Environmental DNA monitoring of invasive zebra mussels: Method design, monitoring tool comparisons, refinement of methods, and considerations for management

By

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Abstract

Interest in environmental DNA (eDNA) for the detection of aquatic invasive species (AIS) is increasing. Considering the invasion of zebra mussels *Dreissena polymorpha* into Lake Winnipeg in 2013, the development of eDNA monitoring methods may help managers prevent further spread of this AIS. For this thesis, sensitive and species-specific eDNA quantitative PCR assays and field/laboratory protocols were developed to be used for monitoring in western North America. These eDNA methods were found to be more sensitive for detection than was plankton netting for presence/absence of veligers. Environmental DNA target gene concentration and veliger abundance appear to be positively correlated. Furthermore, veliger abundance may account for much of the variation in eDNA detections. Refinement of eDNA methods is also presented here to improve detection. The work in this thesis provides considerations and guidelines for managers using eDNA as a detection tool for zebra mussels.

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CHAPTER 1. GENERAL INTRODUCTION

There is increasing interest in the use of molecular detection methods such as environmental DNA (eDNA) as a monitoring tool for aquatic macroorganisms such as aquatic invasive species (AIS). Method development and interpretation of eDNA data for monitoring are not well understood. Explicit quality assurance and control protocols (QA/QC) for all aspects of eDNA data collection are therefore required for molecular detection to be considered a viable tool for managers. The invasion of zebra mussels *Dreissena polymorpha* into Lake Winnipeg furthers the need for such protocols. Ultimately, these will help prevent further spread of invasive mussels by improving collection of occurrence data and informing control efforts by managers.

Considering the lack of thoroughly tested invasive mussel eDNA detection methods for monitoring, zebra mussels provide an opportunity to examine the effectiveness of eDNA during the early stages of an invasion. The themes of this thesis are as follows: 1) the development and testing of field and laboratory protocols for zebra mussel eDNA testing (Chapter 2); 2) comparisons between conventional zebra mussel sampling methods and newly developed eDNA methods (Chapter 3); and 3) the refinement of methods to improve detection of low-concentration eDNA samples. Finally, the goals of this thesis, aside from providing deliverables such as tested zebra mussel eDNA detection methods, are as follows: 1) determine where zebra mussels were located in Lake Winnipeg at the time of this thesis; 2) determine how zebra mussels may spread in the near future; and 3) provide considerations for a monitoring program which addresses occurrence, geographic spread (including abundances in focal systems), and deliver adaptation and control options for zebra mussels.

1.1 Aquatic invasive species detection

As global human activities expand and continue to remove natural barriers which previously isolated aquatic species, aquatic invasive species (AIS) will continue to be a threat to ecologically and economically important ecosystems (Elton 1958). AIS are a leading cause of native biodiversity loss (Wilcove et al. 1998; Sala et al. 2000), can have devastating socio-economic effects on commerce (Pimental et al. 2001; Reaser et al. 2007), can result in a shift in community structure and massive changes to ecosystem function (Elton 1958; Simberloff 1981; Coblentz 1990), and cause alterations to habitat conditions and food webs which facilitate further invasions and disturbances (Simberloff and Von Holle 1999).

Freshwater systems are considered especially vulnerable to AIS due to their greater degree of isolation and endemism in the environment (Richter et al. 1997; Dudgeon et al. 2006). Due to this threat, resource agencies and governments are developing programs and management strategies to reduce AIS introductions.

Management priorities and tools which inform priorities must be developed, as (in the case for Canada) at-risk habitats are expansive and funding for management activities is limited. Management and control of AIS includes several objectives: 1) eradication in localized areas; 2) reducing spread; 3) reducing impact; 4) education and outreach; and 5) developmental research on control methods. While eradication in localized areas is considered a key management component (Davison et al. 2017), biological invasions which result in established populations are generally considered irreversible. Agencies therefore focus resources on prevention (i.e., preventing the entry or secondary spread of AIS into a new range). Methods of determining the distribution of AIS are central to identifying potential invasion pathways and controlling spread.

Recently, interest in molecular methods of detection that can measure the distribution of aquatic macroorganisms has been increasing.

1.2 Environmental DNA

Molecular detection of aquatic organisms is built upon the use of "*DNA barcoding*". It was first suggested by Herbert et al. (2003) that individual species could be routinely identified based on short and specific fragments of DNA; specifically, a 658 base pair (bp) region of the mitochondrial (mt) cytochrome oxidase c subunit I gene. This method, termed "*DNA barcoding*", has become a commonly used technique due to the need to identify species rapidly, the reduced cost of DNA sequencing, and a decrease in available taxonomic expertise (Armstrong 2005; Valentini et al. 2009; Hajibabeai 2012; Taberlet et al. 2012). "*DNA barcoding*" combines polymerase chain reaction (PCR), a technique that amplifies (increases copy numbers) of targeted DNA thus increasing detectability, and non-specific "universal" molecular markers (i.e., primers that will amplify a wide range of taxa) to produce DNA fragments that can be used to identify the species of a specimen via Sanger sequencing.

Molecular markers that target the barcoding regions of the mt genome can also be designed to be species-specific so that only DNA from the target species will be amplified and subsequently detected. Tissue-derived DNA is most commonly used for species identification and genetic/genomics research; however, DNA can also be isolated from environmental sources (e.g., soil, water). These environmental sources contain the DNA of a target macroorganism via vectors such as epidermal cells, decomposition, feces, urine, and mucus (Lydolph et al. 2005; Waits and Paetkae 2005; Haile et al. 2009; Taberlet et al. 2012). Environmental DNA (eDNA) analysis was first described as a method for isolating microbial DNA for microbial community profile analysis (Ogram et al. 1987).

Ficetola et al. (2008) were the first to apply eDNA as a tool for detecting aquatic macroorganisms using invasive American bullfrog *Lithobates catesbeianus* as a model organism. Since then, interest has increased in the molecular detection of aquatic macroorganisms (Jerde et al. 2011, 2013; Dejean et al. 2012; Thomsen et al. 2012a; Mahon et al. 2013; Pilliod et al. 2013). Several independent research groups have developed methods of eDNA analysis for endemic and/or endangered aquatic macroorganisms (Goldberg et al. 2011; Foote et al. 2012; Olsen et al. 2012; Thomsen et al. 2012a, 2012b; Deiner and Altermatt 2014) or invasive (Ficetola et al. 2008; Jerde et al. 2011; Dejean et al. 2011; Collins et al. 2013; Goldberg et al. 2013; Mahon et al. 2013; Takahara et al. 2013; Ardura et al. 2017; Gingera et al. 2016, 2017).

Benefits of eDNA sampling compared to conventional sampling methods include a relatively low financial cost (Ardura et al. 2017; Lacoursière-Roussel et al. 2016), greater capacity to collect multiple samples over a large geographic area (McKelvey et al. 2016), a reduced impact on the environment (Goldberg et al. 2016), and greater detection sensitivity when sampling for elusive and/or rare organisms (Schmelzle and Kinzinger 2016). However, some studies have found that eDNA was less sensitive than conventional methods such as scuba diving (Ulibarri et al. 2017). Environmental DNA has been used to determine the distribution of aquatic organisms (McKelvey et al. 2016; Schmelzle and Kinzinger 2016) and monitor invasion fronts of certain AIS species (Jerde et al. 2011). Ultimately, eDNA may provide managers with a new tool to improve management and control of AIS.

1.3 Zebra and quagga mussels

The zebra mussel and quagga mussel *D. bugensis* 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; USACE 2015).

These species of dreissenid mussels have two distinct life forms: 1) the pelagic microscopic larval form (i.e., veliger), and 2) the sessile mytiliform juvenile and adult form. These species are dioecious and reproduce via external fertilization into the water column. Zebra mussel spawning is limited by temperature and has a lower limit of 12°C (Sprung et al. 1989; Ram et al. 1996; McMahon et al. 1996) but spawning in temperatures as low as 10°C has been recorded (Mantecca et al. 2003). Typically, temperatures of the south and north basin of Lake Winnipeg are above 12°C by late May and June, respectively (WSD 2011; *see* Section 1.4). It can therefore be expected that spawning may begin during these periods. Both the south and north basin temperatures drop below 12°C in October (WSD 2011). Spawning would likely not occur in Lake Winnipeg so late in the season. Fertilized zebra mussel eggs develop into veligers within three to five days and are free floating in the water column for up to one month (Mackie and Schloesser 1996). Although veligers have a limited capacity for active movement, they primarily move passively through the environment along with water currents and flow (Mackie and Schloesser 1996). The passive movement and extended period in which the veliger is in the water column improves the potential for dispersal. Veliger development typically occurs between 12°C and 24°C (Pollux et al. 2010). Greater abundances of veligers have been collected in Lake Winnipeg in September (pers. comm. Laureen Janusz, WSD). Near the end of their larval development (two to three weeks), veligers attach themselves to substrates with byssal threads (MacIsaac 1996) where they develop into juvenile mytiliform mussels. Principal mytiliform

development occurs between May and September and sexual maturity typically coincides with a shell length of eight to 10 mm (Benson et al. 2012).

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}) over short time periods (Ludyanskiy et al. 1993; Effler and Siegfried 1994; Patterson et al. 2005). These invasive mussels can therefore severely damage submerged mechanical equipment, clog pipelines, and suffocate native mussel species (Ludyanskiy et al. 1993; Tucker et al. 1993). The habitat preferences of zebra and quagga mussels are very similar, 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). Quagga mussels can be distinguished morphologically from zebra mussels by their rounded shell surface versus the more angled dorsal and ventral surfaces found on zebra mussels (May and Marsden 1992). The two species can also be distinguished genetically (Stepien et al. 1999). Current evidence suggests 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 an invaded ecosystem (Pace et al. 1998; Wong et al. 2003; Higgins and Vander Zanden 2010). Filtration rates are influenced by several factors, such as mussel size, temperature, and size/type of algae and bacteria (Mackle and Schloesser 1996; Benson et al. 2012). Also, food concentration has been found to be positively correlated with filtration capacity (Baldwin et al. 2002). 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* (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 which can potentially alter the structure of lake substrates (Reeders et al. 1989; Reeders and Bij De Vaate 1990; MacIsaac 1996).

The primary vectors for veliger spread between waterbodies are human-mediated mechanisms such as commercial vessels, recreational boats and float-planes (Carlton 1993; Johnson and Padilla 1996; Padilla et al. 1996; Schneider et al. 1998; Buchan and Padilla 1999). This is due to the potential for 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). 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).

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) to eradicate zebra mussels in Lake Winnipeg. Bioassays showed 100% mortality of zebra mussels within the treated areas. 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. Likewise, an understanding of the hydrology of Lake Winnipeg can be used to help inform the potential pathways of spread for zebra mussels and therefore also inform targeted sampling efforts during monitoring.

An eDNA assay for zebra mussels has been reported by Ardura et al. (2017). This cPCR assay incorporates a novel forward marker and a reverse marker developed by Palumbi (1996) to amplify a 258 bp fragment of zebra mussel 16s rRNA gene. During assay development, Ardura et al. (2017) performed both *in silico* and *in vitro* testing to ensure species-specificity and sensitivity. Assays were tested on water samples collected from the south-eastern part of the Baltic Sea where more closely related species are present (compared to Lake Winnipeg). However, no other species within the genus *Dreissena* were included, so that it is not yet clear if the assay is zebra mussel-specific or *Dreissena*-specific. Furthermore, this assay was only tested on a limited number of samples (total of nine samples across four separate locations) and, although it was reported as being highly sensitive, it was only able to detect two of three samples

at the site with high zebra mussel abundance. High-Throughput-Sequencing (HTS) was found to be more sensitive (all three high abundance sites yielded positive results). Also, the detection limit of the assay was reported as 0.7 ng/µL under ideal conditions (i.e., no inhibition); however, detection limits in ng/µL units is not especially useful for eDNA analysis. Providing information on copies/µL is more useful as it provides an absolute measurement of sensitivity compared to ng/µL. Thus, although promising, a more sensitive and well tested assay is required for monitoring efforts.

1.4 Lake Winnipeg

Zebra mussel veligers have significant potential for passive dispersal via water flow and have been recorded to disperse up to 300 km downstream of spawning sites (Bially and MacIsaac 2000). Thus, the hydrology and movement of water in Lake Winnipeg has direct implications on zebra mussel spread and therefore sampling site locations. Three major river systems flow into Lake Winnipeg: 1) the Red River (11%); 2) the Winnipeg River (45%), and 3) the Saskatchewan River (35%). In total, these three rivers account for more than >90% of the total flow into Lake Winnipeg (pers. comm. Jeff Long, WSD). Of these three rivers, zebra mussels were only found in the US portion of the Red River prior to the first Lake Winnipeg zebra mussel sighting in 2013. Zebra mussels were later visually identified in the Manitoban portion of the Red River in 2015. Assuming passive dispersal as the primary vector of spread in Lake Winnipeg, zebra mussel veligers would have entered Lake Winnipeg through the Red River and into the south basin. Presumably, this vector of spread would have resulted in a gradual movement of zebra mussels northward from the US; however, zebra mussels were not recorded in the Manitoban portion of the Red River until two years after they were found in the south

basin of Lake Winnipeg. This evidence suggests that vectors other than passive spread (e.g., human transport) may have caused the introduction of zebra mussels in Lake Winnipeg.

Water movement and flow within Lake Winnipeg is a major vector that will influence the distribution and spread of zebra mussels in the Lake. Generally, water flow within the south basin moves in a counter-clockwise pattern, eventually flowing northward through the Narrows between the south and north basins (pers. comm. Jeff Long, WSD). Currently, the largest concentration of zebra mussels is found in the south basin. Passive dispersal via water movement in Lake Winnipeg mirrors the patterns of spread seen thus far (i.e., spread from south basin harbours into the narrows followed by the north basin. Spread of zebra mussels will continue in the north basin, especially in areas with submerged infrastructure such as Limestone Bay and Grand Rapids.

The Nelson River drains into Hudson Bay and is the only river that flows out of Lake Winnipeg. The Nelson River accounts for 82% of water loss out of the lake with evaporation accounting for the other 18% loss (WSD 2011). Eventually, passive veliger dispersal will lead to spread into the Nelson River at which point passive spread will be limited by low temperature and salinity tolerances (Therriault et al. 2012). Dispersal into areas upstream of invaded sites will most likely be facilitated by recreational boating and commercial transport of boats overland (Therriault et al. 2012).

1.5 Factors that influence eDNA detection

Detection of eDNA from an aquatic system is dependent on two categories of factors: 1) environmental factors that affect the concentration and distribution of sources of eDNA in an aquatic environment; and 2) laboratory processes, which affect the ability of a researcher to carry

enough eDNA through each process to enable detection and ensure confounding factors do not influence the results.

1.5.1 Natural factors

Two natural factors that affect detection during eDNA monitoring are: 1) concentration of eDNA at the sample site; and 2) the distribution of the eDNA within the aquatic environment that is being sampled.

The concentration of eDNA is influenced by two competing processes, the rate of eDNA production by the target organism(s) and the rate of degradation/removal (e.g., adsorption of eDNA source to sediment) of eDNA due to environmental factors. Rates of eDNA production will vary depending on the physiology (Eichmiller et al. 2014; Laramie et al. 2015) and abundance (i.e., density, biomass) of the target organism (Takahara et al. 2012; Thomsen et al. 2012a; Goldberg et al. 2013; Doi et al. 2015; Klymus et al. 2015; Eichmiller et al. 2016; Gingera et al. 2016).

Rates of eDNA degradation/removal increase with exposure to the environment. Factors such as microbial activity influenced by temperature and UV exposure will increase eDNA degradation (Zhu 2006; Schapiro 2008; Takahara et al. 2012; Thomsen et al. 2012b; Pilliod et al. 2014; Barnes et al. 2014; Strickler et al. 2015). Factors that influence dispersal of eDNA within an aquatic environment will affect detection and must also be considered when inferring the scale and presence of the target organism (Deiner and Altermatt 2014; Goldberg et al. 2016; Wilcox et al. 2016).

Animal behaviour (i.e., where the target organism is in the system) is a key factor that influences eDNA distribution. For example, Takahara et al. (2012) found that common carp

Cyprinus carpio would aggregate to the warmest waters within small ponds and that samples collected from these locations had a higher frequency of detections. Water movement and flow affect eDNA dispersal by increasing diffusion via advection (Ficetola et al. 2008; Pilliod et al. 2013; Deiner and Altermatt 2014). For example, Gingera et al. (2016) found that eDNA detection frequency increased with distance from a natural spawning barrier for sea lamprey *Petromyzon marinus*, which they suggested was due to an accumulation of eDNA downstream.

1.5.2 Field/laboratory processes and quality assurance/control

Sample processes used in the field and laboratory have significant influence on the detection of eDNA sources present in the environment (*see* Appendix A). Unlike environmental variables that affect eDNA detection, these processes can be optimized to improve likelihood of detection, reliability of results, and result interpretation. While conventional monitoring methods for aquatic organisms typically require direct visualization of the target to determine geographic occurrence; eDNA methods are a form of "remote detection" and is a "blind" sampling method (i.e., indirectly infers occurrence and distribution). Although the remote detection of organisms is considered the central strength of eDNA (*see* Appendix A), it also inherently increases the likelihood of inaccurate data collection. Understanding the diagnostic effectiveness of eDNA data is essential to determine where the risks lie during data collection. Quality Assurance and Control (QA/QC) during field/laboratory processes is therefore essential for producing accurate eDNA data. Inaccurate data collection can be divided into two categorical results: 1) false positives; and 2) false negatives. This section will discuss types of false results and provide a brief overview of how field/laboratory processes affect eDNA detection. Optimization of these

processes and their implication regarding eDNA analysis is discussed in the comprehensive methods review (*see* Appendix A).

1.5.2.1 False positives

False positive (henceforth abbreviated to FP) results occur when positive detections are obtained from eDNA samples even though the target organism was not present in the sample area. Generally, FP results can be attributed to three causes: 1) contamination during sample processing; 2) assay non-specificity; and 3) confounding sources of eDNA. This section describes the potential causes and implications of FP results and provides a brief justification for the QA/QC methods used in this thesis.

The most common cause of FP results is contamination of the eDNA sample from other sources (e.g., other samples, nucleic acids on working surfaces or equipment, and/or PCR products present in the work environment). Contamination can be reduced using stringent "clean laboratory" protocols (*see* Appendix A). A review of QA/QC procedures is presented in the comprehensive methods review (*see* Appendix A). The methods presented there should be considered obligatory when conducting eDNA detection work as they minimize the risk of FP results due to method issues.

Methods of identifying contamination are equally important. Contamination can be identified at each stage of eDNA sample processing by including no-template controls (NTC). NTCs do not contain any target DNA and therefore should not test positive during analysis. An NTC which tests positive is therefore a sign of contamination. Also, implementing synthetic positive controls that can be distinguished from target template will allow those using eDNA detection to determine if the source of contamination was from target template or the synthetic

controls (Wilson et al. 2016). This would likewise eliminate the need to remove samples from a data set if contamination was caused by controls.

Secondly, non-target amplification due to non-specific eDNA assays may cause FP results. Therefore, proper assay design and testing are required to ensure FP results do not occur due to poor assay development (Gingera et al. 2017).

Finally, potential causes of FP results are confounding sources of eDNA within a water sample. While these parameters are not field/laboratory processes, they may result in FP results and therefore were included here. “Allochthonous eDNA” is a source of confounding eDNA described as target template present within a system which originated from outside of the sampled area (Darling and Mahon 2011; Mahon et al. 2013). Speculative vectors of allochthonous eDNA includes the deposition of fecal matter containing prey species DNA via wide-ranging predators (Merkes et al. 2014) or the transport of carcasses via predatory species, human traffic, or flow. These sources remain speculative, however, and no confirmed case exists in the literature. Sediment may also be considered a contaminating source of eDNA as nucleic acids can persist longer in sediment compared to in the water column (Turner et al. 2015). Therefore, in the case of when the target species is extirpated or seasonally absent, transportation and/or disturbance of sediment into the water column may introduce confounding sources of eDNA (Turner et al. 2015). Sediment sampling for aquatic organisms may be a potential tool for developing historical presence of a target (Turner et al. 2015). Also, water movement and flow within a waterbody can relocate an eDNA signal which may skew occurrence data collected by eDNA detection (Ficetola et al. 2008; Pilliod et al. 2013; Deiner and Altermatt 2014; Gingera et al. 2016). These factors present an issue related to interpretation of the results as a positive detection may be “true” in that eDNA of the target is present but the organism is not. Results due

to these confounding sources would likely be weak and inconsistent; therefore, more research is required to determine the extent to which they should be considered during monitoring.

1.5.2.2 False negatives

False negative (henceforth abbreviated to FN) results occur when there are no positive detections obtained from an eDNA sample, but the target is present in the sample area and eDNA is available in the sample collected. Current methods of eDNA analysis for aquatic macroorganisms, although varied, follow a general workflow outlined in Ficetola et al. (2008). Enough eDNA must be carried through each stage of the workflow to enable detection; likewise, laboratory parameters at each stage affect the likelihood of FN results. These stages/parameters are: 1) collection of eDNA-containing material for eDNA extraction and eDNA capture efficiency; 2) sample preservation; 3) eDNA extraction processes and extraction efficiency; 4) concentration of eDNA in the extracted sample; and 5) molecular analysis of eDNA extract (including assay sensitivity and inhibition). FN results are more difficult to identify and assess compared to FP results, but likelihood of occurrence can be reduced by optimizing the above parameters and ensuring proper QA/QC (e.g., positive template controls, internal positive controls). This section provides a brief overview and justification for the methods used in this thesis and describes how the above parameters influence eDNA detection (i.e., may cause FN results). As an overview of the current methods used in eDNA analysis was considered an inherent objective of this thesis, a more comprehensive review of methods can be found in Appendix A.

The collection of DNA-containing material for eDNA extraction and the concentration of eDNA material in the water is important to consider when conducting eDNA monitoring. For

example, low eDNA concentrations, like those presumably found during the early stages of an invasion (Gingera et al. 2017; Chapter 2), will be more likely to cause a FN result. It is therefore important that there is efficient eDNA capture during water sampling as this ensures the few detectable eDNA fragments present are captured. In this thesis, water filtration through a glass fibre filter was chosen for collection of DNA-containing material for eDNA extraction. A greater volume of water can be processed in a single sample when filtered, therefore increasing the likelihood of capturing target eDNA. Glass fibre filters were chosen because they are cost effective and have been used successfully in previous studies (Jerde et al. 2011; Gingera et al. 2016, 2017; Lacoursière-Roussel et al. 2016).

Preservation of the collected eDNA sample prior to extraction has an intuitive influence on the likelihood of FN results. Optimized preservation reduces the degradation of detectable eDNA prior to extraction and analysis. In this thesis, sample (i.e., filter) preservation methods were dependant on where sampling took place. Ethanol was used when available/appropriate (Chapter 2, 4) and desiccant beads were used when ethanol was logistically difficult to use (Chapter 3). Preserved samples were kept at -80°C prior to extraction.

Nucleic acid extraction efficiency of eDNA from samples is critical, as low efficiency would result in loss of eDNA from the sample subsequently increasing the likelihood of FN results. Loss of template due to low extraction efficiency can be identified with the inclusion of a positive control during extraction. If a positive control tests as negative, this would be an indication of potential FN results in other samples. The inclusion of positive controls during extraction should be considered an essential QA/QC measure when conducting eDNA monitoring. (Wilson et al. 2016). In this thesis, eDNA extraction was done using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). This method has been found to be

effective in other studies (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011, 2013; Foote et al. 2012; Thomsen et al. 2012a, 2012b; Takahara et al. 2013; Gingera et al. 2016, 2017), is easily scaled-up for many samples, and is cost-effective.

The final eDNA concentration of a sample post-extraction is dependent on all the factors described above. If the final concentration is low, the likelihood of a FN result is increased. For example, ultra-low concentrations of eDNA (i.e., < 1 target copy/μL) may result in < 1 target copy/PCR reaction (rxn), therefore no detection will be made as no DNA is available. Sample enrichment could be used to increase eDNA concentration; however, this has not been explored in the literature. The most common way to ensure detection of low eDNA concentrations is to add large volumes of extract into a reaction and to develop highly sensitive PCR assays.

Assay sensitivity is measured as a limit-of-detection (LoD) and is crucial for detecting small concentrations of eDNA. Sensitivity can be increased by targeting small fragments of 50-150 base pairs. This increases the likelihood of amplifying degraded DNA (Goldberg et al. 2016). Targeting the mt genome is also advisable as mtDNA is more abundant in cells than genomic DNA (gDNA). Assay sensitivity can also be improved by ensuring the target fragments are consistent across a wide geographic range so that FNs do not occur because different haplotypes of the same organism are present. Inhibitory substances may also reduce assay sensitivity and lead to FN results. Inhibitory substances are a large and diverse group of molecules that are abundant in aquatic environments and can reduce the efficiency of PCR based molecular detection methods which (McKee et al. 2015). These substances are co-eluted alongside nucleic acids and are therefore typically present in eDNA samples. FN results may occur if the concentration of inhibitors is great enough. The effects of inhibition on a PCR can be identified with the addition of an Internal Positive Control (IPC) into a reaction (Dingle et al.

2004). IPCs are exogenous DNA that are not amplified by the target assay but will amplify alongside the target assay via a second set of molecular markers (i.e., a multiplex). If a reduction in PCR efficiency is recorded for the IPC compared to a control sample, it is likely that inhibitory substances are present. However, the inclusion of an IPC may not provide an accurate assessment of inhibition if the inhibitor interacts with the target DNA, assays, and/or IPC sequence differently (Chapter 4). Several methods of inhibitor removal exist; a review of these method can be found in the comprehensive methods review (*see* Appendix A). Finally, quantitative PCR (qPCR) methods were developed in this thesis as it is a common PCR method, is compatible with digital droplet PCR (ddPCR), required less expertise than High-Throughput-Sequencing (HTS), and is more sensitive than cPCR.

1.6 Thesis objectives

The overall focus of this thesis is to develop eDNA methods that further the capacity to establish an effective monitoring program for zebra mussels and quagga mussels in Manitoba and western Canada. The approach discussed in this thesis is also applicable to eDNA monitoring of AIS generally.

The first theme of this thesis is to develop and test field and laboratory zebra mussel eDNA methods to ensure accurate distribution data collection during the early stages of an invasion event. Functional eDNA qPCR fluorescent probe assays were developed for molecular monitoring of zebra mussels and quagga mussels in North American freshwater systems. Currently, qPCR is the most common method of molecular detection for eDNA analysis (Takahara et al. 2012; Pilliod et al. 2013; Wilcox et al. 2013; Amberg et al. 2015; McKee et al. 2015; Gingera et al. 2017). Multiplexing several qPCR assays allows for the parallel detection of

>1 target sequence and for the incorporation of an IPC. For these reasons, qPCR fluorescent probe assays were developed in lieu of other molecular detection methods.

The second theme of this thesis is to assess the difference in positive detections between the developed zebra mussel eDNA assays and veliger plankton netting, a commonly used zebra mussel monitoring tool. This comparison analysis will provide insight into the effectiveness of eDNA and if there are benefits to using eDNA alone or in conjunction with plankton net sampling. This comparison may provide an opportunity to analyze the relationship, if any, between eDNA detection and abundance of veligers captured. Direct comparisons of eDNA detection methods and other detection tools for invasive mussel species have not yet been investigated in the literature; therefore, this work will provide valuable insight for managers who are tasked with reducing the spread of invasive mussels.

The third theme of this thesis is to investigate how previously untested (for eDNA research) methods of sample enrichment may affect eDNA assay performance and if an increase of sample DNA concentration may reduce the likelihood of false negatives. This research will adapt methods used in other fields of research which commonly use poor quality and low concentration DNA samples, such as ancient DNA (aDNA) research and forensics. This work will be directly applicable to eDNA detection of AIS/at-risk species and will be applicable to eDNA research, not solely AIS monitoring.

Overall, these themes and the results herein will help address the biological issues of where zebra mussels are in Lake Winnipeg, how their spread has progressed since the first sighting in 2013, how best to design an eDNA monitoring program to address occurrence, geographic spread, and abundance, as well as deliver adaptation and/or control options in the future.

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CHAPTER 2. ENVIRONMENTAL DNA QUANTITATIVE PCR ASSAY DEVELOPMENT FOR ZEBRA MUSSEL AND THE GENUS *DREISSENA*

2.1 Abstract

Zebra mussel *Dreissena polymorpha*, an invasive bivalve in North America, was first recorded in Lake Winnipeg in 2013. Quagga mussel *D. bugensis*, 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, two species-specific and one *Dreissena*-specific qPCR-based environmental DNA (eDNA) assays were developed and 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%. 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 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, this work demonstrates 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.

2.2 Introduction

Zebra mussels *Dreissena polymorpha* (Pallas, 1771) and quagga mussels *D. bugensis* are two bivalve aquatic invasive species (AIS) which have gained notoriety due to their biofouling properties. Dreissenid mussels have two distinct life forms: 1) the microscopic larval form (i.e., veliger), and 2) the sessile mytiliform juvenile and adult form. Veligers predominantly float passively within the water column, whereas juveniles and adults adhere to solid surfaces such as rock, wood, watercraft, 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). Coupled with a high reproductive potential (Walz 1978; Sprung 1990, 1993) and quick maturation rates (Borcherding 1991), zebra and quagga mussels can reach very high densities (> 1 million individuals m^{-2}) (Ludyanskiy et al. 1993; Effler and Siegfried 1994; Patterson et al. 2005) and potentially damage anthropogenic structures and suffocate native species (Ludyanskiy et al. 1993; Tucker et al. 1993). The primary vectors of spread for dreissenid mussels are human-mediated transportation 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). Because of this, dreissenid mussels have been able to extend their range throughout Europe and North America (Hebert et al. 1989; Johnson and Padilla 1996; Vanderploeg et al. 2002; USGS 2016). Since their initial introduction in 1988 in Lake St. Claire (Hebert et al. 1989), zebra mussels have spread to all 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; Fig 2.1); 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 (WSD 2014) to eradicate zebra mussels in Lake Winnipeg. After treatment, zebra mussels re-established in all four harbours, suggesting that individuals recolonized from outside of the harbours. 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 water bodies 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 mussels cannot be differentiated at the veliger stage (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 et al. 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 comprise 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, which amplify and detect fragments of DNA specific to a target organism, are particularly 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 monitor species' distributions quickly and accurately 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, two species-specific genetic assays for zebra mussel and one presumptive genus-specific assay for *Dreissena* (able to detect both zebra and quagga mussels) were developed and tested, resulting in 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 (FN), while simultaneously allowing for limited indirect detection of quagga mussel. Following the evaluation of all three individual assays two of them were incorporated 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 FNs. Sites were sampled 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.

2.3 Methods

2.3.1 Marker design

A series of genetic markers and probes were designed to identify zebra mussel eDNA diagnostically. 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 many 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 (Table 2.1). 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; GenBank 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 the province of Manitoba Water Stewardship Division (WSD) 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*, fatmucket clam *Lampsilis siliquoidea*, flutedshell *Lasmigona costata*, giant floater *Pyganodon grandis*, mapleleaf *Quadrula*, plain pocketbook *Lampsilis cardium*, pink heelsplitter *Potamilus alatus*, threeridge *Amblema plicata*, Wabash pigtoe *Fusconaia flava*, and white heelsplitter mussel *Lasmigona complanata* collected from either the La Salle River, Manitoba or Sydenham River, Ontario (Table 2.2). 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.

2.3.2 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

free of zebra mussels were sampled. Balsam Bay, Gimli, Silver, and Winnipeg Beach harbours, known to contain zebra mussels, were sampled on May14 (prior to eradication efforts) 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 on October 1, 2014, only. 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 ranged from 2.25 to 22.87 km) (Fig 2.1). These locations enabled testing 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. A new pair of nitrile gloves was worn for 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 fibre 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.

All DNA extraction steps were performed in a designated UV sterilized PCR hood equipped with HEPA (High Efficiency Particulate Arrestance) 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 56ºC 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.

2.3.3 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 95ºC; 35 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s, elongation at 72ºC for 30 s; and a final elongation step at 72ºC 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 (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.

2.3.4 Internal positive control validation

The effects of incorporating the hematopoietic cell transcript (HemT) IPC on the Ct values of the Cyt *b* and COI assays as described in Xue et al. (1999) 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* Section 2.4.2). The qPCR final volume, component concentrations, and program were the same as previously described except for 0.2 nm of the HemT forward and reverse primer, 0.1 nm HemT primer, 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 2.4.2) 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).

2.3.5 PCR amplification and evaluation

The detectability of zebra mussel eDNA in Lake Winnipeg was examined by the developed zebra mussel and *Dreissena* assays (Table 2.3). 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 2.3), 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 (NTC) 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.

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).

2.3.6 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 were 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 fibre 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 molecular-grade water were also incorporated with every qPCR reaction to identify any contamination occurring during reaction setup.

2.4 Results

2.4.1 Marker design

PCR cross-amplification tests and BLAST searches confirmed the species- and genus-specificity of the COI, Cyt *b*, and 16S rRNA assays. 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.

2.4.2 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 (Fig 2.2). 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 2.4). 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 2.4). 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 2.4). 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 2.4). 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 2.4). 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 2.4).

2.4.3 Internal positive control

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 (Fig 2.3). 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 2.4) 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 2.4). 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 2.4). 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 2.4).

2.4.4 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) (Fig 2.1; Table 2.5). 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 2.5). 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 2.5). 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 (Fig 2.1; Table 2.6).

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.

2.5 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 detect 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. 2017) 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. 2017), this work examines the applicability of eDNA during an invasion event, when zebra mussel abundance is still 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 detect quagga mussels indirectly 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, 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 it would not have been possible 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, in regions where quagga mussels are a concern, the development of one or more quagga mussel-specific eDNA qPCR assays will also be important; quagga mussels have similar negative impacts on ecosystems and can supplant zebra mussels (Orlova et al. 2005; Ricciardi and Whoriskey 2004; 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 2.4). The 16S rRNA assay performed the poorest in a 16S/COI/Cyt *b* triplex compared to the other two assays (Fig 2.2). Therefore, 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 2.4) 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.

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 principal 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 WSD 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 (FP; i.e., the amplification of non-target DNA; *see* Section 1.5.2.1). 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* and silver carp *H. molitrix*. 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, this is likely to be a true positive detection for the presence of zebra mussel at this location in 2014 independent of direct detection approaches. Mytiliform zebra mussels were first visually detected 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 eDNA 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. Provided here are 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 FN. 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. Reported here is 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 to evaluate how the eDNA signal for zebra mussels changes throughout a growth and reproductive season. WSD 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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2.8 Tables and Figures

Table 2.1 Accession numbers of sequences used in assay development for the cytochrome oxidase *c* subunit 1 (COI), cytochrome *b* (Cyt *b*), and16S rRNA mitochondrial genes.

	Cyt *b*	COI	16S rRNA
Dreissena polymorpha	DQ072117-DQ072126	AF120663	AF038997
	GQ988724-GQ988731	AF474404	AF507049
	JQ762619	AF479636	DQ280038
	JX239088	AF492005	DQ333747-DQ333748
		AF510508-AF510510	EF414464-EF414466
		AM746677	
		AM748975-AM748992	
		AM748996-AM748997	
		AM748999-AM749001	
		DQ333701-DQ333702	
		DQ840121-DQ840131	
		EF414493-EF414495	
		EU484431-EU484435	
		EU484437-EU484456	
		HM210079-HM210081	
		JN543685-JN543687	
		JQ435817	
		JQ771951-JQ771953	
		JX099437	
		JX239087-JX239088	
		KC429149	
		U47653	
Dreissena bugensis			AF038996
			AF507047-AF507048

		AY302247
		DQ333745-DQ333746
		JQ348913
		JX099457
Pyganodon sp.	EF418021.1	EF488181.1
	EF418020.1	EF488183.1
	EF418018.1	
Pyganodon grandis	EF418024.1	EF488182.1
	EF418022.1	
	EF418019.1	
	AF156504.1	
Pyganodon fragilis	EF418017.1	EF488180.1
Pyganodon cataracta	EF418023.1	EF488179.1
	EF418016.1	
Epioblasma triquetra	AF156528.1	
Epioblasma brevidens	AF156527.1	
Lampsilis siliquoidea	AF156522.1	
	AF156521.1	
Lampsilis fasciola	AF156520.1	
Lampsilis cardium	AF156519.1	
	AF156518.1	
Villosa vanuxemensis	AF156526.1	
	AF156525.1	
Villosa iris	AF156523.1	
Actinonaias cacinata	AF156517.1	
Ligumia recta	AF156516.1	
Ligumia nasuta	AF156515.1	
Ptychobranchus fasciolaris	AF156514.1	
Truncilla truncate	AF156513.1	

Amblema plicata	AF156512.1
Quadrula quadrula	AF156511.1
Fusconaia flava	AF156510.1
Plevrabema coccineum	AF156509.1
	AF156508.1
Elliptio dilatata	AF156507.1
	AF156506.1
Strophitus undulates	AF156505.1
Lasmigona compressa	AF156503.1
Alasmidonta marginata	AF156502.1
Unio caffer	AF156501.1
	AF156500.1
Unio pictorum	AF156499.1
Anodonta beringiana	EU327357.1
Anodonta californiensis	EU327356.1
Anodonta nuttalliana	EU327355.1
Anodonta oregonensis	EU327354.1
	EU327353.1
	EU327352.1
	EU327350.1
Anodonta kennerlyi	EU327351.1

Table 2.2 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

Table 2.3 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	115
	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		
	Probe	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		
	Probe	VIC-CCG TAG GGA TAA CAG C-MGBNFQ		

Table 2.4 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

Table 2.5 Sample site coordinates, number of replicates (filters tested), and percent of samples that detected zebra mussel DNA within harbours in the south basin of Lake Winnipeg. Samples were collected on May 14 and October 1, 2014. All sites sampled were considered to contain zebra mussels based on data collected the previous year by Manitoba Water Stewardship Division (WSD) (DFO, 2014).

	Location name	Sample site coordinates	Number of replicates	Detection Rate (%)
May 14, 2014	Balsam Bay Harbour	50.4740660 °N, 96.5839050 °W	4	0
		50.4739470 °N, 96.5833130 °W	4	
	Gimli Harbour	50.630832 °N, 96.962544 °W	3	0
		50.6317870 °N, 96.9823540 °W	3	
		50.6303250 °N, 96.9805450 °W	3	
	Silver Harbour	50.8113640 °N, 96.9616460 °W	2	0
		50.8121290 °N, 96.9636470 °W	2	
	Winnipeg Beach Harbour	50.5081290 °N, 96.9653990 °W	2	33.3
		50.5090210 °N, 96.9675280 °W	2	
		50.5079660 °N, 96.9669650 °W	2	
October 1, 2014	Balsam Bay Harbour	50.4739333 °N, 96.5834167 °W	3	62.5
		50.4739333 °N, 96.5832500 °W	3	
		50.4736500 °N, 96.5832167 °W	3	
		50.4737000 °N, 96.5830167 °W	3	
		50.4738500 °N, 96.5839167 °W	3	
		50.4740717 °N, 96.5838167 °W	3	
		50.4735570 °N, 96.5830090 °W	3	
		50.4753500 °N, 96.5829000 °W	2	
	Gimli Harbour	50.6256833 °N, 96.9878833 °W	4	42.9
		50.6308167 °N, 96.9825167 °W	4	
		50.6293500 °N, 96.9825833 °W	4	
		50.6292833 °N, 96.9842000 °W	4	
		50.6297000 °N, 96.9833500 °W	4	

	50.6317833 °N, 96.9829667 °W	4	
	50.6293833 °N, 96.9801500 °W	4	
Silver Harbour	50.8112833 °N, 96.9617667 °W	4	50
	50.8116500 °N, 96.9612500 °W	3	
	50.8121000 °N, 96.9629667 °W	3	
	50.8136167 °N, 96.9649333 °W	3	
Winnipeg Beach Harbour	50.5076500 °N, 96.9647833 °W	4	71.4
	50.5082667 °N, 96.9652333 °W	4	
	50.5079333 °N, 96.9653667 °W	4	
	50.5092833 °N, 96.9676667 °W	4	
	50.5084333 °N, 96.9680167 °W	4	
	50.5080333 °N, 96.9668500 °W	4	
	50.5075333 °N, 96.9656000 °W	4	
Hnausa Harbour	50.9111500 °N, 96.9817333 °W	4	100
	50.9118500 °N, 96.9812333 °W	4	

Table 2.6 Sample site coordinates, number of replicates, 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. Samples were collected from the Red River November 4, 2014. Samples collected from Grindstone, Gull, and Hecla Village harbours were collected on November 5, 2014. All sites sampled were considered to not contain zebra mussels based on local observation and veliger data collected by Manitoba Water Stewardship Division (WSD) during fall, 2014.

Location name	Sample site coordinates	Number of replicates	Percent of positive samples (%)
Selkirk Waterdome (Red River)	50.169234 °N, 96.861925 °W	4	50
	50.169303 °N, 96.861866 °W	4	
Selkirk Harbour (Red River)	50.149476 °N, 96.855785 °W	4	0
	50.149581 °N, 96.855650 °W	4	
Lockport Floodgate (Red River)	50.085543 °N, 96.941298 °W	4	0
	50.086240 °N, 96.940933 °W	4	
Redwood Bridge (Red River)	49.917692 °N, 97.126324 °W	4	0
The Forks (Red River)	49.885952 °N, 97.132420 °W	4	0
	49.886063 °N, 97.129189 °W	4	
St. Norbert Floodgate (Red River)	49.750839 °N, 97.135083 °W	4	0
	49.750578 °N, 97.134231 °W	4	
Grindstone Harbour	51.2649500 °N, 96.6782500 °W	4	0
	51.2649500 °N, 96.6779000 °W	4	
	51.2649833 °N, 96.6772333 °W	4	
	51.2652000 °N, 96.6778667 °W	4	
Gull Harbour	51.1973667 °N, 96.6171500 °W	4	0
	51.1971000 °N, 96.6179500 °W	4	
	51.1969167 °N, 96.6183167 °W	4	
	51.1963350 °N, 96.6173510 °W	4	
Hecla Village Harbour	51.1322333 °N, 96.6636333 °W	4	0
	51.1321667 °N, 96.6638333 °W	4	
	51.1319667 °N, 96.6633167 °W	4	
	51.1329333 °N, 96.6627667 °W	4	

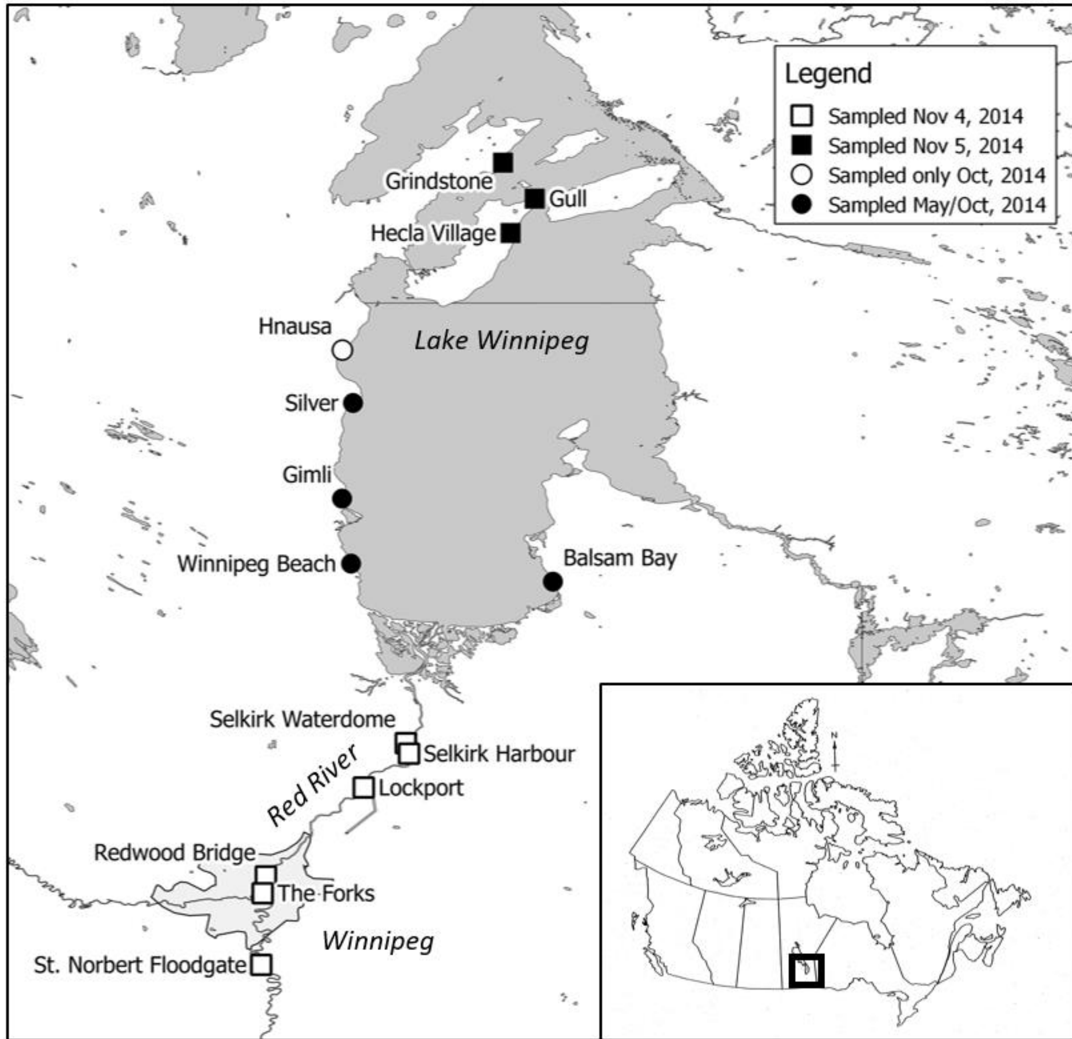

Figure 2.1 Harbour and collection sites for zebra mussel eDNA for Lake Winnipeg and the Red River. Labels indicate general position of harbours and collection sites within the study system. Inset represents approximate location of study sites in Canada.

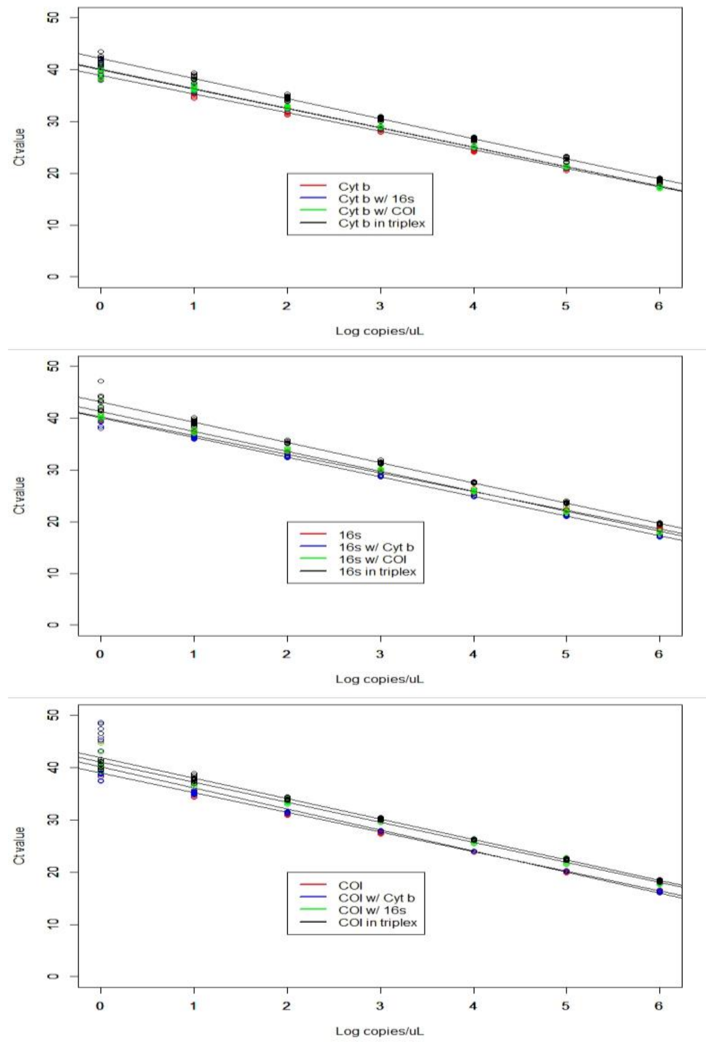

Figure 2.2 Ct values of the cytochrome b (Cyt b) assays (A), 16s rRNA assays (B), and cytochrome oxidase c subunit 1 (COI) candidate assays (C) plotted against log of copies/µL. Each assay was run as either a singleplex, duplex (indicated with companion assay), or as a triplex with a replicate number of n=12 at each copies/µL concentration

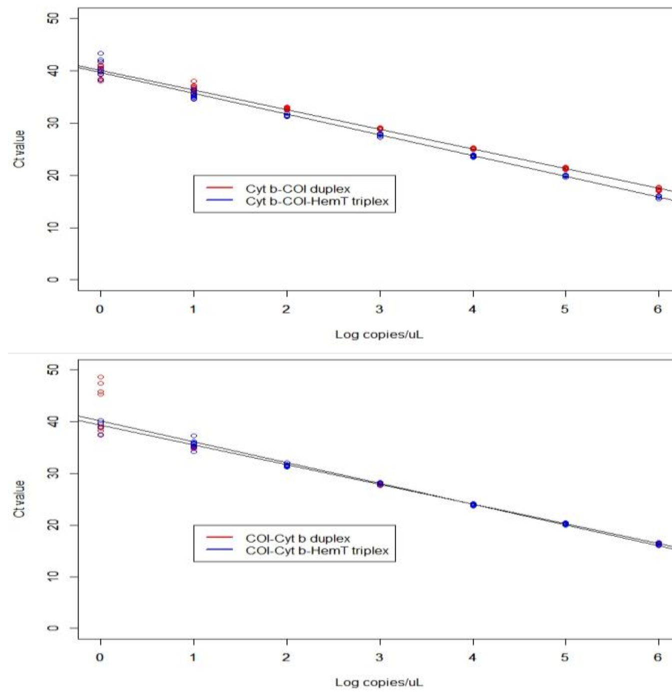

Figure 2.3 Ct values of the cytochrome b (Cyt b) assays (A) and cytochrome oxidase c subunit 1 (COI) candidate assays (B) plotted against log of copies/µL. Each assay was run as either a duplex or as a triplex with the HemT IPC. Replicate number of n=12 at each copies/µL concentration.

CHAPTER 3. QUANTIFYING AN INVASION: ENVIRONMENTAL DNA AS A TOOL FOR ESTIMATING RELATIVE VELIGER ABUNDANCE

3.1 Abstract

Plankton net tows are a common method for invasive mussel detection and monitoring. This method involves the physical capture of the mussel larvae (i.e., veliger) onto plankton nets towed through the water column. Microscopic techniques are subsequently used for visual detection of the veligers. In this study, three environmental DNA (eDNA) assays developed for the detection of gene fragments of Cytochrome *b* (Cyt*b*) and Cytochrome Oxidase *c* subunit I (COI) for zebra mussels *Dreissena polymorpha* and a gene fragment of 16S rRNA for the genus *Dreissena* were compared to veliger count data obtained from plankton net tows, to determine differences in presence/absence detection between the two methods. Parallel eDNA and plankton net samples were collected during the *MS Namao* Fall 2015 survey from 18 sites within Lake Winnipeg (Manitoba, Canada). Environmental DNA methods made novel detections of zebra mussel DNA at three separate sites where no veligers were captured using plankton nets. Current veliger data (at the time) suggested that no zebra mussels were present at these sites. Zebra mussels were subsequently detected in the north basin in 2016, which supports the interpretation that these are not false positives. Environmental DNA methods failed to detect zebra mussel DNA at one site where veligers were captured by plankton net tows. A single site tested negative for both veligers and zebra mussel eDNA; however, both eDNA and plankton net methods were equally effective at detecting presence/absence of zebra mussels at all other sample sites (veliger count ranged from 2 to 2620 individuals). Recent studies have demonstrated that measures of eDNA quantity (e.g., abundance, concentration) can be positively correlated with measurements of target organism quantity (e.g., abundance, biomass, catch-per-unit-effort). Multiple linear

regression (MLR) models and generalized linear mixed (GLM) models showed that target eDNA concentration was positively correlated with veliger count data. Adjusted Akaike's Information Criterion (AICc) and Bayesian Information Criterion (BIC) values calculated for global parameters of MLR models suggest that Cyt*b*+16S and Cyt*b* concentrations best predicted veliger abundance, respectively. AICc and BIC values for GLM models agreed that COI concentration best predicted veliger abundance, accounting for 60.4% variation. The data presented in this study supports that eDNA concentration may be an effective tool for inferring relative veliger abundance.

3.2 Introduction

Monitoring and management of aquatic invasive species (AIS) requires accurate presence/absence and abundance data on broad spatial and temporal scales. Invasive bivalves, such as zebra mussel *Dreissena polymorpha* and quagga mussel *D. bugensis*, have been a concern for both governments and private agencies in North America due to the rapid spread since their discovery in Lake St. Claire in 1988 (*see* Section 1.3) and detrimental biofouling effects (Hebert et al. 1989; Johnson and Padilla 1996; Vanderploeg et al. 2002; USGS 2016). The invasive bivalves can impact the structure and function of the ecosystems they invade (Pace et al. 1998; Wong et al. 2003; Higgins and Vander Zanden 2010) and can cause significant damage to submerged infrastructure (e.g., hydraulic engineering structures, water craft, water intake facilities) (Rajagopal et al. 2005; Brazee and Carrington 2006; Grutters et al. 2012).

Dreissenid mussels have two distinct life forms: 1) the microscopic planktonic larval form (i.e., veliger), and 2) the sessile mytiliform juvenile and adult form. Due to the morphological and behavioural differences between these two life stages, different capture methods are required. For veliger capture, plankton netting is typically used (Mackie and Claudi 2010). Plankton netting methods involve filtering large volumes of water through plankton nets to capture veligers which are suspended in the water column. The resulting samples are processed using the sugar separation method and analyzed under a microscope for visual confirmation of the presence of veligers (Schaner 1990). Settling plate monitoring is a common method used when surveying for mytiliform individuals. Settling plates are physical substrates (e.g., cinder blocks, plastic pails) which are suspended in the water column. These samplers encourage the attachment of mytiliform mussels and are regularly checked for attached individuals (Mackie and Claudi 2010). The plankton net method is usually the preferred method

of detection during the early stages of invasion as it results in earlier detection compared to settling plates (Nalepa and Scholoesse 1993).

Environmental DNA (eDNA) analysis is a molecular method of detection which targets specific fragments of DNA. Sources such as feces, mucus, sloughed off cells, and decomposition introduce eDNA into waterbodies. Sources of eDNA are collected in water sampls and detected using molecular techniques such as quantitative polymerase chain reaction (qPCR) (Takahara et al. 2012; Thomsen et al. 2012b; Pilliod et al. 2013; Wilcox et al. 2013; Gingera et al. 2017, Chapter 2). Detection of a target organism is therefore independent of life stage and can be applied to an aquatic environment regardless of the target's life history traits. This method of detection has been applied successfully as a method of presence/absence detection for both AIS (Darling and Mahon 2011; Jerde et al. 2011, 2013; Amberg et al. 2015; Gingera 2016) and several other freshwater and marine species (*reviewed in* Goldberg et al. 2016).

Molecular detection of environmental DNA (eDNA) from water samples has been demonstrated to be a useful tool for the detection of invasive mussels (Gingera et al. 2017, Chapter 2). Gingera et al. (2017) have used zebra mussel/*Dreissena* qPCR eDNA assays for detecting the presence/absence of zebra mussels from water samples collected from Lake Winnipeg and Red River (Manitoba, Canada) early in an invasion event. This novel detection method for invasive mussels has not been compared quantitatively to the most common method of zebra mussel sampling (i.e., plankton netting). It is therefore unclear how these two methods differ regarding sensitivity and detection. Parallel sampling and analysis of these two methods may inform how eDNA detection can be used to improve detection and control of invasive mussels in North America.

One advantage of data generated via plankton net detection for invasive mussels is that veliger density and biomass can be measured and used to determine the progression of an invasion. Whereas with eDNA detection using qPCR, only the relative concentration of target eDNA in a sample can be measured. Biomass of a target organism has been demonstrated to have a positive correlation with eDNA concentration and subsequent detection in the lab (Takahara et al. 2012; Thomsen et al. 2012a; Goldberg et al. 2013; Doi et al. 2015; Klymus et al. 2015; Gingera et al. 2016; Eichmiller et al. 2016). The same has also been demonstrated for several fish species in the Chicago area waterway system (Mahon et al. 2013) and for lamprey larvae in aquarium experiments (Gingera et al. 2016). Previous studies have observed positive correlations between eDNA sample concentrations in natural ponds and the density of endemic amphibian species (Thomsen et al. 2012b), density and biomass as well as the occupied portion of transects in streams of amphibians (Pilliod et al. 2013), and carp biomass from two pond systems (Takahara et al. 2012). However, this relationship has not consistently been observed in field samples (Spear et al. 2015). Several biotic and abiotic variables such as pH, temperature, UV exposure, target species behaviour, and microbial activity can affect eDNA concentration and detection (Zhu 2006; Schapiro 2008; Takahara et al. 2012; Thomsen et al. 2012b; Barnes et al. 2014; Pilliod et al. 2014). It is currently not well understood how these factors affect the production and degradation of eDNA within aquatic systems (Rees et al. 2014).

Current research suggests that eDNA may still potentially be used for inferring relative biomass and abundance of aquatic organisms under similar conditions. For example, Lacoursière-Roussel et al. (2016) have shown that eDNA concentration is positively correlated with lake trout *Salvelinus namaycush* catch per unit effort (CPUE) and biomass per unit effort (BPUE) generated by gill net capture data collected across 12 lake systems under comparable

environmental conditions prior to lake stratification. Comparison between eDNA detection and veliger count data may provide insights into differences of detection potential. Comparisons may also allow for relative estimates of invasive bivalve abundance. Such estimates could facilitate rapid response and provide managers with more accurate data regarding the extent of an invasion event.

This study aims to: 1) determine the relative effectiveness of plankton netting for zebra mussel veligers compared to the eDNA methods developed in Gingera et al. (2017); and 2) test the relationship between eDNA concentration and veliger abundance. Specifically, eDNA data was compared to plankton net data collected in Lake Winnipeg. In this context: 1) parallel eDNA and plankton net samples were collected; 2) presence/absence for each data type was compared; and 3) the relationship between eDNA concentration and veliger abundance was tested.

3.3 Methods

3.3.1 Sample Collection

Water samples for eDNA analysis were collected from 18 sites within the north and south basins of Lake Winnipeg (Table 3.1) from the research vessel the *MS Namao* between September 14 and October 1, 2015. Prior to sampling, the *MS Namao* was visually inspected for zebra mussels attached to the hull; any attached zebra mussels were removed to prevent spread within the lake. Lake water for eDNA sampling was collected from the surface using a sterile bucket. Water was filtered on site using a peristaltic pump, cordless drill, and Nalgene disposable filter funnels (Nalgene, Rochester, New York, USA) modified to contain a Whatman 1.5 µm pore 47 mm diameter glass fibre filter (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Negative

controls were collected at each site by rinsing the sterilized bucket with 0.5 L of distilled water and subsequently filtering the water. Samples were collected in triplicate at each site (i.e., 3 filters collected). Up to 1.0 L of lake water was filtered for each sample or until the filter became clogged; the same volume of water was filtered for all sample replicates at each site. Filters were preserved in 50 mL conical tubes containing 25 mL of Sorbead Orange silica desiccant beads (BASF, Ludwigshafen, Germany) and kept at room temperature for 24 h before being moved to 4°C until extraction.

All water sampling equipment was sterilized using 10% bleach solution. Equipment was kept exposed to the bleach solution for ≥ 15 min then rinsed with distilled water. Forceps were sterilized by being kept in a 10% bleach solution bath for ≥ 15 min, rinsed with distilled water, and then baked at 190°C for ≥ 5 h.

Parallel plankton net tows were performed by WSD at each site (Table 3.1) as per a regularly scheduled monitoring effort for zebra mussel veligers. Plankton nets with a 63 µm mesh and 30 cm diameter were hauled at a speed of 0.5 m/sec from either 1 m above the sediment or 15 m from the surface, whichever was deeper. A minimum of 1000 L was filtered for each net sample with no maximum limit on water volume. Each veliger sample was preserved in 95% ethanol. The absolute number of veligers per sample was determined using sugar separation and visual survey with microscopy methods (*methods in* Mackie and Claudi 2010). Identification to species of veligers was not required as all native freshwater mussel species in Manitoba belong to the order Unionoida (Graf and Cummings 2007), which do not have a veliger life-stage. All veliger counts were performed provided by WSD.

DNA was extracted from filters using DNeasy Blood and Tissue Kit and Qiagen Lyse and Spin Baskets (Qiagen Inc., Valencia, CA, USA). Filters were folded three times and placed

in a Lyse and Spin Basket using sterile forceps, then were suspended in 360 µL ATL buffer and 40 µL proteinase K and incubated at 56°C and agitated overnight. Samples were centrifuged for 1 min at 18,000 g to remove the extraction solution from each of the filters. Extraction solute was suspended in 400 µL of absolute ethanol and AL buffer, respectively. The remainder of the extraction process was performed following the manufacturer's protocols. An unused and sterile glass fibre filter was included in a separate DNA extraction for each extraction set as a negative extraction control. A positive extraction control was collected by spiking a sterile glass fibre filter with 20 µL of bluegill *Lepomis macrochirus* tissue slurry. All samples were held at -20°C until treatment and analysis.

3.3.2 Analysis

Samples were analyzed on an Applied Biosystems StepOne Plus Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using the cytochrome *b* (Cyt *b*), cytochrome oxidase *c* subunit I (COI), and 16S rRNA qPCR assays described in Gingera et al. (2017; Chapter 2). The Cyt *b*, COI, and 16S rRNA assay probes were labelled with a FAM, VIC, and NED fluorophore, respectively; all probes used had minor-groove binding (MGB). 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 probe, and 5 µL of diluted (1:4) eDNA sample. 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. Each qPCR plate contained a 10-fold standard curve from 10^0 copies/µL to 10^6 copies/µL and no template control (NTC) reactions. All samples,

standards, and NTCs were run in triplicate. Samples were considered a positive detection if a minimum of one assay amplified eDNA from a minimum of one reaction replicate.

Adjusted Akaike's Information Criterion (AICc) and Bayesian Information Criterion (BIC) values were calculated for global parameters of both multiple linear regression models (MLR) and generalized linear mixed (GLM) models to determine the best fitting model. Within each model, the mean concentration of triplicate Cyt *b*, COI, and 16S rRNA reactions per sample site replicate were included as independent variables, ln (1+veliger count) was included as the dependent variable, and volume of water filtered was included as the random variable (GLMM only). For these analyses, target concentration was considered the independent variable to test the predictive power of eDNA concentration to infer veliger abundance. All analyses were done using R v.3.2.3 (R Development Core Team 2015). MLR models were performed using the lm function in base R, GLMMs were performed using the lmer function of Lme4 package, AIC_c values were determined using the AICc function of the AICcmodavg package, BIC values were determined using the BIC function of the MuMln package, and R^2 values for GLMMs were determined using the r.squaredGLMM function in the MuMln package.

3.4 Results

Of the 18 sites sampled, 13 locations (all of which are in the south basin of the lake) were positive for both zebra mussel veligers and eDNA. No veligers were collected from the three sites sampled within the north basin; however, all three sites tested positive for zebra mussel eDNA. Veligers were collected from one site where no zebra mussel eDNA was detected. For an additional one site, neither zebra mussel veligers nor eDNA were collected (Fig 3.1).

AICc values for MLR models indicated that the concentration of Cyt*b* and 16S as predictor variables explain the greatest amount of variation and that Cyt*b* and 16S concentrations were positively correlated with ln(veliger count + 1) (AICc = 154.415, w_{AICc} = 0.289, R^2_a = 0.298, $F_{2, 51}$ = 12.26, P = 4.5e^{-05}) (Table 3.2; Fig 3.2). BIC values for the same MLR models, however, indicated that the concentration of Cyt*b* as the only predictor variable explained the greatest amount of variation and that Cyt*b* concentration was positively correlated with ln(veliger count +1) (BIC = 160.677, w_i = 0.328, R^2_a = 0.2708, $F_{1, 52}$ = 20.69, P = 3.272e^{-05}) (Table 3.2; Fig 3.2).

Both AICc and BIC values for global GLM models suggested that the concentration of COI as a predictor variable explained the greatest amount of variation and that COI concentrations were positively correlated with ln(veliger count + 1) (AICc = 154.644, w_{AICc} = 0.334, BIC = 161.783, w_{BIC} = 0.349, marginal R^2 = 0.202, conditional R^2 = 0.604) (Table 3.2; Fig 3.2).

3.5 Discussion

The research described in this chapter provides a quantified comparison of absolute veliger count collected via plankton netting and eDNA detection data collected via zebra mussel assays described in Chapter 1 and Gingera et a1. (2017). Overall, eDNA detection appears to provide findings comparable to those from plankton netting while having a much smaller effective volume of water tested. Determining zebra mussel presence via eDNA methods may be more reliable than plankton netting considering the detection of eDNA at three sites in the north basin where no veligers were found and that zebra mussels were later confirmed in the north basin the following year. However, there was one instance where plankton nets captured two

veligers and eDNA analysis indicated no detection (i.e., an eDNA false negative, FN; *see* Section 1.5.2.2). During the time of sampling, zebra mussels had not yet been confirmed to be in the north basin of Lake Winnipeg based on similar veliger netting samples collected during the spring and summer of 2015. Considering their growing distribution and rapid spread, their presence within the north basin was not unexpected (Gingera et al. 2017).

Each best fitting model as selected by AICc and BIC demonstrates that eDNA concentration is positively correlated with ln(veliger count +1). Veliger count data transformation was required for model analysis due to the lack of data between 849 and 2620 veliger samples. The data presented in this chapter specifically supports that eDNA may be used to infer relative veliger abundance and generally supports evidence that eDNA methods may be used to infer relative abundance, density, and/or biomass for a wide range of taxa (Takahara et al. 2012; Thomsen et al. 2012b; Mahon et al. 2013; Pilliod et al. 2013; Gingera et al. 2016; Lacoursière-Roussel et al. 2016). Evidence to the contrary exists, however, as Spear et al. (2015) did not find a statistically significant correlation between eDNA quantity and organismal abundances. Ulibarri et al. (2017) also found that eDNA detection of rare bluehead suckers *Catostomus discobolus* was less sensitive than visual surveys done by scuba divers. The data presented in this chapter, however, suggests that eDNA detection may be correlated with veliger abundance in large open waters such as Lake Winnipeg during the Fall (September-October).

AICc and BIC values were not in agreement regarding which MLR model fit best; and these were also different compared to which GLM model fit best. It is common for model selection to differ between AICc and BIC as AICc will often select more complex models (i.e., the model with both Cyt*b* and 16S) whereas BIC inherently penalizes large sample sizes to a greater degree and selects less complex models (i.e., the model with only Cyt*b*) (Aho et al.

2014). Both AICc and BIC selected the GLM model with COI and water volume as the best fitting model, strengthening confidence that this is the most appropriate model of those tested.

The concentration of COI eDNA and volume of water filtered could account for 60.4% (i.e., conditional R^2) of the variation in zebra mussel veliger abundance. The conditional R^2 value was greater than the marginal R^2 value because it incorporates the effect of both COI concentration and the volume of water filtered (i.e., the random variable). Intuitively, the volume of water filtered would influence eDNA detection thus accounting for the greater variation explained by this mode. This high R^2 value suggests that perhaps veliger abundance is the primary parameter which influences eDNA concentration and detectability of invasive mussels. This hypothesis is supported by the confirmed eDNA FN where veliger density was presumably low (two individuals captured via plankton net). Conversely, eDNA detections were recorded at three sites within the northern basin where veliger density was also presumably low. These data do not conflict with the hypothesis that veliger density is the primary parameter influencing eDNA detection of zebra mussels. These detections may be explained by eDNA sources other than veligers or an unlikely capture of veligers in the relatively small volume of water filtered. Novel detections such as these may also be false positives (FP; *see* Section 1.5.2.1); however, the fact that zebra mussels eventually were detected in the north basin of Lake Winnipeg in 2016 (Wong et al. 2016; CBC 2017) supports the interpretation that these are not FP. Environmental DNA detection methods appeared to be more sensitive than plankton netting at sites in the north basin.

This greater sensitivity may be because eDNA sampling methods non-specifically amplify different life-stages whereas plankton netting only targets the veliger life-stage. Specifically, the detected eDNA for the north basin detections may have been sourced from cell

debris of both veligers and mytiliform individuals. Due to the large difference in water volume sampled between eDNA and plankton net methods, it does not seem likely that veligers were the source of detection for the positive eDNA detections at these three sites. Although veliger density may be the most influential variable regarding eDNA detection of invasive mussels, other sources of eDNA may be prevalent enough to allow for detection where veliger density is low. Likewise, these sources of eDNA detection may account for some of the variation not accounted for in the MLR and GLM models. However, because eDNA sources are not being observed directly, it is important to ensure that the novel north basin detections are not FP results.

Variance in eDNA detection is likely reduced where there is an abundance of eDNA source material and degradation is minimal (Dejean et al. 2011). The production, concentration, and distribution of eDNA varies depending on the target organism's physiology, life-cycle, and behaviour (Goldberg et al. 2011; Barnes et al. 2014; Eichmiller et al. 2014; Turner et al. 2014; Laramie et al. 2015; Gingera et al 2016). Environmental DNA detections of actively mobile species have been correlated with movement (Erickson et al. 2016). Movement may account for more variable shedding of eDNA for these species. Mytiliform zebra mussels are non-motile, and therefore shedding rate may be less variable. Also, unlike burrowing species (e.g., lampreys), zebra mussels spend their entire life-cycle in the water column; therefore, all shedding of eDNA sources will be directly in the water column. Ultimately, this would suggest that eDNA detections may be less variable for zebra mussels than other species and may be a better proxy for relative abundance than eDNA detections for more mobile or burrowing species.

Contamination due to allochthonous eDNA (i.e., eDNA originating outside of a system of sampling area) may result in FP and should be considered when novel eDNA detections are

made (Goldberg et al. 2016). Sources such as fecal deposition by predators (Merkes et al. 2014), transport of individuals by predators or human vectors, relocation of sediment, and flow (Darling and Mahon 2011; Mahon et al. 2013) are confounding factors which may influence eDNA distribution data. Allochthonous eDNA is not directly observable and therefore it is difficult to ascertain how such parameters may influence eDNA detection. In the case of this research, zebra mussels have few predators in Lake Winnipeg and so human traffic and allochthonous eDNA due to flow within Lake Winnipeg are the most likely stochastic factors which could have caused FP. For example, water flowing from the south basin and into the north basin could bring with it allochthonous eDNA (*see* Section 1.5.2.1). The detections of zebra mussels in the north basin in 2016, however, support the interpretation that the novel eDNA detections in the north basin were not FP caused by allochthonous eDNA.

One concern during sampling was the possibility of FP due to contaminating factors such as attached juveniles and adults on the hull of the research vessel. Prior to the survey of Lake Winnipeg, the *MS Namao* was surveyed and cleaned of any attached zebra mussels to reduce the likelihood of zebra mussel spread and contaminating veligers into the uninvaded portions of the lake. No eDNA signal was detected at two sites, which suggests that the number of contaminating zebra mussels was sufficiently low as to not produce a consistent eDNA signal. These sites were also sampled near the end of the survey, when contamination would be most likely due to the extended period that the vessel had not been inspected for attached individuals, allowing more of an accumulation of mussels on the hull of the vessel. Alternatively, any attached zebra mussels may have detached themselves due to sufficient agitation. Supposedly, all samples would test positive for zebra mussel eDNA had there been a sufficiently large enough

group of contaminating mussels whereas a low density of mussels could produce an inconsistent eDNA signal.

Overall, veliger density appears to be the most influential parameter affecting eDNA detection of invasive mussels. The physical location of the veligers may also be a major factor affecting eDNA detection. Comparisons between veliger capture and eDNA detection between different seasons may provide insights into the utility of eDNA for invasive bivalve detection. Veligers will likely be found in Manitoban waters as soon as late May and will be prevalent in the system until October-November. Most sampling during this period will be detecting gametes, veligers, and mytiliform mussels. Sampling outside of May-November will likely result in detection of adults only. As eDNA cannot currently distinguish between life-stages, managers will need to sample around these timelines to target only mytiliform mussels. Further investigations with greater sample sizes comparing plankton netting and eDNA methods when veliger concentration is low may help elucidate whether eDNA detections at low veliger concentrations are due to higher sensitivity or chance. Controlled laboratory experiments which aim to determine concentration with a known absolute number of veligers per sample prior to extraction, while also considering inhibition, may also provide insight into interpreting the sources of eDNA signals.

The assays developed in this thesis (Gingera et al. 2017; Chapter 2) appear to be highly sensitive and should be considered as a monitoring tool available to managers. Based on the detections in this chapter, zebra mussels will likely continue their spread into the north basin due to the passive movement of veligers via flow. Sites with submerged infrastructure and human traffic will be the first areas to have confirmed zebra mussel sightings. Eventually, zebra mussels will become prevalent in the north basin and will extend into the Nelson River according to

survival estimates based on calcium concentrations (Therriault et al. 2013). All other spread outside of Lake Winnipeg will likely be due to human transport vectors.

In conclusion, the data collected for this study supports that veliger density is perhaps the most influential parameter affecting the eDNA detection methods described in Gingera et al. (2017) when environmental conditions are relatively consistent (samples were collected over short period of time). These methods may be more sensitive than plankton netting, especially so if the abundance of zebra mussel eDNA source material other than veligers is significant. Also, because shedding rates may vary based on movement for large mobile species (Erickson et al. 2016), zebra mussel eDNA detection may be less variable as the adults are non-mobile. Therefore, eDNA methods may be used to infer relative veliger abundance; however more data collection across different systems are required to determine if this is possible in sites with greater variation. Environmental DNA sampling will yield more accurate results during periods when veligers are present in the water column (i.e., between June and October in Manitoba). Overall, eDNA appears to be more sensitive than plankton netting and should be considered as a monitoring tool for managers.

3.6 Literature cited

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3.7 Tables and Figures

Table 3.1 Sample site coordinates, water volume filtered, assay, intercept, slope, R^2, efficiency (%), mean concentration (copies/µL) for each replicate (triplicate reactions), and absolute veliger count at each site. Samples were collected between September 14 and October 1, 2015. Slope, intercept, R^2, and efficiency were generated by Applied Biosystems StepOne Plus Real-Time PCR System (Life Technologies, Grand Island, NY, USA). All veliger data was collected and provided by the province of Manitoba Water Stewardship Division (WSD).

Site number	Site name	Coordinates	Volume of water filtered (mL)	Target Gene	Slope	Y-intercept	R^2	Efficiency	Mean eDNA concentration (copies/µL)			Absolute veliger count
									Rep 1	Rep 2	Rep 3	
1	W8	51.7748 °N, 89.8629 °W	1000	Cyt *b*	-3.717	34.653	0.99	85.792	0.000	0.000	0.000	2
				16S rRNA	-3.504	37.175	0.985	92.925	0.077	0.151	0.075	
				COI	-3.495	36.747	0.998	93.244	0.089	0.000	0.000	
2	W3	53.1710 °N, 97.8372 °W	1000	Cyt *b*					0.000	0.000	0.000	0
				16S rRNA					0.000	0.109	0.000	
				COI					0.000	0.000	0.000	
3	W6	52.6421 °N, 97.7335 °W	1000	Cyt *b*					0.000	0.000	0.000	0
				16S rRNA					0.081	0.000	0.000	
				COI					0.000	0.000	0.022	
4	13NS	52.136 °N, 97.542 °W	1000	Cyt *b*					0.000	0.000	0.000	0
				16S rRNA					1.431	0.221	0.000	
				COI					0.000	0.000	0.000	
5	3NS	50.6385 °N,	500	Cyt *b*	-3.584	39.453	0.996	90.126	12.567	2.305	15.957	212
				16S rRNA	-3.43	39.125	0.995	95.668	10.255	1.650	14.514	
				COI	-3.728	40.19	0.989	85.462	11.199	1.887	14.757	

		96.9829										
		°W										
6	57B	50.9748	1000	Cyt *b*					0.581	0.645	0.217	16
		°N,		16S rRNA					0.569	0.474	0.317	
		96.8752		COI					0.253	0.529	0.439	
		°W										
7	W10	50.8458	1000	Cyt *b*					0.000	0.000	0.000	52
		°N,		16S rRNA					0.000	0.000	0.000	
		96.7686		COI					0.000	0.000	0.044	
		°W										
8	59	50.6960	1000	Cyt *b*					0.000	0.000	0.000	1
		°N,		16S rRNA					0.000	0.075	0.000	
		96.7898		COI					0.000	0.136	0.000	
		°W										
9	2	50.4336	400	Cyt *b*	-3.775	37.869	0.985	84.044	0.212	0.451	0.029	257
		°N,		16S rRNA	-3.475	39.406	0.984	93.989	0.089	0.22	0.000	
		96.8342		COI	-3.548	39.084	0.992	91.345	0.045	0.000	0.000	
		°W										
10	1	50.3858	250	Cyt *b*					0.411	8.609	0.077	47
		°N,		16S rRNA					0.259	6.42	0.043	
		96.8121		COI					0.137	5.586	0.073	
		°W										
11	4NS	50.4096	250	Cyt *b*					7.506	7.719	2.508	486
		°N,		16S rRNA					5.541	5.335	1.182	
		96.9028		COI					5.041	3.443	0.856	
		°W										
12	3B	50.4582	500	Cyt *b*					11.321	68.896	40.924	2620
		°N.		16S rRNA					8.917	64.695	34.648	
				COI					6.853	45.856	25.268	

		96.6887 °W										
13	60C	50.5587 °N, 96.6669 °W	750	Cyt *b* 16S rRNA COI					14.829 12.785 7.531	2.307 1.271 0.839	1.521 1.005 0.603	849
14	5	50.6794 °N, 96.6373 °W	700	Cyt *b* 16S rRNA COI	-3.86 -3.76 -3.571	40.944 38.485 39.909	0.988 0.97 0.995	81.583 84.499 90.571	0.027 0.003 0.099	0.000 0.000 0.000	0.000 0.101 0.065	2
15	5NS	50.6706 °N, 96.5592 °W	1000	Cyt *b* 16S rRNA COI					13.118 16.213 7.431	11.447 13.899 6.681	13.496 14.210 7.785	178
16	7	50.6495 °N, 96.3784 °W	1000	Cyt *b* 16S rRNA COI					0.000 0.000 0.000	0.000 0.000 0.000	0.000 0.000 0.000	2
17	W11	50.7581 °N, 96.4491 °W	500	Cyt *b* 16S rRNA COI					0.000 0.000 0.000	0.000 0.000 0.000	0.000 0.000 0.000	0
18	9	50.8795 °N, 96.4249 °W	1000	Cyt *b* 16S rRNA COI					0.097 0.371 0.000	0.189 0.261 0.205	0.667 0.352 0.178	23

Table 3.2 Model type (multiple linear regression = MLR, generalized linear mixed model = GLMM), variables included (Parameters), number of variables (K), log-likelihood, AICc (Akaike information criterion), and BIC (Bayesian information criterion) values where ln(veliger count + 1) was considered the dependent variable and target concentration was considered the independent variable in order to determine the predictive power of concentration on veliger count. All values were obtained using base R v.3.2.3 (R Development Core Team 2015).

Model	Model number	Parameters	K	Log-likelihood	AICc	ΔAICc	AICc weight	BIC	ΔBIC	BIC weight
MLR	1a	Null	2	-83.397	171.029	142.664	0.000	174.8	143.53	0.000
	2a	Cyt *b*	3	-14.399	35.759	7.393	0.017	38.9	7.66	0.016
	3a	16S rRNA	3	-43.026	92.802	64.437	0.000	96.8	65.56	0.000
	4a	COI	3	-30.159	67.241	38.875	0.000	70.5	39.28	0.000
	5a	Cyt *b* + 16S rRNA	4	-11.399	32.54	4.171	0.086	36.1	4.88	0.062
	6a	Cyt *b* + COI	4	-9.183	28.366	0.000	0.692	31.2	0.00	0.716
	7a	16S rRNA + COI	4	-26.830	63.400	35.034	0.000	67.0	35.75	0.000
	8a	Global	5	-8.819	30.795	2.430	0.205	33.7	2.49	0.206
GLMM	1b	Null + vol.	3	-78.201	162.882	119.509	0.000	168.4	122.06	0.000
	2b	Cyt *b* + vol.	4	-18.880	47.426	4.053	0.108	51.2	4.92	0.074
	3b	16S rRNA + vol.	4	-41.459	92.209	48.836	0.000	97.3	50.94	0.000
	4b	COI + vol.	4	-32.570	74.740	31.367	0.000	78.7	32.44	0.000
	5b	Cyt *b* + 16S rRNA + vol.	5	-18.215	49.157	5.784	0.0460	53.1	6.78	0.029
	6b	Cyt *b* + COI + vol.	5	-15.108	43.373	0.000	0.822	46.3	0.00	0.867
	7b	16S rRNA + COI + vol.	5	-30.200	73.128	29.755	0.000	77.1	30.75	0.000
	8b	Global	6	-16.8816	50.4299	7.0570	0.0241	53.1	6.77	0.029

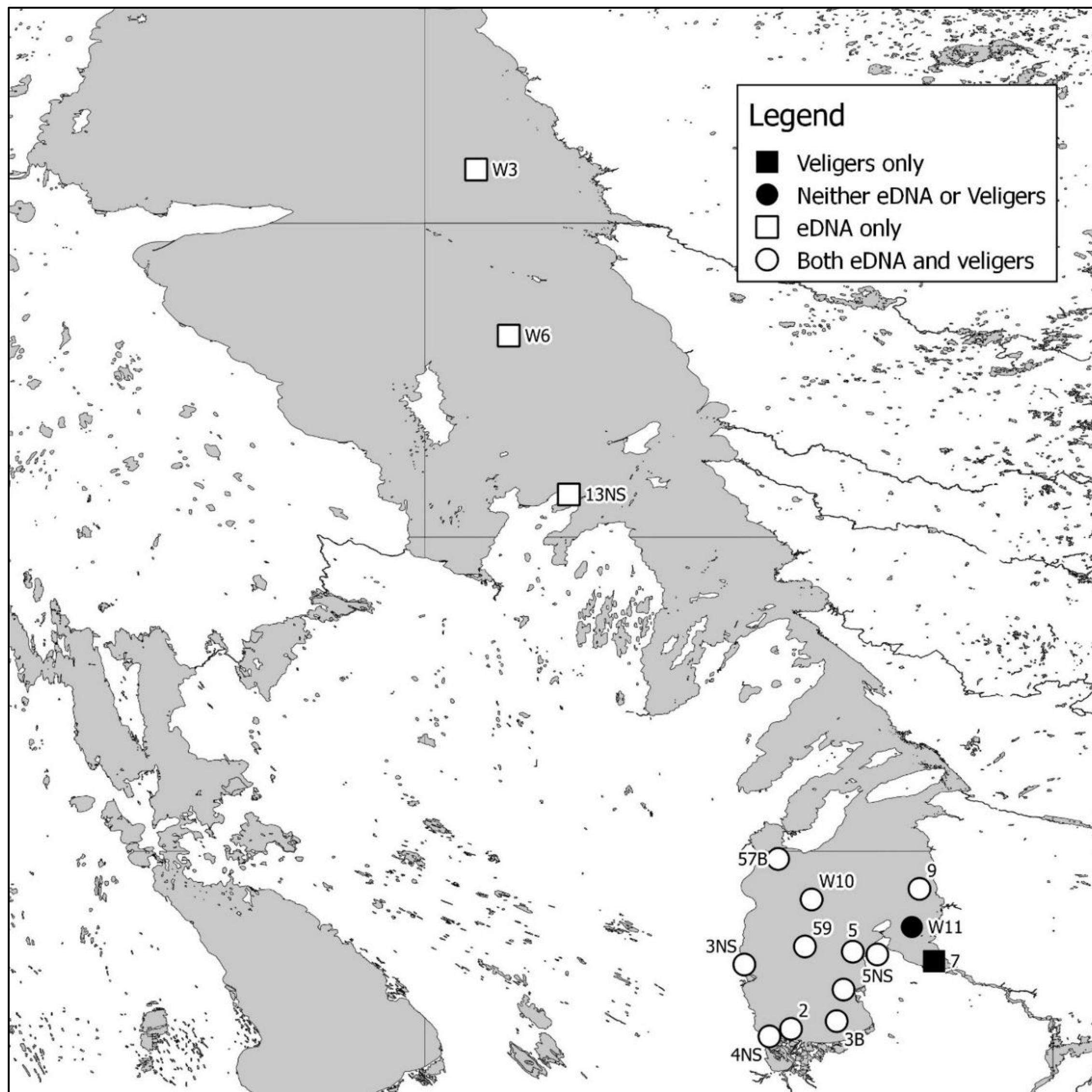

Figure 3.1 Collection sites where parallel eDNA and plankton netting samples were collected within Lake Winnipeg. Samples were collected between September 14 and October 1, 2015 during the fall survey on the *MS Namao* conducted by the Lake Winnipeg Research Consortium. Labels indicate general position of collection sites within the study system and represent which detection methods resulted in a positive detection.

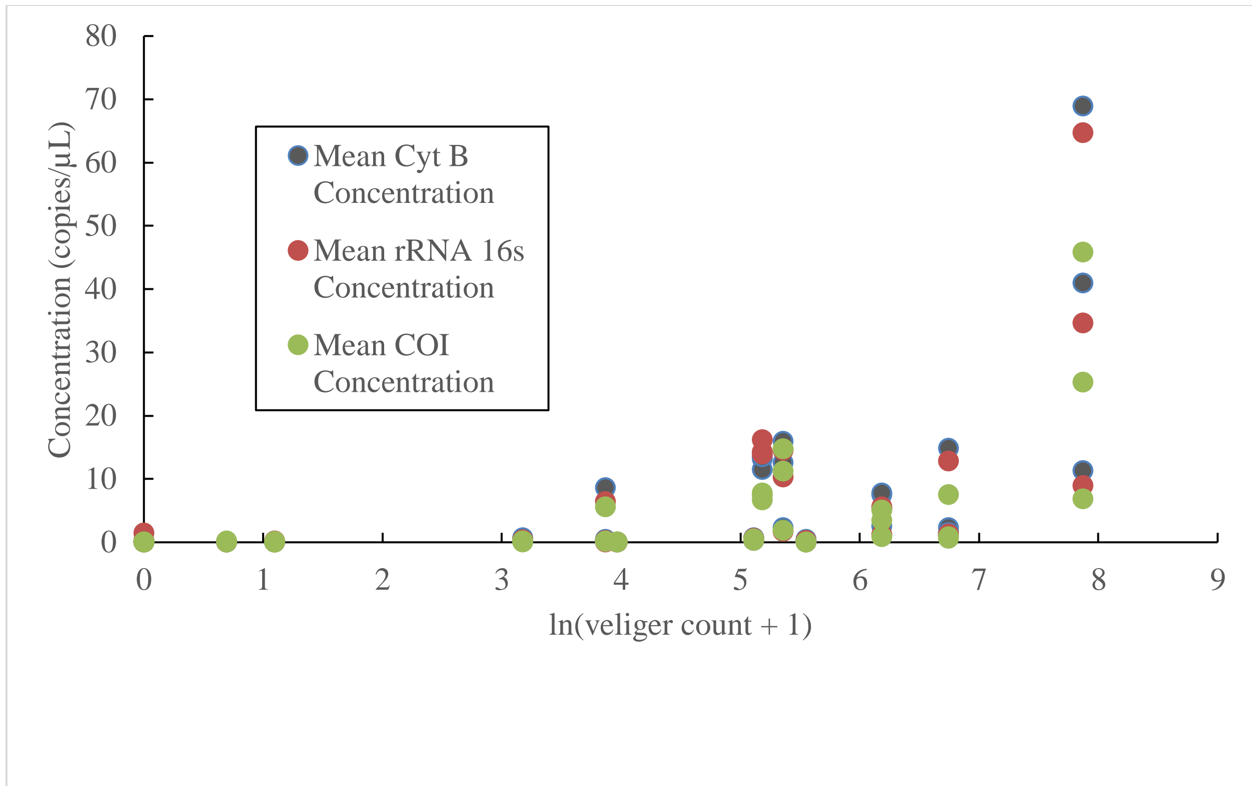

Figure 3.2 Mean concentration of the Cyt B, rRNA 16s, and COI genes (based on triplicate qPCR reactions) plotted against ln(veliger count + 1). Parallel veliger samples and eDNA samples were collected from 18 sites within Lake Winnipeg from the *MS Namao* between September 14 and October 1, 2015.

CHAPTER 4. HOW FORENSICS, VIROLOGY, AND ANCIENT DNA CAN INFORM ENVIRONMENTAL DNA METHODS: A CASE FOR SAMPLE PURIFICATION AND ENRICHMENT

4.1 Abstract

Environmental DNA species detection and monitoring requires the collection of samples from environmental sources, such as soil and water. Nucleic acid amplification and analysis of these sources is intrinsically difficult due to the presence of PCR inhibitors and low target DNA concentration, which both decrease assay sensitivity and the likelihood of detection; this ultimately increases the likelihood of false negatives. Steps therefore must be taken to improve assay performance since the potential of false negatives reduces the precision and accuracy of eDNA data. Applicable methods of nucleic acid purification and concentration often used in the fields of forensics, virology, and ancient DNA (aDNA) were assessed to increase assay performance in eDNA samples collected from a large freshwater lake. The methods tested were: 1) centrifugal filtration units; 2) evaporation; 3) isopropanol precipitation; 4) magnetic bead purification; 5) polyethylene glycol (PEG) precipitation; and 6) silica column purification. Effects of inhibition were observed in all treatment groups except for silica column purification, which consistently enriched eDNA samples 4x without exacerbating inhibitory effects. Thus, concentrating samples a priori is an appropriate method of improving assay performance and should be further investigated in future studies. eDNA researchers should consult forensics, virology, and ancient DNA methods when investigating approaches to sample processing.

4.2 Introduction

The analysis of nucleic acids has been widely adopted within several fields of research since the emergence of polymerase chain reaction (PCR) technology (Armstrong 2005; Valentini et al. 2009; Hajibeabea 2012; Taberlet et al. 2012). Sources of nucleic acids typically include biological samples such as tissues, fluids, and cultured cells. Several fields of research (e.g., forensics, microbial-community analysis) require samples collected from the environment, such as from the soil or waterbodies (Alaeddini 2012; Schrader et al. 2012). Similarly, environmental DNA (eDNA) research uses PCR-based methods on environmental samples to detect target species DNA and infer presence/absence of said species within a system (Taberlet et al. 2012). Quantitative PCR (qPCR) has become a common tool for species detection using eDNA as it is generally considered more sensitive than conventional PCR methods (Higuchi et al. 1992). Also, multiple DNA fragments can be analyzed within a single qPCR (i.e., multiplexed) with the incorporation of several species-specific assays and fluorescent probes (Alaeddini 2012; Shrader et al. 2012). Monitoring programs for several aquatic invasive species (AIS) have successfully implemented eDNA methods to infer species presence/absence (Jerde et al. 2011). For example, eDNA assays have successfully been implemented for zebra mussel *Dreissena polymorpha* detection in Lake Winnipeg, MB (*see* Chapter 2; Gingera et al. 2017).

Because eDNA methods are an indirect form of species detection, quality assurance and quality control (QA/QC) is critical to ensure reliable data collection. In this respect, false positives (hereby abbreviated to FP; i.e., the detection of target DNA when the target organism is not present) and false negatives (hereby abbreviated to FN; i.e., when the target species is present, but no target DNA is detected) are the two concerns when conducting research or during monitoring efforts. The likelihood of FP can be reduced with the application of “clean”

techniques and can be identified with the incorporation of negative controls at each stage of sample processing. FNs are more difficult to address and are a concern of AIS monitoring efforts which use eDNA tools (Jerde et al. 2011; Bohmann et al. 2014; *see* Section 1.5.2.2). Lack of detection when the target organism is present may permit further colonization of invaders if they are not identified and appropriate management measures are not taken. The likelihood of FN can increase due to factors such as low target DNA concentration and the presence of PCR inhibitory substances which can reduce the performance of qPCR assays, thus reducing the likelihood of detection. Low DNA concentration and high inhibitor concentration is characteristic of environmental samples (McKee et al. 2015) and must be considered when conducting research which requires the analysis of DNA from the environment, such as in forensics and eDNA.

PCR inhibitors are a heterogeneous group of chemical substances that vary depending on the source of the sample and are defined as having a negative effect on PCR (McKee et al. 2015). Environmental samples collected from sources of fresh water may contain substances shown to be PCR inhibitors such as algae (Schrader et al. 2012), debris, fulmic acids, humic acids, humic material, metal ions, polyphenol (Tsai and Olson 1992; Abbaszadegan et al. 1993; Ijzerman et al. 1997; Watson and Blackwell 2000; Rådström et al. 2004; Schrader et al. 2012), and polysaccharides (Demeke and Adams 1992; Monteiro et al. 1997), each of which have various mechanisms of PCR inhibition and interfere with different PCR components. These substances typically originate from the sample and are retained during nucleic acid extraction, depending on the method of extraction used (Alaeddini 2012; Shrader et al. 2012). Indeed, eDNA samples are often reported as having a dark-brown pigmentation by researchers and these samples are assumed to contain inhibitors (McKee et al. 2015).

Inhibition of a sample can be measured within a multiplexed qPCR by implementing an internal positive control (IPC), where exogenous genetic markers are used to target known nucleic acid sequences unrelated to the target organism (Dingle et al. 2004; Hoorfar et al. 2004; Dreier et al. 2005; Swango et al. 2006, 2007; Villanova et al. 2007). PCR inhibitors are likely present if performance of the IPC assay is reduced when analyzing an environmental sample compared to a no-template-control (NTC). However, the co-amplification of multiple targets within a single reaction can reduce target assay performance (Raeymaekers 1995; Hofmann 2003; Kontanis and Reed 2005; Gall et al. 2007), which should be considered when incorporating an IPC.

Various methods of PCR inhibitor removal exist and can be implemented at different stages of sample processing. For example, pre-extraction PCR inhibitor removal methods can be used depending on the sample type (i.e., soil, sewage, water) and types of PCR inhibitors present (Alaeddini 2012; Shrader et al. 2012). Typical methods of PCR inhibitor removal for water samples require processing whole samples through gel-packed columns and affinity beads such as Sephadex G-50, G-100, and Sepharose (Rogan and Salvo 1990; Jackson et al. 1997; Shutler et al. 1999). Very few of these methods are implemented in eDNA research or monitoring programs, as water samples are typically filtered to concentrate solutes on glass fibre filters prior to eDNA extraction. These methods, although extensively investigated in other research areas, have not yet been adapted for eDNA sample processing prior to filtration.

During extraction, phenol-chloroform and guanidinium thiocyanate have been shown to be effective at removing different types of PCR inhibitors (Shieh et al. 1995; Wiedbrauk et al. 1995; Hale et al. 1996; Abolmaaty et al. 2007; Chaturvedi et al. 2008). The most common methods of nucleic acid extraction for eDNA from freshwater samples are PowerWater DNA

Isolation Kit (Mo Bio, Carlsbad, CA, USA) (Jerde et al. 2011; Olsen et al. 2012; Mahon et al. 2013; Eichmiller et al. 2016) and DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) (Ficetola et al. 2008; Dean et al. 2011; Foote et al. 2012; Goldberg et al. 2011, 2013; Thomsen et al. 2012a, 2012b, Takahara et al. 2013, Gingera et al. 2016, 2017), with the latter being more commonly used in monitoring programs (USGS 2015; Goldberg et al. 2016) due to relative cost. Although methods such as PowerWater DNA Isolation Kit and phenol-chloroform may be more likely to remove PCR inhibitors (Wiedbrauk et al. 1995; Abolmaaty et al. 2007; Chaturvedi et al. 2008; Eichmiller et al. 2016), these methods are not easily scaled up for high throughput of samples required in a monitoring program.

Often, methods of nucleic acid extraction are not sufficient for the complete removal of PCR inhibitors from a sample and post-extraction methods of inhibition relief and removal are used. PCR additives such as bovine serum albumin (BSA) and T4 bacteriophage gene 32 product (gp32) are commonly used in conventional PCR (Kreader 1996; Al-Soud and Rådström 2000; Scipiono et al. 2008; Opel et al. 2010). The most common additive used in eDNA research and monitoring is TaqMan Environmental Master Mix 2.0 (EMM 2.0) (Life Technologies, Grand Island, NY, USA), which has been shown to relieve qPCR from inhibition (Verhaegen et al. 2016). Alternative to the use of additives, dilution of DNA samples can reduce the concentration of inhibitors and improve assay performance (Widjojoatmodjo et al. 1992; Monteiro et al. 1997; Eckhart et al. 2000; Alonso et al. 2001; Scipiono et al. 2008a, b). However, this method also dilutes the target nucleic acid fragments which may decrease assay performance.

Post-extraction purification for inhibitors likely to be found in water samples are frequently used in forensics. These methods include isopropanol and polyethylene glycol (PEG) precipitation which are commonly used for the removal of humic acids (Hanni et al. 1995;

LaMontagne et al. 2002). The use of magnetic beads (Maher et al. 2001; Rutjes et al. 2005; Ngazoa et al. 2008; Sur et al. 2010) and salt induced absorption of nucleic acids to silica (Nagy et al. 2005; Kemp et al. 2006; Davoren et al. 2007; Vanek et al. 2009; Lee et al. 2010) have also been shown to remove inhibitors, with the latter being the most commercially available purification method (e.g., QIAquick PCR purification kit). Typically, DNA purification techniques which remove inhibitors also result in a loss of DNA which is undesirable as target species DNA concentration is already relatively low.

Concentration of an eDNA sample may increase target assay performance for samples which have lost DNA during PCR inhibitor removal and/or are suspected of low target DNA concentration. Many post-extraction PCR inhibitor removal methods can efficiently enrich samples by reducing the volume of elution buffer. Sample evaporation is also a "straight-forward" sample concentration method which involves limited sample manipulation. Such methods may increase inhibition alongside DNA concentration; however, additives such as EMM 2.0 increase tolerable concentrations of PCR inhibitors for a reaction. Sample concentration may be viable if an appropriate amount of PCR inhibitors are removed prior or during concentration.

In this study, potential methods of eDNA purification and concentration which may improve eDNA as a detection and monitoring tool by reducing the likelihood of FN during sample analysis were identified. This was done by: 1) treating samples with a known concentration of DNA to determine DNA recovery and potential loss; 2) treating eDNA samples and comparing the treated and untreated samples using qPCR to determine if treatment affects assay performance; and 3) comparing the performance of an IPC assay between treatment groups to determine if there is an increase in inhibitory effects. The methods tested were: 1) centrifugal

filtration units (Amicon Ultra 0.5 mL 30 kDa units (Millipore, Billerica, MA, USA)); 2) evaporation; 3) isopropanol precipitation; 4) magnetic bead purification; 5) PEG precipitation; and 6) silica column purification (QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA)). These methods were selected due to their ability to reduce the volume of the input sample, thus concentrating the target DNA, as well as potentially remove inhibitors (excluding evaporation). The goal of this research is to assess the ability of existing DNA purification methods, some of which have been used in forensics, to increase qPCR assay performance.

4.3 Methods

4.3.1 Nucleic acid recovery

4.3.1.1 Sample preparation

Samples for assessing DNA recovery of the six methods were prepared by diluting GeneRuler 50bp ladder (Thermo Fisher Scientific, Waltham, MA, USA) with water at a 1:24 ratio. The diluted ladder stock was aliquoted into 50 µL samples for each treatment (n=16 per treatment, n=96 total). Samples were again diluted to a final volume of 200 µL with water. The diluted samples were concentrated 4x (back to a volume of 50 µL) with each method and subsequently compared to an untreated control to determine DNA recovery. Specific treatments are outlined in the following subsections.

4.3.1.2 Centrifugal filter columns

Amicon Ultra 0.5 mL 30 kDa units (Millipore, Billerica, MA, USA) were used to enrich ladder samples. Each centrifugal filter column was first rinsed with 500 µL of water and

centrifuged for 1 min at 14,000 g then inverted in the collection tube and centrifuged for 1 min at 1,000 g to remove excess water. Ladder samples were aliquoted into each column and centrifuged for 30 min at 3,000 g. The remaining sample was adjusted to 50 µL.

4.3.1.3 Evaporation

Ladder samples were centrifuged in a Vacufuge (Eppendorf, Hamburg, Germany) until the entirety of the sample had been evaporated (approximately 3 hours). Ladder samples were subsequently re-suspended in 50 µL of water. Prior to this study, the possibility of cross-contamination was assessed by evaporating 12 samples with a known quantity of DNA alongside several NTC samples. No cross-contamination was observed in these QA/QC assessments (data not shown).

4.3.1.4 Isopropanol precipitation

Sodium acetate and isopropyl alcohol was added to each ladder sample for a final concentration of 0.3 M and 40%, respectively. Samples were then mixed gently, incubated overnight at -20°C, and centrifuged for 30 min at 15,000 g. The resulting pellet was washed with 70% ethanol and re-suspended in 50 µL of water.

4.3.1.5 Magnetic particle purification

A stock solution of magnetic beads was made with 18% PEG 8000, 1 M NaCl, 10 mM tris-HCl, 0.1 mM EDTA, 0.055% tween 20, and 1 mg/ml Sera-Mag SpeedBead Carboxylate-modified magnetic particles (GE Healthcare Life Sciences, Pittsburgh, PA, USA) following Faircloth and Glenn (2011). On a Qiagen microplate MP (Qiagen Inc., Valencia, CA, USA), 600

µL of the magnetic bead solution was added to 16 separate wells; ladder samples were then added directly to the magnetic particle solution for a total volume of 800 µL. Samples were then incubated overnight at 4°C. Magnetic particle purification was done on a Biosprint 96 (Qiagen Inc., Valencia, CA, USA) platform using the following custom protocol: step 1) collect beads (20 collect counts, no premix); steps 2-4) wash (no action, 1 min wash time, very slow, no bead collection); step 5) dry (10 min, position outside well); step 6) elution (release beads for 5 min on very slow, elution time 2 min on very slow, no heating, remove beads for 20 counts). All wash steps were done in 500 µL of 80% ethanol and all samples were eluted in 50 µL of water. Once purification was complete, each sample was adjusted to 50 µL volume as some of the elution would be maintained in the magnetic beads.

4.3.1.6 Polyethylene glycol precipitation

A stock PEG solution was made with a concentration of 20% PEG 8000 and 1.2 M NaCl. Ladder samples were suspended in PEG solution at a 1:1 ratio, mixed gently, held overnight at -20°C, and centrifuged for 30 minutes at 15,000 g. The resulting pellet was washed with 70% ethanol and re-suspended in 50 µL of water.

4.3.1.7 Silica column purification

Ladder samples were purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol with the exception that all samples were eluted from the column with water. The volume of each sample was adjusted to 50 µL to ensure uniformity.

4.3.2 Ladder sample analysis

The concentration of ladder for each treatment sample and control was determined using Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) high sensitivity (HS) assay following manufacturer's protocol and using 5 μL of sample. Control ladder samples were quantified in triplicate; the resulting average was used to determine the percent recovery of each treatment replicate as follows:

$$\left(\frac{Sample\ conc.}{Control\ conc.\left(\frac{conc.1 + conc.2. + conc.3}{3}\right)}\right)(100) = \%\ DNA\ recovery$$

Comparisons among treatments were performed using Analysis of Variance (ANOVA) with the treatment as the categorical variable and the percent recovery as the dependent variable using the aov function in base R v.3.2.3. Post hoc Tukey analysis was used to determine differences among treatment groups using the TukeyHSD function in base R v.3.2.3.

4.3.3 eDNA purification and enrichment

The ability of each of the six purification methods to: 1) enrich eDNA; and 2) remove inhibitors was assessed using zebra mussel eDNA assays known to be highly sensitive and samples collected from locations known to contain zebra mussels and natural inhibitors (*see* Chapter 2, Gingera et al. 2017).

4.3.3.1 Sample collection

Two or three sites were sampled from five harbours located in the south basin of Lake Winnipeg (Balsam, Gimli, Hnausa, Selkirk, and Winnipeg Beach harbours) for a total of 14 sites sampled (Table 4.1). Water was filtered on site using a peristaltic pump, cordless drill, and a

Nalgene disposable filter funnel unit (Nalgene, Rochester, New York, USA) modified to contain a Whatman 1.5 µm pore 47 mm diameter glass fibre filter (GE Healthcare Life Sciences, Pittsburgh, PA, USA). One negative control and seven samples were collected at each site. For each sample, water was filtered until the filter was near clogging; the volume of water filtered between each site varied but all sample volumes were consistent within a site. Filters were stored in 95% ethanol and held on ice in the field and subsequently stored at -80°C until extraction (*see* Chapter 2).

DNA was extracted from filters using DNeasy Blood and Tissue Kit and Qiagen Lyse and Spin Baskets (Qiagen Inc., Valencia, CA, USA). Filters were folded three times and placed in a Lyse and Spin Basket using sterile forceps, then were suspended in 360 µL ATL buffer and 40 µL proteinase K and incubated at 56°C and agitated overnight. Samples were centrifuged for 1 min at 18,000 g to remove the extraction solution from each of the filters. The remainder of the extraction process was performed following the manufacturer's protocols except for the elution stage where 200 µL of Low TE buffer was used for elution. A negative extraction control was collected using a sterile fibre glass filter and a positive extraction control was collected by spiking a sterile fibre glass filter with 20 µL of bluegill *Lepomis macrochirus* tissue slurry. All samples were held at -20°C until treatment and analysis. Bluegill tissue and assays were used as the standard positive control similar to the Asian Carp eDNA Monitoring Program in the USA (USGS 2015).

4.3.3.2 Treatment and analysis

To compare each treatment method directly, all seven samples collected from each of the respective 14 sites were combined to ensure that the concentration of DNA and inhibitors within

the samples were the same. This resulted in 14 samples which were separated into six 200 µL sub-samples (one for each treatment) where they were subsequently processed using one of the six methods described above (*see* Section 4.1.3.1). All samples were concentrated 4x from 200 µL down to a final volume of 50 µL. A negative control for each treatment method was collected using distilled water. All samples were held at 4°C until analysis. An outline of relative cost for each treatment method is provided in Table 4.2.

Samples were analyzed on a QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using the zebra mussel cytochrome *b* (Cyt *b*) and cytochrome oxidase *c* subunit I (COI) assays described in Gingera et al. (2017) with minor-groove binding (MGB) FAM and VIC fluorophore probes. The HemT IPC (Gingera et al. 2017; Xue et al. 1999) was also used with an ABY fluorophore. The total reaction volume was 20 µL with 1x EMM 2.0, 0.2 µM of each forward and reverse primer, 0.1 µM minor groove binder (MGB) probe, 10^2 copies/µL HemT DNA, and 5 µL of eDNA. 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. Each qPCR plate contained a 10-fold standard curve from 10^0 copies/µL to 10^6 copies/µL and no template control (NTC) reactions. All samples, standards, and NTCs were run in triplicate.

To compare the ability of the six treatments to increase DNA concentration, ΔCt values were calculated by averaging the Ct values of the triplicate reactions for each sample and subtracting the average Ct of the untreated sample from the treatment samples, as follows:

$$Mean\ treatment\ Ct\ \left(\frac{Ct1 + Ct2 + Ct3}{3}\right) - \ Mean\ untreated\ Ct\ \left(\frac{Ct1 + Ct2 + Ct3}{3}\right) = \ Treatment\ \Delta Ct$$

With this ΔCt value, direct comparisons of samples across multiple plates while accounting for variations in reaction efficiencies can be made. Here, a negative ΔCt value indicates an increase in eDNA concentration and detection while a positive ΔCt value indicates lower detection, either due to loss of DNA or more likely effects of inhibitors.

To determine if inhibition of the reaction was increased by treatment, ΔCt values were calculated by averaging the HemT Ct values of the triplicate reactions for each sample and subtracting the average HemT Ct of the NTC sample from the treatment samples using the following equation:

$$Mean\ treatment\ HemT\ Ct\left(\frac{Ct1 + Ct2 + Ct3}{3}\right) - Mean\ NTC\ HemT\ Ct\ \left(\frac{Ct1 + Ct2 + Ct3}{3}\right) = Treatment\ HemT\ \Delta Ct$$

Here, a HemT ΔCt value near or around 0.00 indicates no increase of inhibition, whereas a positive HemT ΔCt value indicates increased inhibition and lower detection.

Comparisons between treatments and untreated samples were performed using Analysis of Variance (ANOVA) with the treatment as the categorical variable and the percent recovery as the dependent variable using the aov function in base R v.3.2.3 for both the ΔCt values of the zebra mussel target genes and HemT IPC. Post hoc Tukey analysis was used to determine differences between treatment groups using the TukeyHSD function in base R v.3.2.3. *A posteriori* p-value adjustments for multiple comparisons were done using the Honest Significant Difference (HSD) Tukey adjustment, as part on the TukeyHSD function.

4.4 Results

4.4.1 Nucleic acid recovery

The percent recovery of DNA varied significantly across all six concentration methods (ANOVA, $F_{5,90} = 47.94$, $P < 0.05$). Amicon filtration units and evaporation performed equally well and had significantly greater recovery than did all other methods ($P < 0.05$) (Fig 4.1). The QIAquick PCR Purification Kit had the second highest recovery which was significantly better than isopropanol precipitation, magnetic bead purification, and PEG precipitation ($P < 0.05$). Isopropanol precipitation and magnetic bead purification performed equally well and had significantly higher recoveries than PEG precipitation, which had the poorest recoveries ($P < 0.05$).

4.4.2 Sample concentration

Fluorescent data generated via qPCR was used to determine the effects of sample concentration methods on the ability of assays to amplify a sample. Positive ΔCt values for any given treatment imply a decrease in assay sensitivity compared to the untreated control sample. Significant variations between ΔCt values of each treatment were found (ANOVA, $F_{5,162} = 9.159$, $P = 1.08e^{-7}$). The QIAquick PCR Purification Kit had the greatest improvement in assay performance (lowest ΔCt values); however, this treatment was not significantly different from Amicon centrifugal units, magnetic bead purification, and PEG precipitation. Isopropanol precipitation resulted in the second greatest reduction of assay performance following evaporation which had the greatest reduction in assay performance with ΔCt values which were significantly higher than the QIAquick PCR Purification Kit ($P < 0.05$) but not significantly

different from Amicon centrifugal units, magnetic bead purification, and PEG precipitation. Evaporation treatment samples were significantly less sensitive than were all other treatments ($P < 0.05$), except for isopropanol precipitation where there was no significant difference (Fig 4.2).

No negative control samples collected at any step of the sample collection, treatment, and analysis process tested positive for contaminated DNA.

4.4.3 Relief of inhibition

Positive ΔCt values of the HemT assay for any given treatment implies a higher concentration of inhibitory substances compared to the NTC reactions where there was likely no inhibition. HemT ΔCt values of treated eDNA samples varied significantly (ANOVA, $F_{6,91} = 5.714$, $P = 4.45e^{-5}$). Evaporation treatment samples had significantly greater inhibition than did all other treatments ($P < 0.05$) (Fig 4.3). There were no significant differences between the Amicon centrifugal units, isopropanol precipitation, magnetic bead purification, PEG purification, QIAquick PCR Purification Kit, and untreated control groups.

4.5 Discussion

This study demonstrates that methods of DNA concentration and purification traditionally used in other fields of research can be applied to eDNA sample analysis to improve detection and reduce the likelihood of FN. The ability to enrich eDNA samples and improve assay performance varied between treatments; however, silica column purification resulted in consistently improved zebra mussel detection and no increase in qPCR inhibition. Although not statistically significant, silica column purified samples were the only treatment group which demonstrated ΔCt values of – 2.0; a Ct value of 1.0 represents a doubling of PCR product

(Higuchi et al. 1992), suggesting that amplification of silica column purified samples was improved almost 4x (i.e., equal to the concentration factor, *see* Section 4.3.2.2). Silica column purification was the most optimal method tested in this study.

Silica column purification is also well suited for high throughput of large sample numbers, typical of monitoring programs. Of the methods tested, silica columns are moderately priced, have a relatively low processing time, require little specialty equipment, and can be purchased in 96-well format. Although silica column purification DNA recovery was not significantly different from isopropanol precipitation, magnetic bead purification, and PEG precipitation, and was significantly less than centrifugal units and evaporation, it is likely more effective at removing inhibitors from eDNA samples compared to all other methods tested due to the consistently improved zebra mussel assay performance after treatment. There was, however, no significant difference in IPC assay performance for these methods.

Amicon centrifugal units and evaporation methods resulted in the greatest recovery of DNA; however, inhibitors were similarly concentrated, which resulted in a reduction of assay performance. Unlike evaporation, the Amicon centrifugal units displayed greater zebra mussel and IPC assay performance, suggesting there was some removal of PCR inhibitors compared to evaporated samples. Amicon centrifugal unit treatment is therefore more favourable than evaporation for concentrating samples; however, it is not recommended for concentration of samples where PCR inhibitors are present. These results demonstrate that DNA recovery and improved assay sensitivity are not mutually inclusive concepts. Methods such as PEG precipitation, which displays poor DNA recovery, but improved PCR inhibitor removal, can result in similar assay performance compared to centrifugal units, which demonstrates high DNA recovery but a low capacity for PCR inhibitor removal. For example, silica columns may

improve assay performance because lower DNA recovery results in the loss of PCR inhibitors with similar chemical properties, such as humic acid.

In all treatments, excluding silica columns, there was a reduction in zebra mussel assay performance without a significant reduction in the IPC assay performance. Presumably, there would be an equal reduction in IPC performance if the target assay performance was reduced, but this was not the case. Areas within a single freshwater system will have varying concentration of certain sources of PCR inhibitors, such as vegetal debris and algae (Schrader et al. 2012). The difference in zebra mussel and IPC assay performances when inhibitors are clearly present may indicate that certain inhibitors may not be affecting the IPC assays in the same way that they are affecting the zebra mussel assays. Research has shown that inhibitors affect various genetic markers and DNA sequences differently (Stahlberg et al. 2003; Villanova et al. 2007; Huggett et al. 2008; King et al. 2009). The inclusion of an IPC may not guarantee an accurate assessment of whether inhibition is present if the PCR inhibitor interacts with the target DNA, assays, and IPC sequence specifically. This effect should be examined in future studies to further the understanding of the usefulness of IPCs and develop methods which address potential shortcomings of IPCs.

This study did not investigate ranges of acceptable concentration factors; instead, all treated samples were concentrated 4x for all methods. Depending on the type and concentration of PCR inhibitors within a sample, different enrichment factors will be more appropriate than the 4x used here. Samples with a lower PCR inhibitor concentration could be enriched to a greater degree than highly inhibited samples, without increasing inhibition. Commonly used additives for eDNA analysis, such as EMM 2.0, improve the robustness of a qPCR and therefore a small increase in PCR inhibitor concentration due to enrichment may be acceptable (depending on

starting concentration). Future studies should assess ranges of enrichment factors for varying concentrations of PCR inhibitors based on acceptable inhibition thresholds for commonly used qPCR additives, such as EMM 2.0.

Often, researchers will simply infer which types of PCR inhibitors are present based on the sample source (*reviewed in* Schrader et al. 2012). All samples in this study had a dark-brown pigmentation which was seemingly co-eluted during extraction. Indeed, highly-pigmented samples exhibited greater inhibition than did more lightly-pigmented samples. Similar properties have been observed in other eDNA studies, and these are common for eDNA samples collected from freshwater lentic and lotic systems (McKee et al. 2015). The cause for the pigmentation has been assumed to be humic substances and sediment (McKee et al. 2015). The relative abundance of certain PCR inhibitors can be determined with spectrophotometric data; for example, light absorbance at 230 nm (A_{230}) has been used to determine the concentration of humic acids in environmental samples (Hanni et al. 1995; LaMontagne et al. 2002). Therefore, silica column purification and enrichment/purification methods which efficiently remove these substances should be preferentially applied to eDNA analysis.

OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) is a commercially available nucleic acid purification method which is specifically marketed as a humic acid removal system for eDNA analysis. This method has been shown to relieve inhibition of eDNA samples to a greater degree than sample dilution (1:4 and 1:9) (McKee et al. 2016); however, some labs have observed poor DNA recovery from this method (pers. comm. Gordon Luikart, University of Montana). This method was not tested in this study as it lacks the capability to enrich samples by lowering elution volume. Future research should assess a coupled approach where OneStep treatment of eDNA samples is followed by either centrifugation (i.e.,

with Amicon units) or evaporation, as these methods demonstrated the greatest DNA recovery of the methods tested. Although less cost-effective, centrifugal units (e.g., Amicon) would be more time-efficient and may also aid in removing additional inhibitors, whereas enrichment via evaporation would be more cost-effective but less time-efficient. These methods may be used effectively together in future studies to improve eDNA target detection and reduce the likelihood of FN. New methods of inhibitor removal, such as the Aurora System (Boreal Genomics, Vancouver, BC, CA) (Engel et al. 2012; Schmedes et al. 2013), should also be considered for future use regarding QA/QC in eDNA research.

This study demonstrates that sample enrichment, coupled with DNA purification methods, can viably be implemented for eDNA analysis to improve detection and reduce FN. Nucleic acid purification techniques often result in some loss of target sequences, which is problematic when starting DNA concentrations are low. Loss of target sequences is undesirable for the purposes of species detection using eDNA as it will increase the likelihood of FN, thus reducing the accuracy and reliability of the data collected. There is a significant amount of technical overlap between forensics, virology, aDNA research, and eDNA research. Many issues common to eDNA research are actively being investigated in these fields, which have developed several solutions to common technical problems shared by these seemingly disparate areas of research. eDNA researchers should become more aware of developments in these fields and vice versa. In this study, silica column purification was most effective at concentrating eDNA samples and improving zebra mussel assay performance in all samples tested. The silica column tested, the QIAquick PCR Purification Kit, also has fewer time requirements and is comparable in cost to the other methods tested in this study. Suggested here is a novel method of sample concentration and purification by coupling the use of OneStep columns followed by

concentration using either evaporation or Amicon units, as recovery is high and would result in little DNA loss. Inhibition of zebra mussel assays was still observed in samples where the IPC showed no inhibition. IPCs should be further investigated as they may not be an accurate tool for determining inhibition as certain inhibitory substances may react to added IPC DNA differently than in-sample eDNA. Further novel methods of eDNA concentration and purification should be examined which could be applied and assessed in future eDNA research. Ultimately, this work will further efforts made to increase the reliability and effectiveness of eDNA detection of aquatic species, which is integral to the establishment of eDNA as a monitoring tool.

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4.7 Tables and Figures

Table 4.1 Sample site coordinates and volume of water filtered for samples collected in the south basin of Lake Winnipeg. Samples were collected August 31 and September 1, 2016. All sites sampled were considered to contain zebra mussels based on data collected by Gingera et al. (2017), *see* Chapter 2.

Harbour	Site number	Coordinates	Volume of water filtered (mL)
Selkirk Harbour	1	50.6592 °N, 99.98106 °W	250
	2	50.6592 °N, 99.98106 °W	250
Winnipeg Beach Harbour	1	50.14935 °N, 96.85574 °W	1000
	2	50.50835 °N, 96.96517 °W	750
	3	50.50908 °N, 96.96761 °W	750
Gimli Harbour	1	50.63041 °N, 96.98347 °W	750
	2	50.63126 °N, 96.98354 °W	1000
	3	50.63118 °N, 96.98359 °W	1000
Hnausa Harbour	1	50.91118 °N, 96.98174 °W	750
	2	50.91087 °N, 96.98199 °W	750
	3	50.91074 °N, 96.98093 °W	500
Balsam Bay Harbour	1	50.4737 °N, 96.58292 °W	500
	2	50.47383 °N, 96.58333 °W	500
	3	50.473838 °N, 96.583873 °W	250

Table 4.2 Nucleic acid purification and concentration method parameters and logistics

Method	Approximate cost per sample ($)[a]	Approximate processing time (h)	Consumables/additional reagents	Reaction temperatures required	Additional equipment required[b]	Target amplification average (mean ΔCt ± SE)	IPC amplification average (mean ΔCt ± SE)
Amicon	4.39 (<5.00)	0.75	Tubes, suspension buffer	RT	N/A	-0.152 ± 0.332	0.152 ±0.130
Evaporation	0.00 (<0.10)	3.0 (for 200 μL sample)	Tubes, suspension buffer	RT	Vacufuge	1.608 ± 0.669	0.993 ± 0.376
Isopropanol precipitation	<1.00	0.5-overnight (incubation) + 0.3	Tubes, suspension buffer, Isopropanol	4°C, RT	N/A	0.604 ± 0.452	0.203 ± 0.197
Magnetic bead	2.10 (<2.50)	0.5-overnight (incubation) + 0.5	Tubes, suspension buffer, magnetic bead solution, ethanol	4°C, RT	Magnetic tube rack or Biosprint (or similar platform)	-0.382 ± 0.089	-0.214 ± 0.052
PEG	<1.00	0.5-overnight (incubation) + 0.3	Tubes, suspension buffer, PEG solution, ethanol	-20°C, RT	N/A	0.778 ± 0.189	-0.089 ± 0.078
QIAquick silica column	1.82 (<2.00)	0.25	Tubes, suspension buffer	RT	Centrifuge	-1.588 ± 0.037	-0.167 ± 0.054

[a] Approximate free market price.

[b] In addition to centrifuge

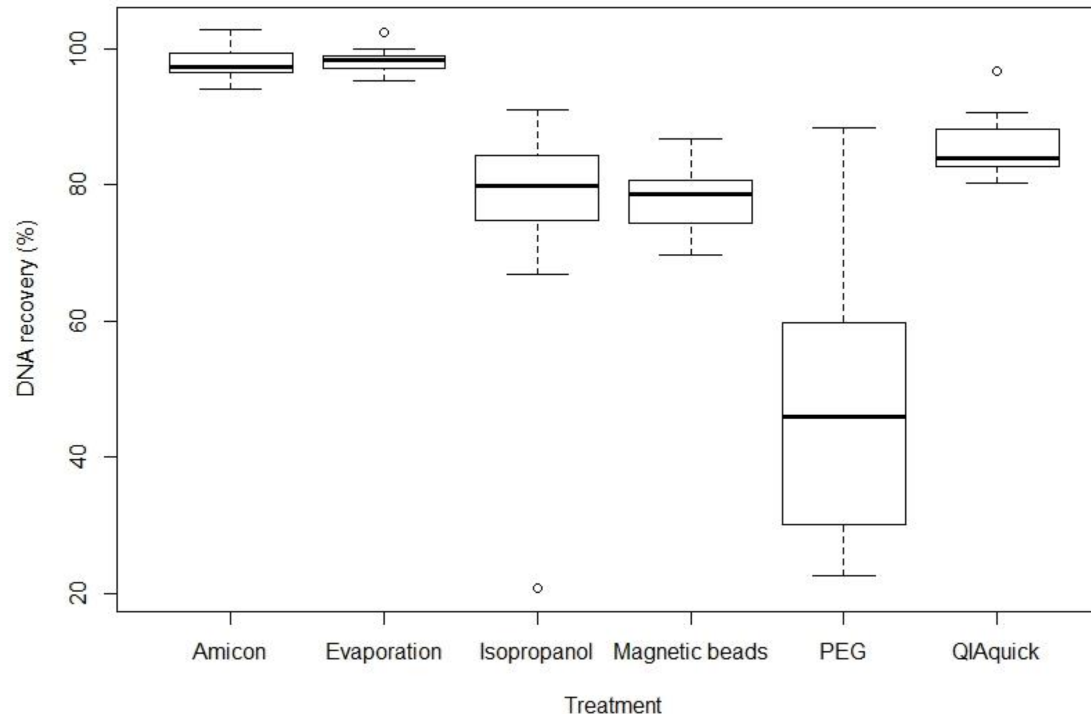

Figure 4.1 DNA recovery (%) of six enrichment treatment methods: 1) centrifugal filter units (Amicon Ultra 0.5 mL 30 kDa); 2) sample evaporation; 3) isopropanol precipitation; 4) magnetic bead purification; 5) polyethylene glycol (PEG) precipitation; and 6) silica column purification (QIAquick PCR Purification Kit). GeneRuler 50bp DNA ladder aliquots (n=16 per treatment) were concentrated from 200 µL down to 50 µL and compared with untreated control sample. DNA recovery was determined using Qubit 2.0 Fluorometer HS assay. DNA recovery was significantly greater for centrifugal filter units and sample evaporation than all other methods. PEG precipitation had significantly lowest DNA recovery. Maximum and minimum values represented by edges of whisker plots, 25% and 75% quartiles represented by edges of box plots, central line of box plots represent median value, outliers (>2 sd) represented by points above/below whisker box plots.

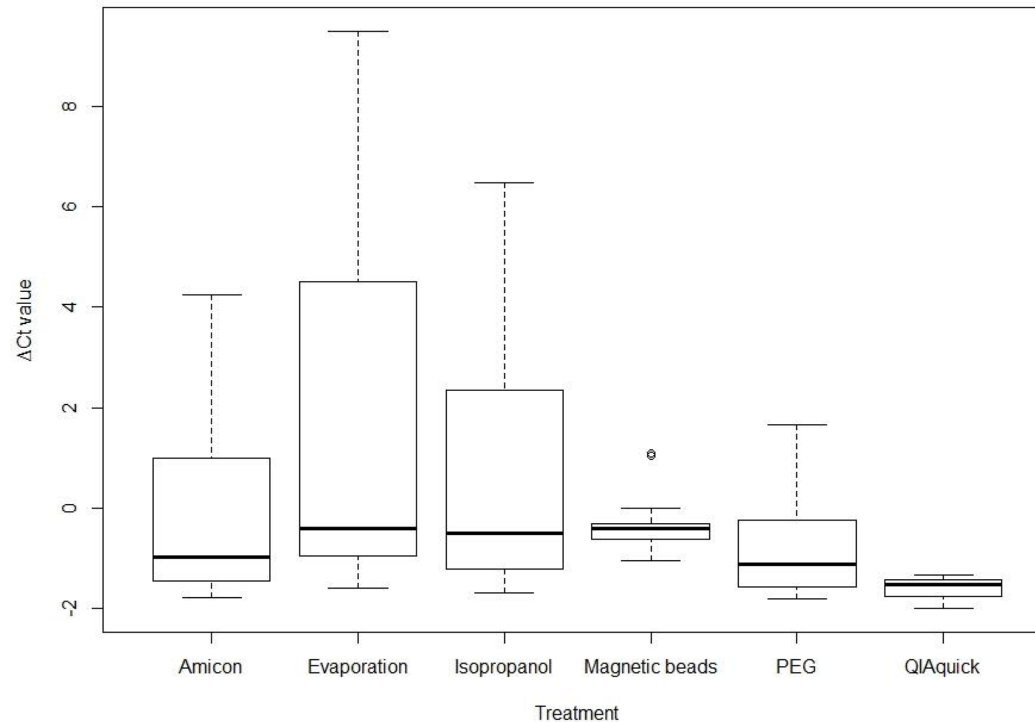

Figure 4.2 ΔCt values of cytochrome *b* (Cyt *b*) and cytochrome oxidase *c* subunit I (COI) zebra mussel assay reactions were calculated by averaging the Ct values of the triplicate reactions for each sample and subtracting the average Ct of the untreated sample from the treatment samples generated via qPCR. Six enrichment treatments were tested: 1) centrifugal filter units (Amicon Ultra 0.5 mL 30 kDa); 2) sample evaporation; 3) isopropanol precipitation; 4) magnetic bead purification; 5) polyethylene glycol (PEG) precipitation; and 6) silica column purification (QIAquick PCR Purification Kit) for eDNA samples collected from 14 sites within Lake Winnipeg (n=28 per treatment) which were enriched from 200 µL down to 50 µL (4x). Sample evaporation and isopropanol precipitation had statistically higher ΔCt values compared to all other treatments. Differences in other treatments were not statistically significant. Maximum and minimum values represented by edges of whisker plots, 25% and 75% quartiles represented by edges of box plots, central line of box plots represent median value, outliers (>2 sd) represented by points above/below whisker box plots.

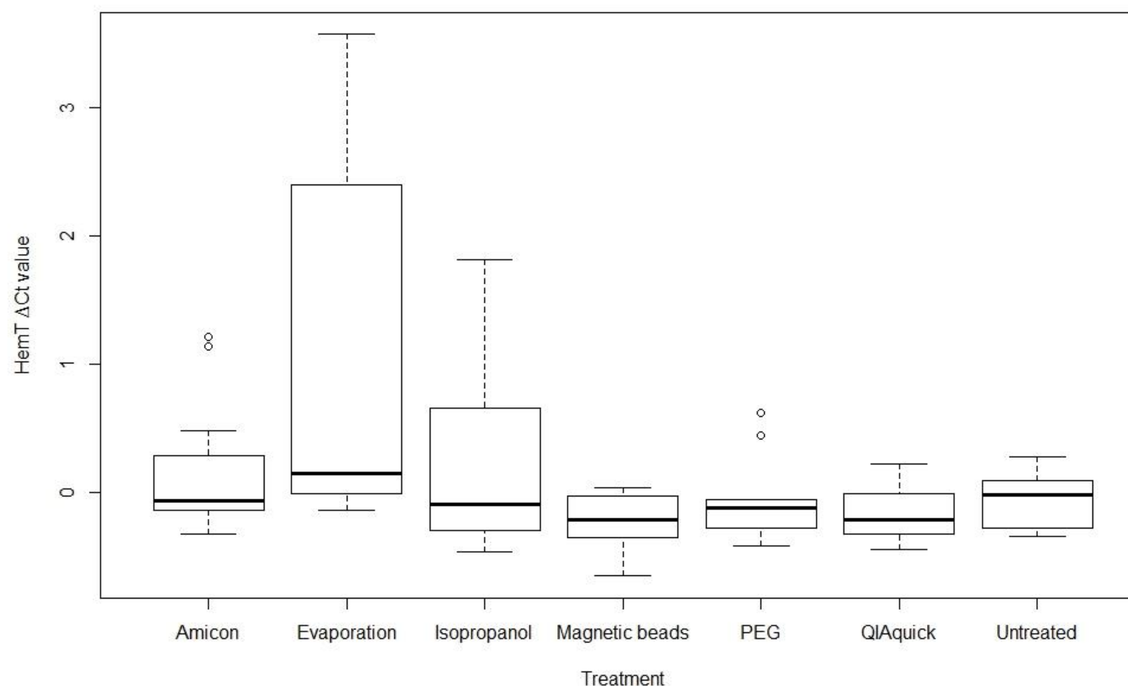

Figure 4.3 ΔCt values of the HemT internal positive control assay reactions were calculated by averaging the Ct values of the triplicate HemT reactions for each sample and subtracting the average Ct of NTC sample generated via qPCR. Seven treatments groups were tested: 1) centrifugal filter units (Amicon Ultra 0.5 mL 30 kDa); 2) sample evaporation; 3) isopropanol precipitation; 4) magnetic bead purification; 5) polyethylene glycol (PEG) precipitation; 6) silica column purification (QIAquick PCR Purification Kit); 7) untreated samples, for eDNA samples collected from 14 sites within Lake Winnipeg (n=14 per treatment) which were enriched down from 200 µL to 50 µL (4x). Sample evaporation and isopropanol precipitation ΔCt values were significantly greater than untreated samples, whereas there was no significant difference between other treatment groups. Differences in other treatments were not statistically significant. Maximum and minimum values represented by edges of whisker plots, 25% and 75% quartiles represented by edges of box plots, central line of box plots represent median value, outliers (>2 sd) represented by points above/below whisker box plots.

CHAPTER 5. GENERAL DISCUSSION

The spread of zebra mussels *Dreissena polymorpha* into Lake Winnipeg poses a significant economic and ecological threat to freshwater ecosystems in Manitoba and western Canada (Rajagopal et al. 2005; Brazee and Carrington 2006; Grutters et al. 2012). Conservation managers require diagnostic detection methods for zebra mussels that can produce efficient, economical, and accurate occurrence data to aid in limiting the spread of zebra mussels into uninvaded waterbodies. Due to the successful implementation of eDNA analysis for AIS in previous studies (Ficetola et al. 2008; Dejean et al. 2011; Jerde et al. 2011; Collins et al. 2013; Goldberg et al. 2013; Mahon et al. 2013; Takahara et al. 2013; Gingera et al. 2016; USACE 2015), eDNA analysis provides a sensitive and economical diagnostic tool for zebra mussel monitoring. The work herein provides the foundation necessary for the development of an invasive mussel eDNA monitoring program for Manitoba and western Canada by: 1) developing and testing field and laboratory protocols for zebra mussel eDNA detection; 2) providing a comparison between eDNA detection and conventional plankton netting for veligers; and 3) refining detection methods by investigating eDNA enrichment methods. These themes provide insight into where zebra mussels presently occur in Lake Winnipeg, how their spread has progressed since the first sighting in 2013, how to best design an eDNA monitoring program to address occurrence, geographic spread, and abundance, as well as deliver adaptation and/or control options in the future.

5.1 Zebra mussel environmental DNA detection

Prior to establishing a monitoring program for AIS, it is paramount that the diagnostic detection methods are well tested and reliable. A central deliverable of this thesis is the field and

laboratory methods for detection zebra mussel eDNA and tested set of eDNA assays capable of reliably detecting zebra mussels and dreissenid mussels (Chapter 2). These three assays are highly sensitive and can out-perform conventional plankton netting methods in large waterbodies late in the season (September-October; Chapter 3). Indeed, these assays provided four novel detections of zebra mussels in areas in/around Lake Winnipeg (i.e., Selkirk in 2014 and Lake Winnipeg north basin in 2015; Chapter 2, 3). Both detections were confirmed the following years with visual sightings of zebra mussels near those locations. Detections do decrease along with a low abundance of target species as evidence by the May harbour samples (Chapter 2) and single location in the south basin of Lake Winnipeg where veligers were captured (Chapter 3). However, a reduction in reliability along with low abundance is not unique to eDNA and affects most diagnostic detection methods. Environmental DNA detection should not replace conventional detection methods, but it provides managers with another tool which is highly sensitive.

It is important to understand how eDNA detection methods compare to conventional methods of aquatic species detection. In this case, plankton net sampling for veliger capture is typically used during monitoring (Mackie and Claudi 2010). Overall, eDNA detection was equally effective at detecting presence/absence compared to plankton netting and was more effective when veligers were in low abundance/absent as other life stages may have been present (Chapter 3). Other studies that have directly compared eDNA to conventional sampling methods have also found that eDNA was more sensitive than past electrofishing results for bull trout *Salvelinus confluentus* (Mckelvey et al. 2016) and seining for tidewater goby *Eucyclogobius newberryi* (Schmelzle and Kinziger 2016). Environmental DNA may not always be the most sensitive method, however, as visual snorkeling surveys of bluehead sucker *Catastomus*

discobolus and Zuni bluehead sucker *C. discobolus yarrow* yielded more detections than did qPCR eDNA methods (Ulibarri et al. 2017).

Zebra mussel eDNA assays already exist in the literature. Ardura et al. (2017) have developed a conventional PCR (cPCR) assay and Mahon et al. (2011) have developed a Light Transmission Spectroscopy (LTS) assay, whereas the assays reported here are probe-based qPCR assays. Neither of the previous analytical approaches are as sensitive as are quantitative PCR (qPCR) or digital-droplet PCR approaches (ddPCR) (Pilliod et al. 2013; Takahara et al 2012; Wilcox et al. 2013; Nathan et al. 2014; Amberg et al. 2015; Doi et al. 2015; McKee et al. 2015; Gingera et al. 2017; *see* Appendix A). The assays reported in this thesis are compatible with ddPCR and thus will not become obsolete as ddPCR becomes more prevalent. These assays also allow for the option of being multiplexed for several target DNA markers (i.e., used simultaneously within a single reaction) either with each other or with an internal positive control (IPC). This benefits monitoring programs by increasing redundancy and thus improving likelihood of detection, albeit while allowing for some measurement of inhibition (Dingle et al. 2004). The effects on Ct-values due to interactions between assays in a multiplex are well recorded. This allows for more informed decisions to be made when applying these methods for invasive mussel monitoring. As these are the most tested zebra mussel assays in the literature (Mahon et al. 2011 Ardura et al. 2017) and can be equally/more reliable than conventional sampling (Chapter 3), they should be considered a monitoring tool by managers.

5.2 Present and future of zebra mussels in Lake Winnipeg

The novel detections in Selkirk (Chapter 2) reflect a pattern of spread which could be predicted by human vectors. The north basin detections (Chapter 3) moreso reflect a pattern of

spread predicted by the hydrography of Lake Winnipeg. The Selkirk detection occurred in the Red River, which feeds into Lake Winnipeg (*see* Section 1.4). Because no detections were recorded upstream of this site, passive dispersal of veligers down the Red River from the US is less likely. However, because the detection was near a high human-traffic area (i.e., a float plane dock), it reflects how human vectors will be a major cause for spread into upstream or isolated areas. The north basin detections reflect the flow of water in Lake Winnipeg. Although the actual vector of spread cannot be determined with any certainty, the passive movement of veligers from the south basin into the north basin via flow would have inevitably led to the spread of zebra mussels into the north basin.

Future spread of zebra mussels within Lake Winnipeg will likely continue, according to survivability estimates based on calcium concentrations (Therriault et al. 2013). An eradication treatment for zebra mussels in Lake Winnipeg is not viable due to the lake's size (Therriault et al. 2013). Although efforts can be made to reduce spread via human vectors within the north basin, passive spread via flow will inevitably lead to wider distributions and movement into the Nelson River. Confirmed sightings of zebra mussels will likely initially occur where there is submerged infrastructure and human traffic. Currently, the north basin is not as heavily invaded and is less frequented by humans compared to the south basin. It is therefore less likely to be the source of secondary spread into upstream systems via human vectors, apart from the few higher traffic areas in the north basin. Currently, zebra mussels are ubiquitous throughout the south basin, apart from areas near the Winnipeg River where calcium concentrations are too low to support shell development (Therriault et al. 2013). The south basin is therefore the greatest source of secondary spread of zebra mussels; management should therefore consider allocating resources towards monitoring traffic from the south basin into other waterbodies.

5.3 Considerations for zebra mussel eDNA monitoring

5.3.1 When/where to sample

Seasonal variation in detection frequency is also useful information for monitoring programs, as it can allow resources to be allocated towards sampling schedules which will result in the greatest likelihood of detection (Marsden 1992). In Manitoba, spawning likely occurs between June and October. During this period gametes, veligers, and mytiliform mussels will be present and probabilities of eDNA detections will increase. Outside of this period, mytiliform mussels will account for the majority of the eDNA signal. Late season (September-October) sampling should be prioritized if the objective is to maximize likelihood of detection. Zebra mussels will have benefited from a reproductive and growth season by this time, further improving detections due to the abundance of veligers and mytiliform mussels. Veliger abundance appears to influence eDNA detections as samples collected in May yielded few positive detections (Chapter 2) and veligers accounted for a large amount of variation in eDNA concentrations (Chapter 3). Late season distribution data may also be used to inform management decisions and strategies for early spring after freeze/thaw of freshwater systems, as there will likely be minimal spread during the winter due to die-off via desiccation and lack of spawning (Sprung 1989; McMahon 1996; Ram et al. 1996; Grazio and Montz 2002; Werner and Rothhaupt 2008; Sousa et al. 2012; Leuven et al. 2014).

Sampling efforts must focus on locations which are at-risk of invasion, as Lake Winnipeg is beyond eradication treatment. Only periodic sampling should occur in heavily infested areas as these areas will likely be the first to be invaded by quagga mussels (Ricciardi and Whoriskey 2004; Orlova et al. 2005; Wilson et al. 2006; Zhulidov et al. 2010; Matthews et al. 2014). Most

waters surrounding Lake Winnipeg drain into the Lake, therefore human vectors will be the major cause of spread throughout Manitoba, and perhaps further west. Therefore, sampling must target surrounding waterbodies with human traffic and submerged infrastructure such as harbours, docks, boat launches, etc. The zebra mussel Risk Assessment (Therriault et al. 2013) should be consulted when designing a sampling plan as it is important to target areas with high survivability estimates.

When attempting to confirm existing negative detections in a waterbody, late season sampling (September-October) provides stronger evidence of absence compared to early season samples (May) (Gingera et al. 2017; Chapter 2, 3). The priority for these "before the fact" areas will be prevention of spread into these areas along with continued monitoring. Redundancy in sampling via multiple assays (Chapter 2), replicate samples (Chapter 3), and inhibitor removal/enrichment methods will improve the likelihood of detection for these samples (Chapter 4).

For attempting to detect progressing positive occurrences, regular temporal sampling between June and September is ideal as it is during this period that spread will occur. The priorities for these "after the fact" areas are issues such as spread within the waterbody (see above), relative abundance estimates (*see* Section 5.4.1), prioritization of control areas (see above), and needs to adapt to changing circumstances between seasons (see above). Regardless, the occurrence of false positive (hereby referred to as FP) and false negative (hereby referred to as FN) results must be considered when conducting monitoring with eDNA.

5.3.2 False results and subsequent implications for monitoring

The potential for FP and FN results have a profound influence on how eDNA data is interpreted, especially when such data are being considered for decision making by managers. Although both should be considered as unfavourable, the implications of each false result have different implications when monitoring for AIS.

FP results may lead to an increased allocation of funds to prevent further spread from the falsely identified invaded area. This would divert resources from other management initiatives and potentially reduce the efficacy of the monitoring program. FP results may also trigger an eradication treatment which can often be devastating to local biota. Eradication treatments for AIS often negatively impact native biota and can be particularly devastating if native species are considered at-risk and would provide no benefit to management. For example, 3-trifluoromethyl-4-nitrophenol (TFM) is a pesticide used to eradicate invasive sea lamprey *Petromyzon marinus* from streams within the Great Lakes basin; however, treatment also eradicates at-risk native lamprey species in the treatment area (Siefkes et al. 2012). Likewise, potash is a common treatment used for eradicating zebra mussels; however, it also eradicates local mussel species in the treatment area (DFO 2014).

FN results may promote further spread of AIS because invaded areas would go unidentified. These areas would likely go untreated and funds would not be allocated to prevent spread from the area. FN results therefore lead to further ecological degradation due to spread of AIS. FN results cannot be detected, only inferred via the inclusion of positive controls and Internal Positive Controls (IPC) (*see* Appendix A). However, IPCs may not always be effective at detecting inhibition (Chapter 4). Critical samples which are suspected to have high inhibition

and low eDNA concentration may be good candidates for sample enrichment/purification (Chapter 4).

5.4 Ongoing topics/concerns for eDNA monitoring

5.4.1 Relative abundance estimates

Relative abundance estimates of AIS become an important aspect of eDNA sampling once the target is first detected in a new area (*see* Section 5.3.1) and when attempting to infer additional “aspects” of the target. Currently, eDNA data are limited to presence/absence detection of aquatic organism (Darling and Mahon 2011; Jerde et al. 2011, 2013; Amberg et al. 2015; Mckelvey et al. 2016; Schmelzle and Kinzinger 2016). It is difficult to infer absolute abundance with eDNA quantification as environmental factors which affect eDNA concentration are often numerous, complex, and not well understood. However, when environmental conditions are more constant (e.g., samples collected within a single system or within a short period of time) eDNA may be used as an indicator of relative abundance.

Veliger abundance was correlated with eDNA concentrations from samples collected within a short period in Lake Winnipeg (Chapter 3). Several studies have also demonstrated a correlation between eDNA concentration and target abundance (Takahara et al. 2012; Thomsen et al. 2012; Goldberg et al. 2013; Doi et al. 2015; Klymus et al. 2015; Eichmiller et al. 2016). Therefore, temporal relative abundance estimates for a target within a system should be a primary concern when monitoring spread of AIS with eDNA. These estimates could be used to infer spread and times of reproduction in an area. Such estimates could also be used to infer if invaders fail to become established in a new area. Presumably, this would manifest as a weak

initial detection followed by negative detections. Treatment for invasive mussels is considered more successful during early stages of an invasion (Mackie and Claudi 2010). Therefore, low abundance estimates may be considered a good indication that treatment of a newly invaded area is viable.

Relative abundance estimates using eDNA could also be used to assess the efficacy of eradication efforts for AIS (Davison et al. 2017). There is also considerable interest in using relative abundance estimates to infer target behaviour, specifically when spawning is occurring. Erickson et al. (2016) found that eDNA concentration was correlated with movement in bighead carp *Hypophthalmichthys nobilis* and suggested that mass movement could be used as a proxy for predicting spawning; however, no correlation was found with spawning activity inferred from drifting eggs. Gingera et al. (2016), however, found that eDNA detections increased along with presumed spawning period and subsequent die-off of invasive sea lamprey in the Great Lakes basin. Clearly, life history of the target organism affects the eDNA detection profile during spawning and will vary between taxa; it is therefore important to consider these aspects of an organism's biology when developing relative abundance estimates. For example, zebra mussels have a drastically different life history compared to most aquatic vertebrates (i.e., long spawning period between June and September, non-motile adults, larvae which move passively along with lake/stream flow). The samples collected in this thesis were collected during short periods; therefore, regular sampling between May and November is required for a more complete eDNA detection profile. However, the results presented in this thesis suggest that detections increase after May and would likely drop in November due to the fewer number of veligers (Chapter 2, 3).

5.4.2 Guidelines for eDNA assay development

Assay development is the identification and testing of oligonucleotide sequences which have potential use with eDNA samples. Three aspects of assay design were focused on in this thesis: 1) species-specificity; 2) assay sensitivity; and 3) multiplexing. This section provides guidelines for the development of eDNA assays with an emphasis on species-specificity and well-defined assay sensitivity, which are essential factors for proper QA/QC during AIS monitoring. Proper testing of eDNA assays must be done to ensure target-specificity; otherwise non-target eDNA fragments may be detected, resulting in FP results. Genetic variation will inevitably be present across different haplotypes of the target organism; therefore, the assay must target fragments which are likely conserved across individuals, populations and species to reduce the possibility of FN results. Sensitivity of an eDNA assay can be measured as the limit-of-detection (LoD) which is defined as the concentration of target template that can be reliably measured (Armbruster and Pry 2008). Due to the low concentration of target typically found in eDNA samples (McKee et al. 2015), a high LoD is detrimental and may result in FN results. For example, Gingera et al. (2016) and Ardura et al. (2017) both reported on cPCR assays which only detected target species where there was a moderate to high abundance of the target DNA. Multiplexing (i.e., incorporating several eDNA assays into a single reaction for simultaneous detection) can reduce the likelihood of FN results by increasing the number of potential targets. Multiplexed reactions increase redundancy within a reaction, which makes it possible that at least one target eDNA fragment will be amplified even if another is not detected either due to low concentration of target or inhibition. Multiplexing can also be done by including an IPC in the qPCR to function as an indicator for inhibition. More research regarding the effectiveness of IPCs is required as they may not always be effective at detecting inhibition (Chapter 4).

Inhibitory substances may interact with different DNA targets differently (Stahlberg et al. 2003; Villanova et al. 2007; Huggett et al. 2008; King et al. 2009) and other factors such as temporal exposure and freeze/thaw may affect IPC template differently compared to the target template (Chapter 4). The simultaneous use of more than one assay will increase competition for qPCR resources, as well as increase the possibility of heterodimer formation, all of which will result in decreased assay performance (Raeymaekers 1995; Hofmann 2003; Kontanis and Reed 2005; Gall et al. 2007, Chapter 2). Increasing the cycle number for a qPCR may compensate for reduced efficiency; however, a drastic reduction of efficiency will increase the likelihood of FN results. Multiplexing must only be implemented in monitoring if proper testing has been done.

5.4.2.1 Assay development: Identification of oligonucleotide sequences (stage one)

The primary goal at this stage of eDNA assay development is to ensure that: 1) oligonucleotide sequences are complementary to all accumulated target sequence data; 2) melting temperatures (Tm) between separate assay nucleotides are comparable; and 3) that the ΔG values for various secondary structures (i.e., heterodimers, homodimers, and hairpins) are within an acceptable range.

Prior to assay design, it is essential to select multiple target fragments and collect the largest set of gene sequences possible. Nucleic acid sequence databases are not complete (Kwong et al. 2012); therefore, some database development may be required (Goldberg et al. 2016). Target gene selection may depend on which genes sequences are available. Mitochondrial (mt) genes should be prioritized as mtDNA is more plentiful in a cell and is more prevalent in sequence databases. It is also important to select sequences from a wide geographic range. This will help avoid FN results due to haplotype differences within the target species.

Oligonucleotide sequences should be identified in areas which are conserved within the species but are sufficiently variable compared to other species. Sequence alignment software (e.g., MEGA) is useful in this regard. Parameters should be set as to what an acceptable ΔG value is for the formation of secondary structures. For this thesis, assays were eliminated if hairpin $\Delta G \geq -5.00$, heterodimer or homodimers $\Delta G \geq -9.00$, and total secondary structure $\Delta G \geq -60.00$ kcal. Also consider if a minor-groove-binding (MGB) site will be incorporated in the probe as it will affect probe Tm. If assays are to be multiplexed, Tm values for the oligonucleotides for the respective assays must be within ≤ 2°C of each other. Assay design software (e.g., primer 3, primer BLAST, Primer Express) could be used to identify oligonucleotides; however, these programs often provide several similar assays which target a small section of the gene and do not always produce a good selection of assays. Therefore, assays designed "by hand" will ensure most possible oligonucleotides are considered; however, this method can be very time consuming. It is important to produce a large pool of candidate assays, as this pool will gradually be culled following each of the following steps until only a few, well tested, assays remain.

5.4.2.2 Assay development: In silico species-specificity (stage two)

The primary goal at this stage is to test candidate assays for target-specificity *in silico*. Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov) and/or primer BLAST searches are currently the most expedient methods of determining non-target amplification of ambiguous and distantly related species. These searches will also allow for the identification of nucleotide mismatches between the oligonucleotides and non-target species. In this thesis, ≥ 7 mismatches between each oligonucleotide and the non-target sequence were

considered enough deviation so as to not result in amplification; however, mismatch location must also be considered. Specifically, mismatches near or at the 3' end of the oligonucleotide will greatly reduce the likelihood of amplification. A high number of mismatches were possible in this study because North America has no native species which are closely related to zebra and quagga mussels. When closely related non-target species are present in the sample area, ≥3 mismatches may provide sufficient species-specificity (Gingera et al. 2016).

5.4.2.3 Assay development: In vitro species-specificity (stage three)

At this stage, assay species-specificity is assessed *in vitro* against: 1) tissue-derived DNA from a wide geographic range of the target; and 2) tissue-derived DNA from closely-related species.

Testing tissue-derived DNA from a wide geographic range of specimens of the target organism reduces the likelihood of FN results (*see* Section 5.4.2.1). Testing tissue-derived DNA from closely-related species in the sampling area ensures non-target amplification for these species will not occur and cause FP results. As several assays are to be tested during this stage, cPCR and gel electrophoresis are the most economical options. Assays which do not amplify all target samples and/or amplify non-target DNA must be eliminated from the pool.

Some AIS may not have closely related species present in the sampling area (such was the case for this thesis); it is therefore up to the discretion of the researcher to determine which native taxa should be tested. For example, the closest related species to zebra mussels in Manitoba are freshwater mussels which belong to a separate order (Unionoida) (Graf and Cummings 2007). It was therefore unlikely non-target amplification for these species would occur following *in silico* analysis (Chapter 2). If closely-related species are expected to be

present in the sample area, as is the case for some AIS and endemic species, this step is crucial. Assays which amplify non-target species may be addressed in one of two ways: 1) if amplification is weak, the PCR annealing temperature (Ta) can be increased to decrease sensitivity; or 2) eliminate the assay. If the pool of candidate assays is reasonably large, elimination of the assay is recommended. Simply put, a sufficiently species-specific assay will amplify all target species samples and no non-target species.

5.4.2.4 Assay development: Limit of detection, qPCR efficiency, and multiplexing (stage four)

The primary goal at this stage of eDNA assay development is to: 1) determine the qPCR LoD of each assay; 2) determine assay efficiency; and 3) determine how the incorporation of other assays affect both above (multiplexing only).

The LoD for each assay should be determined using serial dilutions of purified target template measured in copies/µL (Chapter 2). Understanding the LoD of an assay provides insight into the performance with eDNA samples, as detection limits will certainly be lower than values recorded here due to inhibitory substances (McKee et al. 2015; Chapter 2, 4). Assay efficiencies measured via standard curves will provide insight into how many cycles are required to reach the LoD. In the case of this thesis, assay efficiencies between 90% and 110% were considered acceptable.

The tests conducted at this stage identify the best performing candidate assay(s). If multiplexing with other eDNA assays or if an IPC is being developed/incorporated, assays must be tested using serial dilutions (like above) as multiplexed reactions to determine any decrease in LoD and/or efficiency. This will determine if FN results will occur due to an increase in qPCR resource competition (Raeymaekers 1995; Hofmann 2003; Kontanis and Reed 2005; Gall et al.

2007). If the LoD for an assay does not significantly decrease while in a multiplex, it can safely be used but only with those other assays with which it was tested. The LoD and efficiency of each assay must be well understood prior to use in a monitoring program. This phase can be ignored if multiplexing eDNA assays is not of interest. Future steps involve testing the developed assays in field pilot studies and/or the effect of abundance and the presence of inhibitors on detection in aquaria or mesocosms (Goldberg et al. 2016).

5.5 Directions for future research

Although this thesis provides monitoring strategies which can be adopted by managers for eDNA monitoring, it is limited by only providing data for early (May) and late (October) season sampling (Chapter 2, 3). Regular sampling of zebra mussel eDNA throughout an entire season in Manitoba (between May and November) will help develop a more detailed eDNA temporal detection profile. Such a project would provide data which allows managers to define the timing of the greatest likelihood of eDNA detection. Also, parallel veliger sampling along with regular eDNA sampling could provide a more robust dataset for analyzing the relationship between veliger count and eDNA concentration over time or across several areas with varying ecology.

The data presented in this thesis support a positive correlation between eDNA concentration and veliger abundance when ecological factors are relatively consistent across sites (Chapter 3). Controlled laboratory experiments which aim to determine concentration with a known absolute number of veligers per sample prior to extraction, while also considering inhibition, may provide insight in interpreting the source of the eDNA signal. Related to this, research which follows up weak eDNA signals in previously uninvaded areas with intense

sampling via conventional detection methods will help elucidate how much unit effort is required to confirm a novel eDNA detection. This research would provide data on differences of unit effort required for an eDNA or conventional sampling method detection. Again, such research could be done in conjunction with regular monitoring efforts.

Finally, it has been well documented that inhibitory substances can negatively affect eDNA detection (McKee et al. 2015) and that various inhibitory substances may affect PCR based methods differently (Stahlberg et al. 2003; Villanova et al. 2007; Huggett et al. 2008; King et al. 2009). These differences in mechanisms of inhibition cause differences in how target eDNA and IPCs are amplified which reduce the usefulness of current methods of inhibition detection in eDNA studies and monitoring programs (Chapter 4). Very little eDNA research has addressed the identification of inhibitory substances within a sampled area beyond conjecture. Studies which aim to develop methods of characterizing the inhibitory substances present in different geographical water bodies and determining how those substances may inhibit PCR will aid in designing field sampling and laboratory protocols. Research which investigates how these inhibitory substances may be co-eluted with eDNA, how they differentially affect target template and IPCs due to exposure time, and how increased volume of water filtered may increase inhibition (i.e., the relationship between target template and inhibitor concentration) will also improve confidence in eDNA data. This thesis identified some methods of eDNA sample purification and enrichment (*see* Section 4.5) which should be investigated further.

5.6 Conclusion

This thesis has developed thoroughly tested methods for invasive mussel eDNA detection as part of a monitoring program (Chapter 2). The methods described herein appear to be highly

sensitive compared to conventional monitoring methods (Chapter 3) and should be considered by managers as a monitoring tool. Furthermore, a refinement of methods based on practices used in forensics, ancient DNA (aDNA), and virology, is provided here (Chapter 4). These refinements which involve sample enrichment/purification are applicable to eDNA detection in general, and not specific to zebra mussel monitoring. These refinements have potential of improving detection of target from highly inhibited and low concentration samples.

The above themes have helped address where zebra mussels were located within Lake Winnipeg at the time of this research (*see* Section 5.2). Also, the above work has allowed for some speculation of where eDNA detections may continue to arise in Lake Winnipeg and surrounding waterbodies (*see* Section 5.2). Related to this, suggestions are provided in this thesis regarding eDNA sampling regimes as part of a monitoring program (*see* Section 5.3.1) and how result interpretation may affect management efforts (*see* Section 5.3.2). General guidelines for eDNA assay development are also presented here, as they arose organically from methods development (Chapter 1). Likewise, a comprehensive review of eDNA methods arose organically during methods development and is included here in the appendices (*see* Appendix A).

5.7 Literature cited

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APPENDIX A: COMPREHENSIVE OVERVIEW OF ENVIRONMENTAL DNA METHODS

Herein is a comprehensive review of the environmental DNA (eDNA) methods present in the literature. This appendix was produced as an explicit deliverable to determine which methods would be used in this thesis (Chapter 1). The advantages and disadvantages of the various methods are discussed briefly in Chapter 1 and more thoroughly below. These comparisons among methods were used to select those most likely to reduce potential errors.

A.1 Good molecular practices

Good molecular practices are required when conducting eDNA analysis. Implementation of these quality assurance/control (QA/QC) measures will reduce the likelihood of false positive (FP) and false negative (FN) results (*see* Section 1.5.2).

Separate eDNA laboratories, dedicated to an individual stage of eDNA sample processing and separated from spaces where high-quantities of DNA are handled (Taberlet et al. 1999; Goldberg et al. 2016), are recommended. For example, amplification of eDNA samples must be done in a separate laboratory from other processes. Workstations which are equipped with ultraviolet (UV) sterilization and laminar flow and High Efficiency Particulate Arrestance (HEPA) filtration can also be implemented to reduce the exposure of an eDNA sample from the surrounding environment. All equipment used during eDNA processing should be dedicated solely to eDNA work and not used for other molecular work which involves high-quantity DNA samples such as DNA extracts and PCR products.

All equipment and work surfaces must also be cleaned regularly and after each use to ensure removal of contaminating nucleic acids. Common decontamination solutions include sodium hypochlorite (bleach) and other commercially available reagents (e.g., ELIMINase). A

10% bleach solution is the most common decontamination reagent used (Prince and Andrus 1992; Champlot et al. 2010); however, some studies have found sources of contamination remaining post sterilization with 10% solutions and therefore investigators have begun using 50% solutions to eliminate contamination (Kemp and Smith 2005; Champlot et al. 2010).

Metals and glassware should, in addition to bleach treatment, be baked at a minimum of 185°C for 4 hours as this has been shown to destroy contaminating nucleic acids and nucleases (Blumberg 1987; Sambrook et al. 1989). Autoclaving should not be used as it is not sufficient to eliminate nucleic acid contamination (Unnithan et al. 2014).

Filtered pipette tips should always be used to prevent aerosols from contaminating pipettes. Gloves should be changed liberally, especially when encountering a potential source of contamination. At any point during sample processing, only one sample tube should be opened at any one time and repeater pipettes should not be used when tubes contain sample; this reduces the likelihood of cross-contamination between samples.

FP results due to contamination of eDNA samples can be identified with the inclusion of No Template Controls (NTC) at each stage of sample processing. Contamination can be identified if NTCs test positive. Conversely, FN results due to low efficiency of the methods can be identified by including Positive Template Controls (PTC). PTCs should test positive and indicate loss of template when they are tested negative. However, PTCs can be a source of contamination, therefore synthetic PTCs can be used to identify if contamination of a PTC occurs (Wilson et al. 2016).

These recommendations should be considered obligatory when conducting eDNA detection work and should be considered when conducting all the five stages of eDNA sample processing.

A.2 Environmental DNA sample processing

Current methods of eDNA analysis for aquatic macroorganisms, although varied, follow a general workflow outlined in Ficetola et al. (2008). These stages are: 1) collection of eDNA-containing material for eDNA extraction and eDNA capture efficiency; 2) sample preservation; 3) eDNA extraction processes and extraction efficiency; and 4) molecular analysis of eDNA extract (including assay sensitivity and inhibition). Overall, effectiveness of varying eDNA analysis methods seems to be situationally dependant (Goldberg et al. 2011; Amberg et al. 2015; Deiner et al. 2015; Renshaw et al. 2015; Takahara et al. 2015; Ardura et al. 2016; Eichmiller et al. 2016). Therefore, there is no single prescribed method of eDNA sampling and analysis. The methods chosen for this thesis were considered optimal considering the thesis themes and were chosen to minimize potential sampling errors (Chapter 1).

A.2.1 Collection of environmental DNA material for extraction

Capture and extraction efficiency can vary depending on factors such as target species, water source, and methods used (Deiner et al. 2015; Eichmiller et al. 2016). The three most common methods of collecting eDNA from water samples are: 1) precipitation of nucleic acids directly from water samples; 2) pelleting of suspended particles within a water sample; and 3) filtration of a water sample. Direct precipitation of nucleic acids from small volumes of water (≤ 15 mL) was described by Ficetola et al. (2008). This method has been used successfully in several studies (Ficetola et al. 2008; Goldberg et al. 2011; Dejean et al. 2012; Thomsen et al. 2012; Klymus et al. 2015; Renshaw et al. 2015); however, other studies have found this method to produce lower eDNA yields (Gingera 2013; Eichmiller et al. 2016).

Pelleting of suspended particulate matter from small volumes of water (15-50 mL) via centrifugation is used for the US Asian Carp Monitoring Program (USACE 2015, Ulibarri et al. 2017). Nucleic acids are extracted from swabs taken from the pellets. Both precipitation and pelleting methods facilitate water sampling in the field, as no filtration step is required. This reduces sample collection time in the field and allows sampling teams to cover larger sampling areas within a limited timeframe. Large pellets of suspended particles can also be achieved by dragging plankton nets in the water column; the eDNA is subsequently extracted from the homogenized concentrated particle slurry (Ardura et al. 2016). This method is less common in the literature and its advantages are less clear; however, it allows for a very large volume of water to be filtered.

Filtration is a common method of water sample collection as the volume of water is often much larger than that possible by the above methods (Goldberg et al. 2011; Jerde et al. 2011; Takahara et al. 2012; Thomsen et al. 2012b; Takahara et al. 2013, 2015; Renshaw et al. 2015; Gingera et al. 2016, 2017; Lacoursière-Roussel et al. 2016; Spens et al. 2016). This is because large volumes of water can potentially be sampled and concentrated onto a filter compared to centrifugation methods. The volume of water successfully filtered can vary drastically depending on the abundance of suspended particulate matter (e.g., algae, sediment) (*see* Chapter 2-4; Valentini et al. 2016) and the pore size of filter used. High particulate abundance and small pore size will result in clogging of a filter. Filter pore sizes between 1 and 10 µm are commonly used as this size of pore will sufficiently capture from the water column sloughed off cells; these cells are currently thought to be the predominant source of detectable macroorganism eDNA (Turner et al. 2014; Deiner et al. 2015) as opposed to extracellular DNA. Filters composed of various materials have been used for eDNA analysis from aquatic systems. The most common filter

material used is glass fibre due to its low cost (Jerde et al. 2011; Gingera et al. 2016, 2017; Lacoursière -Roussel et al. 2016); however, cellulose nitrate (Goldberg et al. 2011), polycarbonate (Takahara et al. 2012), nylon (Thomsen et al. 2012b), polyether sulfone (Renshaw et al. 2015; Spens et al. 2016), and cellulose acetate (Takahara et al. 2013, 2015) filters have also been used. Few comparisons have been done to determine how different filter types and pore size may affect eDNA capture. Eichmiller et al. (2016), however, found that glass fibre filters with a pore size of 1.5 µm provided optimal results for presence/absence analysis, whereas polycarbonate filters with a pore size of 0.2-0.6 µm were ideal when quantifying eDNA concentration for cyprinids in the lab. Further testing of these methods from field samples has not been done.

Filtration can occur on-site in the field or samples can be kept on ice and transported to a designated facility where filtration should occur within 24 h of collection (Pilliod et al. 2013). Laboratory filtration reduces field time, can be done in sterile facilities, and DNA can be kept at -80°C after filtration; however, water samples must be kept at 4°C in the field and filtered within 24 h prior to significant eDNA degradation (Pilliod et al. 2013). On-site filtration is common during sampling of remote locations and allows for immediate preservation of the filter in preservatives such as ethanol; however, sample collection time increases, and cross contamination of samples and negative controls are more likely. Currently, however, there is no optimal method of eDNA water sampling as capture efficiency seems to depend on several parameters such as water source, target organism, and purpose of the sampling (Deiner et al. 2015; Eichmiller et al. 2016).

A.2.2 Sample preservation

Preservation methods for eDNA samples prior to nucleic acid extraction is like the preservation of tissue samples collected in the field. Sample preservation is typically applied after water samples have been prepared for eDNA extraction (i.e., pelleted or filtered). Pellets produced from centrifugation of water samples are typically preserved via suspension in ethanol (Goldberg et al. 2011), freezing (Jerde et al. 2011), or a combination of the two (USACE 2015). Desiccation and preservation in cell lysis buffer has also been used for pellet preservation (Renshaw et al. 2015; McKelvey et al. 2016; Chapter 3). Filters have been stored using a wide variety of preservation methods which include freezing at -80°C, desiccation (Chapter 3), suspension in ethanol (Gingera et al. 2016), and RNAlater (Lance and Carr 2012; Spens et al. 2016). Little research is available regarding how preservation methods affect eDNA quantity and quality. However, Takahara et al. (2015) demonstrated that detection of common carp *Cyprinus carpio* eDNA was lower in whole water samples which were frozen at -30°C and then thawed compared to samples which were not frozen. Spens et al. (2016) suggest that the addition of ethanol and/or Longmire's buffer to the filter may improve eDNA yields.

A.2.3 Environmental DNA extraction

Several protocols for eDNA extraction have been used for eDNA analysis with little consistency (Goldberg et al. 2016). The DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) is the most commonly used extraction method (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011, 2013; Foote et al. 2012; Thomsen et al. 2012a, 2012b; Takahara et al. 2013; Gingera et al. 2016, 2017) and has been found to surpass the Ultra-Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) (Goldberg et al. 2011), the PowerWater DNA Isolation Kit (Amberg et al. 2015), and improved eDNA detection compared to sample

precipitation (Deiner et al. 2015). Eichmiller et al. (2016), however, found that the FastDNA SPIN Kit (MP Biomedicals, Sanata Ana, CA, USA) and the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) were more optimal than DNeasy for presence/absence and quantification, respectively, of carp eDNA in lab experiments. CTAB-chloroform and phenol-chloroform extraction (i.e., phase separation and precipitation methods) also resulted in a greater yield of eDNA compared to silica column extraction methods (Deiner et al. 2015; Renshaw et al. 2015).

Optimal extraction protocols appear to be dependent on water source, water sample collection method (Eichmiller et al. 2016), target organism (Deiner et al. 2015), and molecular detection method (Eichmiller et al. 2016). Thus, there is no universally agreed upon method of extraction. It is therefore suggested that the eDNA capture and extraction methods should be optimized towards the goals of the study (Eichmiller et al. 2016) with pilot studies done beforehand to ensure efficient eDNA capture and analysis (Goldberg et al. 2016).

A.2.4 Molecular analysis of environmental DNA extract

Several methods of molecular analysis exist, and all are affected by detection parameters such as assay sensitivity and inhibition. The following is a comprehensive overview of the molecular detection methods available and how inhibitory substances affect eDNA detection.

A.2.4.1 Molecular detection methods

A.2.4.1.1 Conventional PCR

Detection of aquatic organisms in early eDNA studies used conventional PCR (cPCR) fragment analysis and agarose gel electrophoresis for amplicon visualization (Ficetola et al.

2008; Dejean et al. 2011; Goldberg et al. 2011; Jerde et al. 2011; Ardura et al. 2016; Gingera et al. 2016). These early studies demonstrated that eDNA can be an effective tool for identifying the presence/absence of AIS, as well as endangered or otherwise rare organisms, by using common PCR based methods. The studies also began to develop the importance of assay sensitivity, specificity, and methods of reducing PCR inhibition via inhibitory substances (Ficetola et al. 2008; Dejean et al. 2011; Goldberg et al. 2011; Jerde et al. 2011; Gingera et al. 2016). Conventional PCR methods are generally considered to be less sensitive and with a lower capacity for species-specificity compared to quantitative PCR (qPCR) and other methods. PCR amplification and gel electrophoresis are also more time consuming and increase the likelihood of contamination due to the increased amount of sample handing. For these reasons, fluorescence-based qPCR has become more widely adopted in lieu of cPCR.

A.2.4.1.2 Quantitative PCR

A fluorescent-based quantitative PCR method is the most commonly used method of eDNA molecular analysis for a single or small number of target species. Quantitative PCR represents a technological advance in PCR amplification as the results can be monitored in “real-time” as variations in fluorescence within a reaction are measured periodically during the PCR process. The fluorescence signal is produced via a non-specific intercalating fluorescent-dye (e.g., SYBR Green) or via an internal probe containing a reporter dye (i.e., TaqMan). Quantitative PCR is a more sensitive method of analysis and allows for the ability to quantify the concentration of an eDNA sample relative to a control with a known concentration.

When the fluorescence signal is produced via SYBR Green or another non-specific intercalating fluorescent-dye, the fluorescent dye is intercalated with the elongated DNA

fragments during amplification; as the amplicon is replicated, the fluorescence increases (Higuchi et al. 1992). Because these dyes are non-specific and will bind to all DNA within a reaction, dissociative melt-curves can be produced to visualize if co-amplification of non-target products occurred. Intercalating dye qPCR uses the same molecular markers as cPCR, thus cPCR assays are more easily adapted to this method of analysis; however, validation of the assay will still be required (Goldberg et al. 2016; Gingera et al. 2017). Although more sensitive, this method of qPCR is equally specific as are cPCR methods but is not frequently used.

In contrast, probe-based qPCR incorporates an internal probe which is situated between the forward and reverse oligonucleotides and emits fluorescence upon hydrolysis of the probe. The probe contains a 5' fluorescent reporter and a 3' quencher molecule which is cleaved through the 3' exonuclease activity of the Taq polymerase, thereby releasing the fluorescent reporter from proximity with the quencher (Heid et al. 1996). Fluorescence of the reporter dye therefore increases at each replication cycle and is measured by the real-time PCR instrument. Probes can be modified through the incorporation of a minor-groove binding (MGB) linker that raises the melting temperature of the probe (Kutyavin et al. 2000). MGB probes are therefore shorter in length, which provides increased sensitivity and reduces the size of the target fragment. Probe-based qPCR allows for >1 assay to be run simultaneously (e.g., for more than one target species or multiple fragments from one target species), if none of the probes fluoresce at similar wavelengths (i.e., multiplexing). This approach allows for two or three unique eDNA fragments to be analyzed simultaneously. Probe-based qPCR is the most commonly used method of eDNA analysis due to its improved specificity generated by the probe (Takahara et al. 2012; Pilliod et al. 2013; Wilcox et al. 2013; Amberg et al. 2015; McKee et al. 2015; Gingera et al. 2017).

Both qPCR methods reduce the amount of handling required for data analysis as electrophoresis through an agarose gel is not required for product visualization. In turn, reduced handling of PCR products reduces the likelihood of contamination.

A.2.4.1.3 Digital droplet PCR

Digital droplet PCR (ddPCR) is a relatively new method of eDNA analysis which incorporates the fluorescent-based methods (i.e., intercalating dyes, fluorescent-probes) of qPCR, and is therefore useful for a single or small number of target species. Prior to amplification, samples are partitioned into up to 20,000 individual droplets via oil-water emulsification. After amplification, fluorescence data are collected from each droplet individually (Pinheiro et al. 2012; Whale et al. 2012). The likelihood of multiple fragments of target template being co-located within the same droplet is low due to the large number of droplets. An absolute concentration of the target fragment is therefore provided during ddPCR analysis without the need for controls with a known concentration of target (Doi et al. 2015).

Few studies have used ddPCR for eDNA analysis (Nathan et al. 2014; Doi et al. 2015); however, this method will become more common as the technology becomes more prolific and cost decreases. Indeed, ddPCR is currently the best option for eDNA analysis if quantification is the goal of the study and already may be more cost efficient for large numbers of samples (Nathan et al. 2014; Doi et al. 2015). Because ddPCR relies on the same fundamental PCR chemistry as cPCR and qPCR, it is still subject to inhibition, which may result in false negatives (*see* Section A.2.4.2).

Quantitative PCR and ddPCR are highly sensitive methods of eDNA detection and are best applied to situations where high sensitivity is needed and immediate results (<1 hour) are not needed. This include monitoring and distribution data collection efforts.

A.2.4.1.4 Light Transmission Spectroscopy

Light Transmission Spectroscopy (LTS) is a method of DNA detection which measures shifts in the size of carboxylated nanobeads of a known size that are bound to oligonucleotide tags which are, in turn, designed to bind to a target sequence. The beads increase in size as target DNA binds to oligonucleotides, which is subsequently detected by a portable instrument in a short timeframe relative to PCR methods (approx. 1 hour) (Marquette and Blum 2006; Basuray et al. 2009; Mahon et al. 2011).

This method of eDNA analysis involved the use of specialized molecular markers designed to target specific DNA sequences and so it is useful for detection of a single target species. Current sensitivity of LTS analysis does not approach those available with probe-based qPCR or ddPCR and is therefore a promising method for detecting eDNA from samples collected from contained sources of water (e.g., ballast, bait containers) or other scenarios where getting rapid results is more important than sensitivity. This method has been successfully used for detecting invasive species in field samples with a known presence of target AIS as well when the sample sources was ballast water and bait containers (Mahon et al. 2011; Egan et al. 2013, 2015).

A.2.4.1.5 High Throughput Sequencing

High throughput sequencing (HTS) is a general term used to describe several sequencing technologies which provide large amounts of data due to massive parallel sequencing of several nucleic acid fragments simultaneously (Shendure and Ji 2008; Glenn 2011). Unlike traditional targeted Sanger sequencing which is restricted to sequencing a single amplicon with an average read length of approximately 700 bp, HTS is capable of sequencing millions of different short 50-400 bp fragments in a matter of hours (Metzker 2010).

Non-specific molecular markers can be coupled with HTS to sequence many fragments across many taxa when nucleic acids of several organisms may be present in a sample (Taberlet et al. 2012). This is referred to as '*metabarcoding'* and was first described for the use of identifying microbial communities from environmental and cultured samples (Tringe et al. 2005). This method is advantageous when target organisms are not identified *a priori* or if the study aims to characterize the biodiversity within an ecosystem (Hajibabaei et al. 2011).

HTS has been used to detect the presence of several species from samples collected in the field (Thomsen et al. 2012a, 2012b, Ardura et al. 2016), controlled mesocosm experiments (Thomsen et al 2012a; Evans et al. 2016), and bait containers (Mahon et al. 2014). However, HTS has also failed to detect organisms which were known to be present in a sample site (Thomsen et al. 2012b). Few comparisons between HTS and PCR eDNA detection exist (Ardura et al. 2016), and so comparable sensitivity it is not well understood. The application of HTS for the study of aquatic biodiversity using eDNA is still in its early stages but holds enormous potential for conservation efforts in the future (Valentini et al. 2016).

A.2.4.2 Inhibition

Inhibitory substances are a large and diverse group of molecules which can reduce the efficiency of PCR based molecular detection methods which are abundant in aquatic environments (McKee et al. 2015).

Inhibitors present in eDNA samples include algae (Schrader et al. 2012), debris, fulmic acids, humic acids, humic material, metal ions, and polyphenol (Tsai and Olson 1992; Abbaszadegan et al. 1993; Ijzerman et al. 1997; Watson and Blackwell 2000; Rådström et al. 2004; Schrader et al. 2012), and polysaccharides (Demeke and Adams 1992; Monteiro et al. 1997). The most common method to relieve PCR inhibition is to dilute the sample either 1:5 or 1:10 with water (Tsai and Olson 1992; McKee et al. 2015). However, dilution will also reduce target template concentration, and therefore increase the likelihood of FN results. Using PCR additives such as Bovine Serum Albumin (BSA) (Wilson 1997) or commercially available reagents such as Environmental Master Mix 2.0 (EMM) (Life Technologies, Grand Island, NY, USA) is also common. The effects of inhibition on a PCR can be identified with the addition of an Internal Positive Control (IPC) into a reaction (Dingle et al. 2004). IPCs are exogenous DNA which will not interfere with the target assay and will amplify alongside the target assay via a second set of molecular markers (i.e., a multiplex). If a reduction in PCR efficiency is recorded for the IPC compared to a control sample, it is likely inhibitory substances are present (Chapter 4). This thesis has demonstrated, however, that IPCs may not always indicate when inhibition is present (Chapter 4).

Nucleic acid purification methods can be used to remove inhibitory substances from samples, although this is not typically done during eDNA sampling. However, nucleic acid purification is commonly used in ancient DNA (aDNA) and forensic research (Alaeddini 2012;

Schrader et al. 2012). These methods include polyethylene glycol (PEG) (LaMontagne et al. 2002) and isopropanol precipitation (Hanni et al. 1995), silica column purification (Nagy et al. 2005; Kemp et al. 2006; Davoren et al. 2007; Vanek et al. 2009; Lee et al. 2010), and purification via magnetic particles (Maher et al. 2001; Rutjes et al. 2005; Ngazoa et al. 2008; Sur et al. 2010). However, purification methods inevitably result in a reduction of nucleic acid concentration alongside inhibitory substances (Alaeddini 2012).

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