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Comparison of traditional microscopy and automated imaging flow cytometry (FlowCAM) for detecting and identifying rare zooplankton

Keara Michelle Stanislawczyk
University of Windsor

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By

Keara Stanislawczyk

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Keara Stanislawczyk

APPROVED BY:

_____

Dr. Trevor Pitcher
Department of Biological Sciences

_____

Dr. Christina Semeniuk
Great Lakes Institute for Environmental Research

_____

Dr. Hugh MacIsaac, Advisor
Great Lakes Institute for Environmental Research

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Abstract

Non-indigenous (NIS) have negatively impacted ecosystems worldwide. When a species is introduced, its population will generally be small. This is the best time to eradicate NIS, however, detection at this stage is difficult. I hypothesize that rare species will be more easily found as they become abundant, when sampling effort is increased, and morphologically distinct species are more likely to be detected. I spiked different densities of NIS into zooplankton samples from Hamilton Harbour to simulate rarity and assess detection rate with both microscopy and FlowCAM. My results indicate a positive relationship between detection and abundance, counting effort, and distinctiveness. FlowCAM can process more data, but morphologically similar taxa will be distinguished more readily with microscopy. This study provides tools to monitor rare aquatic species as well as a means to combat NIS at the frontiers of invasion and provides a way to further test hypotheses of establishment and invasion.

Dedication

This thesis is dedicated to my family and friends

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Introduction

Non-indigenous species (NIS) are those that have been anthropogenically introduced to an environment that is outside of their native range. Nuisance NIS (also called invasive species) have caused numerous negative impacts worldwide including significant changes to natural habitats and reduction of biodiversity (Vitousek et al. 1997). For example, the Emerald ash borer, *Agrilus planipennis*, was introduced to North America where it quickly proliferated and has since killed millions of native ash trees (Herms and McCullough 2014). Likewise, establishment of Burmese python, *Python bivittatus*, in the Everglades has resulted in the loss of virtually all mammalian species from inhabited areas (Dorcas et al. 2012). NIS also have negative effects on human health and economy (Colautti et al. 2006), such as spread of disease (e.g. Zika and West Nile viruses), as well as loss of recreational revenue and reduction of trading efficiency due to increased regulations. Economic losses are estimated to cost approximately $120 billion annually in the United States alone (Pimentel et al. 2005). Additionally, it is projected that Canadian fisheries, forestry, and agriculture potentially suffer an average of $16.6 billion CDN in damages each year due to nuisance NIS (Colautti et al. 2006).

The Great Lakes of North America contain the largest volume of available freshwater on Earth (Mills et al. 1994). Considering that these lakes support a $450 million fishing industry, and supply drinking water for 43 million people, of whom 8.5 million are Canadian, the region is essential for Canada's economy and human health (Environment and Climate Change Canada, 2013).

Additionally, this region is crucial for both international and domestic commerce. For example, 39,887,042 tonnes of cargo passed through the St. Lawrence Seaway on shipping vessels in 2014 (St. Lawrence Seaway Management Corporation and St. Lawrence Seaway Development Corporation 2014). Regrettably, these same vectors of trade serve as vehicles for introduction of NIS. In the Great Lakes, ballast water from trading vessels has been recognized as the primary source of new NIS (Grigorovich et al. 2003; National Research Council 2011). Expert opinion has placed invasive species from ballast water - especially zebra and quagga mussels (*Dreissena spp.*) and gobies (*Neogobius spp.*) - among the leading stressors in the Great Lakes (Allan et al. 2013).

Non-indigenous species have been present in the Great Lakes and the St. Lawrence Seaway since European settlement occurred in the area in 1830, and today there are more than 180 known invaders in the region, making the Great Lakes one of the most invaded freshwater systems in the world (Mills et al. 1993; Ricciardi 2006). These species include fish such as the round goby *Neogobius melanostomus* (Kornis et al. 2013), and sea lamprey *Petromyzon marinus* (Fetterolf 1980), plants including water hyacinth *Eichhornia crassipes* and water lettuce *Pistia stratiotes* (Adebayo et al. 2011), and invertebrates including the zebra mussel *Dreissena polymorpha* (Berkman et al. 2000) and fishhook waterflea *Cercopagis pengoi* (MacIsaac et al. 1999). Invasive species have caused a wide range of negative economic and environmental impacts in the region since their introduction. For example, the zebra mussel has cost an estimated $1 billion annually in the United States of America due to damages

including fouling of watercraft as well as closures for both physical and chemical removal of colonies to prevent clogging (Pimentel et al. 2005), in addition to a suite of ecological changes (MacIsaac 1996). Furthermore, some invasive species spread from the introduction site to colonize additional areas (Colautti and MacIsaac 2004). The round goby was introduced to the St. Clair River from the coastal Black Sea (Stepien and Tumeo 2006), and has since spread throughout the region causing declines in both native fish and invertebrate populations (Kornis et al. 2013).

It is difficult to predict how and where an invasive species will affect the Great Lakes because a multitude of factors influence NIS and, in consequence, their impact. These include temporal variation in vector strength (e.g. changing trade routes), as well as variable environmental conditions in the location where NIS are deposited (e.g. seasonal temperature differences). Blackburn et al. (2011) formulated a model of the obligatory and sequential steps taken and barriers crossed during the invasion process before an NIS can become an invasive species. Initially, a species must be able to survive uptake, transport, and release into a new environment. Species can be transported directly from their native range or released from captivity. For example a lionfish can be introduced to a new area by being transported from its native habitat (Indo-Pacific region), or it can be moved secondarily, such as when in 1992 lionfish escaped from an aquarium in Biscayne Bay, Florida during a hurricane (Courtenay 1995). Once an individual is released into its new surroundings, it must be able to survive extant environmental conditions, find the resources it needs, and

reproduce in order to become established (Blackburn et al. 2011). Often when a non-indigenous population is established, it will have an opportunity to spread to nearby environments. A species can fail to move on to the next stage (pass through the barriers) at any point in this framework (Blackburn et al. 2011). While a species can have impacts at any stage, impacts are typically greatest when a species becomes abundant and spreads to new locations (Lockwood et al. 2013). Not all NIS will, however, become invasive. In fact, it is expected that only 10% (range 5-20%) of all species released into a new area will become established, and that of those, only 10% will become problematic or undergo invasive spread (Williamson and Fitter 1996). For freshwater fish, bird, and mammal invaders this 10% rule was later revised upwards; it is expected that an average of 50% of invaders may be successful at each stage (Jeschke and Strayer 2005). A lack of knowledge about which species will become problematic forces us to treat all NIS as though they are potentially invasive.

When aquatic NIS are introduced to a new location, their population numbers are generally initially low, effectively making them amongst the rarest of all species in the system. This is the easiest time to eradicate possible invasive species (Mehta et al. 2007) or to suppress spread via containment (Hulme 2006). Therefore, detecting these rare species, particularly those that might become problematic, is of paramount importance. Historically, zooplankton samples have been analyzed with a microscope. For instance, the NIS *Cercopagis pengoi* was identified in Lake Ontario using traditional microscopy (MacIsaac et al. 1999). However, this approach makes the detection of rare aquatic species difficult and

labor intensive. For example, using random site samples, Hoffman et al. (2011) estimated that in order to detect 95% of all zooplankton species present in Duluth-Superior harbour, Lake Superior, 776 samples and examination of 500,000 individuals would be required. This pattern is consistent with Harvey et al.'s (2009) finding that even with intensive (n=100) sampling, zooplankton species present at very low abundance are exceptionally difficult to detect. The high risk associated with aquatic NIS requires a more rapid way to screen samples and monitor aquatic systems.

The problem of false negatives in environmental screening has led to the deployment of several new technologies to detect organisms at very low abundance. Cross-polarized light microscopy is a recent development that has been successfully employed to detect zebra mussel veligers in plankton samples owing to their forming shell (Johnson 1995). Environmental genomics has been proposed as another effective method for finding rare aquatic species (e. g. Ficetola et al. 2008; Valentini et al. 2009). Indeed, this method has been used monitor the invasion front of Asian carp in Chicago's waterways and to assess their presence in the western basin of Lake Erie (Jerde et al. 2013). Additionally, Dejean et al. (2012) determined that environmental DNA was a far more effective means of detecting invasive bullfrogs than traditional methods. A key limitation of metagenomic analysis of communities is the creation of artefact sequences and identification of species that are not present (false positive) or, if using sequence clustering analyses, loss of distinct but closely related species (false negative). To address the former issue, Zhan et al. (2013) spiked very low numbers of

known species (and known sequences) into samples where they could not have naturally existed to determine the minimum abundance threshold for true species detection.

Alternatively, a digital imaging system (Zooscan) was recently developed as a way to quickly identify and enumerate zooplankton in mixed-species samples (Grosjean et al. 2004). Furthermore, automated imaging flow cytometry (FlowCAM) is an emerging technology that has been shown to be a quick and accurate tool for identification of phytoplankton (Álvarez et al. 2011), however its published use with zooplankton is limited. In one study, FlowCAM was able to distinguish between copepods and phytoplankton in the same culture (Ide et al. 2008), however no known studies have demonstrated its capability to distinguish between closely related zooplankton taxa nor has it been applied to early detection of NIS.

In this thesis I employed a variant of Zhan et al.'s (2013) approach by spiking very 'rare' cladoceran zooplankton species into Hamilton Harbour samples to determine detection thresholds using both traditional microscopy and FlowCAM image analysis. I hypothesized that the likelihood of finding spiked species will increase: 1) as more total individuals are counted, 2) as spiking abundance increases, and 3) in relation to the distinctiveness of the spiked species relative to those in the resident community. I also expected FlowCAM would be able to detect morphologically distinct species in the sample even when they are present at low abundance, though the opposite would be true for non-distinct species even when they were abundant.

Methods

I collected a large zooplankton sample from Hamilton Harbour in June 2014 (Figure 1). Hamilton Harbour is adjacent to an international port in Hamilton, Ontario, which receives a large volume of shipping traffic (598 vessels entered in 2013; Hamilton Port Authority 2013) and thus is at a high risk for introduction of new NIS (Grigorovich et al. 2003). Additionally, the harbour is listed as a Canadian port of environmental concern owing to large amounts of metal and organic pollution from both steel refineries and city waste (International Joint Commission United States and Canada 1987). Hamilton Harbour has been very well studied, particularly by Canada's Department of Fisheries and Oceans who has routinely sampled the Harbour in accordance with the Great Lakes Water Quality Agreement (International Joint Commission United States and Canada 1987). A 50-cm mouth, Wisconsin-style plankton net with 100-µm mesh was used for collection. I towed the plankton net vertically from the near-bottom of the harbour to the surface (8 m); depth was determined using a "Hummingbird wide one hundred" fish finder device. The plankton sample was placed in club soda for approximately five minutes to narcotize animals and to reduce bloating before preservation in 95% ethanol (Prepas 1978). This large sample was then randomized and subdivided into five replicate subsamples 'jars' using a 5-ml Hensen-Stempel pipette. A YSI 85 meter was used to collect environmental variables; date and time of collection were also recorded (Table 1). I used the recorded depth to calculate the approximate volume of water sampled (V = 6.29m^3). The equation:

$$V = \pi r^2 h \qquad \text{Equation 1}$$

was used to calculate approximate volume, where V is the volume of the sample, r is the radius of the plankton net used (0.5 m) and h is depth sampled (8 m).

The focal group for this project is Cladocera, also known as water fleas. These freshwater zooplankton are common in the Great Lakes (Johnson and Allen 2005) and virtually all freshwater bodies. This group contains the genera *Daphnia*, *Bosmina*, *Diaphanosoma*, and *Cercopagis*, among many others (Balcer et al. 1984; MacIsaac et al. 1999; Korovchinsky 2002). Cladocerans are an ideal study group because of their importance to aquatic food webs, their high abundance in Hamilton Harbour and, for the purpose of this study, because species within the order exhibit varying distinctiveness. Distinctiveness can be defined by how morphologically different a species is when compared to other taxa in their environment. More distinctive taxa are expected to be more readily recognized by a taxonomist than less distinctive taxa.

Spiked sample experiment

In this blind experiment, four species of non-indigenous cladocerans that have never been reported in Hamilton Harbour were spiked into aforementioned plankton samples. I sought to determine the probability of finding at least one of each species in relation to spiking abundance and counting effort. Spiked cladocerans were selected for their varying degrees of distinctiveness and were not known to the experimenter (myself) until the completion of the experiment. An assistant received monocultures of each spiked species preserved in 95% EtOH

(Appendix 1), and placed a designated number of each species into each sample jar using a dissecting microscope (Table 2).

Prior to addition of the spiked species, I subsampled the five sample jars four times each with a 2 ml Hensen-Stempel pipette. I homogenized the samples before each pipetting to randomize zooplankton. I enumerated cladocerans in each subsample in order to estimate the total number in each sample using the formula:

$$E=\mu\left(\frac{Z}{S}\right) \qquad \text{Equation 2}$$

where E is the total number of cladocerans in the jar, μ is the average number of subsampled individuals (4 trials), Z is the sample volume (60 mL), and S is the subsample volume (2 mL). I performed an analysis of variance (ANOVA) using the program R, and determined that there were no significant differences between the total number of cladocerans in each of the five jars $F(4,15) = 1.77$, $p = 0.188$. In other words, each sample jar contained about the same total number of native cladocerans, and therefore adding the same number of spiked species will result in the same density of each spiked species in each jar.

Before spiking samples, I used a hypergeometric distribution to calculate the expected probability of finding at least one of the spiked individuals for a variety of spiking densities and for designated counting efforts (100, 300, 1000, 5000, and total individuals per sample) in order to choose spiking densities with differing probabilities (Wroughton and Cole 2013). Mean total abundance was ~ 8000 individuals in each of the five samples (Appendix 2 – example problem; Appendix 3 - expected probability tables). The formula:

$$P_a = 1 - P_{nota} \quad \text{Equation 3}$$

$$P_{nota} = \frac{\binom{R}{X}\binom{I}{n-x}}{\binom{R+I}{n}} \quad \text{Equation 4}$$

where

$$\binom{R}{X} = \frac{R!}{X!(R-X)!} \quad \text{Equation 5}$$

$$\binom{I}{n-x} = \frac{I!}{(n-x)!(I-n-x)!} \quad \text{Equation 6}$$

$$\binom{R+I}{n} = \frac{(R+I)!}{n!(R+I-n)} \quad \text{Equation 7}$$

was used to calculate probabilities where P_a is the probability of finding at least one spiked species, and P_{nota} is the probability of finding no spiked species. I is the number of native individuals in the jar, R is the number of spiked individuals added to the jar, n is the total number of individuals counted, and x is the total number of rare individuals found (Wroughton and Cole 2013).

In this blind experiment, each sample jar was spiked with four Cladocera species at up to five different densities (1, 5, 10, 25, and 50). Unfortunately, not enough individuals of each spiked species were procured to test each proposed spiking abundance. No sample contained the abundance 1 *Daphnia lumholtzi*, nor was any sample spiked with 50 *Eubosmina longispina*. 25 and 50 *Evadne nordmanni* were not tested in this experiment. I processed each sample with a microscope at five different efforts (100, 300, 1000, 5000 and, total individuals

[≈8000]) and subsequently processed each entire sample with FlowCAM, both in triplicate, to discover the probability of finding at least one of each spiked species.

Traditional Microscopy

I used both a dissecting and compound microscope, as well as knowledge of the native zooplankton community to identify Cladocera to the lowest possible taxonomic level, which was species level in most cases. Scientific journal articles and plankton keys (Ward et al. 1918; Hebert 1977; Smirnov and Timms 1983; Muzinic 2000; Witty 2004; Johnson and Allen 2005; Haney et al. 2013) were used to identify cladocerans by morphological and anatomical characteristics. Taxonomy of the zooplankton native community (reference samples) was verified by an additional taxonomist for several months until I (the experimenter) became proficient in identification. Individuals that I discerned as potential spiked species were photographed and identified. Only after all samples were completely counted were the names and abundances of each unknown spiked species revealed to me (Table 2).

Distinctiveness

After completion of the microscopic experiment, I hypothesized the distinctiveness of the four spiked species in order to best compare the abilities of both traditional microscopy and FlowCAM to detect rare species of varying distinctiveness. As this was a blind experiment, my assistant initially chose the

four spiked species to have a range of morphological distinctiveness. After I completed the microscopic processing, but before I started analyzing data, my assistant and I proposed ranked distinctiveness of the four spiked species based on how morphologically different we thought they were relative to the native cladoceran community. *Evadne nordmanni* was thought to be the most distinctive of the four spiked species, because this is a marine organism (Smirnov and Timms 1983), and there are no similar species present in Hamilton Harbour (Dermott et al. 2007). *Daphnia longicephala* and *Daphnia lumholtzi* were hypothesized to have medium distinctiveness, because although there are native *Daphnia* species present in Hamilton Harbour (Dermott et al. 2007), the former is very large when compared to other daphnids (Hebert 1977) whereas the latter has distinctive head and tail spines (Haney et al. 2013). Finally, *Eubosmina longispina* was considered the least distinctive of the four spiked species, because it shares many anatomical characteristics and general morphology with native animals in both *Eubosmina* and *Bosmina* genera (Haney et al. 2013).

FlowCAM Analysis

I used automated imaging flow cytometry (FlowCAM) technologies model number VS1 in autoimage mode during this experiment. This machine combines a flow cytometer with a camera and a microscope (Álvarez et al. 2011), and was created for use with phytoplankton (Poulton 2016). In autoimage mode FlowCAM will take a user-defined number of photos each second; this mode is recommended for both high density samples (Fluid Imaging Technologies Inc. 2011) and for preserved samples (Poulton 2016). Each zooplankton sample was

processed in its entirety with FlowCAM, with analyses repeated three times. For each experiment, I used a 2X magnification with the flow cell type FC1000FV (1000 µm depth, 3000µm width). The same tubing size was used in all experiments (inner diameter 0.4cm). I used a 12.5ml pump for all trials. I set each sample to “manual prime with non-sample”, and used 95% EtOH to prime all samples in order to ensure the entire sample was photographed during the experiment.

Before processing, I diluted samples to decrease animal density and added 5% Polyvinylpyrrolidone (PVP) solution to increase viscosity so that large, fast-moving zooplankton could be imaged properly (Table 3). I stirred samples for the duration of the experiment to maintain homogenization.

Throughout this experiment, samples needed to be processed in multiple parts for reasons including computer malfunction and clogging of particles in FlowCAM tubing. Large zooplankton tended to aggregate both on top of the flow cell and at the sphincter that leads to the pump. Often, I was forced to create several run files “subsamples” representing one entire sample in order to compensate for these problems (Table 4).

Each sample was associated with a set of context settings created by FlowCAM. Settings that are relevant to this experiment are listed in Appendix 4. Frames per second (fps) refers to the number of images the camera is set to take each second on autoimage mode. The flow rate (mL/min) represents how quickly the particles are pulled through the flow cell by the pump, and the efficiency (%) represents the amount of fluid that has been photographed divided by the total

amount of fluid that has passed through the system. In these experiments, I increased efficiency past the point recommended by the software, meaning that although all particles were imaged, many of them were photographed multiple times, which is not ideal for quantifying all of the images in the set. However, I used this setting because I was primarily interested in photographing at least one of each spiked species in these samples and the increased efficiency ensures I was photographing the entire sample.

After each run, I manually post-processed the samples using VisualSpreadsheet software version 3.7.5 (Fluid Imaging Technologies Inc. 2011) to sort and eliminate unwanted “noise”, which may include photos of phytoplankton, filaments, and air bubbles. Because FlowCAM samples could not be processed in smaller trials (100, 300, 1000, and 5000 individuals) due to deformation of the zooplankton from multiple runs, I replicated the subsampling process using images from the total sample runs in VisualSpreadsheet. I used a random number generator in Microsoft Excel to select images for each of these computer generated trials which were then analyzed by VisualSpreadsheet as separate entities.

I created image training sets with VisualSpreadsheet by choosing high quality photos representing each spiked individual in varying orientations, which were captured *in situ* throughout the experiment. Example photos of each of the spiked species as were captured with the FlowCAM are shown in appendix 5. Next, I used auto classification, which consisted of the computer comparing image training sets to each sample image, and flagging images that were

statistically similar to each training set (Fluid Imaging Technologies Inc. 2011). Flagged images were expected to portray each spiked species. After the automatic classification was complete, I manually sorted through all sample photos to find images of spiked species in the interest of comparing automatic classification to a manual classification technique.

Statistical Analysis

I analyzed the effect of trial number (triplicates), the total number of individuals counted, the abundance of spiked species, distinctiveness, and identification technique used (microscopy versus manual FlowCAM) on the probability of detecting at least one of each spiked species using backward elimination stepwise multiple logistic regression using the “glm” function in the program R (basic package) (R Development Core Team 2016). At each step, the least significant predictor (highest p value) was removed from the model until all of the remaining variables were significant. I used a generalized linear model (GLM) to predict probabilities for each species and spiking abundance over the full range of counting efforts in order to visualize the results of the stepwise multiple logistic regression analysis. Next, in order to quantify differences in distinctiveness, I used these GLMs to compare the predicted probability of finding at least one of each spiked species at each spiking abundance when 300 individuals were counted for both microscopic and manual FlowCAM analysis. The value 300 was chosen as it is the subsample size commonly used by zooplankton ecologists when counting plankton samples for community analyses

(Zhan et al. 2013). Additionally, I compared the observed and expected probabilities (expected as was calculated from a hypergeometric distribution; Wroughton and Cole 2013) of finding at least one spiked species for both techniques when 1 and 5 total individuals were spiked into the sample (as not all spiked species were represented at 1 individual spiked) and 300 individuals were counted.

Finally, I graphically compared the total number of spiked species that were detected using microscopy to the totals detected when using manual FlowCAM classification analysis using a 1:1 plot. I used a paired t-test to compare the proportion of individuals detected for each spiking abundance when the entire samples were counted.

Results

I observed a positive relationship between the probability of finding at least one of each of the four spiked species and the total number of individuals counted for both microscopic analysis and manual FlowCAM classification analysis (hereafter referred to as FlowCAM analysis) (Table 6; Figures 2, 3). Similarly, I found that the probability of finding at least one individual was significantly affected by the number of individuals spiked into samples (Table 5). I discovered a positive relationship between spiking abundance and probability of finding at least one spiked individual with the microscopic analysis (Figure 2). Generally, I found a positive relationship between spiking abundance and probability of finding at least one of each spiked species with FlowCAM analysis,

however, for all four spiked species I found a higher probability of finding at least one spiked individual when five individuals were spiked into the sample than when 10 were added (Figure 3). FlowCAM performed significantly better than traditional microscopy at finding at least one of each spiked species (Table 5). I did not find any significant effect of trial number (triplicates) for any analysis.

I discovered that species distinctiveness was a significant predictor regarding the probability of finding at least one spiked species (Table 5). *Daphnia longicephala* was the most likely of the four spiked species to be discovered using both microscopy and FlowCAM analysis when 300 individuals were counted, closely followed by *Daphnia lumholtzi* (Figure 4). *Eubosmina longispina* was the least likely of the four species to be found when using microscopy, while *Evadne nordmanni* had the lowest probability of detection when 300 total individuals were enumerated using FlowCAM (Figure 4). I created a probability table showing the values illustrated in Figure 4 (Appendix 6).

When 1 individual was spiked into our sample and 300 total individuals were counted, the average expected probability of finding at least one spiked individual was 3.60% (see Appendix 3 for expected probability tables). When using microscopy, the observed percentage of detecting at least one individual ranged between $4.58x10^{-3}$% and 1.00% whereas with the FlowCAM it ranged between 0.10% and 3.00% (Figure 4; Appendix 6). When 5 individuals were spiked into the sample, and 300 total individuals were counted, the average expected probability of finding at least one spiked individual was 16.2% (see Appendix 3). With microscopy, the empirical probability was always much lower, ranging

between 1.00% and 14.00%, whereas with FlowCAM it ranged between 4.00% and 56.00% (Figure 4; Appendix 6).

When I compared the proportion of total numbers of spiked zooplankton detected with microscopy (mean ± SEM = 0.919 ± 0.079) to the proportion of totals detected with the FlowCAM (mean ± SEM = 0.789 ± 0.117) for all spiking abundances, when the entire sample was counted, no significant difference was found: $t(103) = 0.924$, $p = 0.358$, 95%CL [-0.149, 0.410] (Figure 5).

Automatic FlowCAM classification of the four spiked species resulted in an average accuracy of 33.4%, with a range between 27.1% and 40.7%. Percent accuracy was generated by FlowCAM for each sample, and is a measurement of how statistically similar each flagged particle was compared to the training set that was used to identify it. In other words, percent accuracy is a measurement of how well the training sets were able to find and resolve spiked species in the natural samples.

The identities of spiked species were not known to me until after microscopic processing was completed. Therefore, plankton keys and scientific literature were used to identify the unknowns as I encountered them (and other species) in samples. Although I was able to identify all four spiked species as NIS, I only correctly named one of the four spiked species, *Daphnia lumholtzi*. I selected the correct genus but incorrect species name for both *Daphnia longicephala* and *Evadne nordmanni*. I incorrectly identified *Eubosmina longispina* as the morphologically similar genus *Bosmina* (Table 6).

Discussion

Plankton ecologists have struggled for decades with the problem of identifying the full complement of species present in lakes or marine waters (e.g. Harvey et al. 2009; Gotelli and Colwell 2011). Species present at very low abundance or those with marked spatial or temporal heterogeneity may be exceedingly difficult to find (Delaney and Leung 2010). Furthermore, plankton samples are rarely completely counted, so it is possible to collect a very rare species but not enumerate it owing to small subsample size and count number (see Figure 6). Here I have demonstrated that the ability to find a rare species in the water column is positively related to the total number of individuals counted (e.g. fraction of the total sample examined), the number of individuals spiked into the sample (e.g. density of rare species), and the distinctiveness of the target species.

In these experiments, I found a positive relationship between both the number of individuals counted and spiking abundance on the probability of finding at least one spiked zooplankton. Similarly, Harvey et al. (2009) found that increased sampling effort and increased density of an invasive cladoceran in Lake Ontario resulted in a higher probability of detection. These results are also consistent with the species accumulation theory as described by Hoffman et al. (2011), who predicted that rare species would require a larger effort for detection as they are most likely to be found as the rarefaction curve approaches its asymptote.

The detection likelihood of the spiked species was related to their distinctiveness, however not in the way that I had hypothesized. Both *Daphnia longicephala* and *Daphnia lumholtzi* were predicted to have medium distinctiveness, however these two species were the easiest of the four to find with both techniques. This may have been because although there are many native *Daphnia* species present in Hamilton Harbour, *Daphnia longicephala* is very large in size (up to 5mm; Hebert 1977), which allows for easy identification, while *Daphnia lumholtzi* has a very large head helmet and large tail spine that allow it to be readily distinguished from other species (Haney et al. 2013). *Eubosmina longispina* was thought to be the least distinctive of the four species, and results indicate that *Eubosmina longispina* was the least likely of the four spiked species to be found using classical microscopy. *Eubosmina longispina* is morphologically similar to many native species in Hamilton Harbour, which may have caused it to be misidentified. However, *Evadne nordmanni,* which I hypothesized to have high distinctiveness, was the least likely to be found when FlowCAM was utilized. The low probability of finding at least one *Evadne nordmanni* may stem from its small size and very clear body (Smirnov and Timms 1983), which makes it easy to overlook even though there aren't any morphologically similar species in Hamilton Harbour. It is important to point out that distinctiveness is a measure of how morphologically different the target species is relative to the native species, thus distinctiveness of a NIS will vary depending on the community into which it is introduced (Warwick and Clarke 1995). A recent study detected distinct differences in the 'background'

communities of several high-risk ports throughout Canada, and suggested continued understanding of species present in each port will enhance detection of rare species (Chain et al. 2016).

A recent study rated distinctiveness of invaders based on phylogeny, where more distinctive NIS belong to a genre not already present in the recipient region (Ricciardi and Atkinson 2004). Although this analysis is similar to the one used in this study in that it compares the target species to the recipient community, it brings to light the differences between phylogenetic distinctiveness and detectability. In this study, *Evadne nordmanni* could be considered the most phylogenetically distinct species, however it was found to have low detectability because of its small size and clear body. Ricciardi and Atkinson (2004) proposed that the most phylogenetically distinct species would have a higher impact on the native community, however, this may not correlate with the ease of morphological identification in the recipient region.

I compared the calculated expected probability to the observed range of probabilities of finding at least one spiked species using both techniques when either 1 or 5 individuals were spiked into the sample and 300 total individuals were counted. I found that when using traditional microscopy the range of observed probabilities was lower than the expected probability. For the FlowCAM, the range of observed probabilities was lower than expected when one individual was spiked into the sample, but was very large when 5 individuals were added. These differences may have been because of limitations of both techniques used and because of varying distinctiveness, as our expected

probability calculation did not factor for distinctiveness. Both of our methods exhibit markedly less sensitivity than molecular methods, as Zhan et al. (2013) were able to detect 1 (belonging to a spiked NIS) out of 26639 sequences (0.0037%) when using 454 pyrosequencing.

At the beginning of the double blind microscopic experiment, I did not know the identities of any of the spiked individuals. Reference samples lacking spiked species were counted first, so I was familiar with the taxonomy of the native community. However, as the experiment progressed, and I discovered more of the spiked species, I may have developed a taxonomic 'search image' for species I thought to have been spiked into the samples. In other words, the spiked species were no longer unknowns and I was able to search for their particular morphologies in the samples. Alas, the manner in which samples were systematically processed precludes the possibility of testing this idea. Search image may have increased the probability of finding spiked species in the counting efforts which were completed chronologically later than those which were completed earlier. However, it is possible that taxonomic search image is beneficial for monitoring as it may be more likely for a taxonomist to find a rare species with known morphology in the water column. If this were true, the probability of finding at least one NIS could be increased by incorporating risk analysis into a monitoring program. Knowledge of the morphology of organisms likely to invade may decrease the occurrence of false negatives due to misidentification. Finally, because the FlowCAM experiment was not a blind

experiment, no effect of taxonomic search image could be applied to FlowCAM analysis.

I postulated that FlowCAM would be able to detect distinctive unknown species even at low abundance, but that it would not be able to differentiate between native species and non-distinct spiked species even if the latter were spiked into a sample at high abundance. This hypothesis was supported by the high probability of finding at least one *Daphnia longicephala* and *Daphnia lumholtzi* (high distinctiveness) at low counting efforts regardless of spiking abundance. However, I was unlikely to find at least one *Evadne nordmanni* (least distinctive when using FlowCAM) at any spiking abundance (1, 5, and 10), unless counting effort was very high.

It is interesting to note that when using FlowCAM, the probability of finding at least one of each spiked species was higher when 5 rather than 10 individuals were spiked into the sample. This is probably an effect of a small sample size, where in all five sample jars I identified more photos of the spiked species when 5 individuals were spiked into the sample than when 10 were used. Perhaps if more sample jars were added to this experiment, this effect would not occur and instead I would find more spiked individuals when a higher abundance is spiked into the sample, as was predicted by my initial “expected” probability tables.

The automatic FlowCAM classification, whereby the computer identified images of the spiked species in the natural samples, was considered a failure for this experiment because the average percent accuracy (33.4%), was much lower than ideal operating percent accuracy (80%) (Heather Anne Wright, Fluid

Imaging, pers. comm.). One reason for this could be that the image training sets were not sufficient for this project. I used the highest quality photos of the spiked species in a variety of orientations that were taken during sample processing to create my training sets. However, because the highest spiking abundance was 50, and not all individuals resulted in ideal photos, my image training sets were substantially smaller than reported elsewhere (Zarauz et al. 2009). This *in situ* technique for creating image libraries and training sets was developed and presented for use with phytoplankton (Poulton 2016), and no previous studies have tested this technique on Cladocera. It would be ideal to compare this technique to one that uses monocultures of the target species, which allow creation of more in-depth training sets, as was done by Zarauz et al. (2009). However, it may not be feasible to create monocultures of rare species, particularly if their identities are unknown. Finally, due to constraints of the FlowCAM hardware, it is not currently possible to share image libraries between machines. However, if this obstacle is overcome it would be possible to create a data base of image libraries, which would reduce limitations and drastically improve detection while eliminating the time consuming task of creating a new image library for each study. A second possibility is that the image recognition software does not have the capability to distinguish between Cladocera taxa, as it was created to identify and quantify phytoplankton (Poulton 2016). Indeed, research has found that Automatic FlowCAM classification was ideal for identifying and enumerating phytoplankton from natural samples (Álvarez et al.

2011; 2012; 2014), however no known studies have demonstrated FlowCAM's ability to classify rare Cladocera.

A study of copepods and phytoplankton concluded that FlowCAM had similar abundance counts when compared to traditional microscopy (Ide et al. 2008). Despite the fact that FlowCAM manual classification relies on taxonomic knowledge, it is less time consuming than traditional microscopy (D'anjou et al. 2014), but not as efficient as automatic classification (Ide et al. 2008). Additionally, Le Bourg et al. (2015) compared traditional microscopy to manual FlowCAM classification for a metazooplankton community (including calanoid, *Oithona*, and harpacticoid copepods, nauplii, gelatinous zooplankton, and meroplanton; 80 -1000 µm in size), and concluded that there was no significant difference in abundances between the two techniques. Similar results were found for the total number of individuals detected in this study (Figure 5), although I discovered that the FlowCAM was able to detect at least one of each spiked species at a greater rate than microscopy. It must be noted that in this study I increased the efficiency of the FlowCAM to nearly 100%, which increased the number of double or multiple photos taken of an individual, whereas Le Bourg et al. (2015) ran the machine under normal conditions. However, Le Bourg et al. (2015) processed their entire samples with microscopy, whereas they only processed subsamples with the FlowCAM and this may have biased their detection of rare species.

Although FlowCAM analysis is quicker, I found that it had less resolution than traditional microscopy (Álvarez et al. 2011). For example, one rare species

(not an experimental spiked species), *Moina sp.*, was detected in very low abundances when using microscopy, however it was not detected when using the FlowCAM. However, microscopy, is known to be susceptible to human bias and results can be skewed due to factors including fatigue or inadequate knowledge of taxa. Manual FlowCAM analysis relies on good images that clearly show the identifying characteristics of each species, which was not always possible in the case of blurry photos or photos only containing part of an individual. Unidentifiable images may account for 20-30% of total images when processing phytoplankton (Ide et al. 2008). FlowCAM analysis also relies on taxonomic knowledge to correctly identify the unknown individuals, which makes it susceptible to the same human bias as microscopy. In this experiment, I had taxonomic knowledge of the native species in Hamilton Harbour, but not of the non-indigenous spiked species. Three of the four spiked species were incorrectly identified using traditional microscopy in this study (Table 7). Despite the fact that I was not able to correctly name the spiked species, I was able to classify them as NIS rather than native species. Although it may not be feasible to know the identities of each rare NIS entering a system, a taxonomic knowledge of the background community can enhance identification of an individual as a rare NIS.

Although the FlowCAM analysis was not a blind experiment, I expect that this identification problem would be magnified when analyzing an unknown spiked species with FlowCAM, because of both the high number of unsuitable images and the inability to zoom and orientate target individuals so that all identifying characteristics can be clearly seen. Finally, because my results

showed an increased chance of finding a rare species when more total individuals were examined, I recommend that a combination of approaches be used for monitoring purposes to decrease processing time while maintaining a high probability of finding a rare aquatic species.

Specifically, I recommend that when monitoring for an unknown rare species in the water column, the experimenter should create many small subsamples, and initially use FlowCAM to process these individually. A knowledgeable taxonomist can then look through the resulting photos for each sample, and flag anything that is morphologically unusual. A microscopic analysis will only be performed on the subsamples that were flagged, or that may have a rare species in them. Identification of rare NIS can be increased by coupling this monitoring technique with a risk analysis including examination of both high-risk pathways, recipient ports, and taxa deemed to have a high invasibility (Hulme 2006).

When monitoring for rare species, one of the biggest challenges is the occurrence of false positives and false negatives in data. A false positive (e.g type I error) occurs when a zooplankton species is falsely identified as present in the community, whereas a false negative (e.g. type II error) fails to detect a species that is present. I created a graphical depiction of the false positives and false negatives that may have occurred in this experiment (Figure 6). A false negative may have transpired when sampling in the field due to either spatial or temporal heterogeneity. Spatial heterogeneity can occur when rare species are only located in an unsampled part of the harbour or when a rare species that is

present in the sampling area is not collected in the plankton net. A rare species that is in the sampling area could be missed due to factors including that it is in a position that is either vertically or horizontally outside of the reach of the plankton net, or it could be pushed out of the net due to water pressure when the net is collected. This problem can be minimized by collecting samples across a series of sites. Temporal heterogeneity occurs when the rare species is not present in the water column at the time of sample collection (in this study: June). This problem can be addressed by repeated sampling across seasons. In the laboratory, a false negative can occur when a rare species that was collected in the field is not enumerated due to inappropriate or insufficient subsampling. Both false positives and false negatives can occur when a rare species is misidentified. In this experiment the species *Daphnia longicephala* was misidentified as *Daphnia carinata* which gave both a false negative for *Daphnia longicephala* and a false positive for *Daphnia carinata* (see Table 7). A false positive can be costly since it may set off false alarms and rapid response by managers even though the species isn't present. Whereas, a false negative may allow a newly colonizing NIS time to increase its population size before it is finally detected, allowing it to possibly establish and likely increasing the cost and difficulty of an eradication attempt (Blackburn et al. 2011). Eradication of a NIS is easiest when population numbers are low, and a successful eradication is much less costly than control of a NIS over time (Mehta et al. 2007).

Environmental genomics has been proposed as another technique to monitor for rare aquatic organisms (see Valentini et al. 2009; Jerde et al. 2011;

Bronnenhuber and Wilson 2013; Jerde et al. 2013; Zhan et al. 2013). This technique has been used to monitor the invasion front of both bighead and silver carp in Chicago Area Waterways connecting the Mississippi River to Lake Michigan (Jerde et al. 2011). Evidence of Asian carp DNA in the Great Lakes has been demonstrated using this technique (Jerde et al. 2013). Furthermore, DNA barcoding was estimated to be at least 30% cheaper and four times faster than traditional methods (Briski et al. 2011). Additional research has concluded that a broader approach, metagenomic sequencing (e.g. 454 pyrosequencing), is sensitive for detection of rare zooplankton species in a freshwater environment (Zhan et al. 2013). In spite of the extreme sensitivity of this method, artifacts can be created during processing, creating opportunities for false positives. For example, rare species are often represented by singletons or doubletons (one or two sequence copies, respectively), though these rarest of rare sequence types are usually spurious artefacts (Brown et al. 2015). By coupling this technique with morphological analysis, researchers would have a better idea of whether singletons represent false positives or detection of very rare species.

The purpose of this thesis was to discover the effort required to detect a rare cladoceran and to access the relative merits of classical microscopy versus FlowCAM for detecting rare species in the water column. My results demonstrated that with an increased number of individuals counted, the probability of finding a rare aquatic NIS increased, and that both abundance and distinctiveness can have a positive effect on the likelihood of a species being found. Additionally, I discovered that the range of detection of a spiked species at

very low spiking abundance (1 and 5) and counting effort (300) was determined both by the technology used and by the distinctiveness of the target species. Traditional microscopy is extremely time consuming, and thus expensive, so it is not always possible to have a large enough sample size to detect a rare species. FlowCAM was introduced into this project as an alternative approach to early detection and species identification even though the current generation of equipment is not really designed to handle zooplankton-sized organisms. I observed that automatic FlowCAM classification was ineffective for cladocerans, though with a manual classification technique, the likelihood of finding at least one distinct spiked species was higher than it would be with traditional microscopy. Therefore, I would recommend that natural samples be processed quickly with FlowCAM, following which subsamples with unexpected or unusual images be processed using the traditional approach. I expect that using these techniques in combination when monitoring for rare species will minimize processing time, while maintaining the higher resolution of microscopic analysis. However, this technique is not foolproof, and detection of potential invaders would increase with the addition of risk assessments incorporating knowledge and analyses of high-risk recipient ports as well as known invasive organisms worldwide. Furthermore, zooplankton ecologists need to be cognizant that they may severely underestimate species richness if species are present in low abundance and relatively low sampling effort is used (e.g. subsample counts of <1000 individuals when ~8000 individuals in total are present)(see Figure 4). In conclusion, the use of both FlowCAM and traditional microscopy in combination

will increase the probability of finding and identifying rare NIS in aquatic systems, so that appropriate actions can be taken before invasive establishment occurs.

Table 1: Temperature “temp” (C°), conductivity (µS), salinity (ppt), and dissolved oxygen “Dissolved O_2” (% and mg/L) readings collected just below the surface of the water at the time of sample collection. Date and time of sample collection were recorded as well. The sample was obtained from Hamilton Harbour: 43° 16' 20.3"N, 79° 51' 01.6"W.

Date	Time	Temp (C°)	Conductivity (µS)	Salinity (ppt)	Dissolved O_2 (%)	Dissolved O_2 (mg/L)
19-Jun-14	17:18	20.5	775	0.4	108.4	9.85

Table 2: Names of the four spiked species used in the experiments as well as the abundances spiked into each sample jar.

Spiked species	**Jar 1**	**Jar 2**	**Jar 3**	**Jar 4**	**Jar 5**
Daphnia longicephala	1	10	25	50	5
Daphnia lumholtzi	10	25	50	5	25
Eubosmina longispina	25	5	5	10	1
Evadne nordmanni	10	5	10	1	5

Table 3: Sample volumes used in this experiment as well as dilutions and amount of PVP (5% Polyvinylpyrrolidone solution) added to each FlowCAM sample. FlowCAM dilutions were performed in order to reduce animal density and clogging of the machine, whereas PVP was added to increase sample viscosity in order to slow particles for accurate photographs. Total sample volume includes the addition of the diluted sample volume and the PVP volume, and is the volume that was processed by FlowCAM.

Sample	Original volume (ml)	Diluted volume (ml)	PVP added (ml)	Total volume (ml)
Jar 1 Trial 1	60	300	150	450
Jar1 Trial 2-3	60	350	150	500
Jar 2	60	350	150	500
Jar 3	60	350	150	500
Jar 4	60	350	150	500
Jar 5	60	350	150	500

Table 4: Number of subsamples each sample was split into during FlowCAM processing. Subsample parts were created because of either clogging of FlowCAM tubing, or computer malfunction. Each entire sample jar was processed three times.

Sample Jar	Trial	Number of subsamples
Jar 1	Trial 1	6
Jar 1	Trial 2	5
Jar 1	Trial 3	4
Jar 2	Trial 1	4
Jar 2	Trial 2	5
Jar 2	Trial 3	3
Jar 3	Trial 1	4
Jar 3	Trial 2	5
Jar 3	Trial 3	5
Jar 4	Trial 1	8
Jar 4	Trial 2	6
Jar 4	Trial 3	5
Jar 5	Trial 1	5
Jar 5	Trial 2	4
Jar 5	Trial 3	5

Table 5: Stepwise multiple logistic regression analysis deviance table showing the best fit model for all four spiked species - *Daphnia longicephala, Daphnia lumholtzi, Eubosmina longispina,* and *Evadne nordmanni* – comparing data from both microscopic and manual FlowCAM classification analysis. In this backward elimination analysis, I analyzed the effect of several variables on the probability of finding at least 1 of each spiked species. At each step, the least significant predictor was removed from the model until all remaining independent variables (number counted, distinctiveness, number spiked, and technique) were significant.

Parameter	Degrees of Freedom	Deviance	Residual Degrees of Freedom	Residual Deviance	p-value
# Counted	1	276.77	598	554.98	<0.0005
Distinctiveness	3	95.99	595	458.98	<0.0005
# Spiked	4	92.20	591	366.79	<0.0005
Technique	1	5.60	590	361.19	0.0180

Table 6: Names of the four spiked species used in this experiment along with the name that was identified for each species during taxonomic processing with a microscope.

Spiked species	Taxonomic Identification
Daphnia longicephala	*Daphnia carinata*
Daphnia lumholtzi	*Daphnia lumholtzi*
Eubosmina longispina	*Bosmina liederi*
Evadne nordmanni	*Evadne spinifera*

Figure 1. Map of Hamilton Harbour, Ontario indicating where the sample was collected (White pin) on June 19 2014. Its coordinates are 43° 16' 20.3"N, 79° 51' 01.6"W.

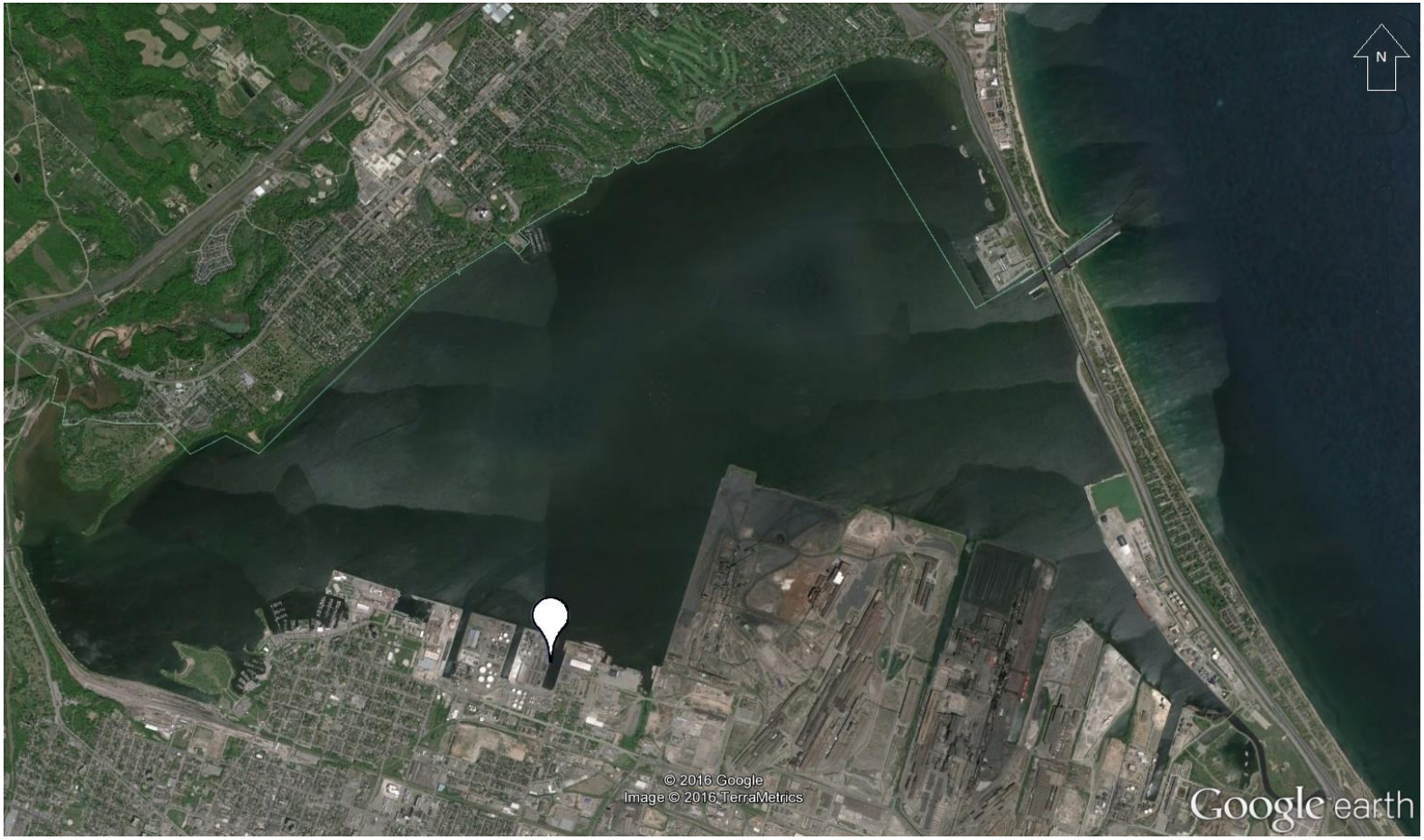


Figure 2: Generalized linear model exploring the effect of the number of zooplankton spiked into each sample and the total number of individuals counted on the probability of finding at least one of each spiked species (1 = blue, 5 = black, 10 = green, 25 = red, 50 = violet). Figures are based on microscopic analysis. It must be noted that not all spiked species were represented at all spiking abundances within the experiment.

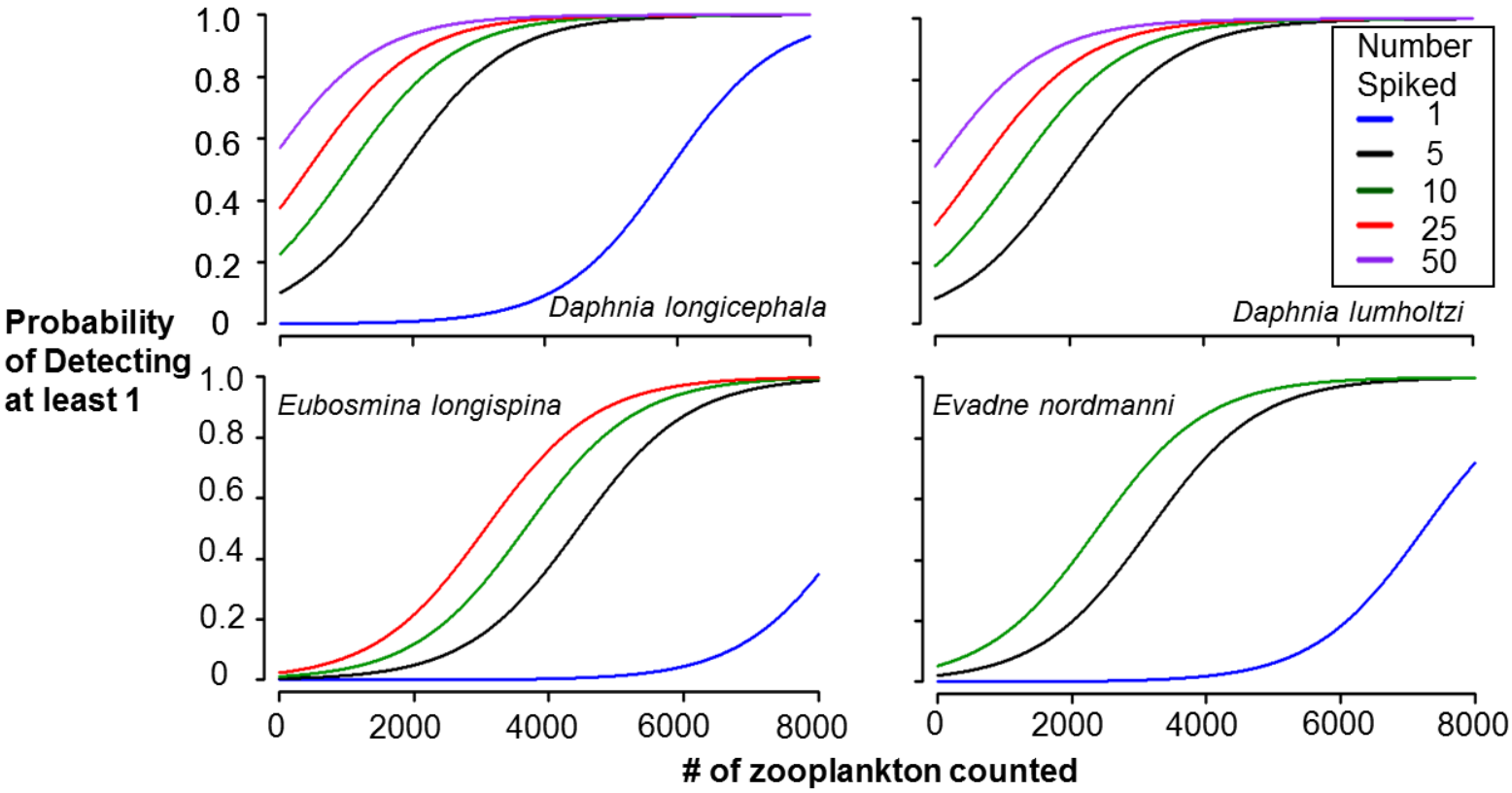


Figure 3: Generalized linear model exploring the effect of the number of zooplankton spiked into each sample and the total number of individuals counted on the probability of finding at least one of each spiked species (1 = blue, 5 = black, 10 = green, 25 = red, 50 = violet). Figures are based on manual FlowCAM classification analysis. It must be noted that not all spiked species were represented at all spiking abundances within the experiment.

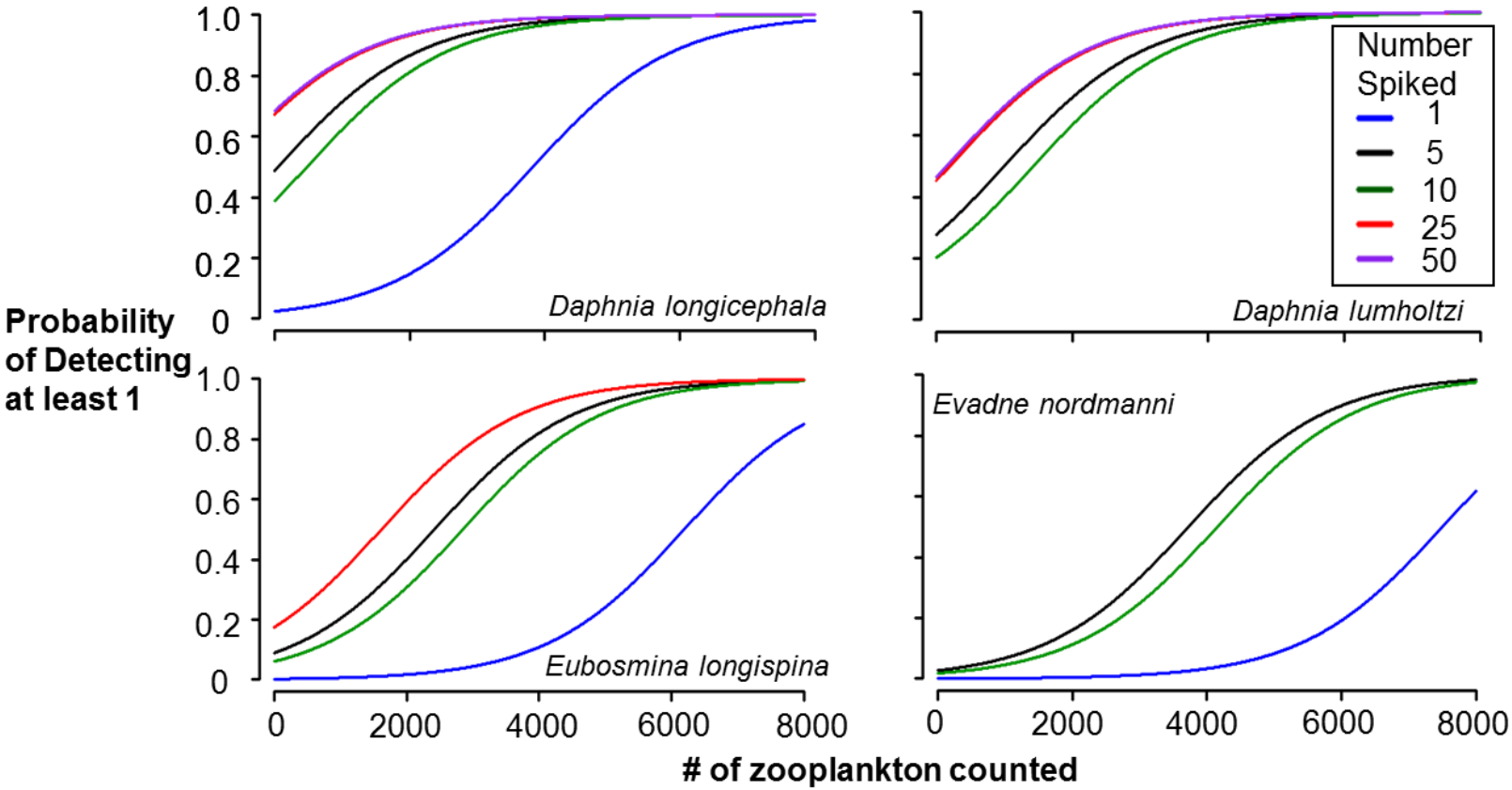


Figure 4: Probability of finding at least of one of each of the spiked species when 300 individuals were counted. This figure encompasses both microscopic analysis (top panel) and manual FlowCAM analysis (bottom panel), as well as all spiking abundances (1 = blue, 5 = black, 10 = green, 25 = red, 50 = violet). It must be noted that for not all spiking abundances were utilized with all species. *Daphnia lumholtzi* did not have a spiking abundance of 1, *Eubosmina longispina* did not have a spiking abundance of 50, and *Evadne nordmanni* did not have a spiking abundance of either 25 or 50 (see Appendix 6 for probability tables).

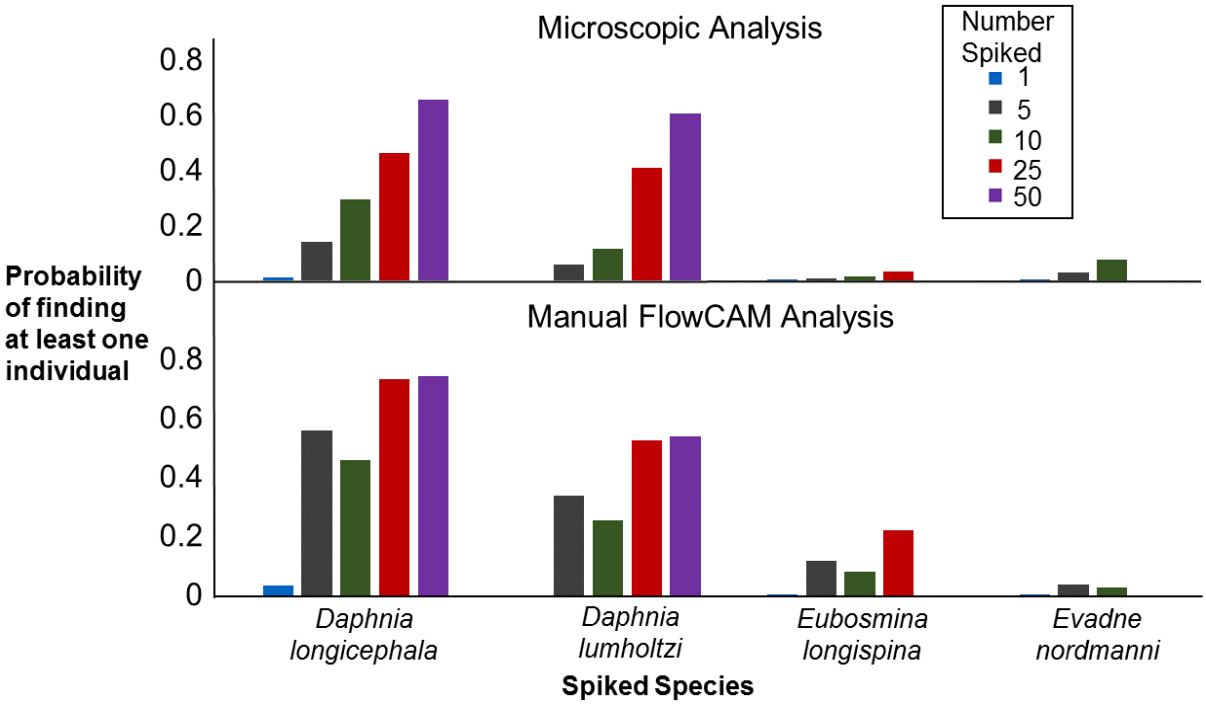


Figure 5: Comparison of the total numbers of spiked zooplankton detected with microscopy (x-axis) and manual FlowCAM classification analysis (y-axis) for all spiking abundances when the entire sample is counted. The solid line shows the 1:1 line.

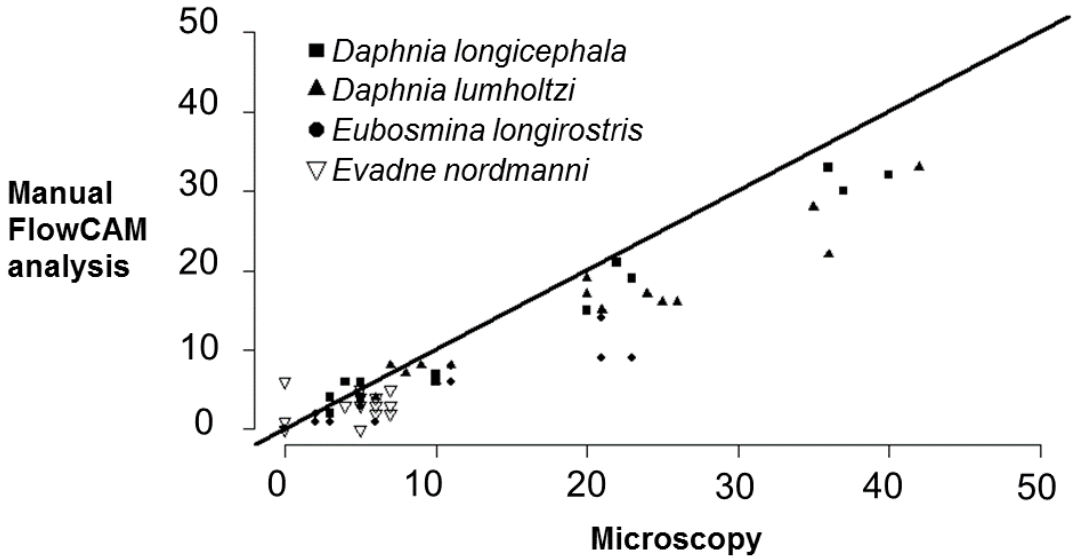
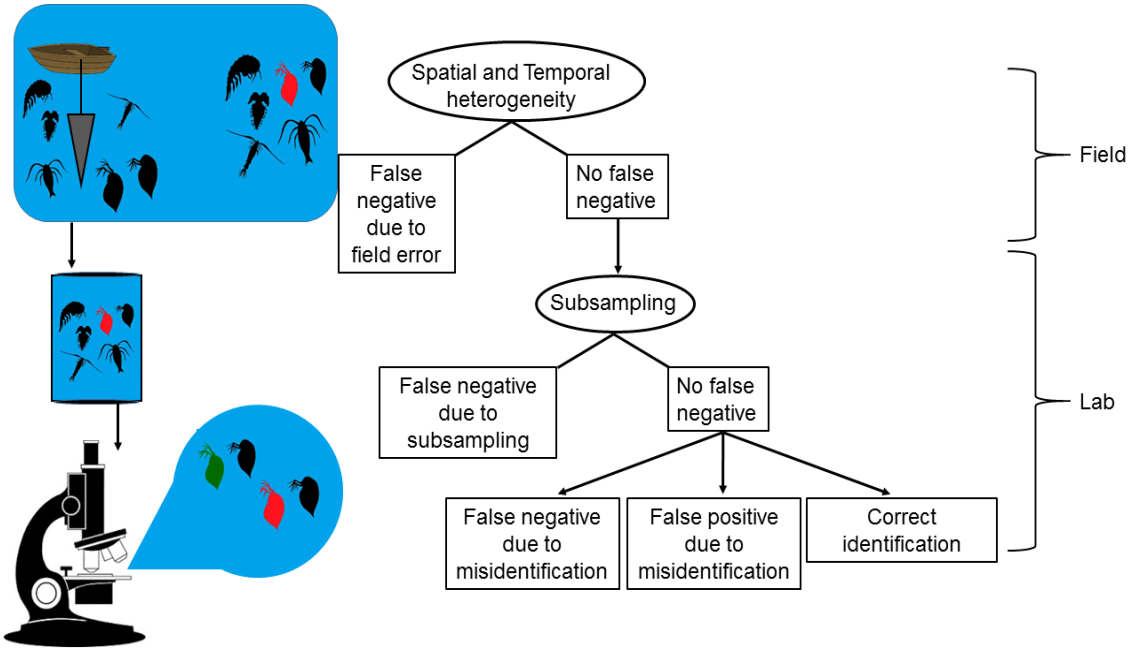


Figure 6: Graphical depiction of false positives and false negatives in zooplankton sampling and identification. A false negative can occur in the field due to spatial and temporal heterogeneity. Spatial heterogeneity can occur when rare species are only present in an unsampled part of the lake or when a rare species that is present in the sampling area is not collected in the plankton net. Temporal heterogeneity occurs when the rare species is not present in the water column at the time of sample collection. In the laboratory, a false negative can occur when a rare plankton that was collected in the field is not enumerated due to subsampling. Both a false positive and a false negative can occur when a rare species is misidentified.



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Appendix 1. Obtained information for spiked species monocultures that were added to each sample jar by an assistant in this blind spiked species experiment. Spiked species were preserved in 95% EtOH. Dr. Linda Weiss is from Ruhr University Bochum, Universitätsstr, Bochum, Germany, whereas Dr. Marina Manca is from "CNR" Institute of Ecosystem Study, Pallanza, Italy. This information was not revealed to the experimenter (myself) until after the completion of the microscopic experiment.

Species	Donor	Source Region	Length of Adult female (mm)	Citation/ Key
Daphnia longicephala	Weiss	Lara Pond, VIC, Australia	up to 5	Hebert 1977
Daphnia lumholtzi	Weiss	Fairfield Reservoir, TX, USA	5.0 to 5.7 (including spines)	Muzinic 2000
Eubosmina longispina	Manca	Lake Maggiore, LOM, Italy	0.55 to 0.84	Witty 2004
Evadne nordmanni	Manca	Lagoon of Venice, VEN, Italy	0.4-0.7	Smirnov & Timms 1983

Appendix 2. Equation and example problem for the expected probability of finding at least one of each spiked species at different abundances and counting treatments (Wroughton and Cole 2013). This formula based on a hypergeometric distribution was used to explore the probability of finding at least one of the spiked individuals for each jar (5) and counting effort (100, 300, 1000, 5000 and, total individuals [≈8000]) for a variety of possible spiking abundances.

The formula:

$$P_a = 1\text{-}P_{nota} \qquad \text{Equation 3}$$

$$P_{nota} = \frac{\binom{R}{X}\binom{I}{n-x}}{\binom{R+I}{n}} \qquad \text{Equation 4}$$

where

$$\binom{R}{X} = \frac{R!}{X!(R-X)!} \qquad \text{Equation 5}$$

$$\binom{I}{n-x} = \frac{I!}{(n-x)!(I-n-x)!} \qquad \text{Equation 6}$$

$$\binom{R+I}{n} = \frac{(R+I)!}{n!(R+I-n)} \qquad \text{Equation 7}$$

was used to calculate probabilities where P_a is the probability of finding at least one spiked species, and P_{nota} is the probability of finding no spiked species, I is the number of native individuals in the jar, R is the number of spiked individuals added to the jar n is the total number of individuals counted, and x is the total number of rare individuals found (Wroughton and Cole 2013).

Example: A sample contains 500 (I) Cladocera. I spike 5 (R) unknown Cladocera individuals into this sample. What is the probability of finding the spiked individual if 100 (n) Cladocera from the sample are counted, where (x) is the number of rare individuals found (x = 0 when looking for P_{nota}).

$$P_a = 1\text{-}P_{nota}$$

$$P_{nota} = \frac{\binom{R}{X}\binom{I}{n-x}}{\binom{R+I}{n}} = \frac{\binom{5}{0}\binom{500}{100}}{\binom{505}{100}}$$

$$P_{nota} = \frac{\binom{R}{X}\binom{I}{n-x}}{\binom{R+I}{n}} = \frac{\left(\frac{R!}{x!(R-x)!}\right)\left(\frac{I!}{(n-x)!(I-n-x)!}\right)}{\left(\frac{(R+I)!}{n!(R+I-n)}\right)}$$

$$P_{nota} = \frac{\binom{5}{0}\left(\frac{500!}{(100-0)!(500-100-0)!}\right)}{\left(\frac{(5+500)!}{100!(5+500-100)!}\right)}$$

$$P_{nota} = \frac{(1)\left(\frac{500!}{(100)!(400)!}\right)}{\left(\frac{(505)!}{100!(405)!}\right)} = 0.33$$

$$P_a = 1\text{-}P_{nota}$$

$$P_a = 0.67$$

Appendix 3: Expected probability tables for the five sample jars in the spiked sample experiment which were used in the exploration of spiking abundances. The total number of individuals for each jar was estimated using equation 2. These probability tables were created using equations 3-7 in the software R (R Development Core Team 2016). The values 1, 5, 10, 25, and 50 were chosen as the final spiking values.

Jar 1 - Expected probabilities of finding at least one spiked species

# Spiked	100 counted	300 counted	1000 counted	5000 counted	Estimated total (7434)
1	0.01	0.04	0.13	0.67	1.00
5	0.07	0.19	0.51	1.00	1.00
10	0.13	0.34	0.76	1.00	1.00
25	0.29	0.64	0.97	1.00	1.00
30	0.33	0.71	0.99	1.00	1.00
50	0.49	0.87	1.00	1.00	1.00
75	0.64	0.96	1.00	1.00	1.00
100	0.74	0.98	1.00	1.00	1.00

Jar 2 - Expected probabilities of finding at least one spiked species

# Spiked	100 counted	300 counted	1000 counted	5000 counted	Estimated total (9270)
1	0.01	0.03	0.11	0.54	1.00
5	0.05	0.15	0.43	0.98	1.00
10	0.10	0.28	0.68	1.00	1.00
25	0.24	0.56	0.94	1.00	1.00
30	0.28	0.63	0.97	1.00	1.00
50	0.42	0.81	1.00	1.00	1.00
75	0.56	0.92	1.00	1.00	1.00
100	0.66	0.96	1.00	1.00	1.00

Jar 3 - Expected probabilities of finding at least one spiked species

# Spiked	100 counted	300 counted	1000 counted	5000 counted	Estimated total (8213)
1	0.01	0.04	0.12	0.61	1.00
5	0.06	0.17	0.48	0.99	1.00
10	0.12	0.31	0.73	1.00	1.00
25	0.26	0.61	0.96	1.00	1.00
30	0.31	0.67	0.98	1.00	1.00
50	0.46	0.85	1.00	1.00	1.00
75	0.60	0.94	1.00	1.00	1.00
100	0.71	0.98	1.00	1.00	1.00

Jar 4 - Expected probabilities of finding at least one spiked species

# Spiked	100 counted	300 counted	1000 counted	5000 counted	Estimated total (8550)
1	0.01	0.04	0.12	0.58	1.00
5	0.06	0.16	0.46	0.99	1.00
10	0.11	0.30	0.71	1.00	1.00
25	0.26	0.59	0.96	1.00	1.00
30	0.30	0.66	0.98	1.00	1.00
50	0.45	0.83	1.00	1.00	1.00
75	0.59	0.93	1.00	1.00	1.00
100	0.69	0.97	1.00	1.00	1.00

Jar 5 - Expected probabilities of finding at least one spiked species

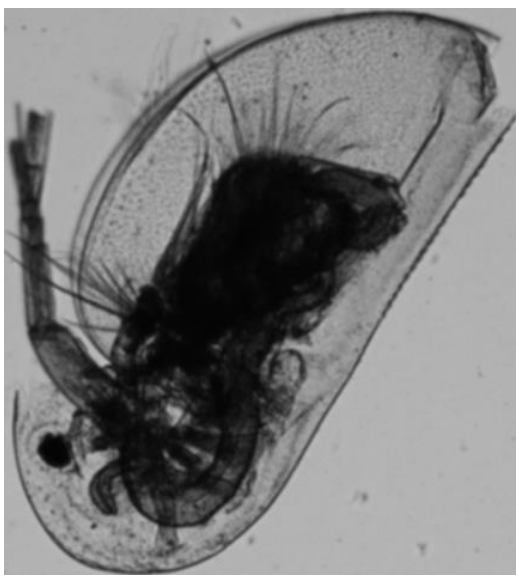
# Spiked	100 counted	300 counted	1000 counted	5000 counted	Estimated total (9818)
1	0.01	0.03	0.10	0.51	1.00
5	0.05	0.14	0.42	0.97	1.00
10	0.10	0.27	0.66	1.00	1.00
25	0.23	0.54	0.93	1.00	1.00
30	0.26	0.61	0.96	1.00	1.00
50	0.40	0.79	1.00	1.00	1.00
75	0.54	0.90	1.00	1.00	1.00
100	0.64	0.96	1.00	1.00	1.00

Appendix 4: FlowCAM context settings used for the spiked (jar) samples. These samples were all collected from Hamilton Harbour on June 19, 2014. Frames per second refers to the number of images the camera takes each second on Autoimage mode and is user-defined. The flow rate represents how quickly the particles are pulled through the flow cell by the pump, and the efficiency represents the amount of fluid that has been photographed divided by the total amount of fluid that has passed through the system.

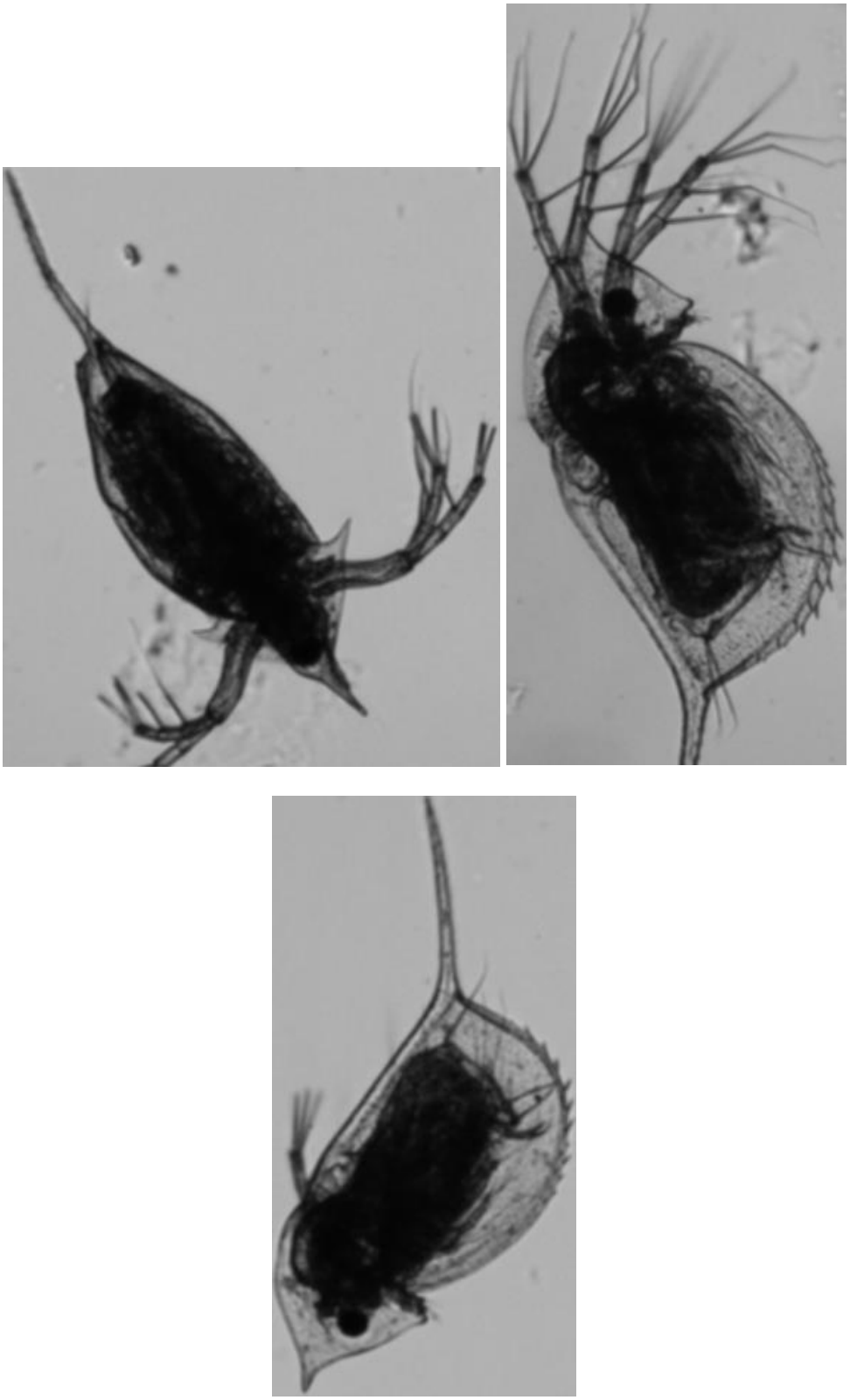
Sample	Date processed	Frames per Second (fps)	Flow rate (mL/min)	Efficiency (%)
Jar 1 Trial 1	19-Oct-15	20	7	100.2
Jar 1 Trial 2	19-Oct-15	20	7	100.1
Jar 1 Trial 3	19-Oct-15	20	7	100.1
Jar 2 Trial 1	20-Oct-15	20	7	99.9
Jar 2 Trial 2	20-Oct-15	20	7	99.9
Jar 2 Trial 3	20-Oct-15	20	7	100.1
Jar 3 Trial 1	26-Oct-15	20	7	100.1
Jar 3 Trial 2	26-Oct-15	20	7	100.0
Jar 3 Trial 3	26-Oct-15	20	7	100.0
Jar 4 Trial 1	28-Oct-15	20	7	100.1
Jar 4 Trial 2	28-Oct-15	20	7	100.1
Jar 4 Trial 3	28-Oct-15	20	7	99.9
Jar 5 Trial 1	29-Oct-15	20	7	100.2
Jar 5 Trial 2	29-Oct-15	20	7	100.1
Jar 5 Trial 3	29-Oct-15	20	7	100.1

Appendix 5: Example photos of each of the four spiked species - *Daphnia longicephala, Daphnia lumholtzi, Eubosmina longispina, and Evadne nordmanni* - that were captured with the FlowCAM.

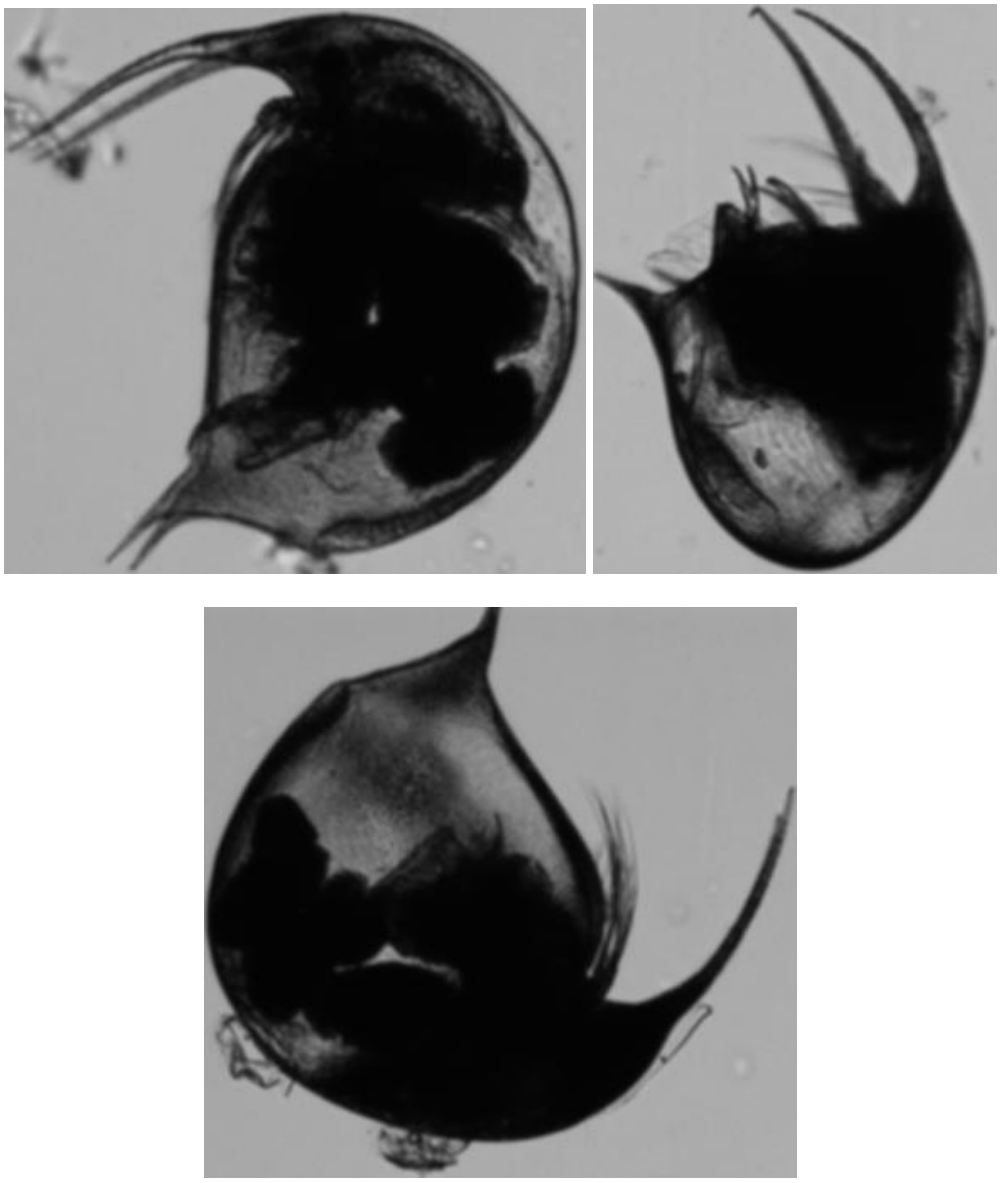
Daphnia longicephala



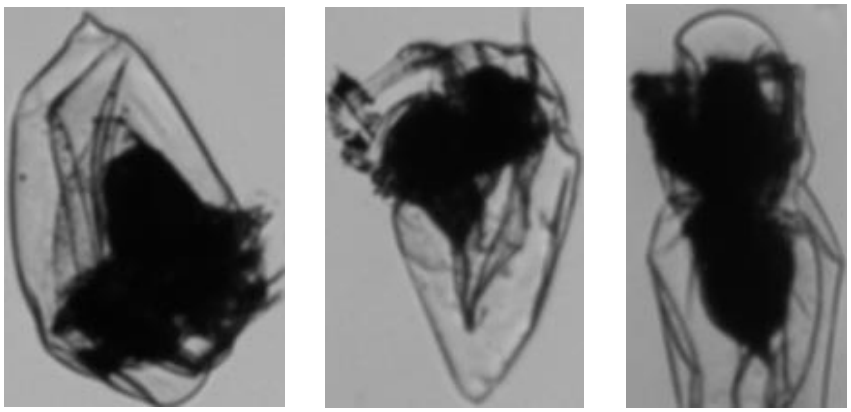
Daphnia lumholtzi



Eubosmina longispina



Evadne nordmanni



Appendix 6: Probability of finding at least of one of each of the spiked species when 300 individuals were counted for both microscopic (top panel) and manual FlowCAM (bottom panel) analysis. It must be noted that for not all spiking abundances were utilized with all species, and that "N/A" is used in the absence of a spiking abundance. See Figure 4 for a graphic representation of this data.

Microscopy

Number spiked	*Daphnia longicephala*	*Daphnia lumholtzi*	*Eubosmina longispina*	*Evadne nordmanni*
1	0.01	N/A	$4.58x10^{-05}$	$2.18x10^{-04}$
5	0.14	0.06	0.01	0.03
10	0.30	0.12	0.02	0.07
25	0.46	0.41	0.03	N/A
50	0.66	0.61	N/A	N/A

Manual FlowCAM

Number spiked	*Daphnia longicephala*	*Daphnia lumholtzi*	*Eubosmina longispina*	*Evadne nordmanni*
1	0.03	N/A	$3.48x10^{-03}$	$9.99x10^{-04}$
5	0.56	0.34	0.12	0.04
10	0.46	0.25	0.08	0.02
25	0.73	0.52	0.22	N/A
50	0.74	0.54	N/A	N/A

Vita Auctoris

Name:	Keara Michelle Stanislawczyk
Place of Birth:	Cleveland, Ohio
Year of Birth:	1991
Education:	Strongsville High School, Strongsville, Ohio 2005-2009, diploma with honors
	Miami University, Oxford, Ohio 2009-2013, BSc, Zoology
	Great Lakes Institute for Environmental Research University of Windsor, Windsor, Ontario 2014-2016, MSc, Environmental Science