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INTENSIVE SAMPLING AND COMPARISON OF METHODS IN DETECTION OF
NON-INDIGENOUS SPECIES

by

Sharon Lavigne

A Thesis

Submitted to the Faculty of Graduate Studies
through the Great Lakes Institute for Environmental Research
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

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NON-INDIGENOUS SPECIES

by
Sharon Lavigne

APPROVED BY:

A. Fisk
Department of Earth and Environmental Sciences

G. Haffner
Great Lakes Institute for Environmental Research

H. MacIsaac, Advisor
Great Lakes Institute for Environmental Research

December 19, 2017

DECLARATION OF ORIGINALITY

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ABSTRACT

Non-indigenous species (NIS) newly introduced to a novel environment usually experience a lag time before the population grows to a detectable level. Management of the NIS during the lag phase provides a better opportunity for eradication than at later stages when the population is larger and established. However, low population density limits detection by conventional methods. Here I tested the effect of intensive sampling on a population of a newly introduced NIS, zebra mussels (*Dreissena polymorpha*), in Lake Winnipeg. Zebra mussel presence can be determined by the presence of their larvae (veligers). I hypothesized that veligers will be detected in the south basin where they were previously reported, but not in the north basin where they were never reported. I also compared detection success as well as the cost and time of three methods of analysis of plankton samples: cross-polarized light microscopy (CPLM), flow cytometry and microscopy (FlowCAM), and environmental DNA (eDNA). I detected veligers throughout Lake Winnipeg, even in the north basin, with varying abundances. As expected, veliger abundance was highest in the south, and very low in the north. Abundance and prevalence were significantly lower with FlowCAM and eDNA analysis, indicating lower success when compared to CPLM. FlowCAM is the most expensive method used, while eDNA is the least expensive. eDNA represents the cheapest and fastest method, and combined with intensive sampling, is the best candidate for wide scale zebra mussel monitoring programs for rapid response.

DEDICATION

This thesis is dedicated to my parents.

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INTRODUCTION

Non-indigenous species (NIS) are species moved from their native ranges into new regions, typically through anthropogenic processes (Ricciardi 2007). The rate of movement of NIS across the globe is increasing due to human activities, such as trade and travel (Ricciardi 2007; Hulme 2009). While most NIS do not adversely impact environments they are introduced into, some species can cause economic and/or ecological harm. NIS that cause harm are termed invasive species (Lockwood et al. 2013; Ricciardi and Cohen 2007).

Invasive species have impacted ecosystems and economies of many countries (Vitousek et al. 1997; Horan and Lupi 2010; Havel et al. 2015). In the United States, invasive species have been estimated to cost almost \$120 billion per year in damages, losses and control (Pimentel et al. 2005). Invasive species prey on native species, compete with them for food and space, or parasitize or infect them (Nalepa et al. 1996; Courchamp et al. 2003; Pimentel et al. 2005; Vila et al. 2011). Invasive species have also been identified as the highest impact stressor in the Laurentian Great Lakes based on expert opinion (Smith et al. 2015).

Only a fraction of NIS will, however, become invasive (Williamson and Fitter 1996). Successful invasions happen in a step-wise fashion, overcoming barriers at each step (see Figure 1; Blackburn et al. 2011). To become a successful invader, a species must undergo uptake at the donor region, survive transport, and be released into the recipient region. As an example, the round goby (*Neogobius melanostomus*) was most likely picked up in ships' ballast from Eurasia (uptake), transported to North America, and released in 1990 into the Laurentian Great Lakes during ballast water discharge

(release) (Charlebois et al. 2001). To establish a reproducing population, the NIS needs to survive and reproduce. In the case of the round goby, the species established populations Lake Saint Clair, preying on zebra mussels - a species with which they co-evolved (Ray and Corkum 1997; Clapp et al. 2001; Djuricich and Janssen 2001) - and other species. In the final step of many invasions, the now-established NIS will spread from the site of original release to other locations in the recipient region (Lockwood et al. 2013). The round goby has since spread throughout the Great Lakes (Kornis et al. 2012).

Species that are introduced in one region can fail to invade in another (Zenni and Nuñez 2013). Conditions in some environments may be incompatible with the growth requirements of the introduced species. For example, ocean currents were detrimental to larval deposition of introduced fishes (Johnston and Purkis 2016). Plants that thrive in dry areas and nutrient-poor soil could fail to invade areas with waterlogged soil and high nutrient levels (Closset-Kopp et al. 2011). The Chinese mitten crab (*Eriocheir sinensis*) requires water with at least 15% salinity to reproduce. This means that Chinese mitten crabs are unable to establish in freshwater systems that are far from salt or brackish water (Herborg et al. 2007). Invasion failure can also occur when the introduced, dioecious species cannot find a mate at low population abundance (Allee effect), and thus is unable to establish a reproducing population (Contarini et al. 2009).

In cases of successful invasions, management measures can become costly, so eradication of an invasive species before it establishes or spreads is preferable (Leung et al. 2002; Mehta et al. 2007). Many species exhibit a lag phase after introduction - where their population remains low - before increasing in size (Kowarik 1995; Crooks and Soule 1999; Sakai et al. 2001; Rilov et al. 2004; Murren et al. 2014). Eradication of

invasive species can be most effective during this lag phase (Mehta et al. 2007). For example, *Caulerpa taxiflora*, a filamentous alga that has colonized many areas of Mediterranean Sea (Meinesz and Hesse 1991), was discovered off the coast of California in 2000 (Jousson et al. 2000). A rapid response team was assembled and successfully exterminated the small populations before they could expand (Williams and Schroeder 2004; Anderson 2005).

While low population levels during lag phase can facilitate the eradication of NIS, it also makes it difficult to detect individuals (Gu and Swihart 2004). Increasing sampling intensity can increase the chances of successful detection during lag phase, and reduce the possibility of false negatives in detection (Harvey et al. 2009; Counihan and Bollens 2017). Detection accuracy – the ability to successfully determine true presence or absence (Václavík and Meentemeyer 2009) – of NIS is important for rapid response programs. Accurate early detection of a NIS can potentially reduce unwanted impacts of that species via successful eradication programs (Bogich et al. 2008; vander Zanden et al. 2010; Horan and Lupi 2010). Detection sensitivity – probability that a species is correctly identified when present – is crucial for the effective eradication and control of NIS (Hayes et al. 2005).

Importantly, we must be aware of, and attempt to reduce, Type I and Type II errors (Banerjee et al. 2009). Type I errors are false positives, where a species not present in the area/sample is reported as present. False positives are serious issues as efforts can be wasted seeking to validate presence of the species, or in attempts to eradicate it (Wilson et al. 2016). If eradication programs are initiated because of false positive detection of an NIS, non-target species can be harmed. For example, chemicals like Clamtrol used to

control zebra mussels could affect non-target aquatic species (Cope et al. 1997; Fernald and Watson 2014). Type II errors refer to false negatives, where we fail to detect a species that is present. False negative errors in the detection of invasive species are particularly problematic as they allow species present at low abundance to remain undetected (MacIsaac et al. 2002; Hayes et al. 2005; Fitzpatrick et al. 2009). A false negative could lead to failure to deploy eradication or control efforts in sufficient time to effectively manage the species (Myers et al. 2000).

Zebra mussels and quagga mussels, *Dreissena rostriformis bugensis*, are invasive species introduced to Laurentian Great Lakes in the mid- to late 1980s (Mills et al. 1993; Carlton 2008). These aquatic bivalves bio-foul submerged structures and clog pipes that draw water from infested waterways (Prescott et al. 2014). Presence of dreissenid mussels can cause an increase in the running costs of water treatment and power generation plants (Sarrouh and Ramadan 1994; Pimentel et al. 2005; Connelly et al. 2007; Prescott et al. 2014). The mussels have also caused declines in populations of native unionid mussels in the Great Lakes (Nalepa et al. 1996; Zanatta et al. 2015). In this study, I focused on zebra mussels. Zebra mussels reproduce by releasing gametes into the water when temperature exceeds 12°C (Borcherding 1991). Zebra mussel larvae, called veligers, form after external fertilization, and stay suspended in the water for 10 to 15 days or more (depending on temperature) before settling onto hard substrate (Hebert et al. 1989). Veligers can be used as an indicator of the presence of dreissenid mussels in the water (Johnson 1995; Frischer et al. 2005).

While eradication of dreissenids from a large lake is infeasible (Nalepa 2014), early management strategies could alleviate costs. For example, water treatment plants can

prevent fouling by installing antifouling materials on submerged surfaces. There are also treatments such as dissolved air flotation, ozonation, deep bed biological activated carbon, chlorine and UV to prevent zebra mussels from entering drinking water reservoir, thereby preventing further cost downstream of removal or mussels from the reservoir (Chakraborti et al. 2014). Early detection of zebra mussels allows decisions to be made to curb the downstream cost of dealing with the species (Hosler 2011).

Lake Winnipeg is the tenth largest freshwater lake in the world by surface area (Wassenaar and Rao 2012). Located in Manitoba, the lake is divided into two basins, connected via a narrow channel. At a volume of 294km³, Lake Winnipeg is smaller by volume as well as shallower compared to the Laurentian Great Lakes, and has little or no summer stratification (Leon et al. 2012; Zhao et al. 2012).

Even though geographically isolated from the Laurentian Great Lakes, Lake Winnipeg is not invulnerable to invasive species. At least three NIS that can affect food webs – rainbow smelt (*Osmerus mordax*), white bass (*Morone chrysops*), and spiny water flea (*Bythotrephes longimanus*) – invaded Lake Winnipeg previously (Hobson et al. 2012; Sheppard et al. 2012; Hann and Salki 2017). There are historically 11 native unionid mussels in Lake Winnipeg (Pip 2006), all of which produce glochidia larvae (which attach to fish), rather than free-floating veligers (Coker and Surber 1911; Trdan 1981), thus any veligers present in plankton samples must be either quagga and/or zebra mussels.

Zebra mussels were detected in the Red River, North Dakota, which flows into Lake Winnipeg, in 2009 (Wassenaar and Rao 2012). In 2013, the species was found in the southern basin of Lake Winnipeg (DFO 2014). It is possible that they spread to the

lake by boaters moving bio-fouled vessels overland (Janusz 2014) or via advective flow from the Red River (L. Janusz, pers. comm.). Periodic sampling by the Manitoba Aquatic Invasive Species Program, Fisheries Branch, Conservation and Water Stewardship reported that there were no zebra mussels detected in the in the north basin of Lake Winnipeg before October 2015 (CWS 2015). The reported presence of zebra mussels in the south basin but seeming absence in the north basin provided an opportunity to test detection limit for a variety of different methods of sampling and analysis.

When a species is present in very small numbers, large efforts are needed to detect them. This can be a problem if resources are limited. That is why we must make detection sensitivity (ability to detected species at very low abundance) by increasing sampling effort and efficiency (Hoffman et al. 2016). While intensive sampling can increase the chance of capturing an individual of a species that is present in low abundance, the method of sample analysis can affect detection success (Trebitz et al. 2017; Stanislawczyk et al. 2017).

New methods are always emerging to improve success and accuracy of NIS detection. For example, use of light-based traps instead of plankton tows increased the chance of capturing the mysid *Hemimysis anomala* (Brown et al. 2017). Invasive plants can be detected and mapped in shorter time using remote sensing imagery than conventional field surveys (Bradley 2014). Use of environmental DNA for species detection is a relatively new technique that appears to be successful for many species (Taberlet et al. 2012). Sampling of 16 marine and freshwater ports in Canada showed the ability of DNA detection in identifying multiple NIS that have been previously detected and unreported (Brown et al. 2016).

In this study, I explored effectiveness of three methods of sample analysis for zebra mussel detection, with a focus on veliger larvae. The veliger is the planktotrophic stage of development, lasting from 10 to 90 days (Reed et al. 1998). This stage of development allows dispersal via currents or movement by water moved by boaters (Padilla et al. 1996). Veliger abundance is a function of adult zebra mussel abundance, fecundity, and water temperature (Reed et al. 1998). The first method of analysis was CPLM. The birefringent properties of veligers' shells cause them to show up brightly lit under cross-polarized light (Johnson 1995; Figure 2), and veligers can be efficiently enumerated using this approach. Prior to development of cross-polarized light, veligers were identified under microscope by taxonomists, which, in addition to being tedious and time consuming (Becerra and Valdecasas 2004), can be difficult because the animals appear visually similar to ostracods (Marsden 1991). Because it is much easier than taxonomic identification, CPLM is widely used for veliger detection in monitoring and surveillance programs (Claudi and Mackie 2010; Hosler 2011; Evans et al. 2011).

The second method of analysis was use of a semi-automated particle visual analyzer (Culverhouse et al. 2006). Two popular systems are Flow Cytometer And Microscope (FlowCAM) (Hosler 2011; Day et al. 2012) and Zooscan (Gorsky et al. 2010). I used FlowCAM for plankton analysis in this thesis. FlowCAM captures images of microscopic particles as they pass through the flow cell (Fluid Imaging Technologies 2011). The images were stored and can be sorted by variables such as size and shape. Plankton imaging systems were developed to reduce plankton processing time and can overcome the problem of difficult taxonomic identification and human error from fatigue (Culverhouse et al. 2003; Benfield et al. 2007). FlowCAM has been widely used in

different applications e.g., monitoring the change of plankton community structure in the face of climate change (Graham and Camp 2017), study of protein structures in drugs prepared for pharmaceutical applications (Zölls et al. 2013), detection of grazing protozoa in algae cultured for biofuels (Day et al. 2012), and identification of metazooplankton (Le Bourg et al. 2015). FlowCAM has also been used to identify red tide cells which are associated with harmful algal blooms, as it requires less time and effort than conventional microscopy (Buskey and Hyatt 2006). FlowCAM differed little with microscope in analyzing plankton samples (Alvarez et al. 2013). Fitted with a cross-polarizing filter (XPL attachment), the FlowCAM can be enhanced for veliger detection (Spaulding 2009). The XPL attachment works the same way as the cross-polarizing lenses on microscopes; it will cause veligers to shine brightly against the background, making them easier to see. Highly-visible, birefringent veligers increase the chance of capture by FlowCAM software.

Finally, I used a molecular method of detection by polymerase chain reaction (PCR), amplifying environmental DNA (eDNA) (Ram et al. 2011; Egan et al. 2015). eDNA is DNA shed from organisms into the environment, which can then be isolated and analysed (Rees et al. 2014). Detection by eDNA makes use of species-specific primers to amplify the DNA of target species, allowing cross-referencing against established online databases for rapid identification. eDNA can be used for the identification of species in microbial communities (Venter et al. 2004), as well as macro-organisms (Ficetola et al. 2008). It has been used to detect many different organisms: fish, amphibians, mammals and reptiles (Rees et al. 2014). Increasing the number of field replicates in eDNA detection is recommended to reduce the possibility of false negatives (Ficetola et al.

2015). Mitochondrial DNA (mtDNA) is a specific fragment of DNA often used in the detection of species. mtDNA is present in higher copies than nuclear DNA because mitochondria are numerous within a cell. Both mtDNA, 16S rRNA and cytochrome oxidase I gene (COI) have been used for detection of zebra mussels (Egan et al. 2015; Ardura et al. 2017). Most eDNA assays test water samples but I increased the chance of detection by testing plankton samples (Peñarrubia et al. 2016; Ardura et al. 2017). eDNA detection has been successfully optimized for detection of another invasive bivalve golden mussel (*Limnoperna fortunei*) in water from both field-collected and laboratory tanks with mussels (Xia et al. 2017). Methods of detecting eDNA vary; earlier research used conventional PCR (Jerde et al., 2011), while more recently there has been a shift toward real-time PCR (RT-PCR) or quantitative (qPCR) (Rees et al., 2014) and next-generation sequencing (Shokralla et al., 2012).

I hypothesized that I would be able to detect the presence of zebra mussels in the south basin where they have been reported, but not in the north where the species is not known to occur. I tested this hypothesis by comprehensively sampling both north and south basins and analyzed samples using all three methods. I hypothesized that FlowCAM equipped with cross-polarizing lenses will perform better than CPLM in detecting veligers in samples (Stanislawczyk et al. 2017). I also hypothesized that eDNA detection will detect zebra mussels in the samples better than either of the alternatives (Egan et al. 2015). I tested these hypotheses by analyzing the samples obtained from intensive sampling of Lake Winnipeg using three different methods.

METHODS

I sampled Lake Winnipeg, Manitoba intensively to increase veliger detection success (Marsden 1991; Counihan and Bollens 2017). I sampled the lake in July at the presumed time of zebra mussel spawning season to ensure the capture of veligers. I sampled five sites from 23 July to 27 July 2015 in the following order: Winnipeg Beach, Grand Rapids, Dauphin River, Calder's Dock and Hnausa (Map shown in Figure 3; coordinates provided in supplementary materials). The five sites were selected to determine the distribution of veligers population from south to north of the lake. Samples were collected with vertical hauls using plankton nets (63 μ m mesh, 50cm diameter mouth and 150cm length). New nets were used at each site to prevent cross-contamination between sites. To decrease the chance of Type II error of missing a veliger that is present in the lake water, I sampled intensively at each site. At each site, 100 plankton tows were made as the boat drifted with the current, yielding a total of 500 samples. The GPS coordinates of the start and end points for each sampling location were recorded. Vertical hauls were collected from just above lake bottom (5.5m to 15m depths) and hauled back up using a hand over hand motion at a rate of about 0.5m/second (Marsden 1991). Depth of each haul was recorded. After each haul, the net was rinsed to limit contamination between hauls. The boat bilge was drained and dried between each site to prevent contamination. All samples were immediately concentrated in a 40 μ m sieve then preserved in 95% ethanol and stored at ambient temperature until processing in the laboratory.

In the laboratory, all samples were filtered through a sieve with 300 μ m Nitex mesh to remove larger particles. *Dreissena* veligers settle from the water column when animals

are between 200-250µm (Hebert et al. 1989), so filtering out the >300µm fraction of plankton in the samples should not reduce veliger abundance in samples while making detection of the species easier (Johnson 1995). To prevent false positives and cross-contamination, sieves were washed and soaked in five percent bleach solution for five minutes between samples. Filtered samples were resuspended in 50mL of 95% ethanol and stored in 50mL polypropylene conical tubes. All samples collected were then analyzed using methods described below for optimal veliger/mussel detection.

Cross-polarized light microscopy

Following (Johnson 1995), I used cross-polarized lenses on a stereomicroscope to detect veligers in samples. Filtered whole samples were studied under cross-polarized light on a Leica transmitting light stereomicroscope at 16× magnification. Polarizing lenses were custom-made by Joseph F.J. Zeman, A-Z Microscope Limited (291 Cheapside Street, London, Ontario, N6A 2A3). Because plastic counting trays are also birefringent and interfered with veliger visualization, glass petri dishes were used. Usually, all taxa in a sample are identified and a rare-fraction curve is formed to establish species richness (Counihan and Bollens 2017). However, to increase efficiency, I only identified and counted *Dreissena* veligers. Numbers of veligers were recorded using laboratory tally counters.

Abundance of veligers in samples was calculated using equations 1 and 2.

$$A_m = \frac{c_m}{V} \quad \text{Equation 1}$$

A_m = microscopic abundance (Ind. m^{-3}), c_m = total counts from microscopy (Ind.), and V = Volume of lake water sampled (m^3) is shown in Equation 2.

$$V = \pi r^2 h$$

Equation 2

Where r = radius of plankton net opening (0.5m) and h = depth of plankton tow (m).

To determine the effect of subsampling on success of detection of veligers in our samples, I also analyzed different subsample volumes extracted from the 50mL sample tubes using graduated pipettes. The subsample volumes were: 3mL, 5mL, 10mL, 15mL, 20mL, 25mL, 30mL, and 40mL.

FlowCAM

The plankton samples in 50mL polypropylene conical tubes were shaken vigorously by hand, and a three-mL subsample was removed using a disposable plastic pipette. The subsample was mixed with about three mL of polyvinylpyrrolidone (PVP) solution to increase viscosity and to slow the movement of particles through the flow cell. The objective used on the FlowCAM was 4× magnification. The flow of plankton through the flow cell was set to the lowest setting to ensure that the maximum number of particles was captured. An XPL (cross-polarizing lens) filter was attached to the FlowCAM to increase visibility of veligers. Particles were imaged using the Image Management System (IMS) under autoimaging mode. Captured images of plankton were analyzed using Visual Spreadsheet (VS). Veligers were easily confirmed from manual, visual inspection of saved images (Fluid Imaging Technologies 2011).

Abundance by FlowCAM, A_f , was calculated as:

$$A_f = \frac{\frac{50}{3} \times c_f}{V} \quad \text{Equation 3}$$

Where A_f = abundance by FlowCAM (Ind. m^{-3}), c_f = counts from FlowCAM (Ind.), and V = volume of lake water filtered (m^3) (as defined in Equation 2).

To assess the possibility that three ml subsamples created volume-based artifacts, I resampled the same samples using subsample volumes of 1mL, 5mL, 10mL, 15mL, and 25 mL using one high abundance sample and one low abundance sample.

Environmental DNA

Total genomic DNA was extracted from bulk plankton samples obtained from plankton tows (Zaiko et al. 2015; Ardura et al. 2017). 1 mL volume of was removed from hand-shaken 50ml tubes using a disposable plastic pipette and placed in 1.5mL microcentrifuge tubes. The plankton was centrifuged to concentrate all solids at the bottom of the microcentrifuge tube. Ethanol was discarded, and solid contents of the tubes were dried in a Vacufuge Concentrator. Digestion buffer and proteinase K was placed into the tubes for overnight digestion before plate-based extraction (Elphinstone et al. 2003). I used conventional PCR instead of real time quatitative PCR (qPCR) (Heid et al. 1996) because I was only looking at absence/presence of the species, not abundance.

The COI gene was PCR amplified using primers DpCOI-F (5'-GGGATTCGGAAATTGATTGGTAC-3') and DpCOI-R (5'-GAATCTGGTCACACCAATAGATGTGC-3') (Egan et al. 2015). All PCR reactions were performed in 11 μ L reactions containing 1 μ L of template DNA, 0.8 μ L of Mg₂SO₄ (BioBasic), 0.2 μ L dNTPs, 0.2 μ L of each primer, 1 μ L of 10 \times buffer, and 0.06 μ L of Taq

polymerase (BioBasic). Cycling conditions for PCR were: an initial denaturation step of 95°C for 1 min, 30 cycles of 94°C for 30 sec, 59°C (COI) for 45 sec, and 72°C for 1 min, and a final extension at 72°C for 8 min. To assess positive detections, PCR products were visualized on a 2% agarose gel.

I also included a sensitivity test for zebra mussel veligers. One, three, five and ten individual veligers were extracted and amplified in the same PCR protocol described to determine the minimum number of veligers needed to amplify the DNA.

Statistical analysis

I conducted a multiple means comparison (Tukey contrasts) after ANOVA on microscopic abundances between Winnipeg Beach (n=100), Hnaua (n=100), Calder's Dock (n=100), Dauphin River (n=99), and Grand Rapids (n=100) using the "multcomp" and "sandwich" package in R (Herberich et al 2010; R Development Core Team 2016). This statistical test is robust for comparing means when data are not normally distributed.

I also conducted conducted Chi-square tests with Yates' correction on contingency tables on prevalence at different locations obtained by the three different methods: CPLM (n=487), FlowCAM (n=487), and eDNA (n=487); Winnipeg Beach (n=97), Hnaua (n=97), Calder's Dock (n=97), Dauphin River (n=97), and Grand Rapids (n=97).

Prevalence data from whole sample CPLM (n=499), 1mL subsample eDNA (n=487) and 3ml subsample FlowCAM (n=487) was analyzed using R package "GLM" (R Development Core Team 2016) to determine the probability of detecting at least one veliger with increasing number of subsamples processed.

I used one-tailed paired t-test to compare the prevalence obtained from Calder's Dock 3mL subsample volumes using eDNA (n=49), FlowCAM (n=49) and CPLM

(n=49). 3mL subsample prevalence data from Calder's Dock was used to calculate the probability of detecting at least one veliger with increasing samples analyzed with basic R (R development Core Team 2016). I did this by sampling the prevalence data at random 100 times with the corresponding number of samples to obtain the probability of detecting at least one veliger.

RESULTS

I successfully detected veligers at all five sites that I sampled for plankton using CPLM. The prevalence (percentage of samples that had at least one veliger) for all samples from all sites was 94% (Table 1). As expected, 100% of samples from the south basin (Winnipeg Beach and Hnausa) tested positive for veligers. Surprisingly, in the north basin, Dauphin River also had 100% prevalence, while Grand Rapids was very close (99% prevalence). Prevalence was substantially lower (63%) in the lake's mid-channel (Calder's Dock) (Table 1; Figure 8). One sample was lost from the Dauphin River site due to leakage and was not analyzed.

30% of the samples tested positive for the presence of veligers using FlowCAM analysis of 3mL subsamples (Table 1). There was a range of FlowCAM prevalence across the sites, with the highest prevalence in Winnipeg Beach and the lowest prevalence in Calder's Dock (Figure 8). There were significant differences between prevalence at all locations (Chi-square test with Yates' correction, $p < 0.05$ for all pairwise comparisons) except for Grand Rapids and Calder's Dock ($p = 0.65$), and Hnausa and Dauphin River ($p = 0.16$). There was a significant difference in prevalence between CPLM and FlowCAM (Chi-square test with Yates' correction, $p < 0.0001$).

Detection using DNA yielded only presence/absence of zebra mussels. While abundance of NIS tells us the stage of invasion, the presence/absence data can alert monitoring programs to the sites (Catford et al. 2012). Sensitivity testing indicated that positive detection was possible with one veliger digested (Figure 7). I obtained positive detection of zebra mussels at all but one site in Lake Winnipeg; there were no positive detections at Calder's Dock using eDNA. eDNA prevalence differed significantly (i.e. was lower) than microscopic prevalence at all locations (Chi-squared test with Yates' correction, $p < 0.05$ for each pairwise comparison) except for Hnausa (Chi-squared test with Yates' correction, $p = 0.48$). At all locations, microscopic prevalence was highest, while eDNA prevalence was higher than FlowCAM prevalence at the high abundance (south basin) locations. For the low abundance (north basin and channel) locations, eDNA and FlowCAM had lower or similar prevalence (Table 1).

I observed a range of microscope abundances of veligers across the sites in Lake Winnipeg (Table 2; Figure 4 and 5). The highest microscopic abundance was recorded at Winnipeg Beach, the southernmost site, followed by Hnausa, which is also in the south basin. The third highest abundance was Dauphin River in the north basin, followed by Grand Rapids, the northernmost site. The lowest microscopic abundance was observed in the channel at Calder's Dock. There was a significant difference in microscopic abundance between sites (Tukey contrasts, $p < 0.001$ for all pairwise site comparisons).

FlowCAM abundance was significantly lower than microscopic abundance at all sites (Tukey contrasts, $p < 0.001$ for all pairwise method comparison). There was significant difference in FlowCAM abundance between sites (Tukey contrasts, $p < 0.05$ for all pairwise site comparison), except for Grand Rapids and Calder's Dock ($p = 0.727$), and

Hnausa and Dauphin River ($p=0.980$) (Figure 6). In the test for volume-based artifacts, I found no significant difference in the number of veligers detected with increasing subsample volume for either the low abundance location (ANOVA, $df = 1, 16$, $F = 0.540$, $p = 0.473$) or high abundance location (ANOVA, $df = 1, 16$, $F = 0.355$, $p = 0.560$). Many of the images captured by FlowCAM IMS software were not veligers, and many veligers were captured only as partial images. Manual inspection of the captured FlowCAM images using VS software confirmed that most images were debris or other plankton. For example, in sample #1-91, I observed only two veligers out of 2182 captured images, while sample #3-57 contained only one veliger out of 1330 captured images, and sample #5-51 contained one veliger out of 1318 captured images.

For samples from all sites, there was a significant difference between 3ml FlowCAM and 1ml eDNA in detection success (generalized linear model, $p < 0.01$); detection success increased more quickly for eDNA than FlowCAM with increasing microscopic abundance (Figure 9).

In our analysis of 3ml subsamples from Calder's Dock, prevalence was significantly higher with microscopy than either FlowCAM (two-tailed paired t-test, $p < 0.005$) or eDNA (two-tailed paired t-test, $p < 0.005$). However, prevalence did not differ significantly between FlowCAM and eDNA in 3 ml subsamples (two-tailed paired t-test, $p = 0.32$). Probability of detecting at least one veliger increased with number of samples analyzed for all three methods (Figure 10). Probability of detecting at least one veliger using CPLM increased significantly faster than the two other methods with increasing number of samples analyzed (GLM, $p > 0.001$, for all pairwise method comparison).

Microscopic analysis of 3mL subsamples resulted in the highest probability of detecting at least one veliger at all numbers of samples analyzed.

DISCUSSION

Increasing sampling effort in plankton sampling can increase the success of detection of zebra mussel veligers (Counihan and Bollens 2017). However, most monitoring programs are limited by cost and time of sampling. So how much sampling is enough? Here, I have shown successful detection of zebra mussel veligers in a newly invaded system via intensive sampling and different methods of analysis. Despite being only recently discovered in Lake Winnipeg, veligers were detected at all five lake sites using all three methods except for Calder's Dock using eDNA (Table 1). By sampling intensively, I detected veligers in the north basin where they were previously unreported. This indicated that sampling intensity of monitoring programs should be increased to improve success of detection of veligers. Zebra mussel veligers were found in the north basin of Lake Winnipeg as early as July 2015. These northern sites - Dauphin River and Grand Rapids - had veliger prevalence rates of 100 and 99%, respectively. Despite these high prevalence rates, mussel abundance in the north basin and channel was very low, universally lower than 300 Ind. m⁻³. The south basin, by contrast, was considered high abundance, with an average of 800 Ind. m⁻³. Veliger abundance in Lake Winnipeg remains very low, with a maximum abundance of 1075 Ind. m⁻³. In contrast, Lake Erie had about 3000 Ind. m³ in 1989 at the initial stages of invasion (Riessen et al. 1993), increasing to 40,000 to 400,000 Ind. m⁻³ in the summer of 1990 (MacIsaac et al. 1992). The very low abundance in Lake Winnipeg might be an indication of the lag phase of the invasion. Zebra mussels can tolerate water temperature up to 30°C (Spidle et al. 1995).

Summer mean surface water temperature in the lake is well within the reported thermal tolerance of zebra mussel. Mean summer water temperature from 1999 to 2007 was 19.7°C in the north basin and 21.5°C in the south basin and channel, with little stratification (Environment Canada 2011). Of all the Great Lakes, Lake Erie is the most similar in volume and surface area to Lake Winnipeg. Water temperature of Lake Erie can reach 25°C in the summer and 0°C in winter (NOAA 2016). Zebra mussels can survive and thrive in Lake Erie (Schloesser et al. 1996). Zebra mussels have the potential to do the same in Lake Winnipeg (Therriault et al., 2013).

Low abundance and high prevalence of veligers in Lake Winnipeg may indicate that there are small populations of adult zebra mussels throughout the lake, even in the north basin. Veligers can be produced by these populations, causing veligers to be detected in all the sample tows, albeit in low abundance. Adult zebra mussels were detected by monitoring programs in the south basin long before reports of detections in the north basin (CWS 2015). As expected, abundance of veligers was higher in the south basin where they were established compared to the north basin where they only recently invaded. Samples from Calder's Dock had the lowest abundance, possibly due to high flow rate through the narrow channel (Zhao et al. 2012). Veligers experience high mortality rates when exposed to turbulent water or hydrodynamic forces in laboratory experiments (Rehmann et al. 2003; Horvath and Crane 2010). Veligers might experience increased mortality at the channel because of turbulence from the high flow rate.

Veligers could spread from the initial site of invasion throughout Lake Winnipeg via water currents or as postveligers attached to drifting macrophytes or debris (Johnson and Carlton 1996; Bobeldyk 2005). Water flow in the lake occurs from the south basin,

through the central channel, and into the north basin (Zhao et al. 2012). High abundance of veligers in the south basin and lower abundance in the north basin (Figure 4) are consistent with this view.

Effective monitoring is needed for the early detection of invasive dreissenid mussels to prevent costly management efforts. While intensive sampling can increase the chance of detection, methods of analysis needs to be quick, sensitive and cost effective. Ideally, methods of analysis must be able to process large numbers of samples obtained from intensive sampling effectively (i.e. with high sensitivity) (Counihan and Bollens 2017). Most research has focused on the optimization of one method of detection. Even when comparing the three methods, different samples were analyzed (Frischer et al. 2012). In this experiment, I tested all three methods on the same samples.

I observed significantly lower prevalence and abundance with FlowCAM and eDNA as compared to CPLM, indicating that the former two approaches were more prone to false negatives. False negatives with FlowCAM were caused by the IMS software not recognizing veligers. I noted that some veligers passed through the flow cell in the FlowCAM but were not captured by the software, causing the FlowCAM values to be lower than microscopic identification even though both approaches used cross-polarizing lenses. Visual inspection of captured FlowCAM images revealed that many particles captured were not veligers, raising the possibility of false positives. Possible type I errors with the FlowCAM could occur when the software captures images of birefringent sand particles or microplastics (Jaeger 2005; Lusher et al. 2017). Newer software is touted to be better at detect veligers (Fluid Imaging Technologies 2017). While some studies have shown that FlowCAM is better at identifying plankton

(Stanislawczyk et al. 2017), in this case FlowCAM did not outperform the microscopic method.

All sites had at least one positive result for the presence of zebra mussel eDNA except Calder's Dock (Table 1). This indicated that eDNA detection did not work well at sites where animal abundance was very low. It is possible that inhibition occurred in some of the sites with prolific phytoplankton, which would lead to false negatives. As preliminary work determined that one veliger was enough to obtain a positive detection result, overall abundance at Calder's Dock must have been very low (Figure 7). However, for low abundance samples, one mL of subsample (taken from a 50mL sample) may have been insufficient as it had a low probability of containing a veliger. 1 mL subsampling at Calder's Dock resulted in 0% prevalence (zebra mussel DNA detection) in all samples. I recommend digesting larger volumes, and whole samples if possible, of plankton for eDNA analysis.

False negatives from eDNA in aquatic environments can result from DNA shed by species swimming through the sampling field but departing the location. The sedentary nature of adult zebra mussels means that there was a smaller chance of false positives from migrating individuals. While there exist many markers and techniques (Ardura et al. 2017; Gingera et al. 2017), I found that eDNA detection combined with intensive sampling resulted in a high chance of detection at high abundance sites. eDNA protocols vary with the study organism and environment (Goldberg et al. 2016). In my study, I was able to obtain positive detection of zebra mussels from one veliger using conventional PCR for eDNA detection. the use of qPCR is increasingly common for eDNA detection

(Xia et al. 2018), conventional PCR was sufficiently sensitive to DNA concentration as low as 7.25×10^{-11} ng/ μ L (Jerde et al. 2010).

Microscopic analyses of 3mL samples had the highest probability of detecting at least one veliger (Figure 10). Microscopic analysis will give the best chance of veliger detection between the three methods when only small numbers of subsamples are analyzed. For 3mL subsampling of samples from Calder's Dock, eDNA had the lowest probability of detecting at least one veliger. Again, eDNA does not perform better than CLPM when the abundance is very low.

While accuracy and reliability of the three methods of analysis are important and have been discussed (Frischer et al. 2012), feasibility, ease of use, and economics of using different methods have not been explored. Cost-effectiveness of these methods is important, as it may inform which detection method should be utilized in future (Roos et al. 1998). Depending on budget, time constraints, and availability of equipment, one of these three methods is better suited for the needs of their monitoring programs. Resources such as time and money can be limited for the management of ecological systems so making informed decisions is important (Chadès et al. 2017; Kling et al. 2017). NIS monitoring programs can benefit from lowering costs and increasing efficiency (Bogich et al. 2008; Hauser and McCarthy 2009). The time and money saved could then be used to manage or prevent an invasion. To that end, I examined the cost and time taken for each method to analyzed 500 samples. I collected the startup cost of buying new equipment for each method. All the necessary paraphernalia for each method was also included in the cost. The cost of the microscope was sourced from a vendor (Leica 2017).

FlowCAM costs were obtained from an invoice from Fluid Imaging. The cost of running PCR and qPCR was obtained from Riedel et al. 2014 and Schlatter et al. 2015.

CPLM was mid-range in startup cost, when compared with eDNA and FlowCAM. Although FlowCAM cost the most, if you are using it for other purposes, it would be justified (because it can be used for a variety of other applications.) FlowCAM is particularly good at phytoplankton identification (Camoying and Yñiguez 2016). eDNA detection was the fastest method and most cost effective. This method would be the best choice for time and budget constraints. Its ability to detect zebra mussels was also significantly better than FlowCAM (Figure 9).

I calculated the cost needed to process each sample. I also calculated the labor and time I took to analyze 500 plankton samples (Table 3). The startup cost of purchasing FlowCAM and accessories was by far the highest. The second most expensive startup cost was CPLM, while the lowest in startup cost was eDNA.

The cost of analyzing one sample by CPLM was CAD4.30 for labour only. The cost of running a sample through FlowCAM was CAD6.90, including labor and consumables. The cost per sample analyzed using conventional PCR was calculated to be CAD6.40 (Table 3). I assumed that one PCR reaction gave us the definitive present/absence result for each sample and training takes the same amount of time for all. I have also included the cost of using qPCR instead of conventional PCR. The cost of using qPCR is CAD8.60 per sample analyzed (Table 3).

eDNA was the fastest method of analyzing many samples (Table 3). It was possible to DNA extract and PCR amplify many samples in three days. This requires a molecular biology laboratory and training in molecular methods. Samples were digested and cannot

be reused after this method. eDNA paired with intensive sampling was a very quick way to confirm presence, because one positive test was required to confirm that the species was present (more positives is, of course, better). This method should be explored in future, in particular the use of qPCR for eDNA detection. While qPCR is more sensitive than conventional PCR at detecting eDNA (Xia et al. 2018), the cost of using qPCR is higher than the cost of conventional PCR (Riedel et al. 2014). When made possible by funding, monitoring programs that utilize eDNA detection for NIS should opt of qPCR methods.

The abundance of veligers can greatly change in one spawning season (Riessen et al. 1993). The abundance and prevalence obtained from this study was from a single sampling event. Increasing the number of sampling events can provide a better idea of variation in veliger abundance and prevalence over the course of a year.

While I rinsed plankton nets between tows to prevent cross-contamination of veligers between samples, there was a chance of transfer of veligers. If this occurred, the veliger from one sample ends up in another, potentially causing a false negative in the first and a false positive in the second. In addition, the presence of sand (which are also birefringent) in the samples could cause a type I error. Finally, it was possible that a veliger could be missed when conducting microscopy, causing a type II error.

CONCLUSION

Detection of zebra mussels through veligers is most successful using CLPM due to their birefringence. Although time-consuming, whole sample CPLM gave the highest probability of detecting at least one veliger at the lowest number of samples processed. CPLM also gave us abundance of veligers in the lake when whole samples of plankton

tows are analyzed. eDNA with conventional PCR was the fastest and lowest-cost in detection of zebra mussels. While not as good as the CPLM at detecting veligers at low abundance, eDNA detection will be useful for monitoring programs involving large areas with multiple lakes. Many samples can be analyzed in a timely and cost-efficient manner. If large samples of plankton can be effectively processed for DNA extraction and PCR amplification, this method can be highly effective at zebra mussel detection.

Table 1: Prevalence (%) of zebra mussel detection in the samples using CPLM, FlowCAM+XPL, and eDNA. 100 samples were collected from each five 5 sites sampled for plankton in Lake Winnipeg. eDNA detection was of DNA amplification of zebra mussels COI mtDNA.

Site	CPLM (n=499)	FlowCAM with XPL attachment (n=487)	eDNA (n=487)
Grand Rapids	99	3	4
Dauphin River	100	34	7
Calder's Dock	63	2	0
Hnausa	100	45	94
Winnipeg Beach	100	63	91

Table 2: Whole counts of veliger in samples were divided by volume of water filtered per plankton tow to obtain lake abundance (Ind. m⁻³). Median and means of microscopic abundance of veligers were calculated for five sampled sites in Lake Winnipeg.

Site	Median	Mean	Standard Deviation
Winnipeg Beach	448.2	493.9	212.7
Hnausa	254.3	256.2	71.9
Calder's Dock	0.3	0.5	0.7
Dauphin River	85.7	92.4	41.5
Grand Rapids	5.6	12.1	24.2

Table 3: Cost and labour comparison of three methods of analysis of 500 plankton samples collected from Lake Winnipeg for the detection of zebra mussels. Cost of equipment compiled from our own purchases and invoices. PCR costs from (Riedel et al. 2014; Schlatter et al. 2015). All costs are in Canadian dollars (CAD).

	Microscopy		FlowCAM		eDNA (Conventional PCR)		eDNA (qPCR)	
Initial costs (CAD)	Microscope	10000	VS-I-C B3 FlowCAM (Fluid Imaging)	77500	BIO RAD T100™ Thermal Cycler (Low End)	5145	StepOnePlus PCR System	37487
	AZ custom-made cross polarizing lenses	430	XPL (Cross- polarizing lenses add-on)	40000	Micropipettors (FisherScientific)	590	Micropipettors (FisherScientific)	590
	Leica Cross- polarizing lenses	1062			Microwave	100		
	Glass Petri Dish	12			Flash Gel Camera and Docking system	1306		
Total start up		11504		81500		7141		38077
Consumables			Disposable Transfer Pipets and PVP	52	Reagents used per reaction	4	Reagents used per reaction	6
Labour (Hours of processing 500 3mL subsamples)		108		167		72		72
Cost of labour (\$/hour)		20		20		20		20
Running cost per 3ml subsample		4.30		6.90		6.40		8.60

Figure 1: Simplified step wise progression of a successful biological invasion. The arrows represent each step of an invasion. Boxes represent barriers to be overcome for a species to be a successful invader. Overcoming a geographic barrier, transport is the movement of the species from the donor region to the recipient region, usually by anthropogenic means. Introduction is the release of the species from captivity into the environment at the recipient region. Species can also be released directly into the novel environment after transport (e.g. ballast water discharge). During establishment, the species must be able to survive and reproduce to form a population. After a reproducing population is formed, they can spread from the site of introduction. Adapted from Blackburn et al. (2011).

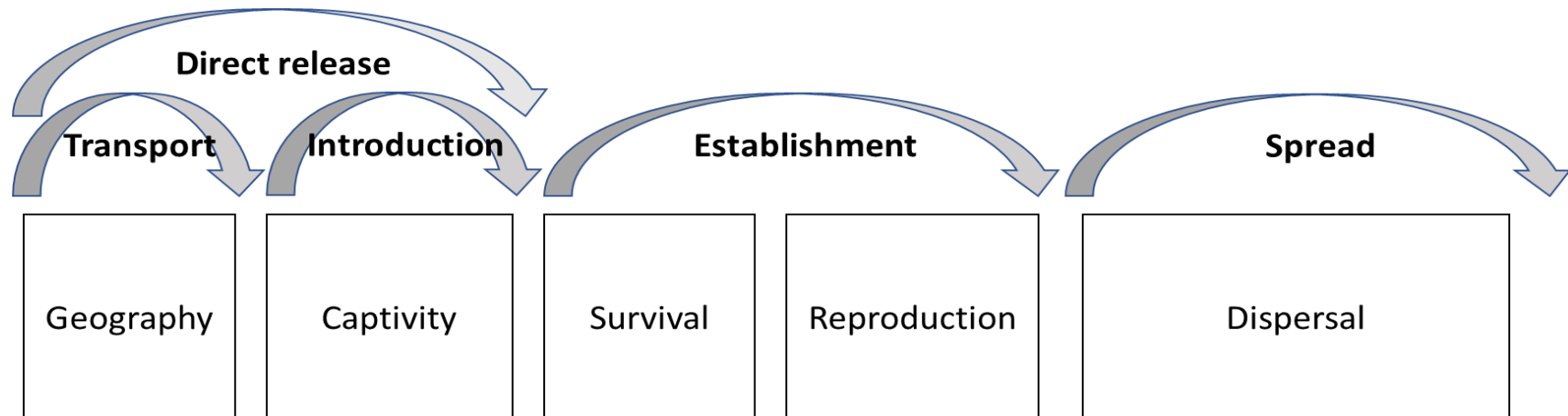


Figure 2: Image of plankton under transmitted light and under cross-polarized microscopy. The pictures show the same field of vision on a microscope. Pictured left, veliger (indicated by arrow) is difficult to distinguish from other plankton. Pictured right, the same veliger (indicated by arrow) under cross-polarized light appears bright owing to the animals' birefringent properties.

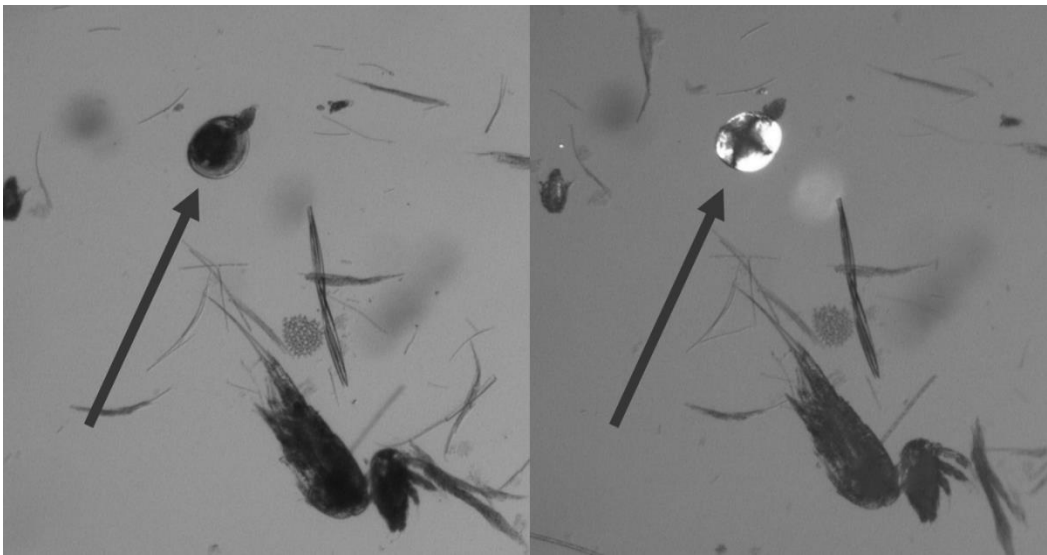


Figure 3: Map of Lake Winnipeg, Manitoba. Black dots indicate plankton tow sampling sites. Grand Rapids and Dauphin River are sites in the north basin of the lake. Calder's Dock is the site in the channel that connects the north and south basin. Hnaua and Winnipeg Beach are in the south basin of the lake.

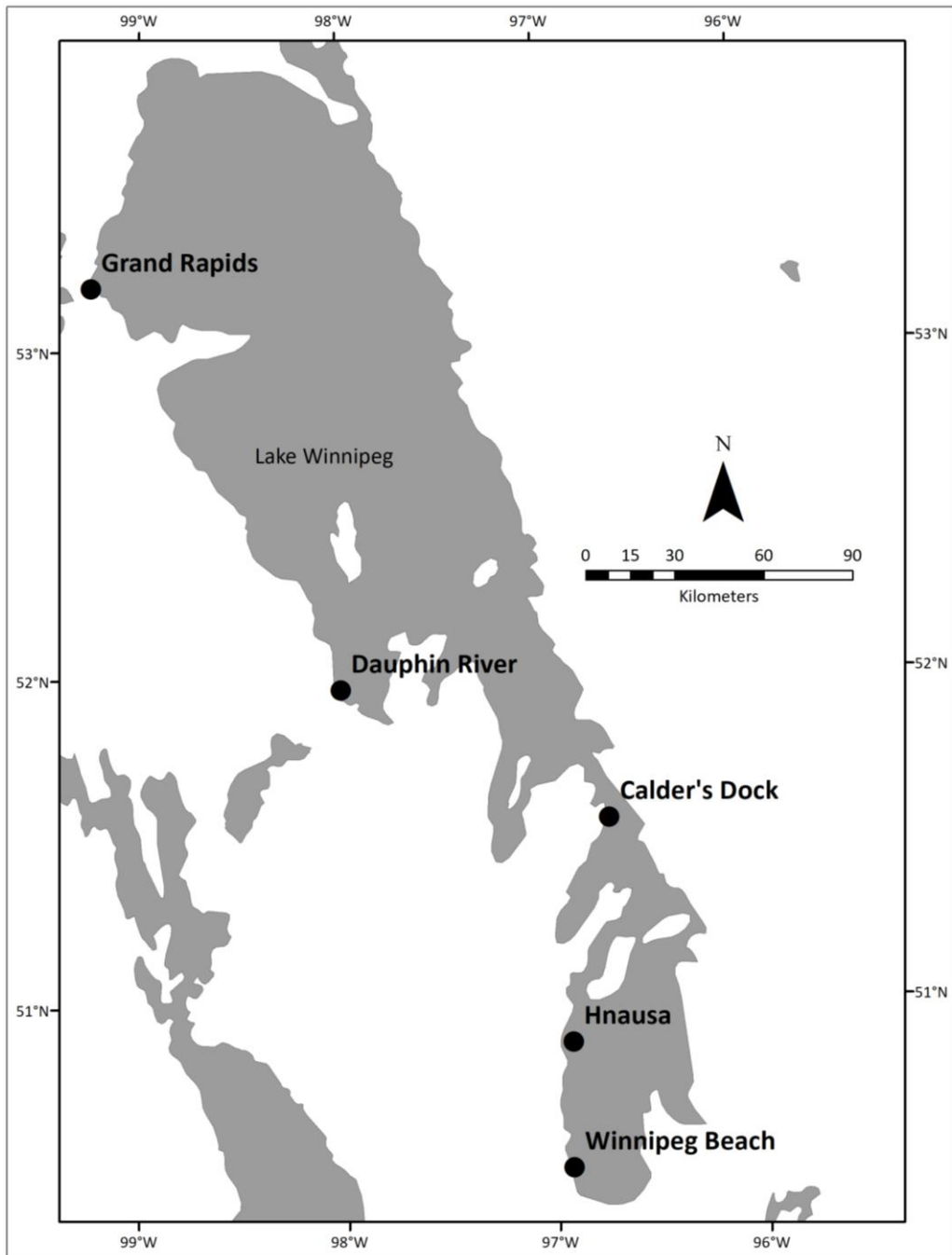


Figure 4: Box-and-whiskers plot displaying median and range of veliger abundance obtained using cross-polarized microscopy at each site. The thick line represents the median abundance. The box indicates 50% of the data closest to the median. The whiskers (the lines on either side) are maximum and minimum. Dots are outliers. There were significant differences in abundance between sites (Tukey test, $p < 0.05$).

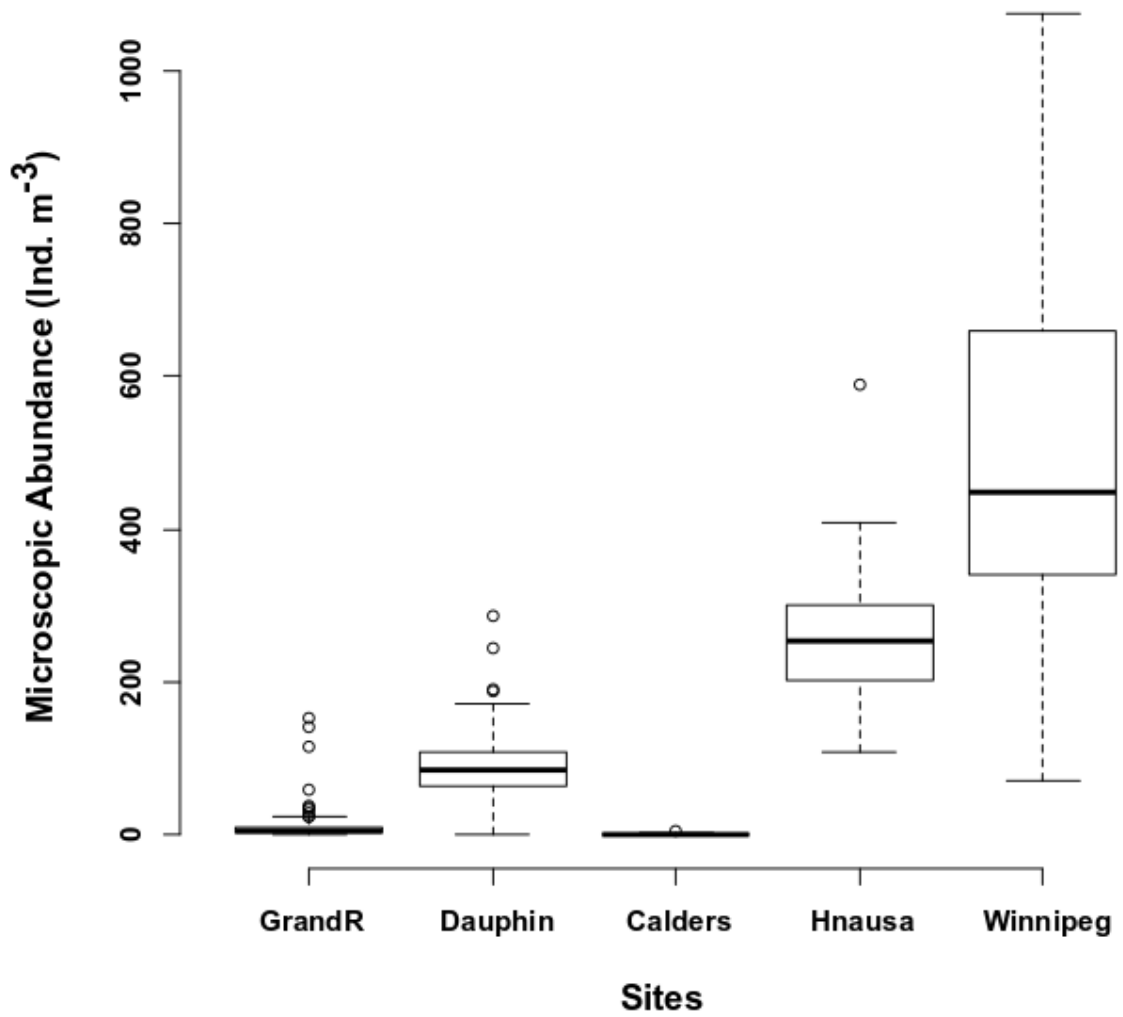


Figure 5: Distribution of microscopic veliger abundance at the five sampled sites, starting from the southernmost site to the northernmost site. The columns represent the number of tows that yielded the abundances at different sites.

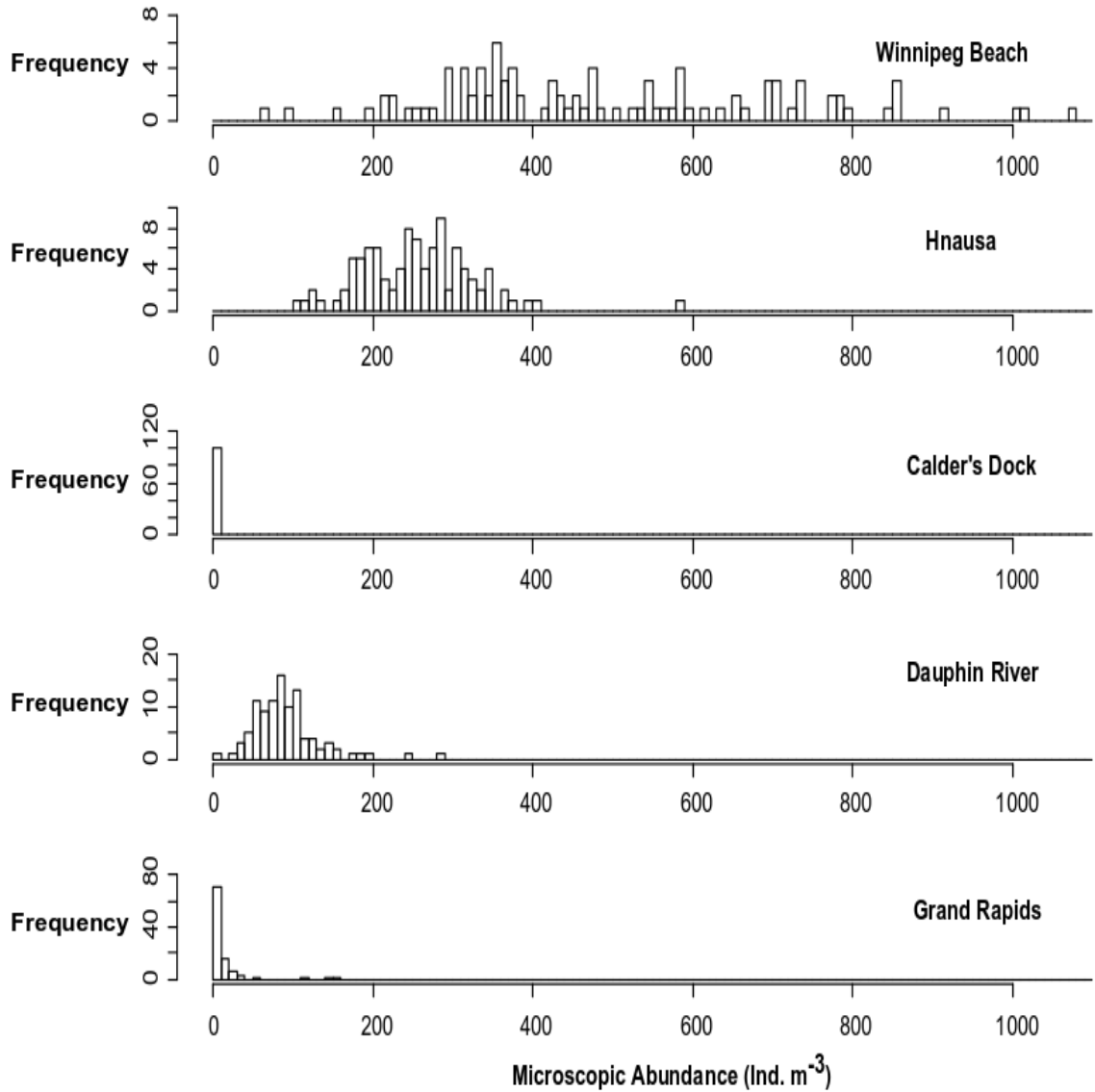


Figure 6: Comparison of mean FlowCAM (3mL subsample) and microscopic (whole sample) veliger abundance. There was significant difference between FlowCAM and microscopic abundance at each site (Tukey contrast of multiple means, $p < 0.05$). Error bars are standard deviation of the mean.

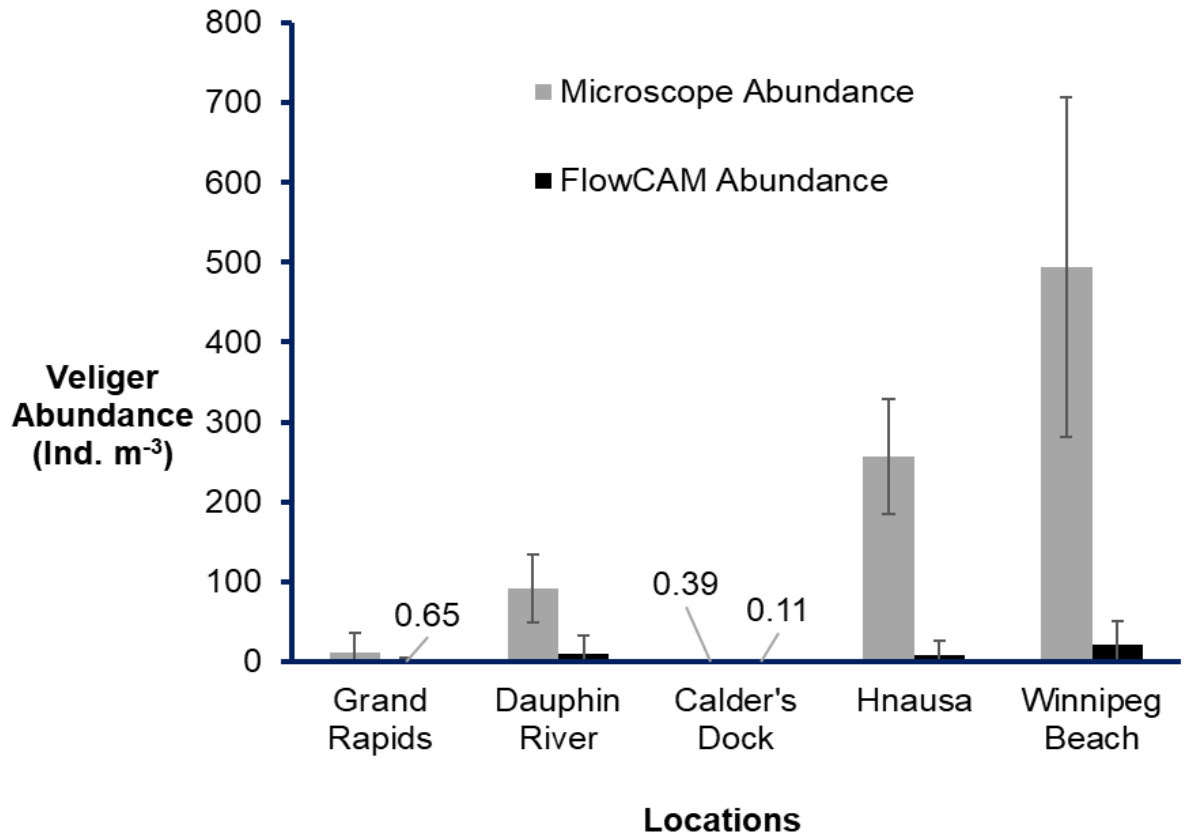


Figure 7: Positive detection of zebra mussel DNA (mtDNA COI amplification) in sensitivity test. Bands on agarose gel are amplified DNA (arrow). Lane 1: Ladder (100bp); Lane 2 to 5: One veliger; Lanes 6 to 9: One veliger + other plankton and particles; Lane 11 to 14: Three veligers; Lanes 15 to 18: Three veligers + other plankton and particles.



Figure 8: Prevalence of zebra mussels in samples analyzed using CPLM, FlowCAM and eDNA. There was significant difference in prevalence of all methods at each location (Chi-squared test with Yates' correction, $p < 0.05$ for all pairwise comparison) except for eDNA and FlowCAM at Grand Rapids ($p = 0.50$) and Calder's Dock ($p = 0.48$).

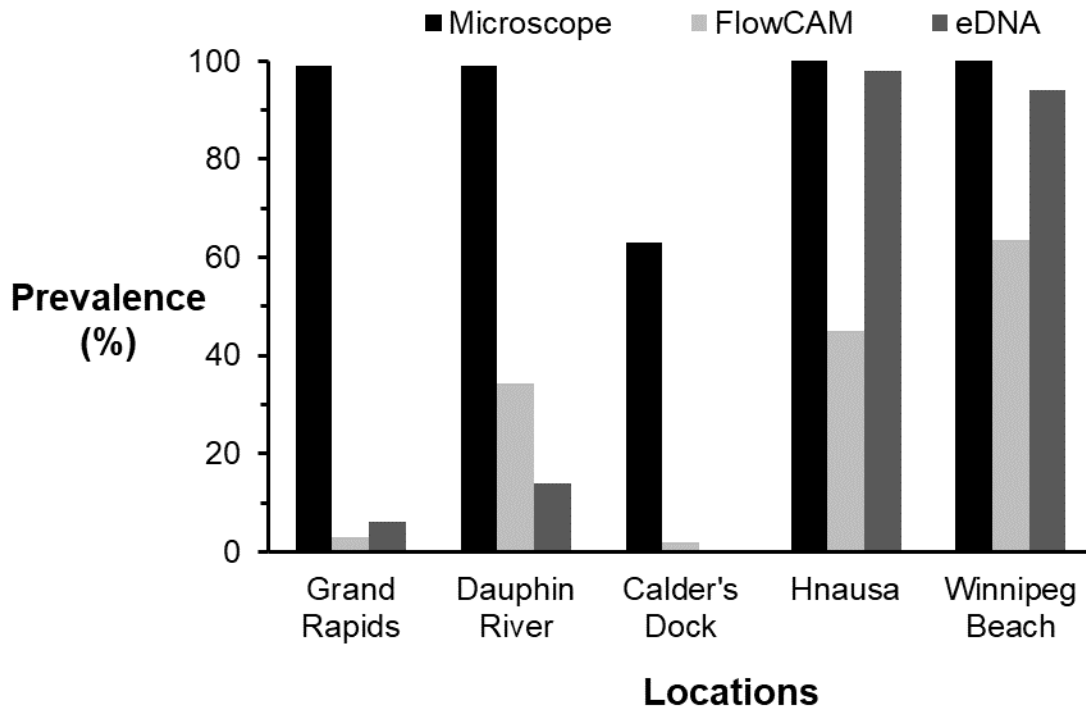


Figure 9: Detection of zebra mussel presence using eDNA (1mL) and FlowCAM (3mL); Detection was plotted as 1, failure to detect as 0. There was a significant difference in detection using FlowCAM versus eDNA (generalized linear model logistic regression, $p < 0.01$). eDNA detected zebra mussels more effectively than microscopic abundance.

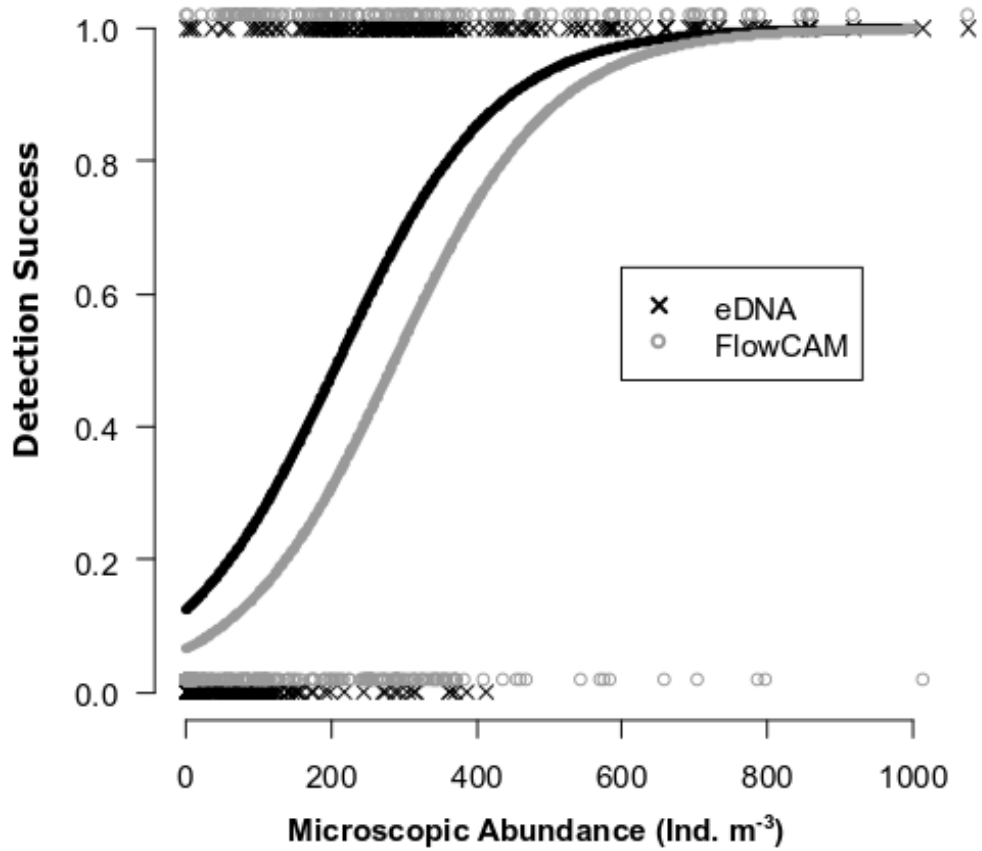
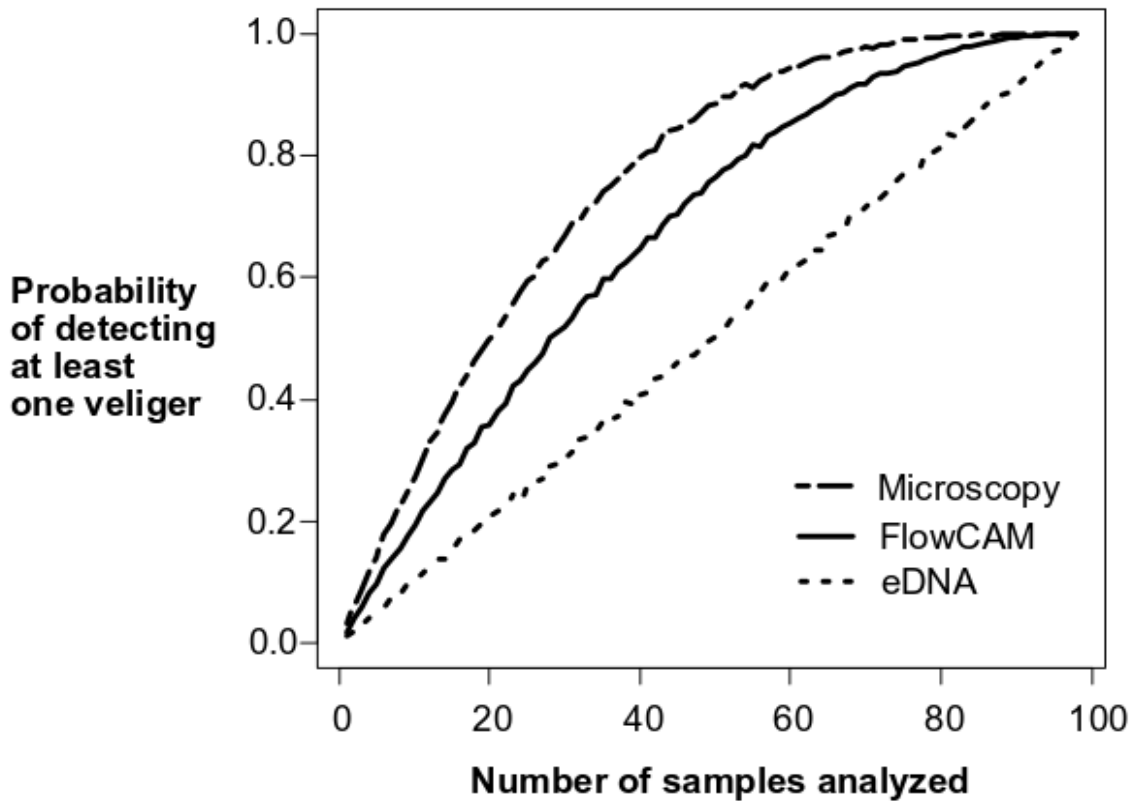


Figure 10: Probability of detecting at least one zebra mussel veliger with increasing number of samples analyzed. Three different methods (FlowCAM, eDNA and CPLM) were tested using 3mL subsamples (of 98 total) of plankton collected from Calder's Dock in Lake Winnipeg. Prevalence was randomly sampled using R to determine detection probability with increasing number of samples analyzed. There were significant differences in the probability of detection between three methods (generalized linear model logistic regression, $p < 0.01$).



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VITA AUCTORIS

NAME: Sharon Lavigne, née Yong (杨盈盈)

PLACE OF BIRTH: Kuala Belait, Brunei

YEAR OF BIRTH: 1987

EDUCATION: Sekolah Menengah Sayyidina Ali, Kuala Belait,
Brunei, 2005

University of Windsor, Bachelor of Science with
Honours, 2012

Great Lakes Institute for Environmental Science,
Master of Science, 2017