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At the forefront: evidence of the applicability of using environmental DNA to quantify the abundance of fish populations in natural lentic waters with additional sampling considerations

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At the forefront: evidence of the applicability of using environmental DNA to quantify the abundance of fish populations in natural lentic waters with additional sampling considerations

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<A> Abstract

Environmental DNA (eDNA) sampling has proven to be a valuable tool for detecting species in aquatic ecosystems. Within this rapidly evolving field, a promising application is the ability to obtain quantitative estimates of relative species abundance based on eDNA concentration rather than traditionally labor-intensive methods. We investigated the relationship between eDNA concentration and arctic char (*Salvelinus alpinus*) abundance in five well-studied natural lakes, and additionally, we examined the effects of different temporal (e.g., season) and spatial (e.g., depth) scales on eDNA concentration. Concentrations of eDNA were linearly correlated with char population estimates ($R^2_{adj} = 0.78$) and exponentially correlated with char densities ($R^2_{adj} =$ 0.96 by area; 0.82 by volume). Across lakes, eDNA concentrations were greater and more homogeneous in the water column during mixis; however, when stratified, eDNA concentrations were greater in the hypolimnion. Overall, our findings demonstrate that eDNA techniques can produce effective estimates of relative fish abundance in natural lakes. These findings can guide future studies to improve and expand eDNA methods while informing research and management using rapid and minimally invasive sampling.

<A> Introduction

Reliable estimates of fish abundance are necessary for making effective conservation and management decisions (Dudgeon et al. 2005). However, obtaining these estimates can be expensive and time consuming, and often requires multiple sampling events (Jerde et al. 2011). Until recently, describing fish populations, even at the presence/absence level, required invasive methods (e.g., gill nets, electrofishing), and these methods can be ineffective or harmful for certain habitats or species, and overall costly and laborious (McDonald 2004). Environmental DNA (eDNA) is increasingly being used as a tool to detect fishes in a more efficient, non-invasive manner (Barnes and Turner 2016; Wilcox et al. 2016). In aquatic systems, organisms release DNA into the environment via life processes (e.g., feces, skin cells, carcasses), and molecular techniques can detect this genetic material from water samples (Ficetola et al. 2008).

Methodologies of eDNA sampling are rapidly evolving and improving (e.g., Furlan et al. 2015), especially with regard to species detection. A next logical step towards advancing eDNA techniques would be to achieve estimates of fish abundance and biomass. Accordingly, there is growing evidence that relates eDNA concentration (e.g., qPCR copies$\cdot L^{-1}$) to fish abundance and/or biomass in laboratory settings (e.g., Klymus et al. 2015) and lotic systems (e.g., Baldigo et al. 2017). However, there is little known about the effectiveness of this application in natural lentic waters In lakes, the distribution and concentration of eDNA likely varies as a function of processes that affect DNA directly (e.g., degradation due to temperature, light, pH; Strickler et al. 2015) or indirectly via lake physical characteristics that can alter species distribution (e.g., temperature; Takahara et al. 2012) or biological activity that can affect eDNA production (e.g., spawning; Barnes and Turner 2016). Further, with these considerations, location (e.g., spatially, depth of sample) and timing (e.g., season) of sampling is an important consideration in lakes,

especially if an estimate of fish abundance is the goal. Here we monopolize on long-term study lakes to verify the application of eDNA to quantify relative abundance of arctic char (*Salvelinus alpinus*) in lakes of northern Alaska and examine differences in detection probability and eDNA copy number concentration (hereafter, eDNA concentration) across sites, depth, and season.

<A> Methods

In July and September 2016, we collected depth-specific water samples at spatially-explicit sites in each of five lakes (Lakes E5, Fog1, Fog2, Fog3, and Fog5) near Toolik Field Station, North Slope, Alaska (Table 1). Additional study site information can be found in Budy and Luecke (2014; see also http://arc-lter.ecosystems.mbl.edu/). All lakes except Lake Fog5 (only two sites) were divided into quadrants and sites were approximately even-spaced with one site in each quadrant. Each site included a shallow (1.0 m) and deep (approximately 2.0 – 3.0 m from lake bottom) sampling depth (see Table 1). During July, the lakes were thermally stratified, while in September, the lakes were isothermal. These lakes are part of the Arctic Long-Term Ecological Research site (http://arc-lter.ecosystems.mbl.edu/), and their fish communities are known to contain only arctic char and slimy sculpin (*Cottus cognatus*). We have conducted extensive fish sampling via traditional methods (i.e., gill nets, hook-and-line) to quantify population abundance in each lake using mark-recapture techniques (e.g., Budy and Luecke 2014; Table 2). For lakes where the times series was >5 years (Lakes E5, Fog1, Fog2), we estimated abundance using a Huggins closed-capture model in Program MARK (White and Burnham 1999). For Lakes Fog3 and Fog5, with shorter mark recapture time series, we used a modified Schnabel estimate (Krebs 1999). Overall, arctic char abundance is relatively low across all lakes, but follows a natural gradient from relatively low to relatively high density. For example, our abundance and density by volume estimates span greater than an order of

magnitude (see Table 2), such that these lakes provide an excellent template to investigate relationships of eDNA concentration and fish abundance.

To sample for eDNA, at each site, we filtered lake water through vinyl tubing lowered to shallow and deep depths using an in-line peristaltic pump (GeoTech Environmental Equipment, Inc: GeoPump). We used 25 mm nylon net filters with 10 µm pore size, housed in a sterile luer-lock filter holder, and filtered a measured amount of lake water (usually 5L). We used the specific amount of water filtered for each sample to correct for eDNA concentration (e.g., copies·L^{-1}). We also carried 1L of distilled water into the field, and filtered this sample using a clean collection hose to serve as a collection negative control. Between lakes, all equipment was sterilized using 10% bleach solution. Prior to attaching filter holders, we flushed the hoses with lake water to remove bleach residue, and also flushed hoses before starting a new site within the same lake. After filtering, we placed intact filter holders, double-bagged, on ice in a dark container until storage at -80 °C at the field station. We shipped frozen samples overnight from the field station to the Molecular Ecology Lab at Utah State University for DNA extraction and qPCR analyses.

eDNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, California). Filters were incubated in 360 µL buffer ATL and 40 µL proteinase K for one hour at 56 °C, with vortexing every 15 minutes. Then, 300 µL buffer AT was added, followed by 300 µL 99% ethanol. Extractions then proceeded following the manufacturers recommendations, with a final elution volume of 100 µL. Each round of extractions included a blank negative control that was later run in qPCR to test for contamination.

Quantitative PCR (qPCR) reactions for arctic char eDNA detection and quantification were carried out using species specific primers and Taqman® Minor Groove Binding probe

targeting 145 bp of the mitochondrial gene *cytochrome b* as described in Rodgers et al. (2017). All samples were initially run in triplicate. For a subset of samples that did not show amplification in the first 3 qPCR replicates (n=10), an additional 3 replicates were run. qPCR reactions were run on an Applied Biosystems QuantStudio three thermocycler (Foster City, California). Each reaction included 7.5 µL Taqman® Environmental Master Mix (Thermo-Fisher; Waltham, MA), 100nM of forward primer, 600nM of reverse primer, 250nM of Taqman® MGB probe, and 4 µL of template DNA in a total reaction volume of 15 µL. Additionally, each reaction included a VIC labeled Taqman® exogenous internal positive control (Life Technologies, Grand Island, NY) to monitor for PCR inhibition. Samples that showed signs of inhibition (6 samples all from July) were treated with Gene Releaser (Bioventures inc., Murfreesboro, TN) to remove inhibitors, and re-run. qPCR thermal cycling conditions were 10 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. All qPCR runs included a minimum of 3 no-template negative controls to test for contamination.

For quantification, each qPCR run included a 5-step, 5-fold standard curve run in triplicate. This standard curve was constructed from a serial dilution of a MiniGene plasmid ordered from Integrated DNA Technologies (Coralville, Iowa, USA) containing the target sequence. The plasmid was suspended in 100 µL of IDTE (10 mM Tris, 0.1 mM EDTA) buffer, linearized by digestion with the enzyme *Pvu1*, and then purified with a PureLink PCR Micro Kit (Thermo-Fisher; Waltham, MA) following manufacturer protocol. The product was then quantified and diluted to create reactions of 10, 50, 250, 1250, and 6250 copies for the standard curve. Resulting copy number quantities for each qPCR reaction were converted to eDNA copies per liter of lake water for further analyses, taking into account the number of qPCR replicates that amplified for each sample. Briefly, this conversion was accomplished by multiplying the

number of DNA copies per qPCR reaction by the proportion of the total extraction volume run in each qPCR reaction, divided by the water filtration volume. As arctic char are known to occupy all study lakes, we calculated detection probability as the percentage of samples that detected char DNA for each sampling event and depth. Across lakes, we used paired Student's t-tests to compare eDNA concentration between seasons and depths and we used linear regression models (fit through the origin) to test for a relationship between eDNA concentration and fish abundance. We assessed model fit by evaluating residual-expected value plots and log-transformed eDNA concentrations when necessary to improve fit and appropriately describe the observed relationship (e.g., density by area, density by volume). Due to a relatively low sample size, we compared relationships using adjusted R^2. We used R statistical package (version 3.3.2; R Development Core Team, 2016) for all analyses.

<A>Results

We collected a total of 38 eDNA samples across all lakes in both July (stratified) and September (mixis). Across all samples, mean eDNA concentrations (copies·L^{-1} ± 2se) were greater in September than in July (78.26 ± 69.71 vs. 9.38 ± 7.87; t = 1.96, df = 37, p = 0.05; Figure 1), and while variable, eDNA concentrations were generally greater in the deep samples relative to the shallow samples in July (15.77 ± 14.94 vs. 3.00 ± 3.63; t = 1.66, df = 18, p = 0.11) but not September (84.05 ± 101.84 vs. 72.46 ± 97.95; t = 0.17, df = 18, p = 0.87).

When pooled across all sites, depths, and lakes, eDNA concentrations were highly correlated with fish abundance (total individuals; R^2_{adj} = 0.78, $F(1,4)$ = 18.40, p = 0.01) as well as density by area (fish·ha^{-1}; R^2_{adj} = 0.96, $F(1,4)$ = 118.1, p < 0.001) and density by volume (fish·m^{-3}) (R^2_{adj} = 0.82, $F(1,4)$ = 23.17, p < 0.01; Figure 2, Table 3). Lake-specific eDNA

168 concentrations (copies·L^{-1} ± 2se) were variable across sites and depths (73.25 ± 114.89, 22.93 ±
169 27.02, 3.70 ± 4.98, 97.17 ± 120.69, 14.80 ± 14.12 for Lakes E5, Fog1, Fog2, Fog3, and Fog5,
170 respectively). Detection probabilities between depths varied between sampling periods with the
171 greatest detection probability in the deeper depths during July, when the lakes were stratified
172 (63.2% deep samples vs. 21.1% shallow samples). In September, there was little difference in
173 detection probabilities between the shallow and deep samples (57.9% deep samples vs. 52.6%
174 shallow samples).

175 <A> Discussion

176 Our results add to the limited body of knowledge for quantification of fish abundance in
177 natural lentic systems using eDNA. To our knowledge, no other study has related eDNA
178 concentration to lake-wide population estimates of fish abundance under natural conditions,
179 though others have come to similar conclusions for other metrics of abundance and biomass
180 (e.g., catch-per-unit-effort; Lacoursiere-Roussel et al. 2016). Our study lakes were ideal for
181 addressing this as they are relatively small and closed to emigration and immigration of fishes,
182 with simple and well-known fish communities (e.g., only two species). On the North Slope,
183 Alaska, similar lakes are extremely abundant and can comprise up to 48% of the landscape's
184 surface. While obtaining lake-specific population estimates for each lake would be logistically
185 challenging and time consuming, we provide a first attempt towards assessing relative abundance
186 of fishes in lesser studied lakes using this study as a baseline. Future work should address the
187 spatial extent of relationships between eDNA concentration and fish abundance across a broader
188 landscape (e.g., multiple watersheds) where environmental variability could be greater.

189 In our study, natural fish abundance is relatively low, and thus, mean eDNA
190 concentrations and detection probabilities were unsurprisingly also relatively low. Ensuring

191 sufficient detection across sites, depths, and lakes with known fish community species diversity
192 and abundance can require significant sample volumes. Further, reducing the number of false-
193 negatives would likely result in a dramatic reduction in variability across sites, depths, and lakes.
194 However, in our study, during only one sampling period, at one lake, did we fail to detect arctic
195 char (Lake Fog2 in July). Fish density in Lake Fog2 is five-fold lower than the next lowest (Lake
196 Fog5) across our study lakes (~21 fish·ha^{-1} vs. ~104 fish·ha^{-1}). To achieve near 100% detection
197 probabilities, the minimum volume of water for a single sample using our sampling method
198 would be 25 - 30 L for Lake E5, Fog1, Fog3, and Fog5, while Lake Fog2 would require greater
199 than 40 L (based on the total volume of false negatives from a given lake). Other studies have
200 used much smaller sample volumes to achieve reasonable detection probabilities, but fish
201 abundance in those studies was also much greater (e.g., Baldigo et al. 2017). Further, filter type
202 and pore size can affect eDNA capture, which could potentially decrease the total volume
203 required in our study lakes (Barnes and Turner 2016). To build upon our work here, future
204 studies that aim to estimate fish abundance from eDNA concentration should further consider
205 necessary sample size (e.g., spatially, volume filtered per sample) and equipment to best achieve
206 these relationships and reduce overall variability, especially in larger lentic systems or with
207 greater fish densities when concentration-abundance relationships may not be exponential.

208 Various factors could influence differences of eDNA concentration and detection
209 probability between sampling periods and sampling depths in this study. Across high latitudes
210 regions, including the North Slope, Alaska, summer 2016 was the warmest on recent record,
211 such that epilimnetic temperatures during July (18 – 20 °C) were greater than the thermal
212 optimum for arctic char (15.2 °C; Lyytikäinen et al. 1997). In contrast, in average years,
213 epilimnetic temperatures rarely exceed this optimal temperature (Luecke et al. 2014). Water

temperatures in July 2016 likely limited thermal habitat for arctic char above the thermocline, such that much of their time was spent in deeper water. Thus, it is reasonable to expect concentrations of eDNA to be higher in samples from deeper depths. With epilimnetic water temperatures in July 2016 approaching 20 °C (Table 1), degradation of DNA due to direct and indirect temperature effects (e.g., microbial metabolism) could also limit the total amount of genetic material in epilimnetic waters. Additionally, in lentic systems, settling of genetic material can result in eDNA concentration in deeper the water column (e.g., Turner et al. 2015). Others have observed increased rates of DNA degradation at similar temperatures (Stickler et al. 2015). Degradation due to UV-B exposure could further decrease July epilimnetic eDNA concentrations relative to: 1) July hypolimnetic concentrations (e.g., less photoexposure); and, 2) September epilimnetic eDNA concentrations (e.g., shorter day length). In Arctic regions during July, there are 24-hrs of daylight, while average day length during our September sampling period was approximately 14 hrs. In September, the entire water column was recently mixed and cooler overall (isothermal) which: 1) allows char to move more freely throughout the lake; 2) decreases the rate of degradation of genetic material; and, 3) could re-suspend eDNA that was concentrated in deeper depths throughout the summer period. While we cannot parse these effects in our current study, the increased and more homogeneous eDNA concentrations we observed during September are likely interactions of physical and biological factors. Overall, when considering physical (e.g., stratification) and biological (e.g., species' temperature preference) factors, we demonstrate that autumn is better than summer to sample these type of oligotrophic, monomictic lakes for fish eDNA.

Overall, for eDNA studies, there is limited information in regard to sampling depth for natural, true lentic waters. We demonstrated that thermal stratification can affect eDNA

concentrations between stratified and isothermal periods (e.g., higher eDNA concentrations in deep samples during the summer). For species detection, many 'early' eDNA studies used surface samples (e.g., Jerde et al. 2011 in lotic systems), while others have sampled during isothermal periods to decrease heterogeneity across depths, but without comparison to a stratified period (Lacoursiere-Roussel et al. 2016). Eichmiller et al. (2014) found no difference between surface and subsurface samples in Lake Staring, Minnesota, but sub-surface sampling depths were less than 1 m deeper than the surface. In contrast to our findings, in a controlled lentic setting, African jewelfish were more readily detected from surface water samples than samples taken from the bottom, even though these fishes were located most often near the bottom (Moyer et al. 2014). However, these controlled systems were much smaller, shallower, and warmer, with greater fish densities than the natural Arctic lakes in our study. In deep, natural lakes, especially those that thermally stratify, understanding seasonal depth-specific concentrations is important for future studies and effective sampling design. Nonetheless, due to the remote location of these lakes, a rapid, non-invasive method of assessing relative abundance will allow us to address pressing ecological questions (e.g., lake connectivity) and be important for helping to guide subsistence fishing, as well as larger-scale monitoring of population persistence, especially in a changing climate.

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<A> Tables

Table 1. Summary of five northern Alaska study lakes and conditions during each eDNA sampling period in 2016.

Lake	Latitude	Longitude	Surface area (ha)	Maximum depth (m)	Mean depth (m)	No. of sampling sites	Depth of deep sample (m)	July shallow water temp (°C)	July deep water temp (°C)	Sept. shallow water temp (°C)	Sept. deep water temp (°C)
E5	68.642	-149.458	10.9	12.9	6.3	4.0	8.0	14.7	6.0	5.6	5.6
Fog1	68.684	-149.082	3.5	19.7	8.4	4.0	10.0	19.0	4.8	7.4	7.3
Fog2	68.679	-149.091	5.9	19.8	7.8	4.0	10.0	18.1	5.2	7.4	7.3
Fog3	68.673	-149.088	3.9	21.0	7.9	4.0	10.0	18.8	4.8	6.7	6.6
Fog5	68.678	-149.065	0.7	9.9	3.5	2.0	6.0 or 7.0	14.4	5.5	5.7	5.7

Table 2. Summary of abundance (number of fish), density by area (fish·ha^{-1}), and density by volume (10^{-3}; fish·m^{-3}) estimates for arctic char (*Salvelinus alpinus*) populations in five study lakes in northern Alaska. Values in parentheses represent lower and upper 95% confidence intervals for each estimate.

Lake	Abundance	Density by area	Density by volume	Time series start	Abundance estimate method
E5	1207 (987 - 1476)	111 (91 - 136)	1.7 (1.4 - 2.1)	1999	Huggins
Fog1	448 (290 - 693)	127 (82 - 197)	1.6 (1.0 - 2.4)	2011	Huggins
Fog2	163 (105 - 288)	29 (19 - 51)	0.3 (0.2 - 0.7)	2007	Huggins
Fog3	666 (477 - 1073)	171 (123 - 276)	2.2 (1.6 - 3.5)	2013	Schnabel
Fog5	75 (55 - 119)	104 (76 - 164)	3.0 (2.2 - 4.7)	2014	Schnabel

380 **Table 3.** Summary statistics of linear models fit to predict eDNA concentration from known metrics of relative fish abundance across
381 five lakes in northern Alaska. Bold text signifies the significant relationship that are presented in Figure 2.

Predictor	Response	β	*SE*	*p*	R^2_{adj}
Fish abundance	eDNA copies	0.077	0.018	**0.01**	**0.78**
	log(eDNA copies)	0.005	0.001	0.01	0.77
Fish density (by area)	eDNA copies	0.422	0.106	0.02	0.75
	log(eDNA copies)	0.028	0.003	**<0.001**	**0.96**
Fish density (by volume)	eDNA copies	21967	9108	0.07	0.49
	log(eDNA copies)	1611	335	**<0.01**	**0.82**

382 <A> Figure Captions

383

384 Figure 1. Mean eDNA copies (L^{-1}) for all lakes pooled (E5, Fog1, Fog2, Fog3, Fog5) by season

385 (white = July 2016, gray = September 2016) for shallow and deep samples. Combined represents

386 the overall mean between shallow and deep depths. Error bars represent standard error.

387

388 Figure 2. Relationships between A) fish abundance (top), B) density by area (bottom left), and C)

389 density by volume (bottom right) and mean eDNA concentration (copies·L^{-1}) across five study

390 lakes in northern Alaska sampled in 2016. Note: density by area and density by volume

391 relationships are back-transformed from log(eDNA concentration).

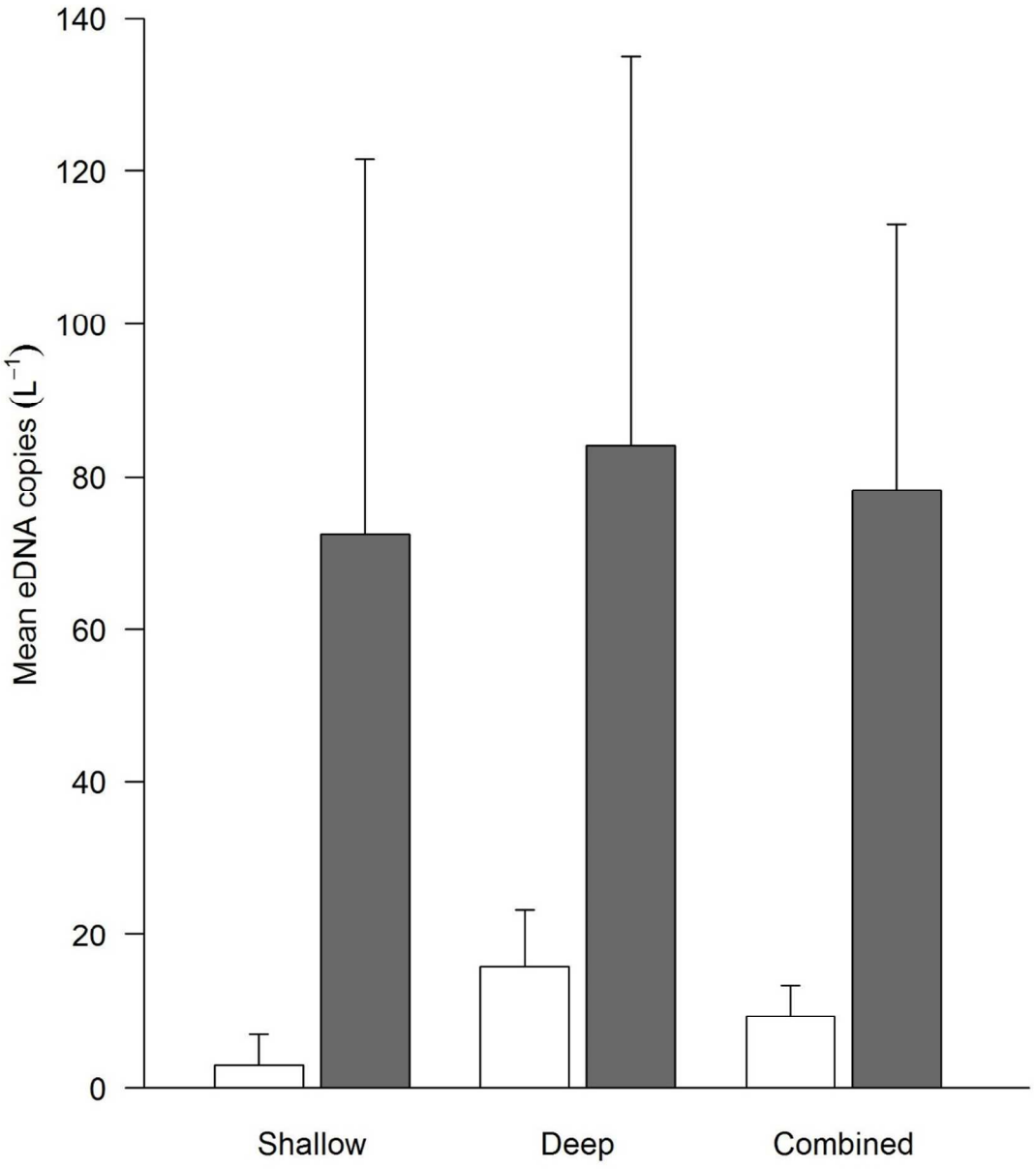
140
120
100
80
60
40
20
0
Mean eDNA copies (L^{-1})
Shallow
Deep
Combined

Figure 1.

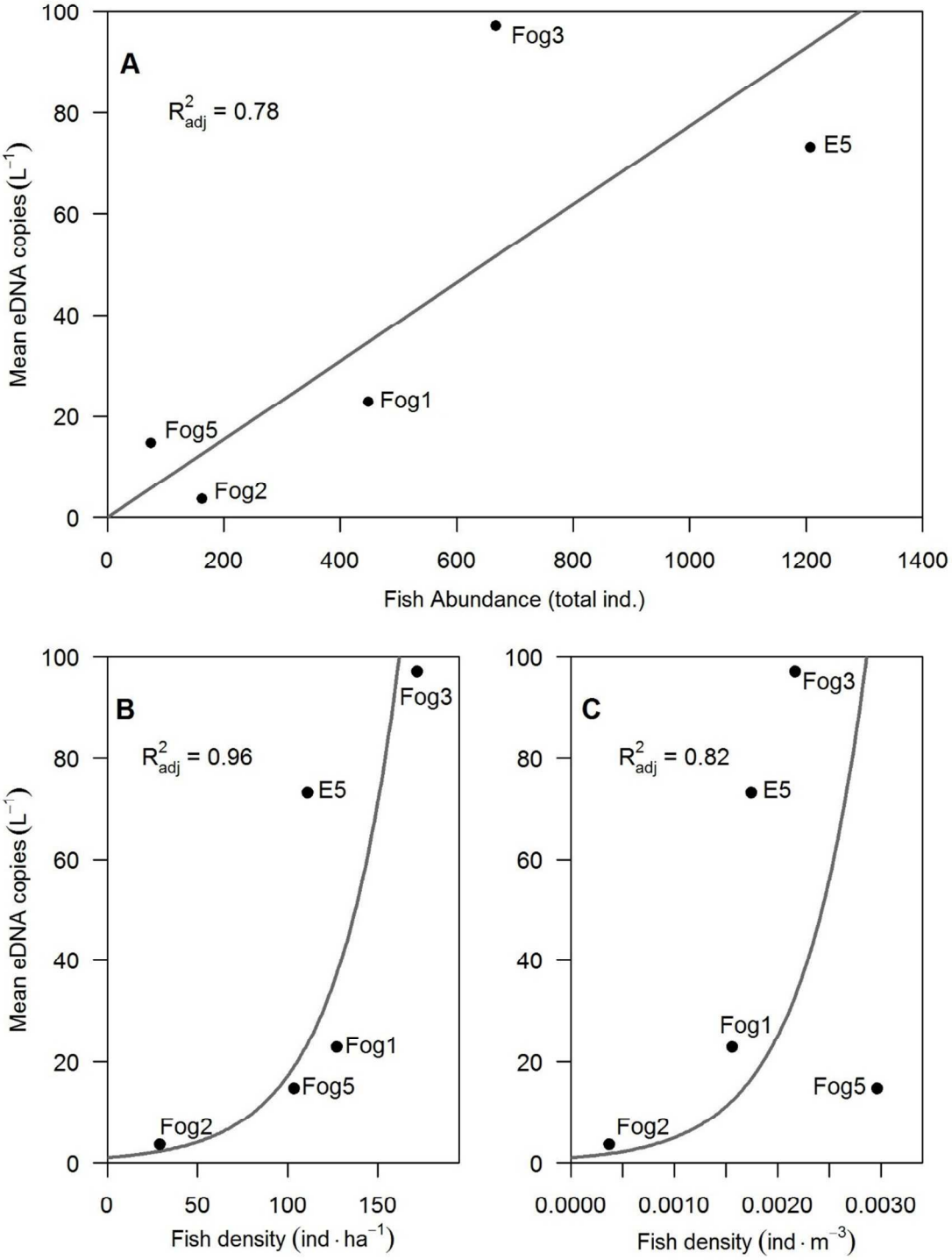
A
R^2_{adj} = 0.78
Fog3
E5
Fog1
Fog5
Fog2
Mean eDNA copies (L^{-1})
Fish Abundance (total ind.)
B
R^2_{adj} = 0.96
Fog3
E5
Fog1
Fog5
Fog2
Mean eDNA copies (L^{-1})
Fish density ($ind \cdot ha^{-1}$)
C
R^2_{adj} = 0.82
Fog3
E5
Fog1
Fog5
Fog2
Fish density ($ind \cdot m^{-3}$)

Figure 2.