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Experimental assessment of optimal lotic eDNA sampling and assay multiplexing for a critically endangered fish

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Abstract

Designing eDNA tools to detect and quantify rare species includes inherent assumptions about the spatial distribution of the organism, spatial nature of eDNA dynamics, and the real-world performance of alternate assays under field conditions. Here, we use cage experiments with small numbers of Atlantic salmon (*Salmo salar*), to reveal that eDNA detection rates and eDNA quantities follow a predictable, but nonlinear relationship with distance from a point source. In contrast to the common assumption of consistent eDNA degradation moving away from a source, eDNA detections and concentrations increased up to roughly 70 m downstream before declining steadily. We apply our eDNA distance functions to selection of stream sampling intervals for detecting fish without known locations and find that even a single juvenile salmon can be reliably detected with intervals up to 400 m spacing. Finally, we show that two different qPCR eDNA assays provide very different detection probabilities in nature despite similar efficiency in laboratory testing, demonstrating the importance of experimentally assessing assay efficiencies in the wild as well as the capacity for multiplexing as a strategy to ensure high detection efficiency when monitoring rare species.

KEYWORDS

eDNA multiplexing, environmental DNA, population abundance, *Salmo salar*, species detection, stream eDNA

1 | INTRODUCTION

Detecting the environmental DNA (eDNA) of an organism can in principle be easier and more efficient than detecting the organism itself, particularly for rare or cryptic species (Hinlo et al., 2018; Jerde et al., 2011; Pfleger et al., 2016). eDNA detection is particularly valuable for threatened fishes, which are otherwise most often surveyed with labor-intensive capture surveys (e.g., electrofishing, netting), and might be harmed during the capture process (Dolan and Miranda, 2004; Miranda and Kidwell, 2010; Rummer and Bennett, 2005). A growing body of work is also now moving beyond detection to quantify or localize sources (Carraro et al., 2018; Eichmiller et al., 2014; Pilliod et al., 2013; Thomsen et al., 2012; Yates et al., 2019). However, while eDNA offers great promise, few surveys are presently guided by empirical power considerations that apply to field settings. This is especially problematic for critically threatened

or harmful species where high-power detection or quantification of very few, or even single individuals, may be necessary. Pushing these limits of field detection requires answering foundational questions about interactions between eDNA production, transport, dissolution, degradation, settlement, resuspension, and detection processes in nature (Shogren et al., 2017). In this study, we used caged fish experiments to assess these interacting processes as part of developing eDNA surveys for a critically endangered stream fish, the Maine Distinct Population Segment of Atlantic salmon (*Salmo salar*) (US Federal Register, 2009).

Understanding the outcome of eDNA production, transport, and loss processes is especially important for high-resolution studies, which may seek to detect or quantify organisms in very local areas, such as stream reaches (Hinlo et al., 2018; Pilliod et al., 2013; Tillotson et al., 2018). Attaining such high resolution in stream systems is complicated by how these time-dependent eDNA processes play out over a spatial scale. DNA released from an organism is rapidly transported downstream and in turn degraded by microbes, diluted by mixing into the larger stream volume, and adsorbed onto substrates (Strickler et al., 2015). Barring major changes in hydrology (e.g., tributary inputs, slack water), the outcome of these interacting processes is typically assumed to be a linear decline in eDNA concentration with increasing distance from the source. However, eDNA-distance relationships are rarely quantified (but see Jane et al., 2015; Jerde et al., 2016; Pilliod et al., 2014; Shogren et al., 2017, 2018), and eDNA is unlikely to behave like salt tracers. We suggest the linear loss model may only be a suitable approximation under certain conditions.

When eDNA is shed from a fish, or other point source, it is likely often in the form of a plume of relatively large particles (e.g., tissue fragments, cells) that are more spatially constrained the closer they are to the source (Wilcox et al., 2015). Samples taken immediately downstream of such a source might often miss this plume, but have high eDNA concentrations when successful. Over time and distance, eDNA may in turn undergo a "breakout phase" wherein particle fragmentation and mixing result in smaller, more evenly dispersed particles. The expected outcome is that the highest eDNA detection probabilities and mean concentrations may actually occur some distance downstream of the source. Beyond this breakout phase, we might expect to see a more steady decrease in detection due to DNA degradation, dilution, and settlement (Barnes et al., 2014; Jerde et al., 2016; Shogren et al., 2017). Understanding the specific form of this eDNA-distance function can in turn be used to either (a) establish where eDNA might best be sampled downstream of a specific location of concern (e.g., road crossing for restoration) or (b) establish the optimal spatial interval of sampling for high-power detection of a rare occurrence of a target species along a stream corridor.

Importantly, eDNA detection and quantification are not just a function of actual eDNA dynamics in nature, but they are also a function of the particular molecular approaches applied to detect eDNA (Darling and Mahon, 2011). The design of particular eDNA assays (primer sets, probes) and detection method can substantially influence the power of detection and quantification of eDNA, particularly at lower eDNA concentrations (Jerde et al., 2016; Wilcox et al., 2013, 2015). Although many eDNA assays undergo basic laboratory screening for target specificity, amplification efficiency, and limits of detection/quantification using known quantities of target DNA, a few studies ever contrast alternate assays under actual field conditions. Moreover, given that detection probability should improve with the number of target fragments available to detect, multiplexing assays may provide greater detection and quantification power than a single assay, but by how much?

Here, we experimentally examine eDNA detection and quantification over distance from a point source by introducing varying numbers of Atlantic salmon into a single cage in an otherwise salmon-free stream in Maine, USA. We tested several alternative eDNA detection rate and quantity over distance models and used these curves to estimate optimum detection downstream eDNA sampling distance for this system as well as demonstrate how to set stream sampling intervals for high-power detecting of a very rare target. We also assessed the relative field power of two alternative eDNA assays, as well as their combined power when multiplexed.

2 | METHODS

2.1 | Salmon experiment

We placed fixed numbers of Atlantic salmon into a cage in the Sunkhaze Stream in Milford Maine, USA (45°00′34.2″N, 68°30′52.6″W) from August 19–25, 2017. The Sunkhaze Stream is a small tributary of the Penobscot River (average summer discharge: 0.34–0.42 m^3/s (Rupp, 1955)) and flows through a mixture of mixed conifer–northern hardwood forest, floodplain forest, fen, and bog (US Fish and Wildlife Service, 2013). While Atlantic salmon currently migrate through the Penobscot River (Izzo et al., 2016) and were historically presented in Sunkhaze Stream (US Fish and Wildlife Service, 2013), salmon are no longer presented in the system due to regional declines, and we detected no salmon eDNA in control samples taken over the entire survey reach prior to salmon additions or in samples taken 10 m upstream of the cage following each successive addition of salmon to the cage (Supplementary methods). Working with Maine Department of Inland Fisheries and Wildlife, 1, 4, 8, and then 20 hatchery-reared salmon (average weight = 27.17 ± 7.15 g) were placed into a cage every 2–3 days, with a flushing period of at least 24 hr between each density treatment. These numbers are akin to the extreme low end of abundances that might be representative of the progeny resulting from a single spawning event (redd) or from fish collecting in a refuge habitat (e.g., cold water seep) during harsh environmental conditions. On each sampling day, we collected 1 L of water from the left bank, right bank, and middle channel at 10, 100, 500, and 1,000 m downstream (into 6 × 500 ml water bottles). We also collected field contamination controls during each sampling event which were transported and processed identically to environmental samples. Stream discharge was very consistent over the study

period, as captured in hydrographs for nearby streams (Figure S1a). Water temperature was recorded via a stationary probe located approximately 3 km upstream of the cage and was likewise relatively consistent over the study period (Figure S1b). All salmon were visually inspected following the completion of the study to ensure that they did not exhibit signs of cage-induced superficial stress (i.e., abrasion, scarring).

2.2 | Sample processing

Environmental samples and contamination controls were filtered through 47 mm glass fiber filters with a pore size of 1.5 microns (GE Healthcare). Filters were subsequently stored at −20°C until extraction using a Qiagen DNeasy spin column protocol (Carim et al., 2016; Goldberg et al., 2011; Wilcox et al., 2015) (Supplementary methods).

2.3 | Assay development

We used online databases and sequencing of tissue to align mitochondrial DNA from the NAD5 and CO1 regions of Atlantic salmon and six other salmonids common in Maine, including brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), and lake whitefish (*Coregonus clupeaformis*). From these aligned sequences, we developed two sets of qPCR primers and associated TaqMan MGB-NFQ probes that incorporated multiple base pair sequence differences in their respective binding regions and targeted 194bp and 144bp regions of the NAD5 and CO1 genes, respectively (Tables S2 and S3). The NAD5 and CO1 probes were, respectively, labeled with 6FAM and VIC fluorophores on their 5′ end to permit multiplexing within a single assay. The NAD5 and CO1 primer-probe sets were confirmed for species specificity through qPCR trials using multiple extracted DNA samples for Atlantic salmon and each of the nontarget salmonids, and for sensitivity and efficiency using serial dilutions of synthetic salmon gene fragments (gBlocks) matching our eDNA target sequences.

2.4 | Quantitative PCR

All qPCR reactions were 20 µl in volume and performed on a BioRad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Both primer-probe sets were multiplexed, and reaction concentrations were 1 µM for forward and reverse primers, 500nM for probes, and 1X for Environmental Master Mix 2.0 (Applied Biosystems). For the purpose of this study, a "sample" included the pooled eDNA of the left, middle, and right bank extractions collected at a given sampling location on a particular date and thus represents a cross section of the stream. Each technical replicate of a sample (repeated qPCR) included 1 µl of the left, middle, and right extractions. We also tested for contamination in our collection and qPCR methods by, respectively, running four technical replicates of field contamination controls each using 3µl of extracted DNA and four technical replicates with 3 µl of sterile water substituted for extracted DNA. Inhibition was quantified for each sample by adding 1× Taqman Exogenous Internal Positive Control Mix and 1× Taqman Exogenous Internal Positive Control DNA (Applied Biosystems) to two separate technical replicates. Finally, six separate reactions on each plate were run with serially diluted synthetic gene fragments (gBlocks) which correspond to the two target sequences at concentrations of 31,250, 6,250, 1,250, 250, 50, and 10 copies/reaction. All qPCR reactions were conducted with the following thermocycler profile: 10 min at 95°C followed by 50 cycles of 10 s at 95°C and 30 s at 60°C. Baseline thresholds were set at 25 and 150 relative fluorescent units for the NAD5 and CO1 markers, respectively. Any eDNA amplification of a technical replicate that exceeded these thresholds was treated as a positive detection.

2.5 | Copy number estimation

Three 6-step, 5-fold dilution series of synthetic gene fragments allowed for the construction of standard curves for NAD5 and CO1 target sequences by regressing the C_q values and log-transformed initial concentrations. One 10-copy reaction for the NAD5 fragment failed to amplify, and these data were removed from further analysis. Standard curve y-intercepts and PCR efficiencies were then used to calculate initial eDNA concentrations for each reaction.

2.6 | eDNA detection rates

We fit all models and calculated all statistics using base functions in R (R Core Team, 2016). We examined several alternative models for eDNA detection rate ~ distance relationships. To determine the per technical replicate detection rate, we tested four classes of binomial generalized linear models, each with the rate function:

$$p = \frac{1}{1+\exp\left(-f\left(F,D\right)\right)} \quad (1)$$

p is detection rate in a single technical replicate, F is fish abundance, and D is downstream distance from fish. Each model therefore had a maximum of 1 (guaranteed detection) and minimum of 0 (detection impossible).

We tested models falling into four types, with f (F, D) as either a linear, or quadratic function of distance, and with D either untransformed or ln-transformed. Considering detection rate as a linear function of distance is statistically simple and parameter efficient but assumes that detection rate always decreases

with downstream distance from fish. Considering detection rate as a quadratic function of distance assumes that detection rate increases, then decreases downstream from the fish, but that the decrease happens at a slower rate than the increase. We also tested a null model, which included assay (NAD5 vs. CO1) as the only independent variable. For full model expansions, see Table S4.

We fit a separate Y-intercept within $f(F, D)$ for each detection model for the full multiplex of assays, as well as for each assay singly, for a total of three unique intercepts. For the best eDNA detection model, we tested whether multiplexing and assay identity had a significant effect on detection rate using a type II likelihood ratio test (Burnham and Anderson, 2004).

We calculated the minimum number of technical replicates required for positive detection for the best model:

$$s = \log_p(\alpha) \tag{2}$$

s is number of technical replicates, p is detection rate, α is confidence (95% confidence: $\alpha = 0.05$).

2.7 | eDNA quantification

We examined the relationship between downstream distance, fish abundance, and quantity of eDNA detected (copies/reaction), again with the main goal of determining the shape of the eDNA quantity ~ distance relationship. Due to the heavily skewed distribution of eDNA quantities (see Results), we fit models predicting $\ln(X + 0.02)$ copies. We fit models of the form:

$$\ln(q+0.02) = \ln\left(\frac{aF}{1+\exp(-f(F,D))} + 0.02\right) \tag{3}$$

q is copies of eDNA per technical replicate, F is fish abundance, D is downstream distance from fish, and α is a fish abundance coefficient, which was estimated separately for each assay. The numerator determines the main relationship between fish abundance and copies detected, while the denominator determines the shape of the copies ~ distance relationship, also allowing that shape to be modified by fish abundance. We began with the log-quadratic expansion of $f(F, D)$ due to its high performance in detecting eDNA (see Results). For full model expansions, see Table S4).

2.8 | Optimal sampling distance for detection

Based on findings from the above analyses, we used the log-quadratic eDNA detection model (see Results) to calculate the per technical replicate eDNA detection rates over our entire studied ranges for fish abundance (1–20) and downstream distance (10–1,000 m). We also used the log-quadratic detection model to calculate the optimal downstream sampling distance for detection for various fish abundances.

TABLE 1 Parameter estimates for log-quadratic eDNA detection model (Equation 5)

Parameter	Estimate	*SE*
β_{BOTH}	−9.31	2.46
β_{CO1}	−9.89	2.56
β_{NAD5}	−10.97	2.49
β_1	2.99	0.96
β_2	0.69	0.17
β_3	−0.28	0.10
β_4	−0.010	0.004

2.9 | Optimal sample interval for detection

We used the log-quadratic eDNA detection model to examine the optimal spacing of samples, assuming even-spaced sampling over the entire length of a stream. We simulated one or more fish inhabiting a random (flat distribution) point in a 10 km long stream. When we simulated more than one fish, we assumed all fish were located at the same point in the stream. We then calculated cumulative detection probabilities for the fish, given an evenly spaced sampling regime beginning at the bottom of the stream:

$$P = 1 - \prod_{i}^{N_{Di<DF}} (1 - p(F, D_i))^r \tag{4}$$

P = total detection probability, $N_{Di<DF}$ = number of samples that are downstream of the fish, $p(F,Di)$ = the log-quadratic eDNA detection function (assuming both fluorophores were multiplexed), and r = the number of technical replicates.

We varied the number of fish (1, 4, 8, or 20), the number of technical replicates per sample (1–3), the spacing between samples (50–1,000 m), and the length of the simulated stream (10, 100, or 1,000 km).

3 | RESULTS

Both qPCR primer-probe sets successfully amplified genomic and synthetic salmon DNA targets. The CO1 primer-probe set also amplified one brown trout sample; however, this was not encountered in other brown trout samples. It is likely this single off-target amplification was due to contamination of the trout tissue sample prior to arrival in our laboratory. All other nontarget taxa were negative for both NAD5 and CO1 primer-probe sets, and brown trout are not presented in our study area (their distribution is due to stocking). Serial dilutions of synthetic salmon genes matching our eDNA target sequences confirmed the sensitivity of our qPCR assays to detect eDNA at our lowest test concentration (10 copies/reaction). PCR efficiencies were high for both assays (103% for NAD5 and 98% for CO1), albeit the NAD5 assay amplified our lowest dilution at a slightly later Cq than the CO1 assay (37.5 vs. 36.5 cycles).

3.1 | eDNA detection

The log-quadratic model performed the best of our candidate models:

$$p = \frac{1}{1+e^{-\left(\beta_X+\beta_1 \ln(D)+\beta_2 F+\beta_3 \ln(D)^2+\beta_4 \ln(D)^2 F\right)}} \quad (5)$$

p is detection rate, F is fish abundance, D is downstream sampling distance, and β-terms were determined during the model-fitting process. For estimates of β-terms, see Table 1. This model predicts sharply increasing, then gradually decreasing eDNA detection rate downstream of the fish (Figure 1). The more numerous the fish, the higher the per technical replicate detection rate and the broader the range of distances within which eDNA was likely to be detected.

Based on the log-quadratic model, the number of technical replicates required for a positive detection rate of 95% decreased sharply, then increased slowly over distance from fish (Figure 1). The number of technical replicates required for 95% chance of detection decreased rapidly with increasing numbers of fish, from roughly 30 for 1 fish to 1 for 20 fish (Figure 1).

There was significant variation in detection rate based on number and type of assays(s) included in the analysis (likelihood ratio test: χ^2 = 10.16, df = 2, p = .006). This was mostly driven by differences between the CO1 and NAD5 assays, as multiplexing resulted in significantly higher detection compared to NAD5 alone (likelihood ratio test: χ^2 = 10.45, df = 1, p = .001), but not compared to CO1 alone (likelihood ratio test: χ^2 = 1.46, df = 1, p = .23). Nonetheless, we retained the model including multiplexing for further analyses, as this model did have the highest overall detection rate.

3.2 | eDNA quantification

The best quantification model of our set was as follows:

$$q = \frac{\alpha_X F}{1+e^{-\left(\beta_0+\beta_1 \ln(D)+\beta_2 F+\beta_3 \ln(D)^2+\beta_4 F \ln(D)^2\right)}} \quad (6)$$

q is mean eDNA quantity per technical replicate, F is fish abundance, and D is downstream sampling distance. For estimates of α- and β-terms, see Table 2. This model had an AIC improvement of 179 compared to a null model, which related eDNA quantity only to assay used (Table 3).

Like the log-quadratic detection model, this model predicts increasing, then decreasing quantities of eDNA downstream from fish. The more numerous the fish, the higher the quantity of eDNA found, and the farther downstream relatively high quantities of eDNA could be found (Figure 2). The CO1 assay provided higher estimates of initial eDNA concentrations compared to the NAD5 assay.

3.3 | Optimal sampling distance for detection

Optimal sampling distance for detection was far (200 m) for a single fish, but steadily decreased with increasing salmon density to approximately 30 m for 20 fish (Figure 3). Importantly, the range of distances within which detection rate was relatively high widened rapidly with increasing number of fish: above 15 fish, any sampling distance between 10 and 300 m downstream was predicted to yield >99% detection rate per technical replicate.

3.4 | Optimal sample spacing for detection

Overall simulated fish detection probability was highly dependent on the number technical replicates and fish simulated (Figure 4). With a single fish and a single technical replicate, average detection probability was only above 95% with relatively frequent (<100 m) sample spacing. However, increasing the number of technical replicates to three made any sample spacing <400 m have nearly guaranteed detection of any number of fish at a single location.

4 | DISCUSSION

In situ experiments are a useful, but still rare, tool for understanding the limits and patterns of eDNA detection in stream systems. We found that detection rate and estimated quantity of eDNA both increased, then decreased with downstream distance from fish, supporting the presence of an initial eDNA breakout phase in lotic systems. Beyond this initial breakout window, we observed steady declines in estimated eDNA concentrations and detections rates for all density treatments. The resulting downstream location of optimum detection or maximum concentrations of eDNA depended on the number of source fish releasing eDNA, and the nature of eDNA transport is such that even a relatively sparse sampling interval can have high power to detect low numbers of salmon in systems like the Sunkhaze Stream. We also found field evidence that assay type matters and that a multiplexed assay can provide significant power benefits. These patterns have potentially important implications for the design of eDNA surveys for endangered salmon and other species.

It is noteworthy that other studies examining lotic eDNA dynamics have not quantified the potential existence of an eDNA breakout window in lotic systems. This may largely relate to how prior studies have been conducted. Most eDNA studies of stream organisms have compared stream-to-steam or site-to-site variation in organism density, inferred from traditional capture methods, to eDNA concentrations (Baldigo et al., 2017; Doi et al., 2017; Stanley and Royle, 2005; Wilcox et al., 2015). Such studies would not be able to discern the finer scale spatial trends we examined. The few studies that have measured eDNA concentrations at varying distances from a known source have reported a more or less

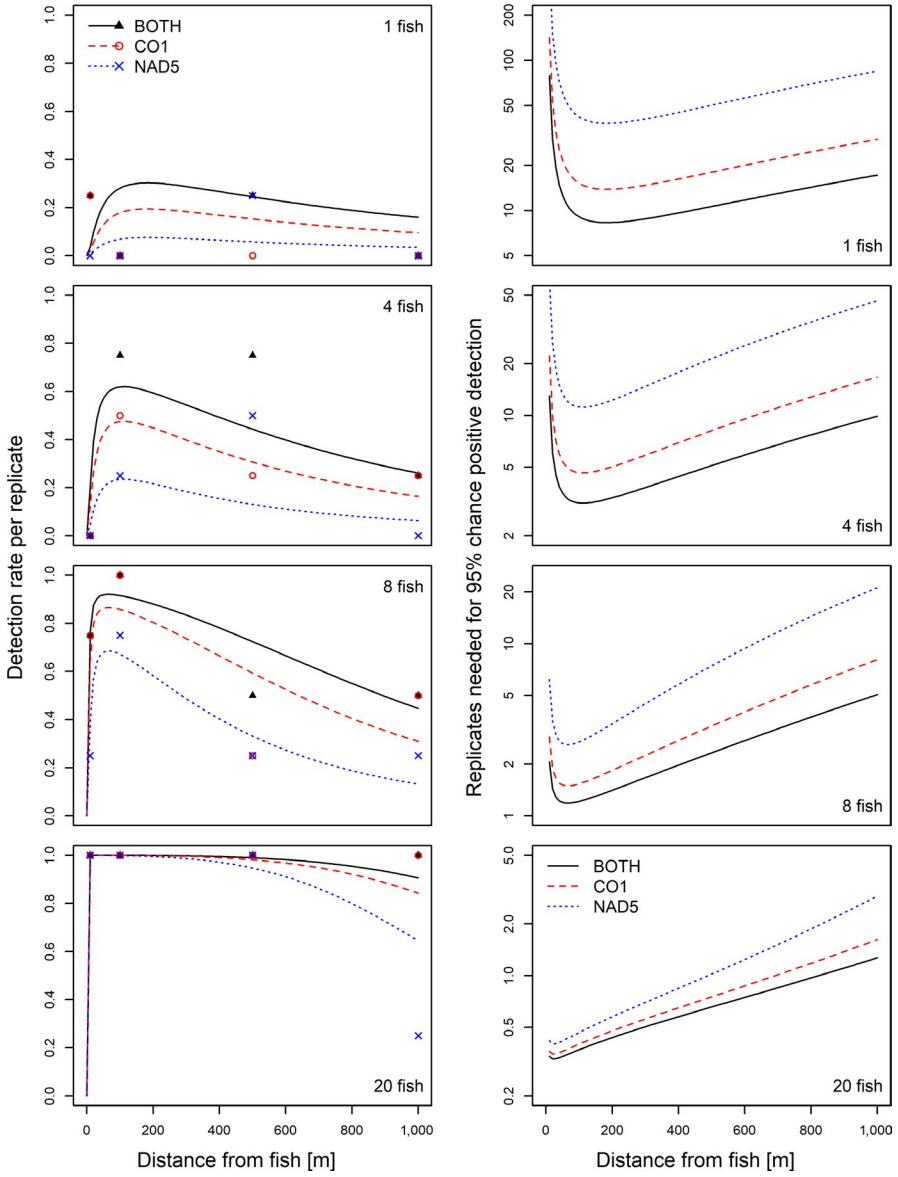

FIGURE 1 LEFT: Environmental DNA (eDNA) detection rate (per technical replicate) using multiplexed or separate assay data. RIGHT: Number of technical replicates required for 95% chance of positive detection using multiplexed or separate assay data

consistent decrease in eDNA quantity with downstream distance (Deiner and Altermatt, 2014; Jane et al., 2015; Jerde et al., 2016; Shogren et al., 2017). Though it should be noted that Jerde et al. (2016) and Shogren et al. (2017) titrated eDNA solutions into small, baffled experimental streams which promoted mixing in the headwaters. Thus, we would not expect to observe a breakout window under these circumstances as the titrated and mixed solution is not representative of eDNA plumes created by organisms. In the case of Deiner and Altermatt (2014), the source was a population of lake zooplankton that could themselves be transported

TABLE 2 Parameter estimates for log-quadratic eDNA quantification model (Equation 6)

Parameter	Estimate	*SE*
α_{CO1}	0.81	0.32
α_{NAD5}	0.0096	0.0040
β_0	−13.85	2.92
β_1	3.71	1.10
β_2	0.54	0.22
β_3	−0.39	0.12
β_4	−0.0063	0.0051

TABLE 3 Relative likelihoods for eDNA detection models. Statistical inferences (i.e., likelihood ratio tests) are not provided, as models are not nested

	Detection		Quantification	
Model	*df*	AICc	*df*	AICc
Null	3	266.1	3	719.3
Linear	6	165.2	7	547.5
Log-linear	6	171.0	7	550.0
Quadratic	7	166.4	8	549.0
Log-quadratic	7	160.4	8	540.3

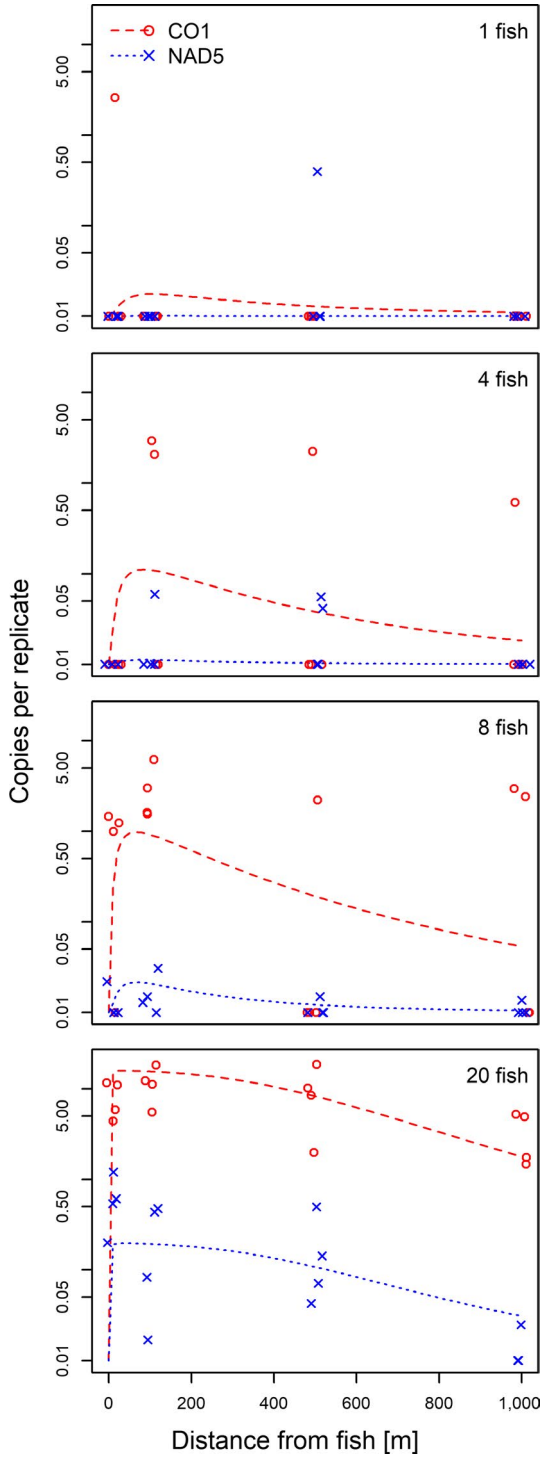

FIGURE 2 Copies per technical replicate increases, then decreases with downstream distance from fish. Line shows log-quadratic model predictions

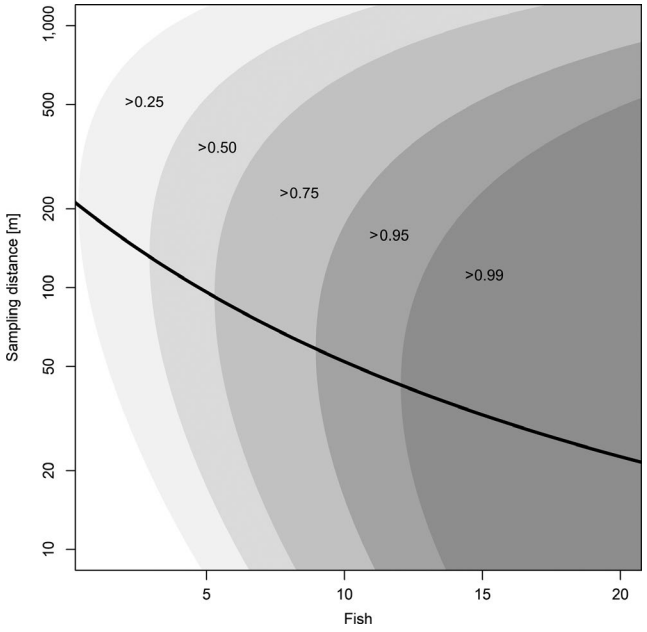

FIGURE 3 Detection rates for a single technical replicate, based on number of fish and downstream sampling distance from fish. Rates shown account for use of both assays. Thick line shows optimum sampling distance for a given fish abundance

downstream, presenting a very different eDNA production and transport dynamic. A more directly comparable brook trout cage study by Jane et al. (2015) collected samples in much smaller (lower order) streams, which may be better mixed at shorter distances. Additionally, Jane et al. (2015) collected mid-channel samples which likely reduced their power to detect an eDNA breakout window as these sampling locations are expected to be the areas of highest eDNA concentrations. We recommend that future studies adopt a cross-sectional sampling approach to gain a more comprehensive understanding of eDNA dynamics in lotic systems. Indeed, such an approach could provide novel insights of eDNA plume dynamics, particularly if cross-sectional samples are analyzed separately.

One important corollary of the eDNA breakout window hypothesis is that the optimal downstream sampling distance will be in closer proximity to the source as the density of a source population increases. The core assumption of the model is that each fish releases a spatially constrained plume of relatively concentrated eDNA particles that will dissociate and mix into the stream volume as downstream distance increases. While each fish releases a confined plume of eDNA particles, increasing the number of fish should increase the concentration of the plume and subsequently the odds that samples collected on the periphery of the plume are positive. As such, the window where breakout processes influence detection will shrink with increasing fish density. Again, our empirical data fit this pattern with the highest rate of positive detections occurring at the 100 and 500 m sample locations for the 4 fish treatment, at 100 m for the 8 fish treatment, and being nearly perfect at the very first sample location 10m downstream of the 20 fish treatment. We hope that our results will encourage other researchers to investigate the presence of an eDNA breakout phase in their systems with particular attention paid to distances nearer to the source population (i.e., <100 m).

As more projects seek to find or census fish within a particular reach (Hinlo et al., 2018; Pilliod et al., 2013; Tillotson et al., 2018), more attention will be paid to distance ~ detection rate and quantity relationships. With this in mind, what biases might exist and how might surveys

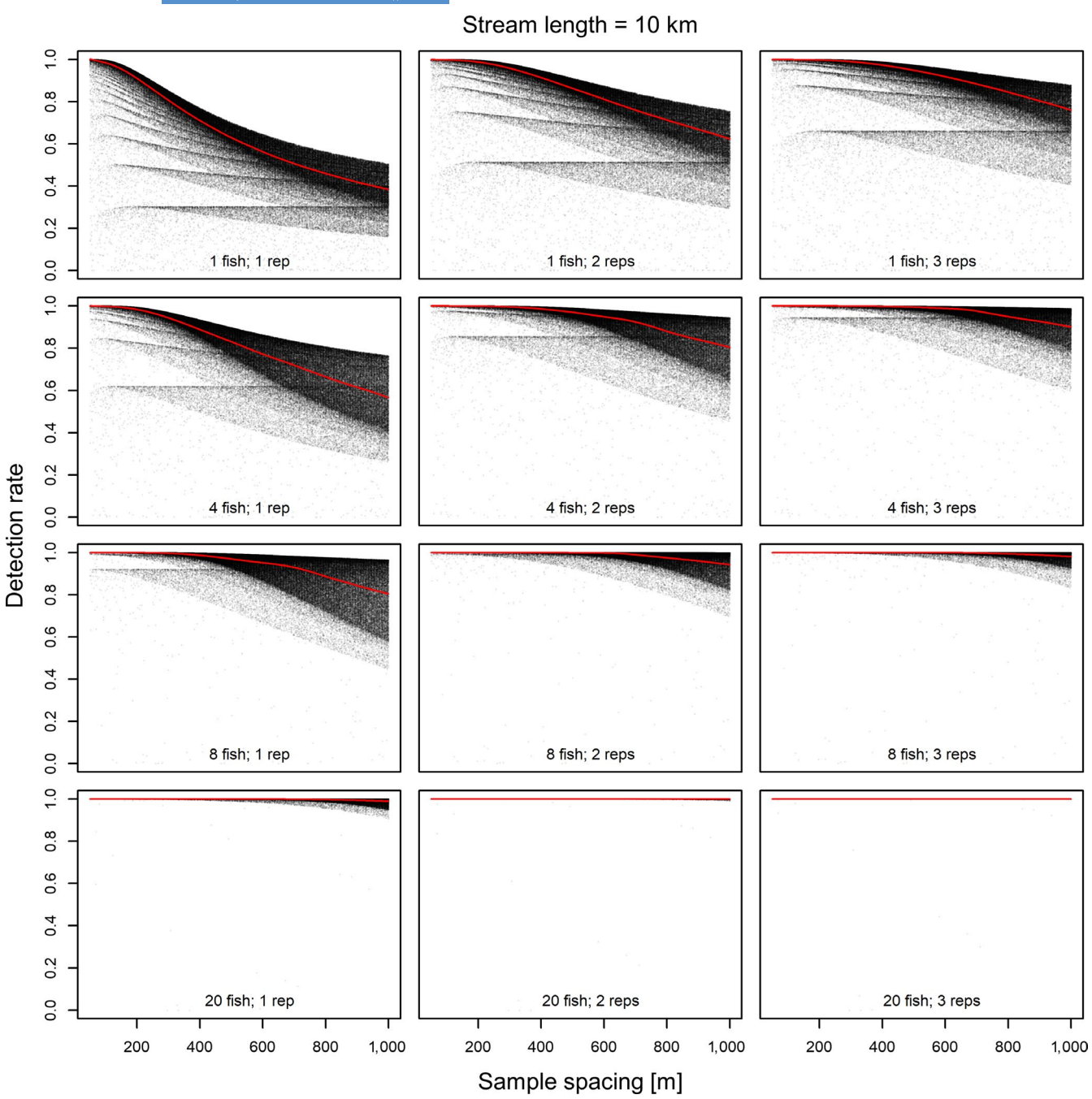

FIGURE 4 Simulated detection rates for various fish abundances and number of technical replicates, assuming evenly spaced stream-long sampling regimes. Using three technical replicates and a <400 m sampling distance nearly guarantees detection of any number of fish. Points show individual simulations; lines show locally weighted scatterplot smoothing estimates. Zebra-like patterning is the result of fish simulated close to the bottom of the stream (i.e., with very few samples taken downstream). Patterning is resolved by increasing simulated stream length (i.e., by reducing the probability, a fish is near the bottom of the stream, Figures S3–S4)

be improved given our models suggest a distinctly nonlinear pattern of eDNA detection and quantification with stream distance? Based on the log-quadratic relationship of our results, studies that take eDNA samples in close proximity to focal sites containing rare organisms are apt to either underestimate local presence, or incidentally detect organisms from farther upstream. eDNA is quickly becoming a preferred tool for detecting rare species (Hinlo et al., 2018; Pfleger et al., 2016), but this spatial bias may be a particularly pragmatic consideration for endangered salmon monitoring in Maine. Endangered salmon monitoring frequently seeks to determine juvenile fish presence on very localized scales in association with U.S. Endangered Species Act permitted activities that pose a risk of contact or harm, such as culvert or bridge improvements. Given our results show that optimal detection rate is most sensitive to distance (i.e., have narrower optimum distance ranges) and that endangered salmon often exist at low abundances (Figure 3), eDNA sampling for such permitted activities may

be recommended well downstream of the proposed impact site rather than immediately nearby. This strategy is apt to impose relatively little cost when fish are more abundant since their detection will generally be higher regardless of sampling distance. In our case, the optimal detection distance for most fish densities was approximately 30–200 m downstream of the source.

While it is useful to know what an optimal distance might be for maximizing detection in an a priori stream location, many surveys for salmon and other rare taxa are interested in determining whether the species exists anywhere along a stream corridor. This inference requires a more systematic survey approach, which can again be optimized around knowledge of eDNA distance functions. While any given stream location sample has low odds of occurring at the peak of the eDNA detection curve for a given target, there should be an optimal sampling interval such that the combined odds of detection from multiple sample locations is large enough to provide high power. Sampling at a smaller interval will not reduce that power, but can be inefficient in expending sampling resources that might be used elsewhere. Based on the eDNA distance functions in this study, it should be readily feasible (>95% probability) to detect even a single salmon with an interval of 400 m or less, assuming three technical replicates per sample. For Sunkhaze stream, which is 44 km long, the entire system could be surveyed for a single juvenile salmon with as few as 110 samples distributed along the stream length, which represents a small proportion of the effort required to survey the same system using traditional methods (e.g., electrofishing). The one caveat to that design is that power can be substantially lower to detect a fish inhabiting the bottom reaches of the survey area because of fewer downstream samples to contribute to the combined detection probability. With that in mind, surveys may opt to increase the frequency of samples collected at the bottom end of survey areas to balance this reduced detection.

In this study, we conducted our surveys during a relatively stable period of late summer flow in a single stream, while manipulating fish numbers and distances of sampling. Optimal distances and intervals are apt to vary for other taxa, and is surely dependent on fish biomass, hydrology, chemical properties of other streams, and interactions thereof. As such, we recommend researchers empirically investigate downstream detection and quantification trends in their study systems using caged populations or known occupancies when possible to help determine optimal survey approaches. Indeed, these studies could improve upon our ability to predict eDNA dynamics by sampling across taxa and stream systems with different environmental parameters (e.g., discharge, velocity) such that future meta-analyses might someday be generalized to more contexts. Finally, field studies that sample at repeated intervals can use the form of distance ~ detection rate and quantity relationships to help improve spatial interpolation (or extrapolation) of likely fish concentration areas.

A second important element of our study was the use of multiplexed eDNA assays. Our NAD5 and CO1 assays differed in their detection rates, with CO1 having a detection rate 25% higher (at 50% detection) than NAD5. Therefore, if one had the option to run only one of these assays, CO1 would be the better option. This finding is interesting in showing how two assays with similar design constraints and high laboratory efficiencies can nonetheless have very different field detection capabilities. However, we also showed that adding even this suboptimal assay to a multiplex with our CO1 assay provided the highest detection rates with a nominal increase 14% at 50% detection. This increase was not statistically significant, likely due to modest contribution possible from the weaker NAD5 assay and limitations of statistical power for this comparison. The benefit from multiplexing two higher power assays would of course likely be greater. Regardless, in this study multiplexing helped guard against incidentally drawing our inferences from a single low-power assay.

Environmental DNA holds great promise for increasing the efficiency of monitoring programs for rare species such as critically endangered Atlantic salmon. Because of their federally protected status, mapping the distribution and the outcomes of recovery actions for Atlantic salmon constitutes a major monitoring effort that bears not only salmon restoration, but on many competing land and water uses. Despite the scope of this effort, most salmon habitat in Maine goes unmonitored due to limited personnel with the mandatory qualifications and permitting to capture salmon. Considering these constraints, eDNA offers the opportunity for increased participation in salmon monitoring as the sampling methodology does not require any specialized equipment or permitting. However, realizing this potential for more expansive and cost-effective salmon monitoring will require matching salmon eDNA surveys and inference to the ecology (production, destruction, immigration, and emigration) of salmon and salmon eDNA. This study provides useful insights toward this overarching goal.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

MTK and JGT designed the experiment and obtained funding. GY collected the data. ZTW and BFE led data analysis and manuscript writing.

DATA AVAILABILITY STATEMENT

All data analyzed in this experiment are available in Table S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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