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Microscopy versus automated imaging flow cytometry for detecting and identifying rare zooplankton

Keara Stanislawczyk · Mattias L. Johansson · Hugh J. MacIsaac

Abstract Many zooplankton surveys underestimate species richness owing to difficulties in detecting rare species. This problem is particularly acute for studies designed to detect non-indigenous species (NIS) when their abundance is low. Our goal was to test the difference in detection efficiency between traditional microscopy and image analysis (i.e., FlowCAM). We hypothesized that detection of rare species should become easier as they become abundant in a sample, if they are morphologically distinct, or if counting effort increased. We spiked different densities of Cladocera into zooplankton samples from Lake Ontario to simulate rarity, and assessed detection rate. Our results indicated that there was a positive relationship between the probability of finding at least one spiked NIS and its abundance, distinctiveness, and counting effort employed. FlowCAM processed more subsamples, though morphologically similar taxa were distinguished more readily with microscopy. The expected probability for detecting one individual spiked into a sample with $\sim$ 8000 individuals (300 counted) was 3.60%, though observed values were considerably lower using both classical microscopy (4.58×10^{-3} to 1.00%) and FlowCAM (0.10 to 3.00%). Our experiments highlight that many plankton ecologists use subsample counts too low to detect rare native species and NIS, resulting in low species richness estimates and false negatives.

Keywords Invasive species · Early detection · Risk assessment · Taxonomy · FlowCAM · Great Lakes · Hamilton Harbour

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Introduction

Detecting the full complement of species in an aquatic environment can be daunting. It is particularly difficult to detect the rarest of species, including endangered species (Thomsen et al., 2012) and newly colonized non-indigenous species (NIS; Campbell et al., 2007; Jerde et al., 2011). Missing rare species in biodiversity assessments can have profound implications. For example, Cao et al. (1998) determined that species richness estimates could be severely distorted, and differences between impacted and non-impacted sites artificially minimized if rare species were excluded from analyses.

False negatives—which we define as the failure to detect organisms that are present—may occur when assessing community composition of an aquatic

habitat, particularly for rare species. Hoffman et al. (2011) estimated that detection of 95% of all zooplankton species present in Duluth-Superior harbour, Lake Superior, would require enormous sampling effort (i.e., 776 samples and examination of ∼ 500,000 individuals). This pattern is consistent with Harvey et al.'s (2009) finding that even with intensive ($n = 100$) field sampling, zooplankton species present at very low abundance are exceptionally difficult to detect. Arnott et al. (1998) suggested that zooplankton "patchiness" in the water column can contribute to false negatives, particularly with respect to rare species. The authors suggested that biodiversity is better accessed by collecting multiple samples over time to overcome patchiness of zooplankton in the water column (Arnott et al., 1998). In the lab, zooplankton samples have been traditionally processed using fixed counts, often of relatively low numbers of individuals (see Fig. 1). In some cases, fixed subsample counts are combined with whole sample analysis of large or otherwise conspicuous rare species. However, this approach may be insufficient for small or inconspicuous rare species, resulting in false negatives.

Automated imaging flow cytometry (FlowCAM) is an emerging technology that combines a flow cytometer with a camera and a microscope (Álvarez et al., 2011), and generates phytoplankton counts comparable to traditional microscopy (e.g., Camoying & Yñiguez, 2016). This machine was created for use with phytoplankton (Poulton, 2016). The user is able to quickly classify particles as the FlowCAM records 22 properties including area, circle fit, and transparency (Fluid Imaging Technologies Inc., 2011). FlowCAM accurately identifies phytoplankton and is effective at measuring size classes in natural samples; however, it may not be comparable to traditional counts for preserved samples due to the deformation of preserved particles (Álvarez et al., 2011). FlowCAM's published use with zooplankton is limited. It was able to distinguish between copepods and phytoplankton in a mixed sample (Ide et al., 2008), and recent studies have successfully used FlowCAM to count zebra mussel veligers (Frischer et al., 2012). However, no published reports exist with respect to FlowCAM's capability to distinguish between Cladocera, nor has it been applied to early detection of NIS.

In this study, we spiked very 'rare' Cladoceran zooplankton species into lake samples to determine detection thresholds using both traditional microscopy and FlowCAM image analysis to test the hypotheses that the likelihood of finding spiked species will

Fig. 1 Histogram of minimum zooplankton counts from research publications in journals Freshwater Biology, Hydrobiologia, Journal of Plankton Research, and Limnology and Oceanography
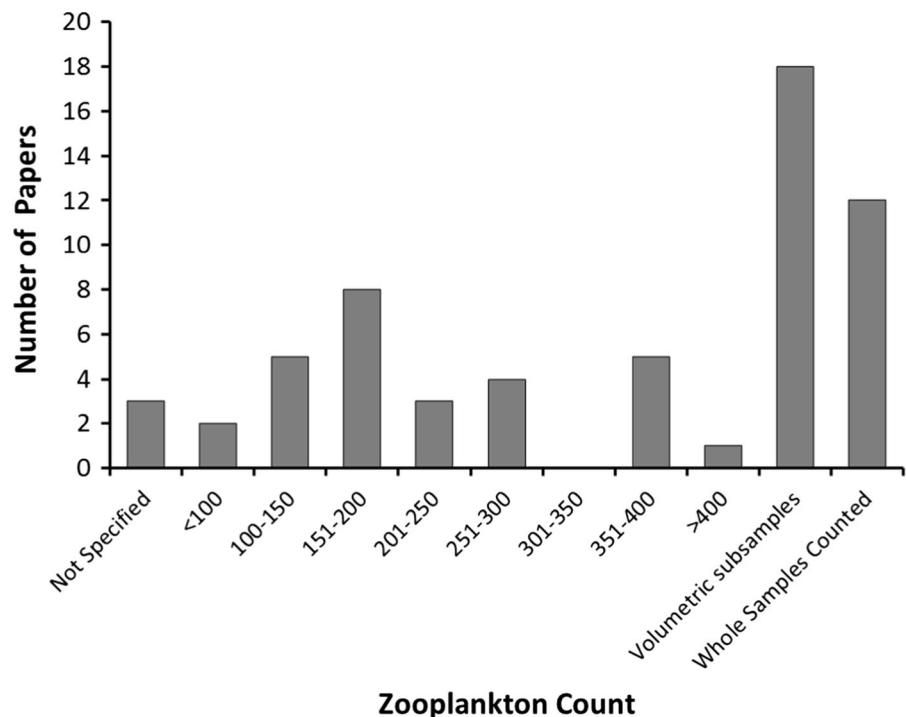
increase (1) as spiking abundance increases; (2) in direct relation to the distinctiveness of the spiked species; and (3) as total individuals counted increases.

Methods

Literature survey

To identify typical sampling efforts for zooplankton studies, we performed a Web of Science search for 'zooplankton sampling' for 2012–2016 focused on the journals Freshwater Biology, Hydrobiologia, Journal of Plankton Research, and Limnology and Oceanography. Of the 61 papers identified, we then examined laboratory counting effort for papers that counted and identified samples using microscopy, plotting the number of papers that processed whole samples, subsamples without specifying a minimum number of individuals identified, and those that specified a minimum number of individuals identified (Fig. 1).

Blind spiking experiment

We collected a large zooplankton sample from Hamilton Harbour, Lake Ontario in June 2014. Hamilton Harbour contains an international port, which receives a large volume of commercial shipping traffic and thus is at risk for introduction of new NIS. We used a 50-cm mouth, Wisconsin-style plankton net with 100-µm mesh for collection. We initially placed live plankton samples in club soda (to reduce EtOH bloat), and subsequently preserved the large sample in 95% EtOH. The sample was filtered through a 250-nm sieve to remove small immature stages of Cladoceran zooplankton. This sample was then randomized and subdivided into five replicate jars.

We used Cladocerans as the focal group because of their importance to aquatic food webs, well-characterized taxonomy, and because species within the order exhibit substantial morphological distinctiveness. We define distinctiveness as a relative measurement; that is, how different a species appears when compared to other taxa in the same sample. More distinctive taxa are expected to be more readily recognized by a taxonomist than less distinctive ones. We identified all Cladoceran species in our sample jars in order to determine Cladocera richness in Hamilton Harbour. Balcer et al. (1984) reported that the Cladoceran community in Hamilton Harbour was dominated by *Daphnia mendotae*, *Daphnia retrocurva*, *Bosmina longirostris*, *Eubosmina coregoni*, and *Cercopagis pengoi*, a finding similar to our own (with the addition of the invader *Cercopagis pengoi*). We subsampled each of the five sample jars four times using a 2-ml Hensen-Stempel pipette ($\sim$ 400 individuals per subsample), and enumerated the Cladocera in order to estimate the mean total number of individuals in each sample. We performed an analysis of variance (ANOVA) using R (R Development Core Team, 2016), and determined that there were no significant differences between the total number of Cladocerans in each of the five jars ($F_{4,15} = 1.77$, $P = 0.188$) before spiking new Cladocerans into each sample.

We used a hypergeometric distribution to calculate the expected probability of finding at least one of the spiked individuals for a variety of spiking densities (1, 5, 10, 25, 30, 50, 75, and 100) and for designated counting efforts (100, 300, 1000, 5000, and complete sample counted), in order to choose spiking densities with differing probabilities of encountering spiked species (Wroughton & Cole, 2013).

We utilized a blind spiking experiment in which four different non-indigenous Cladoceran species— *Daphnia lumholtzi*, *Daphnia longicephla, Eubosmina longispina*, and *Evadne nordmanni,* never before reported in Hamilton Harbour—were spiked into the aforementioned plankton samples at different abundances. The principal taxonomist, who was familiar with the native community, knew neither species identity nor spiking abundance. An assistant received preserved monocultures of each spiked species from taxonomic experts, and introduced a randomly designated number of each species into each sample jar. We then sought to experimentally determine the probability of finding at least one of each of these species in relation to spiking abundance and counting effort. Mean total abundance was $\sim$ 8000 individuals in each of the five samples.

Spiked Cladocerans exhibited varying degrees of morphological distinctiveness when compared to the native community in Hamilton Harbour—*Evadne nordmanni* was thought to be the most distinct, the two daphnids were thought to have medium distinctiveness, and *Eubosmina longispina* was thought to be the least distinctive of the spiked species.

Each sample jar was spiked with the four Cladocera species at one of five abundances (1, 5, 10, 25, and 50 individuals). The total number of each individual spiked into each sample jar can be found in Table 1. Too few individuals were procured to test all spiking abundances for all species. Therefore, we were unable to run trials with 1 *Daphnia lumholtzi*, 50 *Eubosmina longispina*, nor 25 or 50 *Evadne nordmanni*.

Microscopy

We processed each sample with a microscope for all subsample and counting efforts. Each of the five sample jars was counted in triplicate for each counting effort. We used both dissecting microscopy and higher-magnification, compound microscopy using bright-field. We used journal reports and plankton keys (Ward et al., 1918; Hebert, 1977; Smirnov & Timms, 1983; Muzinic, 2000; Witty, 2004; Johnson & Allen, 2005; Haney et al., 2013) to identify Cladocerans to the lowest possible taxonomic level, which was species level in most cases. Only after all samples were completely counted, were the names and abundances of each unknown spiked species revealed. Later, we processed the same samples with automated imaging flow cytometry (FlowCAM) to discover the probability of finding at least one of each spiked species using this instrument.

FlowCAM

FlowCAM technologies model number VS1 was used in autoimage mode. This instrument combines a flow cytometer with a camera and 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. We used a $2\times$ magnification and the flow cell type FC1000FV (1000 μm depth, 3000 μm width) which allows for the study of particles up to 2.5 mm in size (Harry Nelson, Fluid Imaging, Pers. Comm.). The same tubing (inner diameter 0.4 cm) was used in all experiments. We used a 12.5-ml pump for all trials. Before processing, we diluted samples to decrease animal density and reduce clogging of the instrument, and added 5% Polyvinylpyrrolidone (PVP) solution to increase viscosity so that large, fast-moving zooplankton could be imaged properly. We increased FlowCAM's efficiency to 100%, even though it was not recommended by the manufacturer, owing to particles being photographed multiple times. We used this setting because we were primarily interested in photographing at least one of each spiked species in these samples, and increased efficiency ensured that we were capturing the entire sample. Sample images were manually post-processed by a knowledgeable taxonomist before analysis. This included the removal of multiple images of the same individual (which are known by having the same X value and changing Y values as the particle passes through the flow cell) as well as images containing only debris and phytoplankton. Post-processing is an essential part of FlowCAM operation as the instrument does not automatically eliminate images of groups that are not being studied (Fluid Imaging Technologies Inc. 2011). Post-processing was done manually by a knowledgeable taxonomist so that experimental images were not accidentally removed.

FlowCAM samples could not be processed in smaller sampling efforts (100, 300, 1000, and 5000 individuals) due to the deformation of zooplankton from multiple runs, thus we simulated the subsampling process using images from the total sample runs in VisualSpreadsheet (Fluid Imaging Technologies Inc., 2011). We 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.

Table 1 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

Each sample jar was spiked with the four Cladocera species at one of five abundances (1, 5, 10, 25, and 50 individuals). Too few individuals were procured to test all spiking abundances for all species. We were unable to run trials with 1 *Daphnia lumholtzi*, 50 *Eubosmina longispina*, nor 25 or 50 *Evadne nordmanni*

We created FlowCAM 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. Next, we 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, we manually sorted through all sample photos to find images of the spiked species to compare automatic and manual classification.

Statistical analysis

We analyzed the effect of replicates, abundance of spiked species, distinctiveness, total number of individuals counted, and identification technique used (microscopy versus manual FlowCAM) on the probability of detecting at least one of each spiked species using multiple logistic regression in R (R Development Core Team, 2016). Because there was no significant effect of replicate, we eliminated it from the model. We then tested the effect of predictor order on statistical results by reordering predictors in the input model. Three of the predictors [(i.e., abundance of spiked species, distinctiveness, and individuals counted] remained highly significant ($p < 0.001$) irrespective of model order. However, identification technique varied from significant ($p = 0.018$) to non-significant ($p = 0.142$) depending on where it appeared in the model. Therefore, we constructed separate GLMs for each identification technique using the remaining significant predictors separately to predict probabilities for each species and spiking abundance over the full range of counting efforts and visualize the results of the multiple logistic regression analysis. In order to quantify differences in distinctiveness, we 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 taxonomic and manual FlowCAM analyses. The value 300 was chosen as it is a subsample size commonly used by zooplankton ecologists when counting plankton samples for community analyses (Zhan et al., 2013; see Fig. 1). Additionally, we compared the observed and expected probabilities of finding at least one spiked species for both techniques when one individual was spiked into the sample and 300 individuals were counted (Table 2). We created contingency tables to visualize where false positives and false negatives may have occurred in our dataset (Table 3). We graphically compared the total number of spiked individuals that were detected for each spiking intensity and each species, and also compared the total detected using microscopy versus manual FlowCAM classification. We used a linear regression ANOVA to compare the proportion of individuals detected for each spiking abundance when the entire samples were counted.

Results

Literature survey

In our search of recently published papers, we identified a total of 61 papers that utilized microscope identification to characterize the zooplankton community or its components. Of these, the largest fraction (28 papers) specified some minimum number of zooplankton counted. An additional 18 papers were based on processing volumetric subsamples without a target minimum count, 12 counted all the individuals in each sample, and three did not specify what fraction of zooplankton were counted (Fig. 1). Within the 28 papers that specified a minimum number counted, 22 (79%) specified 300 individuals or less.

Hypothesis 1: spiking abundance

The probability of finding at least one individual was positively related to the number of individuals spiked into samples (Table 2). The probability of finding at least one spiked individual using microscopy was positively related to spiking abundance (Fig. 2). We also found a positive relationship between spiking abundance and probability of finding at least one of each spiked species with the FlowCAM analysis, except when comparing five and ten individuals, which was likely due to a higher number of false positives in the former (Fig. 2). FlowCAM performed significantly better than traditional microscopy at finding at least one of each spiked species except for *Evadne nordmanni* (Table 2).

Table 2 Expected percent probabilities of finding at least one of each spiked species when 1 individual is added to the sample, and when 300 total individuals are counted

Sample	Total n	Number spiked	Expected probability (%)	Microscopy					FlowCAM				
				Daphnia longicephala	Daphnia lumholtzi	Eubosmina longispina	Evadne nordmanni		Daphnia longicephala	Daphnia lumholtzi	Eubosmina longispina	Evadne nordmanni
1	7434	1	4.00	1.09	N/A	<0.01	0.02		3.29	N/A	0.35	0.10
2	9270	1	3.00	13.99	5.82	0.65	3.02		55.94	33.77	11.55	3.60
3	8213	1	4.00	29.61	11.59	1.67	7.46		45.72	25.28	7.97	2.42
4	8550	1	4.00	46.32	41.01	3.36	N/A		73.26	52.39	21.99	N/A
5	9818	1	3.00	65.55	60.51	N/A	N/A		74.23	53.64	N/A	N/A

The expected probabilities were calculated with the hypergeometric equation as described in (Wroughton & Cole. 2013), based on the estimated total number of individuals in each sample jar. They do not consider either the varying distinctiveness of spiked species or the effect of taxonomic search image. Experimental percent probabilities of finding at least one of each of the spiked species when 300 individuals were counted for both taxonomic (top panel) and manual FlowCAM (bottom panel) analysis. Not all spiking abundances were utilized with all species; "N/A" reflects an absence of a spiking abundance. See Fig. 3 for a graphic representation of these data

False positives occurred only at the lowest spiking intensities (i.e., 1 and 5 individuals added), whereas false negatives occurred in virtually all samples at higher spiking intensities (Fig. 4). For each data point, the absolute difference between the point and the 1:1 line represents the minimum error.

Hypothesis 2: species distinctiveness

Species distinctiveness was a significant predictor of the probability of finding at least one spiked species (Table 3). *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, followed closely by *Daphnia lumholtzi* (Fig. 3). *Eubosmina longispina* was the least likely to be found when using microscopy, while *Evadne nordmanni* had the lowest probability of detection when 300 total individuals were enumerated using FlowCAM (Fig. 3).

Hypothesis 3: total number counted

We 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 taxonomic analysis and manual FlowCAM classification analysis (hereafter referred to as FlowCAM analysis) (Table 2; Fig. 2) (See Appendix 1 for raw counts).

Differences between microscopy and FlowCAM

When one individual was spiked into our sample and 300 total individuals counted, the average expected probability of finding at least one spiked individual was 3.60%, while the observed frequency ranged between 4.58×10^{-3} and 1.00% for microscopy and between 0.10 and 3.00% with FlowCAM (Fig. 3). Although both techniques performed worse than expected, FlowCAM seemed to perform better than microscopy at detecting species spiked at very low abundance. Visual comparison of the number of individuals detected with each of the two methods (Fig. 5) suggested that FlowCAM underperformed microscopy at detecting all spiked individuals. However, when we assessed the effects of species, counting method, and spiking abundance on the proportion of the total number of spiked zooplankton detected when

Table 3 Mean ± SD number of individuals detected for each species at each spiking intensity

		Daphnia longicephala	Daphnia lumholtzi	Eubosmina longispina	Evadne nordmanni
Taxonomy					
	1	3.0 ± 0.0	NA	0.0 ± 0.0	0.0 ± 0.0
	5	4.67 ± 0.6	7.0 ± 1.0	3.3 ± 1.8	6.2 ± 1.2
	10	10.0 ± 0.0	10.0 ± 1.0	11.0 ± 0.0	5.5 ± 0.8
	25	21.7 ± 1.5	22.7 ± 2.7	21.7 ± 1.2	NA
	50	37.7 ± 2.1	37.7 ± 3.8	NA	NA
FlowCAM					
	1	2.7 ± 1.2	NA	0.0 ± 0.0	2.3 ± 3.2
	5	5.3 ± 1.2	6.3 ± 2.1	1.7 ± 0.8	3.3 ± 3.2
	10	6.7 ± 0.6	7.3 ± 1.2	7.3 ± 1.2	3.2 ± 1.9
	25	18.3 ± 3.1	16.7 ± 1.4	10.7 ± 2.9	NA
	50	31.7 ± 1.5	27.7 ± 5.5	NA	NA

NA indicates that the given species was not spiked at that intensity, due to insufficient samples

the entire sample was counted, we initially found no significant effect of counting method (ANOVA, $P = 0.315$). Lack of difference between counting methods appears to be due to the very high rate of false positives for both methods when spiking abundances were very low (Fig. 5), thus we analyzed the low spiking abundances (1 or 5 individuals added) and high spiking abundances (10, 25, or 50 added) separately. In this case, we found no significant difference between microscopy and FlowCAM for low abundances (Fig. 5, ANOVA, $p = 0.932$), though FlowCAM underperformed when spiking intensity was high (Fig. 5, ANOVA, $P < 0.001$).

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., Arnott et al., 1998; Ficetola et al., 2008; Harvey et al., 2009; Hoffman et al., 2011). Species present at very low abundance or those with marked spatial or temporal heterogeneity may be exceedingly difficult to find (Delaney & Leung, 2010). Here, we have demonstrated that the ability to find rare species in lake plankton samples is positively related to their abundance in the sample, to the distinctiveness of the target species, and to sampling effort (total number of individuals counted). Importantly, we found that

typical subsampling efforts are too small to detect rare species in most aquatic systems.

We found positive relationships between the number of individuals counted and spiking abundance on the probability of finding at least one spiked individual. Similarly, Harvey et al. (2009) found that increased sampling effort and increased density of an invasive Cladoceran in Lake Ontario resulted in higher probabilities of detection. Based on typical subsampling effort used for plankton (< 300 individuals, as shown in our literature survey), our analysis indicates that many rare species or newly colonized NIS are unlikely to be detected (e.g., false negatives). These patterns are consistent with the work by Hoffman et al. (2011), who observed that rare species would require a very large counting effort for detection. In this experiment, we enumerated enough individuals to find the full complement of Cladocera present in Hamilton Harbour as well as all of our spiked species ($n = 14$); however, Hoffman et al. (2011) detected only 38 of an estimated 88 zooplankton species in Duluth-Superior Harbour when enumerating the same total number of individuals. Additionally, we estimated that in order to detect 95% of all species, one would need to count 14,600 individuals (data not shown), whereas Hoffman et al. (2011) estimated it would require 500,000 individuals. This difference may be due to the differences in local communities as well as to the fact that we only enumerated Cladocera, whereas they enumerated all zooplankton. Both

Fig. 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. Figures are based on taxonomic analysis (top panel) and manual FlowCAM classification analysis (bottom panel)
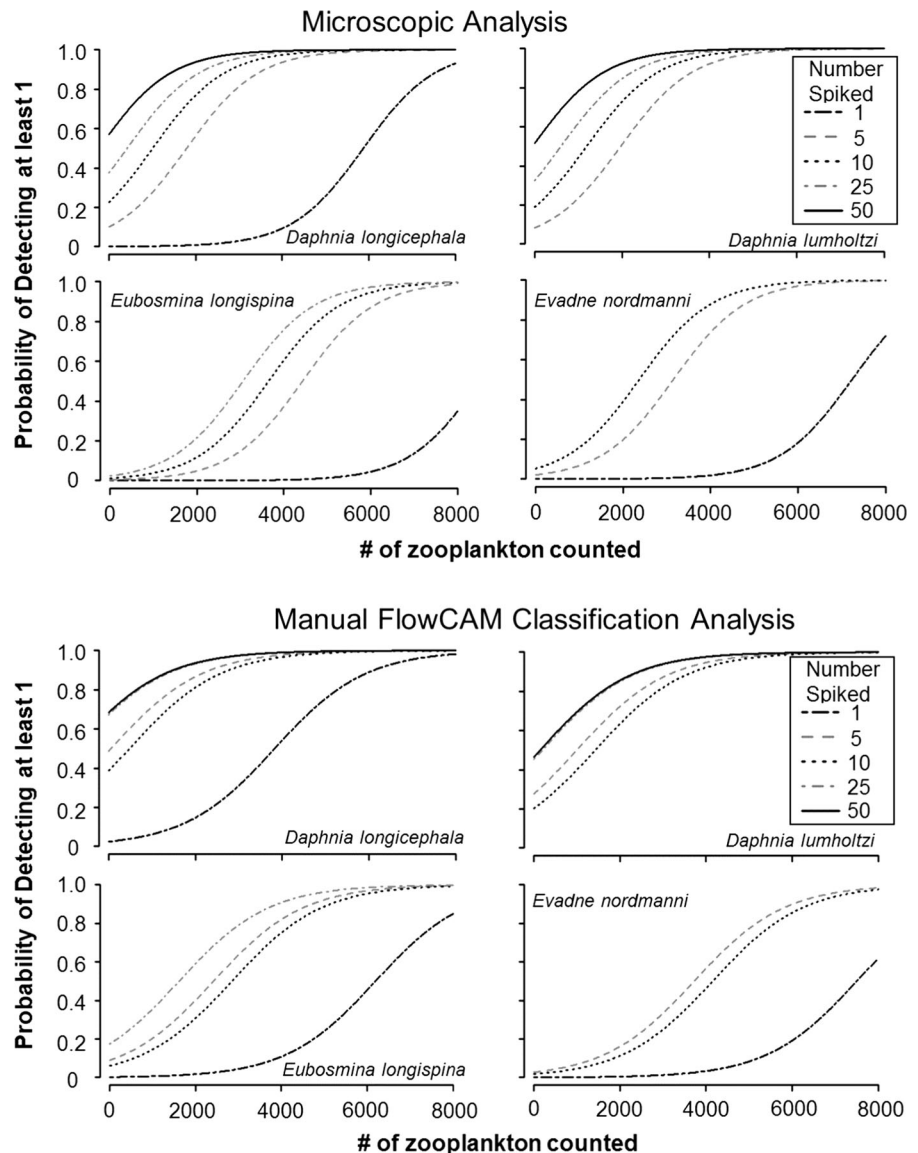

studies illustrate the need to reduce false negatives when looking for rare species; however, it is often time-consuming and expensive to enumerate large numbers of individuals to resolve the problem.

In this study, false negatives were more common than false positives (Fig. 4), especially at higher spiking intensities. Although this figure gives us an overall picture of false or missed identifications, it does not account for hidden offsetting false positives and false negatives which would be impossible to identify based on count data alone. Thus, there may exist cases where some individuals are correctly

identified, others missed (i.e., false negatives), and other cases where other species are misidentified and included (i.e., false positives). Thus, even experimental values that match the spiked-in totals may contain offsetting false positives and false negatives, and differences between the experimental value and spiked-in total represent a minimum number of individuals missed or misidentified. Because both microscopy and FlowCAM ultimately rely on a taxonomist to identify species, the methods are likely equally vulnerable to false negatives. To reduce the risk of missing a potential invader due to a false
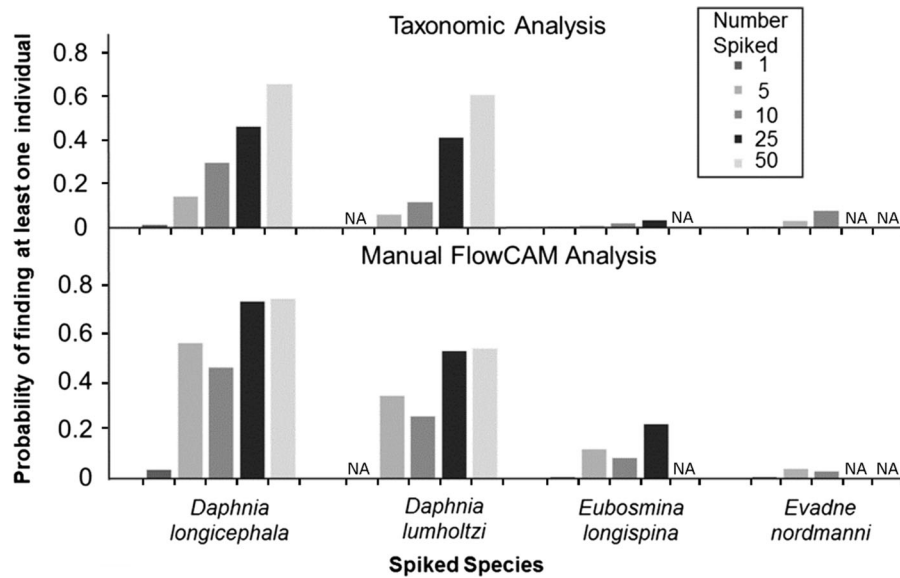
Fig. 3 Probability of finding at least of one of each spiked species when 300 individuals were counted. This figure encompasses both taxonomic analysis (top panel) and manual FlowCAM analysis (bottom panel), as well as all spiking abundances. It must be noted that for not all spiking abundances were utilized with all species. NAs indicate spiking abundances that were not utilized for a particular 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

negative, we suggest combining microscopy and FlowCAM with risk assessment and environmental DNA (eDNA) approaches to flag high-risk species to guide the search. By using multiple methodologies, it may be possible to reduce false negatives, and the harm that may occur if an invasive NIS establishes a new population undetected.

The detection likelihood of spiked species was related to their distinctiveness, though not in the way that we had hypothesized. Both *D. longicephala* and *D. lumholtzi* were predicted to have medium distinctiveness, however, these two species were the easiest of the four to find with both techniques. This finding may stem from the very large size of *D. longicephala* (up to 5 mm; Hebert, 1977), which allows for easy identification, while *D. 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). We expected that *Eubosmina longispina* would be the least distinctive of the four species, and indeed, it was the least likely spiked species to be found using microscopy. *E. longispina* is morphologically similar to many native species in Hamilton Harbour, which may cause it to be misidentified. We hypothesized *Evadne nordmanni* to have high distinctiveness,

although it was the least likely to be found using FlowCAM. The low probability of finding at least one of this species may have stemmed from its small size and very clear body when assessed using bright-field microscopy (Smirnov & Timms, 1983). This made it easy to overlook even though there were no morphologically similar species in our sample. It is important to point out that distinctiveness is a measure of how morphologically different the target species is relative to other species in the sample, thus distinctiveness of a species will vary depending on the community in which it is present. A recent study detected distinct differences in the 'background' communities of several high-risk ports throughout Canada, and suggested that enhanced understanding of species present in each community will facilitate detection of rare species (Chain et al., 2016).

Ricciardi & Atkinson (2004) rated distinctiveness of invaders based on phylogeny, where distinctive NIS belonged to genera not already present in the recipient region. 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 differences between phylogenetic distinctiveness and detectability. In this study, *E. nordmanni* was

Fig. 4 Relationship of number of spiked individuals recovered versus spiking abundance. Dots indicate results from microscopy, open triangles represent results from FlowCAM. False positives occur above the line, false negative below it
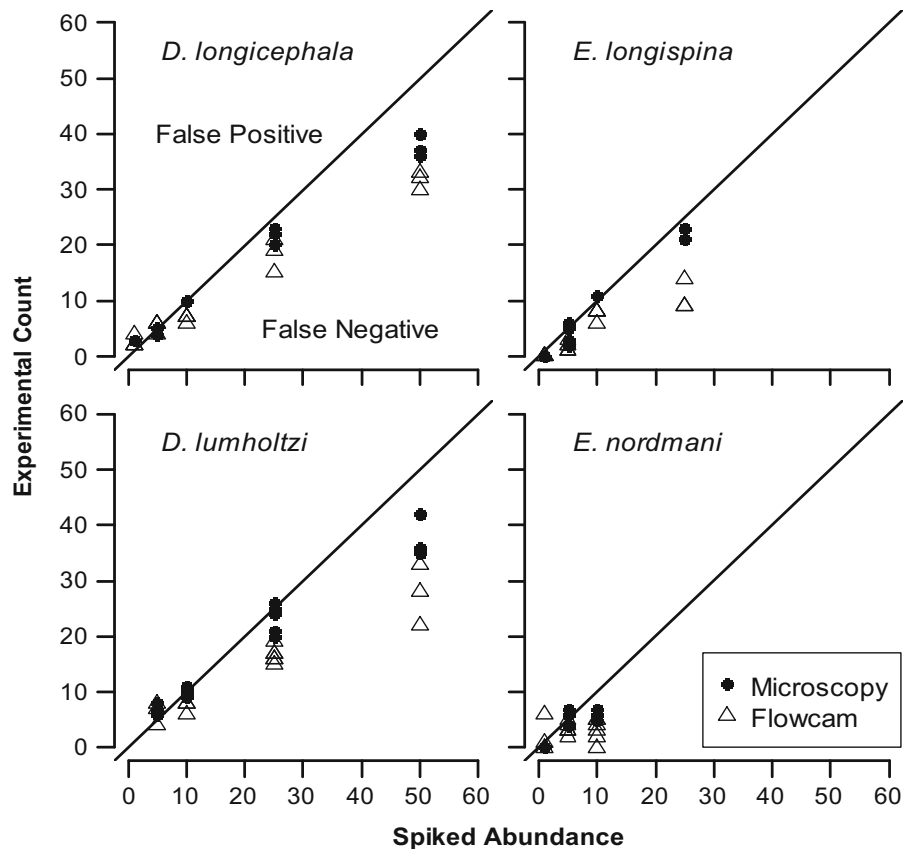

considered the most phylogenetically distinct species; however, it had low detectability because of its small size and clear (under light microscopy) body. Additionally, the sensitivity of the technique used will have an effect on detectability of the target species, although it will not change the morphological distinctiveness of the species.

At the outset of this study, reference samples lacking spiked species were counted first, following which samples were spiked with varying abundances of the four unknown species. As subsample counting progressed and we discovered possible spiked species that were not recorded in the reference sample, it is likely that a taxonomic 'search image' for spiked species was developed. In other words, the spiked species were no longer unknowns and we were able to search for their particular morphologies in the samples. The order in which samples were systematically processed precludes the possibility of testing this hypothesis. The formation of a search image would inflate detection of unknown species in spiked samples. Nevertheless, a taxonomic search image could be

beneficial for monitoring if an experienced taxonomist flags individuals suspected as uncharacteristic of the community they know well.

We compared the calculated expected probability to the range of observed detection frequencies of finding at least one spiked species when one individual was spiked into the sample and 300 total individuals were counted. We found that for both techniques, the observed range of probabilities was relatively low. These results suggest that both species-specific characteristics and the technology used have an effect on the probability of finding low abundance species when counting efforts are low (Fig. 3). Both of our methods exhibit markedly less sensitivity at detecting rare species than Zhan et al.'s (2013) molecular methods, as they were able to detect a single sequence of a spiked species in a mixture of 26,639 sequences (detection to as low as 0.0037%).

Automatic FlowCAM classification, whereby the computer identified images of the spiked species in the natural samples, generated a low average percent accuracy (33.4%); much lower than ideal operating
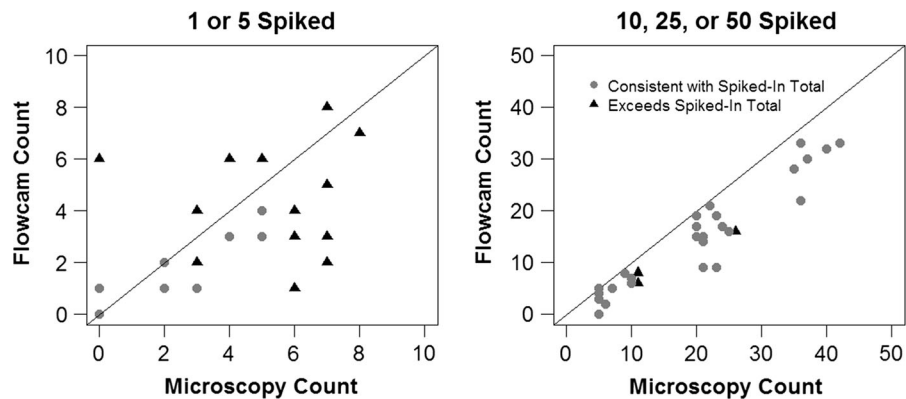
Fig. 5 Comparison of the total numbers of spiked zooplankton detected with microscopy (*x*-axis) and manual FlowCAM classification analysis (*y*-axis) for all spiking abundances. The solid line shows the 1:1 line. All samples were counted in entirety. Circles represent values where counts were consistent with (at or below) the spiked-in total; triangles represent values where counts exceeded the spiked-in total. The lack of significant difference between the counting methods appeared to be driven by a very high rate of false positives for both methods when spiking abundances were one or five

accuracy (80%) (Heather Anne Wright, Fluid Imaging, pers. comm.). We initially expected that Flow-CAM 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 at high abundance. For manual FlowCAM classification analysis, this was supported as both *D. longicephala* and *D. lumholtzi* (high distinctiveness) were readily detected regardless of spiking abundance. However, we were unlikely to find *E. nordmanni* (least distinctive when using FlowCAM) at any spiking abundance unless counting effort was very high (Fig. 2).

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 metazoo-plankton community (including calanoid, *Oithona*, and harpacticoid copepods, nauplii, gelatinous zoo-plankton, and meroplankton; 80–1000 μm in size), and concluded that there was no significant difference in abundances between the two techniques. When considering the total number of individuals detected in this study (Fig. 5), we observed no significant difference between the two techniques when considering

low abundances (once false positives were eliminated); however when spiking intensity was high, microscopy was superior at finding all of the spiked individuals. Therefore, when looking to enumerate the number of rare individuals in a sample, microscopy seems to be the superior technique. However, when looking for at least one rare species, such as looking for a positive identification of a newly colonized NIS, FlowCAM is superior.

We recommend that when monitoring for rare zooplankton, the analyst should analyze multiple subsamples using manual FlowCAM classification. As the automatic classification software becomes more advanced, users will come to rely on it rather than a manual approach. A knowledgeable taxonomist can then flag any organisms that are morphologically unusual. Formal taxonomic (or genetic) analysis would then proceed only on individuals that were flagged. False negatives can be reduced by using a variety of methods including risk assessment to suggest potential high-risk species that could be introduced to the monitored area (Ricciardi & Rasmussen, 1998; Williamson, 1999; Leung et al., 2002) and environmental DNA to identify species that may be present at very low abundance (Valentini et al. 2009; Jerde et al. 2011; Zhan et al. 2014). Only by combining the strengths of the variety of tools available, can we maximize the probability of identifying new invasive species in time for effective management.

References

Álvarez, E., Á. López-Urrutia, E. Nogueira & S. Fraga, 2011. How to effectively sample the plankton size spectrum? A case study using FlowCAM. Journal of Plankton Research 33: 1119–1133.

Arnott, S. E., J. J. Magnuson & N. D. Yan, 1998. Crustacean zooplankton species richness: single-and multiple- year estimates. Canadian Journal of Fisheries and Aquatic Sciences 55: 1573–1582.

Balcer, M. D., N. L. Korda & S. I. Dodson, 1984. Zooplankton of the Great Lakes: A Guide to the Identification and Ecology of the Common Crustacean Species. University of Wisconsin Press, Madison.

Camoying, M. G. & A. T. Yñiguez, 2016. FlowCAM optimization: attaining good quality images for higher taxonomic classification resolution of natural phytoplankton samples. Limnology and Oceanography: Methods 14: 305–314.

Campbell, M. L., B. Gould & C. L. Hewitt, 2007. Survey evaluations to assess marine bioinvasions. Marine Pollution Bulletin 55: 360–378.

Cao, Y., D. D. Williams & N. E. Williams, 1998. How important are rare species in aquatic community ecology and bioassessment? Limnology and Oceanography 43: 1403–1409.

Chain, F. J. J., E. A. Brown, H. J. MacIsaac & M. E. Cristescu, 2016. Metabarcoding reveals strong spatial structure turnover of zooplankton communities among marine and freshwater ports. Diversity and Distributions 22: 493–504.

D'Anjou, R. M., N. L. Balascio & R. S. Bradley, 2014. Locating cryptotephra in lake sediments using fluid imaging technology. Journal of Paleolimnology 52: 257–264.

Delaney, D. G. & B. Leung, 2010. An empirical probability model of detecting species at low densities. Ecological Applications 20: 1162–1172.

Ficetola, G. F., C. Miaud, F. Pompanon & P. Taberlet, 2008. Species detection using environmental DNA from water samples. Biology Letters 4: 423–425.

Fluid Imaging Technologies Inc., 2011. FlowCAM manual version 3.0 [online]. http://www.ihb.cas.cn/fxcszx/fxcs_xgxz/201203/P020120329576952031804.pdf. Accessed 24 March 2016.

Frischer, M. E., K. L. Kelly & S. A. Nierzwicki-Bauer, 2012. Accuracy and reliability of *Dreissena* spp. Larvae detection by cross-polarized light microscopy, imaging flow cytometry, and polymerase chain reaction assays. Lake and Reservoir Management 28: 265–276.

Haney, J. F., Aliberti M. A., Allan E., Allard S., Bauer D. J., and Beagen W. et al., 2013. An-image-based key to the zooplankton of North America. Version 5.0. University of New Hampshire Center for Freshwater Biology. http://cfb.unh.edu/cfbkey/html/references.htm.

Harvey, C. T., S. A. Qureshi & H. J. MacIsaac, 2009. Detection of a colonizing, aquatic, non-indigenous species. Diversity and Distributions 15: 429–437.

Hebert, P. D. N., 1977. A revision of the taxonomy of the genus *Daphnia* (Crustacea: Daphnidae) in south-eastern Australia. Australian Journal of Zoology 25: 371–398.

Hoffman, J. C., J. R. Kelly, A. S. Trebitz, G. S. Peterson & C. W. West, 2011. Effort and potential efficiencies for aquatic non-native species early detection. Canadian Journal of Fisheries and Aquatic Sciences 68: 2064–2079.

Ide, K., K. Takahashi, A. Kuwata, M. Nakamachi & H. Saito, 2008. A rapid analysis of copepod feeding using FlowCAM. Journal of Plankton Research 30: 275–281.

Jerde, C. L., A. R. Mahon, W. L. Chadderton & D. M. Lodge, 2011. "Sight-unseen" detection of rare aquatic species using environmental data. Conservation Letters 4: 150–157.

Johnson, W. S. & D. M. Allen, 2005. Zooplankton of the Atlantic and Gulf Coasts, A Guide to Their Identification and Ecology. John Hopkins University Press, Baltimore.

Le Bourg, B., V. Cornet-Barthaux & J. Blanchot, 2015. FlowCAM as a tool for studying small (80–1000 μm) meta-zooplankton communities. Journal of Plankton Research 37: 666–670.

Leung, B., D. M. Lodge, D. Finnoff, J. F. Shogren, M. A. Lewis & G. Lamberti, 2002. An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species. Proceedings of the Royal Society of London B: Biological Sciences 269: 2407–2413.

Muzinic, C. J., 2000. First record of *Daphnia lumholtzi* Sars in the Great Lakes. Journal of Great Lakes Research 26: 352–354.

Poulton, N. J., 2016. FlowCAM: quantification and classification of phytoplankton by imaging flow cytometry. Imaging Flow Cytometry: Methods and Protocols 1389: 237–247.

R Development Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Ricciardi, A. & S. K. Atkinson, 2004. Distinctiveness magnifies the impact of biological invaders in aquatic ecosystems. Ecol. Lett. 7: 781–784.

Ricciardi, A. & J. B. Rasmussen, 1998. Predicting the identity and impact of future biological invaders: apriority for aquatic resource management. Canadian Journal of Fisheries and Aquatic Sciences 55: 1759–1765.

Smirnov, N. N., and B. V. Timms, 1983. A revision of the Australian Cladocera (Crustacea). Records of the Australian Museum, Supplement 1. P1–132

Thomsen, P. F., J. Kielgast, L. L. Iversen, C. Wiuf, M. Rasmussen, M. T. P. Gilbert, L. Orlando & E. Willerslev, 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology 21: 2565–2573.

Valentini, A., F. Pompanon & P. Taberlet, 2009. DNA barcoding for ecologists. Trends in Ecology & Evolution 24: 110–117.

Ward, H. B., G. C. Whipple & W. T. Edmondson (eds), 1918. Fresh-water Biology, 2nd ed. Wiley, New York.

Williamson, M., 1999. Invasions. Ecography 22: 5–12.

Witty, L. M., 2004. Practical guide to identifying freshwater crustacean zooplankton, 2nd ed. Cooperative Freshwater Ecology Unit, Greater Sudbury.

Wroughton, J. & T. Cole, 2013. Distinguishing between binomial, hypergeometric and negative binomial distributions. Journal of Statistics Education 21: 1–16.

Zhan, A., M. Hulak, F. Sylvester, X. Huang, A. A. Adebayo, C. L. Abbott, S. J. Adamowicz, D. D. Heath, M. E. Cristescu & H. J. MacIsaac, 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. Methods in Ecology and Evolution 4(6): 558–565.

Zhan, A., W. Xiong, S. He & H. J. MacIsaac, 2014. Influence of artifact removal on rare species recovery in natural complex communities using high throughput sequencing. PLoS ONE 9: 1–7.