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COMMENT

Comment: The Importance of Sound Methodology in Environmental DNA Sampling

Environmental DNA (eDNA) sampling—which enables inferences of species' presence from genetic material in the environment—is a powerful tool for sampling rare fishes. Numerous studies have demonstrated that eDNA sampling generally provides greater probabilities of detection than traditional techniques (e.g., Thomsen et al. 2012; ;McKelvey et al. 2016; Valentini et al. 2016; Wilcox et al. 2016). In contrast, Ulibarri et al. (2017) and Perez et al. (2017) reported unusually low rates of eDNA detection at sites known to be occupied by the target species. These authors claim their results demonstrate that traditional sampling methods, based on best practices developed by experienced biologists (Bonar et al. 2009), are more accurate and sensitive for rare species detection than eDNA sampling. We believe the authors would have achieved substantially higher species detection rates and provided more useful comparisons with traditional sampling methods if they had adopted best practices for eDNA sampling (e.g., Goldberg et al. 2016). Here we focus on three elements of eDNA sampling and analysis that are critical to maximizing species detection rates: (1) the volume of water sampled in the field, (2) the volume of each sample that is analyzed in the laboratory, and (3) assessment of potential chemical inhibitors of laboratory eDNA detection.

Volume of water sampled in the field.— A fundamental assumption of any species detection sampling scheme is that the probability of detection increases as species abundance increases. Similar logic applies to eDNA sampling, in which the quantity of a species' DNA collected in the field is assumed to have the greatest influence on detection probability (Schultz and Lance 2015). Maximizing detection probabilities relies on sampling a sufficient volume of water to ensure that the DNA of a species (if it is present) is actually collected. The sample volumes collected by Ulibarri et al. (2017) and Perez et al. (2017) were exceptionally small (15 and 75 mL, respectively). Although such small sample volumes were typical of the initial tests of eDNA sampling related to proof of concept (e.g., for amphibians in small ponds with no or limited flow; Ficetola et al. 2008; Dejean et al. 2012), samples that are orders of magnitude larger are now the norm (typically 1–10 L and as much as 45–100 L [Civade et al. 2016; Valentini et al. 2016]). Several recent studies have determined that filtration of larger water volumes (≥1 L) results in greater fish eDNA capture and detection than precipitation of eDNA from small water volumes (Piggott 2016; Hinlo et al. 2017; Spens et al. 2017). Ulibarri et al. (2017) acknowledged their deficit in sample volume but noted that "a standardized method to compute the volume of water necessary to detect eDNA does not currently exist." However, by the time Ulibarri et al. (2017) and Perez et al. (2017) were accepted for publication, at least three models of eDNA detection probability had been published that explicitly considered the influence of sample volume on eDNA detection (Schultz and Lance 2015; Furlan et al. 2016; Wilcox et al. 2016).

Volume of sample analyzed in the laboratory.— Although critical to detection probability, sample volume in the field only accounts for part of the likelihood of species detection from eDNA sampling. Usually only a portion of the total DNA extracted from an environmental sample is analyzed in the lab (the aliquot), further reducing the total possible amount of DNA assessed for the presence of the target species. To illustrate the twin roles that sample volume and aliquot play in the outcome of eDNA analysis, we used a simple model (Wilcox et al. 2016) to estimate the effective quantities of DNA analyzed in the following four protocols: Ulibarri et al. (2017), Perez et al. (2017), and two of the more widely adopted protocols for eDNA sample collection and laboratory processing produced by the U.S. Geological Survey (Laramie et al. 2015; as parameterized by Pilliod et al. 2013) and the National Genomics Center for Wildlife and Fish Conservation (NGC; as parameterized by Carim et al. 2016a, 2016b; Table 1). This analysis reveals that the more widely adopted protocols effectively sample 10–500× more DNA than the Ulibarri et al. (2017) and Perez et al. (2017) protocols. We recognize that logistical constraints and special conditions may make precipitation-based eDNA sample collection and DNA extraction suitable for some systems (e.g., amphibians in small, turbid wetlands; Biggs et al. 2015). Even compared with established precipitation-based protocols, however, the volumes assessed by Ulibarri et al. (2017) and Perez et al. (2017) were low (5–25

TABLE 1. Effective quantities of DNA analyzed for eDNA sample analysis under five different protocols. The effective quantity of DNA analyzed is the mean proportion of the DNA concentration in the environment (copies/L) that is tested in the laboratory. For example, if there are 100 copies/L in the environment, an effective quantity of 25% translates to a mean of 25 copies (per sample) being analyzed in the lab. The effective water volume sampled in the field is a function of the volume of water collected (L) and the percentage of that volume from which DNA is extracted (Pilliod et al. 2013; Carim et al. 2016b; the National Genomics Center for Wildlife and Fish Conservation [NGC] protocols [Carim et al. 2016a] involve extraction from one-half of a 47-mm-diameter filter). DNA extractions were eluted into 100-μL volumes in all protocols except Biggs et al. (2015; 200 μL), from which 4–36-μL aliquots were used for actual PCR analysis. For example, in the Ulibarri et al. (2017) protocol, 1.5% of 1 L was collected and 100% of that was used for DNA extraction; this was eluted into 100 μL, of which 4% was analyzed (1 μL/PCR with four replicates/sample). Thus, the effective quantity was 0.06% ($0.015 \times 1.0 \times 0.04 = 0.0006$).

Protocol	Volume collected (L)	Volume extracted (%)	Effective volume (L)	Volume eluted (μL)	Aliquot volume (μL)	Effective quantity (%)
Ulibarri et al. (2017)	0.015	100	0.015	100	4	0.06
Perez et al. (2017)	0.075	100	0.075	100	4	0.30
Biggs et al. (2015)	0.09	100	0.09	200	36	1.62
Pilliod et al. (2013)	1	50	0.5	100	6	3.00
NGC	5	50	2.5	100	12	25.00

times more DNA was assessed in the protocol described in Biggs et al. 2015).

These differences in sampling design have a profound effect on the probability of species detection at typical eDNA concentrations (Figure 1). For rare stream fishes such as juvenile Bull Trout *Salvelinus confluentus* in small, cold, natal habitats (McKelvey et al. 2016), we have rarely observed concentrations of >500 DNA copies/L. At such low concentrations, the sampling protocols of Ulibarri et al. (2017) and Perez et al. (2017) would have failed to detect species that are present in 30–75% of samples (see the Appendix to this comment). In contrast, the methods of Pilliod et al. (2013) generate a detection probability >95% at <125 copies/L, and those of the NGC protocol have a detection probability >95% at <13 copies/L. We conclude that the low eDNA detection rates observed by Ulibarri et al. (2017) and Perez et al. (2017) were to be expected given the small field and laboratory sample volumes in their protocols.

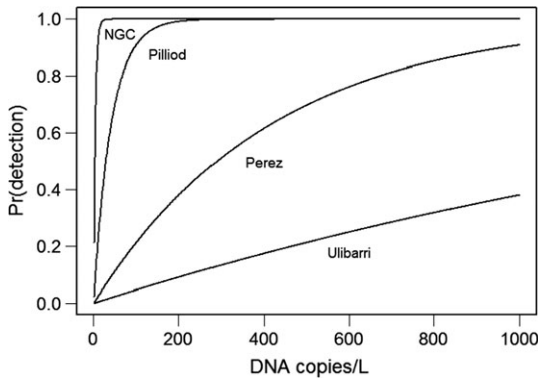

FIGURE 1. Estimated stream fish detection probabilities for the eDNA field and laboratory protocols used by the NGC (Carim et al. 2016a, 2016b), Pilliod et al. (2013), Perez et al. (2017), and Ulibarri et al. (2017) across a range of DNA concentrations in the environment (DNA copies/L). Estimates were based on a simple model of the eDNA detection process described in Wilcox et al. (2016) and the Appendix. The NGC and Pilliod et al. (2013) protocols reflect common, filtration-based designs for fish eDNA sampling. The low detection probabilities of the Perez et al. (2017) and Ulibarri et al. (2017) protocols are largely a function of sampling very small water volumes.

Assessment of inhibitors to laboratory detection.— The presence of PCR inhibitors in an eDNA sample can substantively reduce the rate of species detection. Many naturally occurring substances can act as PCR inhibitors, including compounds found in plant materials and soils. The presence of these compounds in a sample can reduce or completely prevent amplification of DNA during PCR, resulting in downwardly biased estimates of the DNA copy number or outright failure to detect DNA in a sample (Hedman and Rådström 2013). These inhibitors are abundant in aquatic habitats, including small mountain streams (Jane et al. 2015), lakes (Eichmiller et al. 2016), and wetlands (McKee et al. 2015). The ubiquity of PCR inhibitors in the environment has led to the consensus that using protocols to test for and treat PCR inhibitors—and reporting the results along with giving a detailed description of assay development and the validation of these tests —should be standard practice for eDNA-based studies (Bustin et al. 2009; Goldberg et al. 2016). We could not discern whether any measures were taken by Ulibarri et al. (2017) and Perez et al. (2017) to address PCR inhibition, so we cannot determine whether PCR inhibitors reduced the observed detection rates.

Conclusion.— Using eDNA to detect aquatic species is a rapidly expanding technique not yet a decade old (Ficetola et al. 2008), but many of the early adopters have concluded that it provides the most sensitive and cost-effective tool available for detecting rare species. Although environmental DNA sampling is not always the best tool with which to address the myriad issues confronting

aquatic biologists, it is an increasingly important one. For many species, the effectiveness of eDNA sampling relative to that of traditional methods is unknown, necessitating pilot studies such as those attempted by Ulibarri et al. (2017) and Perez et al. (2017) prior to launching large-scale survey efforts (Goldberg et al. 2016; Amberg et al. 2015). These pilot data, particularly when paired with models of detection probability based on eDNA concentration (e.g., Furlan et al. 2016) or detection history (i.e., occupancy modeling, as in Valentini et al. 2016 and others), could be used to optimize sampling designs for further work. However, valid comparisons of sampling methods rely on applying the accepted best practices for those methods. Regarding eDNA sampling, the approaches codified in published protocols may not constitute a single standard method, but they do provide a clear path for designing highly effective eDNA sampling.

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Appendix: A Simple Model of Species Detection from eDNA Sampling

We estimated the probability of eDNA sampling detection for different sampling and analysis protocols using a simple model adapted from equation (6) in Wilcox et al. (2016). Assuming that DNA is randomly distributed in the environment (which may be an optimistic assumption, particularly for small sample volumes; Furlan et al. 2016), the actual number of DNA copies involved in qPCR (k) is a random variable drawn from a Poisson distribution with a mean of λ. For a given DNA concentration in the environment, the mean number of DNA copies involved in qPCR analysis (λ) is a function of the sampling and analysis protocol. The qPCR analysis has an amplification failure rate of f per DNA copy in a reaction. The probability of amplifying at least one DNA copy when k copies are analyzed is $1 - f^k$. As long as $f < 0.5$, we can ignore $k > 10$ ($1 - 0.5^{10} > 0.999$). Expressing this in one equation,

$$\Pr(\text{detection}|\lambda) = 1 - \sum_{k=0}^{10} f^k \times \frac{\lambda^k e^{-\lambda}}{k!}.$$

We assumed that $f = 0.2$ based on the estimates in Wilcox et al. (2016) for a highly sensitive qPCR assay (limit of detection <10 DNA copies, as defined by Bustin et al. 2009). The R script used to create Figure 1 is provided below.

```
### R script
### model to predict probability of detection given:
### 1. lambda (copies/L in the environment)
### 2. protocol (proportion copies/L sampled or effective quantity of DNA analyzed)
### 3. p (probability of qPCR amplificaiton of one DNA copy)

pDNA <- function(lambda, protocol, p) 1 - sum(dpois (0:10, lambda*protocol)*((1-p)^(0:10)))

### vectors to save detection probabilities in
Ulibarri <- c()
Perez <- c()
Pilliod <- c()
NGC <- c()

### "protocol" for each
Ulibarri_protocol <- (0.015/100)*4 #15 mL samples, elute into 100 ul, 4 ul in qPCR
Perez_protocol <- (0.075/100)*4 #75 mL samples, elute into 100 ul, 4 ul in qPCR
Pilliod_protocol <- (0.5/100)*6 #500 mL samples (1/2 of 1 L filter), elute into 100 ul, 6 ul in qPCR
NGC_protocol <- (2.5/100)*12 #2500 mL samples (1/2 of 5 L filter), elute into 100ul, 12 ul in qPCR
Biggs_protocol <- (0.09/200)*36 #90 mL samples, elute into 200 ul, 36 ul in qPCR; not plotted

### loop through 1 to 1000 DNA copies/L in the environment
for(i in 1:1000){
  Ulibarri[i] <- pDNA(i, protocol=Ulibarri_protocol, p=0.8)
  Perez[i] <- pDNA(i, protocol=Perez_protocol, p=0.8)
  Pilliod[i] <- pDNA(i, protocol=Pilliod_protocol, p=0.8)
  NGC[i] <- pDNA(i, protocol=NGC_protocol, p=0.8)
}

### plotting
par(las=1, cex=1.5, mar=c(4,4,1,1))
plot(NULL, NULL, xlim=c(0,1000), ylim=c(0,1),
```

```
    xlab="DNA copies/L", ylab="Pr(detection)")
lines(Ulibarri, lwd=2)
lines(Perez, lwd=2)
lines(Pilliod, lwd=2)
lines(NGC, lwd = 2)
box(lwd=2)

text(200,0.8, cex = 0.8,  "Pilliod")
text(400,0.5, cex = 0.8,  "Perez")
text(600,0.18, cex = 0.8,  "Ulibarri")
text(62, 0.95, cex = 0.8, "NGC")

### probability of detection at 500, 500, 125, and 13 copies/
L for the protocols
Ulibarri[500]
Perez[500]
Pilliod[1125]
NGC[13]
```