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Evidence for Particle Dilution and Dispersion as the Strongest Effects on Reach-Scale Salmonid eDNA Sampling Outcomes in Mediterranean-Climate Rivers and Streams

Shawn A. Melendy

EVIDENCE FOR PARTICLE DILUTION AND DISPERSION AS THE STRONGEST

EFFECTS ON REACH-SCALE SALMONID EDNA SAMPLING OUTCOMES IN

MEDITERRANEAN-CLIMATE RIVERS AND STREAMS

A Thesis

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Master of Science

in

Environmental Science

by

Shawn A. Melendy

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EVIDENCE FOR PARTICLE DILUTION AND DISPERSION AS THE STRONGEST
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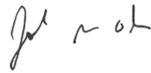
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ABSTRACT

Evidence for particle dilution and dispersion as the strongest effects on
reach-scale salmonid eDNA sampling outcomes in Mediterranean-
climate rivers and streams
by
Shawn A. Melendy
California State University Monterey Bay, 2022

Environmental DNA (eDNA) sampling from rivers has emerged as a promising new
method for monitoring freshwater organisms of management concern. However, to
more confidently interpret eDNA sampling results – and thereby improve eDNA as a
tool for management decision making – the influence of local environmental factors
on eDNA fate (transport & decay dynamics) must be better understood. At nine river
sites across the central California coast, we added a known quantity of novel eDNA
(Brook Trout, *Salvelinus fontinalis*) and collected eDNA at sequential downstream
distances for qPCR analysis. We then used random forest modeling to identify the
most important factors to reach-scale sampling outcomes and characterize salmonid
eDNA fate. Our results offer evidence of particle dilution and dispersion as primary
drivers of salmonid eDNA sampling outcomes at the reach scale. In addition, we
highlight the interplay between discharge, velocity, and cross-sectional area as key to
interpreting eDNA data for future management goals.

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INTRODUCTION

Monitoring the abundance and distribution of wild populations is essential to determine the success of conservation efforts. Such efforts are vital to protect rivers, which are among the most altered ecosystems globally (Dudgeon, 2019; Reid et al., 2019; Vörösmarty et al., 2010). Salmon are among the most important freshwater taxa to monitor, as they play critical ecological roles but have suffered widespread impacts of habitat destruction, pollution, invasive species, overfishing, and climate change (Crozier et al., 2019; Mullan, 1987; Nehlsen et al., 1991). Imperiled (endangered, threatened, vulnerable) salmon species are particularly important targets for monitoring movement of anadromous populations, spawning and juvenile activity, range expansions or contractions, and to further study their ecology. These aims have motivated numerous management efforts and studies in the United States which rely on traditional surveillance methods (e.g., snorkel surveys, electrofishing, weirs) across a vast number of streams. These approaches rely on visual or hand counting of individuals and are inherently time-consuming and disruptive to animals. In recent years, the detection of organisms using environmental DNA has emerged as a potential alternative which could reduce the need for such methods of direct observation.

Environmental DNA (eDNA) refers to DNA that is shed or excreted (e.g., tissue, mucous, saliva, urine, feces) into the environment by an organism (Taberlet et al., 2012). In aquatic environments, eDNA can be analyzed from water samples, revealing the presence of target taxa without direct handling or observation. In this way, eDNA has already shown tremendous utility as a noninvasive biomonitoring tool in rivers. It has proven a viable, cost-effective method for targeted detection of species, including those that are rare, cryptic, or invasive (Bedwell & Goldberg, 2020; Spence et al., 2021; Wittwer et al., 2018; Wilcox et al., 2013), and is increasingly used for assessing biodiversity (Civade et al., 2016; Deiner et al., 2016; Lodge et al., 2012; Pont et al., 2018; Valentini et al., 2016). However, further applications of eDNA in rivers are limited at present. While eDNA detections have shown positive correlations with observed abundance/biomass of freshwater vertebrates, the relationship varies significantly across studies and species, and is less clear at lower eDNA concentrations (Doi et al., 2017;

Lacoursière-Roussel et al., 2016; Pilliod et al., 2013; Sepulveda et al., 2021; Wilcox et al., 2016). Thus, reliably estimating abundance/biomass from eDNA alone remains elusive. Additionally, presence/absence monitoring via eDNA remains limited by the challenge of interpreting negative sampling outcomes. Specifically, it can be difficult to interpret whether a negative sample indicates the absence of the target taxon, or simply a failure to recover target eDNA from the water column due to other factors.

These limitations result from the inability to predict how much eDNA will persist in the water column over time and distance, given a particular target organism and river environment. Accurate predictions of this kind would revolutionize aquatic biomonitoring, but they require a much-improved understanding of two complex phenomena: eDNA release profiles and eDNA fate. A release profile refers to the amount, rate, and particle size distribution of eDNA released by an organism, and can vary by species, life stage, metabolic rate, and activities (Thalinger et al., 2021; Yates et al., 2021). A small number of studies have investigated fish eDNA particle size distribution (Wilcox et al., 2015) and release rate (Andruszkiewicz Allan et al., 2021; Jo et al., 2019; Klymus et al., 2015; Maruyama et al., 2014; Sassoubre et al., 2016), but release profiles cannot yet be reliably predicted for wild individuals or populations. eDNA fate refers to the transport, dispersion, degradation, deposition/adhesion, and/or resuspension of eDNA particles once they are released from an organism. Several studies on eDNA fate have been performed in controlled mesocosms and other environments, offering insight into mechanisms of degradation (Andruszkiewicz et al., 2017; Barnes et al., 2014; Sassoubre et al., 2016; Seymour et al., 2018; Shogren et al., 2017), dispersion (Andruszkiewicz et al., 2019; Laporte et al., 2020), deposition/adhesion, and resuspension (Jerde et al., 2016; Shogren et al., 2017).

However, little progress has been made to understand the cumulative effect of these mechanisms given the complex set of environmental factors that influence them in rivers, which will be necessary to predict eDNA fate. As a start, prior studies have searched for variables that can improve the relationship between target abundance/biomass and the amount of eDNA recovered downstream (Sepulveda et al., 2021; Tillotson et al., 2018). Using model selection by AIC approaches, Tillotson et al. (2018) found a minor effect of water temperature on their correlation between target

abundance and eDNA concentration, and Sepulveda et al. (2021) found minimal support for including stream habitat attributes in their models (with one potentially significant correlation for percent pool). Importantly, both studies saw inclusion of random terms for site improve model performance, suggesting there are environmental factors consistently associated with eDNA concentrations, but they were unable to be identified.

A random forest (RF) modeling approach relating environmental factors to eDNA sampling outcomes is potentially better suited for interpreting the effect of river environment on eDNA fate, given the multitude of potentially relevant variables and their high degree of interaction. Other machine learning algorithms have been used for related purposes: Ogburn et al. (2022) used boosted regression trees (BRTs) to explore the relationship between landscape-scale metrics (e.g., elevation, watershed area, land use) and presence/absence sampling outcomes for anadromous herring, inferring which habitat types within the watershed were preferred for spawning. Here, we took a similar approach, but used RF modeling to explore the relationship between reach-scale environmental factors (e.g., depth, percent riffle/pool, substrate cobble size) and the proportion of positive qPCR technical replicates for water samples collected downstream.

The aim of our study was to (1) identify the most important environmental factors to the amount of salmonid eDNA recovered in reach-scale sampling efforts, (2) characterize the nature of salmonid eDNA fate based on those factors, and (3) determine which, if any, of those factors could be a useful proxy for the eDNA fate profile (i.e., how favorable or unfavorable local conditions are to eDNA recovery) of a given site. At nine river sites across the central California coast, we added 5-gallon buckets containing brook trout (*Salvelinus fontinalis*) eDNA to the water, tracked the subsequent pulse of eDNA visually using fluorescein dye, and sampled from the pulse (leading edge to trailing edge) at sequential distances up to 200m. We then developed a random forest (RF) model of the amount of eDNA recovered at each distance as a function of river environmental factors. Since resuspension seems to primarily occur multiple hours post-deposition/adhesion (Shogren et al., 2017), our experimental design aimed to minimize the effects of resuspension on sampling outcomes. Accordingly, this represents a novel approach to investigate how eDNA fate mechanisms – principally transport, degradation, dispersion, and deposition/adhesion – are influenced by environmental factors at the reach-scale,

offering valuable insight towards eDNA abundance/biomass estimates and presence/absence determinations for salmon or other freshwater vertebrates.

METHODS

SITE SELECTION & DATA

Nine river sites within the central California coast were selected, with the goal of including a range of environmental conditions and corresponding eDNA fate profiles (Table 1, Map 1). We emphasized stream order, discharge, substrate, level of anthropogenic disturbance, and accessibility as selection criteria.

Table 1. Coordinates and select environmental factors for each river site/experimental reach.

Site	Coordinates	Sub-watershed Area (km²)	Discharge (m³/s)	Slope	Temp (°C)	Conductivity (µS/cm)	Dissolved O₂ (mg/L)	pH
Arroyo Seco	36°16'51"N, 121°19'21"W	63.1	0.787	0.11	13.3	353	8.3	6.1
Big Sur River	36°16'29"N, 121°49'29"W	151.3	1.660	0.75	8.4	406	20.1	4.7
Carmel River (Lower)	36°32'05"N, 121°54'33"W	80.1	0.776	0.13	9.7	284	11.9	5.5
Carmel River (Middle)	36°31'24"N, 121°49'50"W	80.1	0.360	0.99	10.8	269	10.0	7.1
Carmel River (Upper)	36°26'04"N, 121°42'35"W	90.3	0.094	1.23	13.9	499	17.6	5.5
Garzas Creek	36°26'14"N, 121°50'42"W	101.7	0.038	1.24	8.1	251	19.0	6.3
Pajaro River	36°53'39"N, 121°38'39"W	135.2	0.564	0.17	14.7	921	8.3	5.6
San Clemente Creek	36°25'34"N, 121°45'14"W	90.3	0.028	6.44	5.8	222	13.5	7.6
San Jose Creek	36°29'13"N, 121°51'50"W	37.2	0.021	3.01	8.4	205	12.0	5.9

Map 1. Locations of the 9 chosen river sites/experimental reaches, labeled with green pins.

SITE CHARACTERIZATION

A series of measurements were taken at each site to characterize the reach ($\leq$ 200m from the eDNA addition point). Measurements were taken after the eDNA trials to avoid disruption of substrate. Discharge (m³/s) was measured at the eDNA addition point using a flow meter (SonTek, S19-02-1219). Macrohabitat types (e.g., riffle, pool) were surveyed throughout the experimental reach (from the eDNA addition point to 200 m downstream), recording the length in meters of each contiguous segment. Channel slope was determined from elevation measurements taken at each riffle head within the reach, using a rotating laser (Topcon, RL-H5A) and measuring rod. Wetted width (m) was measured every 10 meters throughout the reach. Depth (cm) was measured every 10 meters at 5 cross-sectional points (5, 25, 50, 75, and 95% across the wetted width). At each cross-sectional point, the presence/absence of organic substrate and course

particulate organic matter (CPOM) were recorded. Substrate cobble size (mm) was measured with a gravelometer field sieve (Wildco, 3-14-D40) at each of the same cross-sectional points. Biofilm thickness was measured by collecting one substrate particle from the midpoint of each cross-section, scraping a circular area (with a rubber stencil and a toothbrush) centered on the largest face of each particle, and measuring the combined dry mass of this material for each site following Hauer & Lamberti (2017). Total suspended sediment (TSS) (g/L) was sampled upstream of the eDNA addition point using a 1-liter high-density polyethylene bottle secured inside a weighted-bottle sampler (Rickly Hydrological Co., US WBH-96). The 1-liter bottle contents were dried and weighed for each study site. Water chemistry measurements [temperature (°C), specific conductivity (µS/cm), total dissolved solids (TDS) (g/L), salinity, dissolved O2 (mg/L), and pH] were made with a handheld meter (YSI, 6050000) just upstream of the eDNA addition point.

EDNA SOURCE

We selected *S. fontinalis* eDNA because we required a source organism which was not present in our selected rivers, to avoid background detections. eDNA from this species was collected from a 120m x 4m raceway at the California Department of Fish and Wildlife (CDFW) San Joaquin Hatchery. The raceway contained ~4900 trout (671 days old) on the date of our first eDNA collection (11/5/21), and ~500 trout (847 days old) on our final collection date (5/1/22). Following the approach of Snyder et al., 2021, water was scooped into pre-sterilized 5-gallon buckets. The buckets were closed with a lid and transported back to CSUMB campus for room temperature storage before being used at a river site 22-24 hours after collection. Each experimental addition of eDNA occurred within this time window to ensure similar levels of degradation across trials. Just prior to adding the eDNA to each river, we collected 2-L preliminary samples from the bucket to determine the starting concentration.

RIVER SAMPLING

Five gallon buckets (1 for the first 7 sites, 2 for the 8th site, and 4 for the 9th) of *S. fontinalis* eDNA were added to each river at a riffle head and at the thalweg. At the same moment we added the eDNA, we added 2-4 grams (more for higher discharge sites)

of fluorescein dye (Thermo Scientific, 119240250), pre-mixed with river water in a Nalgene bottle. This dye acted as a visual marker of where the eDNA pulse was located as it flowed downstream, thus informing crew members when to begin and end eDNA collection at each sampling distance.

Crew members were positioned to collect eDNA samples from the thalweg (≤2 ft. below the surface) at 10, 50, 100, and 200 meters downstream. If crew needed to position themselves in the water to access the thalweg, they walked and stood downstream of the sampling point. When the leading edge of the fluorescein dye plume first reached each crew member, that person began to pump their first sample. They pumped until 5 L of water had been filtered (or until the filter clogged), and immediately proceeded to take another sample, repeating the process until the entire plume of dye had passed. The sequential order of samples at a given distance were denoted as "A", "B", C", etc. Because the dye plume elongated as it flowed downstream, this approach required fewer samples at the shorter distances (e.g., only "A" at 10m), and more at the longer distances (e.g., "A" through "C" at 200 m).

Samples were collected following Carim et al., 2016, using peristaltic pumps (Geotech, 91350103) to direct river water through 1.6-µm glass microfiber filters (Whatman, 1820-047) and into outflow buckets. The filter holder/collection cup were lowered into the water pointing in the upstream direction. Filters were subsequently folded twice-over and transferred, using plastic forceps, to a 50-mL tube containing approximately 25 mL of silica-bead desiccant. These tubes were protected from sunlight and heat for transport to the lab. Pump tubing, forceps, and other sampling equipment were sterilized with a 20% bleach solution and thoroughly rinsed with DI water prior to collection.

EDNA EXTRACTION

Each filter was split in half, with one side extracted and another archived. Extractions used a combination of the DNeasy Blood and Tissue Kit (Qiagen, 69506), and Qiashredder Kit (Qiagen, 79654) in a protocol developed by Torrey Rodgers and Jim Walton (personal communication). The final elution step used 80 µL AE buffer. Elutes were concentrated using a benchtop centrifugal vacuum concentrator (Labconco,

7810012) at 34-37°C for 30-90 minutes and re-suspended in 20 µL of AE buffer in a shaking incubator at 65°C, 850-1000 rpm, for 10 minutes.

QPCR

We utilized a probe-based qPCR protocol, targeting *S. fontinalis* cytochrome c oxidase I (COI) sequences identified by Hulley et al. (2019).
F: CGGTACGGGGTGAACAGTTT, R: GGAAATGCCAGCTAAATGTAGGG, P: FAM–CTCGCCCACGCAGGAGCTTC–QSY. Primer and probe concentrations followed Hulley et al. (2019). Three technical replicates were run for each eDNA extraction (from a half filter). Each 20 µL reaction contained: 10 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems, 4396838), 1 µL each primer, 1 µL TaqMan QSY probe (Applied Biosystems, 4482777), 2 µL internal positive control (IPC) mix (Applied Biosystems, 4308321), 0.4 µL IPC DNA (Applied Biosystems, 4308321), and 4.6 µL sample. Reactions were run in 96-well plates (VWR, 82006-664) with (i) a triplicate standard curve with 10^1 - 10^6 COI copies inserted to a linearized plasmid (Integrated DNA Technologies), (ii) triplicate no template control with DEPC water, and (iii) triplicate no amplification control for the IPC reaction. Plates were sealed with clear adhesive film for qPCR (VWR, 60941-078). A CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was set to the following thermocycling conditions: 10-min activation at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Samples with a single positive replicate were considered positive for *S. fontinalis* DNA. Positive replicates were confirmed by visually inspecting amplification curve morphology.

STATISTICAL ANALYSIS

NMDS

We used non-metric dimensional scaling (nMDS) ordination plotting to visualize the dissimilarity in river environment across our experimental reaches. nMDS was performed using the Bray-Curtis dissimilarity matrix on 22 environmental factors (Table 2, excluding sampling design group). These include 20 factors measured in our own reach characterization and 2 (watershed- and catchment-level baseflow index) obtained from the EPA StreamCat data set (https://github.com/USEPA/StreamCat). nMDS was

performed using the vegan package and plotted using ggplot2 in RStudio (RStudio Team, 2020).

Table 2. List of predictor variables used in nMDS and RF modelling, categorically grouped. The sampling design group (variables determined by our chosen sampling locations within each experimental reach, and the amount of eDNA we added) was only included in RF, not nMDS. Depth (average), depth (maximum), wetted width (average), percent pool, percent riffle, D50, percent CPOM cover, percent fine particles, and percent organic cover were calculated for the portions of every reach between the eDNA addition point (0 m) and each sampling location (e.g., the average depth between 0–10m, 0–50m, 0–100m, and 0–200m were predictors for the PPTR at each corresponding sampling distance). All other variables had one value to represent the entire reach, so the same predictor value was used for each PPTR within a reach.

Group	Predictor Variable	Unit
Channel morphology	Depth (average)	cm
	Depth (maximum)	cm
	Slope of channel	--
	Wetted width (average)	m
Hydrology	Baseflow index (catchment average)	--
	Baseflow index (watershed average)	--
	Discharge	m^3/s
Macrohabitat	Percent pool	%
	Percet riffle	%
Sampling design	Depth (average) of sampling cross-section	cm
	Depth (maximum) of sampling cross-section	cm
	Distance	m
	Starting quantity / discharge	copy # / (m^3/s)
	Wetted width of sampling cross-section	m
Substrate	Biofilm thickness	g
	Median particle diameter (D50)	mm
	Percent course particulate organic matter (CPOM) cover	%
	Percent fine (<2mm diameter) particles	%
	Percent organic cover	%
Water quality	Conductivity	mS/cm
	Dissolved oxygen	mg/L
	Percent dissolved oxygen	%
	pH	--
	Salinity	--
	Temperature	°C
	Total dissolved solids (TDS)	g/L
	Total suspended sediment (TSS)	mg/L

OCCUPANCY ANALYSIS

We used a Bayesian multiscale occupancy modeling approach to estimate the probability of *S. fontinalis* eDNA detection in a water sample (θ) at the reach-scale for each of our sites. This modeling was performed with the eDNAoccupancy package in R

(Dorazio & Erickson, 2018) using 11,000 Markov chain Monte Carlo iterations. Posterior means and 95% credible intervals for θ were estimated after a burn-in of 1000 iterations.

RANDOM FOREST

We developed a random forest (RF) model (Breiman, 2001) to interpret which river environmental factors have the greatest effect on the amount of eDNA recovered downstream. RF is a nonparametric regression and classification modeling approach that is well suited for numerous predictor variables with interacting effects. We used the proportion of positive technical qPCR replicates across all water samples at a given sampling distance (PPTR) as the response variable. For example, if one site's "A" water sample at 50m had 2/3 positive technical replicates, and the "B" water sample at 50m had 1/3 positive technical replicates, the PPTR for 50m was 3/6 = 0.50.m. PPTR served as a rough proxy for amount of eDNA recovered, since the copy number values from our qPCR results were reliably below the assay's limit of quantification (LOQ = 299.54). 27 predictor variables were initially included prior to variable selection (Table 2). We used the VSURF package in R for predictor and model selection, with default settings except that 200 forests were built for the thresholding step and 100 forests were built for the interpretation step (Genuer et al., 2015). The 8 predictor variables included in the final model were selected by the VSURF interpretation step (comparing all nested models and selecting the single model with the lowest out-of-bag error). If any selected variables were correlated (r > 0.75), the least important of the pair was removed.

RESULTS

SITE CHARACTERIZATION

Our nine chosen experimental reaches captured a variety of environmental conditions. The reaches varied most according to their percentage of fine (<2mm diameter) substrate particles, channel slope, amount of suspended sediment (mg/L), average depth, and water temperature. NMDS1 (horizontal axis) primarily represents river size and NMDS2 (vertical axis) primarily represents substrate particle size and suspended sediment (Figure 1). The most similar reaches (according to proximity in the nMDS plot) aligned with our expectations based on habitat setting, stream order, our observations of the substrate, etc.
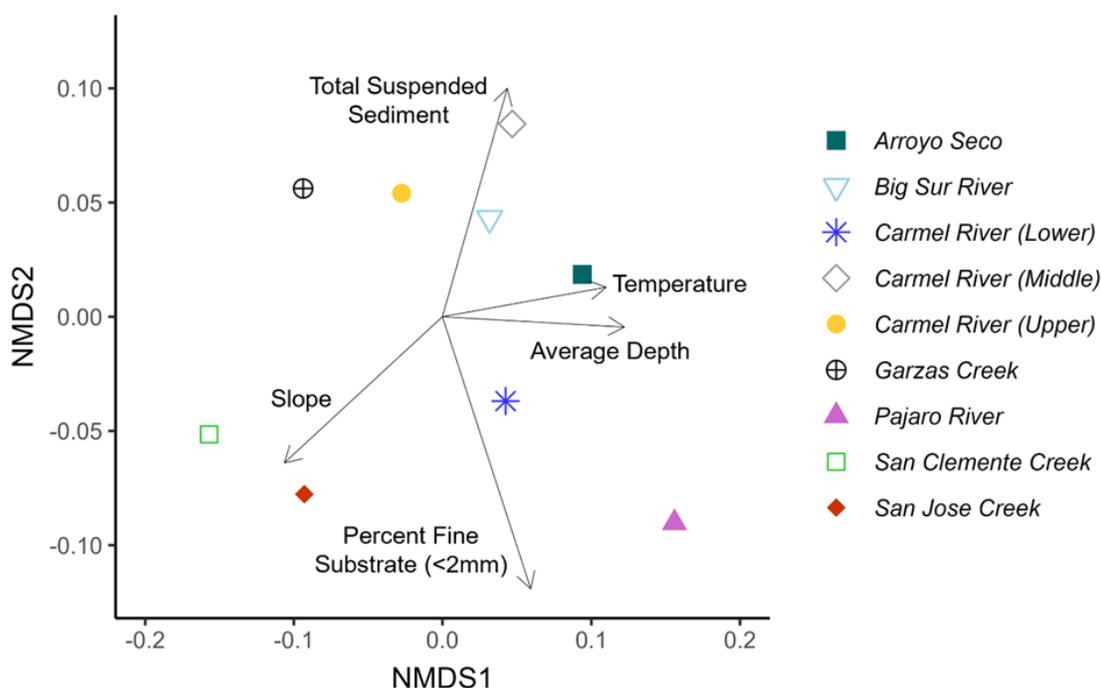
Figure 1. Non-metric multidimensional scaling ordination (nMDS) plot showing Bray-Curtis dissimilarities in river environment of our 9 experimental reaches. Reaches shown by color and symbol. Arrows represent vectors for variables with the 5 greatest loadings from the nMDS.

EDNA ADDED

Our first collection of *S. fontinalis* eDNA from the hatchery yielded 2,800,000 copies (bucket total), which were added to the upper Carmel River site. Each of the eight following collections yielded much lower eDNA quantities, as fish counts declined from the hatchery raceway due to removal and mortalities. The upper Carmel River trial had the highest ratio of starting quantity to discharge, and the Garzas Creek trial had the lowest (Figure 2).
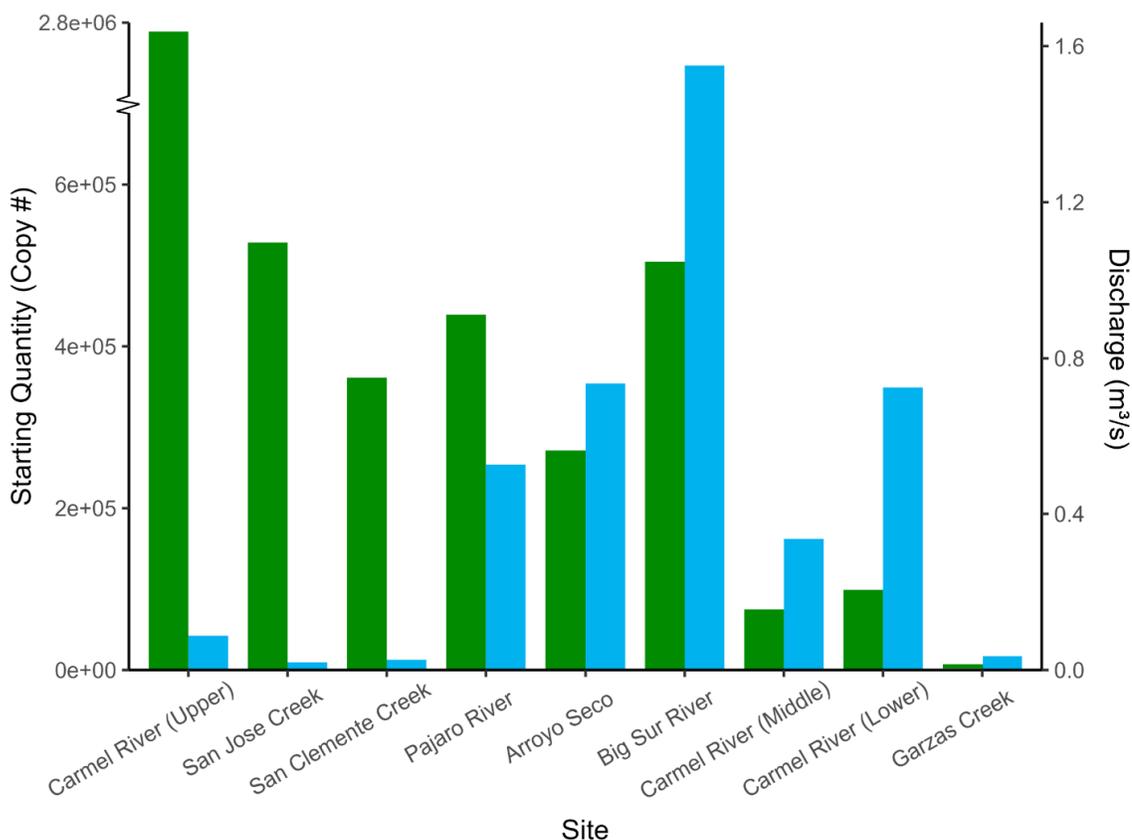
Figure 2. Starting quantity of *S. fontinalis* eDNA added (copy #) and discharge (m³/s) for each experimental reach. Green bars represent starting quantity and blue bars represent discharge. Reaches are shown in decreasing order of starting quantity to discharge ratio, from left to right. The starting quantity axis (left) has a break to accommodate the upper Carmel River, which had a much greater starting quantity than any other site.

EDNA SAMPLING OUTCOMES

We successfully recovered *S. fontinalis* eDNA (i.e., at least 1 qPCR technical replicate amplified) downstream at each of our sites (Figure 3). Cycle threshold (Ct)

values ranged from 33.71 to 43.11. The average positive rate for technical replicates declined over distance from 0.70 at 10m to 0.28 at 200m. The positive rates for water samples and sampling distances increased from 10 to 50m, and then declined through 200m. Regarding individual river sites, the upper Carmel River had the highest positive rate for technical replicates and water samples, while sharing the highest rate for sampling distances with San Jose Creek and San Clemente Creek. The middle Carmel River had the lower positive rate for each level of analysis (Table 3).
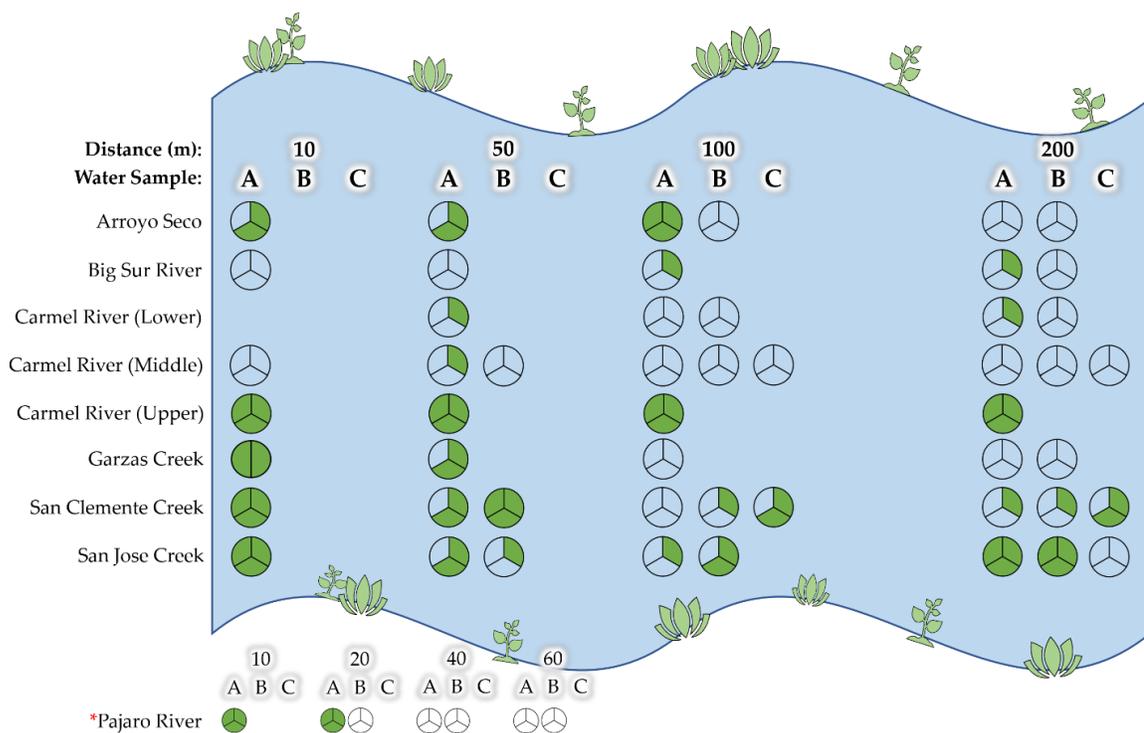
Figure 3. *S. fontinalis* eDNA sampling outcomes for each water sample, at each distance, for every river site. Each circle represents a water sample. The number of divisions within circles represents number of qPCR technical replicates run. Green shading indicates positive replicates. *Pajaro River, shown in bottom left, had samples taken at different distances than every other reach.

Table 3. Positive rates for *S. fontinalis* eDNA sampling, calculated at the level of qPCR technical replicates, water samples, and sampling distances. 1-3 water samples were taken at each sampling distance, and 3 technical replicates were run for each water sample (except Garzas 10A, which had 2). Upper table shows rates for each distance across all sites – excluding the outlier distances sampled at the Pajaro River (20m, 40m, 60m). Lower table shows rates for each river site across all distances, in decreasing order of positive technical replicate rate.

Distance (m)	Positive Rates		
	Technical Replicates	Water Samples	Sampling Distances
10	0.70	0.75	0.75
50	0.52	0.82	0.88
100	0.29	0.47	0.63
200	0.28	0.44	0.63

Site	Positive Rates		
	Technical Replicates	Water Samples	Sampling Distances
Carmel River (Upper)	1.00	1.00	1.00
San Jose Creek	0.63	0.88	1.00
San Clemente Creek	0.56	0.89	1.00
Arroyo Seco	0.39	0.50	0.75
Garzas Creek	0.29	0.40	0.50
Pajaro River	0.29	0.29	0.50
Big Sur River	0.13	0.40	0.50
Carmel River (Lower)	0.13	0.40	0.33
Carmel River (Middle)	0.04	0.11	0.25

OCCUPANCY ANALYSIS

The estimated probability of occurrence of S. fontinalis eDNA in a water sample (θ) was high for all sites (>0.80) but had wide confidence intervals (Figure 4). The Big Sur River had the greatest detection probability ($\theta = 0.98$) while Arroyo Seco had the lowest ($\theta = 0.83$).

RANDOM FOREST MODELING

We modeled the proportion of positive technical replicates (PPTR) across all water samples at a sampling distance, according to environmental predictors from every experimental reach. This response variable was chosen as a rough proxy for amount of eDNA recovered, since all our samples were below the qPCR assay's limit of quantification and copy number could not be used. Of 27 environmental predictors, 8 were identified in our random forest modeling approach as most important to PPTR and

included in the final model (R² = 0.41) (Figure 5). The starting quantity of eDNA, normalized by discharge, was the most important variable (IMP = 20.4), and had a strong positive relationship with PPTR. Average depth (cm) of the reach had the next highest variable importance (IMP = 19.9), showing a strong negative relationship with PPTR. Total suspended sediment (mg/L) (IMP = 17.3), percent pool (IMP = 16.2), and discharge (m³/s) (IMP = 15.0) were the next most important variables and had negative relationships with PPTR. Channel slope (IMP = 14.0) showed a weak positive relationship with PPTR, while percent organic substrate (IMP = 12.9) and percent riffle (IMP = 9.3) showed positive and negative relationships with PPTR, respectively.
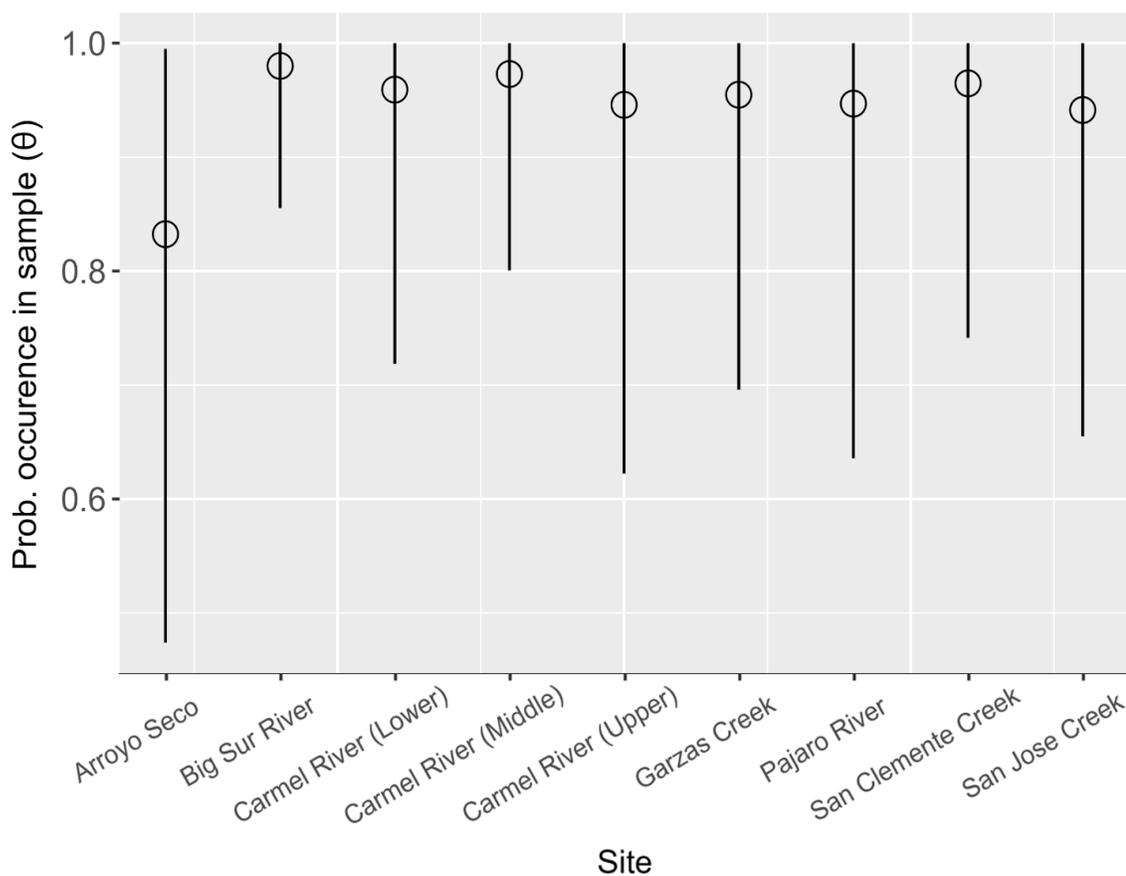
Figure 4. Probability of occurrence of *S. fontinalis* eDNA in individual water samples (θ) for each experimental reach. Estimates are posterior means with 95% credible intervals.
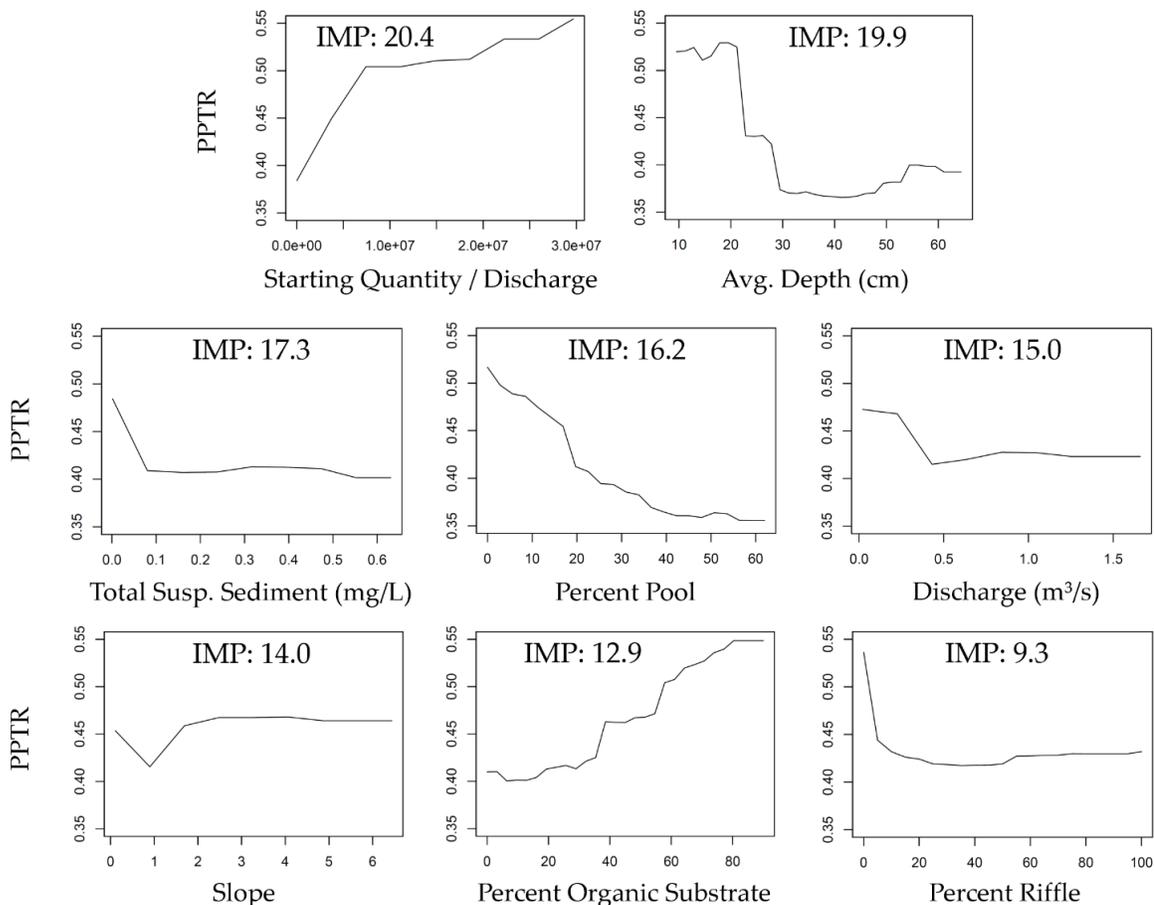
Figure 5. Partial dependence plots (PDPs) of selected random forest model predictors, in decreasing order of importance (IMP). PDPs show, for water samples at a given distance, how the proportion of positive qPCR technical replicates (PPTR) varies in response to individual predictors. The steeper the response curve, the more influential the variable is within that PPTR and variable range.

DISCUSSION

This study uniquely contributes to the investigation of eDNA fate and sampling outcomes in lotic systems, given our novel experimental design in which the starting quantity of target eDNA released into each river was quantified, and the effect of a particular eDNA fate mechanism (resuspension) was largely controlled for. Our results support the sizeable body of evidence that a positive relationship exists between the amount of eDNA recovered downstream in rivers, and the biomass or density of target fish upstream (Baldigo et al., 2017; Levi et al., 2019; Pochardt et al., 2020; and others,

reviewed in Yao et al., 2022), a promising sign for the pursuit of quantitative eDNA-based monitoring. While prior studies have done well to point out the strong dependence of lotic eDNA fate on local conditions (Goldberg et al., 2016; Spence et al., 2021; Wood et al., 2020), an understanding of fate that can be more broadly applied – at least across streams of similar order or within a geographic region – is an essential next step in the development of eDNA-based monitoring. With this step, useful predictions about a site's expected eDNA fate profile can be made, and eDNA sampling outcomes can be interpreted with context - even without thoroughly characterizing the local river environment.

While our discrete pulses of eDNA did not perfectly recreate the eDNA plume produced by an actual fish, it is notable that our direct estimate of target copy number added to each river is positively correlated ($R^2 = 0.67$) with the proportion of positive technical replicates (PPTR) from downstream water samples, even without the contribution of resuspended eDNA. The remaining variability in this relationship, unexplained by starting quantity (SQ), can be attributed to locally determined mechanisms of eDNA fate and sampling/analytical methodology. Using a random forest (RF) modeling approach, we identified river environmental factors important to PPTR (i.e., factors that explain part of the remaining variation) (Figure 5). From these factors and their modeled relationships with PPTR, we gained insight to reach-scale salmonid eDNA fate and identified discharge as a potential proxy variable for the eDNA fate profile of prospective salmonid sampling sites.

The positive relationship between SQ/discharge and PPTR shown in our model, in addition to SQ/discharge having the greatest variable importance (Figure 5A), points to the simple effect of dilution as a significant driver of the amount of eDNA recovered in reach scale sampling efforts. Van Driessche et al. (2022) found a similar effect when comparing sampling outcomes for caged fish placed in high and low discharge river sites: less eDNA was able to be recovered at the higher discharge site. Interestingly, Van Driessche et al. (2022) also found similar detection probabilities between the low and high discharge sites through 300 meters. While this may seem contradictory, they proposed that eDNA may disperse (fragment into smaller particles and mix throughout the water column) faster under higher discharge rates, thereby being less disposed to

decay over time. This explanation is well aligned with the finding of Brandao-Dias (2022): that smaller fish eDNA particles (from both common carp and steelhead) have significantly lower decay rates than larger particles. If it is true that eDNA particles tend to be more dispersed in higher discharge rivers, this suggests a tension between the diluting effect of higher discharge and the dispersing (and thereby stabilizing) effect of higher discharge. Such a tension could explain why higher discharge rivers tend to allow for further downstream detections (Van Driessche et al., 2022; Wilcox et al., 2016; Wood et al., 2021), but can also yield lower amounts of eDNA recovered over distance (Van Driessche et al., 2022).

We initially hypothesized another contributing factor for why higher discharge could lead to similar or even greater detection probabilities: decreased substrate interaction of particles. Deposition of particles to the substrate, particularly organic substrate, has been well-demonstrated as a relevant mechanism of eDNA fate (Jerde et al., 2016; Shogren et al., 2017), but if particles could avoid that interaction all-together by 'riding' along the upper water column of larger rivers, then more of them could travel further downstream. If this effect is strong, one would expect a positive relationship between depth and amount of eDNA recovered. However, our RF model shows the opposite: a strong negative relationship between average depth and PPTR, with average depth as the second-most important variable (Figure 5B). Thus, at the reach-scale in Mediterranean streams, it appears any positive effect on salmonid eDNA recovery associated with less deposition/adhesion in deeper reaches is outweighed by the negative effect of increased dilution and dispersion. Although, this relationship must be considered in context with the macrohabitat profiles of individual reaches (e.g., whether the average depth is representative of the entire reach or is driven by a single deep pool or shallow riffle).

These observations warrant an important distinction between two common end-points of eDNA sampling data: the total amount of eDNA recovered (in concentrations of copies or picograms per liter) and the detection probability (often calculated using the Bayesian occupancy modeling approach described in Dorazio & Erickson, [2018]). The detection probability represents the probability of recovering any detectable amount of eDNA, based on how many replicate water samples are collected and how many PCR

technical replicates are run for a given location. According to the emerging model of lotic eDNA fate, referenced above, higher discharge leads to increased fragmentation of particles, potentially leading to more even mixing throughout the water column and shifting the particle size distribution towards a size class that may degrade more slowly. Thus, the total amount of eDNA able to be recovered in a single water sample will go down with higher discharge, but the probability of capturing any eDNA – even a very small amount – will go up. We observed this decoupling of amount recovered and detection probability in our data, with consistently high detection probabilities (>0.80) (regardless of distance) for all our sites (Figure 4), but an average decline in PPTR over distance (Table 2).

The top environmental factors in our RF model are informative of salmonid eDNA fate dynamics, but the question remains: are any of them a useful proxy variable for managers hoping to assess which sites have favorable eDNA fate profiles? Our results point to discharge and average depth as promising candidates for these proxy variables for fish (and potentially a wide range of taxa) given their top importance in our RF model (Figure 5A, 5B). Our prior expectation was that the best proxy variables for reach-scale eDNA fate may have been related to river substrate (due to the role of substrate in deposition/adhesion) but instead, variables simply relating to the dilution and dispersion of particles were much more important in our model. This implies that the dilution and dispersion of particles has a greater impact on salmonid eDNA sampling outcomes at the reach-scale than deposition/adhesion, and therefore, simply knowing the discharge and depth of a river site may be the most effective way to predict sampling outcomes. However, this must be qualified by the fact that we only collected water samples from the thalweg, near the top of the water column. Calls for sampling eDNA across the wetted width of a river channel have emerged as the importance of dilution and dispersion to sampling outcomes has been revealed (Van Driessche et al., 2022; Wood et al., 2021). We echo this suggestion, and add that sampling up and down the water column vertically – though, admittedly, a logistical challenge – would likely have a positive impact on the amount of eDNA recovered as well. In addition, using dye (e.g., fluorescein) to visually identify the thalweg and degree of dispersion could be useful in deciding where to collect a water sample. If one could hypothetically sample eDNA from the entire cross-section of

a river, the effect of dispersion would be accounted for, and reach-scale sampling outcomes would presumably be driven by deposition/adhesion – making variables related to deposition/adhesion the best proxy variables. Thus, starting quantity/discharge and average depth may be driven in part by only sampling from a small area of the river cross-section. Such concerns for the cross-sectional sampling area should be considered when sampling eDNA in rivers, especially for quantitative efforts.

The third-most important variable in our RF model, total suspended sediment (mg/L), may also implicate the significance of sampling methodology to the amount of eDNA recovered. This factor's negative modeled relationship with PPTR (Figure 5) could be driven by adhesion of eDNA particles to suspended sediment, thereby increasing deposition or some other mechanism of removal from the water column. However, we postulate this relationship points more to the effect of suspended sediment on sample collection and processing: namely, clogging of the filter and an associated drop in pump pressure/flow rate. As eDNA sampling technology has progressed towards professionally engineered sampling units, the importance of controlling pump pressure and flow rate has been increasingly recognized (Thomas et al., 2018), and our results likely further emphasize this point.

Some prior studies have revealed a non-linear relationship with recovered eDNA concentrations over distance, consistent with a 'breakout phase' of plume dynamics: Wood et al. (2020) observed eDNA concentrations peak 70m downstream of caged Atlantic Salmon (Salmo salar), Itakura et al. (2020) observed a peak 50-70m downstream of Japanese eel (Anguilla japonica) capture sites, and Van Dreische et al. (2022) observed peaks at 300m and 1-2km for high and low biomass cages, respectively, of four different freshwater fish species. While our positive rate for technical replicates (across all sites) was highest at 10m (0.70) and then declined over distance, our positive rate for water samples did slightly increase from 10m (0.75) to peak at 50m (0.82). Additionally, two sites had no eDNA recovered at 10m, but did have positive samples further downstream. Thus, our method of adding a discrete pulse of previously shed eDNA may have allowed for 'breakout phase' dynamics for some trials, but there was not a strong indication of such an effect. This may be consistent with using eDNA scooped a day in advance from a

hatchery raceway, where many particles could have already broken down from their largest, original state by the time of the experiment.

Several practical implications for eDNA-based monitoring efforts follow from the strong effect of dilution and dispersion on reach-scale sampling outcomes. As discussed, sampling from as much of the river cross-section as possible, while also accounting for variation in pump pressure and flow rate, are key considerations. Additionally, detecting rare, cryptic, or patchily distributed species is likely to be more successful in smaller rivers and/or higher in watersheds. By the same logic, wet seasons subject to higher discharge or more frequent hydrograph peaks are likely to be less optimal times for eDNA sampling.

In summary, the top importance of SQ/discharge and average depth in our RF model, as well as their individual modeled relationships with PPTR, provide evidence of particle dilution and dispersion as primary drivers of salmonid eDNA sampling outcomes at the reach-scale in Mediterranean-climate streams and rivers. As such, sampling will tend to recover less eDNA in larger rivers (assuming equal target density/biomass), and this proxy can be used to streamline and inform eDNA monitoring efforts. We emphasize the importance of how rivers facilitate dispersal of eDNA particles in the water column, and how that process is likely to affect the amount of eDNA recovered differently from detection probability. Moving forward, we highlight the interplay between discharge, velocity, and cross-sectional area as key to understanding the dynamics of reach-scale eDNA fate and maximize the utility of eDNA data accordingly.

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