LETTER

"Sight-unseen" detection of rare aquatic species using environmental DNA

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Keywords
Asian carp; early detection; environmental DNA; Great Lakes; invasive species; surveillance.

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Received
27 July 2010
Accepted
23 November 2010

Editor
Corey Bradshaw

doi: 10.1111/j.1755-263X.2010.00158.x

Abstract

Effective management of rare species, including endangered native species and recently introduced nonindigenous species, requires the detection of populations at low density. For endangered species, detecting the localized distribution makes it possible to identify and protect critical habitat to enhance survival or reproductive success. Similarly, early detection of an incipient invasion by a harmful species increases the feasibility of rapid responses to eradicate the species or contain its spread. Here we demonstrate the efficacy of environmental DNA (eDNA) as a detection tool in freshwater environments. Specifically, we delimit the invasion fronts of two species of Asian carps in Chicago, Illinois, USA area canals and waterways. Quantitative comparisons with traditional fisheries surveillance tools illustrate the greater sensitivity of eDNA and reveal that the risk of invasion to the Laurentian Great Lakes is imminent.

Introduction

In the absence of tools to detect rare species, uncertainty about when, where, and for how long a management action should be implemented often results in inaction or ineffective use of management resources (Thompson 2004; Lodge *et al*. 2006). Detection probabilities for rare species are typically low in all environments, but particularly in aquatic environments where organisms are hidden beneath the water's surface. Fish surveillance programs, for example, traditionally employ nets or electrofishing gear. Because these tools usually have low capture probabilities per target organism, they are reliable indicators of occurrence only for species present at moderate-to-high abundance (Magnuson *et al*. 1994). In contrast, for rare species, the low detection probability of these tools often leads to an error in inference—concluding a species is absent when it is actually present (Gu & Swihart 2004). In the case of rare species, the only solutions are to increase sampling effort or change to a detection tool with greater detection probability (McDonald 2004). However with traditional surveillance tools, increases in sampling effort sufficient to achieve a usefully high detection probability for rare species are often infeasible.

Here we describe the application of environmental DNA (eDNA) as an effective surveillance method for rare fishes in a large river and canal complex. We show that it is more sensitive than traditional tools, has no risk of harming the species under study (Beja-Pereira *et al*. 2009), and effort can feasibly be increased for species management. The eDNA surveillance method exploits an advantage of aquatic environments: the aqueous environment suspends sloughed tissues, making it easy to sample and detect DNA from even rare organisms that are present but invisible to traditional tools. This conservation application of the eDNA method was inspired by previous identification of whales (*Megaptera novaeangliae,*

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Physeter macrocephalus, and *Eubalaena glacialis*) using DNA in the backwash and feces of diving individuals (Amos *et al*. 1992), and the use of DNA to detect bullfrogs (*Rana catesbeiana*) in ponds in France (Ficetola *et al*. 2008).

We demonstrate the usefulness of eDNA surveillance with a case study involving two species of Asian carps, silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*), which have caused harm to fisheries, recreational use of waterways, and human safety as they have invaded much of North America's Mississippi River basin (Kolar *et al*. 2007). Both carp species now threaten to invade the Laurentian Great Lakes through a set of human-built waterways in and near the city of Chicago, Illinois, which connects the Mississippi River basin and the Great Lakes-St. Lawrence River basin (Garvey *et al*. 2010). Specifically, we target eDNA surveillance at locations on the putative leading edge of invasion, compare the detection capabilities between standard fisheries and eDNA surveillance methods, and report far more advanced invasion fronts for each species. Invasion fronts detected with eDNA surveillance suggest that both species of carps are north of electric barriers installed to prevent fish passage, making clear that management actions to prevent invasions of Lake Michigan and the other Great Lakes are much more urgent than suggested by traditional fisheries methods. The application of eDNA monitoring methods will have broad research and management applicability in freshwater, estuarine, and marine ecosystems for threatened and endangered species and for invasive species.

Methods

The study area was the Chicago area waterway, a large river and canal complex that has been an important invasion pathway for multiple species including zebra mussels (*Dreissena polymorpha*) and round goby (*Neogobius melanostomus*)(Brammeier *et al*. 2008) (Figure 1). The flow of the Chicago River and other connected waterways was reversed in 1900 to redirect Chicago's wastewater from Lake Michigan; throughout the system, water now flows from Lake Michigan into Mississippi River tributaries (Cooley 1913). In the early 1970s, bighead and silver carp escaped fish farms in Arkansas in the southern Mississippi River basin (Kolar *et al*. 2007), with the first recorded observation of bighead carp in the Illinois River, a northern tributary of the Mississippi River, occurring in 1986 (USGS, http://nas.er.usgs.gov). Upstream range expansion and population growth of bighead and silver carp in the Illinois River and its tributaries is ongoing (Williamson & Garvey 2005; Sass *et al.* 2010).

Marker development

Molecular markers (Table 1) were designed for *H. nobilis* and *H. molitrix* using publically available sequence information (GenBank, www.ncbi.nlm.nih.gov). To ensure species specificity, we targeted short fragments of the mitochondrial d-loop region (Taberlet 1996), and used BLAST (Basic Local Alignment Search Tool; Genbank, www.ncbi.nlm.nih.gov/blast) to compare the markers to all available sequence data, including those of closely related species and nontarget species common to the Chicago area water system. We also tested the markers against tissue-derived DNA for a subset of species (Table S1, Supporting Information).

Sample collection, filtration, and extraction

From June 29, 2009 to May 20, 2010, we collected approximately 1,000 2-l surface water samples during 15 sampling trips. Water was collected in autoclaved 2-l Nalgene bottles, and sample locations measured with GPS (Garmin eTrex Venture HC (s.e., <10 m)). Water samples were vacuum-filtered within 24 hours of collection onto 1.5-μm pore size glass fiber filters. Filters containing sample filtrates were placed in 50-mL tubes and stored at -20 °C until further processing. We extracted DNA from the filters using the PowerWater DNA Isolation kit (MO-Bio Laboratories Inc., Carlsbad, CA) following manufacturer's recommendations with one exception: the final DNA elution was done using deionized water instead of the provided buffer solution.

PCR amplification and evaluation

Asian carp DNA presence was detected using a minimum of eight polymerase chain reactions (PCR) per water sample (amplification cocktail described in Supporting Information). PCR results were visualized and photodocumented under UV light on a 1% agarose gel stained with ethidium bromide. Positive reactions were identified for bighead and silver carp by a single, distinct band at 312 bp and 191 bp, respectively. Using these methods, the lowest concentrations we could detect of genomic DNA from pure DNA extracts from bighead and silver carp were 3.30×10^{-8} ng/μL and 7.25×10^{-11} ng/μL, respectively.

Quality assurance and control

During each sampling trip, water samples were collected from upstream to downstream. All equipment, including boats, used in the sampling effort was sterilized using a 10-minute exposure to 10% bleach solution (Prince & Andrus 1992). Two 2-l bottles, filled with deionized

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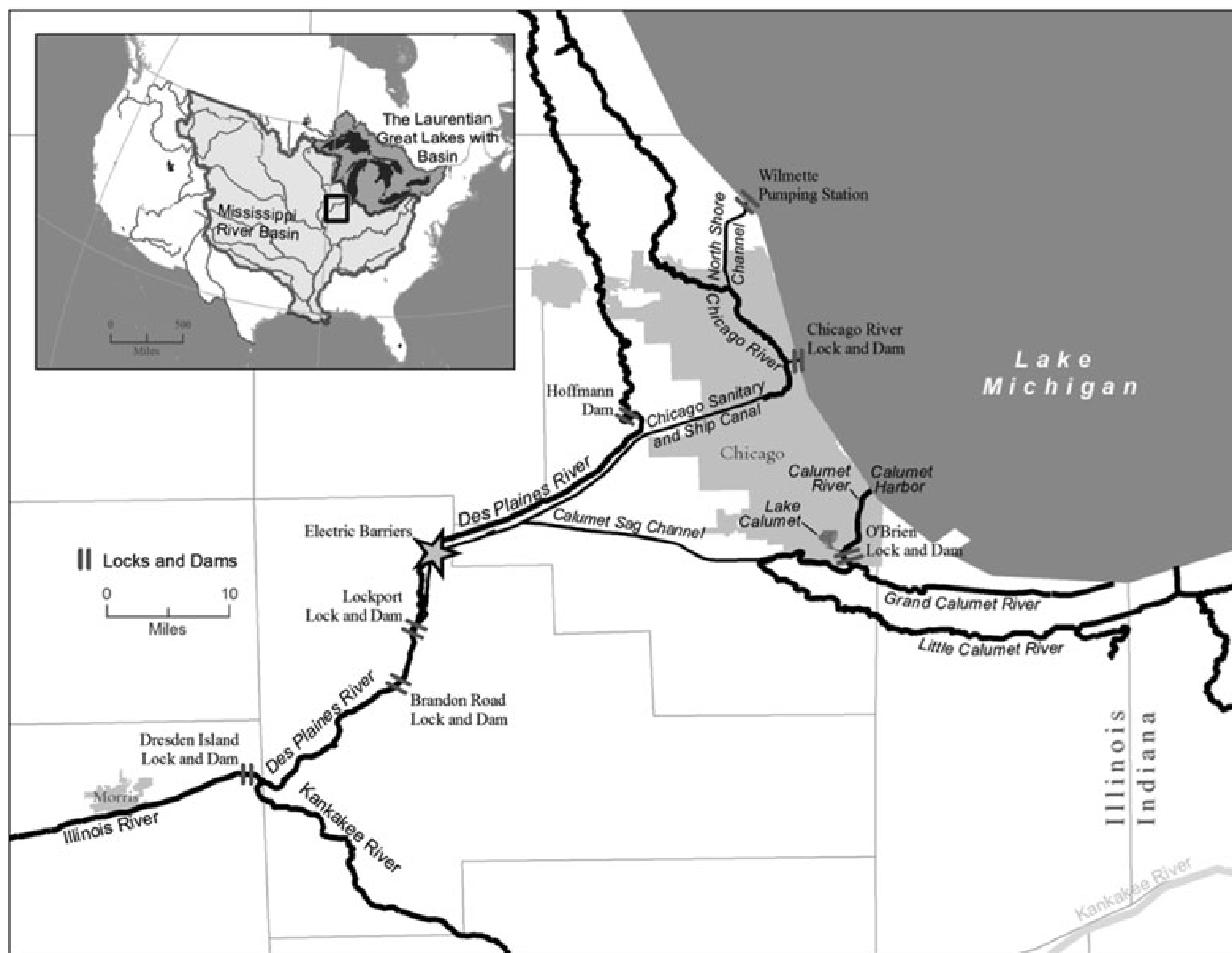


Figure 1 Connection of the Great Lakes-St. Lawrence and Mississippi River basins through the Chicago area waterway system. The system provides a direct hydrological connection between the Great Lakes and Mississippi River watersheds (the Illinois River is a tributary of the Mississippi River). Water flows from Lake Michigan into each part of the waterway but volumes are regulated and limited by control structures at Wilmette Pump Station, Chicago Lock, and O'Brien Lock. Over 70% of the annual flow is driven by storm water and dilute sewerage discharges from four main outfalls that combine with natural tributary inflows to produce a mean annual (2005) discharge at Lockport of approximately 77 m^3/s (2725 ft^3/s) (Brammeier *et al*. 2008). The electric barriers (star) are in the CSSC (not in the Des Plaines River).

water (cooler blanks), were placed in each cooler and taken into the field to test for contamination. Prior to filtering each sample from the field, 1−l of deionized water was passed through each sterilized filter apparatus (equipment control). All cooler blanks were screened for contamination, and all positive detections from field samples had the corresponding equipment control processed. The water for the cooler blanks and equipment controls was sourced from the same tap. Negative and positive controls were run on each gel. For approximately 5% of all positive samples above the electric barrier, positive bands were extracted from the agarose gel using a Qiagen QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) and bidirectionally sequenced on an Applied Biosystems ABI 3730xl DNA Analyzer. Sequence data were screened using the BLAST as further confirmation of marker specificity to each of the two target species.

Negative control reach comparisons

As an additional layer of negative control, we sampled from two adjacent river reaches where both carp species are absent. The Des Plaines River flows into the Brandon Road Pool of the Chicago area waterway system but Hoffman Dam (41° 49′ 15.17″ N, 87° 49′ 20.46″ W) provides a barrier to upstream range expansion by Asian carp

Table 1 Species-specific PCR primers used for amplification of targeted mitochondrial d-loop sequences of bighead and silver carp

Target	Primer name	Primer sequence
Hypophthichthys nobilis	HN203-F	5′-TAACTTAAATAAACAGATTA-3′
	HN498-R	5′-TAAAAGAATGCTCGGCATGT-3′
H. molitrix	HMF-2	5′-CCTGARAAAAGARKTRTTCCACTATAA-3′
	HMR-2	5′-GCCAAATGCAAGTAATAGTTCATTC-3′

(Figure 1). This system has similar species and water chemistry as found in lower sections of the Des Plaines River where we detected carp DNA. Second, we sampled the St. Joseph River, a tributary of Lake Michigan with similar species as found in the upper Chicago area waterway system.

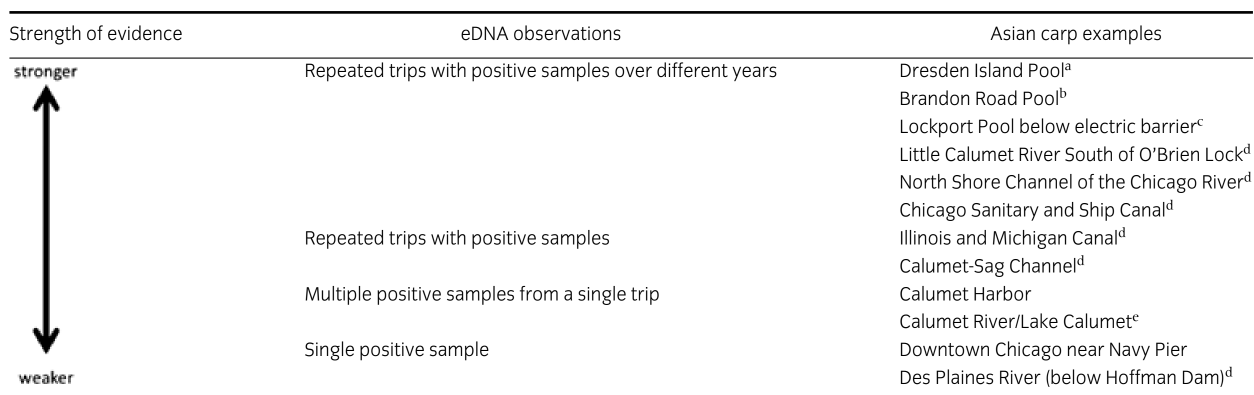
Catch per unit effort comparisons

During our study, the U.S. Army Corps of Engineers, Illinois Department of Natural Resources, Illinois Natural History Survey, and U.S. Fish and Wildlife Service conducted 62 days of electrofishing surveys downstream of the electric barriers from June through November of 2009. To estimate catch per unit effort for electrofishing, we relied on the most detailed information recorded by the management agencies, which consisted of the number of days spent electrofishing. We used a low estimate of effort for electrofishing, with each shock boat consisting of a three-person crew, resulting in 24 person hours needed for an 8-hour electrofishing workday; this results in a catch per unit effort for electrofishing that is upwardly biased because time for travel, preparation, and fieldwork was probably greater than 8 hours and crews may have exceeded three people. Every 100 eDNA samples required 16 person hours for field work and approximately 24 person hours to filter the samples. We averaged 44 samples processed per week. Therefore it took 0.06 person days to collect and 0.114 person days to process a sample, for a total of 0.174 person days per eDNA sample. For electrofishing a catch was defined as a captured or observed Asian carp. For eDNA surveillance, a catch was defined as at least one positive PCR reaction in a sample. We compare the catch per unit effort for electrofishing and eDNA surveillance in three adjacent pools in which both methods were used and which encompassed the presumptive edge of the invasion front in summer 2009. We focused on electrofishing for comparison with eDNA because it has been the most frequently used tool in past fisheries surveillance (Sass *et al.* 2010).

Results

Molecular markers (Table 1) are species specific. Using the BLAST approach and tests against fish tissue samples in the laboratory, there was no binding sufficient to provide a false positive (Supporting Information). The markers designed to detect bighead carp eDNA amplify a 312 bp section of the mitochondrial d-loop (control region). The genetic distance (as uncorrected *p* genetic distance calculated using MEGA 4.0; Kumar *et al.* 2008) between this fragment of DNA for bighead carp and other species is: silver carp, 13.7%; and common carp, 25.2%. The

Table 2 Gradient of evidence for species presence



Strength of evidence	eDNA observations	Asian carp examples
stronger	Repeated trips with positive samples over different years	Dresden Island Pool[a]
		Brandon Road Pool[b]
		Lockport Pool below electric barrier[c]
		Little Calumet River South of O'Brien Lock[d]
		North Shore Channel of the Chicago River[d]
		Chicago Sanitary and Ship Canal[d]
	Repeated trips with positive samples	Illinois and Michigan Canal[d]
		Calumet-Sag Channel[d]
	Multiple positive samples from a single trip	Calumet Harbor
		Calumet River/Lake Calumet[e]
	Single positive sample	Downtown Chicago near Navy Pier
weaker		Des Plaines River (below Hoffman Dam)[d]

[a]Multiple electrofishing captures of bighead and silver carp (2007–2009).
[b]Silver carp observed electrofishing (Summer 2009).
[c]Bighead carp captured in rotenone treatment (December 3, 2009).
[d]No bighead or silver carp captures with electrofishing or netting.
[e]Bighead carp captured in commercial net (June 22, 2010).

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genetic distance between the 191 bp silver carp amplicon and other species is: bighead carp, 8.4%; and common carp, 22.8%.

All sequences ($N = 20$) from positive samples collected above the electric barrier for bighead or silver carp matched the target species. No detections resulted from samples collected from the negative control reaches (Des Plaines River above Hoffman Dam, $n = 15$; St. Joseph River, $n = 60$). An independent, USEPA-led audit, which evaluated the marker specificity, similarly concluded the markers are reliable indicators of bighead or silver carp presence (Blume *et al*. 2010). No cooler blanks tested positive, and no equipment controls tested positive.

We detected DNA from both silver and bighead carps on multiple occasions from multiple reaches of the waterway (Table 2), well upstream of the putative invasion front that had been determined with standard fishing methods (Figure 2). Prior to eDNA surveillance, the management agencies placed the invasion fronts at about river miles 274 and 281.5 for silver and bighead carps, respectively, approximately 10–15 miles south of the electric barriers. Our eDNA surveillance placed the invasion front for silver carp in the Calumet Harbor of Lake Michigan (RM 333.5, Figure 2D) and at least as far north as river mile 325 for bighead carps in the Calumet River, within 13 km of Lake Michigan (Figure 2C). Silver carp DNA was also detected at river mile 341 in the Chicago River's North Shore Channel (Figure 2E) and at river mile 326.5 in the main channel of the CSSC in downtown Chicago (Figure 2B). Both locations are less than 1 km from Lake Michigan.

The catch per unit effort for both electrofishing and eDNA surveillance declined upstream, consistent with approaching the invasion front (Figure 3), but eDNA always had a higher catch per unit effort than electrofishing, despite our bias in calculations that favored electrofishing. For electrofishing, only one silver carp was detected in the Brandon Road Pool after 93 person days of effort that was motivated and targeted by our discovery of Asian carp eDNA. At the lowest Asian carp density (Lockport Pool), both bighead and silver carps were detected only with the eDNA method (Figure 3) until a rotenone application on December 3, 2009 confirmed bighead presence.

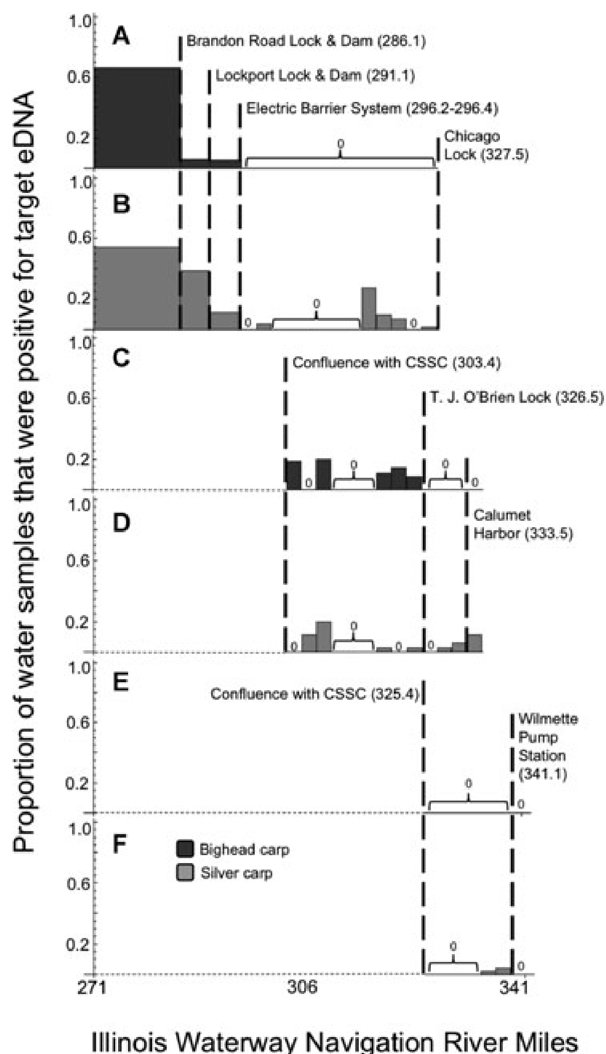

Figure 2 Detection proportions of eDNA for bighead (A,C,E) and silver carp (B,D,F) in the CSSC (A,B), the Calumet Sag Channel (C,D), and the North Shore Channel of the Chicago River (E,F). Locks, barriers, and endpoints of channels are marked with vertical dashed lines. In the reach of the waterway downstream of Brandon Road Lock, both carp species had been collected previously using traditional electrofishing methods (Figure 3). See Supporting Information for the number of 2-l water samples collected within each 2.5 river mile reach.

Discussion

The eDNA evidence reported here was the first indication that both bighead and silver carps had not only reached the electric barriers but were also present north of the barriers, which are intended to prevent their dispersal into Lake Michigan. On June 22, 2010, commercial fisherman caught an adult bighead carp within 13 km of Lake Michigan, only 4 km upstream of the nearest positive eDNA detection, further supporting what the eDNA evidence suggested 8 months earlier. In the Calumet River, silver carp and bighead carp appear to have no impediment to access to Lake Michigan. Regardless of the source of the carps north of the electric barrier (see below), eDNA surveillance has revealed much more imminent risks of invasion of Lake Michigan by both silver and bighead carps than had been indicated by standard surveillance methods.

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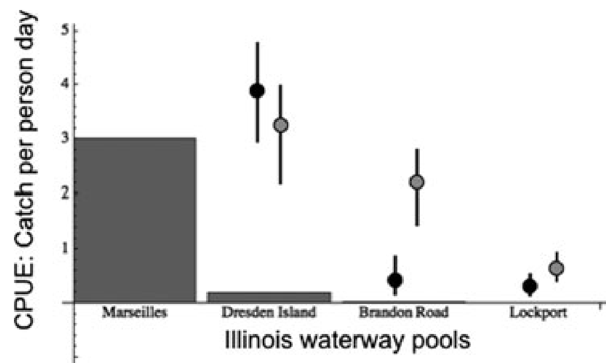

Figure 3 Catch per unit effort for electrofishing (bars) and eDNA surveillance for silver (gray dots) and bighead (black dots) carps from south (downstream) (Marseilles pool) to north (upstream) (Lockport pool); Lockport Lock & Dam is downstream of the electric barriers. Lines are 90% confidence intervals. For electrofishing, silver and bighead carp were not consistently distinguished. A single silver carp was observed with electrofishing in Brandon Road pool (CPUE = 0.01) whereas electrofishing detected no Asian carps in Lockport Pool (CPUE = 0).

Both bighead and silver carps pose a potentially major threat to the fisheries, recreational values, and other ecosystem services provided by the Great Lakes. For example, bighead carp alter trophic structures, resulting in declines of bait, commercial, and game species (Sampson *et al*. 2009), and the presence of leaping silver carp has curtailed recreational uses of heavily infested waterways (Kolar *et al*. 2007). If numbers sufficient to establish a self-sustaining population access Lake Michigan, both species are likely to reproduce, spread, and have substantial negative impacts in portions of the Great Lakes and many of their tributaries (Kolar *et al*. 2007; Herborg *et al*. 2007; Cooke & Hill 2010). However, Allee effects, demographic stochasticity, and environmental stochasticity pose major hurdles to population establishment (Jerde *et al*. 2009); most incipient invasions fail multiple times before they succeed (Drake & Lodge 2006). It is not therefore inevitable that either bighead or silver carps will establish populations in Lake Michigan, especially if the numbers of individuals with access to Lake Michigan can be reduced (Lockwood *et al*. 2005). Thus the urgent management challenge for the state and national agencies responsible for stewardship of Great Lakes resources is to reduce the numbers of Asian carps upstream of the electric barriers and/or reduce access by those fishes to Lake Michigan.

In addition, because eDNA evidence indicates that at least silver carp have entered Lake Michigan, surveillance is warranted within Great Lakes rivers that may be colonized and could support successful spawning (Kolar *et al*. 2007). The eDNA method appears well suited to rapid surveys across the large spatial scale that will be required in the Great Lakes.

Finally, the largest potential source of additional individuals is the abundant populations of both species south of the barriers. Given that the effectiveness of the electric barriers are uncertain (Conover *et al*. 2007; USACE 2010), there is also an urgent need to reduce populations south of the electric barrier to lower the probability of additional upstream dispersal.

Assessing and controlling errors is critical for inferring species presence (Gu & Swihart 2004), especially for an indirect surveillance method like eDNA (Beja-Pereira *et al*. 2009). We controlled false positives by assessing the specificity of the molecular marker, contamination of the samples during transport and processing, and the correct operation of laboratory equipment. No evidence exists of false positives from contamination, molecular marker misspecification, or equipment failure. In addition, waters known to be absent of Asian carp yielded negative results.

On the other hand, the occurrence of false negatives is affected to an unknown degree by many factors including the density of target fish upstream, the mixing of the water column, the flow rate of the river, and the degradation rate of DNA. In reaches where no Asian carp were detected, uncertainty remains about whether the fish are absent or if we failed to detect fish that were present. Only calibration studies that manipulate the factors potentially leading to false negatives will elucidate the false negative rate. What will remain certain, however, is that the false negative rate will be inversely related to the abundance of the target species (Gu & Swihart 2004; McDonald 2004).

Inferences from a positive eDNA result about fish abundance are currently limited, even where a fish has been captured (Table 2). A positive result indicates only presence, not abundance, of the target species. Because it is not yet known how the abundance, size, behavior, or other individual or population characteristics affect the strength of the eDNA signal under different environmental conditions, the only inference that can be drawn with confidence from a positive result is that at least one individual is or has been present in the recent past (England *et al*. 2005). Depending on many variables in any particular site, a positive result could indicate one or many fish. However, under similar environmental conditions (e.g., water temperature), such as those represented during our sampling across the different reaches in the Chicago area waterway system (Figure 2), the strength of the eDNA signal likely correlates positively with abundance of the target species. However, because flow rates and turbulence will undoubtedly affect dilution of the eDNA signal, we believe it is not advisable to make detailed inferences

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about relative abundance of carps on the basis of comparisons of the strength of the eDNA signal between the low flow upstream reaches of the waterway (e.g., the Calumet Sag Channel and the North Shore Channel) and the high flow, turbulent downstream reaches (lower river miles) of the waterway (Table 2 and Figure 2).

It is possible, but not likely, that Asian carp DNA could enter the upper Chicago area waterway (Figure 1) by sources other than living fish, such as sewage and wastewater, bilge water discharge, excrement from predatory fish or waterfowl, or from dead fish carried on barges and boats from downstream. None of these alternate sources explains the overall spatial patterns or repeated detections from independent sampling trips spread across a 12-month period (Table 2). For example, barges and towboats do not typically pump ballast because they are not equipped to exchange large quantities of water; furthermore discharging water from below the electric barriers above the electric barriers is prohibited through federal regulations. Additionally, these vessels do not use some sections of the Chicago area waterway system where we detected eDNA (e.g., North Shore Channel, Figure 2F). To date, no alternative pathway has been demonstrated to transport eDNA in quantities and occurrences sufficient to maintain the pattern of detection reported here (Figure 2 and Table 2). The most plausible explanation for positive detections is the presence of live fish.

Without effective surveillance methods, successful management of rare species is impossible (McDonald 2004). The pattern of greater catch per unit effort using molecular genetic detection compared to more traditional sampling approaches has also been documented in marine systems where invaders are at low abundance (Hayes *et al*. 2005). We believe that improvements to the eDNA method, and applications to additional species and ecosystems, will increase the ability of natural resource managers to foster endangered species and reduce the damages of invasive species in aquatic environments. Targeting management earlier and in more appropriate locations will also improve the cost effectiveness of management (Leung *et al*. 2002).

Acknowledgments

Funding provided by U.S. Army Corps of Engineers (#W912HZ-08-2-0014), Great Lakes Protection Fund (#867), and NOAA CSCOR (#NA09NOS4780192) to the University of Notre Dame, and the Joyce Foundation (#07-30723) to The Nature Conservancy. K. Baerwaldt contributed the electrofishing data, insightful advice on Asian carp habitats, and comments on the manuscript. A. Rohly, S. Mysorekar, M. Brandner, E. Elgin, M. Budny, M. Galaska, M. Barnes, M. Brueseke, C. Turner, and J. McNulty assisted with the research. D. Chapman, J. Hoover, and J. Killgore provided advice about Asian carp biology. The U.S. Fish and Wildlife Service, the Illinois Department of Natural Resources, and the U.S. Army Corps of Engineers contributed logistical support for the water sample collection.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Genetic matching of the species-specific PCR markers used to test eDNA samples in the CSSC comparing percent binding sites of PCR primers. Hnob-F and Hnob-R amplify a 312 bp fragment of the mitochondrial d-loop (control region) for bighead carp. Hmol-F and Hmol-R amplify a 191 bp fragment of the mitochondrial d-loop (control region) for silver carp. The designation "does not bind" indicates negative PCR results of direct tissue analysis (*) or was inferred from *in silico* gaps in the sequence.

Table S2: eDNA results for Des Plaines River, Chicago Sanitary and Ship Canal, Chicago River to the Chicago Lock

Table S3: eDNA results for Calumet-Sag Channel, Little Calumet River, and Calumet River to Calumet Harbor

Table S4: eDNA results for Chicago River and North Shore Channel to Wilmette Pumping Station.

Figure S1: The three main branches of the Chicago area waterway system to Lake Michigan are presented as polygons and the corresponding eDNA detection distributions are provided in Figure 2. Black circles with numbers identify key sighting or capture of bighead carp with netting (1, June 22, 2010), bighead carp with rotenone (2, December 3, 2009), and both species with electrofishing (3, Summer 2007 and 2009). Environmental DNA to the corresponding species of fish was found within 10 m of location three, 100 m of location two, and 4 km of location one. Efforts to recover bighead carp at locations two and one were largely motivated by eDNA detections.

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1 **Supporting information**

2 Table S1. Genetic matching of the species-specific PCR markers used to test eDNA
3 samples in the CSSC comparing percent binding sites of PCR primers. Hnob-F and
4 Hnob-R amplify a 312bp fragment of the mitochondrial d-loop (control region) for
5 bighead carp. Hmol-F and Hmol-R amplify a 191bp fragment of the mitochondrial d-
6 loop (control region) for silver carp. The designation "does not bind" indicates
7 negative PCR results of direct tissue analysis (*) or was inferred from *in silico* gaps
8 in the sequence.

Marker	Comparison species	Percent primer match	Comments
Hnob-F	*H. nobilis*	100	Target species, fully functional
	*H. molitrix**	85	Does not bind
	*C. carpio**	55	Insertion in sequence; does not bind
	Dorosoma sp.	35	Gaps in sequence; does not bind
	*Carassius auratus**	40	Does not bind
	*Channa argus**	55	Does not bind
	*Beta splendens**	45	Does not bind
	Catostomus commersonii	35	Insertion in sequence; does not bind
	Lepomis macrochirus	25	Insertion in sequence; does not bind
	Micropterus salmoides	25	Insertion in sequence; does not bind
	Micropterus dolomieu	30	Insertion in sequence; does not bind
	Carassius auratus hybrid	60	Insertion in sequence; does not bind
	*M. piceus**	45	Does not bind
	*C. idella**	20	Does not bind
Hnob-R	*H. nobilis*	100	Target species, fully functional
	*H. molitrix**	95	Gaps in sequence; does not bind
	*C. carpio**	95	Does not bind

	Dorosoma sp.	†	
	*Carassius auratus**	100	Does not bind
	*Channa argus**	80	Gaps in sequence; does not bind
	*Beta splendens**	40	Does not bind
	Catostomus commersonii	†	
	Lepomis macrochirus	40	Does not bind
	Micropterus salmoides	†	
	Micropterus dolomieu	†	
	Carassius auratus hybrid	100	Functional, but Hnob-F does not bind
	*M. piceus**	100	Does not bind
	*C. idella**	35	Does not bind
Hmol-F	*H. molitrix*	100	Target species, fully functional
	*H. nobilis**	51.8	Insertion in sequence; does not bind
	*C. carpio**	55.5	Insertion in sequence; does not bind
	Dorosoma sp.	51.8	Gaps in sequence; does not bind
	*Carassius auratus**	63	Gaps in sequence; does not bind
	*Channa argus**	56	Gaps in sequence; does not bind
	*Beta splendens**	37	Gaps in sequence; does not bind
	Catostomus commersonii	33.3	Does not bind
	Lepomis macrochirus	0	Unalignable due to gaps; does not bind
	Micropterus salmoides	40.7	Gaps in sequence; does not bind
	Micropterus dolomieu	33.3	Gaps in sequence; does not bind
	Carassius auratus hybrid	70.4	Does not bind
	*M. piceus**	55.5	Insertion in sequence; Does not bind
	*C. idella**	66.6	Does not bind
Hmol-R	*H. molitrix*	100	Target species, fully functional
	*H. nobilis**	88	Does not bind
	*C. carpio**	76	Does not bind
	Dorosoma sp.	80	Does not bind

*Carassius auratus**	72	Does not bind
*Channa argus**	84	Does not bind
*Beta splendens**	36	Does not bind
Catostomus commersonii	†	
Lepomis macrochirus	76	Does not bind
Micropterus salmoides	0	Unalignable, binding not possible
Micropterus dolomieu	0	Unalignable, binding not possible
Carassius auratus hybrid	80	
*M. piceus**	28	Does not bind
*C. idella**	84	Does not bind

† Data were unavailable for comparison.

PCR Amplification

The amplification cocktail consisted of 0.75U Taq Polymerase and 10X PCR buffer (Eppendorf), 2.5 mM $Mg(OAc)_2$, 10 nmol each of dNTP, DNA template, species specific primers (0.2 mM final concentration each; Table 1), and deionized water to a total volume of 25 µL. The PCR thermal program consisted of an initial incubation at 94 °C for 2 minutes followed by 45 cycles of 94 °C for 1 minute, 50 °C (silver; 52 °C for bighead) for 1 minute, and 72 °C for 1.5 minutes. This was followed by a final extension at 72 °C for 7 min.

Hybridization and misspecification of bighead and silver carp

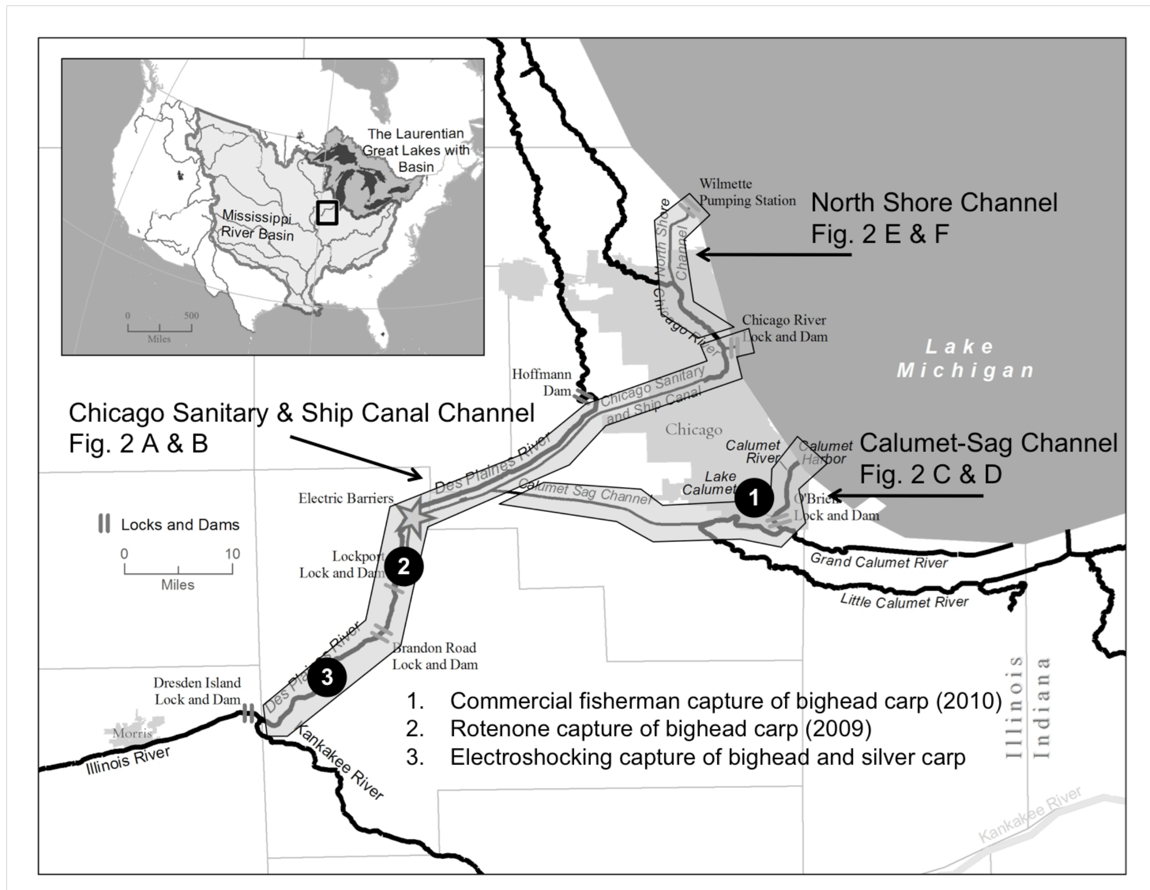
Bighead and silver carp are known to hybridize in the wild and produce viable offspring (Kolar et al. 2007). Although the genetic specificity of the molecular

markers used in this study has been confirmed, positive detections of bigheads could come from phenotypic silver carp with a bighead carp maternal lineage, or a positive silver detection could come from a phenotypic bighead with a silver carp maternal lineage. The extent to which field observations come from hybrid individuals is unknown.

Chicago area waterway system

For purposes of analysis, we evaluated three branches of the waterway. The central connection contains portions of the Des Plaines River, Chicago Sanitary and Ship Canal, and the Chicago River which connects to Lake Michigan through a lock in downtown Chicago (middle shaded polygon in Fig. S1). It has an eDNA detection pattern for bighead and silver carp as presented in Fig. 2 A and 2 B, respectively. This section of the upper waterway is channelized with water depths that can exceed 10 meters. The southern connection to Lake Michigan consists of the Calumet-Sag Channel, which connects to the Little Calumet River, Calumet River, and Lake Calumet. O'Brien Lock (River mile 326.5) controls shipping between the Calumet River and the Little Calumet River (southern shaded polygon in Fig. S1). This section is channelized through the Cal-Sag channel, and the pattern of eDNA detections for bighead and silver carp through the Cal Sag out to Lake Michigan is presented in Fig. 2 C and 2 D, respectively. The third branch, north of downtown Chicago is a connected to Lake Michigan via the Wilmette Pump Station through the North Shore Channel (northern shaded polygon in Fig. S1). The pattern of eDNA detection for bighead and silver carp for the North Shore Channel is presented in Fig. 2 E and 2 D, respectively.

1



2

3 Figure S1: The three main branches of the Chicago area waterway system to Lake Michigan are
4 presented as polygons and the corresponding eDNA detection distributions are provided in Figure 2.
5 Black circles with numbers identify key sighting or capture of bighead carp with netting (1, June 22,
6 2010), bighead carp with rotenone (2, December 3, 2009), and both species with electrofishing (3,
7 Summer 2007 and 2009). Environmental DNA to the corresponding species of fish was found within
8 10 meters of location three, 100 meters of location two, and 4 kilometers of location one. Efforts to
9 recover bighead carp at locations two and one were largely motivated by eDNA detections.

1 Table S2 Des Plaines River, Chicago Sanitary and Ship Canal, Chicago River to the
2 Chicago Lock

River Mile Range	Bighead samples analyzed	Bighead positive detections	Silver samples analyzed	Silver samples positive
<286.11†	24	16	24	13
286.11-291.1‡	34	2	44	17
291.11-296.3§	113	6	142	16
296.31-298.8	14	0	14	0
298.81-301.3	27	0	27	1
301.31-303.8	17	0	17	0
303.81-306.3	10	0	10	0
306.31-308.8	5	0	5	0
308.81-311.3	6	0	6	0
311.31-313.8	9	0	9	0
313.81-316.3	10	0	10	0
316.31-318.8	11	0	11	3
318.81-321.3	21	0	21	2
321.31-323.8	28	0	28	2
323.81-326.3	53	0	53	0
>326.31¶	71	0	71	1

3 † Brandon Road Lock and Dam (River mile 286.1)

4 ‡ Lockport Lock and Dam (River mile 291.1)

5 § Electric Barrier (River miles 296.2-296.4)

6 ¶ Chicago Lock (River mile 327.5)

7

8 Table S3: Calumet-Sag Channel, Little Calumet River, and Calumet River to Calumet
9 Harbor

River Mile Range	Bighead samples analyzed	Bighead positive detections	Silver samples analyzed	Silver samples positive
303.81-306.1†	16	3	16	0
306.31-308.8	9	0	9	1
308.81-311.3	5	1	5	1
311.31-313.8	5	0	5	0
313.81-316.3	6	0	6	0
316.31-318.8	7	0	7	0
318.81-321.3	38	4	38	1
321.31-323.8	77	11	77	0
323.81-326.3	154	13	154	4

326.31-328.8[‡]	57	0	57	0
328.81-331.3	38	0	38	1
331.31-333.8	50	0	50	3
>333.81[§]	9	0	9	1

† Confluence with the Chicago Sanitary and Ship Canal (River mile 303.4)

‡ T. J. O'Brien Lock (River mile 326.5)

§ Calumet Harbor & Lake Michigan (River mile 333.5)

Table S4: Chicago River and North Shore Channel to Wilmette Pumping Station

River Mile Range	Bighead samples analyzed	Bighead positive detections	Silver samples analyzed	Silver samples positive
325.80-326.3[†]	2	0	2	0
326.31-328.8	11	0	11	0
328.81-331.3	0	0	0	0
331.31-333.8	34	0	34	0
333.81-336.3	20	0	20	0
336.31-338.8	53	0	53	1
338.81-341.3	91	0	91	4
>341.31[‡]	1	0	1	0

† Confluence with the Chicago River in downtown Chicago (River mile 325.4)

‡ Wilmette Pumping Station (River mile 326.5)