EVALUATING THE UTILITY OF TRACERS TO CHARACTERIZE ENVIRONMENTAL DNA TRANSPORT AND INFORM DETECTION OF FISHES IN SMALL STREAMS

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ABSTRACT

EVALUATING THE UTILITY OF TRACERS TO CHARACTERIZE ENVIRONMENTAL DNA TRANSPORT AND INFORM DETECTION OF FISHES IN SMALL STREAMS

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This study was motivated by the need to develop a noninvasive and highly sensitive monitoring tool for determining local occupancy of an endangered aquatic species To make inference into the occupancy of fishes within small stream sites, we developed a method to determine whether environmental DNA (eDNA) concentrations from a target species were elevated relative to a tracer, formulated to mimic eDNA and introduced at a single location. We examined patterns in the observed distribution of the tracer to account for the effects of site-specific transport processes and sampling on distributions of naturally occurring eDNA at small spatial scales (400 meters). Concentrations of two tracers, introduced at high and low concentrations, and eDNA from two target species, coho salmon (*Oncorhynchus kisutch*) and steelhead (*Oncorhynchus mykiss*), were simultaneously surveyed in eight study sites across a range of spatial scales (100-400 m). Target species were observed in all study sites during snorkel surveys, thus the expectation was detection of both species, and non-detection would indicate situations where this method required refinement. In total, 128 occupancy assessments were conducted across all study sites, spatial scales, tracers and targets. In 41.4% of assessments, the ratio of target eDNA to tracer significantly increased, providing

evidence for additional sources of target eDNA in the study sites (target presence). Using a higher concentration tracer resulted in an increased number of eDNA detections for both targets (51.6%), compared to the low concentration tracer (31.2%), due in part to the broader range over which the higher concentration tracer could be detected. Spatial scale was identified as a crucial factor for species detection, with higher detection rates (63-88%) in assessments conducted on samples separated by 300-400 m, than for samples separated by 100-200 m (25-38%). This study presents a novel approach employing an eDNA tracer to enable species detection in a non-invasive manner at small spatial scales, emphasizing the utility of using a tracer to account for site-specific transport processes. The approach implemented herein was effective with high concentration tracers and at larger spatial scales, but before the approach could be effectively implemented for conservation and management a more diverse set of environmental factors and occupancy scenarios should be explored to evaluate false positive and false negative detection rates.

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INTRODUCTION

Species monitoring using environmental DNA (eDNA) in water samples is an innovative approach providing high species detection probabilities while causing minimal disruption to the habitat and organisms within it (Spence et al 2021; Schmelzle and Kinziger 2016; Goldberg et al 2016, Penaluna et al. 2021). Utilizing eDNA methods for aquatic species monitoring involves the collection of DNA-bearing particles (mucus, tissue, excrement) released by organisms into their environment (Eichmiller et al. 2014). Collected DNA is then extracted and amplified using targeted and non-targeted methods to estimate the quantity of DNA in a sample (Eichmiller et al. 2014). To date, many studies have implemented this technique in aquatic systems to determine species distribution over broad geographic scales without the need for costly and physically intensive field survey techniques (e.g., Sutter and Kinziger 2019; Ostberg et al. 2019; Duda et al. 2020).

Environmental DNA has been effectively used to monitor aquatic species in rivers (Wood et al. 2021, Hallet et al. 2012, Everets et al. 2022, Shaffer 2024). However accurately determining the spatial scope of the detection of eDNA requires an understanding of its transport dynamics (Ostberg and Chase 2022, Thalinger et al. 2021, Harrison 2019). Generally, concentrations of eDNA decline as they are transported away from their source (Spence et al. 2021; Thalinger et al. 2020), however many interacting local factors influence the transport of eDNA. Hydrological processes greatly affect the transport of DNA through mixing, settling, and resuspension processes (Van Driessche

2022, Wood et al. 2012, Spence et al. 2021, Jo and Minamoto 2021) while degradation of eDNA is dependent upon abiotic factors (temperature and UV exposure) and biotic factors (microbial activity) (Snyder et al. 2023, Caza-Allard et al. 2021, Harrison 2019). Thus, eDNA transport is closely tied to the specific characteristics of the site under study, and can exhibit distinct variations spatially and temporally (Troth et al. 2021, Van Driessche et al 2022, Thallinger et al 2020). Environmental DNA transport models have been developed, however they are limited by the assumption of constant effects of environmental and biological factors, or may not include a sufficient description of the factors enabling effective predictions across systems (Nukazawa et al 2018, Wood et al 2021). In many cases, having a comprehensive understanding of eDNA dynamics for a given system would require significant investment, likely exceeding the capabilities of monitoring initiatives and offsetting the advantages of eDNA methods. Developing a system that allows for a rapid and inexpensive assessment of system-specific eDNA transport would be invaluable for the future of eDNA surveys.

Herman (2023) developed an Autonomous eDNA Introduction Device (ADID) that releases an exogenous eDNA tracer, enabling the system-specific characterization of eDNA transport in small streams. The ADID was specifically was designed to release a tracer that: (1) closely mimics naturally occurring eDNA (NeDNA) and (2) is distinct from any NeDNA present in study systems. A key assumption of this system is that the tracer, once it is fully mixed within a stream, behaves in a similar manner to that of NeDNA and is subject to the same transport and degradation processes. By releasing the tracer from a point source and sampling it at multiple downstream locations, the ADID

facilitates the study of eDNA transport at a specific location. Examining the changes in concentration of the tracer as it is moved away from its source allows for assessment of the vertical and lateral dispersion of eDNA in a stream. By concurrently sampling of NeDNA and the tracer, it is possible to simultaneously evaluate the impact of site specific eDNA transport and eDNA from a native target species.

This research used ADIDs to deliver two exogenous eDNA tracers to characterize eDNA transport over small spatial scales (100 to 400 m), with the goal of determining local-occupancy of fishes in small streams. Tracers were used to examine how eDNA entering a study site is influenced by site-specific transport processes, serving as a system-specific reference for interpreting NeDNA concentrations. The ADID was used for controlled release of exogenous eDNA from a fixed upstream point for several hours, allowing for the tracer to be transported across a study site, and establishing equilibrium conditions. Multiple sampling locations were designated at varying distances from the ADID, resolving concurrent distributions of the tracers and NeDNA. If NeDNA concentrations significantly elevated relative to the tracer between two sampling locations, it suggested an enrichment of NeDNA consistent with the presence of the target species in the site downstream of the ADID.

To assess how NeDNA concentrations are changing relative to the system-specific transport revealed by the tracer, I calculated ratios of the concentrations of target species eDNA relative to the exogenous eDNA tracer. A constant ratio value indicates that concentrations of the target's eDNA and the tracer are declining proportionally with one-another, indicative of no additional input of target eDNA and therefore the targets

absence from the study site. If ratio values increase, it indicates target species presence in the study site, as NeDNA concentrations are enriched and elevated relative to the expectations provided by the tracer (Figure 1, Appendix A: Table A1). Thus, the tracer serves as a baseline for how eDNA is transported through a stream reach without enrichment of eDNA from a target, addressing the potentially misleading influence of target eDNA being transported from upstream of a study site. This allows for distinguishing among four local-scale occupancy scenarios regarding species' presence both upstream and within study sites (Figure 1, Appendix A: Table A1).

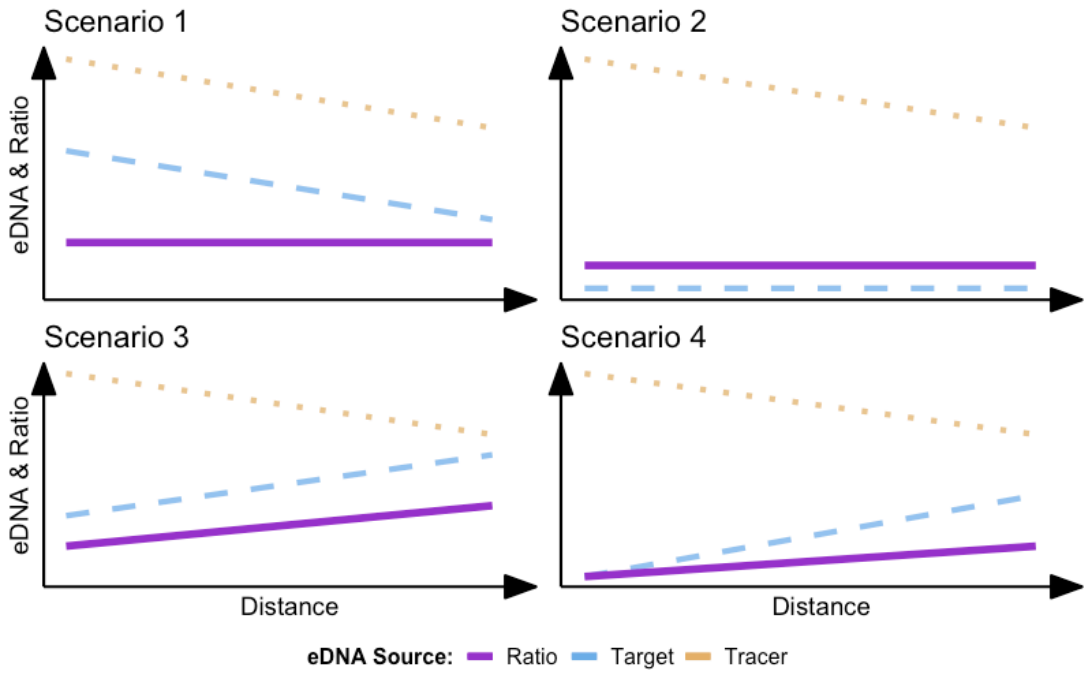

Figure 1:Four occupancy scenarios and the theoretical eDNA concentrations for the tracer (yellow dotted line), target species (blue dashed line), and the resulting ratio between eDNA sources (purple solid line). In Scenario 1 the target species is present upstream of the study site, but absent within it. As a result target eDNA is detected entering the site and declines synchronously and proportionately with the tracer, and the ratio between the two signals remains constant. In Scenario 2, the target species is absent upstream of and within the study site. As a result target eDNA is not detected entering or within the site, and the ratio is a constant zero. In Scenario 3, the target is present upstream of and within the study site. As result target eDNA is detected entering the site and does not exhibit the same decline in concentration as the tracer, and the ratio increases. In Scenario 4, the target is absent upstream of the study site, but present within it. As a result the first detection of target eDNA is within the study site, and the ratio increases starting from zero.

The motivation for this study was to develop an approach for accurate, local occupancy assessments, enabling determination of species presence within small stream reaches (100 to 400 m). The approach leverages the sensitivity of eDNA surveys in

combination with a tracer particle to account for site-specific processes and prevent false detections from occurring. This method could be used for monitoring endangered species, where no take or direct handling of the species is permitted, or within sensitive environments, where precise local scale information is required, such as at bridge-crossings.

This study employed the ADID system to determine if a tracer particle could be used to standardize concentrations of NeDNA for establishing the presence/absence of naturally occurring species within 100 to 400 m study site. Evaluations were conducted in eight study sites, assessing the presence of two target species across differing spatial scales, culminating in 128 assessments of species presence. Data were analyzed to specifically assess: (1) the impact of tracer dosing concentration, high versus low, on occupancy assessments, and (2) the impact of spatial scale, ranging from 100 to 400 m, on detection of native species. Snorkel surveys were conducted at each study site to determine the presence, distribution and relative number of naturally occurring species. Environmental conditions were characterized using standard habitat mapping protocols. I selected study locations where the native species of interest were consistently found across the study sites. This established a context for testing hypotheses, as the null expectation for my approach was detection, whereas deviations from the null would indicate where this method required refinement.

MATERIALS AND METHODS

Autonomous eDNA Introduction Device (ADID) and tracer particle

The methods for this study were reviewed and approved by Cal Poly Humboldt's Institutional Animal Care and Use Committee (IACUC, No. 2020F74-C, No. 2021F29-A).

Exogenous eDNA tracers were introduced into study sites using an ADID (Herman 2023). The eDNA tracers used in this study were derived from muscle tissues of channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*). Freshwater fish were specifically selected to align with the osmolarity of study systems, with the expectation their tissue would closely match NeDNA. Furthermore, channel catfish and common carp do not occur in the study area, thereby minimizing the risk of false positives influencing the characterization of system-specific eDNA transport. Whole specimens of both fish species were obtained from a commercial hatchery (The Fishery, Galt, California), held on wet ice for transport, and then frozen. To produce the tracer, 100 g of skinned filets were homogenized with 1 L of reverse osmosis water in a standard household blender. This mixture was passed three times through five-layer cheesecloth, followed by a single pass through a 200 μm mesh filter, limiting the maximum particle size of the tracer to that of natural eDNA (Turner et al. 2014). The tracer was generated no more than 24 hours prior to use and refrigerated or stored on wet ice prior to use.

The ADID consisted of a programmable Arduino microchip controller, peristaltic pump, and rechargeable lithium battery all housed in a waterproof container (Herman 2023). The eDNA tracers were held in separate five-gallon buckets and placed within coolers filled with wet ice to minimize degradation. Tracers were then diluted to a desired concentration with stream water and battery powered air pumps were used to induce the suspension of particles. The peristaltic pump transferred the tracer from its container into the stream at a constant rate of 0.025 L/minute. Standard operating procedures for producing the exogenous eDNA tracer and constructing an ADID are defined by Herman (2023).

Study Sites

This study was conducted from May to October of 2022 in small coastal streams located in forests of Humboldt County, California, a region dominated by coastal redwoods (*Sequoia sempervirens*). Study sites were defined as 500-meter sections of stream, with ADID systems placed at their upstream end. Water samples for eDNA analysis were collected from multiple downstream cross sections. Study sites had an average width of 5.96 m, average depth of 0.287 m, and an average discharge of 26.0 L/s (See results, Table 1). Sampling was conducted under summer low-flow conditions when distinct pool and riffle habitats are present.

The study determined local occupancy of coho salmon (*Oncorhynchus kisutch*) and steelhead (*Oncorhynchus mykiss*). Coho salmon in the study area belong to the

Southern Oregon/Northern California Coast Evolutionarily Significant Unit, a group designated as threatened under the US Endangered Species Act (NMFS 2014). All coho salmon present in study sites were in the juvenile life history stage (approximately 80-150 mm total length). The study area is inhibited by both anadromous and resident adult (rainbow trout) life history variations, herein referred to as steelhead. Steelhead in the study area belong to the Northern California distinct population segment, and are listed as threatened (NMFS 2014). Within study sites, most steelhead were in the juvenile life history stage (80 – 200 mm total length), however multiple size classes were observed. Both coho salmon and steelhead were present within all study sites.

Study Design

Studies were conducted on eight distinct sites, each spanning 500 m. At each site, two tracers (channel catfish (*I. punctatus*) – IPU, and common carp (*C. carpio*) - CCA) were introduced as discrete single point sources within 15 cm of each other. The use of multiple tracers allowed for comparison between two tracers at different concentrations. The IPU tracer was introduced at a high concentration, at a 1:3 dilution of tracer to stream water, and the CCA was introduced at a low concentration, at a 1:7 dilution (Figure 2). The tracers were released at the head of study sites (0 meters) in the main flow.

The tracers were introduced for six hours to establish a steady state distribution prior to water sample collection (Figure 2; Herman 2023). Water samples were then

collected beginning 500 meters downstream from the ADID, and proceeding upstream to prevent resuspension of settled eDNA from influencing captured eDNA concentrations. Samples were collected at distances of 100 m, 200 m, 300 m, 400 m and 500 m downstream from the head of the site. In similar settings, previous work indicated tracer signals would fully mix about 100 m downstream from the release point (Herman 2023); therefore, the 100 m cross section was considered as the baseline for eDNA entering the study site. At each cross section, triplicate water samples were collected at evenly spaced locations, specifically at 25 (left), 50 (center), and 75% (right) of the stream's width, for a total of 9 water samples per cross section. The equidistant placement of sampling locations did not account for flow patterns or stream habitat. Water sample collection across all cross sections took 30 - 60 minutes depending on site characteristics and access. In total, 45 water samples were collected at each study site. In addition to eDNA samples, one field blank per cross section (five total per site) was collected. Field blanks consisted of one liter of store bought drinking water that was poured into a Whirl-Pak bag in the field. Field blanks were handled in the same manner as water sample throughout all stages of processing, serving as a comprehensive negative control.

At each cross section, stream width (meters) was recorded, and at every sample location, water depth (meters) and water velocity (meters per second) were recorded. Water velocity measurements were recorded using a flowatch flowmeter (JDC Electronics, 41112500). For each study site, an estimate of discharge was developed following Hauer and Lamberti (2006). A cross-section was divided into 10 cells where depth (m) and velocity (m/s) were measured. Section discharge was then calculated by

multiplying velocity by the area of a sample point (determined from the depth and the distance between measurement points). Site discharge was then calculated by summing all sectional discharge estimates.

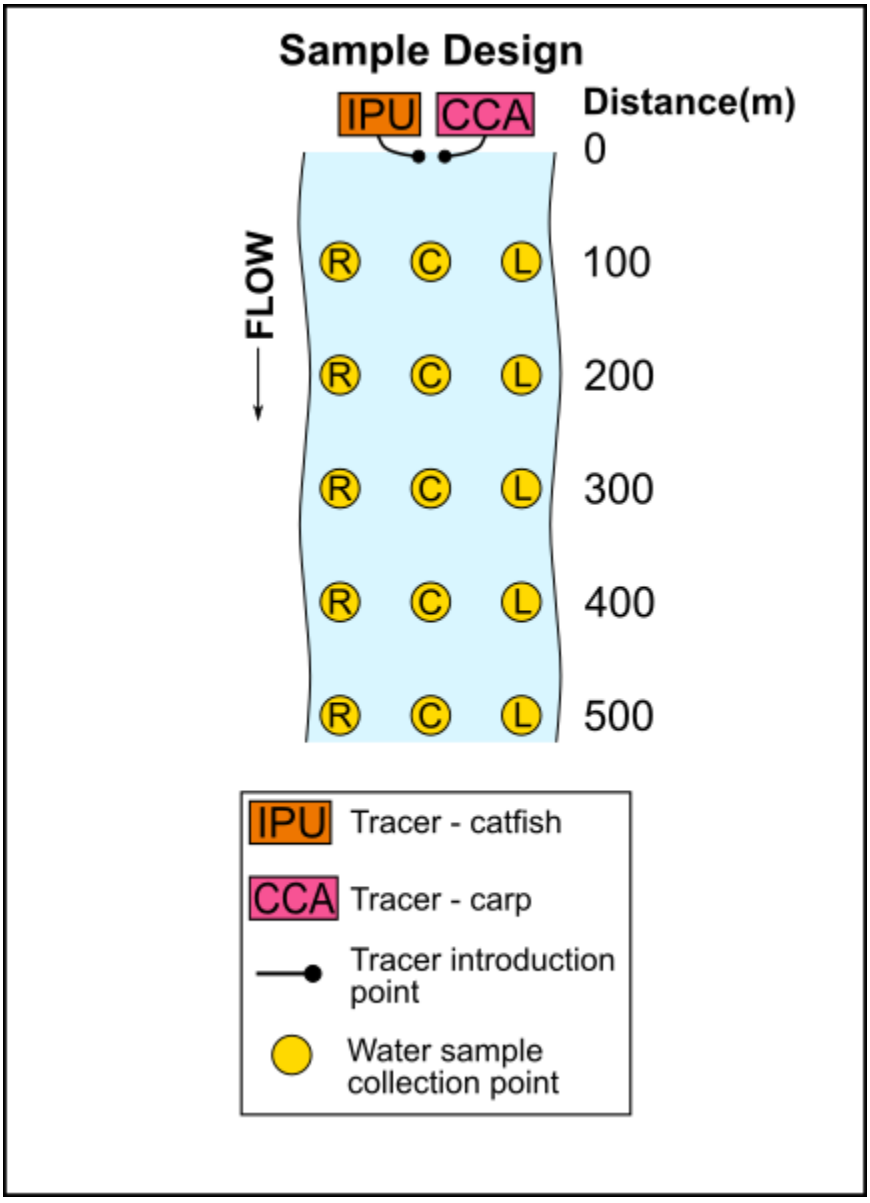

Figure 2: Diagram showing a study site and sampling grid including; tracer release location (IPU & CCA), distance (meters) of cross sections from the head of the study, and locations in cross sections where water samples were collected (L - left, C - center, & R - right).

Snorkel observations

Snorkel surveys were conducted to provide an assessment of coho salmon and steelhead presence and abundance in each study site. The entire study site, encompassing 500 meters of stream, was divided into 50 - 100 meter sections and split between two divers. Each section was surveyed in a single pass by a single diver, starting from the lower extent and progressing upstream. Divers recorded the abundance of coho salmon and steelhead during the survey. Snorkel surveys took place the day prior to eDNA sampling or immediately following water collection.

Environmental DNA Methods

Water Collection and Filtration

Environmental DNA was sampled by pulling a sterile Whirl-Pak bag along the stream's surface to collect approximately 1.75 L of water. Samples were immediately stored on wet ice in a cooler and filtered within 8 hours of collection in a dedicated water filtration laboratory.

Water was filtered under vacuum over 47 mm diameter 0.45 μm cellulose-nitrate-filters placed upon 47 mm diameter filter support pads and inserted into sterilized plastic filter funnels. Up to six samples were filtered at a time using a filter manifold connected to a pneumatic hand pump. The volume of water filtered for each sample was recorded. Filters bearing DNA were removed from the filter funnels, placed into sterile

microcentrifuge tubes using sterilized forceps, and immediately frozen at -20°C until extraction. All filter cups and countertops used in filtering were sterilized with a 10% bleach solution and triple rinsed with reverse osmosis water prior use.

DNA Extraction

DNA was extracted from filters using a combination of acetone dissolution (Hallet et al 2012) and QIAGEN DNeasy Blood and Tissue kits. Filters were dried at room temperature by opening their microcentrifuge tube lids and placing them in a fume hood for one hour. Once the filters were dry, two 3-mm sterile glass beads and 1.5 mL of acetone were added then vortexed every five minutes until filters had dissolved (~30 minutes). Samples were then centrifuged at 8000 rpm for one minute to generate a pellet and the supernatant was discarded. Another 1.5 mL of acetone was added to each sample, and then vortexed every five minutes for an additional 15 minutes, centrifuged at 8000 rpm for one minute, and the supernatant was discarded. Next, 1.5 ml of 200 proof ethanol was added, the samples were vortexed, centrifuged at 8000 rpm for one minute and the supernatant was discarded. The pellet was air-dried at room temperature overnight. Samples were lysed by adding 360 µl of buffer ATL and 40 µl of proteinase k then incubated in a thermoshaker for eight hours at 56°C and agitated at 1000 rpm. Lysed samples were then extracted using QIAGEN DNeasy Blood and Tissue Kits according to the manufacturer's instructions and eluted using 100 µl of buffer AE.

DNA Quantification

The concentration of DNA in each sample was estimated using a Bio-Rad QX200 Droplet Digital PCR system. Each ddPCR reaction included 900 nanomolar (nM) of forward primer, 900 nM reverse primer, 250 nM of probe, 0.27 µL of 300 nM Dithiothreitol, 5 µL of ddPCR Multiplex Supermix, 12 µL of extracted DNA, and a sufficient volume of nuclease-free water to bring the final reaction volume to 22 µL. A total 20 µL of the reaction mix and 70 µl of droplet generator oil were transferred into a Bio-Rad DG8 droplet generation cartridge secured in a Cartridge Holder, covered with a DG8 Gasket, and transferred into a Bio-Rad QX-200 droplet generator where the ddPCR reaction is partitioned into as many as 20,000 nanodroplets. Each sample was transferred into the well of a ddPCR 96-well plate and the plate was sealed with a PCR plate sealer.

Assays used in ddPCR reactions were obtained from the literature and specific to the target and tracer species. The coho salmon assay originally came from Pilliod and Laramie 2016 and was modified by Spence et al 2021. The steelhead assay came from Wilcox et al 2015. For the tracer species the common carp assay came from Eichmiller et al. 2014 and the channel catfish assay was developed by the U.S. Forest Service National Genomics Center for Wildlife and Fish Conservation at the Rocky Mountain Research Station, Missoula, Montana. The four assays were run as two duplex reactions: one combining catfish with coho salmon assays and the other pairing common carp with steelhead assays, using FAM for native species detection and HEX for tracer identification.

Thermocycling was performed on a C1000 Deep Well Touch Thermal Cycler. Thermocycling conditions included a 10-minute enzyme activation at 95℃, followed by 40 cycles of 30-seconds at 94℃ and 60-seconds at 60℃, followed by incubation at 98℃ for 10 minutes, and then held at 4℃ indefinitely. The ddPCR plate was then removed from the thermocycler and placed on a QX200 droplet reader to estimate the concentration of DNA in the reaction. If a reaction contained less than 10,000 droplets and a clear distinction between positive and negative droplet fluorescence did not exist, the sample was rerun. Each plate included positive and negative PCR controls. Negative PCR controls consist of ddPCR reaction mix with nuclease-free water in lieu of extracted DNA. Positive controls consisted of individual ddPCR reactions using DNA extracted from the tissue of each assayed species. Bio-Rad software uses a Poisson algorithm based on the count of negative droplets to calculate the copies per 20 µL reaction. Estimated copies per reaction were corrected to copies per liter in the original sample using:

$$\text{Copies/Liter} = \frac{(\text{Copies/ddPCR reaction}) * (\text{Volume of purified DNA from extraction } (\mu\text{L}))}{(\text{Volume of purified DNA added to reaction } (\mu\text{L})) * (\text{Volume of water filtered } (\text{L}))}$$

Limits of detection and quantification were determined for each assay. Limit of detection (LOD) is defined as the lowest concentration of DNA that can be detected with a 95 percent detection rate. Limit of quantification (LOQ) is defined as the lowest concentration of DNA that can be quantified with a coefficient of variation below 35 percent (Klymus et al. 2020). For each assay, five-fold serial dilutions were made from elution buffer and tissue extracted using a QIAGEN DNeasy Blood & Tissue Kit following the manufacturer's guidelines. Estimated concentrations across all serial

dilutions ranged from 1 to 1 million copies per reaction with 8 - 16 replicates per concentration. LOD and LOQ were determined using the curve-fitting methods presented in Klymus et al. (2020) with the estimated concentrations at each step of serial dilutions.

Data Analysis

Tracer Correlation

To determine the extent to which the two tracers co-varied, I compared paired tracer concentrations from each study reach. Similar patterns between the tracers indicates they are subject to the same transport processes, implying that the tracers are revealing the effects of transport that may be anticipated for small sized cellular material, used herein as an eDNA analog. A linear regression was fit to paired tracer concentrations from within the same sample, to assess the correlation between tracer concentrations across a site. Samples deviating the most from the regression were iteratively removed until the root-mean-square error (RMSE) reached a value below 0.15. This threshold was sensitive enough to remove samples with diverging concentrations, while retaining some of the variability within the data. The number of samples retained from the RMSE screening was used to evaluate the extent of agreement between the two tracers. This approach follows Herman (2023).

Ratios

To evaluate if there was enrichment of NeDNA relative to the tracer, ratios of NeDNA to tracer at the 100 m sampling location were compared to the corresponding ratios measured at each of the cross sections further downstream. A two-sample Wilcoxon Rank-Sum test (R Core Team 2023) was used to conduct occupancy assessments within study sites, examining whether the NeDNA:tracer ratios increased significantly downstream (one-tailed test), as expected if the target was present between cross sections. The Wilcoxon Rank-Sum test is nonparametric (i.e., does not assume any distribution for the data) and is suitable for relatively small sample sizes.

I specifically examined sample-specific ratios to reveal the underlying pattern between NeDNA sources and tracers throughout a study site. Ratios were calculated by dividing concentrations of NeDNA by tracer concentrations within the same sample. In each sample, four ratios were calculated with concentrations of each NeDNA source as the numerator (coho salmon, steelhead) and concentrations of each tracer (IPU, CCA) as the denominator. To ensure an equal sample size in Wilcoxon tests and retention of samples where a source of eDNA was undetected, an arbitrary concentration of one copy per liter was added to all samples (otherwise a zero in the denominator of a ratio would result in an undefined ratio value). Ratios reveal the pattern between eDNA sources by comparing NeDNA to the tracer, the latter serving as a baseline for the transportation of non-enriched eDNA across a study site. Constant target:tracer ratios between cross sections reveal that NeDNA concentrations do not significantly increase from the tracer, suggesting the absence of the NeDNA source species. Conversely, increasing ratios

reveal that NeDNA concentrations have increased relative to the tracer, indicating the presence of the target species. Ratios mitigate variability in concentrations stemming from sample processing efficiencies and hydrological processes, which may decrease or increase eDNA concentrations independent of enrichment from source organisms. Ratios are assumed to account for concurrent, equal effects of these processes on both NeDNA and tracers, thereby canceling out the influence of these processes providing a clear look at the patterns of NeDNA and tracers between cross sections.

Tracer concentration

To evaluate the impact of the tracer dosing levels introduced into study sites on occupancy assessments, I compared the number of species detections using the higher (IPU) and a lower concentration tracer (CCA). Tracer concentrations (high and low) were based upon the initial dilution of tracers before being added to streams. The expectation at all study sites was for both target species (coho salmon and steelhead) to be detected in the analysis given they were visually observed in every site. Ratios of each native species to each tracer were calculated and the number of significant increases in target eDNA, indicative of species presence, were determined using Wilcoxon Rank-Sum Tests. A total of 64 tests were performed per tracer dosing concentration (high and low), including all combinations of two native species (coho salmon and steelhead), eight study sites, and four distances per site (100 to 200, 100 to 300, 100 to 400, and 100 to 500). Both tracers entered the stream at the same location, at the same rate, and for the same amount of

time; thus any difference in the number of species detections is expected to result from varying tracer dosing concentration.

Spatial scale

To assess the impact of spatial scale on the detection of both targets, I determined the number of species detections at distances of 100, 200, 300 and 400 m (the difference between the 100m baseline site and downstream sampling locations) across all study sites. In examining the effect of spatial scale, only concentrations of the high dosed tracer were used to calculate ratios, as it resulted in improved species detection (see results). The number of significant increases in coho salmon and steelhead to high dosed tracer ratio values at each spatial scale were determined using Wilcoxon Rank-Sum tests. At each spatial scale, 16 tests were conducted encompassing all eight study site and both targets.

RESULTS

Site Descriptions

Across five different streams, eight study sites were sampled. In Freshwater Creek, Jacoby Creek, Old Campbell Creek and Prairie Creek one study site was sampled, whereas in Lost Man Creek, four distinct study sites were sampled at separate times and locations. Mean width of cross sections ranged from 3.29 m to 8.02 m, mean depth at sample collection locations ranged from 0.2 m to 0.45 m, mean water velocity at sample collection location ranged from 0.019 L/s to 0.24 L/s. Discharge varied across study sites from 4.32 L/s to 62.35 L/s (Table 1).

Table 1: Stream measurements conducted at all study sites including: stream name, study site name, mean width of cross sections (m), mean depth across all sampling locations (m), mean velocity across all sampling locations (L / second), the single discharge measurement collected at a site (L/s) and the observed abundances of coho salmon and steelhead between the 100 m and 500 m cross sections at each study site.

Stream name	**Study site**	**Mean width (m)**	**Mean depth (m)**	**Mean velocity (L/s)**	**Discharge (L/s)**	**Observed coho salmon**	**Observed steelhead**
Freshwater Creek	FWC1	8.02	0.28	0.02	4.32	1797	39
Jacoby Creek	JC2	4.55	0.22	0.09	33.48	862	77
Lost Man Creek	LMC1	5.80	0.20	0.24	41.64	696	83
Lost Man Creek	LMC2	6.88	0.45	0.21	21.46	526	25
Lost Man Creek	LMC3	5.18	0.29	0.11	21.46	381	80
Lost Man Creek	LMC4	3.29	0.26	0.06	7.95	972	168
Old Campbell Creek	OCC	6.18	0.34	0.16	62.36	82	382

Stream name	Study site	Mean width (m)	Mean depth (m)	Mean velocity (L/s)	Discharge (L/s)	Observed coho salmon	Observed steelhead
Prairie Creek	PRC	7.78	0.26	0.06	15.67	419	123

Snorkel Surveys

Coho salmon and steelhead were observed in every study site and between every eDNA sampling location, as well as above each site. Thus, all sampled reaches conformed to the scenario where the target was present within and above all study sites. (scenario 3; Figure 1).

Target species abundance over each study site ranged from 82 - 1797 coho salmon and 25 - 382 steelhead (Table 1). Coho salmon were more abundant than steelhead across all sites (except for in OCC) with 3.5 (PRC) to 46 (FWC1) times as many coho salmon than steelhead. Observed abundances between 100m sections ranged between 8 - 499 coho salmon and 2 - 117 steelhead.

Tracer

For the high dosed tracer (IPU), none of the field blanks or ddPCR negatives tested positive. The channel catfish assay had a LOD and LOQ of 2.34 and 6 copies/reaction, respectively. Of the 360 water samples examined (8 study site x 45 samples per site), 84.7% were greater than the LOD, and 79.7% were greater than the LOQ. The average concentration of the high dosed tracer at a cross-section never fell below the LOQ.

For the low dosed tracer (CCA), none of the field blanks or ddPCR negatives tested positive. The common carp assay had a LOD and LOQ of 2.55 and 15

copies/reaction respectively. Of the 360 samples examined, 87.2% were greater than the LOD, and 65.8% were greater than the LOQ. The average concentration of the low dosed tracer fell below the LOQ at three cross-sections in the OCC site (300, 400 & 500m), and at two cross-sections in JC2 site fell below the LOQ (400m) and LOD (500m).

Tracer Correlation

The fraction of samples that exhibited strongly correlated tracer concentrations varied across study sites. Within the FWC1, LMC1, LMC2, LMC3, and LMC4 sites, the tracers exhibited consistent patterns in their concentrations, as indicated by the high proportion of samples retained in the RMSE screening (67-76%). Divergent patterns between tracer concentrations were seen in JC2 (11%), OCC (22%), and PRC (42%), as indicated by a low number of samples retained (Table 2). Samples that were not retained in the correlation analysis typically exhibited low concentrations, falling below the LOQ or LOD. The low correlation between tracers at JC2, OCC and PRC was presumed to have occurred because the low dosed tracer was at or below LOQ in these sites, leading to inaccurate estimates of eDNA concentration.

Table 2: Mean concentrations (± SE) of both tracers (CCA & IPU) and NeDNA species (coho salmon & steelhead) across the 45 water samples in each study site. The proportion of correlated samples, determined by linear regression and root-mean-square-error analysis on sample specific tracer concentration, for each study site are also included.

Study site	**CCA mean**	**IPU mean**	**OKI mean**	**OMY mean**	**Proportion retained**
FWC1	373 ± 76	1,056 ± 206	2,003 ± 256	1,277 ± 379	67%
JC2	177 ± 82	1,124 ± 570	222 ± 64	318 ± 94	11%
LMC1	818 ± 95	1,175 ± 152	334 ± 51	327 ± 55	76%
LMC2	724 ± 108	4,120 ± 701	369 ± 68	460 ± 73	71%
LMC3	1,353 ± 329	1,787 ± 494	319 ± 79	577 ± 165	69%
LMC4	6,991 ± 1233	53,728 ± 10,823	2,327 ± 398	2,183 ± 471	73%
OCC	108 ±17	712 ± 176	149 ± 30	705 ± 122	22%
PRC	231 ± 30	983 ± 170	558 ± 85	501 ± 73	42%
Mean	1,347	8,086	785	793	

System-Specific Tracer Patterns

At each study site, the tracers exhibited site-specific spatial patterns in downstream transport. While the exact transport of the tracers was unique to each study site, general trends emerged in their downstream transport. For example, in LMC4 the

tracers generally exhibited a continuous decline in concentration from upstream to downstream (Appendix B: Figure B4**)**. A generally similar pattern was observed in the high dosed tracer at JC2 (Appendix B: Figure B6). In contrast, tracers at most study sites exhibited non-monotonic patterns, without a consistent increase or decrease downstream. For example in FWC1, both tracers decreased in concentration by one-half in the first 100 meters, remained stable across the 200, 300, and the 400 m cross sections, then doubled in concentration at the 500m cross section (Appendix B: Figure B5). While fluctuations in concentrations were captured, the tracers stayed centered around a mean (IPU - ~1000 copies/liter, CCA ~370 copies/liter). Similar patterns were seen in both tracers in LMC1 and LMC2 (Appendix B: Figure B1, Figure B2).

Naturally Occurring eDNA (NeDNA)

For coho salmon, none of the field blanks or ddPCR negatives tested positive. The coho salmon assay had a LOD and LOQ of 4.77 and 18 copies/reaction respectively. Of the 360 water samples examined, 86.1% were greater than the LOD, and 60.3% were greater than the LOQ.

For steelhead, none of the field blanks or ddPCR negatives tested positive. The steelhead assay had a LOD and LOQ of 7.67 and 23 copies/reaction respectively. Of the 360 water samples examined, 89.2% were greater than the LOD, and 63.1% were greater than the LOQ.

Concentrations of coho salmon and steelhead eDNA were similar to one another at six study sites (Table 2). Average concentrations differed by less than 260 copies/liter, despite coho abundances being much greater than steelhead (Table 1 and Table 2). Large differences in concentrations occurred at FWC1, where the average concentration of coho salmon was 726 copies/liter greater than steelhead, while in OCC steelhead concentrations were 556 copies/liter greater. These study sites had the highest observed abundances of coho salmon and steelhead, respectively (Table 1). Concentrations of the tracer were generally greater than that of coho salmon or steelhead; however, the exact differences were site dependent and in some cases, NeDNA concentrations were greater than one or both of the tracers (e.g., PRC and FWC1; Table 2, Appendix B Figure B5 and B7).

Ratio Results

To evaluate if NeDNA concentrations significantly increased relative to the tracer, ratio values from the 100 m cross-sections were compared to ratios from every downstream cross-section within a study site using two-sample Wilcoxon Rank-Sum tests. These occupancy assessments were conducted across all study sites, spatial scales, and ratio types, and they found significant increases in 41.4% of tests (Table 3). Although the null expectation for all occupancy assessments was the detection of both targets, no sites had detections across all Wilcoxon Rank-Sum tests performed. In only four cases, all tests for a given ratio type in a site had significant results (Table 3). The

FWC1 and LMC1 sites had the highest proportion of detections across all ratios (68.8%), while OCC had the lowest (12.5%). Ratios calculated with the high dosed tracer (IPU) had significant test results in 51.6% of occupancy assessment, while the low dosed tracer (CCA) had significant results in 31.2% of assessments. Detections of coho salmon and steelhead were similar for assessments conducted with the high dosed tracer (coho salmon 50%, steelhead 53.1%). In contrast, assessments using the low dosed tracer detected coho salmon more frequently (40.6%) than steelhead (21.9%).

Table 3: The proportion of significant Wilcoxon Rank-Sum test results for each NeDNA:Tracer ratio at each study site. Four tests were performed for each ratio type at a study site, for a total of 16 tests within each study site.

Study Site	**OKI:IPU**	**OKI:CCA**	**OMY:IPU**	**OMY:CCA**	**Overall**
FWC1	100%	75%	75%	25%	68.8%
JC2	25%	0%	100%	25%	37.5%
LMC1	50%	100%	50%	75%	68.8%
LMC2	50%	75%	50%	25%	50.0%
LMC3	25%	50%	0%	0%	18.8%
LMC4	100%	0%	75%	0%	43.8%
OCC	0%	25%	0%	25%	12.5%
PRC	50%	75%	75%	25%	31.3%
Overall	50%	40.6%	53.1%	21.9%	41.4%

Tracer concentration

The high dosed tracer's average concentrations (8,086 copies/L) across all samples in a study site was greater than that of the low dosed tracer (1,347 copies/L) (Table 2). Among study sites, the highest tracer concentrations were observed in LMC4 (high: 53,728 copies/L, low: 6,991 copies/L), while the lowest was in OCC (high - 712 copies/L, low - 108 copies/L). The magnitude of difference between averages varied by site, with the greatest difference between tracer concentrations was in LMC4 (46,737 copies/L), and the smallest difference in LMC1 (357 copies/L).

Analyses based on the higher dosed tracer (IPU) were more effective at detecting both coho salmon and steelhead, as indicated by the number of significant Wilcoxon Rank-Sum Tests conducted on ratios calculated using the higher dosed tracer (Table 4). High dosed tracer's ratios resulted in detections of coho salmon and steelhead more frequently when those calculated with the lower dosed tracer did not (coho salmon 28%; steelhead 38%). In contrast, ratios calculated with the low dosed tracer resulted in detections in far fewer instances where the higher dosed tracer did not (coho salmon 19%; steelhead 6%). Where tests results from both tracers agreed in occupancy assessments, the non-detection of coho salmon or steelhead was more common (coho salmon 31%; steelhead 40%) than detections (coho salmon 22%; steelhead 16%). Given the higher detection provided by the high dosed tracer, subsequent analysis on the effect of spatial scale on occupancy assessments was performed only using the high dosed tracer.

Table 4: The proportion of significant Wilcoxon Rank-Sum tests comparing where tests conducted with each tracer saw agreement or disagreement in results. In the high and low dosed tracer column the "+" indicates a significant Wilcoxon Rank Sum tests while the "-" indicates no significant results. The percentages in the coho salmon and steelhead columns represent the proportion of total tests per species with agreement/disagreement between the tracers. The total column is the proportion of tests with agreement/disagreement between the tracers for both species.

High dosed tracer	Low dosed tracer	Coho Salmon	Steelhead	Total
+	+	22%	16%	19%
+	–	28%	38%	33%
–	+	19%	6%	13%
–	–	31%	41%	36%

Spatial scale

Larger spatial scales resulted in a greater number of detections for both coho salmon and steelhead (Table 5). The larger spatial scales (300 and 400 meters) coincided with the highest number of significant test results (coho salmon 63-88%; steelhead 75%). This contrasts with the smaller spatial scales (100 and 200 meters) that resulted in a decreased number of significant tests (coho salmon 25%; steelhead 25-38%). At the larger spatial scales, study sites where detections of coho salmon and steelhead did not occur coincided with either high discharges (OCC) or low abundances of a target species (LMC3, JC2) (Table 1).

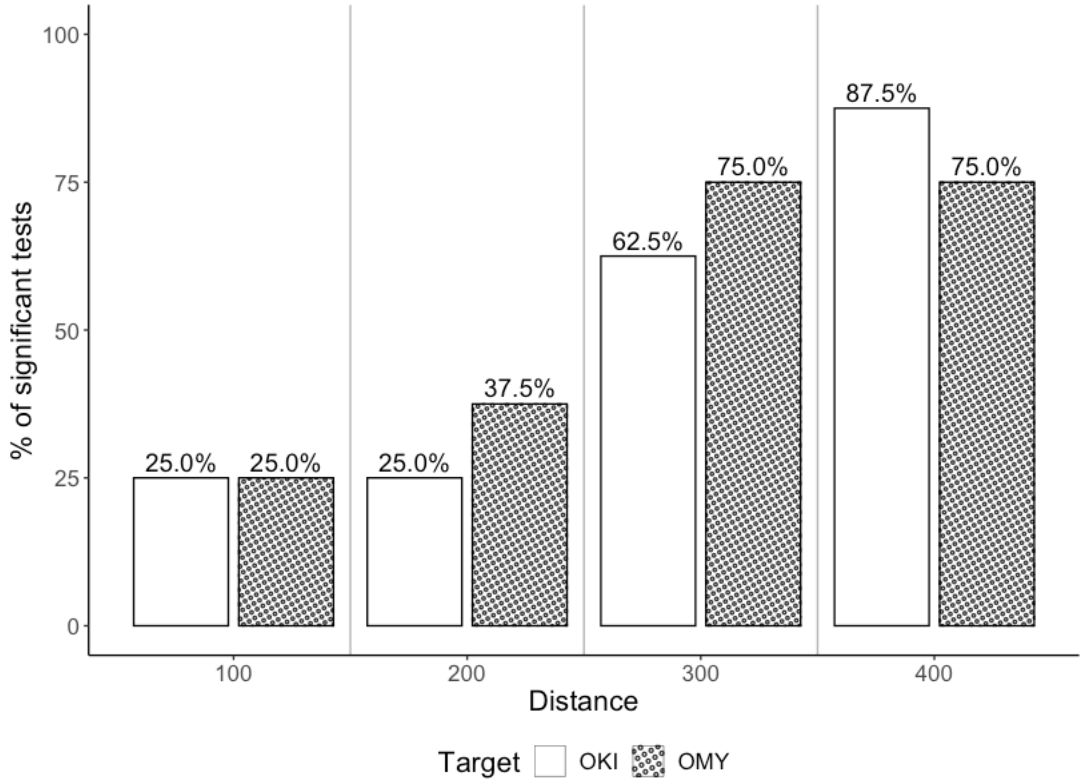

Figure 3:The proportion of significant Wilcox Rank-Sum Tests conducted on high dosed tracer ratios separated by distance between sampling locations. Results are further separated by ratios of coho salmon (empty or left bar) and steelhead (dotted or right bar). The number above each bar represents the percentage of significant tests (out of eight).

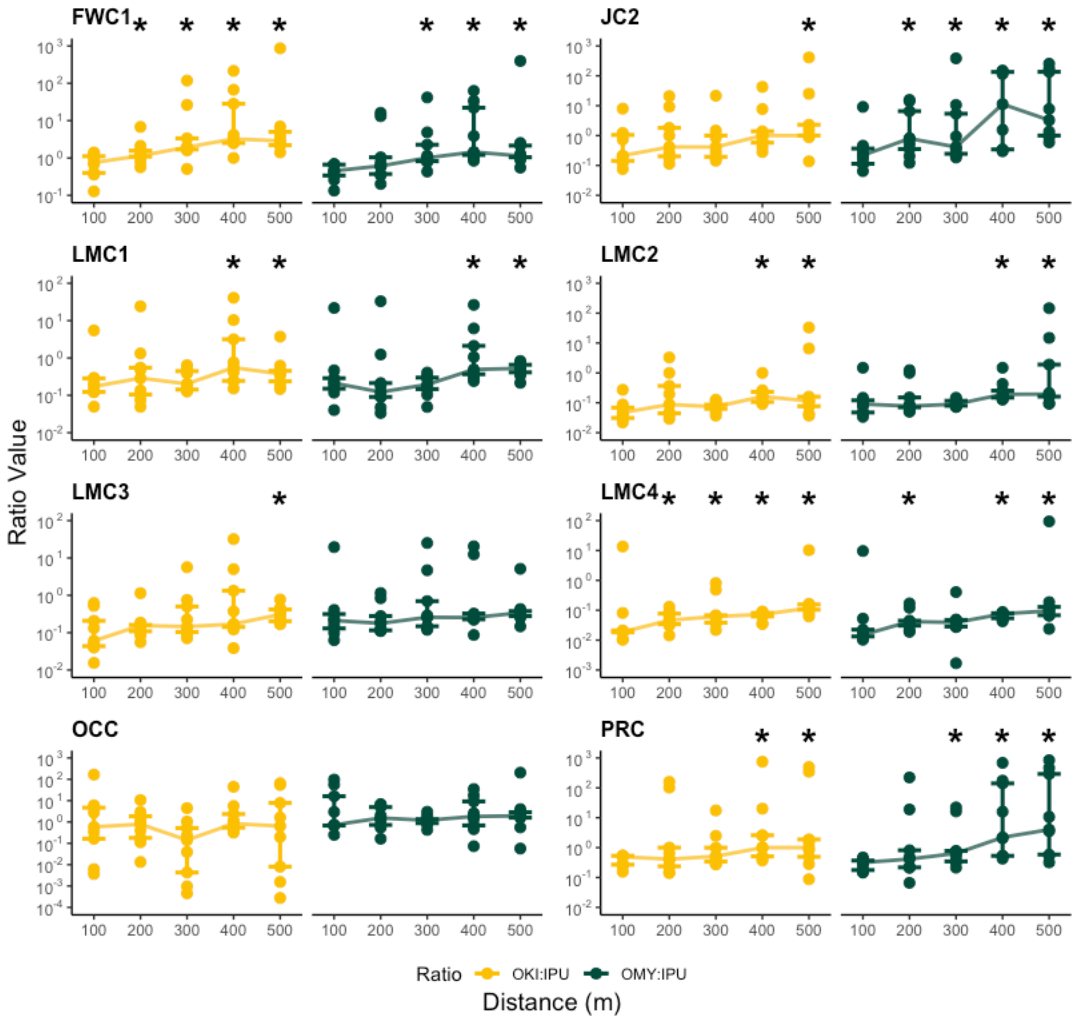

Figure 4: Figure showing NeDNA:high dosed tracer ratio values at each study site. Within each pane, the left plot shows coho salmon ratios (Yellow), and the right plot shows steelhead ratios (Green). The individual point represent the individual ratio values, the error bars have a point in the middle representing the median ratio value, and the upper and lower bars represent the 75th and 25th quantiles respectively for a given cross section's ratios. The "" symbols above the x-axis represents a significant increase in ratio values for that cross section vs the 100 m cross section's ratio values as determined by Wilcox Rank-Sum tests.*

DISCUSSION

This study introduces a unique approach to eDNA surveys - employing an eDNA tracer - to infer the local presence of fishes in stream reaches ranging from 100 to 400 meters, regardless of target presence upstream of a study site. Species presence was indicated through elevated concentrations of target eDNA relative to expected patterns, based on processes resolved by the tracer. In the assessments of this approach herein, the use of a higher concentration tracer and considering spatial scales of 400 m suggested local species presence in 81% of tests. Use of lower dosed tracer concentrations and spatial scales of 100 to 200 m reduced the efficacy of this approach. An analysis of five study site, using similar but not identical methods to those described herein, produced results are consistent with those reported herein (Appendix C). Although these findings suggest the potential for tracer-supported eDNA surveys to detect species at small spatial scales (300-400 m), the range of environmental conditions and occupancy scenarios examined is insufficient for making effective management decisions.

Tracer Correlation

The tracer accounts for the integrated effects of processes that influence the transport of eDNA in a specific stream, without requiring information on the exact nature or intensity of these processes. The tracers were designed with the intent to mimic NeDNA, being made from the tissues of freshwater fish and reduced to the maximum

particle size of eDNA observed in studies (Turner et al. 2014). In this study it was therefore assumed that the processes influencing the tracers would affect NeDNA. Evidence that concentrations of distinct tracers were commonly correlated across samples supports the assumption that eDNA rapidly mixes in natural system and is subject to the same site-specific transport processes. Tracers exhibited strongly correlated trends in five study sites, while in three sites were less strongly correlated as a consequence of imprecise measurements of the low dosed tracer (CCA) resulting from its lower dosing concentration. Concentrations of the lower dosed tracer at these three study sites were frequently at or below levels that could be reliably quantified, while in contrast concentrations of the higher dosed tracer in these sites could be more reliably quantified and were comparable to those observed elsewhere in the study (Table 2). This aligns with previous research that has found higher concentrations of eDNA within streams reduces the variability among replicates (Van Driessche 2022) and remains detectable over larger spatial scales (Herman 2023, Spence et al 2021).

Site specific patterns

Insight gained from the tracer can be used to account for the effects of site-specific and sampling frame processes when evaluating occupancy of target species. While site specific processes influence eDNA concentrations in streams, the way in which eDNA is sampled (the sampling frame) may influence how samples capture the distribution of eDNA across a stream. Across study sites, site specific and sampling

frame dependent patterns were observed, resulting in observed eDNA concentrations increasing and decreasing independently of distance from their source. Studies have identified non-monotonic changes in eDNA concentrations with distance from their source (Herman et al 2023) with differences in the magnitude and spatial scale of changes depending on the initial concentration (Van Driessche 2022). With the deployment of tracers, site-specific and sample frame dependent processes that influence eDNA concentrations can be evaluated through observing concurrent patterns in the change of tracers concentrations between sampling locations (Herman 2023). These patterns, when applied to concentrations of NeDNA, can be used to gain insight into the local-scale occupancy of target species.

With the use of the tracer during sampling, site-specific and sampling effects on eDNA concentrations have been accounted for. Regardless of which direction changes in concentration occur, patterns revealed by the tracer can be used to differentiate between species presence and these processes. The tracer provides a basis for evaluating if observed increases in concentrations of NeDNA are due to the local presence of a target, or an artifact of site specific or sampling effects. For example, in LMC3, concentrations of coho salmon, steelhead and both tracers increase between the 200 and 300 m cross sections. While increases in NeDNA may be indicative of local presence, examining NeDNA:tracer ratios between these locations revealed there was no significant elevations in NeDNA concentrations relative to the tracer (Appendix B: Figure B3, Table B3). In instances like this, without using the tracer one may falsely conclude species presence from increasing NeDNA concentrations.

Conversely, tracer signals can help discriminate whether NeDNA signals decline more slowly than the tracer, reflecting enrichment of eDNA. For example, in FWC1 concentrations of coho salmon and the high dosed tracer decline between the 100 and 200 m cross sections. However, decreases in the tracer concentration are far greater than that of coho salmon. Thus while both eDNA sources declined, coho salmon concentrations increased relative to what would be expected if no enrichment of eDNA occurred, as revealed by the tracer, suggesting species presence. In instances like this, without using the tracer one may falsely conclude species absence from decreasing NeDNA concentrations.

Tracer concentration

Using a high dosed tracer resulted in a greater number of detections than a low dosed tracer. The high dosed tracer also detected targets in more instances where the low doseed tracer failed to, and in sites with low correlation between tracers (Table 2, Table 4). Previous research has identified that higher concentrations of eDNA within streams reduces variability among replicates and remains detectable over larger spatial scales (Van Driessche 2022, Spence et al 2021, Herman 2023). Thus, the high dosed tracer was more precisely quantified and appeared to provide a more robust signal of site-specific processes over a greater spatial scale. While a greater number of detections occurred with the high dosed tracer, only 51.6% of assessments resulted in a detection even though coho salmon and steelhead were present across all study sites and spatial scales. Non-

detections using the high dosed tracer more frequently occurred at smaller spatial scales (table 5)

Spatial scale

The utility of tracer-augmented eDNA surveys to detect the local occupancy of fishes increased with distance over which the tracer and NeDNA signals had scope to diverge. In these study sites, the likelihood of within-site detection increased sharply between 300 and 400 meters downstream from the head of the site. Overall, the frequency of detections at more distant locations was at least double that of locations closer to the tracer source, with samples collected 100-200 m apart having detections in 25-38% of tests, whereas those collected 300-400 m apart detecting in 63-88% of tests.

When considering the spatial scale of detections within the small streams this study was conducted in, it is notable that increasing the distance between sample collection points to 300 m or more proved the most effective. Presumably this occurred because at these larger spatial scales, the cumulative input of eDNA from multiple sources (targets) contributed to the overall NeDNA signal within study sites. While the eDNA from any single target many not significantly augment concentrations above what is already present within a stream, extending the distance between sampling locations enables the inclusion of more points of eDNA introduction. This aggregation over larger scales can potentially elevate the concentration of target eDNA within a study site. Environmental DNA has been observed dispersing away from its

source in a plume, where during transport gradual widthwise mixing leads to greater uniformity widthwise and as long as concentrations remain above LOD, higher detection probabilities (Wood et al. 2021, Thalinger et al 2021). Consequently, placement of collection locations, spaced sufficiently apart, is crucial for accurately reflecting the cumulative increases in eDNA with our study approach, thereby enhancing the detection.

Occupancy Scenarios

The use of a tracer supports assessment of local presence of target species under four distinct occupancy scenarios (Figure 1). Local-occupancy assessments conducted over small spatial scales may be prone to false detections if the target is present upstream of a study site, as their DNA is transported into the site. The tracer was used to account for eDNA generated upstream, serving as an indicator for how a non-enriched source of eDNA disperses over a study site. If concentrations of target eDNA exhibit similar patterns to that of the tracer across sampling locations, it indicates the absence of the target species from the study site.

In this study, coho salmon and steelhead were present above and within every study site, such that only one of the four potential occupancy scenarios (Figure 1) was evaluated in this study. While this provided insight into factors influencing the detections of targets and the occurrence of false negatives, it is imperative for this method to be tested under more occupancy scenarios before the approach can be broadly utilized for conservation and management. Critically, this method needs to be applied in scenarios

where the target species is present above, but not within a study site to assess the degree to which the method is subject to false detections.

CONCLUSION

This study demonstrates a method for determining the local occupancy of multiple target species over small spatial scales using eDNA tracers to provide context of interpretation of natural eDNA patterns. Observations of site-specific patterns influencing concentrations of eDNA within all study sites revealed local occupancy assessments must account for these processes to prevent inaccurate conclusions of species presence or absence. Use of exogenous tracer can account for stream characteristics, and where practical adaptation the method is required, is likely to improve conclusions of occupancy. A clear example of this is the need to consider dosing levels as a function of stream flow, as the higher dosed tracer particle provided a more consistent estimate of site specific patterns, resulting in greater species detections across study sites. Detections tended to increase with distance, owing to a greater chance for more eDNA sources to be included and a greater scope for NeDNA and tracer signals to diverge, such that in the systems studied, detections were increasingly reliable at distances of 300 m or greater between sampling locations.

While limited analysis of environmental conditions was conducted in this research, the lowest number of detections across all ratio types and spatial scales occurred at the highest discharge study site (OCC). The lowest concentrations of both tracers in water samples were also seen within this site. Previous research has shown that as discharge increases the probability of capturing eDNA decrease as a consequence of greater dilution (Pochardt et al. 2020). Future research should examine tracer

introductions into higher discharge scenarios, either through introducing even higher concentrations of tracers or considering larger distances between sample locations.

This method provides an approach to assess the presence of a target species over relatively small spatial scales with minimal to no disturbances to the target or their environment. It has the potential to be implemented in conservation scenarios with endangered species or in especially sensitive environments, and so warrants further development.

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APPENDICES

Appendix A: Occupancy Scenarios

This Appendix contains additional explanations of the four potential occupancy scenarios a study site could have, as outlined in the introduction and figure 1. The table describes patterns in concentrations of target eDNA and the tracer for each occupancy scenario.

Table A1: Table explaining four potential scenarios regarding the presence of a species both upstream and within the study site. This table corresponds to the scenarios depicted in Figure 1.

	Target present above study site	**Target absent above study site**
Target absent in study site	1 Target species eDNA is detected entering the site and declines synchronously and proportionately with the tracer	2 Target species is not detected entering or within the site while the tracer decreases
Target present in study site	3. Target species eDNA is detected entering the site and does not exhibit the same decline in concentration as the eDNA tracer	4. Target species eDNA is not detected entering the site but is detected within the site, increasing in concentration while the eDNA tracer decreases

Appendix B: Supplementary Site Information

This Appendix contains supplementary results from each study site. For each study site, Concentrations figures for both tracers (IPU and CCA), coho salmon (OKI) and steelhead (OMY) are included as well as tables showing Wilcox Rank-Sum test results for each NeDNA:tracer ratio separated by distance between cross-sections (100, 200, 300, and 400 meters).

LMC1

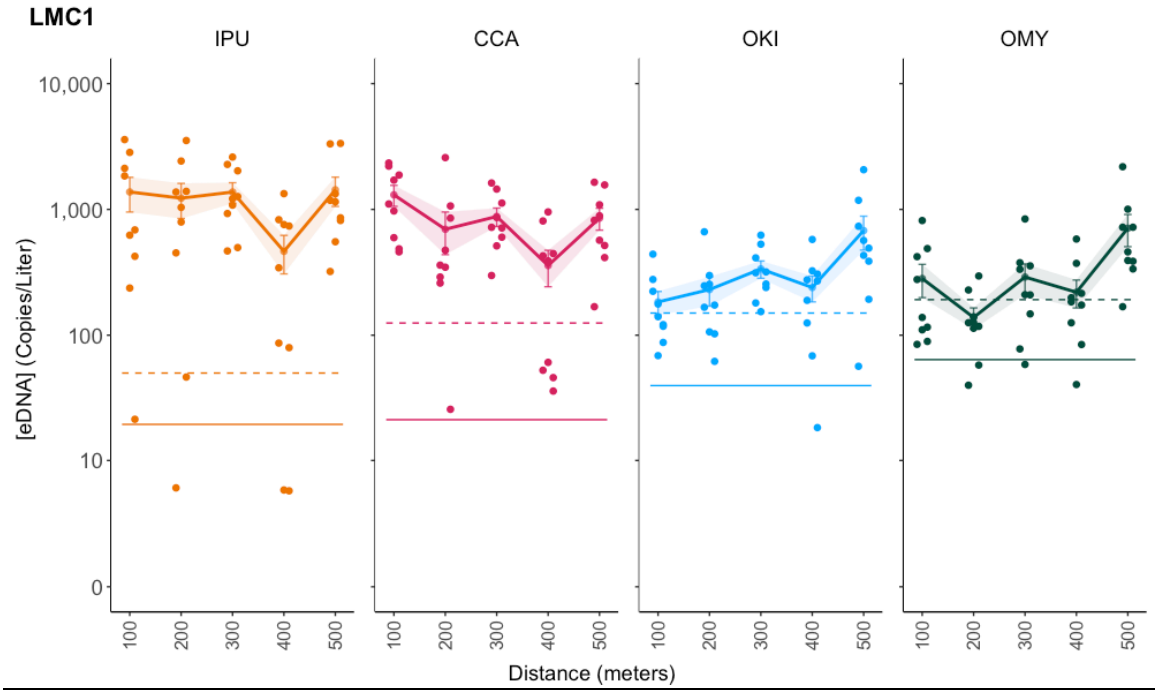

Figure B1:Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in LMC1. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B1: Table comparing the number of detections in LMC1 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are seperated by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 1	0 / 0
200 m	0 / 1	0 / 1
300 m	1 / 1	1 / 1
400 m	1 / 1	1 / 1

LMC2

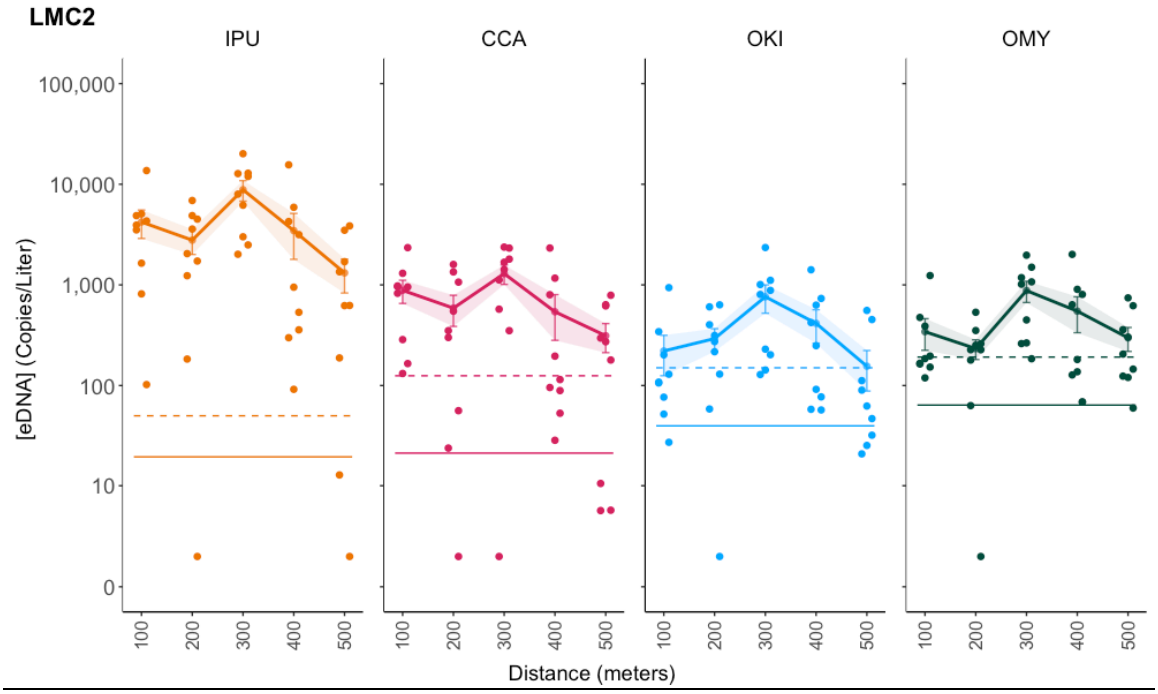

Figure B2: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in LMC2. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B2: Table comparing the number of detections in LMC2 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 1	0 / 0
200 m	0 / 1	0 / 1
300 m	1 / 1	1 / 1
400 m	1 / 0	1 / 0

LMC3

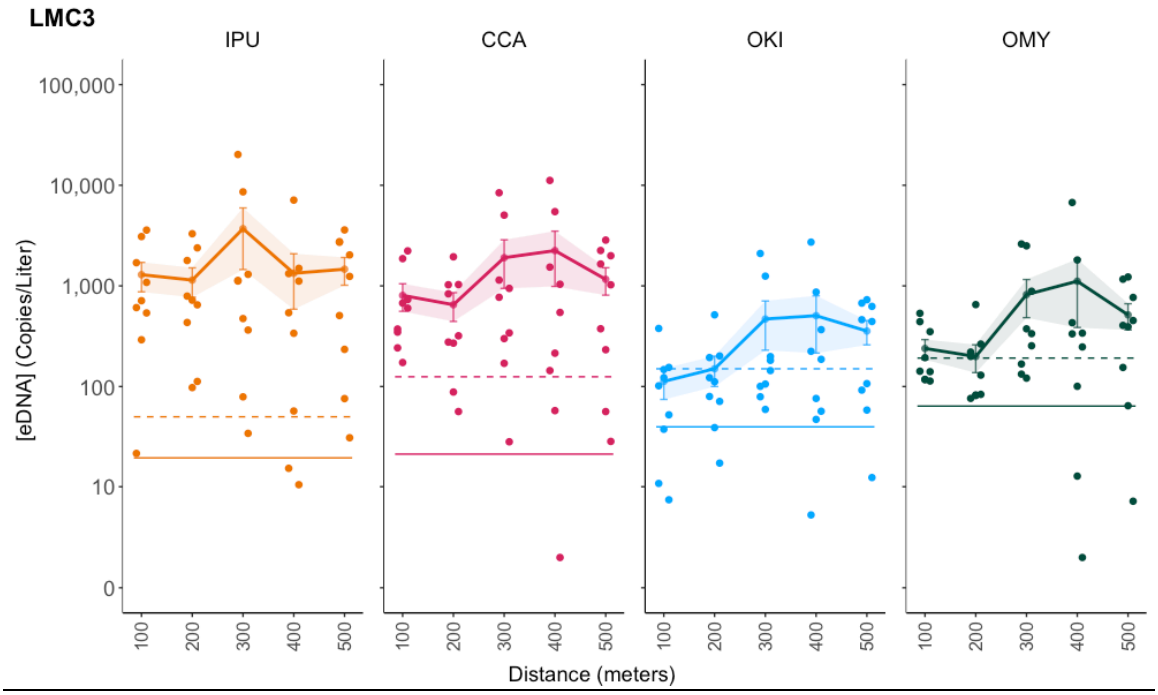

Figure B3: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in LMC3. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B3: Table comparing the number of detections in LMC3 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 0	0 / 0
200 m	0 / 1	0 / 0
300 m	0 / 0	0 / 0
400 m	1 / 1	0 / 0

LMC4

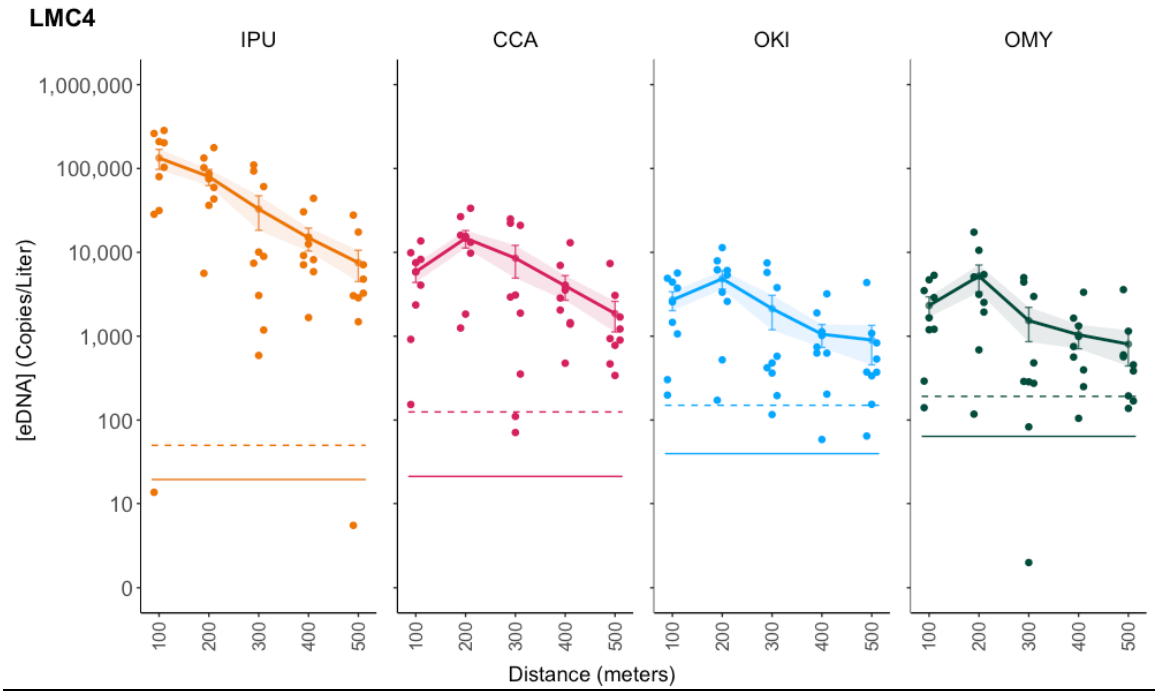

Figure B4: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in LMC4. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B4: Table comparing the number of detections in LMC4 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	1 / 0	1 / 0
200 m	1 / 0	0 / 0
300 m	1 / 0	1 / 0
400 m	1 / 0	1 / 0

FWC1

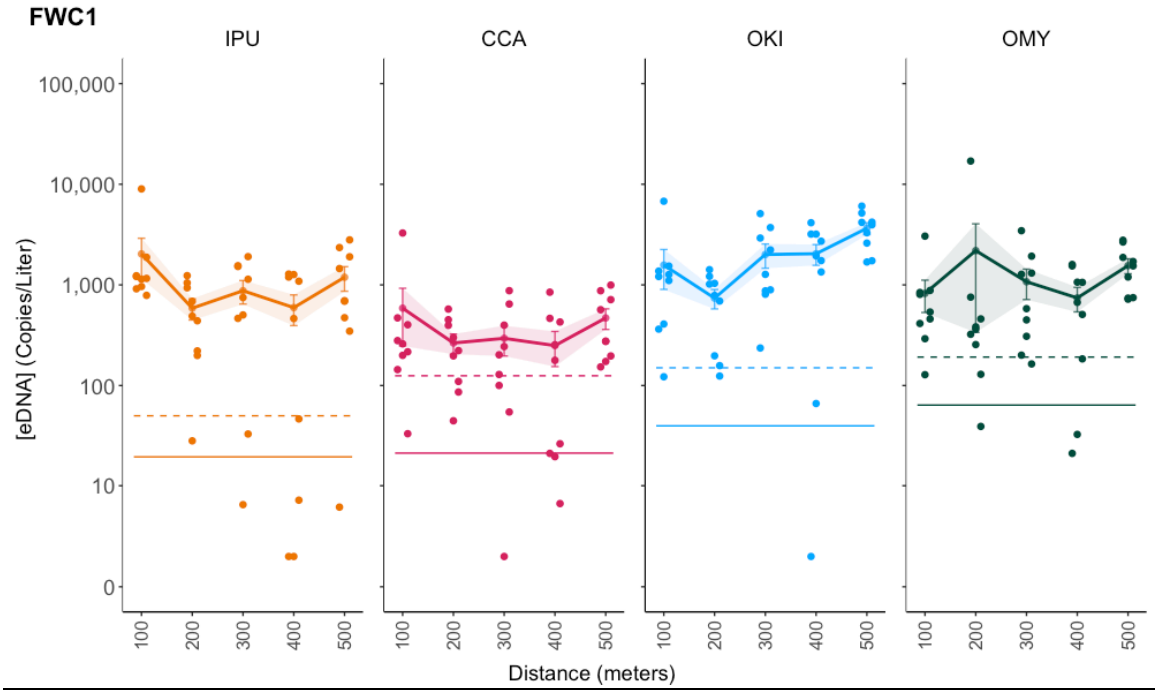

Figure B5: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in FWC1. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B5: Table comparing the number of detections in FWC1 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	1 / 0	0 / 0
200 m	1 / 1	1 / 0
300 m	1 / 1	1 / 0
400 m	1 / 1	1 / 1

JC2

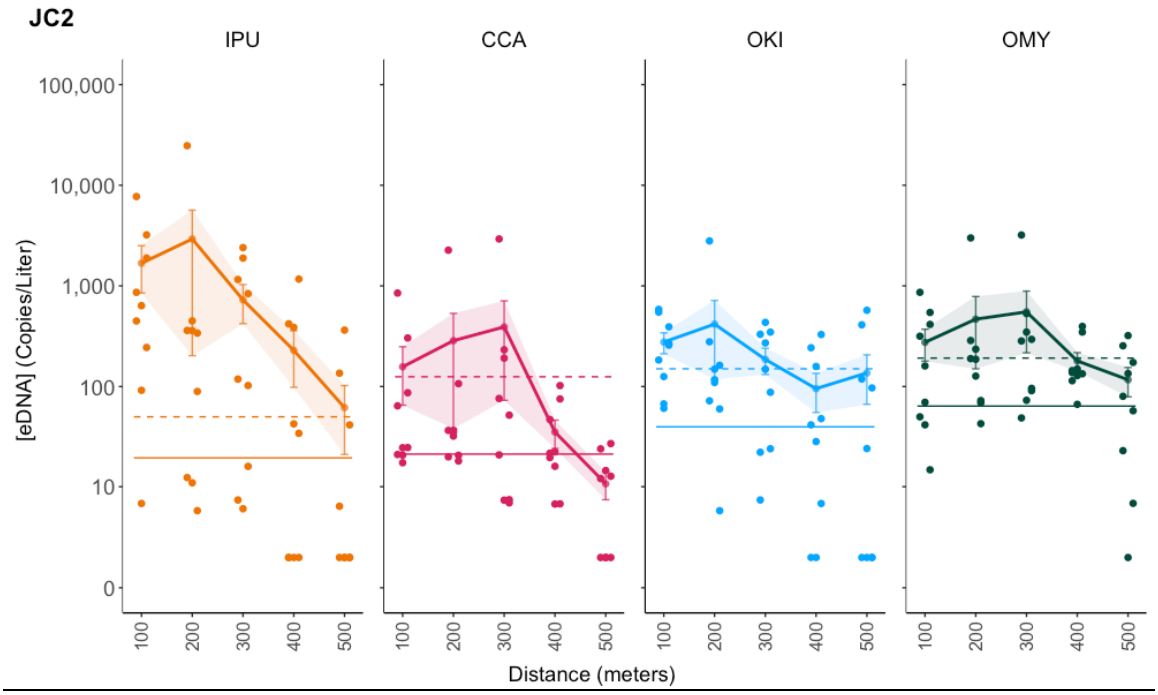

Figure B6: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in JC2. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B6: Table comparing the number of detections in JC2 across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 0	1 / 0
200 m	0 / 0	1 / 0
300 m	0 / 0	1 / 1
400 m	1 / 0	1 / 0

PRC

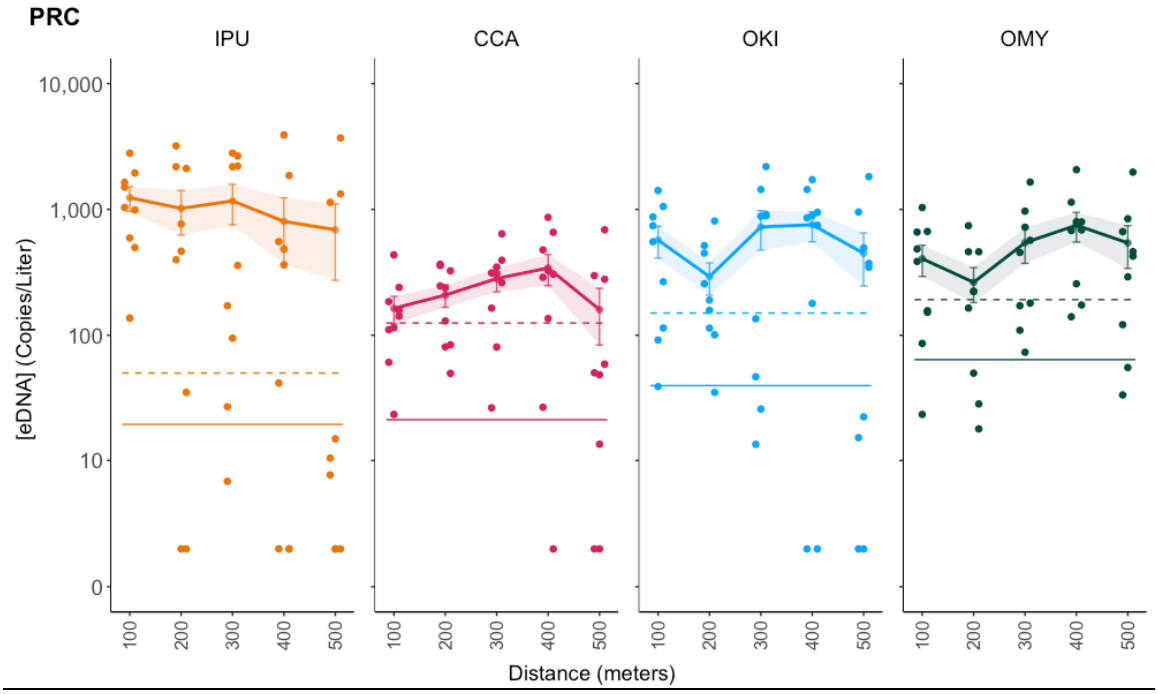

Figure B7: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in PRC. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B7: Table comparing the number of detections in PRC across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 0	0 / 0
200 m	0 / 0	1 / 0
300 m	1 / 0	1 / 0
400 m	1 / 0	1 / 0

OCC

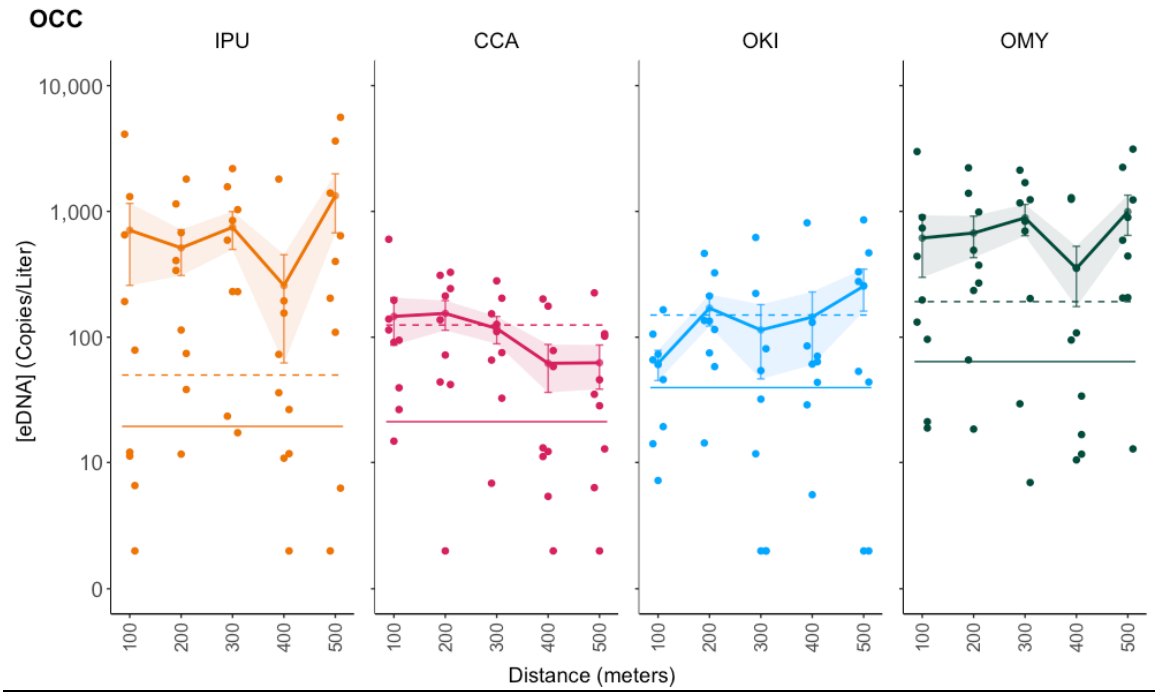

Figure B8: Concentrations of IPU tracer(IPU), CCA tracer(CCA), coho salmon(OKI) and steelhead(OMY) captured in OCC. Individual bold points represent concentrations across the nine samples collected at each cross-section, a jitter is applied for clarity. Light points and error bars are the average concentration ± Standard error at a cross sections. The horizontal lines indicate the species-specific LOD (solid) and LOQ (dashed).

Table B8: Table comparing the number of detections in OCC across distances (100, 200, 300 & 400 meters) as indicated by significant Wilcox Rank Sum Test results. Results are separated by column coho salmon (OKI) and steelhead (OMY). Within columns results are split by tracer species (IPU / CCA) used in ratio calculations. One test per distance per tracer was conducted

Distance between cross sections	Number of significant test (OKI:IPU / OKI:CCA)	Number of significant test (OMY:IPU / OMY:CCA)
100 m	0 / 0	0 / 0
200 m	0 / 0	0 / 0
300 m	0 / 1	0 / 0
400 m	0 / 0	0 / 1

Appendix C: 2021 Study Sites

This appendix contains descriptions of five study sites sampled in 2021 assessing if the eDNA tracer could be used to infer species presence and how the sampling grid should be placed relative to the ADIDs. Key differences between the 2021 and 2022 sampling designs and the limited number of study reaches resulted in the 2021 data being presented separately.

Methods

Study Sites

In 2021, five field trials were conducted in small coastal streams located in Humboldt county. The sites were sampled and the data analyzed following the same methods described in the main text with the following exceptions.

Study sites consisted of 1,000 m of stream. The IPU tracer was introduced at the head of the study site (0 m) in the main flow, while the CCA tracer was introduced 50 m downstream from the head of the study site outside of the main flow. The IPU tracer was introduced at a high concentration, at a 1:1 dilution of tracer to stream water, and the CCA was introduced at a low concentration, at a 1:2 dilution. Water samples were collected beginning 1,000 m downstream of the head of the study site, and proceeding upstream to prevent resuspension of settled eDNA from influencing captured eDNA concentrations. Samples were collected at distances of 100 m, 150 m, 200 m, 300m,

500m and 1,000 m downstream from the head of the site, resulting in 54 water samples per study site. Snorkel surveys were conducted between the 100 and 200 m cross sections to provide an estimate of coho salmon presence and abundance. The 100 m section of stream was surveyed twice by two separate divers. Snorkel surveys were conducted following sample collection.

Environmental DNA methods

After filtration, filters bearing DNA were stabilized in 360 μL of buffer ATL. To dry out filters for dissolution in acetone, each filter was removed from its microcentrifuge tube and placed in a new, sterile tube using sterilized forceps. Filters dried at room temperature for 24 hours. The buffer ATL used to stabilize each sample was retained and used during the lysing phase of the QIAGEN DNeasy Blood and Tissue Kit extraction. The concentration of DNA in each sample was estimated for both tracer species and coho salmon using the same protocol outlined in the methods section.

Analysis

Data from 2021 study sites was analyzed using the same protocol outlined in the methods section, focusing on the effects of tracer concentration and spatial scales

Results

Site Descriptions

In Jacoby Creek, Old Campbell Creek, and Little River one study site was sampled, whereas in Prairie Creek, two field trials occurred at the same site at separate times to sample at high and low flows. Mean width of cross sections ranged from 3.89 m to 7.59 m, mean depth at sample collection locations ranged from 0.2 m to .35 m, mean velocity at sample collection locations ranged from .02 L/s to .43 L/s. Discharge varied across study sites from 1.64 L/s to 142 L/s (Table C1).

Snorkel

Snorkel surveys identified coho salmon between the 100 – 200 m cross-sections across every study site, thus the null expectation for all occupancy assessments was the detection of coho salmon. Observed abundances ranged from 15-97 coho salmon (Table C1).

Table C1: Stream measurements conducted at all 2021 study sites including: stream name, study site name, mean width of cross sections (m), mean depth across all sampling locations (m), mean velocity across all sampling locations (L / second), the single discharge measurement collected at a site (L/s) and the observed abundances of coho salmon and steelhead between the 100 m and 200 m cross sections at each study site.

Stream name	Study Site	Mean width (m)	Mean depth (m)	Mean velocity (L/s)	Discharge (L/s)	Observed coho salmon
Jacoby Creek	JC.21	5.88	0.28	0.02	1.64	97
Little River	LR.21	7.59	0.35	0.54	44.8	58
Old Campbell Creek	OC.21	6.49	0.31	0.11	86.2	38
Prairie Creek	PCH.21	5.13	0.38	0.43	142.0	15
Prairie Creek	PCL.21	3.89	0.20	0.06	6.24	20

Tracer Correlation

The fraction of samples that exhibited strongly correlated tracer concentrations varied across study sites. Within JC.21, LR.21 and PCL.21, the IPU and CCA tracers exhibited similar levels of correlation, as indicated but the samples retained in the RMSE screening (44.4% - 51.9%). Low correlation was in the OC.21 and PCH.21 sites (1.86 – 20.4%), which coincided with the highest discharge sites. Overall lower levels of correlation were seen in 2021 as compared to 2022, which is presumed to have occurred because a greater number of samples had concentrations below quantifiable levels (CCA – 61.3% of samples, IPU 34.1%) compared to 2022 samples levels (CCA – 34.2%, IPU 20.3%). The larger spatial scale of study sites in 2021 likely contributed to a greater proportion of samples having concentrations below the limit of quantification.

Table C2: Mean concentrations of both tracers (CCA & IPU) and coho salmon (OKI) across the 54 water samples collected in each study site ± standard error. The proportion of correlated samples, determined by linear regression and root-mean-square-error analysis on sample specific tracer concentration, for each study site is also included.

Study Site	CCA mean	IPU mean	OKI mean	Proportion retained
JC.21	16,977 ± 5,340	38,691 ± 8,047	324 ± 38.4	51.9%
LR.21	1,808 ± 341	1,077 ± 179	330 ± 57	48.1%
OC.21	541 ± 207	2,422 ± 899	38.3 ± 14.9	20.4%
PCH.21	50.5 ± 25.4	5,092 ± 868	157 ± 83.8	1.86%
PCL.21	17,429 ± 11,451	5,213 ± 1,347	163 ± 39.5	44.4%
Mean	7,361	10,499	203	

Tracer concentration

Two-sample Wilcoxon Rank-Sum tests were performed on ratios from the 100 m cross-section against ratios from every downstream cross-section. In total, 50 tests were conducted encompassing all study sites, spatial scales and ratio types. Significant increases in ratio value, indicative of species presence, were found in 44% of tests. (Table C2) The JC.21 site had the highest proportion of detections across both ratios (90%), while PCL.21 had the lowest (0%). Within study sites, detections of coho salmon between ratio types with similar, and across all sites both ratios had the same proportion of detections.

Table C2: The proportion of significant Wilcoxon Rank Sum Test results for Coho Salmon (OKI):Tracer ratios at each study site. Five tests were performed for each ratio type at each study site, for a total of ten tests within each study site.

Study Site	OKI:IPU	OKI:CCA	Overall
JC.21	80%	100%	90%
LR.21	40%	80%	60%
OC.21	80%	40%	60%
PCH.21	20%	0%	10%
PCL.21	0%	0%	0%
Overall	44%	44%	44%

Directly comparing the performance of ratios revealed that both tracers resulted in similar detections. Both tracer resulted in the same number of detections when the other did not (Table C3). Where both tracers agreed in detections, the non-detection of coho salmon was more common (44%) than detection (32%).

Table C3: The proportion of significant Wilcoxon Rank-Sum tests comparing where tests conducted with each tracer saw agreement of disagreement in results. In the IPU and CCA tracer column the "+" indicates a significant Wilcoxon Rank Sum Tests while the "-" indicates no significant results. The percentage in the detections column represent the proportion of total tests (25) per species with agreement/Disagreement between tracers.

IPU tracer	CCA tracer	Detections
+	+	32%
+	–	12%
–	+	12%
–	–	44%

Spatial scale

Similar occupancy results were seen between both tracers across spatial scales. The lowest proportion of detections coincided with samples collected 50 m apart. Samples collected 400 and 900 m apart had a higher proportion of detections using the higher dosed tracer (IPU) compared to the lower dosed tracer (CCA).

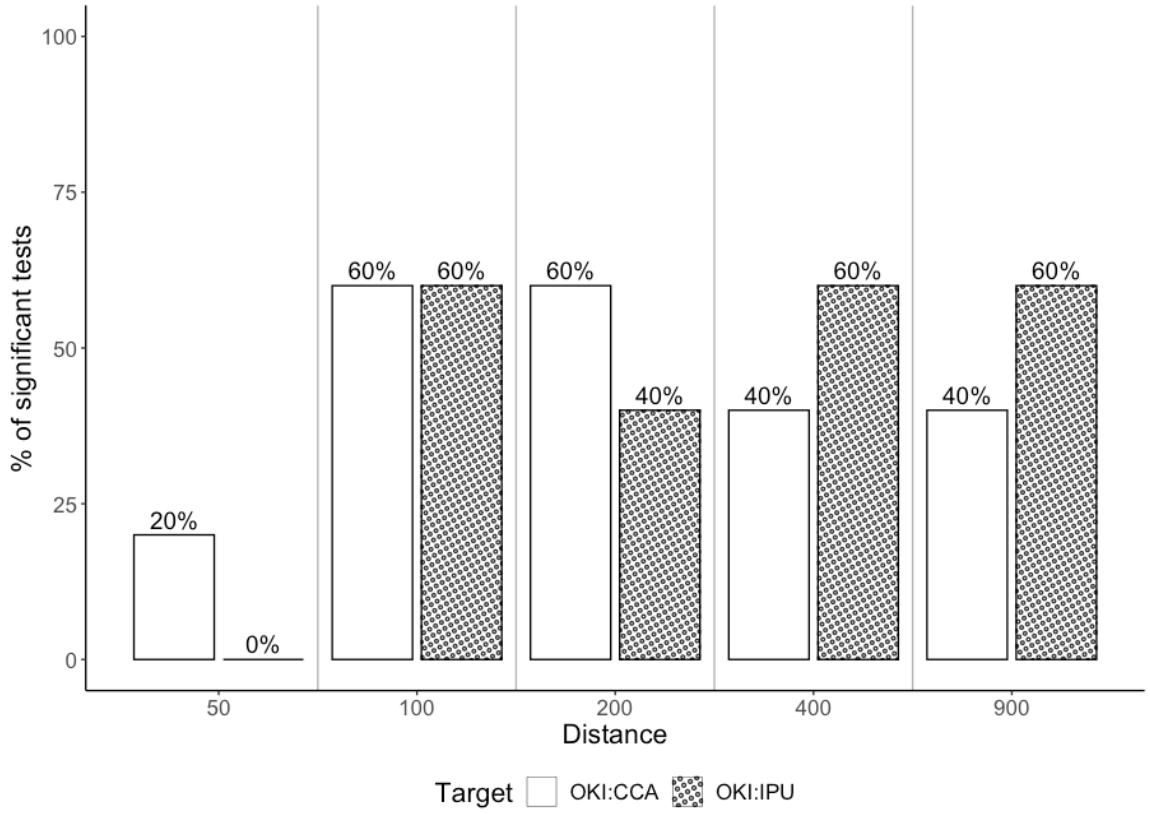

Figure C1: The proportion of significant Wilcox Rank-sum Tests conducted on coho salmon:tracer ratios separated by distance between sampling locations. Results are further broken down by IPU ratios (empty or left bar) and CCA ratios (dotted or right bar). The number above each b bar represents the percentage of significant tests (out of five).