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A sensitive environmental DNA (eDNA) assay leads to new insights on Ruffe (*Gymnocephalus cernua*) spread in North America

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Abstract Detection of invasive species before or soon after they establish in novel environments is critical to prevent widespread ecological and economic impacts. Environmental DNA (eDNA) surveillance and monitoring is an approach to improve early detection efforts. Here we describe a large-scale conservation application of a quantitative polymerase chain reaction assay with a case study for surveillance of a federally listed nuisance species (Ruffe, *Gymnocephalus cernua*) in the Laurentian Great Lakes. Using current Ruffe distribution data and predictions of future Ruffe spread derived from a recently developed model of ballast-mediated dispersal in US waters of the Great Lakes, we designed an eDNA surveillance study to target Ruffe at the putative leading edge of the invasion. We report a much more advanced invasion front for Ruffe than has been indicated by conventional surveillance methods and we quantify rates of false negative detections (i.e. failure to detect DNA when it is present in a sample). Our results highlight the important role of eDNA surveillance as a sensitive tool to improve early detection efforts for aquatic invasive species and draw attention to the need for an improved understanding of detection errors. Based on axes that reflect the weight of eDNA evidence of species presence and the likelihood of secondary

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spread, we suggest a two-dimensional conceptual model that management agencies might find useful in considering responses to eDNA detections.

Keywords Quantitative polymerase chain reaction (qPCR) · Aquatic invasive species (AIS) · Surveillance · Early detection

Introduction

The characteristic lag time between introduction and widespread establishment of biological invaders provides a window of opportunity for early detection and eradication of potentially harmful species in novel environments (Myers et al. 2000; Crooks 2005; Lodge et al. 2006; Mehta et al. 2007). Environmental DNA (eDNA) surveillance is a method for improving early detection efforts for rare aquatic species, including harmful species at the leading edge of an invasion front (reviewed by Rees et al. 2014). Environmental DNA (eDNA) surveillance is especially well suited for aquatic environments because cells and sloughed tissues are suspended in water and can be collected and screened to detect DNA of target organisms that are present but difficult to detect with conventional surveillance tools (Goldberg et al. 2011; Jerde et al. 2011; Sweeney et al. 2011; Dejean et al. 2012; Pilliod et al. 2013; Takahara et al. 2013; Jane 2014). Early applications of eDNA for aquatic invasive species (AIS) surveillance utilized a traditional endpoint polymerase chain reaction (PCR) approach, but eDNA methods are rapidly evolving and recent advances in the development of quantitative PCR (qPCR) assays for sample screening highlight the potential for improved sensitivity of eDNA based surveillance (Beja-Pereira et al. 2009; Bott et al. 2010; Thomsen et al. 2012a, b; Wilcox et al. 2013; Nathan et al. 2014; Turner et al. 2014a, b). Here we describe the application of a qPCR screening assay with a case study for surveillance of Ruffe, a federally designated 'nuisance species,' across the Laurentian Great Lakes (Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, Public Law 101–646).

Ruffe, a percid fish native to western and northern Europe, was first observed in North America in the St. Louis River (the westernmost tributary of Lake Superior) in 1986 (Pratt 1988), having likely been introduced via ballast water of vessel(s) originating from a port associated with the Elbe River drainage (Stepien et al. 2005). A rapidly reproducing population of Ruffe in the St. Louis River, combined with declines in some native fish populations and a persistent eastward advance, led the Aquatic Nuisance Species Task Force, under the authority of the Nonindigenous Aquatic Nuisance Prevention and Control Act, to declare Ruffe a 'nuisance species' in the spring of 1992. This designation triggered formal surveillance efforts by the US Fish and Wildlife Service (USFWS) and the Ontario Ministry of Natural Resources to detect pioneering populations of Ruffe in the Great Lakes. Ruffe is now known to inhabit many tributaries along the US shoreline of Lake Superior, from Duluth, Minnesota to the Tahquamenon River, Michigan. Range expansion into Lake Huron and northern Lake Michigan has also been documented and is presumed to have occurred as the result of larval fish transport in the ballast water of bulk carrier vessels traveling from invaded ports in Lake Superior or abroad (Bowen and Keppner 2013). The most recent USFWS surveillance reports indicated presence of Ruffe in the Cheboygan River (Cheboygan, MI, Lake Huron) and in Green Bay (near Escanaba, MI and Marinette, WI, Lake Michigan; Bowen and Keppner 2013). To date, live Ruffe have not been observed in Lake Erie or the southern basin of Lake Michigan, where it could potentially bridge the hydrological divide that separated the Great Lakes basin from the Mississippi River basin before the construction of the Chicago Sanitary and Ship Canal (US Army Corps of Engineers 2014).

Using current Ruffe distribution data from USFWS and the US Geological Survey Nonindigenous Aquatic Species database (USGS-NAS; http://nas.er.usgs.gov/default.aspx), along with predictions of future Ruffe distribution derived from a recently developed model of ballast-mediated Ruffe dispersal in the US waters of the Great Lakes (Sieracki et al. 2014), we designed an eDNA surveillance study to target Ruffe at the putative leading edge of the invasion. The main objectives of our surveillance effort were to: (1) monitor invasion of high-risk ballast uptake ports in the upper Great Lakes that could seed Lake Erie or the southern basin of Lake Michigan; (2) assess spread of Ruffe in eastern Lake Superior and in the northern parts of lakes Michigan and Huron; and, (3) search for potential incursions of Ruffe in Lake Erie and southern Lake Michigan. Our findings suggest a far more advanced

invasion front for Ruffe in the Great Lakes than has been documented using the conventional fisheries methods employed for formal Ruffe surveillance efforts. We propose a conceptual framework to facilitate application of our results for the management of Ruffe, based on axes that reflect the weight of eDNA evidence of species presence and the likelihood of secondary spread.

Methods

Marker development

Molecular markers for *G. cernua* were designed using publically available sequence information (GenBank, www.ncbi.nlm.nih.gov). We assembled sequences for 23 Percidae species (22 of which are historically found in the Great Lakes basin plus Ruffe) for the cytochrome b, cytochrome c oxidase subunit 1 (COI), and control region as these regions of the mitochondrial genome were best represented in GenBank for the taxa of interest. Three COI primer pairs and two primer pairs from the control region were evaluated with tissue-extracted DNA from *G. cernua* and 17 of the co-occuring Great Lakes Percidae species. DNA extractions were all normalized to 1 ng/μL and tested under the qPCR conditions described below (see *qPCR amplification and evaluation*). Amplifications were post-PCR cleaned with ExoSAP-IT (USB) and submitted to the Genomics and Bioinformatics Core Facility at the University of Notre Dame (GBCF) for unidirectional Sanger Sequencing. Sequences were visually inspected with Sequencher^{TM} (GeneCodes) and submitted to BLAST on NCBI. The optimal pair of primers, Ruffe_COI_Fa (5′-TACCCTCCCCTATC AGGAAACTT-3′) and Ruffe_COI_Ra (5′-TAATTG CGCCCAAGATTGAGGAGAT-3′), targeted a 111 bp fragment of the COI.

DNA extracted from tissues of several nontarget taxa (*Etheostoma caeruleum*, *E. blennoides*, *E. zonale*, *Percina caprodes*, and *P. copelandi*) produced amplicons that were identified as *G. cernua* by Sanger sequencing. As the tissue samples were provided by a museum collection, the potential for contaminating DNA (*G. cernua)* in the samples seemed plausible and the ability of the assay to identify the trace contaminant DNA in an environment dominated by the DNA of the non-target taxon added support for the stringent application of the assay on eDNA samples. Additional details for the design and validation of the assay are given in "Appendix 1: Detailed description of methods for qPCR marker development" section.

Sample collection, filtration, and extraction

From October 25, 2012 to September 11, 2013, we collected 1289 2-L water samples from 24 discrete locations in the Great Lakes basin (Fig. 1). We focused our sampling efforts on 'high-risk' locations at the leading edge of the known Ruffe invasion front (e.g. eastern Lake Superior, northern Lake Huron from Alpena to the Cheboygan River, and Green Bay) and on sites receiving the largest volume of shipping traffic from Ruffe infested ports within the US waters of the Great Lakes (e.g. Chicago, IL and Toledo, OH; National Ballast Information Clearinghouse 2014). Surface-water samples were collected in autoclaved 2-L Nalgene bottles (45 min at 121 °C). Subsurface water samples were collected within one meter of the bottom with a Van Dorn sampler (2.2 L opaque PVC; Wildlife Supply Co., Yulee, FL) or Kemmerer sampler (2.2 L acrylic; Wildlife Supply Co., Yulee, FL) and transferred in the field to autoclaved 2-L Nalgene bottles. All sample locations were geographically referenced with GPS (Garmin Dakota 10; s.e., <10 m). Samples were filtered and extracted as recommended by Mahon et al. (2010). Briefly, samples were vacuum filtered onto 1.5 μm pore-size glass fiber filters within 24 h of collection, filter papers were stored at −20 °C, and DNA was extracted with the PowerWater DNA Isolation kit (MO-Bio Laboratories Inc., Carlsbad, CA).

Apart from the 2-L Nalgene bottles, which were autoclaved as described above, all equipment used in the sampling and screening effort, including boats, was sterilized with a 10 % bleach solution or sourced directly from suppliers (e.g. latex gloves). Cooler blanks, a single 2-L bottle filled with deionized water, were placed in each sample cooler and taken into the field. The cooler blanks were opened in the field, resealed, and then submerged into the waterbody being sampled. Prior to filtering each sample, approximately 500 mL of deionized water was passed through each sterilized filter apparatus onto a filter paper to test for contamination on lab equipment; these samples are referred to as equipment controls. All cooler blanks were screened for contamination and,

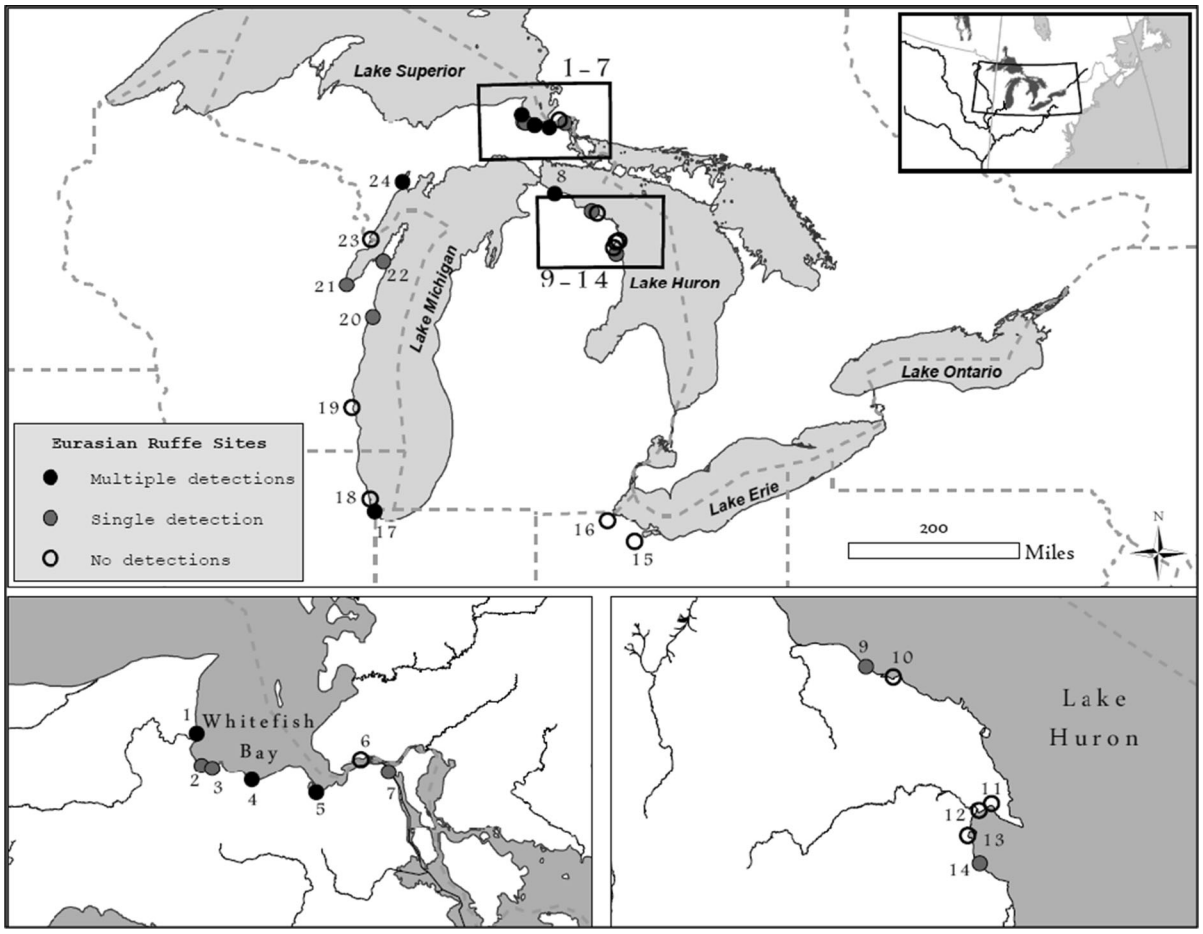

Fig. 1 Sampling locations for 2012–2013 Ruffe eDNA surveillance effort

for every field sample that tested positive, the corresponding equipment control was processed.

qPCR amplification and evaluation

qPCR amplifications were conducted using a SYBR® Green I dye assay. The qPCR amplification cocktail consisted of 1X Power SYBR® Green Master Mix (Life Technologies), 300 nM of each primer, 0.4 μg/μL of Bovine Serum Albumin (Ambion), and 4 μL of extracted DNA in a 20 μL reaction. We performed all reactions on an Eppendorf Mastercycler ep realplex 2 thermocycler. Thermal cycling conditions were as follows: an initial activation step at 95 °C for 10 min; 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min; and, a melting curve analysis transitioning from 60 to 95 °C over 20 min. The fluorescence threshold for each plate was automatically determined by the Eppendorf realplex software using the default Noiseband setting. The fluorescence baseline was calculated for every reaction individually using the default Automatic Baseline setting of the Eppendorf realplex software.

Each eDNA extract was run in triplicate with a single positive control (tissue-derived DNA) and single negative control (1X TE buffer, low EDTA) included on each qPCR plate. Every amplification profile and melt curve profile was visually examined to confirm exponential amplification and a melting temperature matching that of tissue derived reactions. Successful amplifications were post-PCR cleaned with ExoSAP-IT (USB) and submitted to the GBCF for unidirectional Sanger Sequencing. Sequences were checked visually with 4 Peaks (http://nucleobytes.

com/index.php/4peaks) or Sequencher™ (GeneCodes) and specificity to Ruffe was verified by BLAST on NCBI. eDNA extracts were considered positive for the presence of Ruffe DNA only after successful amplification and successful sequence confirmation. Final confirmation for a positive sample required a negative result from the corresponding equipment control.

Assessment of failure to detect target DNA

To assess to what extent our sample screening protocol (i.e. eDNA extract run in triplicate) failed to detect target species DNA when it was present in a sample, we screened samples collected in 2013 a second time. All samples that tested negative in the first round of screening were re-assayed in sextuplet (i.e. 6 technical qPCR replicates) with the appropriate controls in place as in previous analyses. As before, successful amplifications were post-PCR cleaned and submitted for unidirectional Sanger sequencing and equipment controls were screened for final confirmation of a positive DNA detection.

Results

A total of 72 samples across fourteen locations tested positive for Ruffe DNA (Fig. 1). All positive detections were from samples collected over the period May to July 2013 (Table 1). The majority of positive detections (>80 %) were from surface-water samples, however, a larger proportion of total subsurface samples (~14 %; average depth = 3.4 m, min = 2 m, max = 5 m) resulted in positive detections as compared to surface-water samples (~5 %; Table 1). One of the positive detections was a cooler blank from a collection at Escanaba, Michigan in May 2013. Although three other samples tested positive from the May 2013 sampling event at Escanaba, none of these additional positive samples were associated with the contaminated cooler blank (i.e. the three positive samples were not in the same cooler). Because no other samples associated with the contaminated cooler blank tested positive for Ruffe DNA, we did not discard any data.

Positive eDNA detections at the Cheboygan River and Escanaba River corroborate results from conventional fisheries surveillance efforts where at least one Ruffe has been captured at each of these locations over the same time period (Table 2). Ruffe DNA was also detected at seven additional locations where conventional sampling failed to capture live Ruffe in 2012 and 2013, although, at two of these locations, Ruffe have been captured in previous years (site 1 starting in 2006 and site 9 in 2008). Positive detections also occurred from five locations for which conventional surveillance for Ruffe (or with gear capable of incidentally capturing Ruffe) has not been reported. There were no instances where Ruffe were captured using conventional methods in 2012 or 2013 and we subsequently failed to detect Ruffe eDNA.

Of the 72 samples that tested positive for Ruffe DNA, 40 tested positive in the initial assay with three technical qPCR replicates. The remaining 32 (including the positive cooler blank) detected Ruffe DNA only after a secondary screening of six additional technical qPCR replicates. For sites where Ruffe DNA was detected, detection failure (i.e. "initial false negative"; the percent of samples that tested positive for Ruffe DNA only after a secondary screening of additional DNA extract) ranged from 0 to 57 % (Table 3). On average, approximately 9 % of all the samples taken from locations where Ruffe DNA was ultimately detected failed to detect Ruffe DNA during the initial screening.

Discussion

The positive eDNA detections reported here are consistent with the pattern of natural spread of Ruffe, including their ongoing advance towards important ballast-water uptake areas like the St. Marys locks in eastern Lake Superior. In accordance with predictions from a ballast-mediated dispersal model (i.e. Sieracki et al. 2014) our results also provide the first indication that Ruffe is present in southern Lake Michigan, which suggests that Ruffe could be much closer to the Mississippi River than has been indicated by surveillance with conventional sampling gears alone. The potential consequences of a widespread invasion of Ruffe in the lower Great Lakes and the Mississippi River basin (see below) suggest that these results warrant consideration of a management response. However, the uncertainty associated with interpretation of positive eDNA detections (sensu Darling 2014) may impede effective

Table 1 Location, sampling effort, sample date, and number of positive detections for Ruffe DNA

Site no.	Location	No. of samples	Sample date (s)	No. of samples with positive detections
	Lake Superior basin			
1	Tahquamenon River	38 (11)	21 May 2013	29 (8)
2	Roxbury Creek	10	21 May 2013	1
3	Naomikong River	11	21 May 2013	1
4	Pendills Creek	14	21 May 2013	2
5	Waiska River	25 (6)	21 May 2013	11 (3)
6	St. Marys River (West)	50 (6)	20 May 2013	0
7	St. Marys River (East)	26	20 May 2013	1
	Total	174 (23)		45 (11)
	Lake Huron basin			
8	Cheboygan River	45	25 October 2012	0
		37 (7)	22 May 2013	8 (1)
9	Trout River	15	25 October 2012	0
		20	22 May 2013	1
10	Swan River	18 (8)	22 May 2013	0
11	Norwegian Creek	6	23 May 2013	0
12	Thunder Bay River	53	25 October 2012	0
		49 (10)	23 May 2013	0
13	Squaw Bay	5	23 May 2013	0
14	Devils River	24	25 October 2012	0
		5	23 May 2013	1
	Total	277 (25)		10 (1)
	Lake Erie basin			
15	Sandusky River	50	24 June 2013	0
16	Maumee River	48	25 June 2013	0
	Total	98		0
	Lake Michigan basin			
17	Calumet Harbor	32	8 July 2013	10
18	Chicago Waterfront	50	11 September 2013	0
19	Milwaukee	101	7 November 2012	0
		100 (11)	16 May 2013	0
20	Twin Rivers	32	13 November 2012	0
		38 (5)	29 May 2013	1
21	Fox River	48	13 Nov 2012	0
		50 (9)	29 May 2013	1
22	Sturgeon Bay	74	15 November 2012	0
		50 (8)	31 May 2013	1 (1)
23	Menominee River	50	14 November 2012	0
		44 (6)	30 May 2013	0
24	Escanaba River	36 (10)	14 November 2012	0
		35 (6)	30 May 2013	4 (1)[a]
	Total	740 (55)		17 (2)
	Grand total	1289 (103)		72 (14)

Number of benthic samples is indicated in parentheses

[a] One of these four positives was a cooler blank. All remaining cooler blanks (n = 47) and equipment controls (n = 1 for each positive) tested negative for Ruffe DNA

Table 2 Comparison of eDNA versus conventional fisheries surveillance (from both dedicated and incidental capture efforts) for all sites where eDNA surveillance was conducted in 2012 and 2013

Site no.	Location	eDNA		Conventional	
		Effort	# Pos.	Effort[e]	# Ruffe
	Lake Superior basin				
1	Tahquamenon River	38	29	PAT (207)	0
2	Roxbury Creek	10	1	n/a	n/a
3	Naomikong River	11	1	SEN (12)	0
				GN 1 (12)	0
				GN 2 (12)	0
4	Pendills Creek	14	2	SEN (12)	0
				GN 1 (12)	0
				GN 2 (12)	0
5	Waiska River	25	11	n/a	n/a
6	St. Marys River (West)	50	0	FN 1 (34)	0
				BT-4.9 (2.1)	0
				EF 1 (5.8)	0
7	St. Marys River (East)	26	1	BT-4.9 (4)	0
	Lake Huron basin				
8	Cheboygan River	82	8	BT-4.9 (0.5)	0
				EF (2.4)	0
				PT (214)	1
9	Trout River	35	1	EF 3 (1.0)	0
				SPT (102)	0
10	Swan River	18	0	n/a	n/a
11	Norwegian Creek	6	0	n/a	n/a
12	Thunder Bay River[a]	102	0	BT-4.9 (1.8)	0
				EF 1 (6.9)	0
				TN (109)	0
				BT-5.3 (4.3)	0
				BT-11 (10)	0
				GN 4 (16)	0
				GN 5 (18)	0
				GN 6 (1399)	0
				GN 7 (1750)	0
13	Squaw Bay	5	0	n/a	n/a
14	Devil's River	29	1	FN 4 (122)	0
	Lake Erie basin				
15	Sandusky River[b]	50	0	BT-4.9 (2.9)	0
				EF 1 (2.5)	0
				FN 5 (3)	0
16	Maumee River[c]	48	0	BT-4.9 (3.1)	0
				FN 5 (15)	0
	Lake Michigan basin				
17	Calumet Harbor	32	10	n/a	n/a

Table 2 continued

Site no.	Location	eDNA		Conventional	
		Effort	# Pos.	Effort[e]	# Ruffe
18	Chicago Waterfront	50	0	n/a	n/a
19	Milwaukee	201	0	EF 2 (8)	0
				FN 2 (6)	0
				FN 3 (6)	0
				MT (10)	0
20	Twin Rivers	70	1	PAT (79)	0
21	Fox River	98	1	n/a	n/a
22	Sturgeon Bay	124	1	n/a	n/a
23	Menominee River	94	0	PAT (74)	0
				EF 2 (8)	0
				BT-3.7 (1.7)	0
				GN 3 (12)	0
24	Escanaba River[d]	71	4	BT-3.7 (5)	3
				GN EX (4682)	4
				BT-4.9 (1.1)	0
				GN 3 (32)	9

Effort is reported as the composite of all 2012 and 2013 sampling. Data for conventional surveillance is taken from Bowen and Goehle (2012) and Bowen and Keppner (2013). For eDNA, effort is reported as the number of 2L water samples taken and '# pos.' is the number of positive eDNA detections. For conventional methods the type of sampling gear used is reported and the '# Ruffe' is the number of Ruffe captured

[a] Includes Thunder Bay

[b] Includes Sandusky Bay

[c] Includes Maumee Bay

[d] Includes Little Bay de Noc

[e] Gears used for conventional sampling included (with appropriate units of effort in parentheses): FN 1 = paired fyke nets, 4.7 mm mesh with 15 m lead (trapnights); BT-4.9 = bottom trawl with 4.9 m head rope (h); EF 1 = electrofishing (h); SEN = seine, 46 m length (no. of hauls); GN 1 = gillnet, 21 m with 9.5–38 mm mesh (no. of sets); GN 2 = gillnet, 37 m, with 25 mm mesh (no. of sets); PAT = portable assessment trap (trapnights); EF 2 = electrofishing (events); FN 2 = fyke net, 0.9 m × 1.5 m box with 12.7 mm #126 mesh (trapnights); FN 3 = mini fyke net, 0.7 m × 1.0 m box with 3.175 #35 mesh (trapnights); MT = minnow trap array, 5 baited traps spaced 7.6 m apart on one line (arrays); BT-3.7 = bottom trawl with 3.7 m head rope (h); GN 3 = gillnet, 97.5 m including panel of 25 mm stretch mesh (no. of sets); GN EX = gillnet, experimental with 25–127 mm mesh (meters); TN = trapnet, small mesh (trapnights); BT-5.3 = bottom trawl with 5.3 m head rope (h); BT-11 = bottom trawl with 11 m head rope (h); GN 4 = gillnet, graded mesh including panel of 38.1 mm stretch mesh (no. of sets); GN 5 = gillnet, micromesh including panels of 12.7, 15.9, and 19.1 mm stretch mesh (no. of sets); GN 6 = gillnet, graded mesh including panel of 38.1 mm stretch mesh (meters); GN 7 = gillnet, micromesh including panels of 12.7, 15.9, and 19.1 mm stretch mesh (meters); PT = permanent trap (trapnights); EF 3 = backpack electrofishing (h); SPT = semi-permanent trap (trapnights); FN 4 = fyke net (trapnights); FN 5 = paired fyke net (trapnights)

decision-making and, given the reasonable fear of wasting resources if results do not indicate the presence of fish, resource managers may be reluctant to initiate expensive response efforts based only on positive eDNA detections (Finnoff et al. 2007; Darling and Mahon 2011). To allay management concerns, sources of error and uncertainty and the strength of evidence for the presence of live fish need to be communicated (Darling 2014). We attempt to put the results reported above into that context. We present our eDNA surveillance results within a conceptual two-dimensional management response framework based on weight of evidence for species presence and species spread potential (Fig. 2).

Our conceptual model is based, in part, on the idea from Jerde et al. (2011) that a gradient of evidence for species presence exists that is related to number and frequency of eDNA observations. We make the

Table 3 Total number of samples, number testing positive for Ruffe DNA, and percent positive samples for both the initial screening (i.e. with three technical qPCR replicates) and the re-screening (i.e. with six technical qPCR replicates) for eDNA samples collected in 2013

Site no.	Location	Initial screening			Re-screen		Detection failure (%)
		No. samples	No. samples positive	% positive	No. samples	No. samples positive	
1	Tahquamenon River	38	17	44.7	21	12	57.1
5	Waiska River	25	10	40.0	15	1	6.7
17	Calumet Harbor	32	8	25.0	24	2	8.3
8	Cheboygan River	37	3	8.1	34	5	14.7
9	Trout River	20	1	5.0	19	0	0.0
7	St. Marys River	26	1	3.8	25	0	0.0
4	Pendills Creek	14	0	0.0	14	2	14.3
3	Naomikong River	11	0	0.0	11	1	9.1
2	Roxbury Creek	10	0	0.0	10	1	10.0
14	Devils River	5	0	0.0	5	1	20.0
21	Fox River	50	0	0.0	50	1	2.0
20	Twin Rivers	38	0	0.0	38	1	2.6
22	Sturgeon Bay	50	0	0.0	50	1	2.0
24	Escanaba River[a]	34	0	0.0	34	3	8.8
	Total	390	40	10.3	350	31	8.9

The number of samples that were re-screened from each location is the subset of samples that failed to detect Ruffe DNA during the initial screen. Thus, detection failure is equivalent to the percent of samples in which target DNA was detected during re-screening. For all locations included in the table, at least one sample tested positive for Ruffe DNA

[a] Excludes cooler control

assumption that evidence to support species presence is stronger when a pathway of invasion (including natural dispersal) exists and, thus, we incorporate invasion potential as part of the weight of evidence axis. The second axis considers potential for spread from the detection site (e.g. presence of vectors for secondary spread, proximity to vectors, and potential for natural dispersal). Though not explicitly considered in our conceptual model, the potential for negative impacts of species establishment at a site could also be considered as an additional axis, including measures of site vulnerability (i.e. ecological or economic values that could be impacted if the site is invaded, sensu Margules and Pressey 2000).

In three of the six locations where we detected eDNA from more than one sample, our results simply confirm previous records of live fish from the site (sites 1, 8, 24). At Pendills Creek and Waiska River, multiple positive detections are consistent with continued eastward expansion of Ruffe along the southern shoreline of Lake Superior and also probably indicate the presence of live fish (sites 4 and 5; Fig. 3). Pendills Creek and Waiska River are both within 50 km of the Tahquamenon River and, thus, well within range for natural dispersal of Ruffe based on a conservative estimate of Ruffe dispersal distance ($\sim$25 km/year; Sieracki et al. 2014).

Multiple positive detections of Ruffe DNA in southern Lake Michigan at Calumet Harbor suggest a major range expansion, but this result is consistent with predictions from a ballast-mediated dispersal model that indicates high probability of Ruffe introduction in southern Lake Michigan ports (Fig. 3, site 17; Sieracki et al. 2014). We cannot rule out the possibility that the eDNA we detected in Calumet Harbor was introduced into the system via discharged ballast water sourced from a Ruffe-invaded port, but that possibility seems unlikely for two reasons. First, eDNA degradation

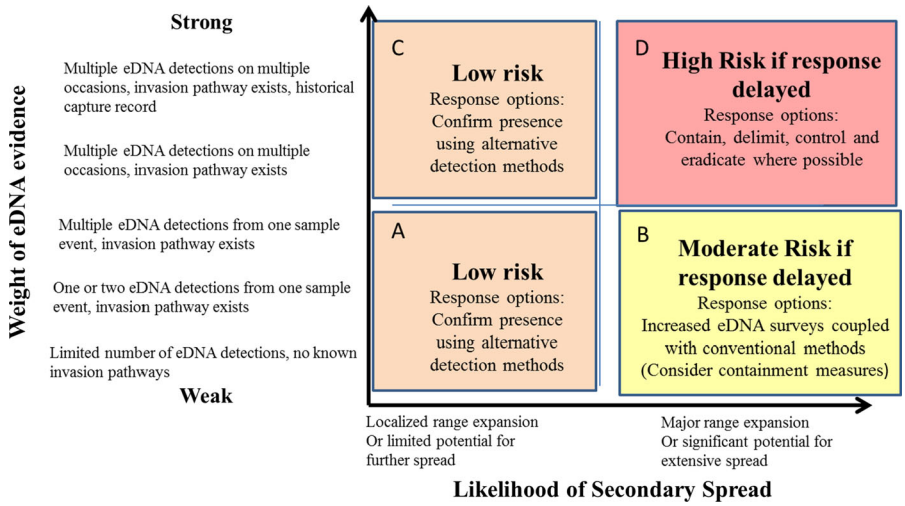

Fig. 2 A conceptual management framework for responding to eDNA surveillance results. Response actions can be influenced by both the weight of evidence in favor of species presence and the likelihood of secondary spread. The strongest eDNA evidence in favor of species presence is evidence that, agrees with predictions from natural spread and/or dispersal models (i.e. presence of an invasion pathway), indicates a pattern of repeated detection over time, and/or corroborates any record(s) of historical live capture. The potential for spread could include consideration of presence of vectors for secondary spread, proximity to vectors, and potential for natural dispersal

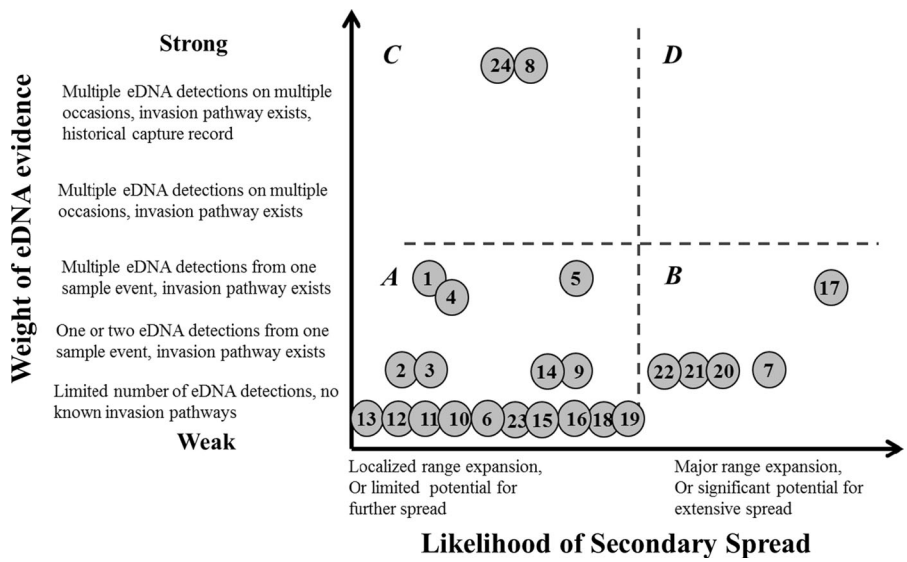

Fig. 3 The conceptual management framework as applied to the eDNA survey results from 2012/2013 Ruffe eDNA surveillance effort (*circled numbers* correspond to site numbers listed in Table 1). Ruffe dispersal to all sampling locations was conceivable based on natural spread or dispersal model predictions

studies using ambient water suggest that the majority of suspended Ruffe DNA would degrade in the time required to transit from the nearest Ruffe invaded port (~24 h; Barnes et al. 2014). Second, although seven commercial vessels entered the port at Calumet in the 1-month period prior to our survey in Calumet Harbor (on July 8, 2013), none of these vessels was traveling directly from a port where Ruffe have been reported (National Ballast Information Clearinghouse 2014). Thus, it seems likely that the DNA detected in Calumet indicates the presence of live Ruffe in the harbor. Any future eDNA sampling that produces repeated

detections over time, especially during intervals with no ballast-water discharge, would strengthen this conclusion (Fig. 2). A large number of eDNA samples collected from Calumet Harbor over a 2- to 4-week window could be especially informative, as such sampling would provide the spatial and temporal replicates required to resolve whether positive detections are a result of a pulse, non-fish vector or indicative of a sustained source of live fish.

At a number of sites, Ruffe DNA was recorded from a single sample, thus, representing the weakest evidence for the presence of live fish (Fig. 2). We cannot rule out that some of these detections may have arisen from low levels of contamination, as can be expected occasionally even when observing strict quality assurance protocols (Apfalter et al. 2005; Turner 2015) and as evidenced by the detection of Ruffe DNA in one of our cooler blanks. However, we found no evidence of systemic contamination in our laboratory or field control samples and all sites where a single positive sample was detected are locations where Ruffe have been captured previously (i.e. site 9) or are within approximately 100 km of sites where live fish have previously been collected and, thus, within range for natural dispersal of Ruffe (e.g. sites 20–22 are plausible given their proximity to the Escanaba and Menominee rivers where Ruffe were first captured in 2002 and 2007, respectively). Together, these observations suggest contamination had little effect on the patterns of detection reported here. It is also a possibility that some of these positive detections are a result of discharged ballast water sourced from a Ruffe-invaded port, although in most cases this seems unlikely given that most of the positive detections we report occurred at sites without an active port or in habitats upstream of existing port facilities. One exception is the positive detection at Fox River (WI), where the sample was taken near the mouth of the river and adjacent to the shipping channel.

Darling and Mahon (2011) make the case that managers should be wary of the risks associated with a failure to detect DNA of target AIS when it is present (i.e. false negative), given that highly sensitive eDNA surveillance methods make detection of incipient invasions a real possibility and, thus, increase the potential to minimize long-term control costs and impacts if establishment can be prevented. In this study, we screened samples using a quantitative PCR assay to reduce the rate of false negatives (i.e. relative to less sensitive traditional PCR methods; sensu Thomsen et al. 2012a, b; Wilcox et al. 2013; Nathan et al. 2014; Turner et al. 2014a, b). Yet, after doubling our level of replication we detected numerous additional positive samples (Table 3). In the Tahquamenon River, where Ruffe have been collected since 2006, 12 out of the 29 samples that eventually tested positive for Ruffe were negative during the initial qPCR screening (3 replicates), suggesting that the concentration of target species DNA is low and highly patchy both within an extracted sample and in the environment (i.e. 25 % of samples collected tested negative despite Ruffe presumably being established at low densities at this site). Patchiness of eDNA is consistent with the occurrence of a substantial portion of eDNA in relatively large particles (i.e. particle sizes corresponding to cells or clumps of cells; Turner et al. 2014a, b). Furthermore, many locations had a relatively low number of positive sample detections (<5 % of samples positive at a location). This is expected if there are few Ruffe releasing eDNA and imperfect mixing of the water column (Jerde and Mahon 2015). If we assume that Ruffe are physically present in these locations with very few positive detections, then presumably there is a large false negative rate (see "Appendix 2: A method for estimating the sensitivity of the Ruffe eDNA survey" section). This is a critical consideration in locations where no positive detections were recorded and where there was a limited sampling effort (locations with fewer than 30 samples collected). The results from our re-screening are consistent with other eDNA studies that highlight the importance of PCR replication and the potential for high prevalence of false negatives from eDNA samples when detection probabilities are low (Ficetola et al. 2015; Furlan et al. 2015).

It follows that decision makers may be more willing to initiate potentially expensive and long-term response programs, where the cost of the failure to act is high (Darling and Mahon 2011). Although effects of Ruffe populations in Lake Superior are equivocal, it is possible that impacts on food webs in other parts of the Great Lakes or connected basins could be larger, especially if populations become large or widespread (Bronte et al. 1998; Gunderson et al. 1998). Ruffe can compete with native Great Lakes fishes for space and food (Edsall et al. 1993; Sierszen et al. 1996; Ogle et al. 2004; Fullerton and Lamberti 2005). It has also been suggested that complete

colonization of the Great Lakes by Ruffe could result in a decrease in Yellow Perch populations by 10–60 % and of Whitefish and Walleye populations by up to 25 % with accompanying annual losses in the range of $24 to $214 million in sportfishing and commercial fishing revenue (Leigh 1998). In addition, the establishment of Ruffe in the southern basin of Lake Michigan would likely accelerate spread out of the Great Lakes and into the Mississippi River basin via the Chicago Area Waterway System. Ruffe was identified as one of 29 species (and one of six fishes) with the potential to cause moderate to severe effects if introduced into the Mississippi River basin (Jerde et al. 2010; US Army Corps of Engineers 2014). The extent of the threat to the Mississippi basin is as yet unquantified, but globally significant freshwater biodiversity is potentially at risk because the Mississippi River and its tributaries contain the largest number of freshwater fishes (260 species) of any region at comparable latitudes (Smith 1981; Fremling et al. 1989) and are a global center of diversity and endemism for crayfish (Lodge et al. 2012) and unionid mussels (Abell et al. 2000). Some native fishes serve as important hosts for endangered unionid mussels and a subset of these host fishes rely heavily on benthic food sources (e.g. Freshwater Drum, Sauger, and Catfishes), and are expected to compete directly with Ruffe (Ed Rutherford, NOAA GLERL, personal communication, December 10, 2013).

Given the detection of Ruffe eDNA in Calumet and the potential risk to the Mississippi River basin there may be value in implementing ongoing surveillance for Ruffe with both eDNA and conventional sampling methods, and managers might consider adopting measures to contain and prevent the movement of Ruffe out of the Great Lakes via the Chicago Area Water System (Fig. 3). In addition, the detection of Ruffe eDNA near important ballast-water uptake areas (sites 7 and 21; Jennifer Sieracki, National Park Service, personal communication, June 24, 2015) highlights further the invasion risk posed by the movement of ballast water within the Great Lakes (Keller et al. 2011; Adebayo et al. 2014; Sieracki et al. 2014). Thus, other slow-the-spread and control options proposed early in the Ruffe invasion, including ballast- water management, might also be productively revisited by managers (Busiahn 1997).

While the capture of a live specimen will always be more compelling than eDNA evidence, we caution against dismissal of eDNA results in the absence of corroborating live captures, especially in the absence of an explicit examination of relative sampling effort and detection sensitivity of conventional gears. There is increasing evidence that genetic surveillance methods are more sensitive than conventional approaches (Jerde et al. 2011; Thomsen et al. 2012a, b; Turner et al. 2012; Biggs et al. 2015; Valentini et al. 2015), which means that eDNA detections are possible even when sampling with conventional gear fails—especially at the putative invasion front when target organisms are rare (Darling and Mahon 2011; Jerde et al. 2011; Dejean et al. 2012). Controlled experiments that directly compare the sensitivity of eDNA sampling and conventional sampling methods when target species are at low abundance would be helpful (Darling and Mahon 2011). Fuller consideration of dispersal, invasion pathways, and other factors affecting invasion risk would provide a stronger basis for management response decisions following the eDNA detection of a potential incipient invasion. Only when eDNA detections are considered alongside other lines of evidence (including probability of spread and potential for negative impacts) can they help guide appropriate and defensible management decisions.

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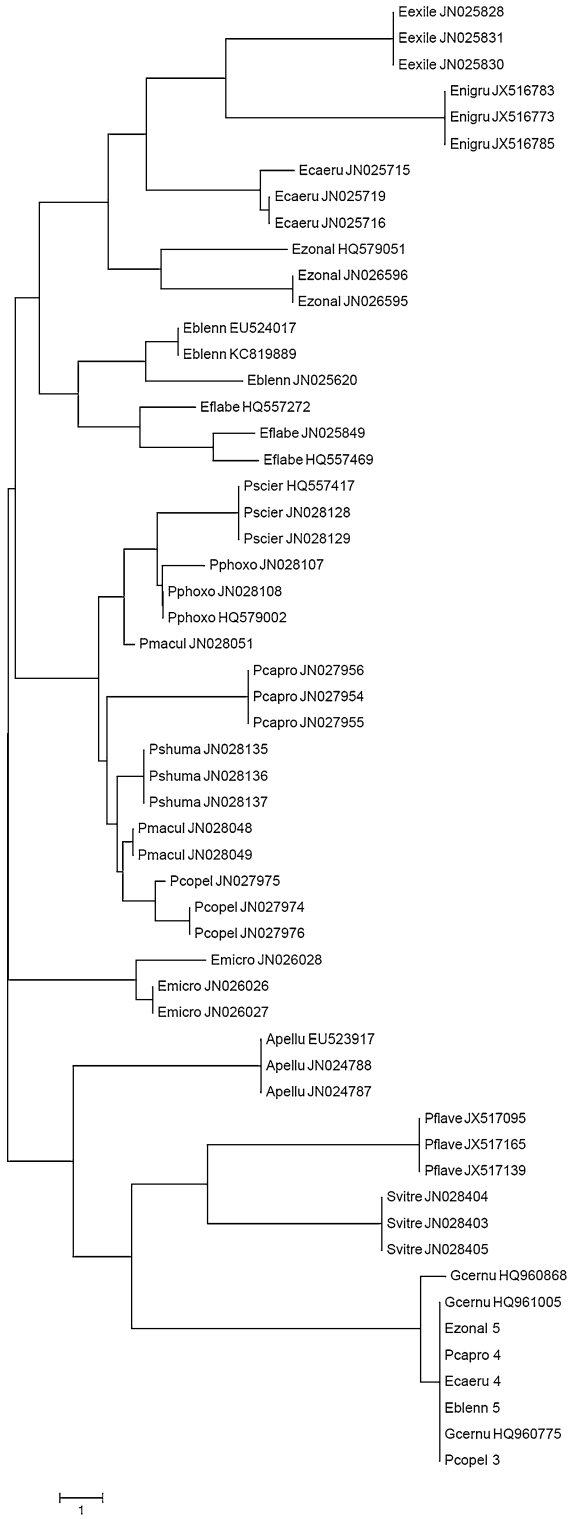

◀**Fig. 4** Neighbor-Joining phylogenetic tree for *G. cernua* and 17 co-occuring Percidae species in the Great Lakes basin. A Sequencher™ (GeneCodes) file was built with COI sequences downloaded from GenBank (accession numbers listed for each sequence in the tree) in addition to the sequences resulting from qPCR amplifications (Eblenn 5, Ecaeru 4, Ezonal 5, Pcapro 4, and Pcopel 3). All sequences were trimmed by eye, leaving a 63-bp consensus sequence. The Neighbor-Joining phylogenetic tree was built in MEGA 5.1 (Tamura et al. 2011) using the number of base-pair differences as the determining factor for the branch lengths. The tree indicates contamination at some level of the tissue collection, storage, or DNA extraction processes (negative controls were included with the qPCR assays and all failed to amplify), but it is evident that the markers are ideal for amplifying only DNA from the target species, *G. cernua*

Appendix 1: Detailed description of methods for qPCR marker development

Species-specific markers for *G. cernua* were generated from each file of sequences with PrimerHunter (Duitama et al. 2009). Potential primer pairs were further evaluated by Primer Express® 3.0 (Life Technologies) to identify primer sets adhering to the stringent parameters of the qPCR assay. A total of 5 potential primer pairs, 3 from the COI and 2 from the control region, were ordered from Integrated DNA Technologies (http://www.idtdna.com). All 5 primer pairs were evaluated with tissue-extracted DNA from both *G. cernua* and 17 additional Percidae species historically found in the Great Lakes basin: *Ammocrypta pellucida*, *E. caeruleum*, *E. blennoides*, *E. exile*, *E. flabellare*, *E. microperca*, *E. nigrum*, *E. spectabile*, *E. zonale*, *Perca flavescens*, *P. caprodes*, *P. copelandi*, *P. maculata*, *P. phoxocephala*, *P. sciera*, *P. shumardi*, and *Sander vitreus* (Fig. 4; Table 4).

Appendix 2: A method for estimating the sensitivity of the Ruffe eDNA survey

The purpose of this exercise is to estimate a false negative rate for the Ruffe samples collected in 2012/2013 and screened using nine qPCR technical replicates. We do not have a solid understanding of the density of Ruffe in any of the locations, so we will

Table 4 Percent similarity of COI primers to Ruffe and co-occurring Percidae species in the Great Lakes basin

Scientific name	Number of sequences	Forward primer (%)	Reverse primer (%)	Together (%)
Gymnocephalus cernua	31	87–100	96–100	92–100
Ammocrypta clara	10	79	80	79
Ammocrypta pellucida	9	83	80	81
Etheostoma caeruleum	71	70–74	64–72	67–73
Etheostoma chlorosomum	8	70–83	76–80	73–81
Etheostoma exile	14	78	76	77
Etheostoma flabellare	32	65–78	64–84	65–81
Etheostoma microperca	23	78	72–76	75–77
Etheostoma nigrum	225	70–83	72	71–77
Etheostoma olmstedi	159	70–83	72–80	71–81
Etheostoma spectabile	80	70–83	68–80	69–81
Etheostoma zonale	25	74–83	76–80	75–81
Perca flavescens	24	70–74	72	71–73
Percina caprodes	53	74–78	72	73–75
Percina copelandi	13	74	72	73
Percina evides	19	70	72	71
Percina maculata	17	83	72	77
Percina microlepida	13	78	72	75
Percina phoxocephala	11	74–78	72	73–75
Percina scieri	13	78	64–76	71–77
Percina shumardi	9	74–78	72	73–75
Sander canadensis	6	78	84	81
Sander vitreus	11	70	84	77

Percent similarity is estimated as [(number of matching bases/total number of bases in primer) × 100]. Table includes the *Number of sequences* downloaded from GenBank per species, percent similarity for the *Forward primer*, percent similarity for the *Reverse primer*, and percent similarity when considering both primers *Together*

necessarily treat each location independently from each other.

First, there are some locations that had no detections of Ruffe. We cannot estimate a false negative rate as we have no indication that Ruffe are present. These locations are: Milwaukee, St Marys River (west), Swan River, Thunder Bay River, Norwegian Creek, Squaw Bay, Menominee, Sandusky River, Maumee River, and Chicago waterfront. This leaves us with 14 locations with at least one positive detection in a sample (Table 5).

Let us start by considering the technical replicates from Tahquamenon. The data of the number of positive technical replicates per sample look as such: {2, 1, 8, 3, 1, 1, 6, 1, 8, 2, 3, 1, 1, 0, 0, 2, 3, 8, 0, 0, 0, 0, 1, 0, 9, 0, 9, 9, 1, 9, 9, 1, 7, 1, 9, 9, 9}. Note that 21 % of samples (8/37) are zero. We can estimate p, the probability any given technical replicate is positive using the Log Likelihood of a binomial function with a maximum of 9 technical replicates per samples as, $LL(p) = \sum_{i=1}^{37} Log\left(\binom{9}{x_i}(p^{x_i})(1-p)^{9-x_i}\right)$ and then finding the value of p that maximizes this function, which results in an MLE, $\hat{p} = 0.4$. With this estimate, the

Table 5 Locations with positive Eurasian Ruffe detections

Location	n	Number of samples with at least one positive replicate	Data: number of positive technical replicates per sample (9 max)
Sugar Island	25	1	1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
Waiska	24	11	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 7, 8, 9, 0, 9, 1, 9, 9, 9, 0, 0, 9, 9, 9
Pendills	13	2	0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 1, 0, 0
Naomikong	11	1	0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0
Roxbury	9	1	0, 0, 0, 0, 1, 0, 0, 0, 0
Tahquamenon	37	29	2, 1, 8, 3, 1, 1, 6, 1, 8, 2, 3, 1, 1, 0, 0, 2, 3, 8, 0, 0, 0, 0, 1, 0, 9, 0, 9, 9, 1, 9, 9, 1, 7, 1, 9, 9, 9
Cheboygan	35	8	1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 2, 6, 1, 0, 0, 1, 1, 0, 0, 0, 4, 0, 0, 0
Trout	19	1	0, 0, 0, 0, 0, 0, 0, 0, 0, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0
Devils River	5	1	0, 0, 0, 0, 1
Fox River	48	1	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
Twin River	35	1	0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,0, 0, 0, 0, 0, 0, 0, 0, 0, 0
Escanaba	33	3	1, 0, 1, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
Sturgeon Bay	44	1	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,0, 0, 0,0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,0, 0, 0
Calumet	29	9	3, 2, 0, 0, 0, 3, 7, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 1, 2, 1

probability of getting nine zeros by chance is $p(0) = 0.009$. If we collected 37 samples then the expected number of samples that are false negatives is the number of collected samples $*p(0) = 37 *0.009 = 0.33$. We would conclude it is unlikely that any of the zeros observed at this location are due to false negatives. As the probability of positive detection decreases, we expect the chance of getting nine technical replicates without a positive to increase. If we apply the same logic to all locations with positive Eurasian Ruffe detections we get the following results (Table 6).

The majority of locations have so few positive detections (1 replicate in 1 sample) that if we assume the one positive detection is due to presence of Eurasian Ruffe, then there was insufficient technical replication to ensure absence of target DNA (or concentrations below detectable levels). However, for Waiska and Tahquamenon, we can safely conclude that most of the samples that had no target DNA detected were absent of the target DNA—that is to say, a very low false negative rate. The inference from Calumet is not as clear as Waiska and Tahquamenon. It suggests that upwards of 65 % of the samples (13.6/20) with no detection may be false negatives, leaving room for some true negatives to be present in the system.

Sensitivity, or the probability of detecting target DNA given it is present at a site, will ultimately depend on the survey method and characteristics of the survey sites, especially the concentration and dispersion of target DNA molecules at a site. Because we do not know the concentration or dispersion of DNA in the sites where we sampled our approach is

Table 6 Sensitivity of the Ruffe eDNA survey for sites with at least one positive eDNA detection based on a Maximum-likelihood estimation of the probability that any given qPCR technical replicate is positive

Location	n	No. of samples with no detection	Prob. positive replicate (p-hat)	Prob. of nine technical replicate by chance ($p(0)$)	Expected number of false negatives by chance	Conclusion
Sugar Island	25	24	0.0044	0.96	24	Insufficient technical replication
Waiska	24	13	0.41	0.009	0.22	Strong evidence for samples without DNA
Pendills	13	11	0.017	0.86	11.2	Insufficient technical replication
Naomikong	11	10	0.01	0.91	10	Insufficient technical replication
Roxbury	9	8	0.012	0.89	8	Insufficient technical replication
Tahquamenon	37	8	0.40	0.009	0.33	Strong evidence for samples without DNA
Cheboygan	35	27	0.044	0.66	23.1	Insufficient technical replication
Trout	19	18	0.012	0.9	17.1	Insufficient technical replication
Devils River	5	4	0.022	0.82	4.1	Insufficient technical replication
Fox River	48	47	0.0023	0.98	47	Insufficient technical replication
Twin River	35	34	0.0032	0.97	34	Insufficient technical replication
Escanaba	33	30	0.01	0.91	30	Insufficient technical replication
Sturgeon Bay	44	43	0.0025	0.98	43.1	Insufficient technical replication
Calumet	29	20	0.08	0.47	13.6	Evidence for some samples absent of DNA

speculative extrapolation based on the best available data and should not be interpreted as quantitative empirical detection sensitivity.

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