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Quantifying Environmental DNA Signals for Aquatic Invasive Species Across Multiple Detection Platforms

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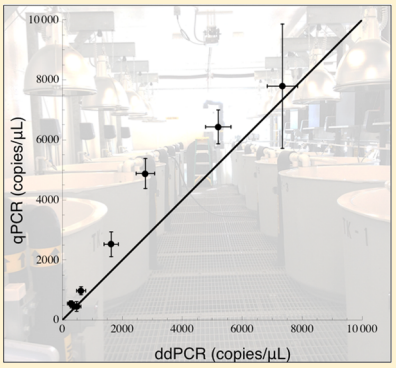
Supporting Information

ABSTRACT: The use of molecular surveillance techniques has become popular among aquatic researchers and managers due to the improved sensitivity and efficiency compared to traditional sampling methods. Rapid expansion in the use of environmental DNA (eDNA), paired with the advancement of molecular technologies, has resulted in new detection platforms and techniques. In this study we present a comparison of three eDNA surveillance platforms: traditional polymerase chain reaction (PCR), quantitative PCR (qPCR), and digital droplet PCR (ddPCR) in which water samples were collected over a 24 h time period from mesocosm experiments containing a population gradient of invasive species densities. All platforms reliably detected the presence of DNA, even at low target organism densities within the first hour. The two quantitative platforms (qPCR and ddPCR) produced similar estimates of DNA concentrations. The analyses completed with ddPCR was faster from sample collection through analyses and cost approximately half the expenditure of qPCR. Although a new platform for eDNA surveillance of aquatic species, ddPCR was consistent with more commonly used qPCR and a cost-effective means of estimating DNA concentrations. Use of ddPCR by researchers and managers should be considered in future eDNA surveillance applications.



INTRODUCTION

Genetic and genomic techniques are being used to detect rare species, including both non-native and threatened and/or endangered organisms.[1−8] These techniques have demonstrated greater sensitivity for detection in systems where traditional fisheries methods have failed (e.g., refs 8−10), and are the focus of intensive research and methodological development for the purposes of conservation management.[11,12] However, as the field of environmental DNA (eDNA) surveillance expands, so does the diversity of tools and assays used to analyze samples. With such rapid evolution of technology, it is imperative to compare the detection sensitivities, time of processing and analysis, and cost of screening a sample across new platforms.

Initial studies of eDNA for detection of multicellular organisms in aquatic systems used end point Polymerase Chain Reaction (PCR) to screen samples for the presence of DNA from single species in water samples.[6,9,10,13−15] As the genetic surveillance tools have advanced, the scientific focus has moved from investigating species presence/absence to quantification of the genetic signal produced by target organisms in the hopes of linking DNA concentration to species abundance or biomass.[2,11,16−18] Primarily, these recent studies have incorporated quantitative (or real time) PCR (qPCR) methodologies to determine the amount of target species DNA in water samples.

A qPCR assay monitors amplification progress after each cycle via the incorporation of fluorescent dyes into the amplification process and estimates the amount of DNA by creating a standard curve, which itself requires quantification via spectrophotometry or intercalating dyes before being used.[19−22] During the real-time amplification, the qPCR signal measurement is based on a Ct value (threshold cycle), that is, the point where the fluorescent signal goes above a threshold.[22] As such, qPCR provides an estimate of total sample target DNA concentration.[23] Previous studies have noted considerable variation in assay performance based on differences in standards and assay systems used between laboratories.[23] For qPCR, rare template detection and determination of copy number variation is possible for rare molecules (e.g., 10 copies from 11 copies) but for the latter, this is only possible with a large number of replicate samples (n = 86).[24] Additionally, qPCR has been noted to suffer from poor reproducibility within and between

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laboratories,[23,25−27] which in application to conservation management can lead to faulty or inconsistent inferences and suboptimal resource management.[28,29]

Digital droplet PCR (ddPCR) provides a new method of sample analysis[30,31] allowing for accurate estimation of low concentrations of DNA. Like standard PCR, ddPCR is a direct method that does not use standard curves to estimate target DNA. Target DNA is randomly allocated into discrete droplets via microfluidics that are then thermally cycled and individually screened via fluorescence measurement for presence of target DNA;[31] (Figure 1).

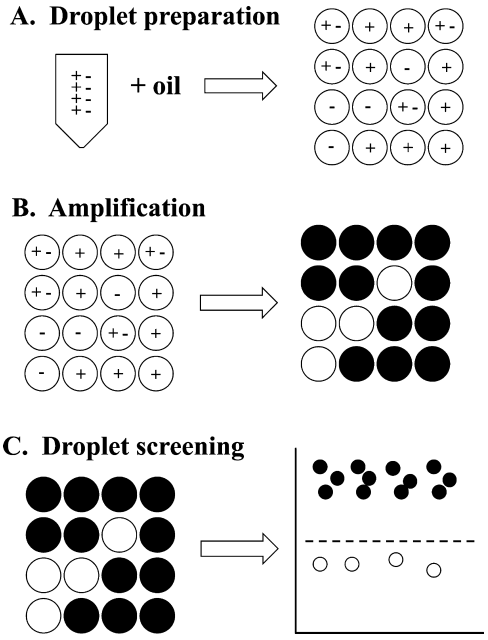

Figure 1. Digital droplet PCR process flow diagram. (adapted from http://bio-rad.com) A. Samples containing target DNA and/or nontarget (background) DNA are partitioned into ∼20 000 individual droplets. Each droplet can contain target (+) or nontarget (−) DNA or both (+ −). B. Droplets are then amplified using standard hydrolysis probe or other fluorescent (e.g., EvaGreen) PCR amplification methods creating positive (shown here as black) and negative (shown here as white) droplets. C. Droplets are then read on the ddPCR instrument and positive and negative droplets are separated and counted. The fraction of positive droplets is used to calculate target DNA concentration.

To demonstrate the efficiency and accuracy of each these detection platforms, we present a comparison of eDNA detection using traditional PCR, qPCR, and ddPCR for the round goby (*Neogobius melanostomus*), an abundant invasive species in the Laurentian Great Lakes poised to expand to inland lakes through direct connections or other anthropogenic vectors such as the commercial bait trade.[8,32] Additionally, we present a time and cost comparison for using ddPCR for genetic surveillance in environmental DNA samples from aquatic systems.

MATERIALS AND METHODS

Sample Collection and DNA Extraction. Mesocosm experiments were conducted in four 2500 L tanks containing approximately 1500 L of water at the Central Michigan University Biological Station (CMUBS; Beaver Island, MI). Prior to any experimental trials, all equipment and tanks were decontaminated to ensure target species DNA was coming from samples and not from any other source (see refs 9 and 33 for the overall quality assurance methods utilized in this study). In short, all surfaces and equipment were treated with 10% bleach solution. Cooler, equipment, and extraction blanks were used to quantify any contamination.[33] Well water was used to fill all mesososms in our experiments. Each mesocosm tank was then stocked at varying densities of round gobies (size range from 10 to 12.5 cm total length) including a negative control tank (C; n = 0 individuals), a low-density tank (LD; n = 3 individuals), a medium-density tank (MD; n = 12 individuals), and a high-density tank (HD; n = 33 individuals). Samples for environmental DNA (eDNA) analyses were taken prior to goby additions to screen for any potential contamination. Experimental samples were collected at eight time intervals during a 24 h period after fish were introduced into the system (1 h, 2 h, 4 h, 6 h, 12 h, 18 h, 24 h) to assess DNA accumulation over time using each of the three analysis platforms (PCR, qPCR, ddPCR). At each sample period, 2 L water samples were collected in autoclave sterilized Nalgene bottles from each tank and filtered through ∼1.5 μm glass filter papers.[7−10,34] Filter papers were then stored at −20 °C until they could be processed in the laboratory. DNA extractions were performed using a MO BIO Power Water DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), following manufacturer's protocols. All DNA extractions were then stored at −20C until screening could be completed.

End Point (Traditional) PCR. Following previously published methods,[9,10,15] standard polymerase chain reactions (PCR) were performed on each sample collected with eight replicates per sample using species-specific primers that target a 150 base pair section of the mitochondrial cytochrome c oxidase subunit I gene (COI) developed by Nathan et al. (2014). Tests for target amplification and specificity are included in Table 1. Individual PCR reactions consisted of 10X PCR buffer, 2.5 mM Mg^{2+} solution, 0.05 mM each dNTP, 0.2 μM each primer, 0.75U *Taq*, 1 μL sample DNA, and water for a final reaction volume of 25 μL. For each PCR trial, three negative controls (sterile water) and four positive controls (i.e., tissue extracted genomic DNA) were included to assess contamination and PCR success, respectively. Thermal conditions for PCR were 94C for 2 min, 35 cycles of 94 °C for 15 s, 53 °C for 15 s, 72 °C for 30 s, and 72 °C for 3 min. Samples were then incubated at 4 °C until they could be screened on a 1% agarose gel stained with ethidium bromide, photographed, and documented.

Quantitative PCR (qPCR). Relative DNA concentrations were analyzed using an Eppendorf Mastercycler ep realplex cycler. Standards for qPCR experiments were prepared using a dilution series of purified PCR amplicons ranging in concentrations from 2 to 200 000 copies/uL. For each eDNA sample, three replicates were analyzed using the same 150 base pair mitochondrial primer as in standard PCR with the addition of an additional hydrolysis probe: 5′-CAG GCA ACT TRG CAC ATG CAG-3′, dual-labeled with a 5′ 6-FAM fluorescent tag and a 3′ Black Hole Quencher. Each reaction consisted of 1× TaqMan Gene Expression master mix (Life Technologies, Inc.), 900 nM each primer, 250 nM of the hydrolysis probe, 2 μL DNA, and deionized water for a final reaction volume of 20 μL. Thermocyler conditions followed the TaqMan gene expression master mix protocol: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Mastercyler realplex software v2.2 (Eppendorf, Inc.) was used

Table 1. Target and Non-Target Species DNA Used to Develop Goby Mitochondrial Markers[a]

species	Goby COI amplification
Target Species	
Round Goby (*Neogobius melanostomus*)	X
Nontarget Species	
Golden Shiner (*Notemigonus crysoleucas*)	-
Fathead Minnow (*Pimephales promelas*)	-
White Sucker (*Catastomus commersonii*)	
Common carp (*Cyprinus carpio*)	-
Goldfish (*Carassius auratus*)	-
Snakehead (*Channa argus*)	-
Largemouth Bass (*Micropterus salmoides*)	-
Brook Stickleback (*Culea inconstans*)	-
Gizzard Shad (*Dorosoma cepedianum*)	-
Silver Carp (*Hypophthalmichthys molitrix*)	-
Bighead Carp (*Hypophthalmichthys nobilis*)	-
Grass Carp (*Ctenopharyngodon idella*)	-
Black Carp (*Mylopharyngodon piceus*)	-

[a]Molecular sequence data were obtained from NCBI's GenBank database (www.ncbi.nlm.nih.gov) and primer sets were produced using NCBI's BLAST program. Goby COI2 was designed to amplify round goby DNA. Non-target species used to test for specificity were selected based on genetic similarities, available DNA, and prevalence in the Great Lakes bait trade. Results indicate species that were amplified (X) or not amplified (−) by designated primers. This table was adapted from Nathan et al. (2014).

to generate concentration estimations with threshold values based on the best R^2 value of the standard curves. For all qPCR analysis, standard curve R^2 values were >0.996 and efficiencies ranged between 0.92 and 0.93.

Digital Droplet PCR. Absolute target species DNA concentrations were measured with a BioRad© QX200 Droplet Digital PCR system. For each sample, a reaction was prepared with 1000 nM of each primer and probe (the same as used in the previous qPCR reactions), 1× BioRad© ddPCR Supermix for probes, 2.5 μL DNA, and water for a total volume of 25 μL. To partition samples into nanodroplets using the BioRad© QX200 droplet generator, 20 μL of the reaction mixture was combined with 70 μL droplet oil (BioRad). After droplet generation, 40 μL of the mixture was transferred to a PCR plate and amplified with the following conditions: 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 60 °C for 1 min, followed by 98 °C for 10 min. Droplet concentrations were analyzed using QuantaSoft software with thresholds set manually.

Analyses. Means and confidence intervals were calculated using the technical replicates of qPCR and ddPCR. To compare the estimates of DNA concentrations between platforms, we plotted the point estimates and confidence intervals across low, medium, and high density treatments with a 1:1 line. If the confidence intervals cross the line, then there is no difference between qPCR and ddPCR estimates. If the confidence intervals do not cross the line, then there is a discrepancy between the point estimates. A 2 × 3 mixed factorial repeated measures ANOVA design was used to evaluate the significance of the platform type across the repeated samples collected within each fish density treatment. We hypothesize that estimated DNA concentrations will not be significantly different between qPCR and ddPCR platforms across the three fish densities. Support for our null hypothesis would result in confidence intervals for most concentrations crossing the 1:1 line and no significance between ddPCR and qPCR platforms as evaluated by a repeated measures ANOVA. To estimate the accumulation rate of DNA, multiple linear regression was performed. Likelihood ratio tests were used to evaluate statistical significance. The slope of the regression is the accumulation rate and confidence intervals are provided. All analyses were performed with R statistical software.

RESULTS AND DISCUSSION

Standard PCR. Round goby DNA was detected in all 24 samples using standard PCR and analyses of all controls and samples collected before introduction of the target species into the mesocosms were negative, indicating no contamination. Three samples, LD 0.5 h, LD 1 h and MD 4 h, had seven of eight total replicate PCR reactions test positive while those from all other time periods for all densities had eight of eight total replicates screen positive for round goby DNA. Although this method does not provide a quantitative estimate of DNA concentration, a general qualitative trend of increasing band brightness was observed over time and across increasing goby densities (Supporting Information Figure 1).

Target Species Density and DNA Concentration. Both qPCR and ddPCR techniques found increases in round goby DNA concentration across varying goby densities through time (Figure 2). The 95% confidence intervals of the qPCR results indicate significant increases at five of eight time intervals where samples were collected. The three discrepancies were found in samples collected at hours 1 and 12 where the HD concentration was lower than the MD and at hour 4 the HD

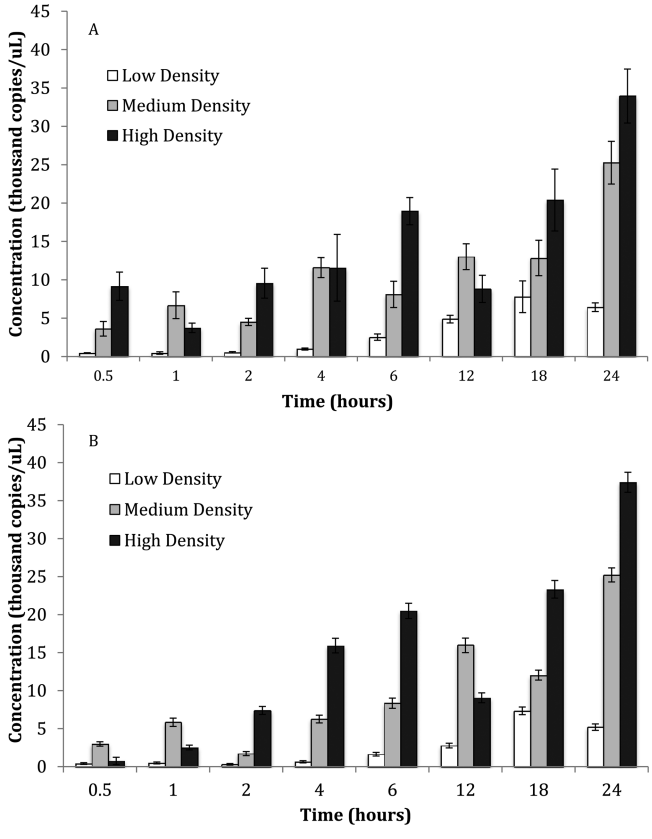

Figure 2. Round Goby DNA concentrations in mesocosm experiments at varying densities, monitored over 24 h and detected with (A) quantitative PCR and (B) digital droplet PCR. Error bars indicate 95% confidence intervals of DNA concentration.

and MD concentrations were not significantly different. Similarly, the ddPCR results from hours 1 and 12 suggest a higher concentration at the MD than the HD (Nonoverlapping confidence intervals), and at hour 4 the HD was not significantly greater than the MD in qPCR (Overlapping confidence intervals; Figure 2). These discrepancies from the expectation are potentially the result of a heterogeneous distribution of DNA in the mesocosm, a phenomenon seen in other studies using eDNA surveillance;[9,35] Mahon unpublished data). Lastly, lowest possible detection concentrations were generated for ddPCR based on a set of known concentrations of target species total genomic DNA that was serially diluted (Supporting Information Figure 2). While previous studies[21,22,30] have shown detection limits using a qPCR platform, we found ddPCR to reliably detect target DNA in purified samples at dilutions of 3 target DNA molecules (Supporting Information Figure 2). However, depending on the amount of nontarget DNA in a given sample from an environmental source, detection limits will vary on all platforms (Mahon unpublished data).

DNA Accumulation Rates. DNA accumulation rates were significantly different across fish density (LRT: χ^2=1.22 × 10^9,d.f. = 2, $p < 0.001$). Inspection of point estimates and 95% confidence intervals of the accumulation rates showed increasing estimated accumulation rates with increasing fish density (Table 2). In these studies, DNA is expected to accumulate as long as DNA shedding rates (through feces, mucus, or urine) are greater than degradation rates of the DNA in the environment.[36] Further, this provides another example of the trend of increasing DNA concentrations with increasing fish density or biomass.

Table 2. Accumulation Rates and 95% Confidence Intervals of Goby DNA over 24 h at Three Densities, Quantified Using Quantitative PCR and Digital Droplet PCR

platform and density	lower 95%	DNA accumulation rate (copies/ul/h)	upper 95%
qPCR low density	210.66	321.64	432.62
ddPCR low density	148.92	274.09	399.26
qPCR medium density	393.53	721.56	1049.59
ddPCR medium density	480.99	823.22	1165.44
qPCR high density	348.23	931.42	1514.60
ddPCR high density	427.28	1111.01	1794.73

qPCR vs ddPCR. While some point estimations of DNA concentrations varied across the two quantitative platforms, indicated by nine of 24 (37.5%) significant differences from a 1:1 ratio across all densities (Figure 3), the overall conclusion from the repeated measures ANOVA is there is no statistical difference between ddPCR and qPCR platforms that measured the DNA concentrations in this experiment (*f-test* = 0.77, p = 0.77). Never the less, point estimate differences were more prevalent at lower fish densities; five (of eight) LD samples differed significantly from a 1:1 ratio, compared to three MD samples and only one HD sample. In eight of the nine comparisons that differed, the qPCR estimation indicated higher concentrations of target species DNA when compared to ddPCR. The only instance when ddPCR reported a higher concentration was the MD sample at hour 12 of the experiment. Although estimates of DNA concentration are largely consistent, one platform may produce a higher estimate of DNA concentration, it may not necessarily indicate a more accurate estimate. Instead, it suggests that occasionally the two platforms generate inconsistent results, particularly at low concentrations. This is of particular concern for the early detection of rare organisms in aquatic environments because of the low abundance of target organism. Previous work has suggested that ddPCR may be better suited for the detection of rare molecules due to the removed necessity for calibration curves and the large number of partitions rather than a single measurement when compared to qPCR.[33] Further calibration

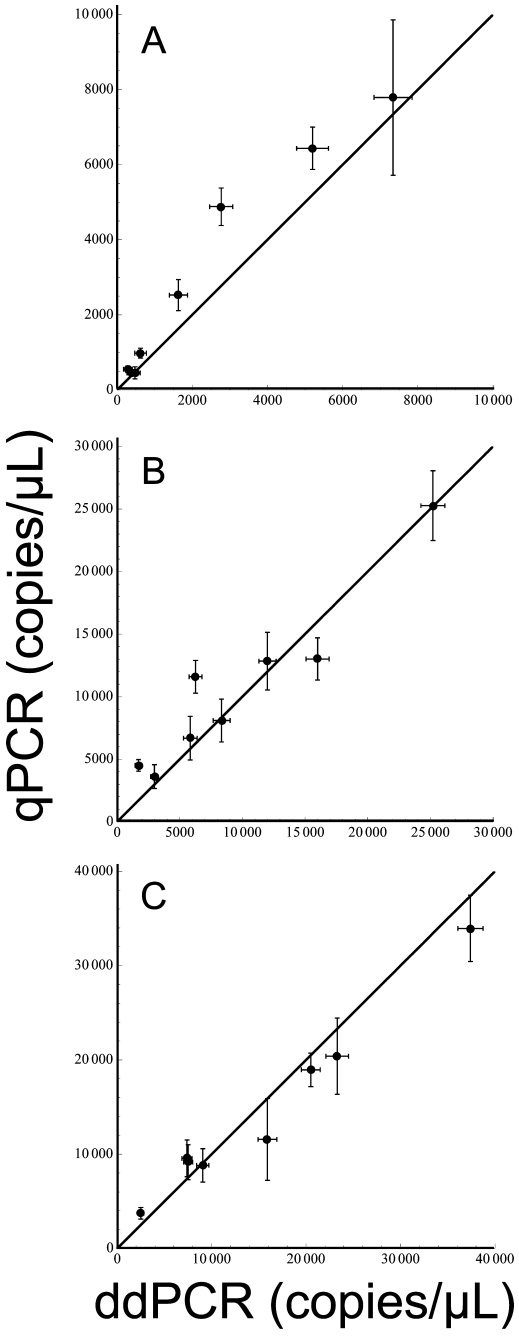

Figure 3. Comparison of eDNA concentrations estimated by quantitative PCR (qPCR) and digital droplet PCR (ddPCR) at low (A), medium (B), and high (C) round goby densities. A 1:1 line is drawn for reference, indicating equal concentration estimates across both detection platforms. Error bars represent 95% confidence intervals for DNA concentrations.

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studies focused on the absolute limits of detection between platforms would serve to assess the application of these platforms under low DNA concentration conditions. With management groups using eDNA results to base large scale responses and decisions,[9,10] it is essential that the most capable and appropriate technologies and techniques be utilized for the given application.

Time and Cost of qPCR vs ddPCR for eDNA Analyses. Screening time and cost of analysis per sample for qPCR and ddPCR screening differed in this experiment. Overall time to prepare, extract DNA, and screen qPCR samples was approximately 3.5 h to completion. For the ddPCR analyses, ddPCR sample screening time (DNA extraction through final data collection) was approximately 2 h. Cost for preparation and screening (DNA extraction through sample analysis) also differed between qPCR and ddPCR in the current study. Cost of analysis per sample was approximately $8.87 for qPCR analysis and $4.02 per sample for ddPCR analysis, respectively (excluding DNA extraction costs which are equal across all platforms; Table 3).

Table 3. Cost Analysis for qPCR and ddPCR Analyses and Do Not Include DNA Extraction Costs That Are Equal Across All Platforms Depending on Type of Extraction Used[a]

	ddPCR	qPCR	
	price per sample	price per sample	price per plate
supermix	$1.00	$2.50	$240.00
primer	$0.02	$0.02	$1.92
probe	$0.15	$0.15	$14.40
cartridge	$1.00		
gasket	$0.25		
PCR plate	$0.85	$0.85	$0.85
foil	$0.10	$0.10	$0.10
droplet oil	$0.20		
machine oil	$0.25		
PCR strip	$0.20		
		qPCR per plate	$257.27
total	$4.02		$8.87 per sample

[a]Cost assumptions include production of a qPCR calibration curve for every plate completed and completion of qPCR reactions in triplicate (as was done for this study). Traditional PCR costs per reaction are not included in the table but were calculated at $4.27/sample.

CONCLUSIONS

While molecular techniques have been applied in aquatic systems for over 30 years,[36] the field of molecular surveillance in aquatic systems remains relatively new, beginning with studies for rare fish and amphibians.[5,9,10,13] From the onset of these investigations, researchers have moved to advance the science of aquatic detection to addressing questions such as what are the best methods for sample collection, DNA preservation, and filtration,[35,37] what environmental conditions influence detection,[38] and how can the science move from a presence/absence detection method to provide managers with more quantitative information relating to species abundance or biomass.[11,12,39] The initial studies using eDNA for surveillance and monitoring for rare species,[9,13] in particular, highlighted questions on accumulation and quantification of target species DNA. This study is the first to compare the use of PCR, qPCR, and digital droplet PCR platforms for analyzing samples for both presence and amounts of target species environmental DNA. The overall observation is that the three platforms, PCR, qPCR, and ddPCR, gave consistent estimates of DNA presence-absence detection, and qPCR and ddPCR gave consistent estimates of DNA concentration. For quantitative applications, the ddPCR platform produced concentrations with reduced variability (as observed from confidence intervals in Figure 2) and lower costs on a per sample basis when compared to qPCR. However, there is some question of sensitivity of ddPCR compared to qPCR at lower concentrations that should be more thoroughly investigated in future studies.

While the traditional PCR platform is less sensitive overall with potential ambiguity in determining low concentration bands in stained agarose gels, as compared to quantitative platforms lacking this ambiguity, PCR may be a sufficient platform in situations where scientists and/or management groups only require data on the presence or absence of target species or in places with limited infrastructure (insufficient funds to purchase more expensive qPCR or ddPCR platforms). In this study we were able to detect target species DNA from our lowest density treatment (3 individuals, ∼5 cm each; 1500 L mesocosm) at our first sampling time (1h). With seven of eight of the PCR replicates screening positive at that time, likely detectable DNA could have been found at a much earlier time interval <1 h. As such, even at low concentrations of DNA, PCR is a reliable presence absence detection platform from which inferences on species' DNA presence can be made.

For studies that necessitate quantification of target species DNA from environmental water samples, ddPCR has both methodological and cost advantages over standard qPCR platforms. While qPCR has been used for analyses of population size,[3,16,17] giving it a distinct advantage over PCR alone, the platform does have its problems. Along with its improved precision, ddPCR does not require the production of a standard curve necessary for qPCR analyses to be performed, consequently decreasing potential for user error (e.g., pipetting error in preparation of standards and accidental contamination). While more experienced laboratories may not have issues with production of the standard curve, they do require both production of standards and increased costs of materials for generation of the curve itself. Less experienced laboratories also have to deal with increased chances of error introduction when generating a standard curve through errors in contamination, pipetting, etc. as noted above. When comparing qPCR and ddPCR, the time to results and cost analyses completed in this study find that ddPCR is both quicker and a more cost-effective procedure.

Environmental genetic and genomic surveillance in aquatic systems have made dramatic steps forward in the types of questions that can be asked when collecting DNA in water samples. The initial presence/absence studies studying invasive species[9,10,13,15] have moved into more quantitative analyses where target species DNA is helping predict abundance.[1,3,16−18,38] Here we have shown the relative performance of PCR, qPCR, and ddPCR in a controlled experimental setting for eDNA surveillance of a targeted invasive species. In general, replication of results by other laboratories, while sometimes inconsistent with qPCR, remains to be determined and will necessitate additional study using the ddPCR platform. As more laboratories deploy this platform, it will be necessary to compare the consistency of this new platform, or any new

platform, for informed conservation management using eDNA. Continuing development of tools such as the application of ddPCR will improve the quality of data on which managers can more confidently take action to address rare species, whether threatened or endangered or invasive, in aquatic environments.[28,29]

■ ASSOCIATED CONTENT

Supporting Information

Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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