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.

54 <A> Introduction

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

65 Methodologies of eDNA sampling are rapidly evolving and improving (e.g., Furlan et al.
66 2015), especially with regard to species detection. A next logical step towards advancing eDNA
67 techniques would be to achieve estimates of fish abundance and biomass. Accordingly, there is
68 growing evidence that relates eDNA concentration (e.g., qPCR copies$\cdot L^{-1}$) to fish abundance
69 and/or biomass in laboratory settings (e.g., Klymus et al. 2015) and lotic systems (e.g., Baldigo
70 et al. 2017). However, there is little known about the effectiveness of this application in natural
71 lentic waters In lakes, the distribution and concentration of eDNA likely varies as a function of
72 processes that affect DNA directly (e.g., degradation due to temperature, light, pH; Strickler et
73 al. 2015) or indirectly via lake physical characteristics that can alter species distribution (e.g.,
74 temperature; Takahara et al. 2012) or biological activity that can affect eDNA production (e.g.,
75 spawning; Barnes and Turner 2016). Further, with these considerations, location (e.g., spatially,
76 depth of sample) and timing (e.g., season) of sampling is an important consideration in lakes,

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

81 <A> Methods

82 In July and September 2016, we collected depth-specific water samples at spatially-
83 explicit sites in each of five lakes (Lakes E5, Fog1, Fog2, Fog3, and Fog5) near Toolik Field
84 Station, North Slope, Alaska (Table 1). Additional study site information can be found in Budy
85 and Luecke (2014; see also http://arc-lter.ecosystems.mbl.edu/). All lakes except Lake Fog5
86 (only two sites) were divided into quadrants and sites were approximately even-spaced with one
87 site in each quadrant. Each site included a shallow (1.0 m) and deep (approximately 2.0 – 3.0 m
88 from lake bottom) sampling depth (see Table 1). During July, the lakes were thermally stratified,
89 while in September, the lakes were isothermal. These lakes are part of the Arctic Long-Term
90 Ecological Research site (http://arc-lter.ecosystems.mbl.edu/), and their fish communities are
91 known to contain only arctic char and slimy sculpin (*Cottus cognatus*). We have conducted
92 extensive fish sampling via traditional methods (i.e., gill nets, hook-and-line) to quantify
93 population abundance in each lake using mark-recapture techniques (e.g., Budy and Luecke
94 2014; Table 2). For lakes where the times series was >5 years (Lakes E5, Fog1, Fog2), we
95 estimated abundance using a Huggins closed-capture model in Program MARK (White and
96 Burnham 1999). For Lakes Fog3 and Fog5, with shorter mark recapture time series, we used a
97 modified Schnabel estimate (Krebs 1999). Overall, arctic char abundance is relatively low across
98 all lakes, but follows a natural gradient from relatively low to relatively high density. For
99 example, our abundance and density by volume estimates span greater than an order of

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

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

115 eDNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia,
116 California). Filters were incubated in 360 μL buffer ATL and 40 μL proteinase K for one hour at
117 56 °C, with vortexing every 15 minutes. Then, 300 μL buffer AT was added, followed by 300 μL
118 99% ethanol. Extractions then proceeded following the manufacturers recommendations, with a
119 final elution volume of 100 μL. Each round of extractions included a blank negative control that
120 was later run in qPCR to test for contamination.

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

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

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

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

157 <A>Results

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

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

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

<A> Discussion

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

In our study, natural fish abundance is relatively low, and thus, mean eDNA 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

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

235 Overall, for eDNA studies, there is limited information in regard to sampling depth for
236 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

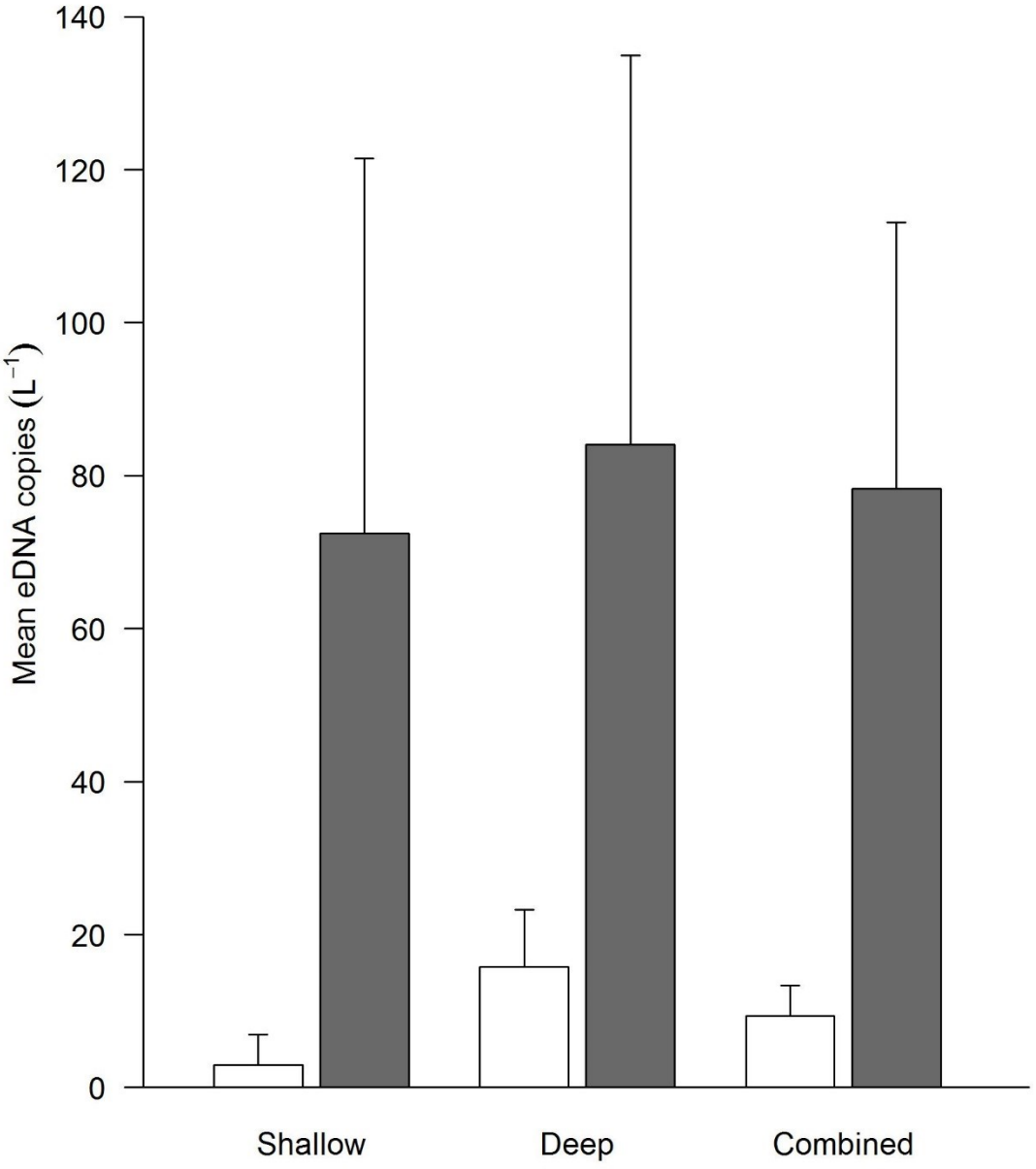
380 **Table 3.** Summary statistics of linear models fit to predict eDNA concentration from known metrics of relative fish abundance across
381 five lakes in northern Alaska. Bold text signifies the significant relationship that are presented in Figure 2.

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**

<A> Figure Captions

Figure 1. Mean eDNA copies (L^{-1}) for all lakes pooled (E5, Fog1, Fog2, Fog3, Fog5) by season (white = July 2016, gray = September 2016) for shallow and deep samples. Combined represents the overall mean between shallow and deep depths. Error bars represent standard error.

Figure 2. Relationships between A) fish abundance (top), B) density by area (bottom left), and C) density by volume (bottom right) and mean eDNA concentration (copies·L^{-1}) across five study lakes in northern Alaska sampled in 2016. Note: density by area and density by volume relationships are back-transformed from log(eDNA concentration).



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404 Figure 1.

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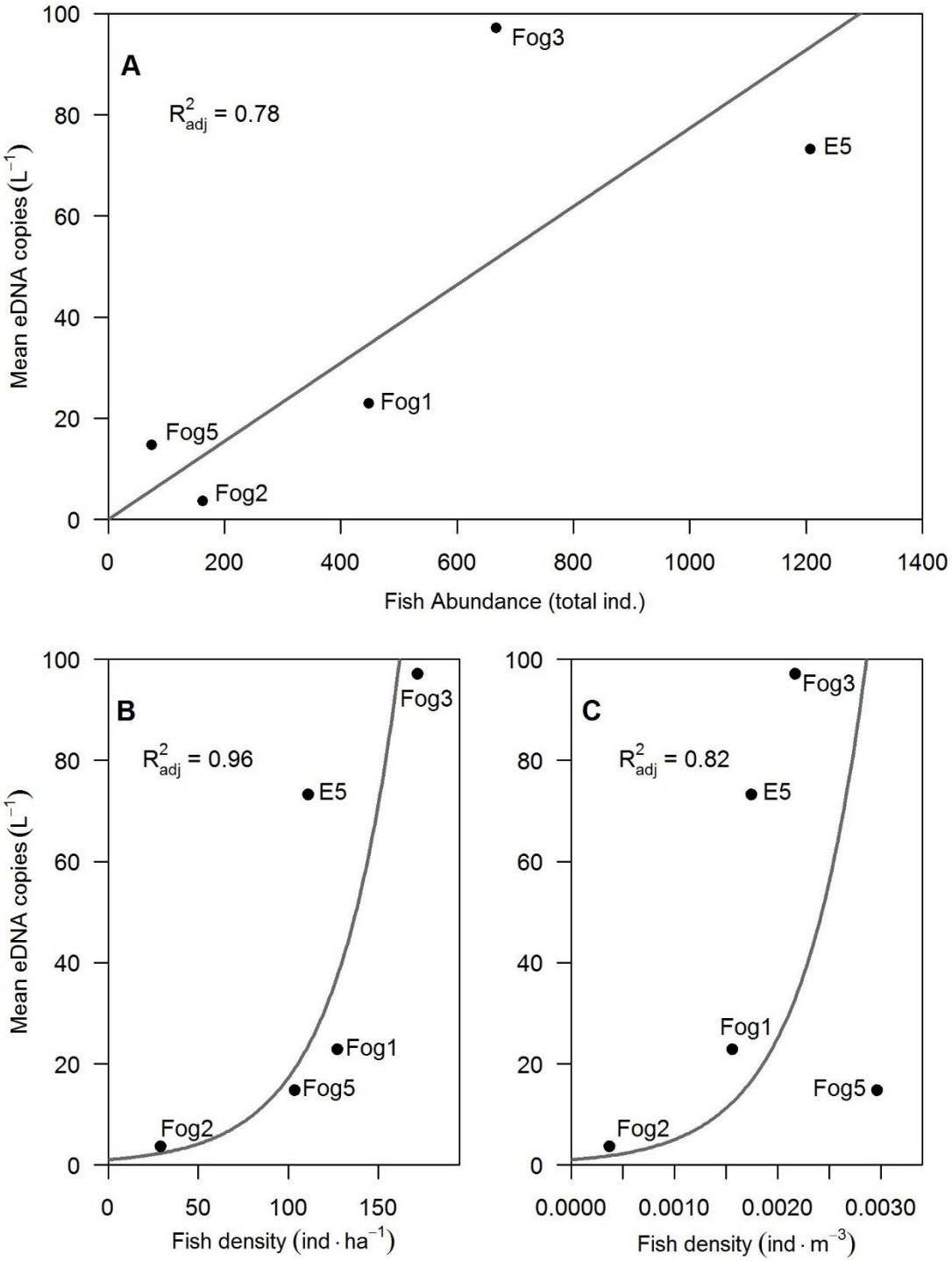

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407 Figure 2.