Management of Biological Invasions (2014) Volume 5, Issue 3: 225–231
doi: http://dx.doi.org/10.3391/mbi.2014.5.3.05

Open Access

Proceedings of the 18th International Conference on Aquatic Invasive Species (April 21–25, 2013, Niagara Falls, Canada)

Research Article

Tracking ghosts: combined electrofishing and environmental DNA surveillance efforts for Asian carps in Ontario waters of Lake Erie

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Received: 23 December 2013 / Accepted: 19 May 2014 / Published online: 24 July 2014

Handling editor: Calum MacNeil

Abstract

Effective control or eradication of invasive species depends on their early detection at low abundance to enable timely management responses. In aquatic environments, the detection of rare or low-abundance species is challenging at best, and discriminating between detection failure (null) and true absence (zero) can be difficult. We developed and applied environmental DNA (eDNA) markers to screen for occurrences of bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*), and grass carp (*Ctenopharyngodon idella*) in Ontario waters of Lake Erie, Lake St. Clair, and tributaries, as part of ongoing efforts to prevent these Asian carps from becoming established in the Great Lakes. A network of 180 sites in nearshore and tributary habitats, selected based on perceived risk of fish access or habitat suitability, were sampled using both boat electrofishing and/or eDNA sampling (948 water samples) throughout the 2012 open-water season. Electrofishing efforts did not capture any Asian carps, and no positive detections of environmental DNA for any of these species were obtained using both conventional and quantitative PCR. These combined results suggest that these Asian carp species are not yet established in Ontario waters of Lake Erie or Lake St. Clair, and highlight the value of combining conventional fisheries assessment and environmental DNA surveillance for assessing the potential presence of invasive species in freshwater systems.

Key words: Asian carps, environmental DNA, quantitative PCR, eDNA surveillance, mitochondrial DNA

Introduction

Early detection and rapid response are critical elements in preventing the establishment and spread of species that may become invasive. Once established, many invasive species cause substantial ecological and economic harm, and are often difficult or impossible to eradicate (Elton 1958; Cox 2004). The North American Laurentian Great Lakes have been irreversibly altered by the establishment of over 180 aquatic invasive species, with significant ecological and economic consequences (Cox 2004; Michigan DEQ 2013). Of these, control and research efforts for sea lamprey (*Petromyzon marinus*) alone cost more than $25,000,000 annually (GLFC 2009). Other species that have caused significant damage to native species and ecosystems are dreissenid mussels (*Dreissena polymorpha* and *D. bugensis*), round gobies (*Neogobius melanostomus*), and *Hemimysis anomala*. Aquatic foodwebs from zooplankton and aquatic macroinvertebrates to forage species and top predators have been heavily impacted by the establishment of Ponto-Caspian invaders (Ricciardi and McIsaac 2000; Ricciardi 2001), with subsequent productivity losses to commercial and recreational fisheries as well as

other ecosystem services (GLFC 2009; Michigan DEQ 2013).

In recent years, concerns have been raised over the potential of several invasive species of Asian carps to invade the Great Lakes (Kolar et al. 2005; Herborg et al 2007; Cudmore et al. 2011; ACRCC 2012, 2013). Since their introduction in the southern United States several decades ago, bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*), and grass carp (*Ctenopharyngodon idella*) have become established in the Mississippi River basin (Kolar et al. 2005) and continue to increase. Two of these species, *H. nobilis* and *H. molitrix*, now comprise a major part of the biomass in the Mississippi River basin and are considered to be at high risk of invading the Laurentian Great Lakes with significant ecological and economic consequences (Kolar et al. 2005; Herborg et al. 2007; Cudmore and Mandrak 2011; ACRCC 2012). Grass carp have been used in inland waterways to control aquatic vegetation (Dibble and Kovalenko 2009), but pose a risk for significantly altering nearshore littoral habitat through removal of aquatic macrophytes (Cudmore and Mandrak 2011), thus impacting habitat availability for littoral forage species and juvenile life stages of exploited species. The probability of establishment with significant ecological impacts for each of these species is considered to be high (Cudmore and Mandrak 2011; Cudmore et al. 2011; Kokovsky et al. 2012). Models suggest there is a significant risk of establishment from low numbers of founders (Cudmore et al. 2011; Cuddington et al. 2014); thus, early detection and rapid response actions will be essential to prevent their establishment and subsequent expansion.

Environmental DNA (eDNA) surveillance has recently emerged as a potent tool for the sensitive detection of waterborne DNA from species of interest, particularly for low-abundance species that are difficult to detect or capture using conventional fisheries assessment methods (Darling and Mahon 2011; Jerde et al. 2011, 2013; Mahon et al. 2013). The method has been proven effective for detecting the presence of aquatic species at low abundances (Ficetola et al. 2008; Darling and Mahon 2011), and has valuable potential for mapping occurrences of invasive species during early colonization stages (Ficetola et al. 2008; Thomsen et al. 2011; Takahara et al. 2012; Mahon et al. 2013). Environmental DNA can be obtained from water samples without directly encountering or capturing the species of interest, as species shed DNA into the environment via feces, urine, and epidermal cells (Ficetola et al. 2008) and gametes. Although eDNA detection has also been used to map occurrences of endangered species (Thomsen et al. 2011; Boothroyd et al. 2014), its primary application has been for the early detection of aquatic invasive species (Ficetola et al. 2008; Jerde et al. 2011; Mahon et al 2013; Takahara et al. 2013). For these reasons, eDNA surveillance has been adopted by many Great Lakes jurisdictions as a proactive measure to detect the presence of Asian carps in the basin (ACRCC 2012, 2013; ECALS 2013).

This study employed boat electrofishing and eDNA surveillance to survey Ontario waters of Lake Erie and tributaries for the potential presence of bighead, silver, and grass carp, in support of Ontario's efforts for early detection of Asian carps. As well as the electrofishing surveys, species-specific eDNA markers were developed for quantitative Polymerase Chain Reaction (qPCR) detection in addition to conventional PCR screening (Bronnenhuber and Wilson 2013). Accordingly, the two facets of this study were 1) field sampling for Asian carps (netting and water sampling), and 2) environmental DNA assessment of water samples, including quality assurance testing (confirmation of true negatives).

Methods

Field sampling

Electrofishing

Sampling was primarily carried out in western Lake Erie and tributaries, based on perceived risk of Asian carp presence from the positive eDNA detections of DNA from bighead and silver carp in Michigan and Ohio waters in 2011 and 2012 (ACRCC 2013; Jerde et al. 2013). Seventy-one Ontario sites in Lake Erie and tributaries were electrofished to search for juvenile and adult bighead and silver carps (Figure 1). Thirty of these sites were resampled one month later, for a total of 101 electrofishing sampling events. At each site, sampling effort consisted of approximately 1000 minutes of shocking along a transect using 500mv and 14 Amps. Geographic coordinates were recorded at the beginning and end of each transect.

Water sampling

Between September 6, 2012 and November 28, 2012, water samples were taken at 124 sites for eDNA analysis (Figure 1, Table S1). Ninety-six of

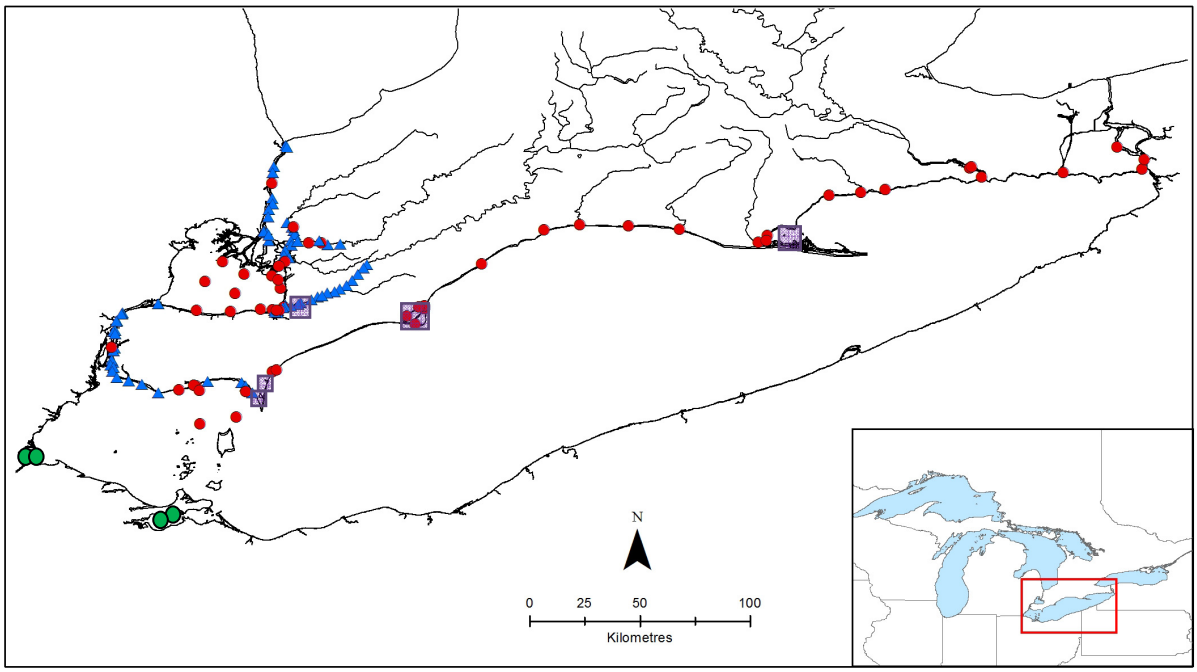

Figure 1. Map of sampling sites in Ontario waters of Lake Erie, Lake St Clair, and tributaries. Green circles indicate positive US eDNA detections for bighead and silver carp from sampling in 2011 and 2012 (ACRCC 2012, 2013; Jerde et al. 2013). Blue triangles represent combined electrofishing and eDNA sampling by OMNR field crews in September to November 2012; red dots indicate eDNA sampling only. Purple squares indicate additional samples provided by the federal Department of Fisheries and Oceans (DFO) for eDNA testing for spotted gar habitat occupancy (Boothroyd et al. 2014) (for details see supplementary material Table S1).

these sites were sampled twice and three water samples were collected during each sampling event for a total of 660 water samples. Water samples were taken from the St. Clair River (14 sites), Lake St. Clair (11 sites), Detroit River (17 sites), Lake Erie western basin (15 sites), Rondeau Bay (5 sites), Sydenham River (18 sites), and Thames River (24 sites), as well as sixteen additional tributaries of the central and eastern basins of Lake Erie (20 sites). Locations were chosen based on potential habitat for Asian carps including backwaters, island side channels, pooled areas, below and around structures, confluence of tributaries and areas with locally high productivity (ACRCC 2012). An additional 288 water samples collected in areas considered suitable for Asian carps (Cudmore et al. 2011), were provided by Fisheries and Oceans, Canada (DFO). These samples were originally collected in June 2012 to test for habitat occupancy of spotted gar (*Lepisosteus maculata*) in Lake Erie [Point Pelee (10 sites), Hillman Marsh (10 sites), Rondeau (10 sites), Long Point Bay (10 sites), and the Thames River (16 sites)] (Boothroyd et al. 2014).

Field sampling protocols followed the University of Notre Dame Environmental DNA Monitoring and Surveillance Standard Operating Procedures (Mahon et al. 2010). Before taking samples, 2L bottles were prepared for water samples by rinsing with a 10% bleach solution followed by tap water. Transport coolers were also rinsed with a 10% bleach solution and dried with paper towels. One 2L bottle was randomly selected from each cooler to serve as a control. This container was filled with tap water and returned to the cooler.

Sampling in rivers began at the most upstream location and proceeded downstream. Surface water samples were taken at the bow of the boat; three replicate 2L samples were taken at each site. Nitrile gloves were worn by the field crew and changed at every site. At a randomly chosen site, the control sample was exposed to the air, resealed and submerged in water. At each site the air temperature, water temperature, water depth, Secchi depth, dissolved oxygen, and geographic coordinates were recorded. Samples were stored on ice in the field, transferred to a refrigerator, and filtered within 24 hours.

Table 1. qPCR primers and probes developed for eDNA surveillance of bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*), and grass carp (*Ctenopharyngodon idella*). Primers and amplification conditions for conventional PCR are detailed in Bronnenhuber and Wilson (2013).

Primer Name	Target species	Primer Sequence (5' to 3')
Hsp-COI_F1	*Hypophthalmichthys* sp.	CGCAGGAGCATCCGTAGAC
Hsp-COI_R1		TTAATAGTTGTGGTGATGAAGTTAATTGC
*Hno*_probe	bighead carp (*H. nobilis*)	VIC-TTCTCCCTCCACTTAGCAG-MGBNFQ
*Hmo*_probe	silver carp (*H. molitrix*)	6FAM-TTCTCTCTTCACCTAGCAG-MGBNFQ
*Cid*_Taqman_COI-F1	grass carp (*C. idella*)	GAGTTTCTGACTTCTACCCCCTTCT
*Cid*_Taqman_COI-R1		CTGTTCACCCTGTTCCAGCTC
*Cid*_Probe1		6FAM-TCCTCCTACTATTAGCCTCT-MGBNFQ

Prior to filtering the samples, all lab equipment was sterilized using a 10% bleach solution and rinsed with tap water. For each set of samples, 2L of tap water were first filtered as a negative control. Samples were pre-filtered using a sterilized metal coffee filter (120µm mesh) to remove any large particles, then filtered using 1.2µm Whatman™ 47mm GF/C glass microfiber filters (http://www.whatman.com). Where necessary, multiple filters were used to filter water from individual water samples, with the number of filters used depending on the amount of sediment in the water. Paper filters were temporarily stored in a -20°C non-frost free freezer, then shipped on dry ice to the OMNR Aquatic Genetics Laboratory at Trent University (Peterborough, ON) and stored at -80°C until extracted for DNA.

Environmental DNA Assessment

DNA was extracted from the filters using MoBio PowerWater™ extraction kits (http://www.mobio.com) following the manufacturer's extraction protocol. The presence of environmental DNA from the extracted filters was confirmed by agarose gel electrophoresis of 2µL DNA per extraction, and visualizing the extracted DNA in a 1.5% agarose gel with SYBR Green (http://www.bioshopcanada.com). Extracted samples were stored at -20°C to prevent DNA degradation until used for PCR and qPCR testing.

The potential presence of eDNA from each species of Asian carp was tested using conventional PCR primers and qPCR primers and probes. Conventional PCR tests used species-specific primers developed for each species (Bronnenhuber and Wilson 2013) based on species-diagnostic 'DNA barcode' sequences for the mitochondrial CO-I gene (Hebert et al. 2003; Ward et al. 2005) available in the Barcode of Life Database (BOLD; Ratnamasingham and Hebert 2007). Amplicon results from conventional PCR assays were visualized by gel electrophoresis in 1.5% agarose gels alongside control samples for each species. Amplification conditions are detailed in Bronnenhuber and Wilson (2013). Three replicate PCR amplifications were done for each extracted filter replicate from each water sample, alongside quantitative PCR standards for each species ranging from 10^6 to 10^0 DNA copies/µL (detailed below).

Species-specific qPCR primers and probes were similarly developed from taxon barcode sequences in BOLD, following the methods detailed in Bronnenhuber and Wilson (2013), and are listed in Table 1. qPCR testing for bighead and silver carp eDNA used a duplex reaction, coupling primers designed to amplify a 96bp CO-I segment from *Hypophthalmichthys* species listed in BOLD (Ratnamasingham and Hebert 2007) with species-specific TaqMan™ detection probes (Table 1). Testing for grass carp eDNA was done separately, using a 83bp species-specific primer and probe set (Table 1) along with a commercially available internal positive control (IPC; Life Technologies). Reaction cocktails for both tests consisted of 10µL of 2X environmental master mix (Life Technologies), 0.4µL of each primer [10µM], 0.4µL of each species-specific probe [10µM], and ddH_2O to make a total volume of 20µL. Cycling conditions consisted of an initial denaturation step at 95ºC for 10min, followed by 40 cycles of denaturation at 95ºC for 15s and copy replication at 60ºC for 1min. Each qPCR

run consisted of two replicate series of species-specific positive DNA controls at 10-fold dilution series ranging from 10^6 to 10^0 DNA copies per reaction to provide quantitate estimates of potential positive detections from environmental samples. Replicate testing was done from each environmental sample for both regular PCR and qPCR to minimize the risk of false negative results (Mahon et al. 2010; Darling and Mahon 2011), with a minimum of three replicate trials each for PCR and qPCR.

Results

The electrofishing survey did not capture or encounter any Asian carps at the sampled Lake Erie and tributary sites (Figure 1). In addition, no Asian carp were reported from commercial fishing activities in Lake Erie (B. Locke, unpubl. data).

All eDNA testing using both conventional and quantitative PCR tests failed to detect any evidence of environmental DNA from bighead, silver, or grass carp. The number of replicate tests per water sample ranged from 3 to 12 for conventional PCR and 3 to 6 for qPCR, and varied with the number of filters required to filter each water sample. The lack of detections did not appear to be due to PCR inhibition from the environmental samples, as the internal positive controls (IPCs) successfully amplified in 657 of the 660 samples. The IPCs failed to amplify in extractions from three water samples (single replicate samples from three different sites), but showed normal amplification from the other replicate samples from these sites. Two sample replicates from the Thames River showed evidence of partial inhibition (45% and 80% reduction in DNA amplification, based on reduced IPC amplification); no other evidence of PCR inhibition was observed. Further testing for PCR inhibition by 'spiking' negative environmental samples with known quantities of species-specific eDNA from qPCR positive controls also showed no evidence of reduced detection or amplification efficiency (data not shown).

Discussion

The lack of physical capture or reporting of Asian carps from Ontario waters was largely consistent with efforts from other Great Lakes jurisdictions. Targeted sampling efforts in recent years by federal and state sampling crews have yet to capture bighead or silver carps from the Great Lakes (ACRCC 2012). Similarly, no Asian carps have been captured or reported by commercial fishermen in Ontario despite an estimated 30,000 km of netting effort for commercial fisheries in Lake Erie (B. Locke, unpubl. data). Thus, the most recent physical evidence for bighead carp in Lake Erie is the single fish captured in 2000 (Morrison et al. 2004). To date, no silver carp have been reported from the Great Lakes, although eDNA sampling by Jerde et al. (2011) led to the capture of one close to Lake Michigan.

Although no evidence of grass carp was seen in Ontario in 2012, Chapman et al. (2013) recently reported naturally-produced grass carp juveniles in the Sandusky River, Ohio. The presence of these juveniles suggests that sexually mature adult grass carp were not only present in 2011, but were able to reproduce in or near the area and that both reproduction and recruitment were successful (Chapman et al. 2013). This evidence supports predictions from risk-assessment models that grass carp would be able to successfully reproduce and recruit in western Lake Erie tributaries (Herborg et al. 2007; Cudmore and Mandrak 2011; Kocovsky et al. 2012). As noted by Jerde et al. (2013), it is also possible that bighead and silver carp may be present in western Lake Erie, but so far at low enough numbers to not yet establish self-sustaining populations. As modelling studies have predicted the potential for population establishment from small numbers of founding adults (Cudmore et al. 2011; Cuddington et al. 2014), there is a clear need for continued surveillance to enable rapid control or eradication responses and minimize the chances for population establishment.

The lack of positive eDNA detections for bighead, silver, or grass carps in Ontario waters was largely consistent with results from sampling efforts in the United States. Jerde et al. (2013) reported positive detections for silver and bighead carps from water samples taken in Maumee Bay and Sandusky Bay in 2011 (Figure 1). Subsequent positive detections for silver carp from Sandusky Bay in 2012 highlight the concern that silver carp may be present, albeit in low enough numbers to evade their physical detection or capture (Jerde et al. 2013). The sensitivity of eDNA for detecting Asian carps has been validated under both controlled and field conditions (Mahon et al. 2010; Jerde et al. 2011; Mahon et al. 2013). Although it is possible that positive detections could result from sources or fomites other than live fish (ACRCC 2012, 2013), the repeated eDNA detections from geographically proximate sampling in successive years heighten concerns

that live fish may be present, and warrant continued monitoring in high-risk areas (Jerde et al. 2013).

Although both false positive and false negative results are risks for eDNA surveillance (Darling and Mahon 2011), neither appeared to be applicable to the current surveillance results. As no positive detections for any of the three Asian carp species were obtained from our samples, false positives were a non-zero risk but did not occur. Conversely, the lack of detections did not appear to be due to false negative results, as the inclusion of internal positive controls in all qPCR reactions showed no evidence of significant PCR inhibition. In addition, the successful detection of eDNA for spotted gar, a federally-listed threatened species in Canada, from the water samples provided by DFO (Boothroyd et al. 2014) indicated that amplification of Asian carp eDNA from these same environmental samples should have been successful if any of these species had been present.

Preventing the arrival of invasive species remains the most effective and proactive defence (Elton 1958; Cox 2004). As well as the potential for natural expansion of Asian carp into the Great Lakes (Kolar et al. 2005; ACRCC 2013), human-assisted movement remains a significant risk (Herborg et al. 2007; Cudmore et al. 2011). Despite existing legislation and regulations prohibiting transport or possession of live Asian carps, the illegal presence of live Asian carps in live food fish markets remains a concern (Herborg et al. 2007; Cudmore et al. 2011; Jerde et al. 2013). Regulations requiring Asian carps to be eviscerated before they are brought into Ontario, as well as ongoing stakeholder outreach and education efforts, should simplify enforcement efforts and help reduce the risk of live transport and release.

Despite the lack of detections from Ontario waters, the electrofishing, eDNA surveillance, and liaising with commercial and recreational fishers are important contributions to the broader Great Lakes binational efforts to monitor and prevent establishment of Asian carps. As it is predicted that populations of each species could establish from small numbers of adults (Cudmore et al. 2011; Cuddington et al. 2014), continued monitoring and contingency plans for rapid response are essential to help minimize the risk of their establishment and subsequent expansion in the Great Lakes basin (Cudmore et al. 2011). The continued cooperation and regular communication among federal, state, and provincial agencies and partner organizations will help achieve mutual surveillance goals and enable timely response actions if needed.

Acknowledgements

Funding for this project was provided through the Canada-Ontario Agreement Respecting the Great Lakes Basin Ecosystem (COA) and the OMNR Biodiversity Policy Section. Logistic support for field sampling (eDNA and electrofishing) was provided by OMNR Lake Erie Management Unit field crews. Caleigh Smith, Margaret Boothroyd, and Cait Nemeczek (OMNR) provided invaluable lab support for DNA extractions and screening. Andrew Mahon (Central Michigan University) provided reference samples for primer development and testing. Sandra Orsatti, Ala Boyd, and Bruce Hawkins (OMNR) provided constructive suggestions for the project during team discussions. The manuscript was improved by constructive suggestions from Jim Reist and an anonymous reviewer.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Sampling locations in Ontario waters for electrofishing and eDNA samples in 2012.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2014/Supplements/MBI_2014_Wilson_etal_Supplement.xls