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Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish

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Abstract

Few studies have examined capture and extraction methods for environmental DNA (eDNA) to identify techniques optimal for detection and quantification. In this study, precipitation, centrifugation and filtration eDNA capture methods and six commercially available DNA extraction kits were evaluated for their ability to detect and quantify common carp (*Cyprinus carpio*) mitochondrial DNA using quantitative PCR in a series of laboratory experiments. Filtration methods yielded the most carp eDNA, and a glass fibre (GF) filter performed better than a similar pore size polycarbonate (PC) filter. Smaller pore sized filters had higher regression slopes of biomass to eDNA, indicating that they were potentially more sensitive to changes in biomass. Comparison of DNA extraction kits showed that the MP Biomedicals FastDNA SPIN Kit yielded the most carp eDNA and was the most sensitive for detection purposes, despite minor inhibition. The MoBio PowerSoil DNA Isolation Kit had the lowest coefficient of variation in extraction efficiency between lake and well water and had no detectable inhibition, making it most suitable for comparisons across aquatic environments. Of the methods tested, we recommend using a 1.5 μm GF filter, followed by extraction with the MP Biomedicals FastDNA SPIN Kit for detection. For quantification of eDNA, filtration through a 0.2–0.6 μm pore size PC filter, followed by extraction with MoBio PowerSoil DNA Isolation Kit was optimal. These results are broadly applicable for laboratory studies on carps and potentially other cyprinids. The recommendations can also be used to inform choice of methodology for field studies.

Keywords: common carp, *Cyprinus carpio*, environmental DNA, invasive species, quantitative PCR

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Introduction

Measurement of environmental DNA (eDNA), the genetic material from environmental samples in the absence of biological source material, is a promising and relatively new technique to detect rare macro-organisms (Taberlet *et al.* 2012; Thomsen & Willerslev 2015). Although eDNA has been used to detect terrestrial animals (Valiere & Taberlet 2000; Andersen *et al.* 2012; Nichols *et al.* 2012), most studies have used this method to detect aquatic animals (Ficetola *et al.* 2008; Jerde *et al.* 2011; Lodge *et al.* 2012; Mahon *et al.* 2013). A large-scale monitoring effort for invasive bigheaded carps (*Hypophthalmichthys* spp.) in the Mississippi River presently uses eDNA for detection (Jerde *et al.* 2011, 2013; USFWS 2013). However, other studies have shown that eDNA may be used to quantify aquatic species (Takahara *et al.* 2012; Thomsen *et al.* 2012; Pilliod *et al.* 2013). While attention has been focused on sampling design (Olson *et al.* 2012; Schmidt *et al.* 2013; Pilliod *et al.* 2014), relatively few studies have compared eDNA capture or extraction methods (See Smith *et al.* 2012; Renshaw *et al.* 2014; Turner *et al.* 2014; Deiner *et al.* 2015). There is a need to move towards 'best practices' for the collection and analysis of eDNA (Darling & Mahon 2011; Goldberg *et al.* 2015).

Capture methods (i.e. methods used to concentrate DNA from the water) used to date have varied widely and include precipitation (Ficetola *et al.* 2008; Thomsen *et al.* 2012), centrifugation (USFWS 2013; Klymus *et al.* 2015) and filtration (Jerde *et al.* 2011; Olson *et al.* 2012; Goldberg *et al.* 2013; Pilliod *et al.* 2013). Moreover, studies that have used filtration capture methods have employed a variety of filter types and a range of pore sizes. Small pore size filters have been shown to retain more eDNA (Liang & Keeley 2013; Turner *et al.* 2014), while larger pore size filters have been shown to be more accurate for biomass quantification (Takahara *et al.*

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2012). Filter material can also drastically affect the recovery of eDNA (Liang & Keeley 2013; Renshaw *et al.* 2014). However, the relative performance of capture methods is not well-studied.

Differences among capture methods in the maximum sample water volume that can be processed may also influence detection. For example, only 15 mL of water is typically used for the precipitation capture method (Ficetola *et al.* 2008), and 50 mL is used for centrifugation (USFWS 2013; Klymus *et al.* 2015). In contrast, filtration in some instances can process up to several litres of water (Olson *et al.* 2012). However, due to the more efficient capture of eDNA on smaller pore sized filters, small water volumes can achieve equivalent eDNA recovery relative to filtration of several litres through a larger pore sized filter (Turner *et al.* 2014). In the only study that directly compared precipitation and filtration eDNA capture methods, Deiner *et al.* (2015) found that filtration resulted in a higher detection rate of macro-organisms than precipitation for both conventional PCR and metabarcoding analysis methods. Although comparison of standardized protocols (USFWS 2013) for centrifugation and filtration methods for capture of bigheaded carp eDNA is underway (E. Monroe, personal communication), a direct comparison of precipitation, centrifugation and filtration methods has not yet been published.

After eDNA has been captured, samples must be extracted to release DNA from intact cells and organelles and remove compounds that can inhibit enzymatic reactions. Inhibitors range from cellular components to materials in waters that are co-extracted with DNA, such as humic substances (Wilson 1997). Similar to eDNA capture methods, DNA extraction methods include in-house formulations (Egan *et al.* 2013) and several different commercially available kits (Ficetola *et al.* 2008; Jerde *et al.* 2011; USFWS 2013). Few comparisons of the various DNA extraction methods have been reported. Smith *et al.* (2012) compared six commercially available DNA extraction kits for detection of clam eDNA (*Corbula amurensis*) from filtered water samples and found differences in both target DNA yield and purity of DNA extract. Detection frequency of Idaho giant salamanders (*Dicamptodon aterrimus*) in small streams ranged from 0% to 100%, depending on the DNA extraction method (Goldberg *et al.* 2011). Optimal extraction method also depends on species (Deiner *et al.* 2015). As extraction methods can impact the ability to detect and reliably measure eDNA, there is a need to evaluate which extraction methods are best for eDNA studies.

As eDNA studies typically have a goal of either detection or quantification, the choice of both capture and extraction methods are crucial and must be optimized towards the objective of the study. For instance, detection methods typically have sought to identify organisms at low densities and, thus, should be optimized for detection sensitivity. Therefore, detection studies should employ methods that optimize the yield (total eDNA recovered) of eDNA whereas the precision and accuracy of eDNA concentration is of less importance. In contrast, quantification may be most useful where populations are established. Consequently, for quantification, the measurement must be precise, and total yield might be of secondary importance. Quantification studies must emphasize consistent recovery of eDNA and the fit and slope of eDNA/biomass relationships to produce accurate and precise estimates of biomass. Inhibition can affect both aims as it lowers the perceived recovery of eDNA, thereby suppressing the ability to detect as well as leading to inaccurate quantification. Ideally, methods would be consistent across all eDNA studies, but in reality there are likely to be trade-offs between eDNA yield, consistent recovery, fit and slope of eDNA/biomass relationships, and inhibition.

This study evaluated capture and extraction methods for eDNA in a series of controlled laboratory experiments. Common carp (*Cyprinus carpio*, hereafter 'carp') was used as the target organism. This species has been widely used as a model organism in eDNA studies (Takahara *et al.* 2012; Mahon *et al.* 2013; Barnes *et al.* 2014; Turner *et al.* 2014). Precipitation, centrifugation and filtration were directly compared, and the effect of pore size and filter material was also evaluated. The range of pore sizes (0.2–5.0 μm) and filter materials [polycarbonate (PC) or glass fibre (GF)] tested was typical of previous studies. DNA from well and lake water stocked with carp in the lab was extracted with six commercially available DNA extraction kits, and DNA recovery, detection sensitivity, and inhibitor removal were evaluated. Each method was evaluated for its utility for detection and quantification of eDNA using quantitative PCR (qPCR) of a previously developed carp mitochondrial DNA marker, CarpCyt*b*, hereafter referred to as 'carp eDNA' (Eichmiller *et al.* 2014). Our results provide recommendations for optimal capture and extraction of eDNA for future laboratory studies and can be used to help guide choice of methods for field studies.

Materials and methods

Experiments were performed to examine eDNA capture and extraction methods for detection and quantification. Experiment 1 evaluated eDNA capture methods and had two components. Part 1A compared the yield and concentration of carp eDNA from precipitation, centrifugation and filtration capture methods. Part 1B examined the relationship of carp eDNA and biomass among precipitation and filtration using PC filters of pore sizes ranging from 0.2 to 5.0 μm. Centrifugation and the use of

depth filters made of GF have been typically used for detection purposes in eDNA studies (Jerde *et al.* 2011; USFWS 2013); therefore, GF filtration and centrifugation methods were not included in experiment 1B. Experiment 2 evaluated six widely used commercial extraction kits with regard to the yield of carp eDNA, extraction efficiency and degree of inhibition.

Experiment 1A: effect of capture method on eDNA yield and concentration

The carp eDNA yield (total number of copies recovered in the DNA extract) and estimation of carp eDNA concentration (copy number per volume of water sample) were compared across widely used capture methods, including precipitation, centrifugation and filtration through five different filters of varying pore sizes and materials. Filters consisted of PC filters of 0.2, 0.6, 1.0 and 5.0 μm pore size and a GF filter of 1.5 μm pore size (Table S1, Supporting information). Comparison of capture methods was conducted using a 400 L tank containing a high biomass (~2000 mg/L) of juvenile carp (average weight = 100 g) held under flow-through conditions at 18 °C. The tank was aerated, which promoted water mixing. Fish were fed once daily ad libitum 2.5 mm pellet feed (Oncor Fry, Skretting USA, Tooele, UT) that did not contain the genetic markers targeted in this study. All fish were held in accordance with the University of Minnesota's Institutional Animal Care and Use Committee (IACUC) (Protocol: 1302-30339A).

Water was sampled from the tank using 1-L HDPE bottles (Nalgene, Rochester, New York) from below the water's surface. Bottles were stored at 4 °C until eDNA was captured via precipitation, centrifugation or filtration. Six replicate samples were processed for each capture method. Bottles were randomly selected prior to eDNA capture. Capture of eDNA by precipitation followed the method of Ficetola *et al.* (2008). Briefly, 1.5 mL of 3 M sodium acetate and 33 mL absolute ethanol was added to each of six replicate 15 mL samples drawn from separate tank sample bottles, and the solutions were stored at −20 °C overnight. The sample was centrifuged at 5500 *g* for 35 min, the supernatant was discarded, and DNA was extracted from the remaining pellet. Capture of eDNA by centrifugation followed the Quality Assurance Project Plan (QAPP) for eDNA monitoring of Bighead and Silver Carps (USFWS 2013). Briefly, six replicate 50 mL samples drawn from separate tank sample bottles were centrifuged at 5000 *g* for 30 min, the supernatant was discarded, and the pellet was stored at −20 °C until extraction. For capture of eDNA by filtration, six tank water samples were added incrementally to separate filters placed within a polyphenylsulfone filter funnel (Pall Corporation, Port Washington, NY) until the filter clogged (Table 1). Filters were folded in half to minimize exposure of the filter surface, placed into a sterile tube, and stored at −20 °C. All eDNA capture methods were completed within 6 h of the tank water sampling. Laboratory equipment and bench surfaces were treated with 10% bleach for a minimum of 10 min to prevent cross-contamination.

All samples were extracted with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) using the protocol for human DNA analysis. This kit was chosen for experiments 1A and 1B, prior to the extraction kit comparison, as it contains inhibitor removal steps, and the protocol is designed for extraction of DNA from eukaryotic cells. For precipitation and centrifugation eDNA capture methods, the pellet was suspended in extraction buffer before proceeding with DNA extraction. For filtration methods, filters were sliced into small (1 × 3 mm) fractions with a sterile razor blade prior to placement in extraction buffer. Extraction control DNA (Ultrapure Salmon Sperm DNA Solution, 50 ng, Life Technologies,

Table 1 Overview of experiments and volumes processed per replicate

	Experiment		
Component	1A	1B	2
Methods compared	Capture	Capture	Extraction
Factor/s examined	eDNA yield and concentration	Biomass determination	eDNA yield, recovery variability, inhibitor removal
Tank volume (L)	400	40	120
Tank biomass (mg/L)	2000	25–150	5000
Volume processed (mL)*			
Precipitation	15	15	NA†
Centrifuge	50	NA	NA
Filtration, PC‡ 0.2 μm	300	300	NA
Filtration, PC 0.6 μm	500	600	50
Filtration, PC 1.0 μm	1000	1500	NA
Filtration, GF§ 1.5 μm	2000	NA	NA
Filtration, PC 5.0 μm	2000	4000	NA

*Filtration volumes determined by volume required to clog filter.
†Not applicable.
‡Hydrophilic polycarbonate track etch membrane.
§Glass microfiber filter.

Grand Island, NY) was added to the extraction buffer to adjust for variability in recovery of DNA as previously described (Haugland *et al.* 2005). Briefly, the concentration of carp eDNA was divided by the per cent recovery of extraction control DNA to reduce variability of replicate samples. DNA was eluted in 50 μL and stored at −20 °C until qPCR analysis for carp eDNA and the extraction control.

Experiment 1B: effect of eDNA capture method on the relationship between biomass and eDNA

Fish biomass in the natural environment is orders of magnitude lower than those typical of the laboratory setting; therefore, the biomass in experiment 1B was reduced to more accurately reflect levels found in the field. Juvenile carp (average weight = 3 g) were added to 80 L tanks at 18 °C at a density of 0, 1, 2 or 4 individuals per tank (biomass = 25–150 mg/L). Locations of individual fish and stocking levels were randomly assigned. Three replicate tanks were established for each density. Tanks were held in flow-through conditions for 1 week, the water was turned off 24 h prior to sampling to allow eDNA to accumulate, and tanks were aerated throughout the experiment to promote mixing. Fish were fed once daily with frozen brine shrimp (San Francisco Bay Brand, Newark, CA) equivalent to 1% body weight that did not contain the genetic markers targeted in this study.

Water was sampled from each tank with 1-L HDPE bottles (Nalgene, Rochester, New York) from below the water surface and stored at 4 °C until eDNA capture. For each tank, water was precipitated or filtered through PC membranes of 0.2, 0.6, 1.0 and 5.0 pore sizes, as described in experiment 1A. A total of three replicate samples per stocking level were processed for each capture method. The volume of water used for each method is shown in Table 1. DNA extraction and analysis was done as described for experiment 1A.

Experiment 2: comparison of commercial DNA extraction kits

The ability to extract carp eDNA was compared across six commercially available DNA extraction kits: (i) MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA), (ii) MoBio PowerWater DNA Isolation Kit (Carslbad, CA), (iii) MP Biomedicals FastDNA SPIN Kit (Santa Ana, CA), (iv) MP Biomedicals FastDNA SPIN Kit for Soil (Santa Ana, CA), (v) Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany), and (vi) Qiagen QIAamp DNA Stool Mini Kit (Hilden, Germany). Both the MoBio PowerWater DNA Isolation Kit and the Qiagen DNeasy Blood and Tissue Kit are prescribed in the QAPP for eDNA monitoring of Bighead and Silver Carps (USFWS 2013). The Qiagen QIAamp DNA Stool Mini Kit is similar to the Qiagen DNeasy Blood and Tissue Kit, but contains several modifications that may reduce the potential for inhibitor co-purification. The remaining kits are widely used in environmental microbiology applications for isolation of bacterial DNA from environmental samples.

To examine the potential effect of water chemistry on extraction kit performance, samples extracted from lake water and well water were compared. Lake water was collected in the fall from Casey Lake (45°1′22″ N, –93°0′35″ W), a small, shallow lake where carp had previously been eradicated. Lake water was collected approximately a month after leaf senescence, and the water was observably coloured, likely due to high amounts of humic substances or phenolic compounds leached from fallen leaves and known to inhibit PCR (Wilson 1997). The lake water was collected several hours prior to the experiment and was warmed to 18 °C, the temperature of the well water, prior to fish addition. Tanks were filled with 120 L of water and 4 juvenile carp (average weight = 150 g) were added to each tank for 8–16 h. One tank was prepared for each source water type, and the water was aerated to promote mixing. Fish were removed, and water was sampled from each tank from below the water surface using 1-L HDPE bottles (Nalgene, Rochester, New York).

For eDNA capture, 50 mL of tank water was filtered through a 0.6 μm pore size PC membrane. Filters were folded once, and stored at −20 °C. All tank water was filtered within 4 h, and bleach treatment of laboratory surfaces and equipment was used to prevent sample contamination. For both well and lake water, four replicate filters were extracted for each kit, except for the MoBio PowerWater DNA Isolation Kit where three replicate filters were extracted. Filters were sliced into small (1 × 3 mm) fractions with a sterile razor blade prior to extraction. Extraction control DNA was also added to compare the extraction efficiency between lake and well water. The extraction efficiency was calculated by dividing the copy number of extraction control DNA recovered at the end of extraction by the initial amount added to the extraction buffer, and it was expressed as a percentage (Haugland *et al.* 2005). The carp eDNA concentration was not adjusted for recovery of extraction control DNA. DNA was eluted in 50 μL of the final elution/storage buffer across all kits and stored at −20 °C until qPCR analysis for carp eDNA and the extraction control.

To test qPCR inhibition, a dilution series of DNA extracts were spiked with an internal amplification control (IAC) to test for suppression of the qPCR reaction. A plasmid containing a *Campylobacter* 16S sequence insert served as an IAC (Lund *et al.* 2004). The *Campylobacter*

16S sequence was not detectable in the DNA extract prior to IAC addition.

qPCR analysis

The genetic markers in this study were amplified using previously published protocols (Table S2, Supporting information). Standards were created by dilution of genomic DNA (extraction control) or cloning the target amplicon (CarpCyt*b* and IAC) using the StrataClone PCR kit (Stratagene, Santa Clara, CA) (Table S3, Supporting information). Standard DNA was quantified with the QuantiFluor-ST Fluorometer (Promega, Madison, WI) and a series of 5, 10-fold dilutions were used to create a standard curve. The assay limit of detection (LOD) was defined as the lowest copy number that all three replicate standards successfully amplified (Table S3, Supporting information). For the multiplex qPCR of carp eDNA (CarpCyt*b*) and extraction control DNA, both standards were combined prior to dilution. Reactions contained 12.5 μL iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA), primers and probes and template DNA in a final reaction volume of 25 μL. Reaction conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing and extension step at 60 °C for 1 min.

The template for the carp eDNA and extraction control multiplex assay consisted of 5 μL of DNA extract for experiments 1A and 1B. To confirm that inhibition was not present, 5 μL of extract from at least two replicates from each experiment was run at four dilutions (undiluted, 1/2, 1/5, 1/10) to confirm that the copy number of the CarpCyt*b* and the extraction control target remained consistent. For experiment 2, the volume of extract added to the reaction was reduced to 0.5 μL to overcome qPCR inhibition by dilution. To test for inhibition in the IAC assay, 5 μL of carp DNA extract was run at five dilutions (undiluted, 1/2, 1/5, 1/10 and 1/20). The template consisted of 6000 copies of IAC target that was added to the reaction. Reactions were considered inhibited if the Cq value (quantification cycle value) was significantly lower than the Cq value of the 6000 copy IAC standard.

Each qPCR run contained triplicate reactions of standards and samples. Triplicate reactions of no-template controls were included in each qPCR run to confirm that contamination by PCR product did not occur over the course of the run (Table S3, Supporting information). Amplifications were performed using the StepOnePlus Real-Time PCR System (Life Technologies, Grand Island, NY), and Cq values were automatically determined using the system software. Sample marker concentrations were calculated on a per-run basis.

Statistical analysis

For experiment 1A, a one-way analysis of variance (ANOVA) was used to compare extraction efficiency across capture methods, the amount of carp eDNA in the total DNA extract volume, and the concentration in experimental tanks. For experiment 1B, the effect of capture method on eDNA concentration in the highest biomass treatment was evaluated using ANOVA. Linear regression was used to examine the relationship of carp biomass and carp eDNA concentration. A linear model was used to examine whether filter pore size had an effect on the slope of the relationship. Slopes were statistically compared using contrasts, and *P*-values were adjusted for multiple comparisons using Bonferroni correction. The sample detection limit for precipitation and filtration methods was calculated based on the per cent recovery of the extraction control, the sample water volume that was processed per replicate, the proportion of total DNA extract that was run in each qPCR assay, and the qPCR assay limit of detection.

For experiment 2, Student's t-test was used to compare the extraction efficiency and carp eDNA concentration between lake water and well water for each extraction kit. Coefficient of variation (CV) of extraction efficiency was calculated across all samples extracted for each kit. ANOVA was used to compare the eDNA concentration and the detection sensitivity. The detection sensitivity was evaluated by calculating the maximum carp eDNA (in copies/assay) that was capable of being run in a single assay, which was calculated by multiplying the total eDNA yield per extract by the proportion of DNA extract that could be run without inhibiting the qPCR reaction.

Post-hoc comparisons for ANOVA were performed using Tukey's HSD. For tests other than linear regression, values of carp eDNA were log-transformed prior to analysis to reduce skewing. Comparison of slopes for experiment 1B was performed with R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2014, http://www.R-project.org). All other analyses were done with JMP, Version 10 (SAS Institute Inc., Cary, NC).

Results

Experiment 1A: effect of capture method on eDNA yield and concentration

All samples had quantifiable carp eDNA. There was no significant difference among capture methods in the extraction efficiency as measured by the recovery of the extraction control ($P = 0.78$). Yield (i.e. the total copies of

carp eDNA in the DNA extract) ranged from 2.0×10^3 to more than 5.0×10^6 copies (Fig. 1a). The 1.5 μm GF filter had the highest yield overall, followed by the 0.6, 1.0 and 5.0 μm PC filters. The GF filter performed well, most likely because a larger volume of water could be filtered before clogging of the filter matrix (Table 1). Centrifugation had a lower carp eDNA yield than all filter types, and precipitation had the lowest carp eDNA yield overall, with a 92% reduction in yield relative to centrifugation.

The estimated concentration of carp eDNA in tank water ranged from 4.0×10^3 to 6.0×10^4 copies/mL, and concentration depended on the eDNA capture method (Fig. 1b). Correction for recovery of the extraction control DNA decreased the coefficient of variation (CV) of carp eDNA concentration by an average of 3% for all but the 5.0 PC filter, which increased by 1%. All filters produced similar concentration estimates, with the exception of the 5.0 μm PC filter, which had an estimate approximately 25% lower than that of other filters. The estimate of carp eDNA concentration using centrifugation was similar to the 5.0 μm PC filter. Precipitation produced the lowest estimates of carp eDNA concentration, with an estimate that was 25% of the concentration of centrifugation and less than 10% of the filtration methods.

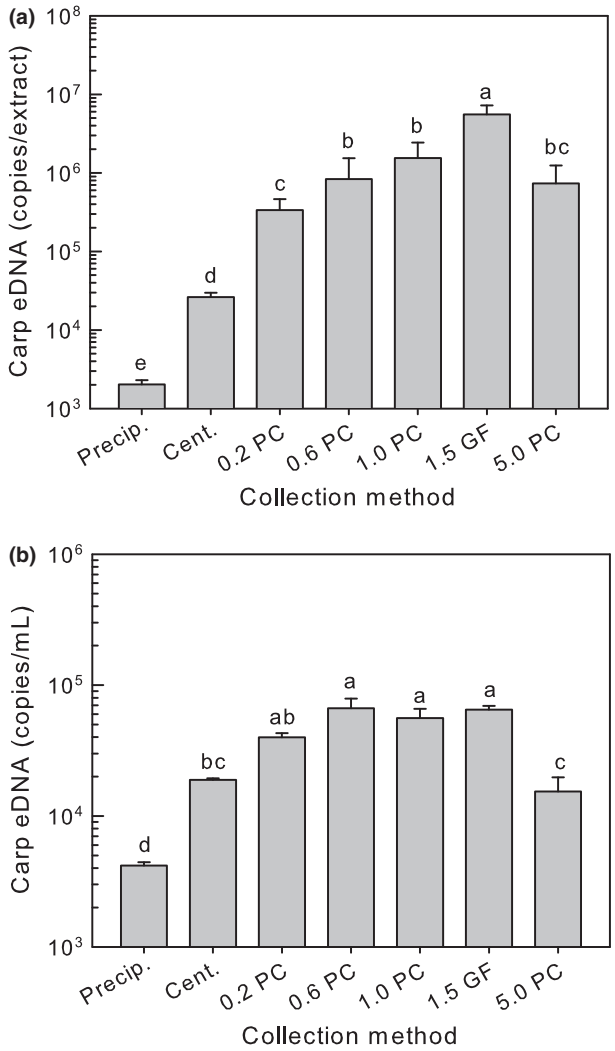

Fig. 1 The yield of carp eDNA for the total volume of DNA extract (a) and the concentration of carp eDNA in tank water adjusted for extraction efficiency (b) across eDNA capture methods. Error bars show 95% confidence intervals of the means. Letters indicate significant differences among means based on Tukey's test ($\alpha = 0.05$).

Experiment 1B: effect of eDNA capture method on the relationship between biomass and eDNA

No carp eDNA was detected in tanks that did not contain fish. The detection rate (proportion of samples that contained fish that were above the LOD) for all filter methods was 1.0, whereas the detection rate for the precipitation method was 0.3, as carp eDNA was only above the LOD in the four fish treatment. The sample detection limit for precipitation was 670 copies/mL, whereas the sample limit of detection ranged from 3 to 33 copies/mL for filtration methods due to the larger volume of water that was capable of being filtered. In tanks containing four fish, precipitation produced an average carp eDNA concentration of 2018 copies/mL (95% CI: 1602, 2541), which was lower than all estimates using filter methods ($P \leq 0.05$).

For filters of all pore sizes, the linear regression of carp biomass and carp eDNA concentration was significant (Fig. 2). The relationship of carp biomass and carp eDNA for 0.2, 0.6 and 5.0 μm pore sizes had similar R^2 values of approximately 0.86, whereas the 1.0 μm pore size had an R^2 of 0.71. Filter pore size affected the slope of the biomass/eDNA relationship ($P < 0.0001$). Slope decreased with increasing pore size; however, there was no significant change in slope from 0.2 to 0.6 μm ($P = 1.0$) or from 1.0 to 5.0 μm ($P = 0.9$) pore sizes. The slope of the biomass/eDNA relationship for the 0.2 and 0.6 μm filter pore sizes were two to four times higher than the slope of the relationship using the 1.0 and 5.0 μm filter pore sizes ($P \leq 0.03$).

Experiment 2: comparison of commercial DNA extraction kits

As the same volume of water was extracted across all kits, carp eDNA yield was proportional to the carp eDNA concentration. Carp eDNA yield varied across kits (Fig. 3). The CV of the carp eDNA concentration (within a kit and water source) was less than 5% for all kits, with the exception of the lake water samples extracted with

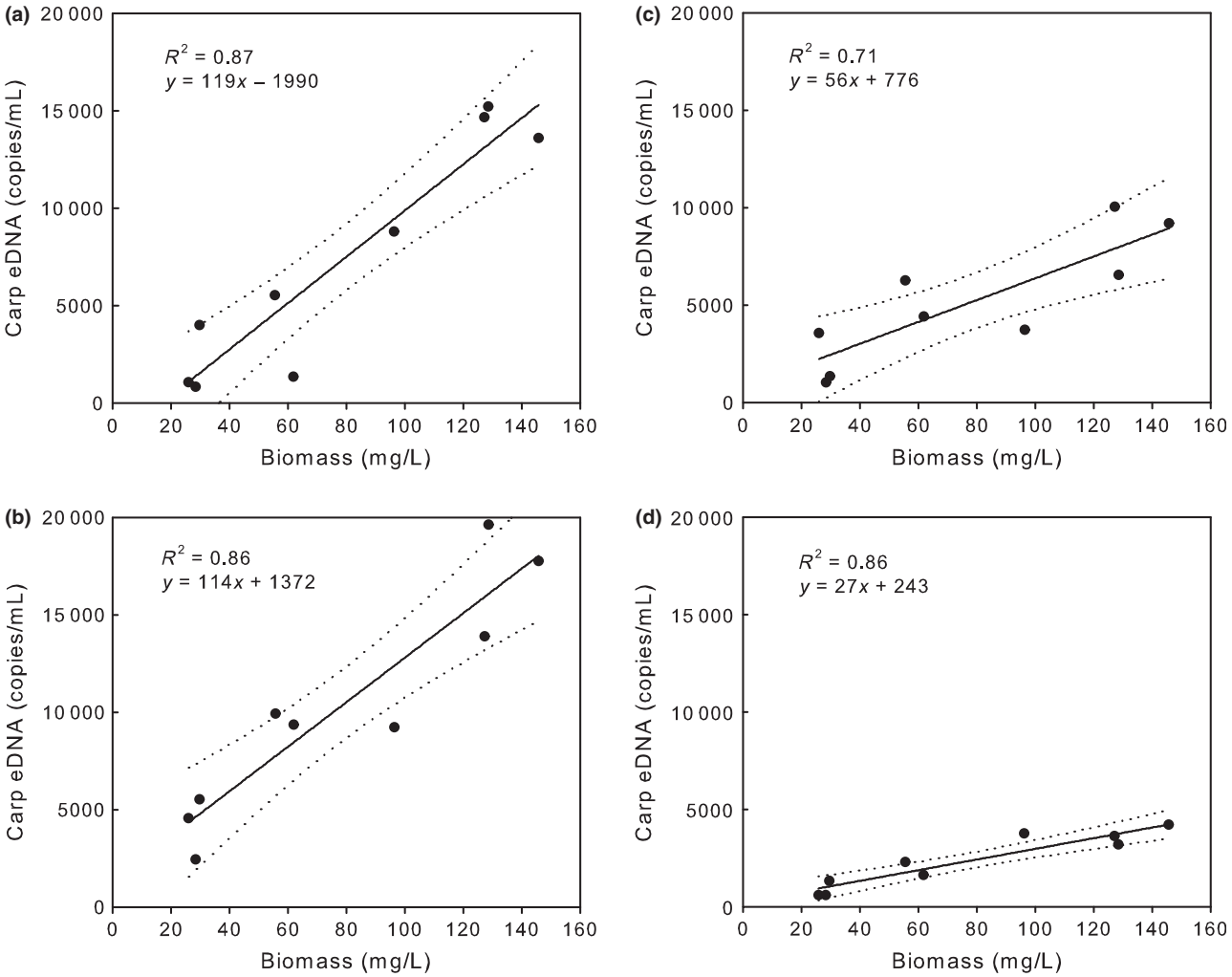

Fig. 2 The linear regression of carp biomass and carp eDNA for polycarbonate (PC) filters of pore sizes 0.2 μm (a) 0.6 μm (b) 1.0 μm (c) 5.0 μm (d). Dotted lines indicate 95% confidence intervals of the regression.

the MoBio PowerWater kit, which was nearly 20% due to the presence of an outlier. The MP Biomedicals FastDNA SPIN Kit yielded the most carp eDNA for well water, and it also had the highest yield for lake water, but the yield was not significantly higher than the MP Biomedicals FastDNA SPIN Kit for Soil and the Qiagen DNeasy Blood and Tissue Kit (Fig. 3). For well water, three kits had a higher yield of carp eDNA than the FastDNA SPIN Kit for Soil, whereas the DNeasy kit had the second highest yield for well water, outperforming four other kits. The MoBio PowerSoil DNA Isolation Kit had lower carp eDNA yield than the FastDNA SPIN Kit for lake water but did not differ significantly in yield from the other kits. The PowerSoil kit had the third highest yield for well water, outperforming half of the kits tested. The MoBio PowerWater DNA Isolation Kit and the Qiagen QIAamp Stool Mini Kit performed poorly overall. The PowerWater kit had the lowest yield for lake water, although, due to variation among replicates the yield was not significantly different than the PowerSoil and QIAamp Stool kits. The PowerWater kit also had lower yield than half the kits tested for well water. The QIAamp Stool kit had the lowest yield for well water, and it had the second lowest yield for lake water, although the yield was not significantly different than most of the kits tested.

The extraction efficiencies, measured by the recovery of extraction control DNA, of well and lake water were different for all kits ($P \leq 0.05$) (Table 2). Therefore, the CV in extraction efficiency was used to compare the robustness of the extraction efficiency across all replicates. The PowerSoil kit was most robust to differences in water source and the FastDNA SPIN Kit and the PowerWater kit were the least robust. The difference in carp eDNA concentration between lake and well water also varied among extraction kits (Table 2). As the PowerSoil

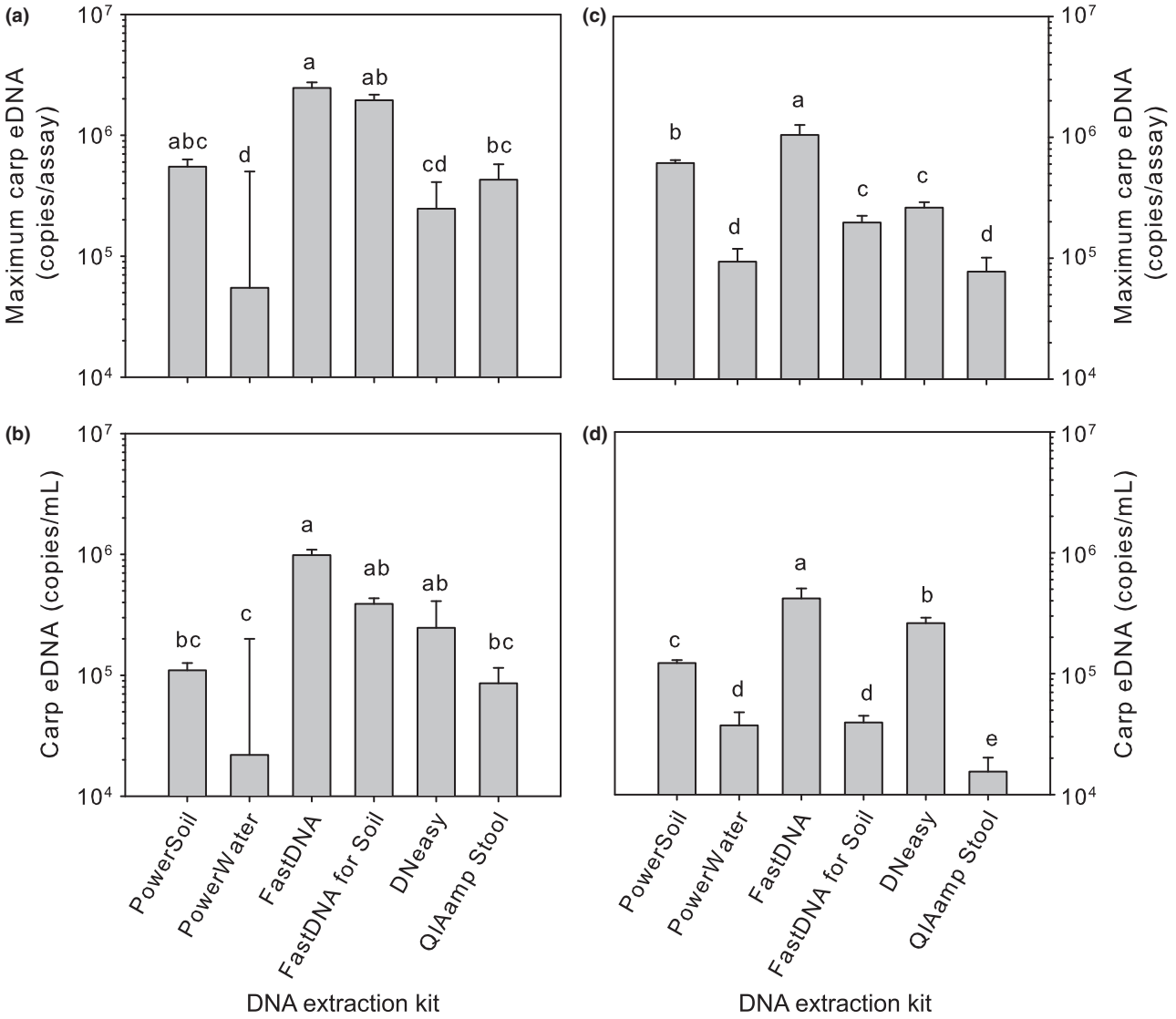

Fig. 3 The maximum carp eDNA copies per assay, a measure of detection sensitivity, for lake (a) and well (c) water, and the carp eDNA concentration for lake (b) and well (d) water. Error bars show 95% confidence intervals of the means. Letters indicate significant differences among means based on Tukey's test ($\alpha = 0.05$).

kit had the lowest variation in extraction efficiency between water types, this kit likely reflects the most accurate relative carp eDNA concentration between the lake and well water. Using the PowerSoil kit, well water contained 1.2×10^4 copies/mL more carp eDNA than lake water, but the difference was not significant ($P = 0.24$). Similar magnitude of difference and lack of statistical significance were observed for the PowerWater kit and the DNeasy kit. The remaining kits yielded 7.0×10^4 to 4.2×10^5 copies/mL more carp eDNA from lake water relative to well water, and the difference was significant ($P \leq 0.05$). Considering the large difference in extraction efficiency between well and lake water for these extraction kits, the observed difference in concentration using these kits may not be accurate.

Inhibition was observed in three kits: the PowerWater kit, the FastDNA SPIN Kit and the DNeasy kit (Table 2). The inhibition observed in the PowerWater and FastDNA SPIN Kit was slight and could be relieved by a 1/2 dilution of DNA extract. The DNeasy kit had the most inhibition, and inhibition was only relieved when extract volume was diluted 1/5. Due to the presence of inhibition, the total DNA yield was not a valid measure of sensitivity. Dilutions required to relieve inhibition affected the detection sensitivity of the kit. Taking into consideration the uncorrected DNA yield and the dilution

Table 2 Extraction efficiency, difference in estimate of carp eDNA for well and lake water samples, and extract dilution required for inhibitor release

Extraction kit	Extraction efficiency (%)		Extraction efficiency CV (%)*	Diff. carp eDNA Lake–Well (copies/mL)†	Extract dilution‡
	Lake	Well			
MoBio PowerSoil DNA Isolation Kit	58	48	15	-1.2×10^4	None
MoBio PowerWater DNA Isolation Kit	1.5	6.8	80	-1.5×10^4	1/2
MP Biomedicals FastDNA SPIN Kit	4.0	42	93	*5.7×10^5*	1/2
MP Biomedicals FastDNA SPIN Kit for Soil	1.9	0.6	57	*3.5×10^5*	None
Qiagen DNeasy Blood and Tissue Kit	27	63	42	-1.5×10^4	1/5
Qiagen QIAamp Stool Mini Kit	31	12	56	*7.0×10^4*	None

*Coefficient of variation.
†Statistical significance based on Student's T-test are indicated by normal ($P > 0.5$) or italicized ($P \leq 0.05$) text.
‡Lowest dilution at which inhibition was not observed. 'None' indicates that inhibition was not observed for undiluted extract.

required, a maximum copy number yield per reaction was calculated for each kit (Fig. 3). This value was a relative measure of the ability to detect carp eDNA. For lake water, the best detection sensitivity was from the FastDNA SPIN Kit, but the PowerSoil kit and the FastDNA SPIN Kit for Soil had comparable sensitivity. For well water, no kit performed as well as the FastDNA SPIN Kit, but the PowerSoil kit had the second highest sensitivity.

Discussion

Our results identified optimal eDNA capture and extraction methods for laboratory studies of eDNA for both detection and quantification. Capture method affected carp eDNA yield, concentration, and relationship to biomass. Likewise, DNA extraction method affected the carp eDNA yield, comparisons across water types and detection sensitivity. Of the methods tested in this study, optimal detection would be achieved by filtration through a 1.5 μm GF filter, followed by extraction with the MP Biomedicals FastDNA SPIN Kit. The 1.5 μm GF filter provided the best yield of DNA due to the large volume of water that could be filtered, and the FastDNA SPIN Kit yielded the highest amount of carp eDNA relative to five other commercial DNA extraction kits. Of the tested methods, those most suitable for quantification purposes include filtration through a 0.2–0.6 μm PC filter, followed by extraction with the MoBio PowerSoil DNA Isolation Kit. The 0.6 μm filter provided a slight advantage in detectability of carp eDNA relative to the 0.2 μm filter and was sensitive to changes in biomass, and the PowerSoil kit had the most robust extraction efficiency across all the extraction kits and did not exhibit qPCR inhibition.

Evaluation of eDNA capture methods for detection

The most important criteria for eDNA detection by PCR is yield, the total number of copies recovered in the DNA extract. In general, the larger the volume of water that was processed the higher the carp eDNA yield. The volume of water that could be processed with centrifugation and precipitation methods is inherently limited, which may have led to lower carp eDNA yield than filtration methods. The filter that produced the highest carp eDNA yield was the 1.5 μm GF filter. The GF filter can be classified as a 'depth filter,' which retains particles within the filter matrix as opposed to the PC membrane filters, which retain all particles larger than the pore size on the filter's surface. Depth filters are less likely to retain large particles, and are therefore also useful in applications where high total suspended solids would lead to rapid filter clogging of a membrane filter (Danielsson 1982; Morrison & Benoit 2001; Hutten 2007).

Centrifugation and precipitation methods for eDNA capture recovered less carp eDNA than filtration. Although not as commonly used as filtration, both centrifugation and precipitation have been used successfully by several research groups to detect eDNA (Ficetola *et al.* 2008; Dejean *et al.* 2012; Thomsen *et al.* 2012; Klymus *et al.* 2015). It is possible that for precipitation and centrifugation methods the pellet was not fully resuspended in the extraction buffer, but an alternative explanation is

DNA loss due to binding to polypropylene tubes used in the experiments. DNA binds readily to polypropylene and can lead to substantial DNA loss, especially at high ionic strength (Gaillard 1996; Gaillard & Strauss 1998). Therefore, sodium acetate would further promote DNA loss in the precipitation method. Due to the low recovery of carp eDNA, the use of precipitation and centrifugation for eDNA capture is not recommended, but these methods may be more successful if alternative materials are used (Gaillard 1996; Gaillard & Strauss 1998). An alternative precipitation method was recently used by Tréguier *et al.* (2014) and Biggs *et al.* (2015). The method calls for homogenization of 20, 40 mL water samples before 6, 15 mL subsamples are taken. The subsamples are precipitated and sequentially suspended in extraction buffer for a total of one extracted sample per site. Due to the larger volume of water per replicate (90 mL), it may be more sensitive for species detection.

The choice of capture methods for field applications is not always straightforward. Differences in water quality can affect the relative volume that may be able to pass through a filter, and cost and ease of sample processing are also a factor. For precipitation and centrifugation methods of capture, water samples are also easier to collect due to the lower sample volume needed. It is also possible that for water with a high amount of suspended particles that the water volume capacity of filtration methods may approach that of precipitation or centrifugation. However, Deiner *et al.* (2015) also found that filtration resulted in higher detection rate of macroorganisms in both lentic and lotic systems relative to precipitation, despite limiting the sample volume to 15 mL for both methods. Our results confirm that filtration is likely preferable for increasing detection probability.

The utility of environmental DNA capture methods for quantification

To successfully quantify eDNA, the relationship between eDNA concentration and organism biomass must be significant and exhibit good fit (R^2). A higher slope is also preferred, as it indicates that the method is more sensitive to changes in biomass. All filter methods satisfied the first two criteria. The slope of carp biomass and carp eDNA was inversely correlated with filter pore size. Therefore, at low filter pore sizes, the method was more sensitive to changes in biomass. Precipitation was not recommended for quantification of biomass outside of the lab setting as it was only able to detect carp eDNA at the highest biomass tested.

Only one other published study has directly compared filter pore size with respect to biomass estimation. Takahara *et al.* (2012) examined the relationship of biomass and eDNA in experimental ponds and concluded that 3.0 μm PC filters performed better than 0.8 μm PC filters. However, water filtered through the 0.8 μm filter was first passed through a 12 μm prefilter, which could potentially remove up to 20% of eDNA according to a study of particle size distribution of eDNA in a carp pond (Takahara *et al.* 2012; Turner *et al.* 2014). Turner *et al.* (2014) also showed that retention of eDNA was greatest at 0.2 μm, the smallest filter size, with an 85% retention of eDNA. Unfortunately, as in the present study, filtration using the 0.2 μm pore size led to rapid clogging of the filter membrane (Turner *et al.* 2014). Clogging led to a slightly lower yield of carp eDNA by the 0.2 μm filter compared to other sizes of PC filters in experiment 1A; therefore, the 0.6 μm filter may be considered an optimal balance between total yield and quantification efficacy.

Filtration though a 0.2–0.6 μm filter was optimal for biomass quantification in the laboratory, but it may be optimal for the field as well. Pilliod *et al.* (2013) was the only study to show a significant correlation between organism biomass and eDNA concentration in the field at natural densities (i.e. not stocked ponds). In their study, 0.45 μm pore sized cellulose nitrate filters were used to collect Rocky Mountain tailed frog (*Ascaphus montanus*) and Idaho giant salamander (*D. aterrimus*) eDNA. Using the same method, Spear *et al.* (2015) did not observe a significant biomass and eDNA relationship, but this was attributed to the difficulty in obtaining accurate estimates of biomass. The range of biomass tested in this study is typical of areas with large carp populations. The biomass of carp in shallow lakes of the upper Midwest has been shown to approach nearly 500 kg/ha (Bajer & Sorensen 2012), whereas densities of up to 3144 kg/ha have been reported in Australia (Driver *et al.* 1997). Therefore, a significant biomass/eDNA relationship may exist for areas with sufficiently large carp populations.

Comparison of commercial DNA extraction kits

The optimal DNA extraction method for detection must balance high yield with inhibition. For example, low to moderate yield may be acceptable if the extract is free of inhibitors, thereby enabling more extract to be added to each qPCR reaction. We found that there was a trade-off between carp eDNA yield and removal of inhibitors. For well water, kits that included 'inhibitor removal' steps, had lower yields than those that did not (MP Biomedicals FastDNA SPIN Kit and the Qiagen DNeasy Blood and Tissue Kit). The MoBio PowerSoil DNA Isolation Kit has been consistently shown to produce DNA extract that is free of inhibitors (Dineen *et al.* 2010; Mahmoudi *et al.* 2011; Smith *et al.* 2012). In contrast, MP Biomedicals

kits, including both the FastDNA SPIN Kit and FastDNA SPIN Kit for Soil, yielded the highest amount of DNA in some comparisons, but both kits also have been shown to co-purify inhibitors, requiring up to a 100-fold dilution to relieve inhibition (Dineen *et al.* 2010; Mahmoudi *et al.* 2011; Kennedy *et al.* 2014). In our study, the FastDNA SPIN Kit had a small degree of inhibition, but was the most sensitive for detection due to the high yield of carp eDNA.

Patterns in extraction control recovery did not reflect carp eDNA recovery. For example, the FastDNA SPIN Kit had the highest overall yield of carp eDNA, but it did not have the highest recovery of the extraction control. The extraction control was added prior to the DNA extraction as extracellular or 'free' DNA, whereas the carp eDNA is presumed to be a combination of free DNA and DNA within intact cells or organelles. Although a study of the particle size distribution of eDNA found that most eDNA is associated with particles greater than 0.2 μm (Turner *et al.* 2014), the eDNA may not necessarily be within intact cells or organelles and may be extracellular DNA bound to particulates. Although the per cent recovery of eDNA within cells and organelles is not known, ideally, recovery of DNA in both bound and extracellular forms should be consistent across sample types for accurate quantification. Therefore, for quantification purposes, the variability in extraction efficiency among water sources should be minimized.

All kits had a significant difference in extraction efficiency between well and lake water. Water quality can affect the recovery of DNA from water samples (Frostegård *et al.* 1999; Liang & Keeley 2013); however, the difference in carp eDNA recovery between lake and well water was inconsistent across kits. This indicates that choice of extraction kit can affect the interpretation and significance of results. The differences in extraction efficiencies across kits may be due to the specific thermal or mechanical lysis method prescribed by each manufacturer. However, the difference in extraction efficiency for well or lake water between kits from the same manufacturer ranged from 2% to 56%. Most of the downstream steps after lysis were relatively consistent within each manufacturer, so the variation was most likely due to differences in extraction buffer formulation. Regardless of the cause, kits that had low CV in extraction efficiency, such as the PowerSoil kit, may be presumed to have more consistent recovery of DNA from intact cells or mitochondria and are therefore more suitable for quantification.

The presence of inhibition can make accurate eDNA quantification problematic; therefore, the extraction method optimally will produce extract with no detectable inhibition. Half of the tested DNA extraction kits, including both MP Biomedicals kits and the DNeasy kit, exhibited some degree of qPCR inhibition for the lake water sample and are not recommended for quantification. The lake water sample was collected in the fall, after leaf senescence and the dark colouration of the water suggested a high amount of dissolved humic substances and phenolic compounds leached from leaf litter. Humic substances and phenolic compounds are the two most common inhibitors in environmental water samples (Wilson 1997). Although the volume of water that was filtered in this study was less than the typical amount filtered for most eDNA studies, less than a ng of humic or tannic acid has been shown to inhibit qPCR (Kontanis & Reed 2006; Green & Field 2012); therefore, it is important to test for inhibition in environmental samples.

Aside from optimization of DNA extraction, a change in the analysis platform, formulation of reaction mixture, use of a postextraction clean-up column, dilution or addition of PCR adjuvants can help to relieve inhibition during PCR. Digital PCR (dPCR) has been shown to be more resistant to inhibition than qPCR for analysis of clinical and environmental samples (Hoshino & Inagaki 2012; Sedlak *et al.* 2014). Dilution or the use of a postextraction nucleic acid clean-up column can alleviate the effect of inhibition to improve the accuracy of eDNA quantification (McKee *et al.* 2015). The use of reaction buffers formulated for environmental samples has also been shown to reduce the effects of inhibition (Cao *et al.* 2012; Jane *et al.* 2014). Finally, PCR adjuvents, such as bovine serum albumin, can relieve inhibition (Kreader 1996; Wilson 1997). Although there are several options to deal with inhibition postextraction, it is preferable to minimize copurification of inhibitors, especially if large volumes of water will be filtered or if dilution will substantially impact the limit of detection.

Conclusions

The results of this study suggest that eDNA capture and extraction techniques must be optimized to meet goals of detection or quantification for laboratory studies. For filtration capture methods, there was a trade-off between efficient capture of carp eDNA at small pore sizes and improved detection capability at larger pore sizes. Precipitation and centrifugation capture methods cannot be recommended due to low eDNA recovery. For DNA extraction, there was a trade-off between carp eDNA yield and effective removal of inhibitors. It remains unclear whether the patterns we observed in the lab setting would hold for natural environments, as laboratory tanks lack many features of natural systems in physical and biological community structure. However, these results reflect optimal methods for eDNA capture and extraction of common carp eDNA in laboratory applica-

tions. Moreover, our results are relevant to other invasive carps and potentially most cyprinids, the largest family of fishes. These results can be used to help narrow the subset of methods to compare for field applications. Although use of eDNA for fisheries management has focused primarily on the monitoring of invasive bighead and silver carps, undoubtedly, eDNA will increasingly be used for routine detection and quantification of fishes. These results provide a step forward in the development of best practices for eDNA sample collection and processing.

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Data accessibility

The copy number of individual samples for experiments 1–3, fish biomass of each tank for experiment 2, individual sample extraction efficiencies for experiment 3, and qPCR standard curves are archived in DRYAD as doi:10.5061/dryad.2f681.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Manufacturer information for materials used in this study.

Table S2 Primers and probe used for quantitative PCR.

Table S3 Quantitative PCR calibration data.