-	+	-	-	-	+	+	+	-	+
		Cyprinella spiloptera	-	-	-	-	+	+	-	-	-	+
		Cyprinus carpio	+	+	+	+	+	+	+	+	+	+
		Erimystax x-punctatus	-	-	-	-	-	-	-	-	-	+
		Hybopsis amblops	-	-	-	-	-	-	-	-	-	+
		Hy. Winchelli	-	-	-	-	-	-	-	-	-	+
		Hypophthalmichthys molitrix	-	-	-	-	-	-	-	-	+	+
		Luxilus cornutus	-	-	-	-	++	-	-	-	-	-
		Lythrurus umbratilis	-	-	-	-	+	+	-	-	-	-
		Macrhybopsis storeriana	-	-	-	-	++	-	-	-	+	+
		Notemigonus crysoleucas	-	+	-	-	++	+	+	+	-	+
		Notropis sp.	-	-	-	-	-	+	-	-	-	+
		Notropis atherinoides	-	+	-	-	+	+	+	+	+	+
		No. buchanani	-	-	-	-	+	+	-	-	-	-
		No. heterolepis	-	-	-	-	-	-	-	-	-	-
		No. hudsonius	-	-	-	-	++	+	+	-	-	-
		No. photogenis	-	-	-	-	-	-	-	-	-	+
		No. stramineus	-	-	-	-	+	+	-	-	-	+
		No. texanus	-	-	-	-	-	+	-	-	-	+
		No. volucellus	-	-	-	-	++	+	-	-	-	-
		No. wickliffi	-	-	-	-	-	-	-	-	-	-
		Phenacobius mirabilis	-	-	-	-	+	+	-	-	-	-
		Pimephales sp.	-	-	-	-	-	-	-	-	-	+
		Pi. Notatus	-	+	-	-	+	+	+	+	-	+
		Pi. Promelas	-	-	-	-	-	-	-	-	-	-
		Semotilus atromaculatus	-	-	-	-	++	-	-	-	-	-
Gobiiformes	Gobiidae	*Neogobius melanostomus*	-	+	-	-	++	+	+	-	-	+
		Proterorhinus semilunaris	-	+	-	-	-	+	+	-	-	-
Hiodontiformes	Hiodontidae	*Hiodon alosoides*	-	-	-	-	-	-	-	-	-	+
		Hi. Tergisus	-	-	-	-	-	-	-	-	-	+
Lepisisteiformes	Lepisosteidae	*Lepisosteus osseus*	+	-	-	-	+	-	-	-	-	-
		Le. Platostomus	+	+	-	-	-	-	-	+	-	+
Perciformes	Centrarchidae	*Ambloplites rupestris*	-	-	-	-	++	-	+	+	-	-
		Lepomis sp.	-	-	-	-	-	+	-	-	-	-
		Lp. Cyanellus	-	+	-	-	+	+	-	-	+	+
		Lp. Cyanellus x macrochirus	-	-	-	-	++	-	-	-	-	-
		Lp. Gibbosus	-	-	-	-	+	+	-	-	-	-

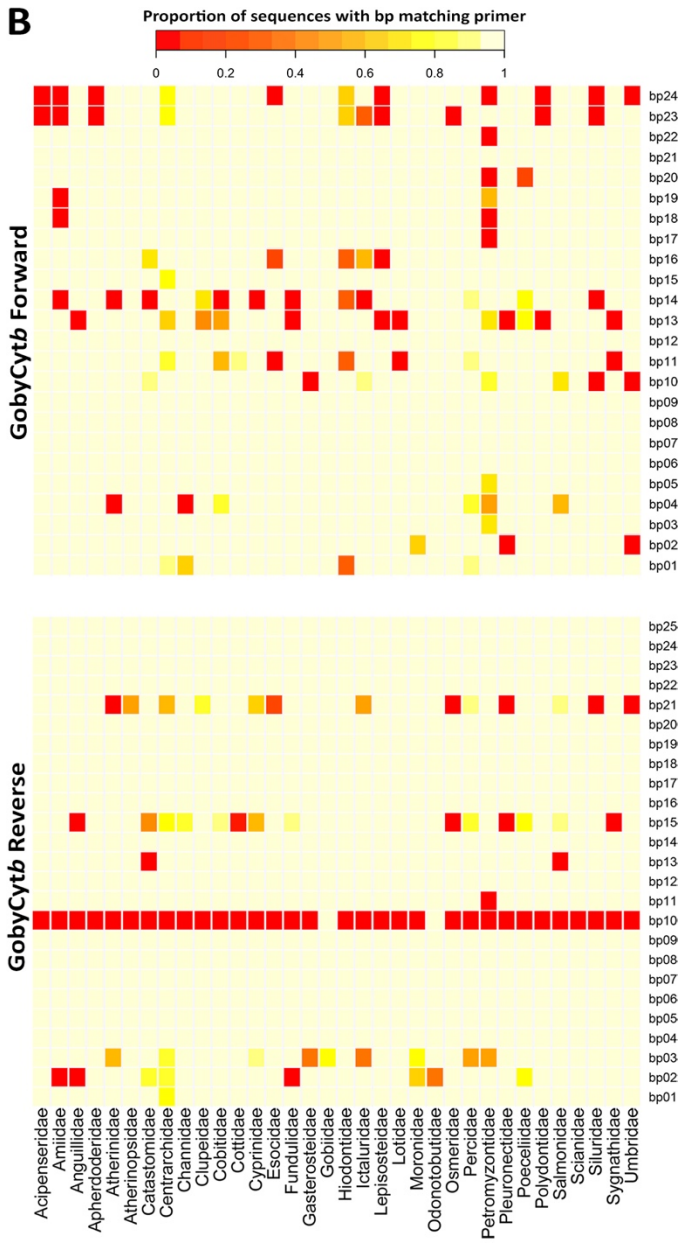
Order	Family	Species	Morph	eDNA	Morph	eDNA	Morph	eDNA	eDNA	eDNA	Morph	eDNA
		Lp. Gibbosus x macrochirus	-	-	-	-	++	-	-	-	-	-
		Lp. Humilis	-	-	-	-	+	+	-	-	-	-
		Lp. Macrochirus	+	+	-	-	+	+	+	-	+	+
		Lp. Megalotis	-	-	-	-	+	-	-	-	+	+
		Micropterus sp.	-	+	-	-	-	-	-	-	-	+
		Mi. dolomieu	+	+	-	-	++	+	+	+	+	+
		Mi. floridanus	-	+	-	-	-	-	-	-	-	-
		Mi. salmoides	+	+	-	-	+	+	+	-	-	-
		Mi. melanops	-	-	-	-	+	+	-	-	-	-
		Pomoxis annularis	-	-	-	-	+	-	-	-	-	-
		Po. Nigromaculatus	+	+	-	-	-	+	-	-	-	-
Perciformes	Moronidae	*Morone* sp.	-	-	+	-	-	-	-	-	-	-
		Mr. americana	-	-	-	+	+	+	-	-	-	-
		Mr. chrysops	-	+	-	+	+	+	+	-	-	-
		Mr. chrysops x saxatilis	-	-	-	-	-	-	-	-	-	-
		Mr. saxatilis	-	-	-	-	-	+	-	+	-	-
Perciformes	Percidae	Percid	-	-	-	-	-	-	-	-	-	-
		Ammocrypta pellucida	-	-	-	-	++	-	-	-	-	-
		Etheostoma blennioides	-	-	-	-	++	-	-	-	-	-
		E. flabellare	-	-	-	-	++	-	-	-	-	-
		E. nigrum	-	-	-	-	++	-	-	-	-	-
		E. spectabile	-	-	-	-	-	+	-	-	-	-
		Perca flavescens	+	+	+	+	-	-	+	-	-	-
		Percina caprodes	-	-	-	-	+	+	-	-	-	-
		Pe. Maculata	-	-	-	-	++	-	-	-	-	-
		Sander vitreus	+	+	-	-	++	+	+	+	-	+
		S. vitreus x canadensis	-	-	-	-	++	-	-	-	-	-
Perciformes	Scianidae	*Aplodinotus grunniens*	+	+	-	-	+	+	-	+	+	+
Salmoniformes	Salmonidae	*Oncorhynchus keta*	-	-	-	-	-	-	+	-	-	-
Scorpaeniformes	Cottidae	*Cottus ricei*	-	-	-	-	-	+	-	-	-	-
Siluriformes	Ictaluridae	*Ameiurus melas*	-	-	-	-	-	-	-	+	-	-
		Am. Natalis	+	+	-	-	++	+	-	-	-	-
		Am. Nebulosus	-	-	-	-	-	-	-	-	-	-
		Ictalurus sp.	-	-	-	-	-	-	-	-	-	+
		It. Furcatus	+	+	-	-	-	-	-	-	-	-
		It. Punctatus	-	-	-	-	+	+	-	-	-	+
		Noturus flavus	-	-	-	-	+	-	-	-	-	-

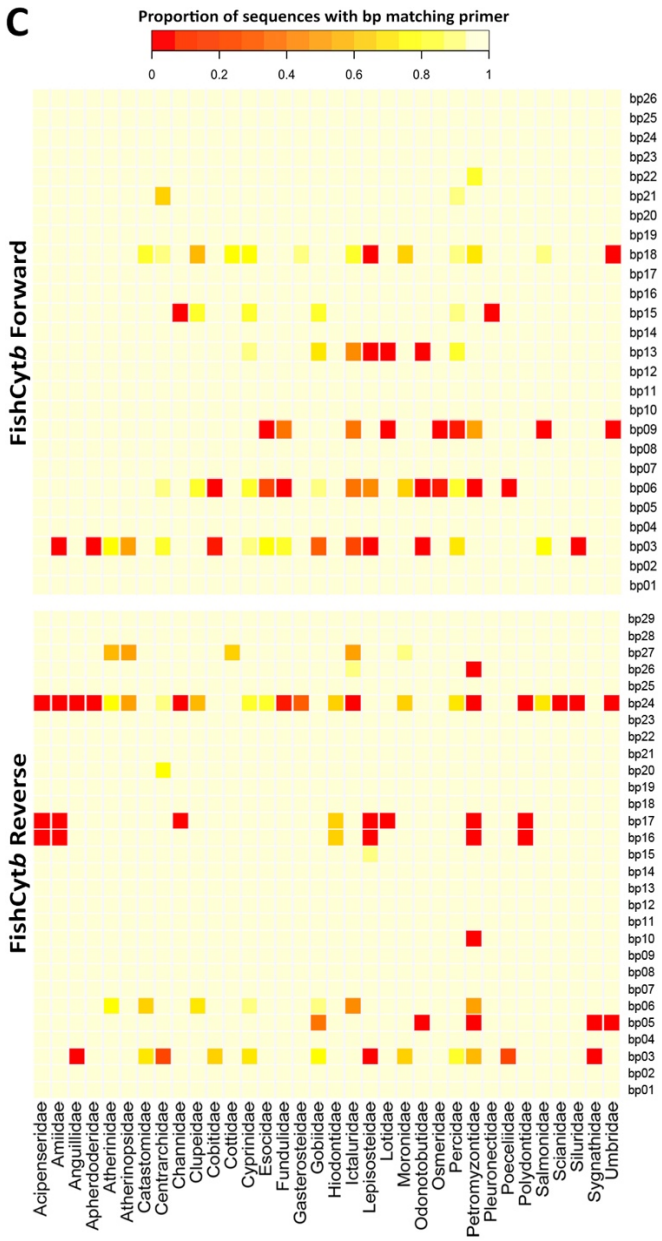
Order	Family	Species	Morph	eDNA	Morph	eDNA	Morph	eDNA	eDNA	eDNA	Morph	eDNA
		Nt. Gyrinus	-	-	-	-	++	-	-	-	-	-
		Nt. Miurus	-	-	-	-	++	-	-	-	-	-
		Pylodictis olivaris	-	-	-	-	+	+	-	-	-	-
		Unknown sp.	-	-	+	-	-	-	-	-	-	-
		Total detections	16	28	7	7	59	48	24	14	18	46
		Genus or higher	0	4	3	1	0	5	2	1	0	9

Appendix B.8 Biomass versus sequence reads. Relationship between log % biomass (g) of species sampled morphologically and reads. Linear regression slope (m) and R^2 and Spearman rank correlation coefficient (ϱ) for each sample and region for each marker. Samples collected in the same region concomitant with electrofishing surveys were combined in the Maumee (1–4) and Wabash (1–2) River. Maumee R. all shows relationships using all summer 2012 electrofishing surveys in the region regardless of whether concomitant eDNA data was processed. Significance for slope is difference from one. *=p<0.05, **=p<0.01, and ***=p<0.001.

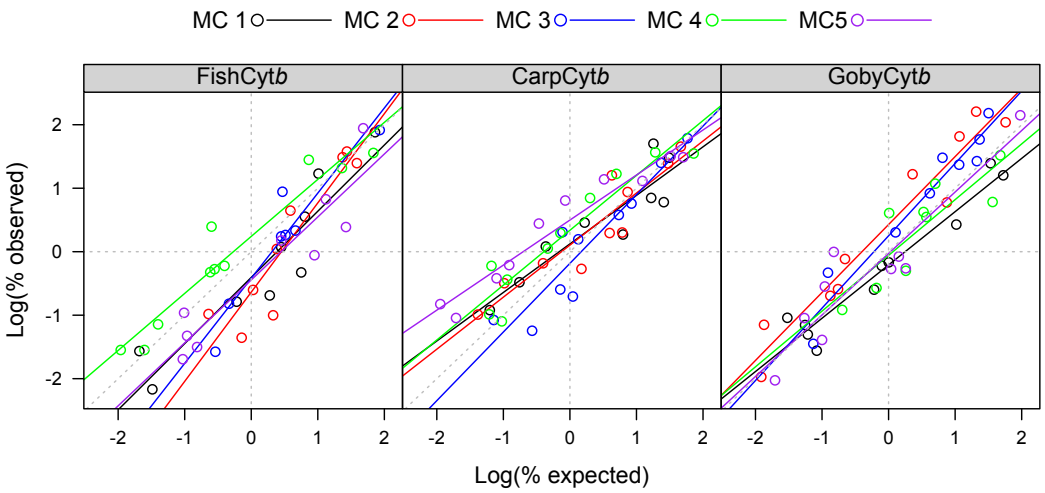
Assay	Sample	Slope	R2	*p*
FishCyt*b*	Display aquarium 3	0.79	0.30	0.27
	Detroit R. larvae	0.68	0.22	0.43
	Maumee R. 1	0.22**	0.21	0.56
	Maumee R. 2	0.08**	0.02	0.13
	Maumee R. 3	0.29	0.06	0.23
	Maumee R. 4	0.15***	0.16	0.46
	Maumee R. 1–4	0.38***	0.42**	0.70***
	Maumee R. all	0.25***	0.32**	0.60**
	Wabash R. 1	0.08**	0.06	0.14
	Wabash R. 2	0.57	0.89	0.6
	Wabash R. 1–2	0.69	0.51*	0.87**
CarpCyt*b*	Display aquarium 3	1.16	0.59*	0.96***
	Detroit R. larvae	0.36	0.22	0.50
	Maumee R. 1	-0.17**	0.22	-0.50
	Maumee R. 2	0.37	0.23	0.00
	Maumee R. 3	0.63	0.23	0.46
	Maumee R. 4	0.13	0.05	0.40
	Maumee R. 1–4	0.33*	0.20	0.44
	Maumee R. all	0.13***	0.08	0.34
	Wabash R. 1	1.11	0.34	0.43
	Wabash R. 2	-0.15	0.02	-0.43
	Wabash R. 1–2	-0.07*	0.00	0.13
GobyCyt*b*	Display aquarium 3	0.61	0.17	0.21
	Detroit R. larvae	0.24	0.07	0.07
	Maumee R. 1	-0.02	0.00	0.41
	Maumee R. 2	0.12	0.02	0.30
	Maumee R. 3	0.13	0.01	-0.11
	Maumee R. 4	0.25**	0.27	0.12
	Maumee R. 1–4	0.36**	0.22*	0.58*
	Maumee R. all	0.26***	0.23*	0.53*
	Wabash R. 1–2	1.56	0.68	0.80
MiFish	Display aquarium 3	0.68	0.22	0.33
	Detroit R. larvae	0.42	0.22	0.30
	Maumee R. 1	0.5	0.16	0.36
	Maumee R. 2	0.21**	0.08	0.39
	Maumee R. 3	0.02*	0.00	0.08
	Maumee R. 4	0.02***	0.00	-0.05
	Maumee R. 1–4	0.25***	0.16	0.44*
	Maumee R. all	0.16***	0.10	0.29
	Wabash R. 1	0.25*	0.24	0.36
	Wabash R. 2	-0.04***	0.01	-0.02
	Wabash R. 1–2	0.05***	0.01	0.10


A
Proportion of sequences with bp matching primer
0
0.2
0.4
0.6
0.8
1
CarpCytb Forward
bp25
bp24
bp23
bp22
bp21
bp20
bp19
bp18
bp17
bp16
bp15
bp14
bp13
bp12
bp11
bp10
bp09
bp08
bp07
bp06
bp05
bp04
bp03
bp02
bp01
CarpCytb Reverse
bp23
bp22
bp21
bp20
bp19
bp18
bp17
bp16
bp15
bp14
bp13
bp12
bp11
bp10
bp09
bp08
bp07
bp06
bp05
bp04
bp03
bp02
bp01
Acipenseridae
Amiidae
Anguillidae
Apherdoderidae
Atherinidae
Atherinopsidae
Catastomidae
Centrarchidae
Channidae
Clupeidae
Cobitidae
Cottidae
Cyprinidae
Esocidae
Fundulidae
Gasterosteidae
Gobiidae
Hiodontidae
Ictaluridae
Lepisosteidae
Lotidae
Moronidae
Odonotobutidae
Osmeridae
Percidae
Petromyzontidae
Pleuronectidae
Poeciliidae
Polydontidae
Salmonidae
Sciaenidae
Siluridae
Sygnathidae
Umbridae

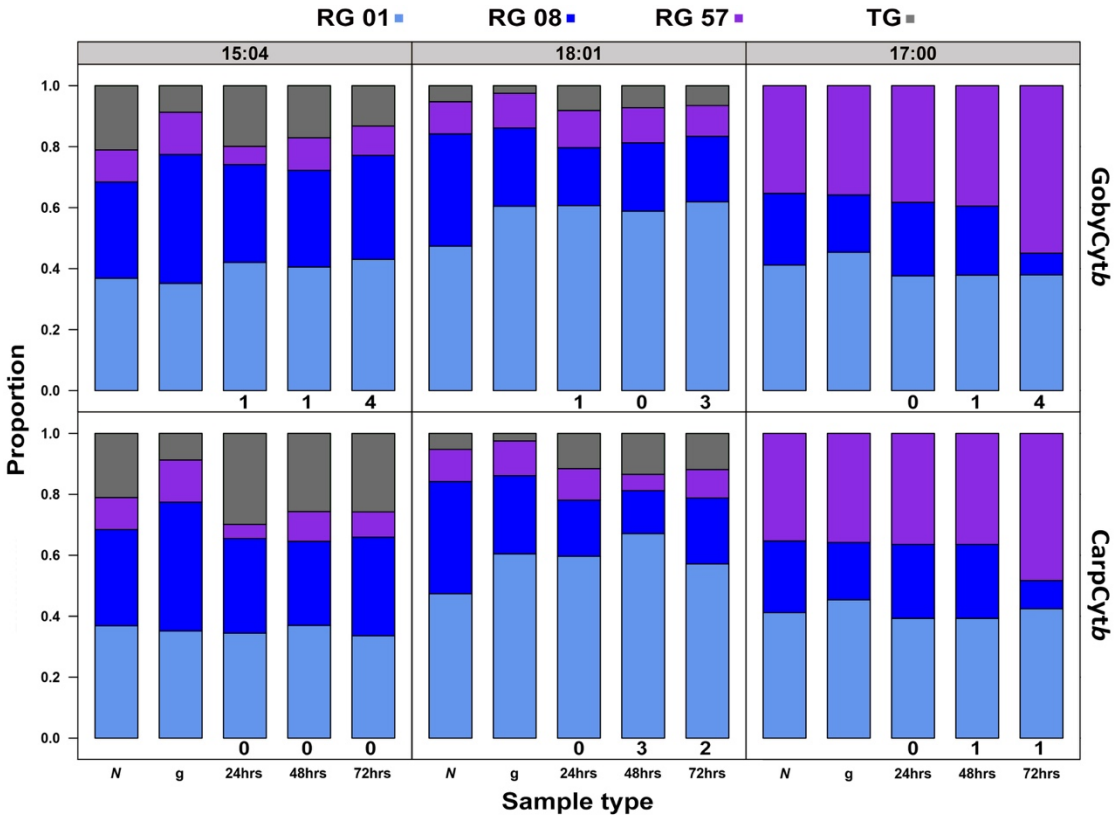

B
Proportion of sequences with bp matching primer
0
0.2
0.4
0.6
0.8
1
GobyCyt*b* Forward
bp24
bp23
bp22
bp21
bp20
bp19
bp18
bp17
bp16
bp15
bp14
bp13
bp12
bp11
bp10
bp09
bp08
bp07
bp06
bp05
bp04
bp03
bp02
bp01
GobyCyt*b* Reverse
bp25
bp24
bp23
bp22
bp21
bp20
bp19
bp18
bp17
bp16
bp15
bp14
bp13
bp12
bp11
bp10
bp09
bp08
bp07
bp06
bp05
bp04
bp03
bp02
bp01
Acipenseridae
Amiidae
Anguillidae
Apherdoderidae
Atherinidae
Atherinopsidae
Catastomidae
Centrarchidae
Channidae
Clupeidae
Cobitidae
Cottidae
Cyprinidae
Esocidae
Fundulidae
Gasterosteidae
Gobiidae
Hiodontidae
Ictaluridae
Lepisosteidae
Lotidae
Moronidae
Odonotobutidae
Osmeridae
Percidae
Petromyzontidae
Pleuronectidae
Poeceliidae
Polydontidae
Salmonidae
Scianidae
Siluridae
Sygnathidae
Umbridae



Appendix B–1 Primer binding region mismatches for cytochrome *b* eDNA assays designed to target native and invasive Great Lakes fishes. Heat maps show the proportion of known variation in sequences with matching bases to the primers for fish families present in the Great Lakes and predicted future invaders. (A) CarpCyt*b*, (B) GobyCyt*b*, and (C) FishCyt*b* assays.



Appendix B–2 Mock community plots. Plots of log % expected (based on input ng/µl of genomic DNA) and observed sequence reads for mock communities (MC) processed with the FishCyt*b*, CarpCyt*b*, and GobyCyt*b* assays. Dotted lines show x and y = 0 and a 1:1 relationship. Solid lines show the actual fitted regression between observed sequence reads and expected (based on genomic DNA dilution).



Appendix B–3 Results of round and tubenose goby aquarium experiments. The three aquaria contained either 15 round and four tubenose gobies (15:4), 18 round and one tubenose gobies (18:1), or 17 round gobies (17:0), whose morphological proportions are indicated (*N* column). The fish comprised three round goby haplotypes (RG haplotype 01 (light blue), 08 (dark blue), and 57 (purple) and one tubenose goby (TG; grey) haplotype (indicated in *N* column). Each fish was measured (mm, TL) and weighed (g), with the latter values indicated in the g column. After the experiment, fish were anesthesized and sacrificed according to our approved IACUC, tissues sampled, DNA extracted, and the entire cyt *b* gene was sequenced. Bars show numbers of individuals (*N*) of each species and their haplotype at various times from the eDNA assay of water samples (24, 48, and 72 hrs). Almost all false haplotypes that were above the calculated error cutoff were <1% of reads, and thus are not shown for clarity. Numbers below eDNA proportion bars represent the number of probable false haplotypes in the sample.

Appendix C

Chapter 4 Supplementary Tables and Figures

Appendix C1 DNA capture and extraction.

250ml of sampled water was divided in labeled sterile 50ml tubes and centrifuged at 4500 rpm for 45 min at 4°C, alongside negative controls containing ddH_2O. Water was decanted, pellets combined from each tube that originated from a single water sample by re-suspending in 1ml 95% EtOH, and stored at -20°C until use. Samples then were centrifuged at 14,000 rpm for 10 min and EtOH decanted and evaporated. DNA was extracted using Qiagen Dneasy kits (Hilden, Germany) following manufacturer's protocol, except using two washes of both wash buffers (AW1 and AW2), and eluted in 150µl loTE (3 mM Tris-HCl and 0.2 mM EDTA, pH 8.0). Negative extraction controls contained reagents only. Extractions were cleaned of potential inhibitors using Zymo (Seattle, WA) OneStep™ PCR Inhibitor Removal kits.

Appendix C2 Library prep and HTS

We employed a two-step library prep protocol. First step PCR reactions (rx) comprised 25 or 50µl (the latter for inhibited samples, assessed by presence of primer dimer in failed reactions, see below) containing 1X Radiant® TAQ reaction buffer (Alkali Scientific

Inc., Ft. Lauderdale, FL), 3mM MgCl2, 0.25mM each dNTP, 0.6mM of each primer (with spacer inserts and an Illumina®, MiSeq sequencing primer tail), and 1.25 units of Radiant TAQ polymerase. Each rx first was attempted using 2µl DNA in a 25µl rx. Failed rxs lacking primer dimer were deemed inhibited, and were re-run using 1µl of diluted DNA (1:10–1:50) in a 50µl rx. Failed rxs that contained primer dimer were interpreted as uninhibited, and were re-run using ≤10µl DNA in a 25µl rx. Conditions were 2 min at 95°C, then 40 cycles of 95°C for 45 sec, primer specific annealing temp (Table 2) for 30 sec, and 72°C for 45 sec, capped by 3 min at 72°C.

Amplifications were attempted on extractions that appeared negative, which were, centrifuged 3X, along with no-template PCR controls (for every rx) d. Rxs whose associated no-template PCR controls from the first step did not amplify, were indexed for HTS. Successful rxs were column cleaned and indexed with unique combinations of 5' and 3' Nextera 96 indices (Illumina®, San Diego, CA), following the manufacturer's protocol except that 2.5µl of each index were added per rx. Indexed samples were visualized on 1% agarose gels stained with ethidium bromide. Successful rxs were column cleaned, sized, and quantified on an Agilent Bioanalyzer (Santa Clara, CA), pooled in equimolar concentrations, and sequenced in eight separate runs on an Illumina® MiSeq at Ohio State Wooster's Molecular and Cellular Imaging Center (website?), with a targeted 40% PhiX spike in. One "clean-up negative control" was generated after each marker was processed for each year of samples by subjecting just the reagents to column cleaning. Indexing was attempted on these controls.

To quantify sequencing error, including index-hops (see below), positive controls were amplified for each marker on each run (Deiner et al., 2017). These were constructed by mixing equal mass of genomic DNA from 10 marine species (from Pacific coastal

ecosystems) that cannot live in freshwater bait or pond store tanks. Each extraction was Sanger sequenced for the region of cyt *b* that contained our eDNA assays. When no appropriate positive control could be constructed (e.g., for the Mollusk16S assay), an error cutoff of 0.1% was used based on the known rate of index-hopping on the MiSeq platform (see below). Species and accession numbers for associated sequences are in Table A2. Complete FASTQ files for all samples sequenced are in the NCBI Sequence Read Archive (BioProject # PRJNA548536).

Appendix C3 HTS eDNA assay bioinformatic pipeline

Primers were trimmed from raw reads with a custom PYTHON v3.7.1 script. Several errors in positive controls occurred at the first base after the primer, presumably during PCR, and thus we trimmed their first and last bases. The trimming script also removed any reads with the wrong spacer primer, which likely resulted from index-hopping, which occurs when the wrong index is incorporated into an HTS library, leading to mis-assignment of the sequence to the sample (MacConaill et al., 2018; Xiong et al., 2016). Due to the use of four sets of spacer primers per assay, this script removed 75% of index-hops among samples per marker. This script also removed non-informative short sequences from primer dimer (Khodakov et al., 2016).

Trimmed reads were merged in DADA2 (Callahan et al., 2016), which employed a de-noising algorithm to correct potential sequencing errors and removed chimeras. DADA2 was run with default parameters except that "maxEE" was set to "(3, 5)". Inputs were truncated using the "truncLen" parameter to the length at which <100% of reads extended or the median Q score was <30, determined with the plotQualityProfile function in DADA2 for the first 10 samples/marker/run.

De-noised sequences that DADA2 grouped by 100% similarity are known as amplicon sequence variants (ASVs), which were subjected to the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the command line, for the top 500 results per sequence. The four fish assays were queried against a custom database of all sequences used for primer design and positive control species. The mollusk assay was BLASTed against the entirety of GenBank using the "–remote" option. A custom PERL script summarized BLAST results, removing hits with <90% (fish assays) or <97% (Mollusk16S) query cover and identity. All hits per ASV that passed this filter and were the lowest e value (best match) were combined into a list of potential taxa. We employed a conservative approach to analyze these BLAST results. If the advertised species was on the list, the hit was considered a detection of this species. If not and a legal bait or pond species was on the list, it was classified as that species. If it was not and a native non-bait or pond species was on the list, it was considered to be that species. In cases that neither was on the list, but an AIS was, it was treated as the latter.

Sequencing error was calculated as the greatest number of sequences belonging to an unexpected ASV in a positive control divided by the total number of reads in the sample. The rate of known index-hoping is <0.1% of sequences per MiSeq run (MacConaill et al., 2018). Error frequencies below this value were rounded up to 0.1%. Sequences with a BLAST result to positive control species in samples from retailers were discarded.

Species findings were considered valid either when their occurrence frequency was greater than the error/marker/run (see above) or if they occurred in multiple markers (at any proportion). This multiple assay approach allowed for detection of more species since each primer set may have some bias, but error resulting in a false positive in two markers for the same sample is highly unlikely (Evans et al., 2017). Due to the large variety of taxa discerned

with the mollusk assay, that assay was evaluated alone and for ASV frequency alone. A custom PYTHON script combined results from each assay, generated a list of valid species detections, and compared them to results based on morphological sampling. Any samples that identified species on the GLANSIS watch list or AIS not known to occur within the sampling area (see Results) at any frequency of reads were re-run for the GobyCytb, CarpCytb, FishCtyb-L, and Mollusk16S assays (pond stores only, including a fish-blocking primer to reduce the amplification of fish DNA; Klymus et al., 2017) to confirm their presence. To mitigate undetectable index-hopping among these repeat samples we avoided shared 5' or 3' indices for a single spacer and primer combination. All custom scripts were deposited in the Dryad online database (XXXX).

Appendix C4 Retailer and angler surveys.

Bait Shop Supply Chain Survey

Who: University of Toledo
PI: Dr. Carol Stepien
Co-I's: Dr. Andrew Solocha Department of Finance
Dr. Kevin Czajkowski, Department of Geography and Planning

Project Title: Invasive Species Prevention from Retailers via Metagenetics, Supply Chains, and Public/Stakeholder Engagement
Funding Agency: EPA Great Lakes Restoration Initiative

1. BOPPS name_____

2. Address_____

3. Within the past thirty days, how many times did you receive live fish bait supplies (e.g., emerald shiners, fathead minnows)?

4. What quantity do you receive (individuals, pounds, or volume)?

5. Who are your suppliers for this bait …
in the past year?

at present (most recent order)?

6. How many times in the last year were there foreign species in your bait? (e.g., if you purchased/sold emerald shiners but there were chubs in the bait, this would be considered a foreign species.)

7. What foreign species have been mixed in with your bait? (Check all that apply)

Species
bought/sold:_____
Other species mixed in:

fathead minnows___ grass carp___ gobies___
spotfin shiner___ common carp___ perch___
spottail shiner___ goldfish___
other:_____

Species
bought/sold:_____
Other species mixed in:
fathead minnows___ grass carp___ gobies___
spotfin shiner___ common carp___ perch___
spottail shiner___ goldfish___
other:_____

Species
bought/sold:_____
Other species mixed in:
fathead minnows___ grass carp___ gobies___
spotfin shiner___ common carp___ perch___
spottail shiner___ goldfish___
other:_____

Species
bought/sold:_____
Other species mixed in:
fathead minnows___ grass carp___ gobies___
spotfin shiner___ common carp___ perch___
spottail shiner___ goldfish___
other:_____

Angler Survey

PI: Dr. Carol Stepien, Director of the University of Toledo's Lake Erie Center
Co-I's: Dr. Andrew Solocha Professor of Finance
Dr. Kevin Czajkowski, Professor of Geography and Planning

Project Title: Invasive Species Prevention from Retailers via Metagenetics, Supply Chains, and Public/Stakeholder Engagement
Funding Agency: EPA Great Lakes Restoration Initiative

ADULT RESEARCH - INFORMED CONSENT INFORMATION

Invasive Species Prevention from Retailers via Metagenetics, Supply Chains, and Public/Stakeholder Engagement

Purpose: You are invited to participate in the research project entitled, Invasive Species Prevention from Retailers via Metagenetics, Supply Chains, and Public/Stakeholder Engagement, which is being conducted at the University of Toledo under the direction of Dr. Carol Stepien, Dr. Kevin Czajkowski and Dr. Andrew Solocha. This project will analyze the potential avenues for introducing invasive fish species and mollusk species from BOPPS (Bait, Outfitter, Pond Suppliers, and Pet Stores). The project will survey and test for invasive species throughout the Huron-Erie Corridor and along the U.S. Lake Erie shore, through Michigan, Ohio, Pennsylvania, and New York. A survey for distributors, retailers, and consumers of bait, pets, and plants will be used to map and analyze supply chains of the bait, fish and pond industries.

Description of Procedures: This survey will be administered online and through paper forms from the summer of 2016 to 2018. In the survey, you will be asked to answer a number of questions. Your participation will take about 5 minutes.

Potential Risks: There is a possible minimal risk to participation in this study although we will take all precautions possible to protect your confidentiality.

Potential Benefits: The direct benefit to you if you participate in this research may be that you may learn more about live fish bait. Others may benefit by learning about the results of this research.

Confidentiality: The researchers will make every effort to prevent anyone who is not on the research team from knowing that you provided this information, or what that information is. We will make every effort to protect your confidentiality.

Voluntary Participation: If you decline to participate in this study will involve no penalty or loss of benefits to which you are otherwise entitled and will not affect your relationship with The University of Toledo or any of your classes. In addition, you may discontinue participation at any time without any penalty or loss of benefits.

Contact Information: Before you decide to accept this invitation to take part in this study, you may ask any questions that you might have. If you have any questions at any time

before, during or after your participation you should contact a member of the research team. If you have questions beyond those answered by the research team or your rights as a research subject, please feel free to contact the IRB Chair at (419) 530-2844.

THE UNIVERSITY OF TOLEDO
SOCIAL, BEHAVIORAL & EDUCATIONAL INSTITUTIONAL REVIEW BOARD

The research project described in this consent has been reviewed and approved as EXEMPT
by the University of Toledo SBE IRB

By beginning the survey, you are stating that you have read and accept the information above and are giving your consent to participate in this research. You are also confirming that you are 18 years old or over.

1. Today's Date _____
2. How many years have you fished? _____
3. What water body do you fish in most often? _____
4. What type of gear do you use to fish? _____
5. What county do you fish in or what county do you launch a boat from?

   _____
6. How often did you fish in the last month? _____
7. How often did you fish in the last year? _____
8. How many other people fish in your family? _____
9. What type of bait do you use? _____
10. What percentage of your live fish (e.g., emerald shiners, fathead minnows, etc.) used for bait do you catch yourself? _____ %
11. If you catch your own fish to use for bait, where do you catch it?
12. If you catch your own fish to use for bait, what species do you catch?

Emerald shiners ____ Estimate the number of fish per month: _____

Fathead minnows ____ Estimate the number of fish per month: _____

Round goby ____ Estimate the number of fish per month: _____

Other ____ Estimate the number of fish per month: _____

List the other species _____, _____, _____

13. Where do you purchase your bait?

Store name: _____

Address: _____

_____

14. If you purchase live fish for use as bait, what species do you buy?

Emerald shiners ____ Estimate the number of fish per month: _____

Fathead minnows ____ Estimate the number of fish per month: _____

Round goby ____ Estimate the number of fish per month: _____

Other ____ Estimate the number of fish per month: _____

List the other species _____, _____, _____

15. Do you purchase bait outside of the state where you fish? ____ yes ____ no

16. What invasive/non-native fish are you aware of (Check all that apply)?

bighead carp____ common carp____ grass carp___ silver carp____

round goby____ tubenose goby____ goldfish____ ruffe ____

snakehead ____bighead carp____ white perch___

other:_____

17. Which of these species have you seen in the wild?

Seen?	Species	Location
____	bighead carp	_____
____	common carp	_____

____ silver carp _____

____ grass carp _____

____ goby _____

____ goldfish _____

____ ruffe _____

____ snakehead _____

____ white perch _____

____ other:_____ _____

18. In the past year, I have released unused live bait fish into the water:
19.
____0 times ____1–5 times ____6–10 times ____11–20 times ____>20 times

What water body did you release them into? _____

20. In the past year, I have thrown unused live bait fish into the trash:
21.
____0 times ____1–5 times ____6–10 times ____11–20 times ____>20 times

22. In the past year, I have flushed unused live bait fish down the toilet:
23.
____0 times ____1–5 times ____6–10 times ____11–20 times ____>20 times

24. What state/province do you live in? _____

25. What county do you live in? _____

26. (Optional) What is your age range?

18-29 ____ 30-39 ____ 40-49 ____ 50-59 ____ 60-69 ____ 70-79 ____ 80+ ____

27. (Optional) What is your gender and/or sex? _____

28. (Optional) What is your ethnic and/or racial background?
_____

29. (Optional) Name: _____

Address: _____

_____

Phone: _____

Email: _____

Your name will not be linked to the answers you give and will not be shared with any outside organizations.

Appendix C.1 Bait and pond fish retailer sampling summary, showing numbers of stores and samples. Bait stores were sampled in two different years. Number of stores unique to a sampling year are in parentheses.

	Bait				Pond	
	Stores		Samples		Stores	Samples
Region	**2016**	**2017**	**2016**	**2017**	**2017**	**2017**
Indiana	4 (0)	4 (0)	4	4	1	3
Michigan	14 (0)	14 (0)	14	14	9	34
Ohio	30 (2)	31 (3)	30	45	11	44
Total	*48 (2)*	*49 (3)*	*48*	*63*	*21*	*81*

Appendix C.2 Species and accession numbers used to design eDNA assays and for the BLAST database. Common name, species name, taxonomy (order and family), and Genbank Accession numbers. Only sequences >1000NT were included. *=non-native species either established or a predicted future invader of the Great Lakes.

Common name	Species	Taxonomy	Accession
Lake sturgeon	*Acipenser fulvescens*	Order: Acipenseriformes Family: Acipenseridae	AJ245829.1, KU985070.1, KU985081.1, KU985082.2, KU985084.1, NC_030325.1
Paddlefish	*Polyodon spathula*	Order: Acipenseriformes Family: Polydontidae	AJ245841.1, AP004353.1, AY442349.1, AY510086.1, KU985085.2, KU985086.2, NC_004419.1
Bowfin	*Amia calva*	Order: Amiiformes Family: Amiidae	AB018999.1, AB042952.1, NC_004742.1
American eel	*Anguilla rostrata*	Order: Anguilliformes Family: Anguillidae	AB021767.1, AF006716.1, AF006717.1, AF485271.1-AF485276.1, AP007249.2, KJ564170.1-KJ564184.1, KJ564186.1, KJ564187.1, KJ564189.1-KJ564217.1, NC_006547.2
Bigscale sand smelt*	*Atherina boyeri*	Order: Atheriniformes Family: Atherinidae	AB848929.1, EU036421.1, EU036422.1, EU253549.1, EU253550.1
Brook silverside	*Labidesthes sicculus*	Order: Atheriniformes Family: Atherinopsidae	JQ282031.1, KC736409.1
Skipjack herring*	*Alosa chrysochloris*	Order: Clupeiformes Family: Clupeidae	EF653231.1, EF653232.1, KJ158092.1, MG958209.1
Alewife*	*Alosa pseudoharengus*		AP009132.1, NC_009576.1
American shad*	*Alosa sapidissima*		EU552616.1, HQ331537.1, KY769128.1, NC_014690.1
Black sea sprat*	*Clupeonella cultriventris*		AP009615.1, NC_015109.1
Gizzard shad	*Dorosoma cepedianum*		DQ536426.1, EU552584.1-EU552586.1, NC_008107.1
Banded killifish	*Fundulus diaphanus*	Order: Cyprinidontiformes Family: Fundulidae	FJ445394.1, FJ445395.1, KX359040.1, KX359041.1, NC_012361.1
Starhead topminnow	*Fundulus dispar*		GQ119707.1, GQ119708.1, L31599.1, U77119.1, U77120.1
Blackstripe topminnow	*Fundulus notatus*		KF245643.1-KF245748.1, KP013106.1, KP059009.1, NC_028293.1
Mosquitofish*	*Gambusia affinis*	Order: Cyprinidontiformes Family: Poeceliidae	AP004422.1, EF017514.1, KP059011.1, NC_004388.1
River carpsucker	*Carpiodes carpio*	Order: Cypriniformes Family: Catastomidae	AB126083.1, AF454867.1, AP006763.1, AY366087.1, JF799431. JN053177.1, JN053185.1, JN053187.1, JN053188.1, JN053190.1 JN053193.1, JN053194.1, JN053208.1, JN053221.1, JN053222.1 JN053237.1-JN053239.1, JN053245.1-JN053253.1, JN053255.1-JN053260.1, NC_005257.1

Common name	Species	Taxonomy	Accession
Quillback	*Carpiodes cyprinus*		JF799432.1, JF799433.1, JN053178.1, JN053179.1, JN053183.1, JN053191.1, JN053195.1-JN053197.1, JN053199.1, JN053203.1, JN053205.1, JN053209.1-JN053220.1, JN053228.1, JN053241.1, JN053242.1, JN053261.1, JN053263.1, JX488761.1
Highfin carpsucker	*Carpiodes velifer*		JF799434.1, JN053180.1-JN053182.1, JN053184.1, JN053186.1, JN053189.1, JN053192.1, JN053204.1, JN053262.1, JX488762.1
Longnose sucker	*Catostomus catostomus*		AF454871.1, EU676808.1, JX258854.1-JX258858.1, KJ441284.1, KP757032.1-KP757038.1, KT203373.1, KT203374.1, KU697931.1, KU761848.1, KU761849.1
White sucker	*Catostomus commersonii*		AB127394.1, HQ446762.1, JF799435.1-JF799437.1, JX488781.1, KP013114.1, KU697932.1, NC_008647.1
Western chubsucker	*Erimyzon claviformis*		No records
Eastern chubsucker	*Erimyzon oblongus*		AF454876.1, AP011228.1, NC_013064.1
Lake chubsucker	*Erimyzon sucetta*		AF454878.1, KU697910.1
Northern hogsucker	*Hypentelium nigricans*		AB242169.1, AF454909.1, AY253341.1-AY253405.1, AY253407.1-AY253413.1, JF799441.1, NC_008676.1
Smallmouth buffalo	*Ictiobus bubalus*		AP009316.1, FJ226281.1, FJ226285.1, FJ226287.1-FJ226290.1, FJ226299.1, FJ226300.1, FJ226302.1, FJ226333.1, FJ226335.1-FJ226364.1, JF799443.1, JX488763.1-JX488765.1, NC_013071.1
Bigmouth buffalo	*Ictiobus cyprinellus*		FJ226256.1-FJ226270.1, FJ226286.1, FJ226291.1-FJ226298.1, FJ226301.1, FJ226303.1-FJ226308.1, JF799444.1, JX488766.1, JX488767.1, KP306894.1, NC_026528.1
Black buffalo	*Ictiobus niger*		FJ226271.1-FJ226280.1, FJ226282.1-FJ226284.1, FJ226309.1, FJ226313.1-FJ226315.1, FJ226321.1, FJ226325.1-FJ226332.1, JF799446.1, JX488768.1, JX488769.1
Spotted sucker	*Minytrema melanops*		AB242166.1, AF454879.1, DQ536432.1, JF799447.1-JF799449.1, KU697909.1, NC_008113.1
Silver redhorse	*Moxostoma anisurum*		AF454880.1, AF454881.1, JF799450.1-JF799452.1
River redhorse	*Moxostoma carinatum*		AF454884.1, JF799455.1-JF799459.1, JX488820.1
Black redhorse	*Moxostoma duquesnei*		AF454894.1, AF454895.1
Golden redhorse	*Moxostoma erythrurum*		AF454886.1, AF454887.1, AY253421.1, JF799463.1-JF799470.1, KJ441285.1, KU697911.1
Copper redhorse	*Moxostoma hubbsi*		AF522289.1, JF799471.1, JF799472.1, JX488821.1
Harelip sucker	*Moxostoma lacerum*		No records
Shorthead redhorse	*Moxostoma macrolepidotum*		AF454890.1, JF799473.1-JF799476.1
Greater redhorse	*Moxostoma valenciennesi*		AF454893.1, JF799487.1

Common name	Species	Taxonomy	Accession
Oriental weatherfish*	*Misgurnus anguillicaudatus*	Order: Cypriniformes Family: Cobitidae	AB473261.1-AB473407.1, AB599977.1-AB599980.1, AB614357.1-AB614359.1, AB674743.1-AB674748.1, AB899670.1-AB899684.1, AF051868.1, AP011291.1, AY625700.1, DQ026434.1, DQ105238.1, DQ105240.1, DQ105241.1, DQ886941.1, EF088651.1, EF376188.1, EF424602.1-EF424608.1, EF508555.1-EF508559.1, EF595974.1-EF595982.1, EU131132.1-EU131140.1, EU145021.1-EU145024.1, EU670766.1, GU583669.1-GU583677.1, HM856629.1, KC509900.1, KC509901.1, KC734881.1, KC762740.1, KC823274.1, KC881110.1, KC884745.1, KF736233.1, KM186181.1, KM576227.1, KM576236.1, KM576243.1, NC_011209.1
Common bleak*	*Alburnus alburnus*	Order: Cypriniformes Family: Cyprinidae	AB239593.1, AF090743.1-AF090745.1, AY026393.1, DQ350253.1, DQ350254.1, HM560060.1-HM560062.1, JQ436541.1, KJ463863.1, NC_008659.1, Y10443.1
Central stoneroller	*Campostoma anomalum*		AF452079.1, DQ324063.1, DQ486786.1-DQ486788.1, DQ486795.1-DQ486801.1, DQ486803.1-DQ486811.1, DQ486813.1, DQ486816.1-DQ486822.1, DQ486824.1, DQ486826.1-DQ486828.1, DQ486837.1, JQ712313.1, KY398932.1
Largescale stoneroller	*Campostoma oligolepis*		DQ324064.1, DQ486793.1, DQ486794.1, DQ486802.1, DQ486812.1, DQ486814.1, DQ486815.1, DQ486823.1, DQ486825.1, DQ486829.1-DQ486836.1, DQ486838.1, HQ446741.1, JQ712314.1
Goldfish*	*Carassius auratus*		AB006953.1, AB111951.1, AF051858.1, AP011236.1, AP011239.1, EF055472.1, EF483931.1, GU086395.1-GU086397.1, GU135503.1-GU135601.1, GU135603.1-GU135605.1, HQ689793.1-HQ689890.1, HQ689910.1-HQ689912.1, HQ875340.1, JN105355.1, JX183534.1-JX183536.1, KF147851.1, KJ476998.1, KJ735886.1-KJ735908.1, KJ874428.1-KJ874431.1, KM657141.1-KM657143.1, KM659025.1, KT756205.1, KU146528.1, KX505165.1, MF443758.1-MF443771.1, NC_002079.1, NC_006580.1, NC_015142.1
Crucian carp*	*Carassius carassius*		AY714387.1, JQ911695.1, KX781320.1, NC_006291.1

Common name	Species	Taxonomy	Accession
Prussian carp*	*Carassius gibelio*		GU138989.1, HM000005.1, HM000006.1, HM000010.1, HM000019.1, HM000022.1, HM000025.1-HM000029.1, KU896991.1, KU896992.1, KX505166.1, KX611160.1
Northern redbelly dace	*Chrosomus eos*		AP009151.1, EU755056.1, NC_015364.1
Southern redbelly dace	*Chrosomus erythrogaster*		AP011276.1, AY281055.1, EU755049.1-EU755055.1, NC_031570.1
Redside dace	*Clinostomus elongatus*		AP011280.1, GU182772.1-GU182776.1, GU182820.1-GU182822.1, JN053201.1, JN053223.1, JN053254.1, NC_031572.1
Lake chub	*Couesius plumbeus*		AF452083.1, AP011274.1, AY281053.1, NC_031568.1
Grass carp*	*Ctenopharyngodon idella*		AB900162.1, AF051860.1, AF420424.1, HM237985.1-HM238043.1, JN673556.1
Satinfin shiner	*Cyprinella analostana*		GQ275236.1, GQ275237.1
Red shiner	*Cyprinella lutrensis*		AB070206.1, DQ324095.1, GQ275183.1-GQ275190.1, GQ275194.1, KR061540.1-KR061551.1, KR061553.1, KR061555.1-KR061557.1, KR061559.1, KR061561.1-KR061563.1, KR061566.1-KR061569.1, NC_008643.1
Spotfin shiner	*Cyprinella spiloptera*		DQ536422.1, GQ275218.1-GQ275223.1, NC_008103.1, U66605.1
Steelcolor shiner*	*Cyprinella whipplei*		GQ275230.1-GQ275233.1
Common carp*	*Cyprinus carpio*		AB126083.1, AB158803.1-AB158807.1, AF454867.1, AP006763.1, AY347276.1-AY347295.1, AY366087.1, EU676848.1, EU689059.1-EU689072.1, HQ443697.1, JF799431.1, JN053177.1, JN053185.1, JN053187.1, JN053188.1, JN053190.1, JN053193.1, JN053194.1, JN053201.1, JN053208.1, JN053221.1-JN053223.1, JN053237.1-JN053239.1, JN053245.1-JN053260.1, KF574485.1-KF574490.1, NC_005257.1
Gravel chub	*Erimystax x-punctatus*		AF117172.1, AF117173.1, AY486043.1-AY486054.1, KC763653.1
Tonguetied minnow	*Exoglossum laurae*		JF949841.1, JQ712316.1, JX442989.1, KY398933.1
Cutlips minnow	*Exoglossum maxillingua*		JX442988.1, KC763683.1
Brassy minnow	*Hybognathus hankinsoni*		AF452080.1, EU811090.1
Eastern silvery minnow	*Hybognathus regius*		EU811087.1, EU811088.1, GQ275151.1
Bigeye chub	*Hybopsis amblops*		EU917316.1-EU917406.1, EU917408.1-EU917417.1, HQ446747.1

Common name	Species	Taxonomy	Accession
Silver carp*	*Hypophthalmichthys molitrix*		AB198974.1, AF051866.1, EU315941.1, JQ231114.1, KJ671449.1, KJ671450.1, KJ679503.1, KJ729076.1, KJ729092.1-KJ729094.1, KJ746938.1-KJ746940.1, KJ746943.1-KJ746957.1, KJ746960.1, KJ746961.1, KJ746964.1, KJ746965.1, KP013119.1, KY126320.1, MF180230.1-MF180232.1, NC_010156.1, NC_034667.1
Bighead carp*	*Hypophthalmichthys nobilis*		AP011217.1, EU343733.1, HM162839.1, JQ346141.1, KJ679504.1, KJ679505.1, KJ710362.1, KJ710363.1, KJ729077.1-KJ729091.1, KJ729095.1-KJ729097.1, KJ746935.1-KJ746937.1, KJ746941.1, KJ746942.1, KJ746958.1, KJ746959.1, KJ746962.1, KJ746963.1, KJ746966.1, KJ756343.1, KY126320.1, MF180233. MF180235.1, NC_010194.1, NC_034667.1
Eurasian ide/orfe*	*Leuciscus idus*		AY026397.1, HM560098.1, HM560099.1
Common dace*	*Leuciscus leuciscus*		AY509823.1, DQ664302.1-DQ664306.1, HM560100.1, HM560101.1
Striped shiner	*Luxilus chrysocephalus*		AF117166.1, AF117167.1, AP012079.1, GQ275161.1, NC_033923.1, U66595.1, U66596.1
Common shiner	*Luxilus cornutus*		AP012090.1, NC_033931.1, U66597.1
Redfin shiner	*Lythrurus umbratilis*		AP012094.1, GQ275160.1, NC_033935.1, U17274.1
Silver chub	*Macrhybopsis storeriana*		KC763654.1, KX139438.1, NC_030485.1
Allegheny pearl dace	*Margariscus margarita*		AF452072.1, AP012081.1, JX443011.1
Northern pearl dace	*Margariscus natchtriebi*		No records
Black carp*	*Mylopharyngodon piceus*		AF051870.1, AP011216.1, DQ026435.1, EU979305.1, EU979307.1, MF687109.1, MF687137.1, NC_011141.1
Hornyhead chub	*Nocomis biguttatus*		AP012082.1, AY486057.1, GQ275149.1, JQ712283.1, JQ712284.1, JQ712322.1-JQ712325.1, KM281559.1-KM281563.1, KM281565.1-KM281583.1, KM281585.1, NC_033924.1
River chub	*Nocomis micropogon*		AF452077.1, GQ275148.1, JQ712294.1-JQ712297.1, JQ712344.1-JQ712348.1, JQ712356.1
Golden shiner	*Notemigonus crysoleucas*		AB127393.1, KP013116.1, MG570412.1, MG570425.1, MG570428.1, MG570438.1, NC_008646.1, U01318.1
Pugnose shiner	*Notropis anogenus*		AY140698.1
Emerald shiner	*Notropis atherinoides*		AF261220.1, AF352272.1-AF352274.1, AP012083.1, AY096008 AY281062.1, HM224297.1, KT834521.1, NC_033925.1

Common name	Species	Taxonomy	Accession
Bridle shiner	*Notropis bifrenatus*		AP012097.1, KC763658.1, NC_033938.1
River shiner	*Notropis blennius*		AF117170.1, AF117171.1
Bigeye shiner	*Notropis boops*		AF352261.1
Silverjaw minnow	*Notropis buccatus*		AF117154.1-AF117157.1, GQ275154.1, KC763688.1
Ghost shiner	*Notropis buchanani*		AY281058.1, GQ275162.1, HM179622.1-HM179630.1
Ironcolor shiner	*Notropis chalybaeus*		KC763697.1
Bigmouth shiner	*Notropis dorsalis*		AF117162.1, AF117163.1, AF117174.1, AF117175.1
Blackchin shiner	*Notropis heterodon*		AY140697.1
Blacknose shiner	*Notropis heterolepis*		AY140696.1
Spottail shiner	*Notropis hudsonius*		HQ446752.1, KT834523.1
Silver shiner	*Notropis photogenis*		AF352280.1, AF352281.1
Swallowtail shiner	*Notropis procne*		KC763670.1
Rosyface shiner	*Notropis rubellus*		AF117194.1, AF117195.1, AF469164.1, EU084794.1-EU084867.1
Sand shiner	*Notropis stramineus*		DQ536429.1, HM179631.1-HM179637.1, NC_008110.1
Weed shiner	*Notropis texanus*		AF352267.1
Mimic shiner	*Notropis volucellus*		AF352268.1, HM179557.1-HM179596.1
Pugnose minnow	*Opsopoeodus emiliae*		AF261221.1, AP012085.1, GQ184496.1-GQ184498.1, GQ275152.1, GQ275153.1, NC_033926.1
Suckermouth minnow	*Phenacobius mirabilis*		DQ536431.1, JF949845.1, NC_008112.1
Finescale dace	*Phoxinus neogaeus*		EU755058.1, EU755059.1
Eurasian minnow*	*Phoxinus phoxinus*		AB671170.1, AP009309.1, AP011272.1, EF094550.1, EU352213.1, EU755036.1, KC992395.1, KX265376.1-KX265402.1, NC_020358.1
Bluntnose minnow	*Pimephales notatus*		AP012101.1, GQ184499.1-GQ184518.1, GQ275155.1, HQ446759.1, KU856827.1-KU856888.1, KU856890.1-KU856949.1, NC_033941.1, U66606.1
Fathead minnow	*Pimephales promelas*		AP011279.1, GQ184519.1-GQ184522.1, GQ275158.1, GQ275159.1, KT278765.1, KT289925.1, KU856825.1, NC_028087.1
Bullhead minnow	*Pimephales vigilax*		AF117202.1, AF117203.1, AP012102.1, GQ184528.1-GQ184534.1, GQ275157.1, KU856822.1-KU856824.1
Stone moroko*	*Pseudorasbora parva*		AB366541.1, AB677449.1, AF051873.1, AY952995.1, EU934500.1-EU934504.1, HM117852.1-HM117901.1, HM224302.1, HM560155.1, JF802126.1, JX472459.1, KJ135626.1, LC098191.1-LC098196.1, NC_015614.1, Y10453.1

Common name	Species	Taxonomy	Accession
Eastern blacknose dace	*Rhinichthys atratulus*		AF452078.1, AP012104.1, JX442984.1, KF640094.1, KF640095.1, KY398975.1, NC_033943.1
Great lakes longnose dace	*Rhinichthys cataractae*		AP012105.1, EU811101.1, FJ744108.1, HQ446760.1, JQ712320.1, JX442982.1, KF640096.1-KF640151.1, KF640153.1-KF640157.1, NC_033944.1
Western blacknose dace	*Rhinichthys obtusus*		DQ990250.1
Common roach*	*Rutilus rutilus*		AF090772.1, DQ061933.1, DQ447727.1, FJ025068.1, FJ025072.1, FJ025074.1, FJ025077.1-FJ025079.1, HM156751.1-HM156759.1, HM560167.1, KC696559.1, KF784808.1, KF784810.1-KF784815.1, KF784819.1-KF784822.1, KF784831.1-KF784833.1, KF784838.1-KF784841.1, KU302643.1, KX583754.1-KX583795.1, KX583814.1-KX583817.1, KX583835.1-KX583919.1, KX588545.1-KX588552.1
Common rudd*	*Scardinius erythrophthalmus*		AP011263.1, AY509835.1-AY509848.1, EF105295.1, EU856057.1, HM560171.1, NC_031561.1
Creek chub	*Semotilus atromaculatus*		AF452082.1, AP012107.1, HM224307.1, HQ446761.1, NC_033946.1
Fallfish	*Semotilus corporalis*		No records
Grass pickerel	*Esox americanus*	Order: Esociformes Family: Esocidae	AY497427.1-AY497436.1
Northern pike	*Esox Lucius*		AP004103.1, AY497445.1-AY497453.1, FJ425091.1-FJ425097.1, HM177469.1, HM177470.1, KT124232.1-KT124235.1, KT203375.1-KT203379.1, KU244688.1, KU244696.1, KU659805.1, KY399416.1-KY399442.1, NC_004593.1
Muskellunge	*Esox masquinongy*		AY497455.1, AY497456.1
Chain pickerel	*Esox niger*		AP013046.1, AY497437.1-AY497441.1
Central mudminnow	*Umbra limi*	Order: Esociformes Family: Umbridae	AY497458.1, KP013095.1, NC_028282.1
Burbot	*Lota lota*	Order: Gadiformes Family: Lotidae	AP004412.1, DQ174052.1, DQ174053.1, KC844053.1, KM201364.1, KM363244.1, KT327178.1, KU244689.1, KU244691.1, KU244692.1, NC_004379.1
Fourspine stickleback	*Apeltes quadracus*	Order: Gasterosteiformes Family: Gasterosteidae	AB445126.1, NC_011580.1
Brook stickleback	*Culaea inconstans*		AB445125.1, NC_011577.1

Common name	Species	Taxonomy	Accession
Threespine stickleback*	*Gasterosteus aculeatus*		AB094606.1-AB094627.1, AB678412.1-AB678418.1, AF356079.1, AP002944.1, AY116004.1, AY787224.1, KJ628012.1, KR912169.1-KR912173.1, KT971020.1-KT971072.1, LC108042.1, LC108074.1, LC108076.1, LC108085.1, LC108093.1, LC108094.1
Ninespine stickleback	*Pungitius pungitius*		AB094628.1, AB445130.1, AF356080.1, GU227740.1-GU227783.1, JF798872.1-JF798929.1, JQ982981.1-JQ983070.1, KJ627975.1-KJ627989.1, KR779233.1-KR779244.1, KT583722.1, KT583723.1, KT989571.1, KX384721.1-KX384725.1, LC108045.1, LC108047.1, LC108050.1, LC108053.1, LC108055.1, LC108069.1-LC108073.1, LC108075.1, LC108086.1, LC108087.1, LC108089.1, LC108090.1, LC108097.1, NC_011571.1
Racer goby*	*Babka gymnotrachelus*	Order: Gobiiformes Family: Gobiidae	EU444667.1, FJ526765.1-FJ526767.1, KC886267.1, KC886268.1, KF415509.1
Starry goby*	*Benthophilus stellatus*		FJ526780.1
Caucasian dwarf goby*	*Knipowitschia caucasica*		FJ526796.1, KF214248.1-KF214256.1, KT809447.1-KT809449.1
Black Sea monkey goby*	*Neogobius fluviatilis*		EU444672.1, FJ526749.1-FJ526753.1, KC886273.1-KC886275.1, KF549991.1-KF549993.1, KJ605175.1-KJ605184.1
Round goby*	*Neogobius melanostomus*		EU331156.1-EU331236.1, EU564119.1-EU564125.1, KC886276.1-KC886278.1, KF549988.1-KF549990.1, KJ605185.1, KT231987.1-KT232004.1, KX619643.1
Caspian Sea monkey goby*	*Neogobius pallasi*		GQ444372.1-GQ444434.1
Bighead goby*	*Ponticola kessleri*		EU444669.1, FJ526768.1-FJ526770.1, KC886259.1, KC886260.1, KJ605186.1-KJ605189.1, KM583832.1, NC_025638.1
Marine tubenose goby*	*Proterorhinus marmoratus*		EU444614.1-EU444617.1, EU444620.1-EU444624.1, EU444629.1, EU444635.1, EU444637.1-EU444648.1, EU444652.1-EU444657.1, EU444666.1, KF415640.1
Freshwater tubenose goby*	*Proterorhinus semilunaris*		EU444604.1-EU444609.1, EU444612.1, EU444613.1, EU444625.1-EU444628.1, EU444632.1-EU444634.1, EU444649.1-EU444651.1, EU444658.1-EU444665.1, KJ605190.1-KJ605212.1

Common name	Species	Taxonomy	Accession
Mooneye	*Hiodon tergisus*	Order: Hiodontiformes Family: Hiodontidae	AP009499.1, NC_015082.1
Spotted gar	*Lepisosteus oculatus*	Order: Lepisisteiformes Family: Lepisosteidae	AB042861.1, AY442350.1, JF912051.1-JF912053.1, NC_004744.1
Longnose gar	*Lepisosteus osseus*		DQ536423.1, JF912057.1-JF912059.1, NC_008104.1
Shortnose gar	*Lepisosteus platostomus*		JF912054.1-JF912056.1
European smelt*	*Osmerus eperlanus*	Order: Osmeriformes Family: Osmeridae	EU492295.1, EU492321.1, FJ010889.1
Rainbow smelt*	*Osmerus mordax*		AB114911.1, FJ010902.2-FJ010904.2, HM106493.1, HQ115272.1, HQ915956.1, KP257703.1-KP257780.1, NC_015246.1
Rock bass	*Ambloplites rupestris*	Order: Perciformes Family: Centrarchidae	AY115977.1, AY115978.1, AY225663.1, EU501059.1-EU501080.1, KY660677.1, NC_035659.1
Redbreast sunfish*	*Lepomis auritus*		AY115969.1, AY115970.1, AY828949.1-AY828957.1, JF742827.1, MF621723.1, NC_036385.1
Green sunfish	*Lepomis cyanellus*		AY115973.1, AY115974.1, AY828958.1, AY828959.1, JF742828.1, KC427094.1, KP013087.1, NC_020359.1
Pumpkinseed	*Lepomis gibbosus*		AY828960.1-AY828962.1, JF742829.1, KJ513207.1, KP013097.1, MF621724.1-MF621726.1, NC_028284.1
Warmouth	*Lepomis gulosus*		AY115971.1, AY115972.1, AY828963.1, JF742830.1
Orangespotted sunfish	*Lepomis humilis*		AY374293.1, AY828964.1, AY828965.1, JF742831.1
Bluegill	*Lepomis macrochirus*		AP005993.1, AY115975.1, AY115976.1, AY225667.1, AY828966.1-AY828968.1, JN389795.2, KP013118.1, MF621712.1-MF621714.1, NC_015984.2
Longear sunfish	*Lepomis megalotis*		AY828973.1-AY828977.1, JF742833.1, KF571551.1-KF571627.1
Redear sunfish	*Lepomis microlophus*		AY828978.1-AY828982.1, JF742834.1
Smallmouth bass	*Micropterus dolomieu*		AB378749.1, AB378750.1, AY115997.1, AY115998.1, AY225685.1-AY225694.1, HM070845.1-HM070849.1, HM070897.1, HM070903.1, HM070904.1, KC819834.1, KU171303.1-KU171330.1, MF621710.1, MF621711.1, NC_011361.1
Florida largemouth bass*	*Micropterus floridanus*		HM070866.1, HM070868.1-HM070881.1, HM070883.1, HM070887.1-HM070889.1, HM070899.1, HQ391897.1, NC_014689.1

Common name	Species	Taxonomy	Accessions
Largemouth bass	*Micropterus salmoides*		AF479273.1, AP014537.1, AY115999.1, AY116000.1, AY225675.1-AY225684.1, DQ536425.1, HM070864.1, HM070865.1, HM070867.1, HM070882.1, HM070891.1, HM070900.1, HM070901.1, HM070910.1, HM070911.1, HQ391896.1, KC819835.1, KX588083.1-KX588092.1, L14074.1, NC_008106.1, NC_014686.1
White crappie	*Pomoxis annularis*		AY115989.1, AY115990.1, JF742839.1
Black crappie	*Pomoxis nigromaculatus*		AY115991.1, AY115992.1, JF742840.1, KP013112.1, MF621715.1, MF621719.1, NC_028298.1
Northern snakehead*	*Channa argus*	Order: Perciformes Family: Channidae	AP006041.1, GU937112.1, JN681169.1-JN681171.1, JX978723.1, KC823605.1, KM077026.1, KT358952.1, KT358953.1, NC_015191.1
White perch*	*Morone americana*	Order: Perciformes Family: Moronidae	KU641485.1, NC_030281.1
White bass	*Morone chrysops*		AF240745.1, AY374295.1, AY770838.1
Yellow bass*	*Morone mississippiensis*		AF045362.1
Striped bass	*Morone saxatilis*		AF240746.1, HM447585.1, NC_014353.1
Amur sleeper*	*Percottus glenii*	Order: Perciformes Family: Odonotobutidae	AB560893.1, AY722208.1, AY722217.1, AY722243.1, AY722244.1, EF031143.1, EF031144.1, KC292213.1, KC493693.1-KC493753.1, KF415632.1, KM657956.1, NC_020350.1
Western sand darter	*Ammocrypta clara*	Order: Perciformes Family: Percidae	AF045350.1, AF183941.1, HQ128065.1
Eastern sand darter	*Ammocrypta pellucida*		AF183943.1, AY374257.1, FJ381008.1
Greenside darter	*Etheostoma blennioides*		AF288426.1, AF386539.1, AY374261.1, EF587846.1-EF587848.1, EU118843.1-EU118896.1, EU296656.1, EU296659.1, EU296664.1, EU296665.1, EU296667.1, EU716042.1, EU716043.1, HQ128092.1, HQ128093.1, KT880218.1
Rainbow darter	*Etheostoma caeruleum*		AY374263.1, DQ465072.1-DQ465226.1, FJ381011.1-FJ381027.1, GQ250800.1-GQ250833.1, KT880220.1, KY660678.1, NC_035660.1
Bluntnose darter	*Etheostoma chlorosomum*		HQ128105.1, HQ128106.1, JQ397531.1
Iowa darter	*Etheostoma exile*		AF386541.1
Fantail darter	*Etheostoma flabellare*		AF045342.1, AF386544.1, AF412526.1, HQ128131.1

Common name	Species	Taxonomy	Accessions
Least darter	*Etheostoma microperca*		FJ381003.1, KM035907.1-KM035910.1, KM035913.1-KM035923.1, KM035925.1-KM035931.1, KM035933.1, KM035934.1
Johnny darter	*Etheostoma nigrum*		AF183945.1, AY374268.1, GQ183642.1-GQ183677.1, KT289926.1
Tessellated darter	*Etheostoma olmstedi*		GQ183678.1-GQ183700.1
Orangethroat darter	*Etheostoma spectabile*		AF045344.1, DQ465068.1, EU046673.1, FJ381042.1-FJ381067.1, FJ381071.1, FJ381072.1, HQ128229.1, KF377052.1-KF377118.1, KF377120.1-KF377122.1
Banded darter	*Etheostoma zonale*		AF288449.1, AP005994.1, AY964705.1, AY964706.1, EU296686.1, HQ128252.1, KF592243.1-KF592245.1, KF592249.1-KF592251.1, KF592253.1-KF592260.1, KF592268.1-KF592271.1, KF592273.1, KF592276.1-KF592279.1, KF592285.1, KF592288.1, KF592292.1-KF592294.1, KF592298.1, KF592299.1, KF592302.1-KF592304.1, KF592308.1-KF592311.1, KF592313.1-KF592316.1, KF592318.1-KF592321.1, KF592328.1, KF592331.1, KF592332.1, KF592336.1-KF592339.1, KF592341.1-KF592346.1, KF592350.1, KF592353.1-KF592355.1, KF592358.1-KF592365.1, KF592370.1, KF592371.1, KF592374.1, KF592379.1, KF592380.1, KF592384.1-KF592387.1, KF592390.1, KF592394.1, KF592398.1-KF592400.1, KF592402.1, KF592404.1, KF592411.1, KF592415.1, KF592419.1, KF592420.1, KF592423.1-KF592426.1, KF592429.1, KF592430.1, KF592447.1
Eurasian ruffe*	*Gymnocephalus cernua*		AF045356.1, AF386598.1, KC819833.1, KM978956.1, NC_025785.1
Yellow perch	*Perca flavescens*		AF045357.1, AF386600.1, AF546115.1, AY374280.1, EU348833.1-EU348838.1, JX629442.1-JX629448.1, KC819830.1, MF621736.1, NC_019572.1
European perch*	*Perca fluviatilis*		AF045358.1, AF386599.1, AF546116.1, AF546117.1, AY374281.1, AY929376.1, EU348839.1-EU348846.1, FJ172663.1, FJ172664.1, FJ788389.1, FJ788391.1-FJ788393.1, FJ788400.1-FJ788411.1, KM410088.1, NC_026313.1

Common name	Species	Taxonomy	Accession
Logperch	*Percina caprodes*		AF045354.1, AF386550.1, AY770841.1, DQ493482.1-DQ493490.1, EF587838.1-EF587841.1, EU046670.1, EU379093.1-EU379095.1, KC211182.1, KT880217.1
Channel darter	*Percina copelandi*		AF386568.1, AY374283.1
Gilt darter	*Percina evides*		AF375938.1-AF375955.1, AY374284.1, DQ493500.1
Blackside darter	*Percina maculata*		AF045353.1, AF386557.1
Slenderhead darter	*Percina phoxocephala*		AF386563.1, AY374289.1, KM209994.1-KM210030.1
River darter	*Percina shumardi*		AF386571.1, AF386572.1
Sauger	*Sander canadensis*		KC663435.1, KC819814.1-KC819818.1, KT211477.1, KT211478.1, NC_021444.1
Zander*	*Sander lucioperca*		AF546122.1, AY374291.1, FJ788390.1, FJ788394.1-FJ788399.1, GQ214532.1-GQ214534.1, HM049965.1, JX025362.1-JX025365.1, KC819823.1-KC819826.1, KC960516.1-KC960521.1, KM410087.1, KP125333.1, NC_026533.1
Walleye	*Sander vitreus*		AF045359.1, AF386602.1, KC819819.1-KC819822.1, KP013098.1, KT211421.1-KT211476.1, NC_028285.1
Freshwater drum	*Aplodinotus grunniens*	Order: Perciformes Family: Scianidae	AY225662.1, KP722606.1, KT880216.1
Pirate perch	*Aphredoderus sayanus*	Order: Percopsiformes Family: Apherdoderidae	AP004403.1, NC_004372.1
Troutperch	*Percopsis omiscomaycus*	Order: Percopsiformes Family: Percopsidae	No records
Chestnut lamprey	*Ichthyomyzon castaneus*	Order: Petromyzontiformes Family: Petromyzontidae	GQ206168.1
Northern brook lamprey	*Ichthyomyzon fossor*		GQ206170.1, KM267716.1, NC_025552.1
Silver lamprey	*Ichthyomyzon unicuspis*		GQ206171.1, KM267717.1, NC_025553.1
American brook lamprey	*Lampetra appendix*		GQ206179.1, KJ684697.1-KJ684704.1, KM267719.1, NC_025583.1
Sea lamprey*	*Petromyzon marinus*		GQ206148.1, KJ684768.1, NC_001626.1, U11880.1
European flounder* (extirpated)	*Platichthys flesus*	Order: Pleuronectiformes Family: Pleuronectidae	AB125334.1, EU224026.1, EU492120.1, EU492121.1, EU492293.1, EU492294.1, FJ515658.1
Cisco	*Coregonus artedi*	Order: Salmoniformes Family: Salmonidae	JX960771.1, JX960772.1, MF621765.1, MF621766.1, NC_036393.1
Lake whitefish	*Coregonus clupeaformis*		JQ390060.1, JQ661482.1-JQ661487.1, JX960775.1, JX960776.1, NC_020762.1
Bloater	*Coregonus hoyi*		JX960777.1, JX960778.1

Common name	Species	Taxonomy	Accession
Ives lake cisco	*Coregonus hubbsi*		No records
Kiyi	*Coregonus kiyi*		JX960780.1
Blackfin cisco	*Coregonus nigripinnis*		JX960788.1
Nipigon tullibee	*Coregonus nipigon*		No records
Shortnose cisco	*Coregonus reighardi*		No records
Siskiwit lake cisco	*Coregonus zenithicus*		JX960796.1
Deepwater cisco (extinct)	*Corregonus johannae*		No records
Pink salmon*	*Oncorhynchus gorbuscha*		EF455489.1, FJ435607.1, FJ435608.1, JX185439.1, JX185440.1, JX185443.1, JX185444.1, JX960805.1, JX960806.1, KU761855.1, KU872713.1, NC_010959.1
Chum salmon*	*Oncorhynchus keta*		AB039896.1, AF125212.1, AP010773.1, FJ435616.1, FJ435617.1, JX960807.1, JX960808.1, KU872716.1, KX958410.1, NC_017838.1
Coho salmon*	*Oncorhynchus kisutch*		EF126369.1, FJ435609.1, FJ435610.1, JX185441.1, JX185442.1, JX258853.1, JX960809.1, JX960810.1, KJ740755.1-KJ740761.1, KP671851.1, KU761856.1, KU761857.1, KU872712.1, MF621749.1, MF621751.1, NC_009263.1
Rainbow trout*	*Oncorhynchus mykiss*		AF125208.1, AF125209.1, AY032629.1-AY032632.1, AY587167.1-AY587185.1, D58401.1, DQ288268.1-DQ288271.1, FJ435586.1-FJ435602.1, HQ167694.1, JX960813.1-JX960815.1, KP013084.1, KP085590.1, KU761858.1, KU761859.1, KU872710.1, L29771.1, LC050735.1, MF621750.1, NC_001717.1
Chinook salmon*	*Oncorhynchus tshawytscha*		AF392054.1, FJ435603.1, FJ435604.1, HQ167695.1, JX960819.1, JX960820.1, KU761862.1, KU761863.1, KU872715.1, KX958411.1, NC_002980.1
Pygmy whitefish	*Prosopium coulterii*		JX960823.1, JX960824.1, KT630746.1-KT630748.1
Round whitefish	*Prosopium cylindraceum*		AP013050.1, JQ390062.1, JX960825.1, JX960826.1, KT630744.1, KU244693.1, KU244694.1, KU761864.1-KU761866.1, MF621759.1, MF621764.1, MF621767.1, MF621768.1, NC_020764.1

Common name	Species	Taxonomy	Accessions
Atlantic salmon*	*Salmo salar*		AF053591.1, AF133701.1, AF202032.1, BT044011.1, EF584212.1, EU492280.1, EU492281.1, FJ435618.1-FJ435620.1, HQ167697.1, JQ390055.1, JQ390056.1, JX960833.1, JX960834.1, KF792729.1, KY122205.1, KY122206.1, LC012541.1, NC_001960.1, U12143.1
Brown trout*	*Salmo trutta*		AM910409.1, D58400.1, EU492108.1, EU492109.1, EU492282.1, EU492348.1, FJ435621.1-FJ435623.1, FJ655773.1, HQ167696.1, JN995186.1, JQ390057.1, JX960835.1-JX960837.1, JX960839.1, KF985666.1-KF985738.1, KT279167.1-KT279175.1, KT279178.1-KT279198.1, KT633607.1, LC011387.1, LC137015.1, LC145638.1, MF621760.1-MF621763.1, NC_010007.1, NC_024032.1
Brook trout	*Salvelinus fontinalis*		AF154850.1, D58399.1, HQ167699.1, JX960851.1, JX960852.1, KU872718.1, MF621737.1-MF621739.1, NC_000860.1
Lake trout/siscowet	*Salvelinus namaycush*		JX960857.1, JX960858.1, KT630743.1, KU761867.1-KU761869.1, MF621742.1, MF621744.1-MF621748.1, NC_036392.1
Arctic grayling (extirpated)	*Thymallus arcticus*		AF319544.1, GQ452036.1, JX960861.1, JX960862.1, KJ866481.1, KT630732.1-KT630734.1, KU258419.1, KU258420.1, KU761871.1-KU761876.1, MF621752.1-MF621758.1
Mottled sculpin	*Cottus bairdii*	Order: Scorpaeniformes Family: Cottidae	AF549123.1-AF549127.1, AF549162.1-AF549167.1, AY116363.1, AY833333.1-AY833336.1, KP013090.1, NC_028277.1
Slimy sculpin	*Cottus cognatus*		AF549118.1-AF549120.1, AY116364.1, AY116365.1, AY833342.1
European bullhead*	*Cottus gobio*		AY116366.1
Spoonhead sculpin	*Cottus ricei*		AY833363.1
Deepwater sculpin	*Myoxocephalus thompsonii*		AY338275.1, AY338276.1, AY833369.1
White catfish	*Ameiurus catus*	Order: Siluriformes Family: Ictaluridae	AF484159.1, AF484163.1, AY184267.1, AY184270.1, EF491729.1, KM264126.1, KM576102.1, NC_028151.1
Black bullhead	*Ameiurus melas*		AY184263.1, AY184273.1, KT804702.1
Yellow bullhead	*Ameiurus natalis*		AF484158.1, AY184255.1, AY184265.1, AY458888.1, MF621735.1, NC_036391.1

Common name	Species	Taxonomy	Accessions
Brown bullhead	*Ameiurus nebulosus*		AY184257.1, AY184264.1, AY184271.1, AY458889.1, MF621731.1, MF621733.1, MF621734.1, NC_036387.1
Blue catfish*	*Ictalurus furcatus*		AF484159.1, EF491729.1, KM264126.1, KM576102.1, NC_028151.1
Channel catfish	*Ictalurus punctatus*		AB045119.1, AB069646.1, AF477829.1, AF482987.1, AY184253.1, AY184254.1, AY458886.1, AY791413.1, EU490914.1, GQ396767.1, GQ396769.1-GQ396773.1, GQ396792.1, GQ396793.1, JN015529.1, MF621716.1-MF621718.1, MF621720.1-MF621722.1, NC_003489.1
Stonecat	*Noturus flavus*		AY327287.1-AY327290.1, AY458892.1, KM264121.1
Tadpole madtom	*Noturus gyrinus*		AY327295.1-AY327297.1, AY458890.1
Margined madtom	*Noturus insignis*		AY327301.1-AY327303.1, AY458891.1
Brindled madtom	*Noturus miurus*		AY327306.1-AY327308.1, DQ790738.1, DQ790739.1, KM264123.1, KM363003.1-KM363065.1
Northern madtom	*Noturus stigmosus*		AY327319.1, AY327320.1
Flathead catfish*	*Pylodictis olivaris*		AF484161.1, AY458887.1, DQ790748.1, GQ396768.1, MF621727.1-MF621730.1, NC_036386.1
Wels catfish*	*Silurus glanis*	Order: Siluriformes Family: Siluridae	AM398435.2, NC_014261.1
Shortsnouted pipefish*	*Syngnathus abaster*	Order: Sygnathiformes Family: Sygnathidae	AF356060.1, JX228141.1

Primers used for HTS eDNA assays in bait and pond stores. Table shows function, name, direction (Dir; F=forward, R=reverse), and sequences for each primer element. Annealing temperatures (T_A) are given for the target specific primers. Primer topology was 5'–Illumina sequencing adapter, Spacer insert, Target specific primer–3'. Mollusk16S primer set and spacer inserts are from Klymus et al. (2017).

Function	**Name**	**Dir**	**Sequence 5'–3'**	T_A
Target specific	FishCytb-S	F	CACACNTCNAAACAACGAGGNCTNACNTTCCG	54° C
		R	GGGTGTTCNACNGGYATNCCNCCAATTCA	
	FishCytb-L	F	GCCTACGCYATYCTHCGMTCHATYCC	50° C
		R	GGGTGTTCNACNGGYATNCCNCCAATTCA	
	CarpCytb	F	KRTGAAAYTTYGGMTCYCTHCTAGG	54° C
		R	AARAAGAATGATGCYCCRTTRGC	
	GobyCytb	F	AACVCAYCCVCTVCTWAAAATYGC	50° C
		R	AGTCANCCRAARTTWACRTCWCGRC	
	Mollusk16S	F	RRWRGACRAGAAGACCCT	58° C
	Mollusk16S	R	ARTCCAACATCGAGGT	
Adapter	Illumina sequencing	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	
	Illumina sequencing	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	
Spacer inserts	e	F	TCCTATG	
		R	CGTACTAGATGTACGA	
	f	F	ATGCTACAGT	
		R	TCACTAGCTGACGC	
	g	F	CGAGGCTACAACTC	
		R	GAGTAGCTGA	
	h	F	GATACGATCTCGCACTC	
		R	ATCGGCT	

Appendix C.4 Positive control species and their GenBank accession numbers. Genomic DNA extractions from marine species were Sanger sequenced and then mixed in equal mass proportions for use as positive controls to assess sequencing error.

Species	Accession
Clinitrachus argentatus	MK990528
Cristiceps argyropluera	MK990529
Eliginus gracilis	MK990530
Gadus macrocephalus	MK990531
Gibbonsia montereyensis	MK990532
Myxodes viridis	MK990533
Gadus chalcogrammus	MK990534
Myxodes cristatus	MK990538, MK990539
Clinus superciliosis	MK990535
Sebastes alutus	MK990536
Ribeiroclinus eigenmanni	MK990537

Appendix C.5 Legal fish species for sale as bait or grown in aquaculture facilities. Family or species scientific names not in parentheses were explicitly defined in state regulations. Taxa that are not explicitly defined or have had taxonomic revisions were interpreted as in parentheses.

	Indiana	**Michigan**	**Ohio**
Legal bait species	Non-endangered native minnows (Cyprinidae), suckers (Catastomidae), Brook stickleback (*Culaea inconstans)*	Minnows, shiners, dace, and stonerollers (Cyprinidae), suckers (Catastomidae), mudminnows (Umbridae), sculpins (Cottidae)	Native minnows (Cyprinidae), mudminnows (Umbridae), sculpins (Cottidae), suckers (Catastomidae), top minnows (Fundulidae), darters (*Etheostoma*, *Percina*, and *Ammocrypta* spp.), brook silverside (*Labidesthes sicculus*), brook stickleback (*Culaea inconstans*), common carp (*Cyprinus carpio*), emerald shiner *Notropis atherinoides*, gizzard shad (*Dorosoma cepedianum*), rainbow smelt (*Osmerus mordax*), skipjack herring (*Alosa chrysochloris*), trout perch (*Percopsis omiscomaycus*)
Legal aquaculture species	Black crappie (*Pomoxis nigromaculatus*), blue catfish (*Ictalurus furcatus*), bluegill (*Lepomis macrochirus*), bluntnose minnow (*Pimephales notatus*), bowfin (*Amia calva*), brown trout (*Salmo trutta*), buffalo (*Ictiobus* spp.), bullhead (*Ameiurus* spp.), burbot (*Lota lota*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), fathead minnow (*Pimephales promelas*), flathead catfish (*Pylodictis olivaris*), freshwater drum (*Aplodinotus grunniens*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), green sunfish (*Lepomis cyanellus*), hybrid striped bass (*Morone saxatilis x chrysops*), hybrid sunfish (*Lepomis* sp. x sp.), largemouth bass (*Micropterus salmoides*), mosquitofish (*Gambusia* spp.), muskellunge (*Esox masquinongy*), northern pike (*Esox lucius*), paddlefish (*Polyodon spathula*), rainbow trout (*Oncorhynchus mykiss*), redear sunfish (*Lepomis microlophus*), rock bass (*Ambloplites rupestris*), smallmouth bass (*Micropterus dolomieu*), striped bass (*Morone saxatilis*),	Lake sturgeon *Acipenser fulvescens*, paddlefish *Polyodon spathula*, Arctic grayling *Thymallus arcticus*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, splake *Salvelinus namaycush* x *Salvelinus fontinalis*, lake trout *Salvelinus namaycush*, Chinook salmon *Oncorhynchus tshawytscha*, Coho salmon *Oncorhynchus kisutch*, pink salmon *Oncorhynchus gorbuscha*, rainbow trout *Oncorhynchus mykiss*, lake whitefish *Coregonus clupeaformis*, lake herring *Coregonus artedi*, muskellunge *Esox masquinongy*, northern pike *Esox lucius*, tiger muskie *Esox masquinongy* x *Esox lucius*, common carp *Cyprinus carpio*, goldfish *Carassius auratus*, creek chub *Semotilus atromaculatus*, bowfin *Amia calva*, redbelly dace *Phoxinus eos*, Finescale dace *Phoxinus neogaeus*, common shiner *Luxilus cornutus*, golden shiner *Notemigonus crysoleucas*, emerald shiner *Notropis atherinoides*, bluntnose minnow *Pimephales notatus*, fathead minnow *Pimephales promelas*, black bullhead *Ameiurus melas*, yellow bullhead *Ameiurus natalis*,	Alewife *Alosa pseudoharengus*, American eel *Anguilla rostrata*, Atlantic salmon *Salmo salar*, bigmouth buffalo *Ictiobus cyprinellus*, black buffalo *Ictiobus niger*, black bullhead *Ameiurus melas*, black crappie *Pomoxis nigromaculatus*, black redhorse *Moxostoma duquesnei*, blackstripe topminnow *Fundulus notatus*, bluegill *Lepomis macrochirus*, bluntnose minnow *Pimephales notatus*, bowfin *Amia calva*, brook stickleback *Culaea inconstans*, brook trout *Salvelinus fontinalis*, brown bullhead *Ameiurus nebulosus*, brown trout *Salmo trutta*, burbot *Lota lota*, central mudminnow *Umbra limi*, chain pickerel *Esox niger*, channel catfish *Ictalurus punctatus*, chinook salmon *Oncorhynchus tshawytscha*, Coho salmon (*Oncorhynchus kisutch)*, common carp *Cyprinus carpio*, common shiner *Luxilus cornutus*, creek chub *Semotilus atromaculatus*, creek chub sucker *Erimyzon oblongus*, cutthroat trout *Oncorhynchus clarkii*, emerald shiner *Notropis atherinoides*, fathead minnow *Pimephales promelas*, flathead catfish *Pylodictis olivaris*, freshwater drum *Aplodinotus grunniens*, gizzard shad *Dorosoma cepedianum*, golden redhorse *Moxostoma erythrurum*, golden shiner *Notemigonus crysoleucas*, goldfish *Carassius auratus*,

	Indiana	Michigan	Ohio
Legal aquaculture species (cont.)	sucker (Catostomidae), tiger muskellunge (*Esox masquinongy*), tilapia (Tilapiine Ciclids), walleye (*Sander vitreus*), warmouth (*Lepomis gulosus*), white bass (*Morone chrysops*), white catfish (*Ameiurus catus*), white crappie (*Pomoxis annularis*), yellow perch (*Perca flavescens*)	brown bullhead *Ameiurus nebulosus*, channel catfish *Ictalurus punctatus*, flathead catfish *Pylodictis olivaris*, burbot *Lota lota*, smallmouth bass *Micropterus dolmieu*, largemouth bass *Micropterus salmoides*, white crappie *Pomoxis annularis*, black crappie *Pomoxis nigromaculatus*, hybrid crappie *Pomoxis annularis* x *Pomoxis nigromaculatus*, warmouth *Lepomis gulosus*, rock bass *Ambloplites rupestris*, green sunfish *Lepomis cyanellus*, bluegill *Lepomis macrochirus*, hybrid bluegill *Lepomis cyanellus* x *Lepomis macrochirus*, pumpkinseed *Lepomis gibbosus*, redear sunfish *Lepomis microlophus*, sauger *Stizostedion* (*Sander*) *canadense*, walleye *Stizostedion* (*Sander*) *vitreum vitreum*, saugeye *Stizostedion* (*Sander*) *canadense* x *Stizostedion* (*Sander*) *vitreum vitreum*, yellow perch *Perca flavescens*, bigmouth buffalofish *Ictiobus cyprinellus*, black buffalofish *Ictiobus niger*, white perch *Morone americana*, white bass *Morone chrysops*, tilapia *Oreochromis*, *Sarotheradom* and hybrids thereof, not *T.* (*Tilapia*) *rondeliia*	grass pickerel *Esox americanus*, greater redhorse *Moxostoma valenciennesi*, green sunfish *Lepomis cyanellus*, highfin carpsucker *Carpiodes velifer*, Iowa darter *Etheostoma exile*, lake chubsucker *Erimyzon sucetta*, lake trout *Salvelinus namaycush*, lake whitefish *Coregonus clupeaformis*, largemouth bass *Micropterus salmoides*, least darter *Etheostoma microperca*, longear sunfish *Lepomis megalotis*, longnose gar *Lepisosteus osseus*, mooneye *Hiodon tergisus*, muskellunge *Esox masquinongy*, northern hog sucker *Hypentelium nigricans*, northern pike *Esox lucius*, orangespotted sunfish *Lepomis humilis*, pumpkinseed *Lepomis gibbosus*, quillback *Carpiodes cyprinus*, rainbow smelt *Osmerus mordax*, rainbow trout *Oncorhynchus mykiss*, redear sunfish *Lepomis microlophus*, redfin shiner *Notropis umbratilis cyanocephalus*, river carpsucker *Carpiodes carpio*, river redhorse *Moxostoma carinatum*, rock bass *Ambloplites rupestris*, sauger *Sander canadensis*, scarlet shiner *Lythrurus fasciolaris*, shorthead redhorse *Moxostoma macrolepidotum*, silver redhorse *Moxostoma anisurum*, silver shiner *Notropis photogenis*, smallmouth bass *Micropterus dolomieu*, smallmouth buffalo *Ictiobus bubalus*, spotfin shiner *Cyprinella spiloptera*, spottail shiner *Notropis hudsonius*, spotted bass *Micropterus punctulatus*, spotted sucker *Minytrema melanops*, steelcolor shiner *Cyprinella whipplei*, tilapia *Tilapia spp. or Oreochromis spp.*, walleye *Sander vitreus vitreus*, warmouth *Lepomis gulosus*, Western mosquitofish *Gambusia affinis*, white bass *Morone chrysops*, white crappie *Pomoxis annularis*, white sturgeon *Acipenser transmontanus*, white sucker *Catostomus commersoni*, yellow bullhead *Ameiurus natalis*, yellow perch *Perca flavescens*

Appendix C.6 Complete morphological results for bait fish retailers. Species advertised and sampled either morphologically or with eDNA in each store from samples taken in 2016 (white rows) or 2017 (grey rows). Most stores advertised explicitly (species A–F; see table header). A small number advertised "ambiguously", using terms such as "minnows" (†), or "shiners" (Δ). Two stores in the Huron-Erie Corridor region advertised mudminnows (Umbridae, ★). Stores in each region are given an ID number to protect their anonymity. Numbers reflect proportion of bait sold.

					Legal bait spp. sold															Non-legal spp. sold					
																				Native				Invasive	
Region	Store ID	Sampling date	Advertised species	Total bait sold	**A. Creek chub** *Semotilus atromaculatus*	**B. Emerald shiner** *Notropis atherinoides*	**C. Fathead minnow** *Pimephales promelas*	**D. Golden shiner** *Notemigonus crysoleucas*	**E. Spottail shiner** *Notropis hudsonius*	**Bluntnose minnow** *Pimephales notatus*	**Brook silverside** *Labidesthes sicculus*	**Brook stickleback** *Culaea inconstans*	**Common shiner** *Luxilus cornutus*	**Cutlips minnow** *Exoglossum maxillingua*	**Johnny darter** *Etheostoma nigrm*	**Ninespine stickleback** *Pungitius pungitius*	**Pugnose minnow** *Opsopoeodus emiliae*	**Yellow perch** *Perca flavescens*	**F. Goldfish** *Carassius auratus*	**Mosquitofish** *Gambusia affinis*
Wabash	**Total**	**2016**	A,C,D,†,Δ	220	0.01		0.91	0.08																	
River	**Total**	**2017**	F,†,Δ	267	0.01		0.97																0.02		
Indiana	1	**07/11/2016**	C	41			0.92	**0.08**																	
	1	**05/25/2017**	Δ,F	59			0.98																0.02		
	2	**07/11/2016**	C	51			0.96	**0.04**																	
	2	**05/25/2017**	†,Δ	70			1.00																		
	3	**07/11/2016**	A,†,Δ	67	0.04		0.96																		
	3	**05/25/2017**	†,Δ	48	0.06		0.94																		
	4	**07/11/2016**	C,D	61			0.80	0.20																	
	4	**05/25/2017**	F,†	90			0.97																0.03		
Maumee	**Total**	**2016**	B–D,F	1264		0.30	0.48	0.21															0.00		
Bay	**Total**	**2017**	B–D,F,†	2644	0.00	0.17	0.27	0.56				0.00	0.00		0.00		0.00						0.00	0.00	0.00
Ohio	**5**	**08/14/2017**	D	307				1.00																	
	6	**08/01/2016**	B	9		0.10		**0.90**																	
	6	**06/28/2017**	B	107		0.56	**0.05**	**0.39**																	
	6	**09/21/2017**	B	104		1.00																			
	7	**08/15/2016**	B,D	66		0.61		0.39																	
	7	**05/25/2017**	B,D	120		0.89	**0.10**	0.00							**0.01**										
	8	**07/08/2016**	D	263		**1.00**		0.00																	
	8	**09/13/2017**	B	97		1.00																			
	9	**07/08/2016**	B,D	244		0.02	**0.01**	0.97																	
	9	**06/28/2017**	C,D	220			0.80	0.20																	
	9	**09/11/2017**	D	175				1.00																	
	10	**06/08/2016**	B,C	70		0.04	0.96																		
	10	**07/19/2017**	C,D	99			0.16	0.83					**0.01**												
	10	**09/12/2017**	C	353		**0.00**	0.98					**0.02**													

Region	Store ID	Sampling date	Advertised species	Total bait sold	**A. Creek chub** *Semotilus atromaculatus*	**B. Emerald shiner** *Notropis atherinoides*	**C. Fathead minnow** *Pimephales promelas*	**D. Golden shiner** *Notemigonus crysoleucas*	**E. Spottail shiner** *Notropis hudsonius*	**Bluntnose minnow** *Pimephales notatus*	**Brook silverside** *Labidesthes sicculus*	**Brook stickleback** *Culaea inconstans*	**Common shiner** *Luxilus cornutus*	**Cutlips minnow** *Exoglossum maxillingua*	**Johnny darter** *Etheostoma nigrm*	**Ninespine stickleback** *Pungitius*	**Pugnose minnow** *Opsopoeodus emiliae*	**Yellow perch** *Perca flavescens*	**F. Goldfish** *Carassius auratus*	**Mosquitofish** *Gambusia affinis*
	11	**08/14/2017**	D	317				1.00												
	11	**09/27/2017**	D	118				1.00												
	11	**10/16/2017**	D	72		**1.00**		0.00												
	11	**10/21/2017**	B,D	116		0.01		0.99												
	12	**08/15/2016**	C,F	90			0.94												0.06	
	12	**05/25/2017**	C,F	151			0.99												0.01	
	13	**07/07/2016**	D	65		**1.00**		0.00												
	13	**09/12/2017**	D	130				1.00												
	14	**06/08/2016**	C	260			1.00													
	14	**09/11/2017**	D	106				0.99												**0.01**
	15	**08/15/2016**	C	197			1.00													
	15	**08/15/2017**	†	13	0.15		0.23										**0.62**			
	15	**09/22/2017**	D	39				1.00												
Huron-	**Total**	**2016**	B–D,Δ	2008		0.40	0.28	0.32												
Erie Corr.	**Total**	**2017**	A,B,D,†,Δ,★	2392	0.00	0.24	0.26	0.47	0.02	0.01		0.00	0.00	0.00		0.00		0.00		
Michigan	**16**	**08/16/2016**	D	116				1.00												
	16	**07/25/2017**	†	67	0.01	0.01		0.98												
	17	**07/07/2016**	D	77		**1.00**		0.00												
	17	**07/26/2017**	E,†	172			0.33	0.37	0.28									**0.03**		
	18	**08/16/2016**	B	87		0.99	**0.01**													
	18	**07/25/2017**	★	211		**0.02**	**0.87**	**0.02**		**0.06**			**0.02**							
	19	**08/19/2016**	B	235		0.26	**0.74**													
	19	**07/25/2017**	B	119		1.00														
	20	**08/16/2016**	D	113			**0.01**	0.99												
	20	**07/31/2017**	†	130		0.25	0.06	0.68												
	21	**07/07/2016**	Δ	136		1.00														
	21	**07/25/2017**	Δ	226			**0.36**	0.63										**0.01**		
	22	**07/07/2016**	D	112		**1.00**		0.00												
	22	**07/26/2017**	†	312		0.79	0.21					**0.00**				**0.00**				
	23	**07/07/2016**	D	187		**1.00**		0.00												
	23	**07/26/2017**	B,D,★	189		0.56	**0.17**	0.23						**0.04**						
	24	**07/07/2016**	D	160			**0.02**	0.98												
	24	**07/26/2017**	D	295			**0.00**	1.00												
	25	**08/19/2016**	D	154			**0.48**	0.52												
	25	**07/25/2017**	D	143				1.00												
	26	**08/16/2016**	B,D	99		0.01		0.99												
	26	**07/31/2017**	D	182		**0.36**	**0.02**	0.62												
	27	**08/16/2016**	C	206			1.00													

Region	Store ID	Sampling date	Advertised species	Total bait sold	A. Creek chub *Semotilus atromaculatus*	B. Emerald shiner *Notropis atherinoides*	C. Fathead minnow *Pimephales promelas*	D. Golden shiner *Notemigonus crysoleucas*	E. Spottail shiner *Notropis hudsonius*	Bluntnose minnow *Pimephales notatus*	Brook silverside *Labidesthes sicculus*	Brook stickleback *Culaea inconstans*	Common shiner *Luxilus cornutus*	Cutlips minnow *Exoglossum maxillingua*	Johnny darter *Etheostoma nigrim*	Ninespine stickleback *Pungitius ...*	Pugnose minnow *Opsopoeodus emiliae*	Yellow perch *Perca flavescens*	F. Goldfish *Carassius auratus*	Mosquitofish *Gambusia affinis*
	27	07/31/2017	A	179	0.00		**0.99**					**0.01**								
	28	08/19/2016	B,C,D	204		0.70	0.00	0.30												
	28	07/25/2017	Δ,†	101				0.96	0.01					0.03						
	29	08/16/2016	C,D	122			0.80	0.20												
	29	07/31/2017	D	66	**0.02**		**0.09**	0.89												
Sandusky	Total	2016	B–D,†	1949		0.21	0.07	0.72												
Bay,	Total	2017	B,D,Δ	3248		0.08	0.02	0.90			**0.00**									**0.00**
Ohio	30	07/01/2016	D	244		**0.99**		0.01												
	30	07/24/2017	†	303				1.00												
	30	09/11/2017	D	180				0.98												**0.02**
	31	07/01/2016	D	175				1.00												
	31	07/24/2017	D	172			**0.01**	0.99												**0.01**
	31	09/14/2017	B,D	261		0.00		1.00												
	32	07/13/2016	B,D	36		1.00		0.00												
	32	09/21/2017	Δ	108				1.00												
	33	07/13/2016	†	124		1.00														
	33	05/22/2017	Δ	196		1.00														
	33	09/18/2017	D	145				1.00												
	34	07/08/2016	D	72				1.00												
	34	07/24/2017	D	139			**0.01**	0.99												
	34	09/22/2017	D	128		**0.01**		0.99												
	35	07/13/2016	D	112				1.00												
	35	07/24/2017	D	198				1.00												
	35	09/14/2017	D	175				1.00												
	36	07/08/2016	D	211			**0.03**	0.97												
	36	07/24/2017	D	159				1.00												
	36	09/21/2017	D	159		**0.01**		0.99												
	37	07/07/2016	D	153				1.00												
	37	07/24/2017	Δ	129			**0.10**	0.90												
	37	09/21/2017	D	152		**0.06**		0.93			**0.01**									
	38	07/08/2016	C,D	87			0.25	0.75												
	39	07/13/2016	D	167		**0.02**		0.98												
	39	07/24/2017	D	215				1.00												
	39	09/14/2017	D	160				1.00												
	40	07/01/2016	D	180				1.00												
	40	05/22/2017	Δ	82		0.40	**0.20**	0.40												
	40	09/212017	D	78		**0.05**		0.95												
	41	07/08/2016	C,D	388			0.27	0.73												
	41	06/28/2017	D	109			**0.27**	0.73												

Region	Store ID	Sampling date	Advertised species	Total bait sold	**A. Creek chub** *Semotilus atromaculatus*	**B. Emerald shiner** *Notropis atherinoides*	**C. Fathead minnow** *Pimephales promelas*	**D. Golden shiner** *Notemigonus crysoleucas*	**E. Spottail shiner** *Notropis hudsonius*	**Bluntnose minnow** *Pimephales notatus*	**Brook silverside** *Labidesthes sicculus*	**Brook stickleback** *Culaea inconstans*	**Common shiner** *Luxilus cornutus*	**Cutlips minnow** *Exoglossum maxillingua*	**Johnny darter** *Etheostoma nigrm*	**Ninespine stickleback** *Pungitius*	**Pugnose minnow** *Opsopoeodus emiliae*	**Yellow perch** *Perca flavescens*	**F. Goldfish** *Carassius auratus*	**Mosquitofish** *Gambusia affinis*
Lake Erie	**Total**	**2016**	B,D,E,F,†	728		0.12	0.13	0.74											0.00	
Central Basin, Ohio	**Total**	**2017**	A–D,F,†	1336	0.00		0.00	0.98		0.00			0.01						0.00	**0.00**
	42	**07/28/2017**	D	165				1.00												
	43	**08/07/2016**	D,E,F	66			**0.61**	0.34	0.00										0.05	
	43	**07/30/2017**	A,D	64	0.02		**0.02**	0.97												
	44	**08/07/2016**	D	182				1.00												
	44	**07/28/2017**	C,D	134			0.01	0.99												
	45	**08/07/2016**	D	63				1.00												
	45	**07/28/2017**	D	58				1.00												
	46	**08/06/2016**	D	88				1.00												
	47	**08/06/2016**	D	37			**0.03**	0.97												
	47	**07/30/2017**	D	102				1.00												
	48	**08/06/2016**	†	122				1.00												
	48	**07/28/2017**	D	126			**0.01**	0.98												**0.02**
	49	**08/06/2016**	B,D	82		0.00	**0.67**	0.33												
	49	**07/30/2017**	F,†	302				0.95		0.00			0.04						0.01	
	50	**08/07/2016**	B	88		1.00														
	50	**07/28/2017**	B,D	256		0.00	**0.01**	0.99												
	51	**07/30/2017**	D	129			**0.01**	0.99												

Appendix C.7 High-throughput sequencing metrics. Assay (FishS=FishCyt*b*-S, FishL=FishCyt*b*-L, Goby=GobyCyt*b*, Carp=CarpCyt*b*, and Mol=Mollusk16S), anonymized store ID (B=bait, P=pond, r=rerun, PC=positive control), year (E=early, L=late for 2017 bait store samples only), library name in the Sequence Read Archive, number of raw reads returned from Illumina MiSeq, successfully trimmed (had both primers and the correct spacer insert), merged without chimeras in DADA2, and proportion of trimmed that merged, and unique ASVs in each library. All FASTQ files are deposited in the Sequence Read Archive (Bioproject # PRJNA548536).

Run 1				*N* reads				
Assay	**ID**	**Time**	**Library**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**ASVs**
FishS	1-B	2016	9BKP01	298,078	279,423	194,392	0.7	7
	2-B	2016	9BKP02	79,121	74,133	50,967	0.69	2
	3-B	2016	9BKP03	116,490	109,843	81,577	0.74	3
	4-B	2016	9BKP04	68,318	63,725	44,571	0.7	6
	6-B	2016	9BKP05	99,436	92,076	67,089	0.73	13
	7-B	2016	9BKP06	147,141	138,068	79,990	0.58	13
	9-B	2016	9BKP07	78,708	73,928	54,560	0.74	6
	12-B	2016	9BKP09	64,753	59,102	37,649	0.64	8
	15-B	2016	9BKP10	85,421	78,671	54,664	0.69	5
	16-B	2016	9BKP11	130,646	122,856	88,108	0.72	5
	17-B	2016	9BKP12	102,043	95,922	51,840	0.54	14
	18-B	2016	9BKP13	89,141	81,236	54,390	0.67	19
	19-B	2016	9BKP14	82,586	75,933	46,580	0.61	9
	20-B	2016	9BKP15	117,430	105,235	69,101	0.66	14
	21-B	2016	9BKP16	181,633	171,829	109,092	0.63	16
	22-B	2016	9BKP17	70,405	66,030	38,335	0.58	10
	23-B	2016	9BKP18	148,612	139,812	80,024	0.57	12
	24-B	2016	9BKP19	168,315	158,369	113,468	0.72	8
	25-B	2016	9BKP20	254,019	235,313	167,991	0.71	16
	26-B	2016	9BKP21	105,771	96,516	68,058	0.71	8
	27-B	2016	9BKP22	200,172	187,905	140,690	0.75	3
	29-B	2016	9BKP24	95,802	89,976	62,847	0.7	15
	30-B	2016	9BKP25	209,933	198,445	122,675	0.62	11
	31-B	2016	9BKP26	95,825	89,661	63,172	0.7	9
	32-B	2016	9BKP27	126,498	118,977	84,058	0.71	11
	33-B	2016	9BKP28	238,654	207,470	107,155	0.52	20
	34-B	2016	9BKP29	112,954	106,342	74,980	0.71	7
	35-B	2016	9BKP30	75,916	71,099	51,333	0.72	5
	36-B	2016	9BKP31	57,964	46,796	28,917	0.62	6
	37-B	2016	9BKP32	181,040	170,093	126,334	0.74	6
	38-B	2016	9BKP33	13,028	10,524	6,203	0.59	4
	39-B	2016	9BKP34	118,641	106,980	72,921	0.68	10
	40-B	2016	9BKP35	133,041	125,252	92,043	0.73	10
	41-B	2016	9BKP36	105,058	99,010	72,031	0.73	7
	43-B	2016	9BKP37	106,286	82,824	61,923	0.75	19
	44-B	2016	9BKP38	110,819	104,390	75,760	0.73	6
	45-B	2016	9BKP39	138,913	111,035	60,778	0.55	11
	46-B	2016	9BKP40	106,551	99,296	56,331	0.57	10
	47-B	2016	9BKP41	128,491	121,001	87,524	0.72	7
	48-B	2016	9BKP42	119,503	111,700	79,634	0.71	9
	49-B	2016	9BKP43	119,182	111,906	78,198	0.7	7
	50-B	2016	9BKP44	107,760	101,291	56,091	0.55	15

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	11-B	2016	9BKP45	96,275	82,526	57,526	0.7	10
			Total FishS	*5,286,373*	*4,872,519*	*3,271,570*	--	--
			Mean FishS	*122,939*	*113,314*	*76,083*	*0.67*	*9.6*
Goby	1-B	2016	gBKP01	48,689	41,491	25,208	0.61	11
	2-B	2016	gBKP02	63,280	52,053	33,228	0.64	8
	3-B	2016	gBKP03	34,669	29,337	18,887	0.64	6
	4-B	2016	gBKP04	69,789	60,719	36,314	0.6	10
	6-B	2016	gBKP05	26,149	18,859	10,361	0.55	6
	7-B	2016	gBKP06	43,260	34,713	20,733	0.6	30
	9-B	2016	gBKP07	93,759	75,351	44,072	0.58	10
	12-B	2016	gBKP09	38,657	27,737	16,531	0.6	6
	15-B	2016	gBKP10	48,243	34,025	19,816	0.58	10
	16-B	2016	gBKP11	100,949	86,552	51,688	0.6	7
	17-B	2016	gBKP12	47,835	36,443	22,295	0.61	16
	18-B	2016	gBKP13	41,088	28,691	16,967	0.59	38
	19-B	2016	gBKP14	53,417	35,589	19,612	0.55	21
	20-B	2016	gBKP15	23,278	18,204	11,534	0.63	13
	21-B	2016	gBKP16	38,663	33,792	20,051	0.59	17
	22-B	2016	gBKP17	48,035	39,106	24,247	0.62	18
	23-B	2016	gBKP18	54,940	36,768	22,218	0.6	32
	24-B	2016	gBKP19	94,751	86,614	53,590	0.62	10
	25-B	2016	gBKP20	39,827	32,878	20,766	0.63	9
	26-B	2016	gBKP21	67,253	34,766	22,035	0.63	9
	27-B	2016	gBKP22	98,829	91,007	59,859	0.66	5
	29-B	2016	gBKP24	43,552	36,219	21,775	0.6	18
	30-B	2016	gBKP25	206,402	183,609	101,310	0.55	52
	31-B	2016	gBKP26	44,503	40,241	26,504	0.66	13
	32-B	2016	gBKP27	21,736	19,259	12,403	0.64	7
	34-B	2016	gBKP29	116,703	106,972	66,866	0.63	7
	35-B	2016	gBKP30	124,523	114,260	70,310	0.62	6
	36-B	2016	gBKP31	103,363	86,398	55,464	0.64	22
	37-B	2016	gBKP32	85,849	39,081	23,810	0.61	7
	38-B	2016	gBKP33	165,845	145,446	83,058	0.57	12
	39-B	2016	gBKP34	46,929	21,166	13,702	0.65	11
	40-B	2016	gBKP35	169,796	141,313	80,231	0.57	10
	41-B	2016	gBKP36	136,570	119,063	66,865	0.56	10
	44-B	2016	gBKP38	161,036	141,061	80,660	0.57	5
	47-B	2016	gBKP41	111,070	97,420	56,847	0.58	11
	48-B	2016	gBKP42	127,486	102,041	59,091	0.58	12
	49-B	2016	gBKP43	179,446	151,360	84,143	0.56	18
	50-B	2016	gBKP44	124,133	75,334	37,588	0.5	35
	11-B	2016	gBKP45	104,316	89,318	48,653	0.54	13
	PC		gBLE00	85,348	75,183	49,170	0.65	2
			Total Goby	3,333,966	2,719,439	1,608,462	--	--
			Mean Goby	83,349	67,986	40,212	0.6	14.1
Run 2								
FishL	30-B	2017L	fBKS01	103,111	87,470	64,242	0.73	13
	10-B	2017L	fBKS04	87,627	77,983	60,899	0.78	17
	6-B	2017L	fBKS07	181,681	170,021	96,009	0.56	44
	36-B	2017L	fBKS13	137,163	113,414	84,699	0.75	18
	15-B	2017L	fBKS17	64,246	61,045	54,934	0.9	10
	3-B	2017E	fBKV01	77,101	69,107	58,579	0.85	19
	2-B	2017E	fBKV04	94,207	85,398	79,986	0.94	13
	15-B	2017E	fBKV07	122,052	109,070	100,135	0.92	21
	24-B	2017E	fBKV14	156,413	139,383	97,536	0.7	15

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	26-B	2017E	fBKV19	193,739	181,836	132,511	0.73	32
	29-B	2017E	fBKV22	106,312	100,333	77,790	0.78	16
	28-B	2017E	fBKV24	124,109	97,247	70,190	0.72	22
	41-B	2017E	fBKV26	196,074	169,679	135,365	0.8	50
	31-B	2017E	fBKV32	130,276	115,141	84,859	0.74	13
	34-B	2017E	fBKV35	69,712	59,583	44,918	0.75	12
	44-B	2017E	fBKV41	98,528	89,425	63,491	0.71	12
	PC		fBLE01	43,082	41,495	35,695	0.86	13
			Total FL	*1,985,433*	*1,767,630*	*1,341,838*	--	--
			Mean FL	*116,790*	*103,978*	*78,932*	*0.78*	*20*
Carp	1-B	2016	cBKP01	63,364	53,723	49,253	0.92	23
	4-B	2016	cBKP04	30,431	26,478	23,892	0.9	11
	9-B	2016	cBKP07	64,606	53,183	43,505	0.82	15
	18-B	2016	cBKP13	248,474	156,823	144,930	0.92	42
	21-B	2016	cBKP16	109,469	89,482	70,770	0.79	25
	25-B	2016	cBKP20	168,152	94,103	79,649	0.85	21
	29-B	2016	cBKP24	65,738	44,441	38,557	0.87	16
	30-B	2016	cBKP25	57,044	41,655	32,537	0.78	24
	35-B	2016	cBKP30	106,884	87,408	60,926	0.7	13
	36-B	2016	cBKP31	176,801	95,909	74,661	0.78	32
	41-B	2016	cBKP36	171,557	154,673	111,858	0.72	25
	48-B	2016	cBKP42	140,613	88,113	68,156	0.77	30
	11-B	2016	cBKP45	86,911	73,187	55,427	0.76	20
	33-B	2017L	cBKS02	142,439	80,729	61,106	0.76	23
	13-B	2017L	cBKS05	140,234	114,162	76,338	0.67	14
	32-B	2017L	cBKS08	224,677	160,904	102,819	0.64	14
	40-B	2017L	cBKS16	102,796	86,050	56,793	0.66	17
	8-B	2017L	cBKS19	144,719	115,635	85,842	0.74	32
	3-B	2017E	cBKV01	47,093	37,175	34,638	0.93	13
	4-B	2017E	cBKV02	299,528	96,967	77,012	0.79	13
	12-B	2017E	cBKV05	33,808	28,351	26,963	0.95	16
	6-B	2017E	cBKV08	62,603	47,497	40,743	0.86	34
	21-B	2017E	cBKV13	64,332	48,470	42,218	0.87	25
	17-B	2017E	cBKV16	30,396	27,486	23,663	0.86	19
	23-B	2017E	cBKV18	47,253	44,714	35,677	0.8	28
	27-B	2017E	cBKV21	144,682	105,402	101,145	0.96	7
	19-B	2017E	cBKV25	28,698	20,552	13,679	0.67	14
	31-B	2017E	cBKV32	172,729	116,736	88,044	0.75	19
	42-B	2017E	cBKV36	192,559	71,842	52,397	0.73	12
	50-B	2017E	cBKV39	141,901	86,271	64,545	0.75	37
	PC		cBLE01	80,651	73,355	67,116	0.91	16
			Total Carp	*3,591,142*	*2,421,476*	*1,904,859*	--	--
			Mean Carp	*115,843*	*78,112*	*61,447*	*0.8*	*21*
Goby	14-B	2017L	gBKS06	56,505	48,793	43,063	0.88	7
	11-B	2017L	gBKS09	93,752	64,043	53,679	0.84	24
	34-B	2017L	gBKS12	44,135	30,373	26,367	0.87	6
	40-B	2017L	gBKS16	112,777	105,150	94,195	0.9	14
	3-B	2017E	gBKV01	101,954	58,237	50,544	0.87	16
	7-B	2017E	gBKV06	245,777	156,829	147,435	0.94	34
	11-B	2017E	gBKV10	153,618	118,161	109,251	0.92	9
	24-B	2017E	gBKV14	165,968	137,633	126,821	0.92	19
	22-B	2017E	gBKV17	84,951	53,143	45,471	0.86	41
	18-B	2017E	gBKV23	125,861	39,453	34,498	0.87	15
	30-B	2017E	gBKV29	148,618	74,184	64,075	0.86	11

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	31-B	2017E	gBKV32	185,570	91,430	80,791	0.88	10
	50-B	2017E	gBKV39	206,416	113,143	97,233	0.86	34
	51-B	2017E	gBKV43	297,789	167,446	130,295	0.78	12
	PC		gBLE01	44,252	42,562	38,187	0.9	12
			Total Goby	*2,067,943*	*1,300,580*	*1,141,905*	--	--
			Mean Goby	*137,863*	*86,705*	*76,127*	*0.88*	*17.6*
Run 3								
FishL	33-B	2017L	fBKS02	76,412	71,670	56,196	0.78	16
	13-B	2017L	fBKS05	95,767	91,093	70,461	0.77	17
	32-B	2017L	fBKS08	71,712	69,170	52,683	0.76	10
	11-B	2017L	fBKS10	45,838	43,788	30,290	0.69	24
	5-B	2017L	fBKS11	56,347	53,689	39,498	0.74	41
	39-B	2017L	fBKS14	150,604	113,216	101,060	0.89	13
	9-B	2017L	fBKS15	129,481	120,374	92,058	0.76	12
	37-B	2017L	fBKS18	80,271	77,718	59,481	0.77	33
	4-B	2017E	fBKV02	177,386	66,838	60,599	0.91	74
	12-B	2017E	fBKV05	123,650	101,738	92,488	0.91	24
	6-B	2017E	fBKV08	50,585	45,369	37,930	0.84	33
	16-B	2017E	fBKV12	87,546	82,129	71,428	0.87	20
	25-B	2017E	fBKV15	77,110	74,741	62,430	0.84	13
	22-B	2017E	fBKV17	74,767	69,189	52,395	0.76	41
	20-B	2017E	fBKV20	105,357	101,084	82,712	0.82	30
	18-B	2017E	fBKV23	50,865	48,756	46,306	0.95	11
	40-B	2017E	fBKV27	102,954	83,907	66,584	0.79	33
	37-B	2017E	fBKV30	67,659	63,268	51,483	0.81	24
	36-B	2017E	fBKV33	152,933	97,741	80,106	0.82	14
	42-B	2017E	fBKV36	91,086	83,026	68,931	0.83	16
	50-B	2017E	fBKV39	83,647	70,273	56,573	0.81	19
	43-B	2017E	fBKV42	28,702	27,257	24,347	0.89	17
	51-B	2017E	fBKV43	76,537	65,655	60,575	0.92	11
	PC		fBLE02	37,018	36,222	32,946	0.91	13
			Total FishL	*2,094,234*	*1,757,911*	*1,449,560*	--	--
			Mean FishL	*87,260*	*73,246*	*60,398*	*0.83*	*22.5*
Carp	2-B	2016	cBKP02	68,235	61,974	59,959	0.97	27
	6-B	2016	cBKP05	48,641	33,084	29,610	0.89	16
	12-B	2016	cBKP09	106,751	93,210	73,726	0.79	16
	22-B	2016	cBKP17	28,279	21,530	17,275	0.8	16
	23-B	2016	cBKP18	132,387	86,735	67,862	0.78	33
	26-B	2016	cBKP21	116,595	83,904	67,994	0.81	12
	31-B	2016	cBKP26	72,784	55,580	39,634	0.71	16
	37-B	2016	cBKP32	99,915	81,676	63,613	0.78	9
	39-B	2016	cBKP34	112,821	71,931	56,406	0.78	24
	44-B	2016	cBKP38	69,782	61,574	41,069	0.67	10
	47-B	2016	cBKP41	204,424	96,420	71,703	0.74	24
	49-B	2016	cBKP43	48,101	39,497	31,965	0.81	17
	14-B	2017L	cBKS06	90,089	86,595	62,560	0.72	8
	11-B	2017L	cBKS09	89,393	76,161	59,301	0.78	32
	5-B	2017L	cBKS11	130,896	78,742	60,255	0.77	32
	36-B	2017L	cBKS13	85,651	66,340	55,609	0.84	18
	39-B	2017L	cBKS14	154,287	96,236	76,328	0.79	16
	15-B	2017L	cBKS17	68,726	59,132	56,577	0.96	16
	1-B	2017E	cBKV03	118,328	45,619	43,352	0.95	34
	7-B	2017E	cBKV06	99,188	91,202	76,364	0.84	23
	11-B	2017E	cBKV10	75,515	66,012	51,692	0.78	6
	24-B	2017E	cBKV14	102,093	91,723	68,719	0.75	19

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	26-B	2017E	cBKV19	77,555	72,386	57,715	0.8	22
	29-B	2017E	cBKV22	67,726	60,724	50,700	0.83	17
	28-B	2017E	cBKV24	215,444	124,098	98,883	0.8	14
	41-B	2017E	cBKV26	178,875	116,544	100,641	0.86	55
	30-B	2017E	cBKV29	144,343	97,537	77,519	0.79	35
	37-B	2017E	cBKV30	113,711	82,524	65,092	0.79	20
	36-B	2017E	cBKV33	70,330	55,301	40,845	0.74	14
	39-B	2017E	cBKV34	137,440	67,990	55,468	0.82	17
	45-B	2017E	cBKV37	79,100	61,094	46,513	0.76	13
	49-B	2017E	cBKV40	63,210	41,548	38,102	0.92	25
	43-B	2017E	cBKV42	37,432	31,439	27,568	0.88	12
	47-B	2017E	cBKV44	131,248	82,645	64,814	0.78	21
	PC		cBLE02	117,103	114,007	105,820	0.93	18
			Total Carp	*3,556,398*	*2,552,714*	*2,061,253*	--	--
			Mean Carp	*101,611*	*72,935*	*58,893*	*0.81*	*20.4*
Goby	35-B	2017L	gBKS03	107,358	94,576	80,971	0.86	10
	6-B	2017L	gBKS07	204,078	140,142	109,212	0.78	55
	11-B	2017L	gBKS10	277,121	164,483	125,295	0.76	81
	36-B	2017L	gBKS13	200,651	143,097	128,379	0.9	12
	15-B	2017L	gBKS17	61,281	54,649	51,462	0.94	8
	8-B	2017L	gBKS19	123,345	78,085	61,203	0.78	56
	2-B	2017E	gBKV04	131,612	100,194	93,964	0.94	26
	15-B	2017E	gBKV07	34,333	21,669	16,700	0.77	11
	5-B	2017E	gBKV11	92,752	87,084	79,140	0.91	13
	25-B	2017E	gBKV15	86,747	66,464	60,368	0.91	8
	23-B	2017E	gBKV18	107,147	89,060	80,598	0.9	47
	20-B	2017E	gBKV20	120,568	96,345	87,985	0.91	18
	27-B	2017E	gBKV21	164,924	103,681	99,659	0.96	8
	19-B	2017E	gBKV25	73,023	48,089	36,796	0.77	24
	37-B	2017E	gBKV30	109,453	95,516	82,625	0.87	18
	36-B	2017E	gBKV33	152,708	94,027	83,452	0.89	7
	34-B	2017E	gBKV35	80,683	68,786	60,012	0.87	14
	42-B	2017E	gBKV36	83,060	70,136	60,561	0.86	10
	49-B	2017E	gBKV40	129,592	85,838	66,947	0.78	27
	44-B	2017E	gBKV41	83,759	76,216	64,681	0.85	14
	47-B	2017E	gBKV44	158,905	123,480	109,266	0.88	14
	PC		gBLE02	172,934	63,820	56,369	0.88	16
			Total Goby	*2,756,034*	*1,965,437*	*1,695,645*	--	--
			Mean Goby	*125,274*	*89,338*	*77,075*	*0.86*	*22.6*
Run 4			**Sample**	**Raw**	**Trimmed**	**Merged**	**Prop.**	**ASVs**
FishL	35-B	2017L	fBKS03	53,997	51,770	37,368	0.72	11
	14-B	2017L	fBKS06	57,736	55,958	40,071	0.72	8
	11-B	2017L	fBKS09	84,163	80,621	59,954	0.74	36
	34-B	2017L	fBKS12	72,131	70,056	52,504	0.75	6
	40-B	2017L	fBKS16	50,333	48,764	38,997	0.8	21
	8-B	2017L	fBKS19	59,962	56,994	40,431	0.71	30
	1-B	2017E	fBKV03	198,419	128,377	117,565	0.92	37
	7-B	2017E	fBKV06	16,157	13,781	11,327	0.82	18
	11-B	2017E	fBKV10	83,257	76,000	59,222	0.78	10
	21-B	2017E	fBKV13	91,928	76,261	62,362	0.82	48
	17-B	2017E	fBKV16	109,603	97,032	77,220	0.8	48
	23-B	2017E	fBKV18	103,468	98,953	69,806	0.71	43
	27-B	2017E	fBKV21	88,493	83,254	81,411	0.98	4
	19-B	2017E	fBKV25	88,988	80,875	55,230	0.68	44
	33-B	2017E	fBKV28	134,841	76,730	62,242	0.81	52

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	35-B	2017E	fBKV31	136,144	114,547	87,923	0.77	20
	39-B	2017E	fBKV34	275,787	102,019	82,067	0.8	14
	45-B	2017E	fBKV37	82,014	62,930	47,733	0.76	9
	49-B	2017E	fBKV40	58,142	53,333	48,252	0.9	19
	47-B	2017E	fBKV44	65,829	60,419	46,724	0.77	13
	10-B	2017E	fBKV45	97,649	85,507	80,921	0.95	10
	PC		fBLE03	117,557	114,294	104,095	0.91	22
			Total FishL	*2,126,598*	*1,688,475*	*1,363,425*	--	--
			Mean FishL	*96,664*	*76,749*	*61,974*	*0.8*	*23.8*
Carp	3-B	2016	cBKP03	68,615	60,770	59,144	0.97	7
	15-B	2016	cBKP10	138,798	66,934	56,640	0.85	47
	16-B	2016	cBKP11	77,704	67,254	51,232	0.76	4
	19-B	2016	cBKP14	101,205	65,390	43,823	0.67	40
	27-B	2016	cBKP22	80,272	68,069	66,689	0.98	10
	34-B	2016	cBKP29	157,753	79,678	61,109	0.77	12
	50-B	2016	cBKP44	90,423	75,652	57,323	0.76	35
	30-B	2017L	cBKS01	124,394	71,321	58,178	0.82	17
	10-B	2017L	cBKS04	171,360	125,146	119,360	0.95	24
	6-B	2017L	cBKS07	71,603	59,083	44,662	0.76	32
	11-B	2017L	cBKS10	101,887	83,890	67,716	0.81	33
	34-B	2017L	cBKS12	128,497	72,857	54,366	0.75	4
	9-B	2017L	cBKS15	101,191	81,464	62,323	0.77	15
	37-B	2017L	cBKS18	88,409	74,530	51,256	0.69	26
	2-B	2017E	cBKV04	53,296	48,225	47,303	0.98	20
	15-B	2017E	cBKV07	46,370	39,561	32,883	0.83	14
	5-B	2017E	cBKV11	81,736	76,205	55,044	0.72	21
	25-B	2017E	cBKV15	79,750	62,302	48,410	0.78	10
	22-B	2017E	cBKV17	91,382	81,509	67,532	0.83	47
	20-B	2017E	cBKV20	99,473	85,104	70,249	0.83	26
	18-B	2017E	cBKV23	229,628	92,271	86,697	0.94	25
	40-B	2017E	cBKV27	286,092	140,214	122,439	0.87	45
	33-B	2017E	cBKV28	146,619	97,662	83,483	0.85	24
	35-B	2017E	cBKV31	69,197	61,359	46,730	0.76	18
	34-B	2017E	cBKV35	189,894	109,693	85,469	0.78	23
	48-B	2017E	cBKV38	142,334	89,437	66,936	0.75	16
	44-B	2017E	cBKV41	120,704	87,078	69,238	0.8	25
	PC		cBLE03	67,060	61,854	56,406	0.91	16
			Total Carp	*3,205,646*	*2,184,512*	*1,792,640*	--	--
			Mean Carp	*114,487*	*78,018*	*64,023*	*0.82*	*22.7*
Goby	30-B	2017L	gBKS01	99,693	70,028	62,127	0.89	20
	13-B	2017L	gBKS05	91,571	87,345	75,002	0.86	9
	32-B	2017L	gBKS08	57,924	53,409	45,838	0.86	8
	5-B	2017L	gBKS11	74,035	62,166	58,644	0.94	21
	9-B	2017L	gBKS15	133,128	116,356	105,378	0.91	12
	37-B	2017L	gBKS18	111,804	103,065	91,327	0.89	17
	12-B	2017E	gBKV05	200,640	97,661	91,481	0.94	23
	6-B	2017E	gBKV08	358,803	156,967	143,476	0.91	54
	16-B	2017E	gBKV12	219,316	134,288	121,526	0.9	14
	17-B	2017E	gBKV16	176,337	131,642	119,697	0.91	46
	26-B	2017E	gBKV19	105,047	92,976	83,783	0.9	21
	29-B	2017E	gBKV22	131,943	88,881	80,748	0.91	15
	41-B	2017E	gBKV26	127,639	90,158	79,109	0.88	32
	35-B	2017E	gBKV31	95,721	79,716	70,641	0.89	14
	39-B	2017E	gBKV34	336,090	120,139	105,447	0.88	35
	45-B	2017E	gBKV37	128,229	77,052	69,789	0.91	18

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	48-B	2017E	gBKV38	82,811	69,048	61,895	0.9	11
	43-B	2017E	gBKV42	87,349	67,462	55,522	0.82	23
	PC		gBLE03	92,649	87,892	79,039	0.9	17
			Total Goby	*2,710,729*	*1,786,251*	*1,600,469*	--	--
			Mean Goby	*142,670*	*94,013*	*84,235*	*0.89*	*21.6*
Run 5								
FishL	6-B-r	2016	fBKP05r	205164	89897	72731	0.81	19
	12-B-r	2016	fBKP09r	166584	145052	108608	0.75	12
	19-B-r	2016	fBKP14r	157473	106763	79018	0.74	24
	20-B-r	2016	fBKP15r	152044	96414	70092	0.73	13
	25-B-r	2016	fBKP20r	213302	110281	83526	0.76	17
	34-B-r	2016	fBKP29r	50169	45494	31619	0.7	11
	45-B-r	2016	fBKP39r	129291	84802	65359	0.77	16
	47-B-r	2016	fBKP41r	152649	83071	50985	0.61	64
	50-B-r	2016	fBKP44r	43920	36814	22858	0.62	34
	11-B-r	2016	fBKP45r	143712	91512	66033	0.72	20
	6-B-r	2017L	fBKS07r	82578	59701	37306	0.62	29
	11-B-r	2017L	fBKS10r	102471	65873	39781	0.6	28
	9-B-r	2017L	fBKS15r	52928	41397	26770	0.65	11
	8-B-r	2017L	fBKS19r	58771	44610	26530	0.59	22
	1-B-r	2017E	fBKV03r	255528	123896	87968	0.71	25
	30-B-r	2017E	fBKV29r	90129	56672	38889	0.69	8
	PC		fBLE04	73846	68023	58998	0.87	21
			Total FishL	*2,130,559*	*1,350,272*	*967,071*	--	--
			Mean FishL	*125,327*	*79,428*	*56,887*	*0.7*	*22*
Carp	6-B-r	2016	cBKP05r	492,654	68,792	41366	0.6	19
	12-B-r	2016	cBKP09r	36,578	33,470	26271	0.78	3
	19-B-r	2016	cBKP14r	82,334	65,460	40726	0.62	24
	25-B-r	2016	cBKP20r	263,297	149,479	127908	0.86	27
	34-B-r	2016	cBKP29r	50,983	43,701	35323	0.81	3
	35-B-r	2016	cBKP30r	41,276	35,111	28715	0.82	15
	45-B-r	2016	cBKP39r	390,309	79,361	50052	0.63	24
	48-B-r	2016	cBKP42r	166,640	73,984	58641	0.79	18
	50-B-r	2016	cBKP44r	59,640	39,548	29813	0.75	22
	11-B-r	2016	cBKP45r	57,326	44,076	32960	0.75	15
	6-B-r	2017L	cBKS07r	46,993	39,348	29122	0.74	17
	32-B-r	2017L	cBKS08r	125,101	70,811	53913	0.76	8
	11-B-r	2017L	cBKS10r	32,965	25,888	20475	0.79	17
	9-B-r	2017L	cBKS15r	46,771	38,704	25492	0.66	8
	8-B-r	2017L	cBKS19r	54,120	42,484	30874	0.73	20
	1-B-r	2017E	cBKV03r	393,734	206,965	144512	0.7	78
	21-B-r	2017E	cBKV13r	95,683	71,753	57117	0.8	15
	29-B-r	2017E	cBKV22r	93,598	63,470	44719	0.7	6
	28-B-r	2017E	cBKV24r	85,464	57,209	44306	0.77	16
	41-B-r	2017E	cBKV26r	91,659	53,052	39790	0.75	11
	30-B-r	2017E	cBKV29r	109,894	62,170	45955	0.74	4
	44-B-r	2017E	cBKV41r	51,975	43,547	30047	0.69	7
	PC		cBLE04	98,305	78,215	67824	0.87	8
			Total Carp	*2,967,299*	*1,486,598*	*1,105,921*	--	--
			Mean Carp	*129,013*	*64,635*	*48,084*	*0.74*	*16.7*
Goby	6-B-r	2016	gBKP05r	355954	81537	69314	0.85	6
	7-B-r	2016	gBKP06r	299908	116115	83005	0.71	24
	19-B-r	2016	gBKP14r	200453	79022	66134	0.84	16
	25-B-r	2016	gBKP20r	475468	193481	158626	0.82	21
	34-B-r	2016	gBKP29r	64632	53714	48096	0.9	5

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
	45-B-r	2016	gBKP39r	280129	105532	81257	0.77	15
	50-B-r	2016	gBKP44r	530752	173400	125941	0.73	31
	11-B-r	2016	gBKP45r	49266	41537	35820	0.86	9
	6-B-r	2017L	gBKS07r	467656	190150	136336	0.72	27
	32-B-r	2017L	gBKS08r	93696	59256	48323	0.82	7
	11-B-r	2017L	gBKS10r	619020	146658	97705	0.67	30
	9-B-r	2017L	gBKS15r	83699	54963	46119	0.84	7
	8-B-r	2017L	gBKS19r	314365	75265	52101	0.69	31
	PC		gBLE04	162196	107599	92718	0.86	9
			Total Goby	*3,997,194*	*1,478,229*	*1,141,495*	--	--
			Mean Goby	*285,514*	*105,588*	*81,535*	*0.79*	*17*
Run 6								
FishL	20-P	2017	f0526A	83,878	79,432	76,510	0.96	12
	8-P	2017	f0531B	55,825	52,376	51,318	0.98	11
	17-P	2017	f0602A	67,200	62,758	61,339	0.98	10
	4-P	2017	f0602B	65,080	63,501	60,648	0.96	9
	5-P	2017	f0602C	66,627	64,517	57,543	0.89	10
	2-P	2017	f0602D	63,034	60,533	59,213	0.98	4
	3-P	2017	f0612A	78,984	73,708	70,729	0.96	22
	19-P	2017	f0612B	61,994	60,908	56,157	0.92	9
	10-P	2017	f0614A	77,748	75,571	65,982	0.87	16
	9-P	2017	f0614B	66,152	63,815	54,174	0.85	7
	7-P	2017	f0614C	63,922	61,808	51,702	0.84	12
	6-P	2017	f0616A	81,771	79,401	71,216	0.9	19
	18-P	2017	f0621A	67,651	65,463	61,122	0.93	47
	1-P	2017	f0621B	78,755	71,925	70,465	0.98	11
	21-P	2017	f0626A	61,159	59,184	54,504	0.92	27
	11-P	2017	f0626C	74,922	72,662	65,125	0.9	4
	13-P	2017	f0627A	57,125	55,614	48,297	0.87	10
	16-P	2017	f0627B	54,275	52,423	44,995	0.86	10
	15-P	2017	f0627C	56,162	54,624	47,667	0.87	17
	PC		fBLE05	117,153	115,380	105,686	0.92	18
			Total FishL	*1,399,417*	*1,345,603*	*1,234,392*	--	--
			Mean FishL	*69,971*	*67,280*	*61,720*	*0.92*	*14.3*
Carp	20-P	2017	c0526A	77,253	71,391	68,621	0.96	11
	8-P	2017	c0531B	69,506	68,033	66,834	0.98	8
	17-P	2017	c0602A	71,208	66,785	65,032	0.97	4
	4-P	2017	c0602B	22,422	21,879	21,149	0.97	3
	5-P	2017	c0602C	58,308	56,962	46,773	0.82	5
	2-P	2017	c0602D	67,826	66,730	65,984	0.99	2
	3-P	2017	c0612A	56,749	55,427	53,720	0.97	21
	19-P	2017	c0612B	54,680	53,762	51,306	0.95	9
	10-P	2017	c0614A	62,666	61,763	51,955	0.84	8
	9-P	2017	c0614B	71,734	70,447	50,688	0.72	6
	7-P	2017	c0614C	77,736	76,128	60,930	0.8	8
	6-P	2017	c0616A	76,769	73,907	61,938	0.84	14
	18-P	2017	c0621A	48,538	47,397	45,376	0.96	50
	1-P	2017	c0621B	82,461	78,472	77,518	0.99	8
	21-P	2017	c0626A	82,671	80,480	75,079	0.93	20
	12-P	2017	c0626B	64,674	63,579	51,929	0.82	4
	13-P	2017	c0627A	94,122	92,483	73,558	0.8	13
	16-P	2017	c0627B	99,491	98,022	80,640	0.82	9
	15-P	2017	c0627C	115,051	113,459	89,933	0.79	9
	PC		cBLE05	102,253	100,553	95,760	0.95	19
			Total Carp	*1,456,118*	*1,417,659*	*1,254,723*	--	--

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
			Mean Carp	*72,806*	*70,883*	*62,736*	*0.89*	*11.6*
Goby	20-P	2017	g0526A	55,437	48,029	45,920	0.96	12
	8-P	2017	g0531B	62,125	49,375	48,148	0.98	3
	4-P	2017	g0602B	74,345	55,689	53,916	0.97	5
	5-P	2017	g0602C	113,348	93,097	77,555	0.83	15
	2-P	2017	g0602D	61,280	54,033	52,192	0.97	12
	3-P	2017	g0612A	128,508	81,698	78,726	0.96	30
	19-P	2017	g0612B	150,312	134,755	122,147	0.91	14
	10-P	2017	g0614A	80,107	73,228	63,072	0.86	13
	9-P	2017	g0614B	93,670	81,560	64,610	0.79	14
	7-P	2017	g0614C	133,941	102,315	78,211	0.76	14
	6-P	2017	g0616A	153,674	108,599	92,507	0.85	20
	18-P	2017	g0621A	78,307	69,674	63,519	0.91	36
	1-P	2017	g0621B	109,721	61,487	58,980	0.96	9
	21-P	2017	g0626A	69,204	60,139	54,834	0.91	24
	11-P	2017	g0626C	89,595	84,886	71,752	0.85	6
	13-P	2017	g0627A	72,844	62,070	51,899	0.84	9
	16-P	2017	g0627B	110,647	93,007	80,285	0.86	9
	15-P	2017	g0627C	90,419	84,967	69,885	0.82	40
	PC		gBLE05	89,904	88,287	78,751	0.89	17
			Total Goby	*1,817,388*	*1,486,895*	*1,306,909*	--	--
			Mean Goby	*95,652*	*78,258*	*68,785*	*0.89*	*15.9*
Run 7								
Mol	20-P	2017	m0526A	171,058	67,857	60,003	0.88	12
	4-P	2017	m0602B	41,055	33,265	29,994	0.9	5
	3-P	2017	m0612A	171,573	26,721	23,731	0.89	16
	19-P	2017	m0612B	116,176	99,933	61,269	0.61	12
	10-P	2017	m0614A	66,315	32,051	29,602	0.92	7
	9-P	2017	m0614B	90,021	71,045	48,732	0.69	10
	7-P	2017	m0614C	116,810	83,563	69,347	0.83	24
	6-P	2017	m0616A	151,542	54,352	44,920	0.83	17
	21-P	2017	m0626A	68,734	18,843	15,174	0.81	10
	12-P	2017	m0626B	18,046	6,821	5,522	0.81	7
	13-P	2017	m0627A	55,672	30,201	22,930	0.76	12
	16-P	2017	m0627B	101,848	46,371	33,321	0.72	9
	15-P	2017	m0627C	117,713	88,723	73,217	0.83	21
			Total Mol	*1,286,563*	*659,746*	*517,762*	--	--
			Mean Mol	*98,966*	*50,750*	*39,828*	*0.81*	*12.5*
Run 8								
FishL	10-P-r	2017	f0614Ar	154,203	146,511	121,280	0.83	25
	6-P-r	2017	f0616Ar	174,826	151,471	134,086	0.89	47
	1-P-r	2017	f0621Br	81,466	70,754	64,815	0.92	22
	PC		fBLE06	107,432	104,842	101,504	0.97	24
			Total FishL	*410,495*	*368,736*	*320,181*	--	--
			Mean FishL	*136,832*	*122,912*	*106,727*	*0.9*	*29.5*
Carp	10-P-r	2017	c0614Ar	103,212	91,109	75,152	0.82	15
	6-P-r	2017	c0616Ar	187,063	144,255	123,398	0.86	26
	1-P-r	2017	c0621Br	196,645	127,813	124,344	0.97	10
	PC		cBLE06	72,667	70,664	64,767	0.92	34
			Total Carp	*486,920*	*363,177*	*322,894*	--	--
			Mean Carp	*162,307*	*121,059*	*107,631*	*0.89*	*21.3*
Goby	10-P-r	2017	g0614Ar	108,566	94,727	87,431	0.92	21
	6-P-r	2017	g0616Ar	187,480	132,975	119,223	0.9	56
	1-P-r	2017	g0621Br	114,364	84,941	83,035	0.98	35
	PC		gBLE06	110,213	107,587	103,466	0.96	24

Assay	ID	Time	Library	Raw	Trimmed	Merged	Prop.	ASVs
			Total Goby	*410,410*	*312,643*	*289,689*	--	--
			Mean Goby	*136,803*	*104,214*	*96,563*	*0.94*	*34*
Mol	3-P-r	2017	m0612Ar	95,812	79,370	64,221	0.81	25
	10-P-r	2017	m0614Ar	84,151	77,542	75,835	0.98	11
	6-P-r	2017	m0616Ar	142,852	115,072	105,281	0.91	25
	12-P-r	2017	m0626Br	114,713	93,126	79,102	0.85	27
	13-P-r	2017	m0627Ar	95,636	88,606	80,968	0.91	27
			Total Mol	*533,164*	*453,716*	*405,407*	--	--
			Mean Mol	*106,633*	*90,743*	*81,081*	*0.89*	*23*

		***N* reads**				
	All runs	**Raw**	**Trimmed**	**Merged**	**Prop.**	**ASVs**
	Total	49,275,676	35,486,381	27,878,530	**--**	**--**
	Mean sample	113,801	81,955	64,385	0.79	18.2
	±SE	±3,624	±1,818	±1,400	±0.01	±0.6

Appendix C.8 eDNA sampling in bait stores. Anonymized store ID, year of sampling (Time), E=early and L=late season (2017 only), *N* libraries successfully sequenced for all markers (*N* assays) and FishCyt*b* (short or long), CarpCyt*b*, or GobyCyt*b* assays. A single pond store failed to amplify with any assays, all others amplified with all three fish assays.

ID	Time	N Assays	FishCyt*b*	CarpCyt*b*	GobyCyt*b*	ID	Time	N Assays	FishCyt*b*	CarpCyt*b*	GobyCyt*b*
1	2016	3	1	1	1	26	2016	3	1	1	1
1	2017E	2	1	1	0	26	2017E	3	1	1	1
2	2016	3	1	1	1	27	2016	3	1	1	1
2	2017E	3	1	1	1	27	2017E	3	1	1	1
3	2016	3	1	1	1	28	2016	0	0	0	0
3	2017E	3	1	1	1	28	2017E	2	1	1	0
4	2016	3	1	1	1	29	2016	3	1	1	1
4	2017E	2	1	1	0	29	2017E	3	1	1	1
5	2017L	3	1	1	1	30	2016	3	1	1	1
5	2017E	2	0	1	1	30	2017L	3	1	1	1
6	2016	3	1	1	1	30	2017E	3	1	1	1
6	2017L	3	1	1	1	31	2016	3	1	1	1
6	2017E	3	1	1	1	31	2017E	3	1	1	1
7	2016	2	1	0	1	32	2016	3	1	1	1
7	2017E	3	1	1	1	32	2017L	3	1	1	1
8	2016	0	0	0	0	33	2016	1	1	0	0
8	2017L	3	1	1	1	33	2017L	3	1	1	1
9	2016	3	1	1	1	33	2017E	2	1	1	0
9	2017L	3	1	1	1	34	2016	3	1	1	1
9	2017E	0	0	0	0	34	2017L	3	1	1	1
10	2016	0	0	0	0	34	2017E	3	1	1	1
10	2017L	3	1	1	1	35	2016	3	1	1	1
10	2017E	1	1	0	0	35	2017L	3	1	1	1
11	2016	3	1	1	1	35	2017E	3	1	1	1
11	2017L	3	1	1	1	36	2016	3	1	1	1
11	2017L	3	1	1	1	36	2017L	3	1	1	1
11	2017E	3	1	1	1	36	2017E	3	1	1	1
12	2016	3	1	1	1	37	2016	3	1	1	1
12	2017E	3	1	1	1	37	2017L	3	1	1	1
13	2016	0	0	0	0	37	2017E	3	1	1	1
13	2017L	3	1	1	1	38	2016	3	1	1	1
14	2016	0	0	0	0	39	2016	3	1	1	1
14	2017L	3	1	1	1	39	2017L	2	1	1	0
15	2016	3	1	1	1	39	2017E	3	1	1	1
15	2017L	3	1	1	1	40	2016	3	1	1	1
15	2017E	3	1	1	1	40	2017L	3	1	1	1
16	2016	3	1	1	1	40	2017E	2	1	1	0
16	2017E	3	1	1	1	41	2016	3	1	1	1
17	2016	3	1	1	1	41	2017E	3	1	1	1
17	2017E	3	1	1	1	42	2017E	3	1	1	1
18	2016	3	1	1	1	43	2016	1	1	0	0
18	2017E	3	1	1	1	43	2017E	3	1	1	1
19	2016	3	1	1	1	44	2016	3	1	1	1
19	2017E	3	1	1	1	44	2017E	3	1	1	1
20	2016	2	1	0	1	45	2016	3	1	1	1
20	2017E	3	1	1	1	45	2017E	3	1	1	1
21	2016	3	1	1	1	46	2016	2	1	1	0
21	2017E	2	1	1	0	47	2016	3	1	1	1

ID	Time	N Assays	FishCyt*b*	CarpCyt*b*	GobyCyt*b*	ID	Time	N Assays	FishCyt*b*	CarpCyt*b*	GobyCyt*b*
22	2016	3	1	1	1	47	2017E	3	1	1	1
22	2017E	3	1	1	1	48	2016	3	1	1	1
23	2016	3	1	1	1	48	2017E	3	1	1	1
23	2017E	3	1	1	1	49	2016	3	1	1	1
24	2016	3	1	1	1	49	2017E	3	1	1	1
24	2017E	3	1	1	1	50	2016	3	1	1	1
25	2016	3	1	1	1	50	2017E	3	1	1	1
25	2017E	3	1	1	1	51	2017E	2	1	0	1

Appendix C.9 Species detected with eDNA that were also present in morphological results. Ninespine stickleback (*Pungitius pungitius*) was identified in one bait store with morphology and was the only species that was never detected with eDNA. †=unestablished aquatic invasive species on the GLANSIS watch list, *=Mollusk, •=Bryozoan.

Bait		Pond	
eDNA detections	Morphology	eDNA detections	Morphology
Ambloplites rupestris		*Carassius auratus*	X
Campostoma anomalum		*Catostomus commersonii*	
Carassius auratus	X	*Cipangopaludina chinensis**	
Catostomus catostomus		*Cipangopaludina japonica**	
Catostomus commersonii		*Ctenopharyngodon idella*	X
Clinostomus elongatus		*Cyprinus carpio*	X
Coregonus sp.		*Dorosoma cepedianum*	
Ctenopharyngodon idella		*Dreissena polymorpha**	
Culaea inconstans	X	*Fredericella indica•*	
Cyprinella spiloptera		*Gambusia affinis*	
Cyprinus carpio		*Gyraulus parvus**	
Dorosoma cepedianum		*Helisoma trivolvis**	
Esox lucius		*Hypophthalmichthys molitrix†*	
Etheostoma spp.	X	*Hypophthalmichthys nobilis†*	
Gambusia affinis	X	*Ictalurus punctatus*	X
Gymnocephalus		*Ladislavella elodes**	
Hybognathus hankinsoni		*Lepomis microlophus*	X
Hypophthalmichthys molitrix		*Leuciscus idus†*	
Hypophthalmichthys nobilis		*Lophopodella carteri•*	
Ictiobus cyprinellus		*Melanoides tuberculata**	
Labidesthes sicculus	X	*Micropterus salmoides*	X
Lepomis spp.		*Morone saxatalis*	
Luxilus cornutus	X	*Neogobius melanostomus*	
Micropterus salmoides		*Notemigonus crysoleucas*	X
Morone americana		*Perca flavescens*	X
Morone chrysops		*Physa acuta**	
Neogobius melanostomus		*Pimephales promelas*	X
Nocomis biguttatus		*Planorbarius corneus**	
Notemigonus crysoleucas	X	*Pomoxis spp.*	
Notropis atherinoides	X	*Sander vitreus*	X
Notropis buchanani			
Notropis hudsonius	X		
Notropis rubellus			
Notropis stramineus			
Notropis volucellus			
Osmerus mordax			
Perca flavescens	X		
Percina spp.			
Phenacobius mirabilis			
Pimephales notatus	X		
Pimephales promelas	X		
Pomoxis spp.			
Proterorhinus			
Rhinichthys atratulus			
Sander vitreus			
Semotilus atromaculatus	X		

Appendix C.10 Complete eDNA results for bait store samples. Anonymized store ID, time (year and E=early and L=late fishing season samples for 2017 only), state, and the number of unique BLAST hits that were any unadvertised legal bait (Bait), native non-bait, aquatic invasive species (AIS), silver or bighead carp (*Hypop.* spp.), round goby (*N. melano.*), and Eurasian ruffe (*G. cernu.*).

			Unadvertised						
ID	**Time**	**State**	**All**	**Bait**	**Native non-bait**	**AIS**	***Hypop.* spp.**	***N. melano.***	***G. cernu***
1	2016	IN	4	2	0	2	0	0	0
1	2017 E	IN	12	7	0	5	1	1	1
2	2016	IN	1	1	0	0	0	0	0
2	2017 E	IN	2	0	1	1	0	0	0
3	2016	IN	2	2	0	0	0	0	0
3	2017 E	IN	4	2	1	1	0	0	0
4	2016	IN	1	1	0	0	0	0	0
4	2017 E	IN	11	7	1	3	0	0	1
5	2017 E	OH	9	6	3	0	0	0	0
5	2017 L	OH	2	1	0	1	0	0	0
6	2016	OH	4	2	1	1	0	0	0
6	2017 E	OH	3	1	2	0	0	0	0
6	2017 L	OH	7	4	0	3	0	1	0
7	2016	OH	10	9	0	1	0	1	0
7	2017 E	OH	14	11	1	2	0	1	0
8	2017 L	OH	3	3	0	0	0	0	0
9	2016	OH	2	2	0	0	0	0	0
9	2017 L	OH	2	1	0	1	0	0	0
10	2017 E	OH	4	4	0	0	0	0	0
10	2017 L	OH	0	0	0	0	0	0	0
11	2016	OH	1	0	0	1	1	0	0
11	2017 E	OH	4	2	1	1	0	0	0
11	2017 L	OH	3	1	0	2	2	0	0
11	2017 L	OH	0	0	0	0	0	0	0
12	2016	OH	1	1	0	0	0	0	0
12	2017 E	OH	10	8	0	2	0	1	0
13	2017 L	OH	2	1	0	1	0	0	0
14	2017 L	OH	2	1	0	1	0	0	0
15	2016	OH	8	5	2	1	0	1	0
15	2017 E	OH	4	2	0	2	0	0	0
15	2017 L	OH	5	2	0	3	0	1	0
16	2016	MI	2	2	0	0	0	0	0
16	2017 E	MI	3	1	1	1	0	1	0
17	2016	MI	10	8	2	0	0	0	0
17	2017 E	MI	12	6	5	1	0	1	0
18	2016	MI	9	7	0	2	0	1	0
18	2017 E	MI	6	4	1	1	0	1	0
19	2016	MI	4	1	1	2	1	0	0
19	2017 E	MI	4	4	0	0	0	0	0
20	2016	MI	1	0	0	1	0	1	0
20	2017 E	MI	3	2	0	1	0	1	0
21	2016	MI	12	9	2	1	0	1	0
21	2017 E	MI	9	7	2	0	0	0	0
22	2016	MI	8	7	1	0	0	0	0
22	2017 E	MI	8	4	3	1	0	1	0
23	2016	MI	10	8	1	1	0	0	0
23	2017 E	MI	10	7	2	1	0	1	0

ID	Time	State	All	Bait	Native non-bait	AIS	*Hypop.* spp.	*N. melano.*	*G. cernu*
24	2016	MI	2	2	0	0	0	0	0
24	2017 E	MI	7	4	1	2	0	1	0
25	2016	MI	10	5	1	4	0	0	1
25	2017 E	MI	2	1	0	1	0	1	0
26	2016	MI	5	2	0	3	0	1	1
26	2017 E	MI	7	6	1	0	0	0	0
27	2016	MI	1	1	0	0	0	0	0
27	2017 E	MI	2	1	0	1	0	1	0
28	2017 E	MI	10	8	0	2	0	1	1
29	2016	MI	10	7	1	2	0	1	0
29	2017 E	MI	4	3	1	0	0	0	0
30	2016	OH	10	8	1	1	0	1	0
30	2017 E	OH	3	1	0	2	0	1	0
30	2017 L	OH	3	2	0	1	0	0	0
31	2016	OH	4	3	0	1	0	1	0
31	2017 E	OH	2	1	0	1	0	0	0
32	2016	OH	3	3	0	0	0	0	0
32	2017 L	OH	3	3	0	0	0	0	0
33	2016	OH	5	4	1	0	0	0	0
33	2017 E	OH	3	2	0	1	0	1	0
33	2017 L	OH	1	1	0	0	0	0	0
34	2016	OH	0	0	0	0	0	0	0
34	2017 E	OH	0	0	0	0	0	0	0
34	2017 L	OH	2	1	0	1	0	0	0
35	2016	OH	2	1	0	1	1	0	0
35	2017 E	OH	2	1	1	0	0	0	0
35	2017 L	OH	6	4	1	1	0	0	0
36	2016	OH	5	4	0	1	0	1	0
36	2017 E	OH	4	2	1	1	0	0	0
36	2017 L	OH	3	2	0	1	0	0	0
37	2016	OH	2	2	0	0	0	0	0
37	2017 E	OH	8	4	2	2	0	0	1
37	2017 L	OH	7	4	1	2	0	0	0
38	2016	OH	1	1	0	0	0	0	0
39	2016	OH	2	2	0	0	0	0	0
39	2017 E	OH	0	0	0	0	0	0	0
39	2017 L	OH	5	2	1	2	0	1	1
40	2016	OH	4	4	0	0	0	0	0
40	2017 E	OH	2	2	0	0	0	0	0
40	2017 L	OH	4	2	1	1	0	1	0
41	2016	OH	1	1	0	0	0	0	0
41	2017 E	OH	8	6	1	1	0	0	0
42	2017 E	OH	3	3	0	0	0	0	0
43	2016	OH	1	1	0	0	0	0	0
43	2017 E	OH	6	5	0	1	0	0	0
44	2016	OH	2	2	0	0	0	0	0
44	2017 E	OH	1	0	0	1	0	0	0
45	2016	OH	6	5	0	1	0	0	0
45	2017 E	OH	4	2	1	1	0	1	0
46	2016	OH	3	2	1	0	0	0	0
47	2016	OH	8	3	1	4	2	1	0
47	2017 E	OH	2	2	0	0	0	0	0
48	2016	OH	4	2	0	2	0	1	0

ID	Time	State	All	Bait	Native non-bait	AIS	*Hypop.* spp.	*N. melano.*	*G. cernu*
48	2017 E	OH	2	1	0	1	0	0	0
49	2016	OH	3	1	0	2	0	1	0
49	2017 E	OH	9	8	0	1	0	0	0
50	2016	OH	9	3	4	2	0	1	0
50	2017 E	OH	2	1	0	1	0	1	0
51	2017 E	OH	2	1	0	1	0	0	1

Appendix C.11 Complete fish assays eDNA results for pond store samples. Anonymized store ID, state, and the number of unique BLAST hits that were any unadvertised species or unadvertised legal pond (Pond), native non-pond, aquatic invasive species (AIS), silver or bighead carp (*Hypop.* spp.), round goby (*N. melano.*), and Eurasian ide (*L. idus*).

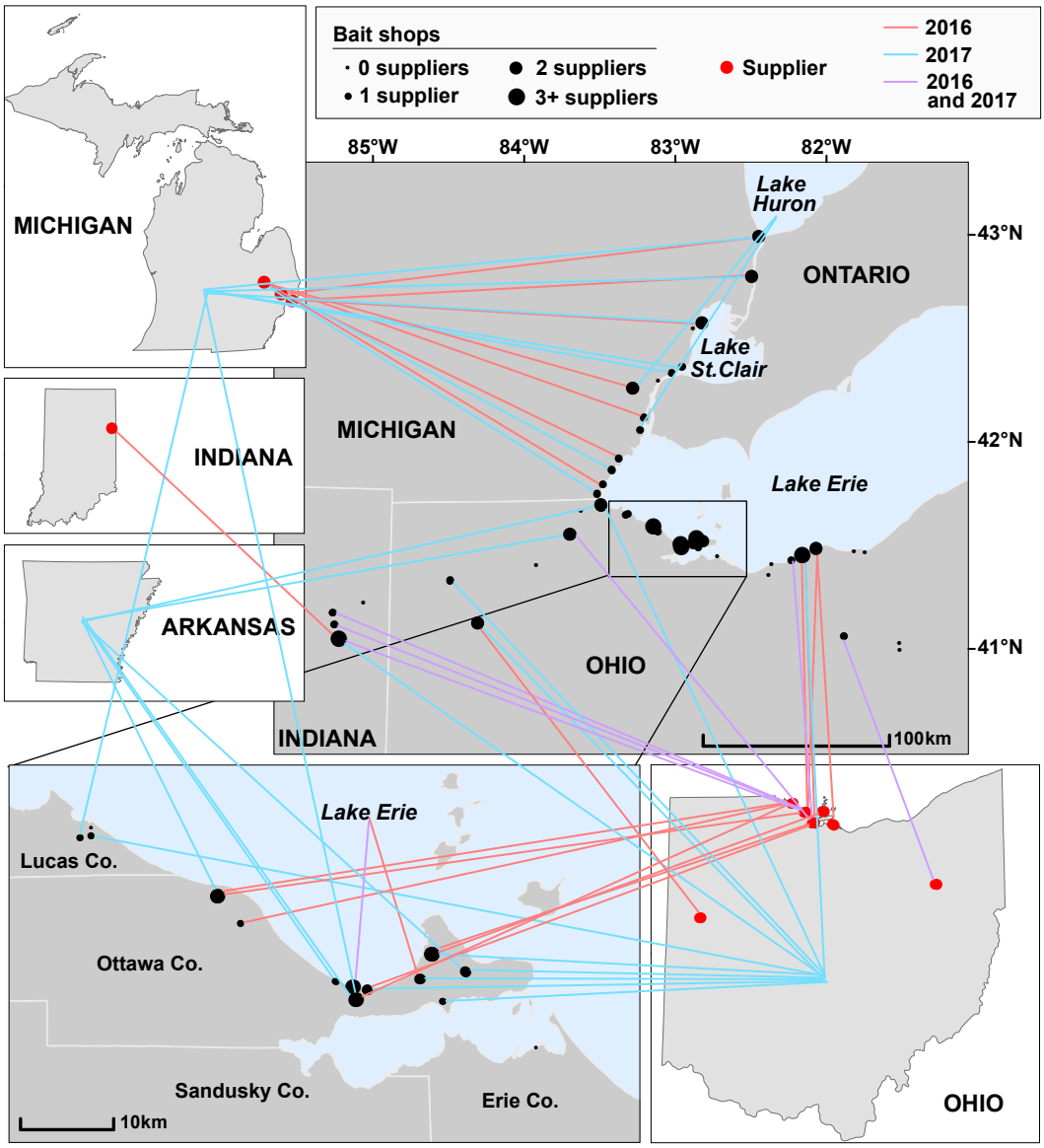
		Unadvertised						
ID	**State**	**All**	**Pond**	**Native non-pond**	**AIS**	***Hypop.* spp.**	***N. melano.***	***L. idus***
1	IN	2	1	0	1	0	0	1
2	MI	0	0	0	0	0	0	0
3	MI	1	0	1	0	0	0	0
4	MI	1	1	0	0	0	0	0
5	MI	2	1	1	0	0	0	0
6	MI	5	1	2	2	1	1	0
7	MI	1	1	0	0	0	0	0
8	MI	1	0	0	1	0	1	0
9	MI	1	1	0	0	0	0	0
10	MI	4	1	0	3	2	0	0
11	OH	1	1	0	0	0	0	0
12	OH	1	1	0	0	0	0	0
13	OH	3	1	2	0	0	0	0
15	OH	3	1	2	0	0	0	0
16	OH	1	1	0	0	0	0	0
17	OH	1	1	0	0	0	0	0
18	OH	1	0	0	1	0	1	0
19	OH	1	1	0	0	0	0	0
20	OH	2	1	0	1	0	1	0
21	OH	2	2	0	0	0	0	0

Appendix C.12 Complete Mollusk16S assay eDNA results for pond store samples. Anonymized store ID, state, and the number of unique BLAST hits that were advertised mollusk AIS or unadvertised mollusk or other invertebrate AIS.

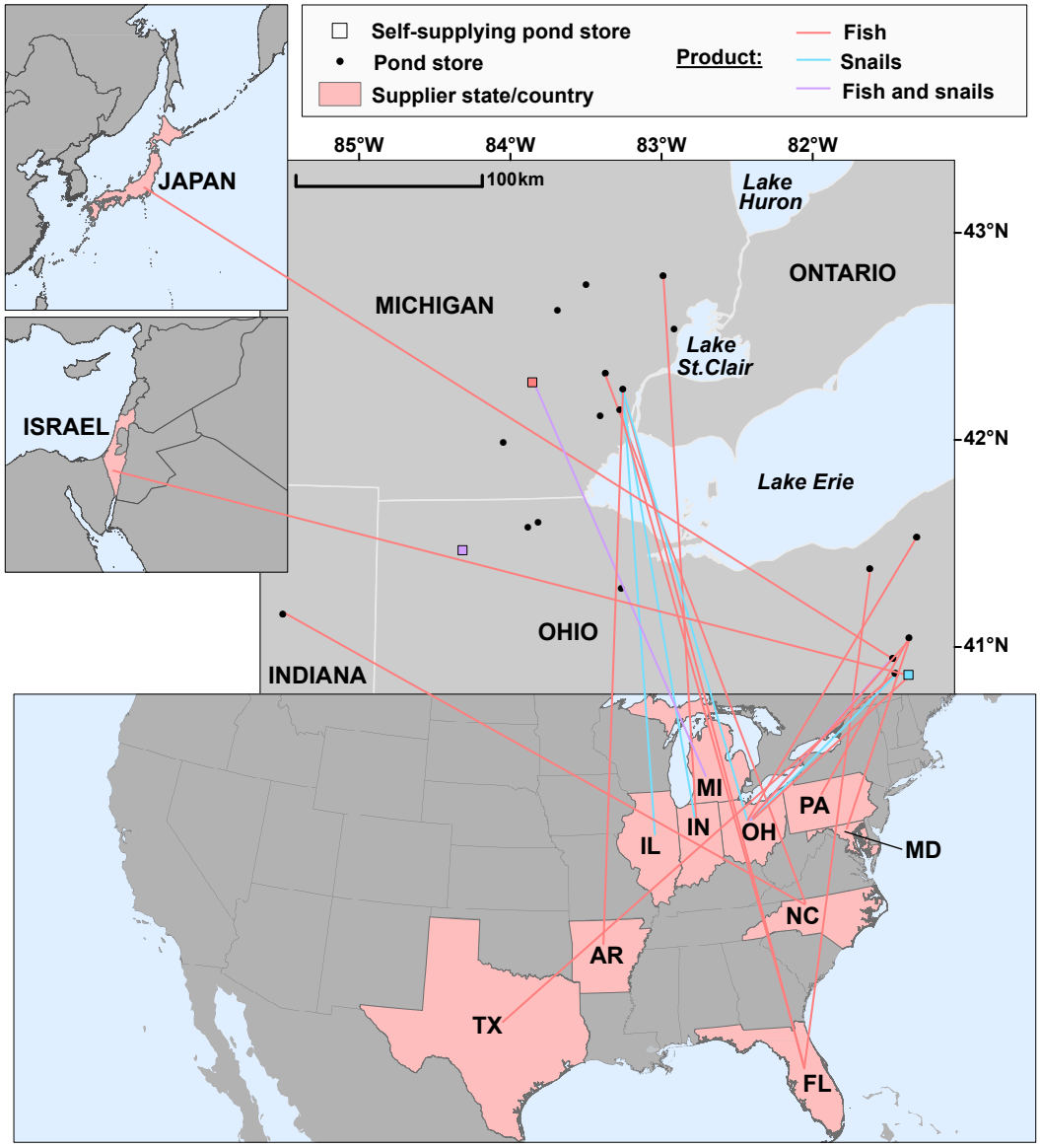
		Mollusks		Other invertebrates
ID	**State**	**Advertised AIS**	**Unadvertised AIS**	**Unadvertised AIS**
2	MI	2	2	0
3	MI	1	0	0
6	MI	2	2	1
7	MI	2	1	0
9	MI	1	0	0
10	MI	2	0	0
12	OH	1	2	1
13	OH	1	0	1
15	OH	1	2	1
16	OH	1	2	0
21	OH	1	0	2

Appendix C.13 Angler survey results. Anglers who reported fishing in Great Lakes and other regions, mean years fishing experience, live bait-fish use, reported dumping, *N* anglers aware of any AIS and mean number of species, and reported transporting live bait-fish out of state of purchase. Parentheses=proportions of anglers by location.

Location fished	Anglers reported fishing	Mean years fishing experience	Use live bait-fish	Dumped live bait-fish	Aware AIS	Mean AIS aware	Transport bait
MN Inland	4	46.5	1 (0.25)	–	4 (1.00)	8.50	–
WI Inland	6	35.7	1 (0.17)	–	6 (1.00)	7.00	2 (0.33)
L. Superior	4	53.3	1 (0.25)	–	3 (0.75)	6.50	1 (0.25)
L. Michigan	10	37.8	4 (0.40)	–	10 (1.00)	7.00	1 (0.10)
MI inland	37	32.9	11 (0.30)	1 (0.03)	33 (0.89)	5.97	2 (0.05)
L. Huron	4	45.8	2 (0.50)	1 (0.25)	4 (1.00)	7.50	1 (0.25)
Huron-Erie Corridor	8	42.3	5 (0.63)	4 (0.50)	8 (1.00)	6.38	1 (0.13)
Indiana	1	20.0	–	–	1 (1.00)	9.00	–
OH Inland	38	37.6	15 (0.39)	9 (0.24)	34 (0.89)	5.39	3 (0.08)
L. Erie	61	37.8	40 (0.66)	20 (0.33)	55 (0.90)	6.43	7 (0.11)
L. Ontario	5	41.6	3 (0.60)	1 (0.20)	5 (1.00)	5.00	1 (0.20)
Canada Inland	10	40.8	3 (0.30)	–	9 (0.90)	6.40	2 (0.20)
PA Inland	3	32.7	3 (1.00)	1 (0.33)	3 (1.00)	3.00	–
NY Inland	3	30.0	1 (0.33)	1 (0.33)	3 (1.00)	5.33	1 (0.33)
Out of GL region	7	30.2	1 (0.14)	1 (0.14)	7 (1.00)	7.14	1 (0.14)
Ocean	4	40.0	–	–	3 (0.75)	3.25	1 (0.25)
Unclear	30	34.6	3 (0.10)	2 (0.07)	22 (0.73)	6.19	2 (0.07)
Total responses	*179*	36.4	*94 (0.53)*	*41 (0.23)*	175 (0.98)	5.90	16 (0.09)



Appendix C–1 Map of bait fish retailer supply chains from the entire sampling region, with inset of the Sandusky Bay region of western Lake Erie, OH.



Appendix C–2 Map of pond fish retailer supply chains.