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Research Article

Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada

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Editor's note:
This study was first presented at the 19th International Conference on Aquatic Invasive Species held in Winnipeg, Canada, April 10–14, 2016 (http://www.icais.org/html/previous19.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Zebra mussel *Dreissena polymorpha* (Pallas, 1771), an invasive bivalve in North America, was first recorded in Lake Winnipeg in 2013. Quagga mussel *D. bugensis* (Andrusov, 1897), a second invasive mussel to North America, has yet to be detected in Manitoba waters. The establishment and continued spread of zebra mussels is of concern. Accordingly, we developed two species-specific and one *Dreissena*-specific qPCR-based environmental DNA (eDNA) assays designed as a single multiplexed reaction able to identify the presence of zebra mussel and infer the presence of quagga mussel in water samples from at-risk and invaded locations. In 2014, samples were collected from four invaded harbours on Lake Winnipeg during the early (May) and late (October) seasons. Zebra mussel eDNA was detected in 0–33.3% of samples per site early in the season, whereas late season sample detection ranged from 42.9 to 100%. In order to confirm that there was no non-specific amplification of DNA from local biota, samples were also collected from sites where neither adult nor veliger-stage zebra mussels had been previously observed. These sites included three harbours on Lake Winnipeg and six sites within the Manitoban portion of the Red River. No amplification of eDNA was recorded at these sites except for that at a float-plane dock in the Red River upstream of Lake Winnipeg. Zebra mussels were subsequently detected at this location by sampling for transformed attached individuals. Thus, we demonstrate that eDNA is an early indicator of the presence of zebra mussels and is a useful detection tool at the forefront of their recent invasion in Manitoba. This work provides the foundation for the development of a zebra mussel eDNA monitoring program for waterbodies in Manitoba and western Canada.

Key words: invasive molluscs, species detection, quagga mussel, quantitative PCR, real-time PCR, survey tools, monitoring

Introduction

Aquatic invasive species (AIS) have had profoundly negative global impacts on aquatic ecosystems, resulting in loss of native diversity as well as devastating socio-economic effects on human commerce (Simberloff 1981; Pimental et al. 2005; Reaser et al. 2007; Pechar and Mooney 2009; Nienhuis et al. 2014). The zebra mussel *Dreissena polymorpha* (Pallas, 1771) and quagga mussel *D. bugensis* (Andrusov, 1897) are two bivalve AIS that have established large populations throughout North America and Europe outside of their natural ranges (Hebert et al. 1989; Johnson and Padilla 1996; Vanderploeg et al. 2002; USGS 2016).

These two species of dreissenid mussels have two distinct life forms: 1) the microscopic larval form (i.e., veliger), and 2) the sessile mytiliform juvenile

and adult form. Dreissenid mussel veligers predominantly exist in the water column whereas late stage larval, juvenile, and adult mussels attach themselves to solid surfaces such as rock, wood, hydraulic engineering structures, water craft, water intake facilities as well as other biota such as plants, crustaceans, and native mussel species (Rajagopal et al. 2005; Brazee and Carrington 2006; Grutters et al. 2012). The ability to attach to solid surfaces in combination with the short maturation time (females can reproduce within 6–7 weeks of settling) (Borcherding 1991), and high fecundity (>1 million eggs per female for each spawning season) (Walz 1978; Sprung 1990, 1993), allows dreissenid mussels to reach very high densities (> 1 million individuals m^{-2}) (Ludyanskiy et al. 1993; Effler and Siegfried 1994; Patterson et al. 2005). This potentially damages submerged mechanical equipment, clogs pipelines, and suffocates native mussel species (Ludyanskiy et al. 1993; Tucker et al. 1993). The habitat preferences of zebra and quagga mussels are very similar to each other, with the exception that quagga mussels are more energy-efficient and can spawn in cooler and more oligotrophic conditions (Roe and MacIsaac 1997; Baldwin et al. 2002). This has led to speculation that zebra mussels are gradually supplanted by quagga mussels following the establishment of the latter species (Ricciardi and Whoriskey 2004; Orlova et al. 2005; Wilson et al. 2006; Zhulidov et al. 2010; Matthews et al. 2014).

Zebra mussels are highly efficient filter feeders and feed primarily on planktonic algae and zooplankton, characteristics that additionally impact the structure and function of the invaded ecosystem (Pace et al. 1998; Wong et al. 2003; Higgins and Vander Zanden 2010). For example, in South Bay, Lake Huron, zebra mussels were implicated in the reduction of growth rate and body condition of lake whitefish *Coregonus clupeaformis* (Mitchill, 1818) (Bousfield 1989; McNickle et al. 2006; Rennie et al. 2009) due to a reduction in the abundance of *Diporeia* populations, an important food source for lake whitefish. The high filtration capacity of zebra mussels can result in increased water clarity and light penetration that can potentially alter the structure of lake substrates (Reeders et al. 1989; Reeders and Bij De Vaate 1990; MacIsaac 1996).

Due to the potential for dreissenid veligers to pass unseen in raw water, the ability of juveniles and adults to attach to solid surfaces, and their ability to survive out of water for up to 18 days in high humidity conditions (McMahon 2002), the primary vectors for their spread between waterbodies are human-mediated mechanisms such as commercial vessels, recreational boats, and float-planes (Carlton 1993; Johnson and Carlton 1996; Johnson and Padilla 1996; Padilla et al. 1996; Schneider et al. 1998; Buchan and Padilla 1999). Zebra mussels were first discovered in North America in 1988 in Lake St. Claire (Hebert et al. 1989), and were likely introduced via the ballast water of international trading vessels (Hebert et al. 1989; Carlton 1993; *but see* Bossenbroek et al. 2014). Since their initial introduction, zebra mussels have spread to all of the Laurentian Great Lakes, many waterbodies in the US, and recently to Lake Winnipeg, Manitoba (USGS 2016).

In October 2013, visual surveys confirmed the presence of zebra mussels in four harbours on Lake Winnipeg (Balsam Bay, Gimli, Silver, and Winnipeg Beach harbours); soon after, 425 mytiliform zebra mussels were removed from these harbours (DFO 2014). Between May and June of 2014, all four harbours were treated with potash (CWS 2014) in an attempt to fully eradicate zebra mussels in Lake Winnipeg. After treatment, zebra mussels re-established in all four harbours, suggesting that individuals either recolonized from outside of the harbours or were not completely killed in the harbour by the potash treatment. Government and industry are now focusing on improved monitoring of "high-risk" waterbodies to prevent the spread of zebra mussels within Manitoba and western Canada. Accordingly, a rapid and sensitive early detection method able to document the presence of zebra mussels and concurrently infer the presence of quagga mussel (a potential invader) in waterbodies would help facilitate inspection, enforcement, and control efforts.

A common sampling tool to assess the density and distribution of zebra mussels is veliger capture using plankton nets and subsequent identification using microscopic methods (Mackie and Claudi 2009), but such methods can be expensive and slow (Lawrence and Cordell 2010). Moreover, zebra and quagga mussel veligers cannot be differentiated (Bott et al. 2010). Typically, plankton nets must be deployed alongside other detection methods, such as substrate sampling, to detect both the veliger and mytiliform stages. Ideally, early detection via "remote-sensing" methods which do not rely on the direct observation of the target organism would not share the same disadvantages as current detection methods. Highly sensitive, species-specific molecular surveillance techniques already used for detection and monitoring of other AIS (e.g., Jerde et al. 2013; Laramie et al. 2015) offer alternative approaches that provide more accurate species distribution data and distinguish between different invasive mussel species.

Environmental DNA (eDNA) is becoming increasingly prevalent in conservation (Schwartz et al. 2007)

and is being rapidly developed in its application to monitoring AIS (Darling and Mahon 2011; Jerde et al. 2011, 2013). For example, the Asian carp eDNA monitoring program of the US has successfully developed and applied eDNA techniques in the Great Lakes basin (Jerde et al. 2011, 2013; Amberg et al. 2015). Other successful monitoring programs such as the Sea Lamprey Control Program, initiated by the Great Lakes Fishery Commission (GLFC), have considered implementing eDNA as a "red-flag" detection system to identify streams which require lampricide treatment (Gingera et al. 2016). DNA from sources such as mucus and feces, sloughed-off cells, and decomposing organisms can introduce eDNA in a system (Valentini et al. 2009; Klymus et al. 2015) which can be detected using methods based on polymerase chain reaction (PCR). These tools amplify and detect fragments of DNA specific to a target organism thus are particularity useful for efficiently assessing the presence of eDNA from water samples. As an alternative to traditional survey methods, eDNA technologies provide a cost-effective and sensitive detection tool to quickly and accurately monitor species' distributions over large geographic areas (Armstrong and Ball 2005; Taberlet et al. 2012; Laramie et al. 2015; Sigsgaard et al. 2015). Use of eDNA techniques to detect zebra mussels during the forefront of an invasion event and for long-term monitoring allows for rapid implementation of protocols to reduce the likelihood of spread to uninfected waterbodies surrounding Lake Winnipeg, across Manitoba and into western Canada and the United States.

In this study, we develop and validate two species-specific genetic assays for zebra mussel and one presumptive genus-specific assay for *Dreissena* (able to detect both zebra and quagga mussels), and report on the first successful use of eDNA for detecting zebra mussels in Lake Winnipeg. The use of multiple assays increases redundancy thus reducing the likelihood of false negatives, while simultaneously allowing for limited indirect detection of quagga mussel. Following the evaluation of all three individual assays, we incorporated two of them into a multiplexed assay that included an internal positive control (IPC). The IPC allows for the identification of samples experiencing PCR inhibition, which is a common problem for environmental samples and can result in false negatives. We then sampled sites within Lake Winnipeg and the Red River where local observation and veliger data had not detected zebra mussels to confirm the ability of these eDNA assays to detect zebra mussels but not non-target local biota.

Methods

Marker design

A series of genetic markers and probes were designed to diagnostically identify zebra mussel eDNA. Three quantitative (qPCR) assays were developed, each targeting a separate mitochondrial gene: cytochrome oxidase c subunit I (COI), cytochrome b (Cyt *b*), and 16S rRNA. COI was included as it is widely used as the "barcode of life" (Hebert et al. 2003) and sequences are widely available for several target and non-target species. Cyt *b* and 16S rRNA were chosen as both genes are well conserved across species and are therefore good candidates for species and genus-specific assays. To increase sensitivity, given the often-fragmented nature of eDNA, assays were designed to amplify short fragments between 80 and 150 bp. Of the three assays developed, two assays (COI, Cyt *b*) were designed to be species-specific to zebra mussel and one (16S rRNA) was designed to be genus-specific to *Dreissena* to amplify DNA from both zebra mussel and quagga mussel. Primer/probe combinations for each gene were developed "by eye" with MEGA v.6 (Tamura et al. 2013) using all sequence data available on GenBank for Cyt *b*, COI, and 16S rRNA of zebra mussel, quagga mussel, and the 31 unionid mussel species for which sequence data was available (Supplementary material Table S1). Primer Express v3.0.1 (Applied Biosystems) and Oligoanalyzer v3.1 (Integrated DNA Technologies; https://www.idtdna.com/calc/analyzer) were used to determine melting temperature (Tm) and identify potential secondary structures. Primer-BLAST (Basic Local Alignment Search Tool; Gen-Bank https://www.ncbi.nlm.nih.gov/blast) searches compared the primer and probe sequences to all available sequence data to test whether they were likely to result in the amplification and detection of non-targeted organisms. Each candidate assay was tested against tissue-derived DNA from 39 zebra mussel specimens collected from Lake Winnipeg during 2014 and 2015, and 173 quagga mussel specimens from sections of the Colorado River within CO, USA. Zebra mussel specimens were collected in accordance with Manitoba Conservation and Water Stewardship (CWS) collection permit SCP 25–15. Each assay was also tested against DNA from 1–9 specimens of the following 10 mussel species native to Manitoba: black sandshell *Ligumia recta* (Lamarck, 1898), fatmucket clam *Lampsilis siliquoidea* (Barnes, 1823), flutedshell *Lasmigona costata* (Rafinesque, 1820), giant floater *Pyganodon grandis* (Say, 1892), mapleleaf *Quadrula quadrula* (Rafinesque, 1820), plain pocketbook *Lampsilis cardium* (Rafinesque,

Table 1. Summary of species used for eDNA assay validation, number of samples for each species, and location of where each sample was collected.

Species	Common name	# samples	Location
Dreissena polymorpha	Zebra mussel	39	Lake Winnipeg, MB, Canada
Dreissena bugensis	Quagga mussel	173	Colorado River, CO, US
Lasmigona complanata	White heelsplitter	1	Sydenham River, ON, Canada
		1	La Salle River, MB, Canada
Lampsilis cardium	Plain pocketbook	1	Sydenham River, ON, Canada
		2	La Salle River, MB, Canada
Quadrula quadrula	Mapleleaf mussel	9	La Salle River, MB, Canada
Lampsilis siliquoidea	Fatmucket clam	2	La Salle River, MB, Canada
Potamilus alatus	Pink heelsplitter	1	La Salle River, MB, Canada
Pyganodon grandis	Giant floater	1	La Salle River, MB, Canada
Fusconaia flava	Wabash pigtoe	3	Sydenham River, ON, Canada
Ligumia recta	Black sandshell	4	Sydenham River, ON, Canada
Lasmigona costata	Flutedshell mussel	3	Sydenham River, ON, Canada
Amblema plicata	Threeridge mussel	3	Sydenham River, ON, Canada

1820), pink heelsplitter *Potamilus alatus* (Say, 1817), threeridge *Amblema plicata* (Say, 1817), Wabash pigtoe *Fusconaia flava* (Rafinesque, 1820), and white heelsplitter mussel *Lasmigona complanata* (Barnes, 1823) collected from either the La Salle River, Manitoba or Sydenham River, Ontario (Table 1). DNA was extracted from each sample using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's protocols. DNA sequences were produced for zebra and quagga mussel samples (n = 8) using both the sense and antisense primers and the BigDye Terminator v3.1 Cycle Sequencing Kit, following standard procedures on an Applied Biosystems 3130xl sequencing platform (Life Technologies, Grand Island, NY, USA) to confirm amplification of the desired product.

Sample collection, filtration, and extraction

To test the utility of the three assays to detect eDNA under natural field conditions, locations in which zebra mussels were known to be present and those which were considered to be free of zebra mussels were sampled. Balsam Bay, Gimli, Silver, and Winnipeg Beach harbours, known to contain zebra mussels, were sampled on May14 and October 1, 2014; Hnausa Harbour, also known to be positive for zebra mussel based on the identification of transformed attached individuals in the harbour, was opportunistically sampled only on October 1, 2014. For sites where the presence of zebra mussels had not yet been recorded based on local observation and veliger data, water samples were collected November 4 and 5, 2014; these sites included Grindstone, Gull, and Hecla Village harbours and six locations along the Red River upstream of Lake Winnipeg (distance between locations spanned between 2.25 and 22.87 km apart) (Figure 1). These locations enabled us to validate the specificity of the three assays in typical ecosystems of the region with native biotic communities. The number of samples collected differed depending on date and location, with 2–3 samples collected from each harbour in May and 2–8 samples collected in October and November at each location. Sample sizes in May were small as they were collected opportunistically and strategically with a distribution which reflects reasonable coverage throughout the harbours. At all field sites, water samples were collected from boat docks with sterile 2 L plastic Nalgene bottles, and a new pair of nitrile gloves was worn between each sampling. Water samples were kept on ice in the field and then placed in a 4 °C refrigerator once brought to the laboratory. Water temperature was recorded at each location with a TidbiT v2 Temperature Logger (Onset, Bourne, MA, USA). All water samples for this study were from public waters and did not require specific permits (except for Silver Harbour, where permission to sample was given by the owner).

Filtration of each water sample occurred within 24 hours of field collection in a sterile laboratory dedicated to water filtration (i.e., where no DNA work is conducted). Water filtration was conducted as described in Jerde et al. (2011); water was filtered onto a Whatman 1.5 µm pore 47 mm diameter glass fiber filter (GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a vacuum manifold (Pall, Ann Arbor, MI, USA). Between two and four filters were used to filter the full volume of the 2 L samples; filtration of water through a filter continued until either the filter became clogged or 1L of water was filtered. Filtered water volume ranged from 0.150 to 1.0 L. All filters were stored in separate 15 mL conical tubes and held at −80 °C until extraction.

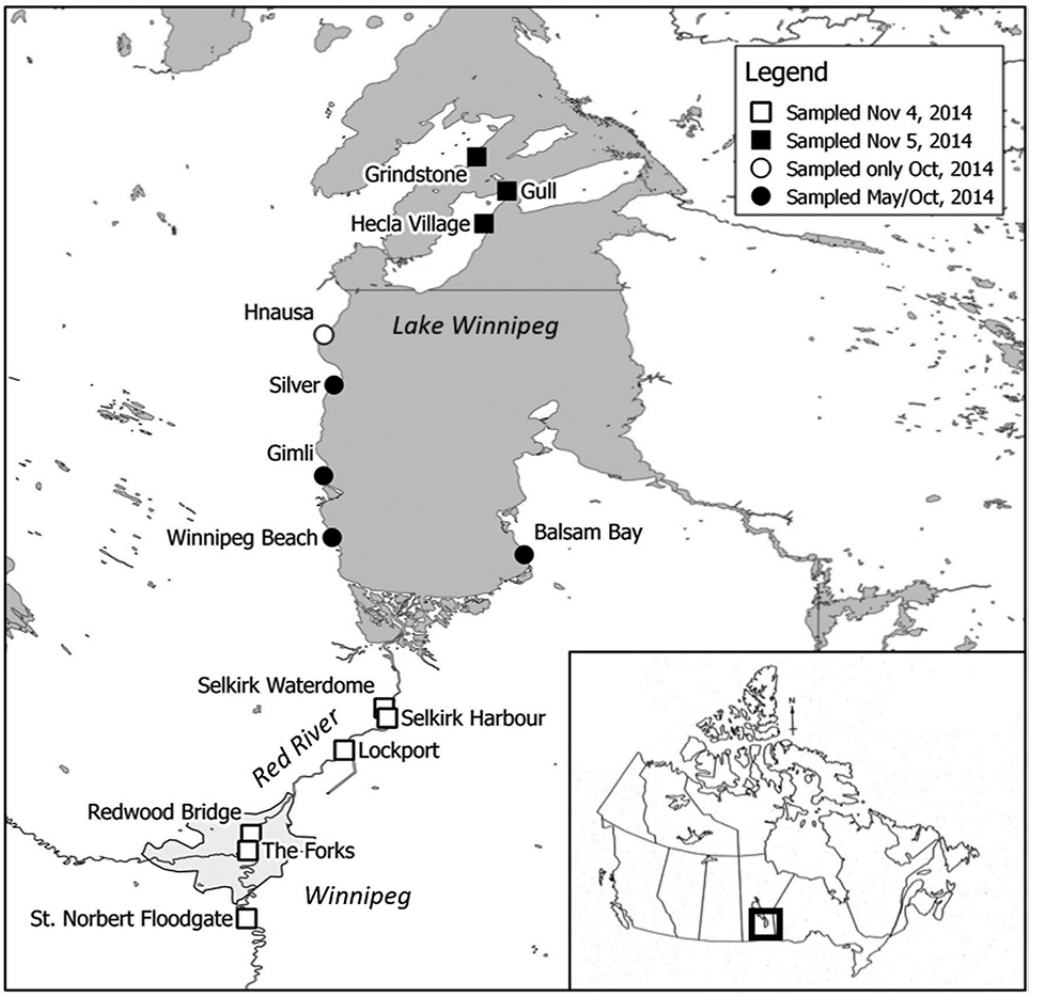

Figure 1. Harbour and collection sites for zebra mussel eDNA for Lake Winnipeg and the Red River. Labels indicate general position of harbours and collection sites within the study system. Inset represents approximate location of study sites in Canada.

All DNA extraction steps were performed in a designated UV sterilized PCR hood equipped with HEPA (High Efficiency Particulate Air) filtration using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA), and eluted to a volume of 200 µL. The filters were folded in half three times with sterile forceps to allow them to fit in a 1.5 mL microtube and suspended in 360 µL ATL buffer and 40 µL proteinase K. Filters were incubated overnight at 56 °C with agitation and subsequently suspended in 400 µL of absolute ethanol and 400 µL AL buffer. All subsequent buffer washes and elution steps followed the manufacturer's protocol.

Multiplex analysis

Evaluation of the performance of candidate eDNA assays was accomplished comparing parameters of qPCR standard curve results. Standards were produced by amplifying tissue-derived DNA of each assay. The total PCR reaction volume was 25 µL with 1X PCR Gold Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.625 U of AmpliTaq Gold DNA polymerase (Life Technologies, Grand Island, NY, USA), and 5 µL of DNA solution containing 1 µL of DNA extract and 4 µL of water. The PCR program included an initial 5 min denaturation step at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. Amplified products were visualized using electrophoresis on a 1.5% agarose gel using GelRed (Biotium Inc., Fermont, CA, USA). For each assay, 20 µL from five PCR reactions were combined (for a total volume of 100 µL) and purified using the QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA,

USA) following the manufacturer's protocols. The concentration (ng/µL) for each purified product was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The number of DNA amplicons within each sample was determined using the Thermo Fisher Scientific DNA Copy Number and Dilution Calculator (http://www.thermofisher.com), assuming an average molar mass per base pair of 618.04, 618.03, or 618.01 (g/mol)/bp for Cyt *b*, COI, and 16S rRNA, respectively. A serial dilution was performed on each purified PCR product to create a standard curve from 10^6 to 10^0 copies/µL. Twelve replicates were performed for each concentration using a QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Grand Island, NY, USA). The total reaction volume was 20 µL with 1x TaqMan Environmental Master Mix 2.0 (Life Technologies, Grand Island, NY, USA), 0.2 µM of each forward and reverse primer, 0.1 µM minor groove binder (MGB) probe, and 5 µL of DNA standard. The qPCR program included an initial 10 min activation step at 50 °C, a 10 min denaturation step at 95 °C followed by 50 cycles of denaturation at 95 °C for 1 min, and a 1 min elongation step at 60 °C. The standard curve intercept and slope were calculated using the linear regression in base R v.3.2.3 (R Development Core Team 2015) with Ct value as the independent variable and log of copies/µL as the response variable. Comparisons between assays were performed using Analysis of Covariance (ANCOVA) with the assay as the categorical variable using the aov function in base R v.3.2.3. Difference in the number of positive reactions at 10^0 copies/µL between assays were analyzed using two-tailed Pearson's chi-squared test in base R v.3.2.3 (R Development Core Team 2015) to determine if any individual assay performed differently at very low concentrations of DNA.

Internal Positive Control (IPC) validation

The effects of incorporating the HemT IPC (Xue et al. 1999) on the Ct values of the Cyt *b* and COI assays were analyzed by performing a triplex reaction of all three assays where the target DNA of the Cyt *b* and COI assays was a 7-point standard curve from 10^6 copies/µL to 10^0 copies/µL with 12 replicates per concentration and 100 target copies of HemT DNA in each reaction. The effect of HemT on the 16S rRNA assay was not examined as the 16S rRNA assay was the poorest performing of the three assays while in a triplex (see results). The qPCR final volume, component concentrations, and program were the same as previously described with the exception of 0.2 µM of the HemT forward and reverse primer, 0.1 µM HemT probe, and a total of 100 copies of HemT target DNA per reaction. HemT DNA concentration was determined using the same protocols described above (see section *Multiplex analysis*) with an average molar mass per base pair of 618.3 (g/mol)/bp. The standard curve intercept and slope were calculated using linear regression with Ct value as the independent variable and log of copies/µL as the response variable, comparisons among assays were performed using ANCOVAs with the assay as the categorical variable, and difference in the number of positive reactions at 10^0 copies/µL between assays were analyzed using a two-tailed Pearson's chi-squared test; all in base R v.3.2.3 (R Development Core Team 2015).

PCR amplification and evaluation

The detectability of zebra mussel eDNA in Lake Winnipeg was examined by the developed zebra mussel and *Dreissena* assays (Table 2). The Cyt *b*, COI, and 16S rRNA assays were multiplexed into a single reaction. Each sample was tested in duplicate using an Applied Biosystems StepOne Plus qPCR platform (Life Technologies, Grand Island, NY, USA). The total reaction volume was 20 µL with 1x TaqMan Environmental Master Mix 2.0 (Life Technologies, Grand Island, NY, USA), 0.2 µM of each assay's forward and reverse primer, 0.1 µM MGB probe for each assay (fluorophores used in each assay were FAM, VIC, and NED, respectively, *see* Table 2), and 5 µL of DNA solution containing 1 µL of DNA extract and 4 µL of water as dilution of eDNA has been shown to be effective at reducing inhibition (McKee et al. 2015). The qPCR program included an initial 10 min activation step at 50 °C, a 10 min denaturation step at 95 °C followed by 40 cycles of denaturation at 95 °C for 1 min, and a 1 min elongation step at 60 °C. Standards ranged from 1.7 ng/µL to 1.7×10^{-4} ng/µL and were made from zebra mussel DNA quantified with a Nanodrop (Thermo Fisher Scientific, Waltman, MA, USA) and diluted with nuclease-free water. Any well that showed no amplification curve during a qPCR run was interpreted as a negative result. A sample was considered positive if at least one of the multiplexed assays produced an amplification curve in at least one of the two sample replicates. Three wells of no-template negative controls were used in all qPCR plates to identify the presence of contamination during analysis. To avoid contamination, all master mix preparation was performed in a designated PCR hood in a pre-PCR room. The addition of the DNA standards and samples were also performed in a designated PCR hood in a separate qPCR room. All standards were prepared in a separate third room.

Table 2. Summary of the eDNA markers developed in this study for zebra mussel and the genus *Dreissena* using qPCR and the cytochrome oxidase *c* subunit 1 (COI), cytochrome *b* (Cyt *b*), and16S rRNA mitochondrial genes; optimal annealing temperature (Tm) and amplicon size for each assay are given.

Target gene	Marker	Sequence (5' – 3')	Tm (°C)	Amplicon size (bp)
Cyt B	Sense	CAT TTT CTT ATA CCT TTT ATT TTA TTA GTG CTT TT	60	114
	Antisense	CGG GAC AGT TTG AGT AGA AGT ATC A		
	Probe	FAM-TAG GTT TTC TTC ATA CTA CTG GC-MGBNFQ		
COI	Sense	SCC TGC GAT AGA TTT TTT GAT TTT A	60	136
	Antisense	GCA GAA CAA AGG GAC CCG		
	Probes	NED-CGT GCT GGA TGT CAT-MGBNFQ		
16S rRNA	Sense	TGG GGC AGT AAG AAG AAA AAA ATA A	60	139
	Antisense	CAT CGA GGT CGC AAA CCG		
	Sense	VIC-CCG TAG GGA TAA CAG C-MGBNFQ		

Table 3. Intercept, slope, R^2, efficiency (%), and number of reactions which amplified at 10^0 copies/µL for cytochrome *b* (Cyt *b*), cytochrome oxidase subunit I (COI), and 16S rRNA assays when incorporated into a duplex and a triplex. All values were obtained using base R v.3.2.3.

Assay		Intercept	Slope	R^2	Efficiency (%)	Amplifications at 10^0 copies/µL
Cyt *b*	Singleplex	38.86188	-3.58103	0.9984	90.22	12/12
	Duplex (COI)	40.1061	-3.7610	0.9951	84.45	11/12
	Duplex (16S)	40.02863	-3.75628	0.9966	84.60	11/12
	Triplex (COI, 16S)	42.14276	-3.87082	0.996	81.28	9/12
	Triplex (COI, HemT[a])	39.694	-3.96971	0.9922	78.61	9/12
COI	Singleplex	38.95973	-3.76413	0.9883	84.36	11/12
	Duplex (Cyt b)	40.1370	-4.0325	0.9589	77.00	7/12
	Duplex (16S)	41.09371	-3.83067	0.9927	82.41	10/12
	Triplex (Cyt *b*, 16S)	41.9062	-3.9160	0.9823	80.04	10/12
	Triplex (Cyt *b*, HemT[a])	39.298	-3.8075	0.9962	83.08	9/12
16S rRNA	Singleplex	40.29144	-3.61142	0.9925	89.19	10/12
	Duplex (Cyt b)	40.06755	-3.78549	0.9945	83.72	11/12
	Duplex (COI)	41.34106	-3.86670	0.9953	81.39	11/12
	Triplex (Cyt *b*, COI)	43.13562	-3.90041	0.993	80.46	12/12

[a]HemT DNA concentration 10^2 copies/µL

Species identity of fragments amplified from field samples which tested positive for zebra mussel eDNA (Cyt *b*, n = 8; COI, n = 6; 16S rRNA, n = 11) was confirmed by performing sequencing reactions with BigDye Terminator v3.1 Cycle Sequencing Kit, using both the sense and antisense primers and following standard procedures on an Applied Biosystems 3130xl sequencing platform (Life Technologies, Grand Island, NY, USA).

Quality assurance and control

Rigorous quality assurance and quality control protocols outlined in the Asian carp monitoring program's 2015 Quality Assurance Project Plan (QAPP; USACE 2015) were used at every stage of this study. Each step of the water sample filtration, DNA extraction, qPCR setup, and qPCR analysis was conducted in a separate sterile laboratory dedicated to that step with sequential work flow to ensure no cross contamination.

Prior to water sampling and filtration, all bottles, manifolds, filter holders, and forceps were sterilized by soaking in a 10% bleach solution for a minimum of 15 min and then thoroughly rinsed with distilled water. All coolers used for water sample collection in the field were washed with a 10% bleach solution and left to sit for a minimum of 15 min at which point they were also rinsed with distilled water. Prior to field sampling, negative controls were collected by taking two sterile 2 L Nalgene bottles filled with distilled water into the field. One control would be opened and exposed to the open air for 10 s, closed, and then completely submerged in water for 10 s. The second negative control was left on ice and never opened. During the filtration stage, negative controls of distilled water were collected to identify contamination of equipment. Prior to filtering a water sample, 0.5 L of distilled water was filtered through an unused glass fiber filter using the same filter holder to be used for the field sample.

All DNA extraction, qPCR setup, and qPCR analysis steps were done in separate PCR work stations equipped with ultra-violet sterilization, HEPA filtration (Ultra-Violet Products Ltd., Upland, CA, USA) and aerosol barrier ART tips (Molecular

BioProducts, St. Louis, MO, USA). Each work station was wiped down with ELIMINase™ (Decon Laboratories, King of Prussia, PA, USA) followed by distilled water and then exposed to UV light for a minimum of 15 min before and after each use. Separate controls of reactions with nuclease-free water were also incorporated with every qPCR reaction to identify any contamination occurring during reaction setup.

Results

Marker design

PCR cross-amplification tests and BLAST searches confirmed the species- and genus-specificity of the COI, Cyt *b*, and 16S rRNA assays (Table 2). The zebra mussel specific assays (Cyt *b* and COI) successfully amplified all zebra mussel samples but did not amplify DNA from the non-target species (quagga mussel and native mussels). The genus-specific 16S rRNA assay amplified both zebra and quagga mussel DNA, whereas native mussel DNA was not amplified. All DNA sequenced PCR fragments from tissue-derived zebra and quagga mussel DNA matched the species and target gene.

Multiplex analysis

Higher Ct values at any given DNA concentration imply a decrease in assay sensitivity. Comparisons between the duplex and triplex reactions demonstrated that the Ct values increased as additional assays were incorporated into the same qPCR reaction (Figure 2). The Cyt *b* assay had lower Ct values when duplexed with the 16S rRNA assay than did the COI assay when duplexed with the 16S rRNA assay at 10^0 copies/µL (ANCOVA, $F_{1,162} = 92.483$, $P < 0.001$) (Table 3). Likewise, the Cyt *b* assay had lower Ct values when duplexed with the COI assay than did the 16S rRNA assay when duplexed with the COI assay at 10^0 copies/µL (ANCOVA, $F_{1,162} = 123.223$, $P < 0.001$) (Table 3). The 16S rRNA assay had lower Ct values when duplexed with the Cyt *b* assay than with the COI assay at 10^0 copies/µL (ANCOVA, $F_{1,163} = 144.975$, $P < 0.001$) (Table 3). The COI assay had lower Ct values when duplexed with the Cyt *b* assay than with the 16S rRNA assay at 10^0 copies/µL (ANCOVA, $F_{1,162} = 62.917$, $P = 3.34\ e^{-13}$) (Table 3). When all three assays were combined into a single reaction (i.e., triplex) the 16S rRNA assay had higher Ct values at 10^0 copies/µL than did the other two assays (ANCOVA, $F_{2,242} = 60.231$, $P < 0.001$) (Table 3). Analysis of the difference of positive detections at 10^0 copies/µL demonstrated that there was no significant difference between the Cyt *b* assay run as a singleplex or as a triplex (Pearson's chi-squared, $x^2_1 = 3.429$, $P = 0.064$), the 16S rRNA assay (Pearson's chi-squared, $x^2_1 = 2.182$, $P = 0.140$), and the COI assay (Pearson's chi-squared, $x^2_1 = 0.381$, $P = 0.537$) (Table 3).

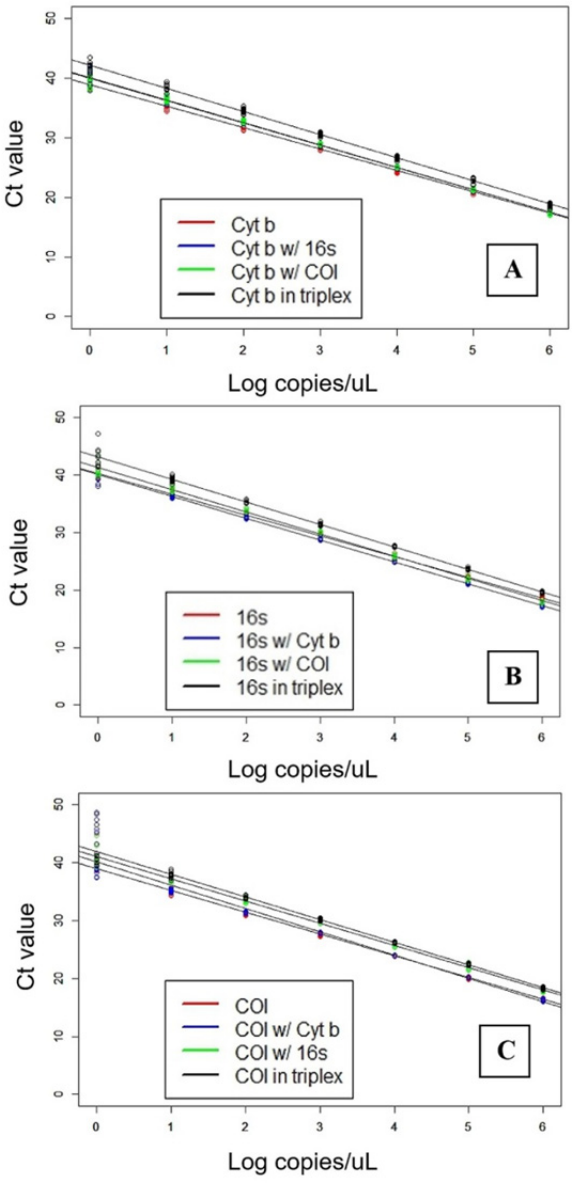

Figure 2. Ct values of the cytochrome b (Cyt b) assays (A), 16s rRNA assays (B), and cytochrome oxidase c subunit 1 (COI) candidate assays (C) plotted against log of copies/µL. Each assay was run as either a singleplex, duplex (indicated with companion assay), or as a triplex with a replicate number of n = 12 at each copies/µL concentration.

Internal Positive Control (IPC)

The incorporation of the HemT IPC (HemT DNA concentration of 10^2 copies/µL) had a significant effect on the Ct values of the Cyt *b* assay but not the COI assay (Figure 3). The Ct values for the Cyt *b* assay at 10^0 copies/µL significantly decreased when in a triplex with COI and HemT (ANCOVA, $F_{1,159} = 121.47$, $P < 0.001$) (Table 3) suggesting increased sensitivity. Analysis for differences in positive detections at 10^0 copies/µL demonstrated that there was no significant difference between the Cyt *b* assay run as a duplex with the COI assay or as a triplex with the COI and HemT assays (Pearson's chi-squared, $x^2_1 = 1.2$, $P = 0.273$) (Table 3). There was no significant difference in the Ct values for the COI assay at 10^0 copies/µL when in a triplex with HemT (ANCOVA, $F_{1,155} = 0.363$, $P = 0.548$) (Table 3). Analysis for the differences in positive detections at 10^0 copies/µL demonstrated that there was no significant difference between the COI assay run as a duplex with the Cyt *b* assay or as a triplex with the Cyt *b* and HemT assays (Pearson's chi-squared, $x^2_1 = 0.75$, $P = 0.386$) (Table 3).

Lake Winnipeg sample testing

Of the four harbours known to contain zebra mussels and sampled in May, only Winnipeg Beach tested positive for zebra mussel eDNA (with 33% of water samples taken testing positive); no positive detections were recorded from the other three harbours (Balsam Bay, Gimli, Silver harbours) (Figure 1; Table S2). Zebra mussel eDNA was detected in all four harbours in October 2014, with 42.9–71.4% of the water samples testing positive. Hnausa Harbour was only sampled in October 2014, at which time 100% of the water samples tested positive for zebra mussel eDNA (Table S2). The presence of zebra mussels in Hnausa Harbour was later confirmed in 2015 via visual survey within the harbour.

Water samples collected from three harbours (Grindstone, Gull, and Hecla Village harbours) in the northern portion of the south basin of Lake Winnipeg on November 5, 2014, did not show indication of zebra mussel eDNA (Table S2). Of the six Red River sites sampled, zebra mussel DNA was detected in 50% (1 of 2) of samples collected at the most northern site (Selkirk Waterdome in the Red River), whereas no zebra mussel DNA was detected from the other five sites (Figure 1; Table S3).

DNA sequencing confirmed all positive qPCR results (Cyt *b*, n = 4; COI, n = 10; 16S rRNA, n = 4) as being zebra mussel. None of the 16S rRNA fragments were identified as quagga mussel.

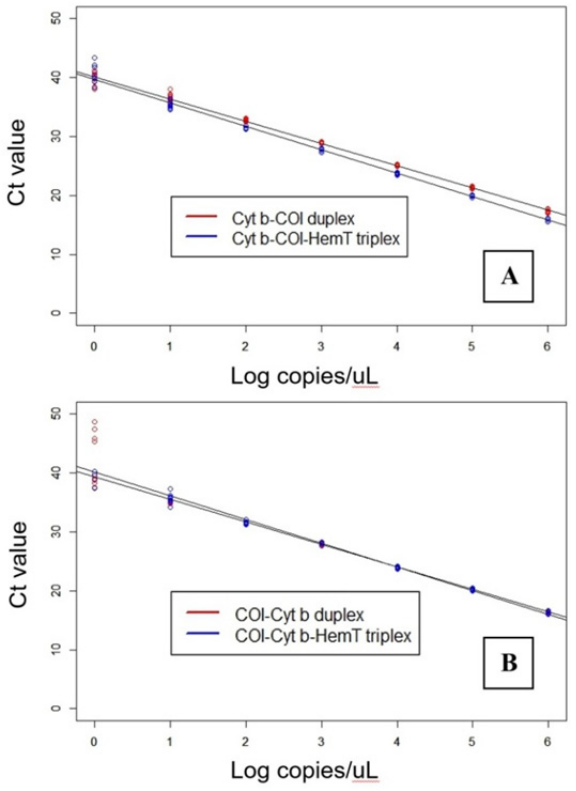

Figure 3. Ct values of the cytochrome b (Cyt b) assays (A) and cytochrome oxidase c subunit 1 (COI) candidate assays (B) plotted against log of copies/µL. Each assay was run as either a duplex or as a triplex with the HemT IPC. Replicate number of n = 12 at each copies/µL concentration.

Discussion

This study demonstrates that eDNA is a promising tool for zebra mussel monitoring in newly invaded waters where the invaders are not yet common. The sensitive and species-specific assays described here could reliably detect concentrations as low as 10^0 copies/µL in triplex. They were successfully used to detected zebra mussels in Lake Winnipeg and the Red River in 2014. These results both confirmed and predicted zebra mussel detection by visual inspection and veliger netting surveys.

The assays described in this study are, to our knowledge, the first zebra mussel eDNA qPCR assays described in the literature. Other studies have developed zebra mussel eDNA assays for conventional PCR (Ardura et al. 2016) and Light Transmission Spectroscopy (Mahon et al. 2011); however, qPCR has become the primary eDNA detection technology due to its increased sensitivity. Also, while other

studies have examined eDNA detection from water sources which have established zebra mussel populations (Egan et al. 2015; Ardura et al. 2016), this work examines the applicability of eDNA during an invasion event, when zebra mussel abundance is presumably low.

The eDNA qPCR assay developed in the current study can successfully detect DNA from both zebra and quagga mussels in aquatic samples. All assays displayed high levels of specificity (to either zebra mussels alone or to both species, as designed) with no instance of cross-amplification when tested with tissue-derived DNA from 10 mussel species native to Manitoba. The applicable range of these assays is likely large and can be effectively applied within North American freshwater systems as dreissenid mussels are distantly related to North American mussels. The genus-specific 16S rRNA assay can be used to indirectly detect quagga mussels when multiplexed with the species-specific zebra mussel assays. If a water sample tests positive for the genus-specific 16S rRNA assay, for example, but tests negative for both species-specific zebra mussel assays, we infer that quagga mussel eDNA may be present. Confirmation of the presence of quagga mussel eDNA can be accomplished with DNA sequencing. This is an imperfect system of detection, as not all assays will amplify target DNA when concentrations approach or are below 10^0 copies/µL. Zebra mussel detection was the priority of this study (and we would not have been able to test a quagga mussel-specific eDNA assay in Lake Winnipeg), but biologists should be mindful of the possibility of quagga mussel invasion. The combined genus- and species-specific assays allow indirect detection of quagga mussels, but the development of one or more quagga mussel-specific eDNA qPCR assays will also be important as quagga mussels have similar negative impacts on ecosystems and can supplant zebra mussels (Ricciardi and Whoriskey 2004; Orlova et al. 2005; Wilson et al. 2006; Zhulidov et al. 2010; Matthews et al. 2014). All three assays successfully amplify zebra mussel DNA from water samples collected in the field and functionally work as a multiplexed reaction (i.e., all three assays run within a single reaction). Due to the possibility that eDNA can be highly degraded, targeting multiple gene fragments allows for the detection of some targets whereas others may be undetectable due to degradation.

Performance of all three assays diminished when incorporated into a multiplex reaction, likely because of increased competition between each assay (Raeymaekers 1995). Also, the likelihood of secondary structure formation, such as heterodimers, increases within multiplexed reactions, which also decreases performance (Hyndman and Mitsuhashi 2003). Both factors may have affected the performances of the multiplexed reactions (Table 3). The 16S rRNA assay performed the poorest in a 16S/COI/Cyt *b* triplex compared to the other two assays (Figure 2). We therefore suggest that if only two assays can be run due to either limitations of the qPCR platform or for the incorporation of the HemT IPC, the COI/Cyt *b*/HemT triplex should be used. Of the seven COI positive detections in the COI/Cyt *b*/HemT triplex when testing detection at 10^0 copies/µL, three detections were below the threshold which was automatically set by the QuantStudio 6 software algorithms. These reactions were well above the reaction baseline, however, and were considered as positives by visually inspecting the multicomponent fluorescent data, which displayed clear amplification. As no Ct values were generated, ANCOVA analysis resulted in no significant differences between COI in a COI/Cyt *b* duplex and COI in COI/Cyt *b*/HemT triplex.

Zebra mussel DNA was not detected in harbours sampled in May 2014 except for Winnipeg Beach. The developed assays may not have been sensitive enough to detect zebra mussel eDNA during the early season. Conversely, assuming all three assays maintained their high sensitivity at low DNA concentrations (Table 3) and that there was sufficient relief of inhibition (via the Environmental MasterMix 2.0 and template dilution), this low detection rate may reflect a low abundance of zebra mussels within each of the sampled harbours due to biotic and anthropogenic factors. Indeed, data collected in 2013 indicated that zebra mussel populations were in an early stage of colonization (DFO 2014). The reduced water levels and freezing temperatures in winter likely resulted in the death of zebra mussels located above the surface water or within the frozen surface due to desiccation (Grazio and Montz 2002; Werner and Rothhaupt 2008; Sousa et al. 2012; Leuven et al. 2014). It is unlikely that spawning had occurred by May 2014, as surface water temperatures for each of the sampled harbours ranged from 2.4 to 7.7 °C and the lower threshold for spawning is 12 °C (Sprung 1989; McMahon 1996; Ram et al. 1996). While lower temperatures would have reduced detectability due to the lack of eDNA input from spawning (Spear et al. 2015; Gingera et al. 2016), eDNA persistence can increase under lower temperature which would have translated to an increased likelihood of detection for eDNA already present in the harbours. Finally, the dilution factor of any eDNA present in the harbours in spring would likely be very high due to the large volume of water in the harbours, especially after snow melt earlier in the season; increased

dilution of eDNA may ultimately be typical for early season sampling.

In contrast to the above, water samples collected in October from all four harbours yielded positive zebra mussel eDNA detections, despite treatment in June 2014 of each of these four harbours with potash. The increase in eDNA detection even after treatment suggests that zebra mussels re-established themselves within the harbours and underwent a growth period between the end of treatment and October. Zebra mussel growth rates, gametogenesis, and spawning are temperature dependent (Ram et al. 1996; Lucy 2006) with their principle growth season extending from May to September (Hecky et al. 2004). In Hnausa Harbour, which was not treated with potash, 100% of the water samples collected in October 2014 tested positive for zebra mussel DNA. eDNA has been demonstrated to persist from 1 to 8 weeks (Dejean et al. 2011; Thomsen et al. 2012a, b; Pilliod et al. 2014); therefore, high detection rates are not likely due to die-off during potash treatment. Future research on the effects of potash treatment on eDNA signals would be beneficial for the future implementation of zebra mussel monitoring using eDNA.

No zebra mussel DNA was detected from the samples collected from Grindstone, Gull, and Hecla Village harbours. At the time of sampling (November 5, 2014), local observation and CWS veliger sampling data had not detected zebra mussels at these locations. This indicates that the DNA of local biota does not result in false positives (i.e., the amplification of non-target DNA). However, it is important to note that these samples were collected late in the season compared to the others (collected May 14 and October 1); abiotic conditions typical of this late in the season, such as surface water temperature < 10 °C, may have directly and/or indirectly affected eDNA detectability because of low source amounts. Although lower temperatures generally reduce metabolic rate in ectotherms, Klymus et al. (2015) demonstrated that variations in water temperature had no effect on DNA shedding rates of two freshwater fish species, bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845) and silver carp *H. molitrix* (Valenciennes 1844). However, lower water temperatures may increase the persistence of DNA in the water column by decreasing microbial enzymatic activity (Zhu et al. 2006; Dejean et al. 2011; Strickler et al. 2015).

Similarly, none of the samples collected from five of the six locations sampled along the Red River in 2014 were positive for zebra mussel DNA. The most northerly location sampled along the Red River, however, yielded positive results for zebra mussel DNA. This location, a float-plane dock, situated north of Selkirk had a positive detection of 50% (1 of 2 samples). Initially, these samples were collected to ensure that DNA from local organisms was not amplified by the zebra mussel and *Dreissena* primers. All field, filtration, and qPCR negative controls were free of zebra mussel DNA and all sequencing data confirmed qPCR amplicon species identity as zebra mussel; thus, we consider this to be a true positive detection for the presence of zebra mussel at this location independent of direct detection approaches. Zebra mussels were first discovered in the Manitoban portion of the Red River in June 2015, in Selkirk Harbour. Prior to this, zebra mussels were not thought to be present in the Manitoban portion of the Red River. The float-plane dock at which the positive detection was found would typically be considered a "high-risk" area for zebra mussel invasion due to the high amount of recreational traffic, the largest anthropogenic vector of spread (Carlton 1993; Johnson and Carlton 1996; Johnson and Padilla 1996; Padilla et al. 1996; Schneider et al. 1998; Buchan and Padilla 1999). This result potentially refines the date of zebra mussel invasion to 2014; additionally, it represents a "real world" example of eDNA techniques being successfully used to detect the leading edge of an invasion event. Similar examples of successful eDNA detection of AIS include the positive detection of Asian carp eDNA from samples collected from the Chicago Area Waterway System and in the western basin of Lake Erie within 6 and 4 km, respectively, from where bighead carp were collected in previous years (Jerde et al. 2013). Moreover, given that this location serves as a float-plane base, zebra mussel detection here raises concerns regarding future spread of this AIS through this vector.

In summary, this study is proof-of-concept that eDNA detection can be used in resource monitoring programs concerned about the initial periods of invasion or colonization where zebra mussel abundance is presumably low (e.g., early spring after winter die-off and in newly invaded areas) as exemplified by this study on Lake Winnipeg and the Red River, Manitoba. We provide three qPCR assays that have been extensively tested, and which can be multiplexed into a single qPCR reaction and readily implemented for wide-scale monitoring of zebra mussels in Manitoba and other North American freshwater systems. This multiplexed reaction incorporates two assays specific to zebra mussels and one *Dreissena* (zebra and quagga mussel) assay, and allows for the detection of three separate mitochondrial genes within each qPCR reaction, which reduces the likelihood of false negatives. Zebra mussel DNA was detected in the early spring at Winnipeg Beach Harbour but not at other harbours. The developed assays may not have been sensitive enough for

detection; however, zebra mussel abundance was potentially low due to potential die-off during the winter season. Detection of zebra mussel DNA increased later in the season, even after die-off due to potash treatment three months prior. We also report the first detection of zebra mussels in the Manitoban portion of the Red River at a float-plane dock north of Selkirk Harbour upstream of Lake Winnipeg. Future efforts should focus on consistent water sampling for eDNA detection over an entire season in order to evaluate how the eDNA signal for zebra mussel changes throughout a growth and reproductive season. CWS has committed resources to implementing mandatory zebra mussel inspection stations for portable watercraft, cleaning stations for recreational watercraft, and has expanded legislation to help reduce zebra mussel spread within the province. Other monitoring programs, such as the Asian carps eDNA program in the United States, have had success in identifying invaded areas using eDNA techniques (Jerde et al. 2011, 2013). Similar efforts implemented in Manitoba could be instrumental in reducing the potential impact of zebra mussels in local waterbodies as well as western Canada.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Accession numbers of sequences used in assay development for the cytochrome oxidase *c* subunit 1 (COI), cytochrome *b* (Cyt *b*), and16S rRNA mitochondrial genes.

Table S2. Sample site coordinates, number of filters tested, and percent of samples that detected zebra mussel DNA within harbours in the south basin of Lake Winnipeg.

Table S3. Sample site coordinates, number of filters tested, and percent of samples for which zebra mussel DNA was detected within harbours in the northern portion of the south basin of Lake Winnipeg and docks from six locations along the Red River.

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