

WILEY

ECOLOGICAL APPLICATIONS

**Quantifying Impacts of an Environmental Intervention  
Using Environmental DNA**

Journal:	<i>Ecological Applications</i>
Manuscript ID	EAP23-0011.R1
Wiley - Manuscript type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Andruszkiewicz Allan, Elizabeth; University of Washington, School of Marine and Environmental Affairs Kelly, Ryan; University of Washington, School of Marine and Environmental Affairs D'Agnese, Erin; University of Washington, School of Marine and Environmental Affairs; Wild EcoHealth Garber-Yonts, Maya; University of Washington, School of Marine and Environmental Affairs Shaffer, Megan; University of Washington, School of Marine and Environmental Affairs Gold, Zachary; Northwest Fisheries Science Center Shelton, Andrew; Northwest Fisheries Science Center,
Substantive Area:	Management < Substantive Area, Molecular Approaches < Methodology < Substantive Area, Community Analysis/Structure/Stability < Community Ecology < Substantive Area
Organism:	Other (specify type in field below) < Fishes < Vertebrates < Animals, Teleost < Fishes < Vertebrates < Animals
Habitat:	Rivers/Streams < Freshwater < Aquatic Habitat < Habitat
Geographic Area:	Northwest US (ID, MT, OR, WA, WY) < United States < North America < Geographic Area
Key words/phrases:	environmental DNA, quantitative metabarcoding, environmental impact assessments, culvert, salmon
Abstract:	Environmental laws around the world require some version of an environmental impact assessment surrounding construction projects and other discrete instances of human development. Information requirements for these assessments vary by jurisdiction, but nearly all require an analysis of biological elements of ecosystems. Amplicon-sequencing - also called metabarcoding - of environmental DNA (eDNA) has made it possible to sample and amplify the genetic material of many species present in those environments, providing a tractable, powerful, and increasingly common way of doing environmental impact analysis for development projects. Here, we analyze a 18-month time-series of water samples taken before, during, and after two culvert removals in a salmonid-bearing freshwater stream. We also sampled multiple control

streams to develop a robust background expectation against which to evaluate the impact of this discrete environmental intervention in the treatment stream. We generate calibrated, quantitative metabarcoding data from amplifying the 12s MiFish mtDNA locus and complementary species-specific quantitative PCR data to yield multi-species estimates of absolute eDNA concentrations across time, creeks, and sampling stations. We then use a linear mixed-effects model to reveal patterns of eDNA concentrations over time, and to estimate the effects of the culvert removal on salmonids in the treatment creek. We focus our analysis on four common salmonid species: cutthroat trout (*\*Oncorhynchus clarkii\**), coho salmon (*\*O. kisutch\**), rainbow trout (*\*O. mykiss\**), and sockeye salmon (*\*O. nerka\**). We find that one culvert in the treatment creek seemed to have no impact while the second culvert had a large impact on fish passage. The construction itself seemed to have only transient effects on salmonid species during the two construction events. In the context of billions of dollars of court-mandated road culvert replacements taking place in Washington State, USA, our results suggest that culvert replacement can be conducted with only minimal impact of construction to key species of management concern. Furthermore, eDNA methods can be an effective and efficient approach for monitoring hundreds of culverts to prioritize culverts that are required to be replaced. More broadly, we demonstrate a rigorous, quantitative method for environmental impact reporting using eDNA that is widely applicable in environments worldwide.

SCHOLARONE™  
Manuscripts

<sup>1</sup> Quantifying Impacts of an Environmental Intervention Using Environmental  
<sup>2</sup> DNA

<sup>3</sup> Elizabeth Andruszkiewicz Allan<sup>1\*†</sup>, Ryan P. Kelly<sup>1\*</sup>, Erin R. D'Agnese<sup>1,3</sup>, Maya N. Garber-Yonts<sup>1</sup>, Megan R.  
<sup>4</sup> Shaffer<sup>1</sup>, Zachary J. Gold<sup>2</sup>, Andrew O. Shelton<sup>2</sup>

<sup>5</sup> <sup>1</sup> University of Washington, School of Marine and Environmental Affairs, 3737 Brooklyn Ave NE, Seattle,  
<sup>6</sup> WA 98105, U.S.A.

<sup>7</sup> <sup>2</sup> Conservation Biology Division, Northwest Fisheries Science Center, National Marine Fisheries Service,  
<sup>8</sup> National Oceanic and Atmospheric Administration, 2725 Montlake Blvd. E, Seattle, WA 98112, U.S.A.

<sup>9</sup> <sup>3</sup> Wild EcoHealth, Tacoma WA, 98465, USA

<sup>10</sup> \* Authors contributed equally to this work.

<sup>11</sup> † Corresponding author: eallan@uw.edu

<sup>12</sup> For submission to: *Ecological Applications*

<sup>13</sup> Manuscript type: Article

<sup>14</sup> Open Research Statement: Data are provided as private-for-peer review (shared privately or publicly on a  
<sup>15</sup> repository). The repository for code can be found at: [https://github.com/eandrusz/quantitative\\_salmon\\_re](https://github.com/eandrusz/quantitative_salmon_re)  
<sup>16</sup> submit.git

<sup>17</sup> Keywords: environmental DNA, quantitative metabarcoding, environmental impact assessments, salmon,  
<sup>18</sup> culvert

19 **Abstract**

20 Environmental laws around the world require some version of an environmental impact assessment surrounding  
21 construction projects and other discrete instances of human development. Information requirements for  
22 these assessments vary by jurisdiction, but nearly all require an analysis of biological elements of ecosystems.  
23 Amplicon-sequencing - also called metabarcoding - of environmental DNA (eDNA) has made it possible to  
24 sample and amplify the genetic material of many species present in those environments, providing a tractable,  
25 powerful, and increasingly common way of doing environmental impact analysis for development projects.  
26 Here, we analyze a 18-month time-series of water samples taken before, during, and after two culvert removals  
27 in a salmonid-bearing freshwater stream. We also sampled multiple control streams to develop a robust  
28 background expectation against which to evaluate the impact of this discrete environmental intervention in the  
29 treatment stream. We generate calibrated, quantitative metabarcoding data from amplifying the 12s MiFish  
30 mtDNA locus and complementary species-specific quantitative PCR data to yield multi-species estimates of  
31 absolute eDNA concentrations across time, creeks, and sampling stations. We then use a linear mixed-effects  
32 model to reveal patterns of eDNA concentrations over time, and to estimate the effects of the culvert removal  
33 on salmonids in the treatment creek. We focus our analysis on four common salmonid species: cutthroat  
34 trout (*Oncorhynchus clarkii*), coho salmon (*O. kisutch*), rainbow trout (*O. mykiss*), and sockeye salmon (*O.*  
35 *nerka*). We find that one culvert in the treatment creek seemed to have no impact while the second culvert  
36 had a large impact on fish passage. The construction itself seemed to have only transient effects on salmonid  
37 species during the two construction events. In the context of billions of dollars of court-mandated road culvert  
38 replacements taking place in Washington State, USA, our results suggest that culvert replacement can be  
39 conducted with only minimal impact of construction to key species of management concern. Furthermore,  
40 eDNA methods can be an effective and efficient approach for monitoring hundreds of culverts to prioritize  
41 culverts that are required to be replaced. More broadly, we demonstrate a rigorous, quantitative method for  
42 environmental impact reporting using eDNA that is widely applicable in environments worldwide.

## 43 Introduction

44 At present, it remains difficult to comprehensively measure the environmental impacts of discrete human  
45 activities, despite such assessment often being required by law. Within the United States, both state and  
46 federal laws often require a form of environmental-impact assessment for medium- to large-scale projects (i.e.,  
47 those that might have a significant impact on the environment) (Morgan 2012). Outside the US, many nations  
48 have their own versions of these same laws. Specifically when measuring impacts on aquatic ecosystems,  
49 assessments generally are based on literature reviews or field measurements of key species selected beforehand  
50 (Rubin et al. 2017). These traditional methods are often expensive, rely on just a few species, and are limited  
51 in spatial and temporal coverage (Martin et al. 2012). Moreover, they often lack pre-project monitoring and  
52 any or sufficient post-project monitoring, given that the goals of a development project normally focus on  
53 construction itself and funding is often extremely limited. For example, a recent literature review of stream  
54 restoration projects cited that more than half of projects evaluated (62%) had no pre-project monitoring and  
55 only sampled once per year (for before, during, and post-project sampling) (Rubin et al. 2017). Thus, current  
56 assessment efforts relying on traditional survey methods often fall short in documenting and quantifying  
57 environmental impacts.

58 A key difficulty in conducting ecosystem assessments is that there is no one way to survey the world and just  
59 “see what is there.” All methods of environmental sampling are biased as they capture a selective portion of  
60 the biodiversity present (Rubin et al. 2017). Net samples for fish, for example, fail to capture species too  
61 small or too large to be caught in the net. Environmental DNA (eDNA), however, comes as close to this  
62 goal as any method yet developed although not without bias (see below): a sample of water, soil, or even  
63 air, contains the genetic traces of many thousands of species, from microbes to whales. Sequencing eDNA is  
64 therefore a means of surveying many species in a consistent and scalable way (Taberlet et al. 2012, Thomsen  
65 and Willerslev 2015). Environmental assessments have begun to make use of eDNA for such work around  
66 the world (Muha et al. 2017, Duda et al. 2021, Klein et al. 2022, Maasri et al. 2022, Moss et al. 2022),  
67 but are not yet common practice. Surveying the world by eDNA has long been commonplace in microbial  
68 ecology (Ogram et al. 1987, Rondon et al. 2000, Turnbaugh et al. 2007) but has recently become popular for  
69 characterizing eukaryotic communities (Taberlet et al. 2012, Kelly et al. 2014, De Vargas et al. 2015, Port et  
70 al. 2015, Valentini et al. 2016, Stat et al. 2017). Techniques generally include an amplification step such  
71 as quantitative PCR, digital or digital-droplet PCR, or traditional PCR from mixed templates followed by  
72 high-throughput sequencing (Ruppert et al. 2019). This last technique is known as eDNA metabarcoding.

73 In a metabarcoding approach, broad-spectrum PCR primers identify hundreds or thousands of taxa across a  
74 very wide diversity of the tree of life (e.g., Leray et al. (2013)). Nevertheless the absence of a taxon from a

75 sequenced sample does not indicate the absence of that taxon from the environment but rather that the taxon  
76 failed to amplify (Shelton et al. 2016, Kelly et al. 2019, Buxton et al. 2021, Gold et al. 2023). In virtually all  
77 comparisons, metabarcoding recovers far more taxa than any other sampling method (Port et al. 2015, Kelly  
78 et al. 2017, Seymour et al. 2021). However, we expect results from metabarcoding to differ dramatically  
79 from non-PCR based sampling methods due to the fundamental differences in sampling residual genetic  
80 material as opposed to whole organisms. Furthermore, eDNA analyses rely on several laboratory processes,  
81 including PCR amplification, all of which contribute to complicating the interpretation of results (see Shelton  
82 et al. (2016) and Kelly et al. (2019)). Specifically, PCR amplification is an exponential process for which the  
83 efficiency varies across species and primer set (Gloor et al. 2016). By understanding these differences, we  
84 can correct for taxon-specific biases to yield quantitative estimates of the community composition prior to  
85 PCR (McLaren et al. 2019, Shelton et al. 2022). Other ways to conduct quantitative metabarcoding include  
86 using qSeq (Hoshino et al. 2021), a process in which a random tag is added to target sequences before PCR.  
87 However, if different species amplify at different rates during PCR, these quantifications would reflect not  
88 just the starting concentration but also the amplification efficiency.

89 After correcting for amplification biases, the resulting dataset is compositional, revealing the proportions  
90 of each species' DNA present in each sample, but importantly, contains no information about the absolute  
91 abundance of DNA present (Gloor et al. 2016, McLaren et al. 2019, Silverman et al. 2021, Shelton et  
92 al. 2022). We can tie these proportional estimates to absolute abundances using additional data such as  
93 a quantitative PCR (qPCR) assay for one of the taxa present. Thus, a single qPCR assay and a single  
94 metabarcoding assay can together provide quantitative estimates of many species as opposed to running  
95 as many qPCR assays as species of interest (see also (Pont et al. 2022)). Together, we can use these data  
96 to assess changes in eDNA concentrations of species over time, and due to environmental impacts, such as  
97 replacing a culvert under a road.

98 As a result of a ruling in a federal court (Martinez 2013), Washington State is under a mandate to replace  
99 hundreds of culverts that allow water to pass under roads and highways, costing billions of dollars. Improperly  
100 designed culverts can lead to many negative consequences for fish, especially anadromous salmon, including  
101 habitat fragmentation, loss of accessibility to spawning and rearing habitat, and genetic isolation (Price et al.  
102 2010, Frankiewicz et al. 2021). These impacts on salmonids furthermore violate the sovereign treaty rights of  
103 the region's indigenous tribes (Martinez 2013). Salmonid species are of cultural and economic importance to  
104 the indigenous peoples of the region, and without restoration of historic salmon-rearing habitat, the continued  
105 decline of salmonids can lead to not only ecological destruction, but the loss of cultural and economic viability  
106 for many indigenous tribes (Schmidhauser 1976, Lackey 2003, Long and Lake 2018).

107 Presently, the prioritization process for replacing culverts preventing fish passage conducted by the Washington  
108 Department of Transportation is a protocol provided by the Washington Department of Fish and Wildlife,  
109 which includes factors such as the amount of habitat blocked by the barrier, the types of species blocked by  
110 the barrier, and estimated cost of repair, among other things (Washington Department of Fish and Wildlife  
111 2019). However, data on fish presence upstream of barriers (i.e., culverts blocking fish passage) are rare and  
112 often not included in these assessments. Using eDNA as a proxy for fish presence could provide important  
113 data for project prioritization and have the potential to be more cost effective.

114 Once a culvert has been designated as in need of repair, the intention is to improve conditions for biota,  
115 including migrating fish, but the construction itself might have a short-term negative effect before the  
116 longer-term improvements are realized. Specifically in culvert replacements, studies have cited the negative  
117 impacts of construction to include sediment accumulation, removal of vegetation, and blocking flow and  
118 stranding fish (Wellman et al. 2000, Washington Department of Fish and Wildlife 2019). However, it is  
119 unclear how long these effects might last and if the long-term benefits of the culvert replacement justify the  
120 short-term costs of the construction. These disruptions also underscore the importance of both properly  
121 assessing culverts to determine if they are blocking fish passage and monitoring after construction to ensure  
122 the replacement actually improved fish passage.

123 Many studies have attempted to quantify when culverts are barriers to fish passage and how effective culvert  
124 replacements are for fish passage, either by measuring physical parameters of the culvert and stream after  
125 replacement (Price et al. 2010), or by measuring biological parameters, including electrofishing (Ogren and  
126 Huckins 2015) or genetic differentiation from fish tissues (Wood et al. 2018, Nathan et al. 2018). In some  
127 cases, culverts deemed blockages did not prove to block fish passage (MacPherson et al. 2012), while in others,  
128 blockages that were replaced were not found to improve fish passage (Price et al. 2010) or improve overall biotic  
129 integrity (Ogren and Huckins 2015). Sampling water for eDNA analysis before, during, and post-restoration  
130 can provide valuable information on if the restoration is needed, how the restoration negatively impacts  
131 communities during construction, and if the restoration efforts did in fact correct the blockage.

132 Here, we report the results of an approximately 18-month eDNA sampling effort before, during, and after  
133 the replacement of two culverts (one small and one large) in a creek, assessing the impact of these projects  
134 on the salmonid species present. We do so using a combination of metabarcoding (12s mtDNA) and qPCR  
135 to yield estimates of the concentrations of DNA present at each time point, and we use parallel samples  
136 from four control creeks to develop a causal analysis of changes in these concentrations. A clear opportunity  
137 for policy-relevant eDNA work is in using its power to survey many species at a time to improve the way  
138 we assess the impacts of human activities. Here, we demonstrate the utility of eDNA for policy-relevant

139 environmental assessments by surveying many species simultaneously and improving the way we assess the  
140 impacts of human activities.

## 141 Methods

### 142 Site and Species Selection

143 We selected sampling locations based loosely on an asymmetrical BACI (Before-After-Control-Impact) study  
144 design (Underwood 1992, 1994, Benedetti-Cecchi 2001) to measure the environmental impact of a culvert  
145 replacement using eDNA. We sampled four control creeks where construction was not occurring, (Figure  
146 1) at monthly intervals, both upstream and downstream of each creek's culvert. The two culverts in the  
147 treatment creek (Padden) were suspected to be partially impassable and thus were removed and replaced  
148 during the course of the study. The four control creeks ranged from preventing fish passage (Barnes and  
149 Chuckanut), partially passable (Squalicum), to allowing fish passage (Portage; see Supplemental Text 1)  
150 (Washington Department of Fish and Wildlife 2019). These creeks were chosen due to their comparable size,  
151 flow, watersheds, and species presumed to be present to constrain as many ecological variables as possible.

152 The first culvert replacement (SR-11) in Padden Creek occurred over about two months and included the  
153 "de-watering" of the creek, removal of the existing culvert, installation of the new culvert, and then the  
154 "re-watering" of the creek from late August 2021 to early October 2021 (Supplemental Text 1; Figure S1.4).

155 The second culvert replacement (I-5) in Padden Creek was a much larger construction project, including  
156 daylighting the creek and building a bridge under a large, five-lane interstate. In-water work for the I-5  
157 culvert replacement began in late June 2022 and was completed in September 2022. By sampling before,  
158 during, and after both construction events, we were then able to isolate the effect of the culvert replacement  
159 itself – controlling for temporal trends, background environmental variability, and sampling variability – using  
160 a linear mixed effects model of eDNA abundances across creeks, time points, sampling stations, and species.

161 Because salmonids are the primary species of management concern in these creeks, we focus the present  
162 analysis on the four salmonid species most common in our data: cutthroat trout (*Oncorhynchus clarkii*),  
163 coho salmon (*O. kisutch*), rainbow/steelhead trout (*O. mykiss*), and sockeye/kokanee salmon (*O. nerka*).

164 Not all four salmonids are expected to be found in all five of the creeks sampled. As documented by WA  
165 Department of Fish and Wildlife SalmonScape (<http://apps.wdfw.wa.gov/salmonscape/map.html>), all creeks  
166 contain cutthroat trout, steelhead trout, and coho salmon. Barnes Creek is the only creek documented to  
167 have kokanee salmon (a freshwater sub-type of sockeye salmon). However, local spawner surveys conducted  
168 by the City of Bellingham from 2015-2020 in Padden Creek documented kokanee salmon, as well as the

169 other three species (City of Bellingham 2015). The four salmonid species in this study have different life  
170 histories and behaviors that would impact when fish (and therefore eDNA concentrations) occur in the creeks.  
171 Furthermore, three of the four species in this study have both freshwater resident and saltwater migrating  
172 behavior. For the fish exhibiting migratory behavior, the run timings vary for each species in the study area  
173 (see Discussion and Figure S1.4). Therefore, our eDNA concentrations might reflect contributions from both  
174 migrating and non-migrating individuals at any given time point in the dataset.

175 **Water Sampling**

176 From March 2021 to February 2022, all five creeks were sampled monthly (n=12). Monthly sampling continued  
177 in Portage Creek, Padden Creek, and Squalicum Creek through August 2022, with one additional sampling  
178 point in December 2022 (n=19). At each sampling station (upstream and downstream of a culvert) at each  
179 creek, we collected three 2-liter water samples. Samples were collected using an eDNA Backpack (Smith  
180 Root; Thomas et al. (2018)), a portable pumping-and-filtering device set to filter at 1 L/min at 82.7 kPa (12  
181 psi). In some months, less than 2 L of water was filtered due to clogging. Water samples were filtered using  
182 single-use inlet tubes through 5 $\mu$ m self-preserving filters (Smith Root, Vancouver, WA), which were then  
183 dried and kept at room temperature until DNA extraction within 1 month of collection (Thomas et al. 2019).

184 Over the course of the sampling, water discharge varied from very low to no flow in summer months to  
185 high flow in winter months (Figure 2). We account for this dilution by converting eDNA concentration  
186 [copies/ $\mu$ L] to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s]  
187 (Tillotson et al. 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey  
188 (USGS) were used for Padden Creek (USGS Gauge 12201905), Chuckanut Creek (USGS Gauge 12201700),  
189 and Squalicum Creek (USGS Gauge 12204010; <https://maps.waterdata.usgs.gov/mapper/index.html>; U. S.  
190 Geological Survey (1994); Figure S1.1). Over the course of sampling, the flow gauges at Chuckanut Creek  
191 and Squalicum Creek became inoperable after a major flooding event. To find discharge rates for Chuckanut Creek  
192 and Squalicum Creeks, five years of historical data (2015-2020) were used to generate a monthly averaged  
193 correction factor based on Padden Creek (Supplemental Text 1, Figure S1.3). No discharge data was available  
194 for Portage Creek or Barnes Creek. Based on field sampling conditions, the discharge from Padden Creek was  
195 used as a proxy for both Portage and Barnes as they are in similarly sized watershed areas and land-cover  
196 characteristics.

197 **DNA Extraction, Amplification, Sequencing**

198 All molecular work prior to sequencing was performed at the University of Washington. Details of the molecular  
199 work can be found in Supplemental Text 1. Briefly, DNA was extracted off filters using a QiaShredder  
200 column (Qiagen, USA) and the DNeasy Blood and Tissue Kit (Qiagen, USA) with an overnight incubation  
201 (Supplemental Text 1, Thomas et al. (2019)). Extracts were stored at -20°C until PCR amplification within  
202 2 months of extraction.

203 For metabarcoding, we targeted a ~186 bp hypervariable region of the mitochondrial DNA 12S rRNA gene  
204 for PCR amplification (MiFish; Miya et al. 2015), but using modified primer sequences as given in Praebel  
205 and Wangensteen (unpublished; via personal communication). The primer sequences, final reaction recipe,  
206 and cycling conditions can be found in Supplemental Text 1. Each month of samples was amplified on a  
207 single plate with the addition of a no template control (NTC; molecular grade water in lieu of template) and  
208 a positive control (genomic DNA from kangaroo, a species not present in the environment). PCR products  
209 were visualized, size-selected, and diluted iteratively if inhibited. After cleaning, a second PCR amplification  
210 added unique indices to each sample using Nextera indices (Illumina, USA) to allow pooling multiple samples  
211 onto the same sequencing run (See Supplemental Text 1 for details). Indexed PCR products were also  
212 size-selected and visualized before pooling for sequencing. Samples were randomized in 3-month blocks and  
213 each block split across 3 sequencing runs to avoid run effects, for a total of 14 sequencing runs. The loading  
214 concentration of each library was 4-8 pM and 5-20% PhiX was included depending on the composition of the  
215 run. Sequencing was conducted using an Illumina MiSeq with v3 2x300 chemistry at the NOAA Northwest  
216 Fisheries Science Center and the University of Washington's Northwest Genomics Center.

217 Here, we used mock communities to determine the species-specific amplification efficiencies for each salmonid  
218 in the study. We constructed five communities with known proportions of starting DNA from different  
219 species (total DNA as measured by Qubit). The communities ranged from having a total of 12 to 20  
220 species, but six salmonid species were included in all five mock communities (Supplemental Table 2). We  
221 sequenced these communities using the same metabarcoding primers and thermocycling conditions above and  
222 then determined the species-specific amplification rates given the discrepancy between the known starting  
223 proportion and the proportion of reads after sequencing. The mock community data were then used to correct  
224 the sequencing reads from the environmental samples to estimate the starting DNA proportions of each  
225 species in environmental samples, which is the metric of interest (Figure 3, green boxes). This is the first  
226 application of the model to correct eDNA data from water samples with mock community data as described  
227 in Shelton et al. (2022) (see Supplemental Text 2 for more information).

228 **Bioinformatics**

229 After sequencing, bioinformatic analyses were conducted in R (R Core Team 2017). A more detailed  
230 description of the bioinformatics pipeline is included in Supplemental Text 1. Briefly, primer sequences were  
231 removed using *Cutadapt* (Version 1.18) (Martin 2011) before *dada2* (Callahan et al. 2016) trimmed, filtered,  
232 merged paired end reads, and generated amplicon sequence variants (ASVs). Taxonomic assignment was  
233 conducted via the *insect* package (Wilkinson et al. 2018) using a tree generated by the developers for the  
234 MiFish primers that was last updated in November 2018. Only species level assignments from *insect* were  
235 retained and ASVs not annotated or not annotated to species level were then checked against the NCBI  
236 nucleotide database using BLAST+ (Camacho et al. 2009). Query sequences that matched a single species  
237 at >95% identity were retained.

238 **Quantitative PCR and Inhibition Testing**

239 We quantified cutthroat trout (*O. clarkii*) DNA in each sample, targeting a 114 bp fragment of the cytochrome  
240 b gene with a qPCR assay (Duda et al. 2021). The primer/probe sequences, final recipe, and thermocycling  
241 conditions can be found in Supplemental Text 1. Each DNA sample was run in triplicate and was checked for  
242 inhibition using the EXO-IPC assay (Applied Biosystems). The majority of environmental samples (60%)  
243 were inhibited and accordingly diluted for analysis. In 80% of inhibited samples, a 1:10 dilution or less  
244 remedied the inhibition, but some samples (11%) required dilution by a factor of up to 1000. Each plate  
245 included a 8-point standard curve created using synthetic DNA (gBlocks) ranging from 1 to 100,000 copies/ $\mu$ L  
246 and six no template controls (NTCs) were included on each plate with molecular grade water instead of  
247 template. All qPCRs were conducted on an Applied Biosystems StepOnePlus thermocycler.

248 All qPCR data was processed in R using Stan (Stan Development Team 2022), relating environmental samples  
249 to the standard curve via a linear model (Figure 3, blue boxes; Figure S2.1). We amended the standard  
250 linear regression model to more realistically capture the behavior of qPCR observations, accommodating  
251 non-detections as a function of underlying DNA concentration, and letting the standard deviation vary with  
252 the mean (lower-concentration samples had more uncertainty). See McCall et al. (2014) and Shelton et al.  
253 (2019) for similar models; see Supplemental Text 2 for full statistical details. Subsequent analysis corrected  
254 for sample-specific dilution if found inhibited and corrected for any variation in water-volume filtered during  
255 sample collection. Samples with standard deviations between technical replicates larger than 1.5 Ct values  
256 were removed from analyses.

257 **Quantitative Metabarcoding**

258 The intercalibration of the mock community samples demonstrated the rank order of amplification efficiencies  
259 for salmonids (Supplemental Figures 10 and 11). Cutthroat trout (*O. clarkii*) and sockeye/kokanee salmon  
260 (*O. nerka*) had similar amplification efficiencies, both of which were higher than rainbow/steelhead trout  
261 (*O. mykiss*) and coho salmon (*O. kisutch*), which had the lowest amplification efficiency. Calibrated  
262 metabarcoding analysis yielded quantitative estimates of the proportions of species' DNA in environmental  
263 samples prior to PCR. We then converted these proportions into absolute abundances by expansion, using  
264 the qPCR results for our reference species, cutthroat trout (*O. clarkii*). We estimated the total amplifiable  
265 salmonid DNA in environmental sample  $i$  as  $C_{\text{amplifiable}_i} = \frac{C_{\text{qPCR reference}_i}}{\text{Proportion}_{\text{reference}_i}}$ , where  $C$  has units of [DNA  
266 copies/uL] and then expanded species' proportions into absolute concentrations by multiplying these sample-  
267 specific total concentrations by individual species' proportions, such that for species  $j$  in sample  $i$ ,  $C_{i,j} =$   
268  $C_{\text{amplifiable}_i} * \text{Proportion}_{i,j}$ . Here, we combine the modeled output of the qPCR model for cutthroat trout  
269 (Figure 3 dashed blue box) and modeled proportions of salmonid DNA from metabarcoding (Figure 3 dashed  
270 green box). Although in the future this could be used as a joint model, here the precision of our modeled  
271 estimates were very high such that we used the mean of the posterior estimates from each model to move  
272 forward as input to the time series model (Figure 3 dashed purple box; see Supplemental Text 2 for more  
273 details). Finally, due to the range of water discharge over the course of the year, we converted from DNA  
274 concentration [copies/L] to a mass flow rate [copies/s] after multiplying by the discharge of each creek [ $\text{m}^3/\text{s}$ ]  
275 (Figure 3, solid purple boxes).

276 **Estimating the Effects of Culvert Replacement and of Culverts Themselves**

277 We sampled four control creeks as context against which to compare the observations in Padden Creek,  
278 our treatment creek where the two culverts were being replaced. At a given station in a given creek, some  
279 DNA concentration exists for each species. For simplicity, we focus on a single species and a single station  
280 (downstream or upstream) for the moment. Our observations of the (log) DNA concentration in creek  $i$  at  
281 time  $t$  are distributed as  $Y_{i,t} \sim \mathcal{N}(\mu_{i,t}, \sigma^2)$ . More complex versions of the model may let  $\sigma$  vary across creeks,  
282 time points, species, or with environmental covariates of interest.

283 We are interested in how the DNA concentration changes over time, so we assert that the expected value  
284 of DNA in a creek at time  $t$ ,  $\mu_{i,t}$ , depends upon its time point, in some way. We considered three ways  
285 of modeling the salmonid eDNA data, each in a Bayesian framework, but each treating non-independence  
286 among time points somewhat differently:

- 287 • A linear auto-regressive (AR(1)) model, written in **stan**. For each species in each creek, the expected

288 concentration of eDNA of each month is a linear function of the expected value from the previous  
289 month. Within a species, the monthly autoregressive parameters are shared across creeks.

- 290 • A generalized additive model (GAM), written in `brms` (which itself writes a `stan` model). For each  
291 species in each creek, an independent set of spline (weighting) parameters describes the temporal trends  
292 in expected eDNA concentration; the number of spline knots is shared across species and creeks.  
293 • A linear mixed-effects (LME) model, written in `rstanarm`. For each species in each creek, sampling  
294 month is treated as a random effect. Each species-creek-month effect is treated as an independent draw  
295 from a common distribution.

296 Ultimately, the three models yielded very similar results (Figure S2.2), and the LME model proved simplest  
297 and most flexible insofar as it could easily handle datasets with uneven sets of observations – for example,  
298 cases in which a species was detected downstream of a barrier, but not upstream.

300 In R code using `rstanarm`, this model is coded as

301 `stan_glmer(log(observed) ~ (1 + time_idx|creek:species) + (1|station:creek:species:time_idx)`

302 See Supplemental Text 2 for more details on the linear mixed-effects model.

## 303 Results

### 304 Metabarcoding and Quantitative PCR

305 In total, we generated ~52 million reads across all environmental samples and 27 mock community samples (3  
306 communities x 9 replicates [6 even, 3 skewed proportions]) for calibration (see below). After quality-filtering  
307 and merging all runs, ~45 million reads remained from ~24,000 amplicon sequence variants (ASVs) in the  
308 environmental samples, of which ~83% of reads were annotated to species level (per sample: mean = 82%,  
309 median = 93%, min = 0%, max = 99.99% of reads annotated). We only focus on the metabarcoding data  
310 from four salmonids for the remainder of this paper. The four salmonids represent ~54% of all reads and  
~64% of the annotated reads found in environmental samples.

311 In the mock community samples, 98.7% of the ~5 million reads after quality filtering were annotated to  
312 species level. Importantly, the target salmonid ASVs in the mock communities were found in environmental  
313 samples, unambiguously linking the taxa in calibration samples with those in environmental samples. The  
314 most common salmonid species found in the environmental samples was cutthroat trout (*O. clarkii*), which  
315 was found in ~85% of samples, followed by coho salmon (*O. kisutch*) found in ~62% of samples, then  
316 rainbow/steelhead trout (*O. mykiss*) found in ~40% of samples, and finally sockeye/kokanee salmon (*O.*

317 *nerka*) found in ~10% of samples. Over half of the samples across all times, creeks, and stations had at least  
318 50% of reads assigned to cutthroat trout.

319 After calibrating metabarcoding data using mock communities (See Supplemental Texts 1 and 2), we estimated  
320 the salmonid composition across time points, creeks, and stations (Figures 4 and 5). The culvert in one  
321 control creek (Barnes) appeared to be nearly a total barrier to salmonid passage, with salmonid eDNA  
322 detected upstream of the culvert at only three time points, in contrast to being detected at every time point  
323 in the downstream station of the same creek. The other four creeks had no such pattern associated with  
324 the culverts, suggesting that fish passage may have been possible through the culverts, or that there were  
325 resident populations upstream of the culverts.

326 All environmental samples were quantified for absolute concentrations of cutthroat trout DNA across 32  
327 qPCR plates, resulting in ~630 samples (~60%) with a positive detection in at least 1 of 3 technical replicates.  
328 The modeled output of cutthroat trout DNA concentrations, ranged from 50 copies/L to  $1.4 \times 10^6$  copies/L,  
329 with a mean value of ~47,000 copies/L (Figure 6).

330 We combined compositional information from metabarcoding with absolute concentrations from qPCR for  
331 our reference species, cutthroat trout (*O. clarkii*), to estimate the total concentration of DNA for each species  
332 (See Supplemental Text 2). These quantitative data for all four target species were then used in the linear  
333 mixed-effects model to assess salmonid trends over time (Figure 7).

### 334 Effects of Culverts

335 Before considering the effect of construction, the difference in trends between upstream and downstream  
336 stations (Figure 7) demonstrates that the culverts themselves have some effect, but generally not a large  
337 effect on the salmonid species surveyed. A notable exception was Barnes Creek, as the culvert was so clearly  
338 a barrier as most time points had no salmonid DNA upstream. Padden Creek upstream of I-5 also was more  
339 clearly a barrier to fish passage, while the culvert across SR-11 seemed to be less of a barrier to fish passage.  
340 In other cases, salmonid DNA is found upstream but not downstream, indicating that the culvert is likely not  
341 a barrier and there are resident individuals upstream of the culvert.

342 Summarizing over all species and the four creeks used in the time series model, the culvert effect was minimal  
343 (Figure 8); the average log-fold change between upstream and downstream sites was not significantly different  
344 from zero. Individual species' patterns were similar, indicating that there is not a species-specific effect where  
345 culverts block the passage of some salmon but not others (Figure S1.12). The maximum positive log-fold  
346 change (i.e., upstream having a higher mass flow rate) was 2.78 in Squalicum Creek for coho salmon (*O.*

347 *kisutch*) in August 2021, while the maximum negative log-fold change (i.e., downstream having a higher mass  
348 flow rate) was -1.11 found in Squalicum Creek for cutthroat trout (*O. clarkii*) in December 2021 (Figure  
349 S1.12). Of all species, creeks, and time points, 23 of the 151 observations were within a log-fold change of -0.1  
350 to 0.1, which corresponds with eDNA mass flow rates upstream within 10% of mass flow rates downstream.

351 We also considered how the log-fold change in eDNA mass flow rates were impacted by the flow rate or  
352 discharge of the creek itself (Figure 9. We found that at months of the lowest flow (summer months), the  
353 log-fold changes between mass flow rates were the highest, while in winter months with highest discharge the  
354 log-fold changes were lower and downstream sites often had higher eDNA mass flow rates than upstream  
355 sites (Figure S1.12).

### 356 Effects of Culvert Replacement

357 By comparing the difference in upstream and downstream mass flow rates before and after construction in  
358 Padden Creek, we can assess how large of an impact the two culvert replacements had on salmonid species  
359 (Figure 10). The effects of the culvert replacement operations appeared to have been transient and fairly  
360 minor for the four salmonid species surveyed. We saw very minor fluctuations in the difference between  
361 upstream and downstream salmonid DNA mass flow rates, and did not see an increase in this difference due  
362 to the culvert removal as the log-fold changes in Padden Creek were similar to those in the control creeks at  
363 the same time points (Figure 10, grey points vs. black points in areas of yellow shading).

## 364 Discussion

### 365 Environmental DNA can provide quantitative measurements of environmental impacts

366 Here, we used both eDNA metabarcoding and a single species-specific qPCR assay to rigorously quantify  
367 both the effect of culverts and the impact of two culvert replacements on salmonids in the same creek. We  
368 observed a clear seasonal pattern in the DNA concentrations of four salmonid species detected in the study.  
369 The sampling design and the linear mixed effects model leveraged information across treatment and control  
370 creeks to integrate the change in eDNA concentrations due to time, whether a sample was collected below or  
371 above a barrier (i.e., culvert), and whether or not there was construction occurring. Thus, we could isolate  
372 the changes in eDNA concentrations as a result of the intervention (i.e., construction) while accounting for  
373 the variance due to time and station (i.e., season and culvert).

374 A few other studies have used eDNA to measure environmental impacts in rivers and streams. Duda et al.  
375 (2021) used 11 species-specific qPCR assays to document the distribution of resident and migratory fish

376 after a large dam removal project (Elwha River near Port Angeles, Washington). No eDNA sampling was  
377 conducted before the dam removal, but the study provided a wealth of information about species returning  
378 after the dam removal, providing a very important dataset to use eDNA to monitor ecological changes due to  
379 human intervention. Similarly, Muha et al. (2017) sampled three locations upstream and three locations  
380 downstream before and after the removal of a weir that was thought to be a barrier to salmonid migrations.  
381 The authors only sampled once before and twice after the removal, spanning about a year, and used eDNA  
382 metabarcoding to look at the presence/absence of species detected. They found that in fact the before sample  
383 demonstrated that the weir was not preventing fish passage (similar to the results found in this study) and  
384 furthermore documented a slight increase in alpha diversity in the first time point after the barrier removal  
385 and then a return to a similar alpha diversity in the second time point after the removal (similar results found  
386 in this study using eDNA concentrations rather than diversity). Finally Yamanaka and Minamoto (2016)  
387 sampled along a river with three barriers, finding some fish able to cross barriers and some not, suggesting  
388 that the eDNA can indicated habitat connectivity for fishes across barriers.

389 Importantly, our study demonstrates the value of combining a single qPCR assay with metabarcoding data  
390 to generate quantitative estimates of eDNA concentrations of many species without requiring  $n$  qPCR assays  
391 for  $n$  species of interest. Here, we ultimately only quantified the impacts of four species, but importantly, we  
392 did not know *a priori* how many species of interest there might be and we reduced our efforts two fold by  
393 only conducting two assays (one species-specific qPCR and one metabarcoding assay) as opposed to four  
394 assays (four species-specific qPCR assays). This can also be particularly helpful for taxa that don't have a  
395 previously published qPCR assay, but are detected using universal metabarcoding assays. Metabarcoding  
396 data alone only gives compositional data, which cannot be used in a time series to quantify environmental  
397 impacts because there is no information about absolute eDNA concentrations. However, by anchoring or  
398 grounding proportions using a single qPCR assay, the proportional data can be turned into quantitative data.  
399 The species for which to run the qPCR assay can be determined after the metabarcoding is completed; the  
400 most commonly found species with a robust qPCR assay should be used to glean the most information.

#### 401 **Fish life histories and expected patterns**

402 The four salmonid species in this study have different life histories and behaviors that would impact when  
403 fish (and therefore eDNA concentrations) occur in the creeks. Three of the four species in this study have  
404 both freshwater and anadromous populations. Cutthroat trout (*O. clarkii*) encompasses both non-migrating,  
405 resident trout in the creeks and coastal run cutthroat that migrate into Padden Creek from saltwater  
406 (Bellingham Bay). Similarly, *O. nerka* includes both anadromous sockeye salmon and freshwater resident

407 kokanee salmon and *O. mykiss* includes both anadromous steelhead trout and non-migrating rainbow trout.  
408 Using eDNA, we cannot distinguish between the migrating and non-migrating subspecies of *O. clarkii*, *O.*  
409 *nerka*, and *O. mykiss*. Therefore, our eDNA concentrations might reflect contributions from both migrating  
410 and non-migrating individuals at any given time point in the dataset.

411 For these four anadromous salmonids, the run timings for the migrating populations vary for each species in  
412 the study area (Bellingham, WA). Adult coastal cutthroat (*O. clarkii*) are documented to run throughout the  
413 entire year, whereas coho salmon (*O. kisutch*) run from September to December, sockeye salmon (*O. nerka*)  
414 run from October to December, and steelhead trout (*O. mykiss*) run from November to June. For migrating  
415 coho (*O. kisutch*) and steelhead trout (*O. mykiss*), juveniles may be present in the creeks year-round (Figure  
416 S1.4). eDNA methods at present cannot distinguish adults versus juveniles from DNA found in a water  
417 sample.

418 Despite the mix of migrating and non-migrating populations and various run timings, our metabarcoding  
419 data demonstrate that in Padden Creek, there was a clear signal of sockeye/kokanee salmon (*O. nerka*)  
420 both upstream and downstream only in November 2021 - February 2022 and again in December 2022.  
421 This signal corresponds well with the documented run timing of October to December and the presence of  
422 out-migrating juveniles in early spring. In contrast, cutthroat trout (*O. clarkii*) and coho salmon (*O. kisutch*)  
423 were found nearly year-round in Padden Creek. The persistent signal from *O. clarkii* could be explained  
424 by resident cutthroat trout. However, *O. kisutch* does not have a resident subspecies and the run timing is  
425 only documented from September to December. This could potentially be due to juveniles maturing and  
426 residing in the creeks for 1-2 years after hatching while adults migrate into the creeks only during the run  
427 time to spawn. Visual surveys (e.g., snorkel surveys, electrofishing, smolt traps) are conducted infrequently  
428 to determine adult and juvenile salmonid abundances. Though *O. kisutch* eDNA was found year round, the  
429 highest concentrations were found near the expected run timing and the life history of *O. kisutch* includes  
430 rearing year-round in freshwater. Finally, though the lowest concentrations on average, rainbow/steelhead  
431 trout (*O. mykiss*) was also found nearly year-round in Padden Creek, which could be contributions from  
432 migrating steelhead (November to June), juveniles maturing and migrating, or from resident rainbow trout.  
433 Though the *O. mykiss* signal is found year-round, the highest concentrations do seem to correspond with the  
434 steelhead run timing.

#### 435 **Interpreting eDNA with respect to fish abundance and flow**

436 By capturing residual eDNA from water samples, we are measuring a different signal than counting how many  
437 fish are in the creek at each time of sampling. We should not expect the eDNA concentration to directly

438 correlate to the number of fish in the creek at the time of sampling. Shelton et al. (2019) used a paired eDNA  
439 sampling and seine netting analysis to demonstrate that eDNA concentrations provide a smoothed biological  
440 signal over space and time. We acknowledge this smoothing effect and emphasize that in the context of using  
441 eDNA for environmental impact assessments, it is preferable to use a survey technique such as eDNA that  
442 integrates signal across a larger spatial and temporal scale.

443 Many previous papers have commented on the “ecology” of eDNA and the various processes that contribute  
444 to eDNA concentrations in environmental samples (e.g., shedding rates, decay rates, transport) (Barnes  
445 and Turner 2015). For example, higher concentrations of eDNA could be the result of a greater number (or  
446 biomass) of fish present, or increased shedding rates, or decreased decay. Many review papers document  
447 the nuances of interpreting eDNA data and we recommend reviewing them for a deeper understanding (see  
448 Andruszkiewicz Allan et al. (2020) for a review on shedding and decay rates and Harrison et al. (2019) for a  
449 review on transport). Other studies have also documented the relative importance of eDNA transport in  
450 streams. Most notably, Tillotson et al. (2018) measured eDNA at four sites with similar discharge rates  
451 to the creeks in this study and specifically addressed spatial and temporal resolutions, finding that eDNA  
452 concentrations reflect short time- (and therefore length-) scales by comparing peaks in eDNA concentrations  
453 to counts of salmon and accumulation by measuring both upstream and downstream sites. The authors found  
454 that the sampling site furthest downstream did not accumulate eDNA and that two tributaries feeding into a  
455 main channel were additive (Tillotson et al. 2018). For more general models and empirical data documenting  
456 transport distances in streams, see Wilcox et al. (2016), Jane et al. (2014), Jerde et al. (2016), Shogren et  
457 al. (2016), and Civade et al. (2016). Certainly eDNA concentrations can arise from different scenarios and  
458 future work should continue to investigate how to tease apart the nuances of relating eDNA concentrations  
459 to fish abundance.

460 In this study, to assess the impact of a culvert on fish passage, we compare eDNA concentrations upstream  
461 and downstream at the same time point in a given creek. The distance between the upstream and downstream  
462 sampling was minimal (~60-300 m, average distance of ~150 m). Therefore, we assume that the small  
463 differences in spatial and temporal scale between sampling locations is minimal such that the impacts of these  
464 various processes will affect the downstream and upstream concentrations equally. That is, in the upstream  
465 station, some amount of eDNA is coming from upstream of that location into the sampling station and leaving  
466 at the same time – in the same way that eDNA would be both entering and exiting the downstream station.  
467 Additionally, at almost every single time point for all creeks and species, the upstream DNA concentration is  
468 higher than the downstream DNA concentration. Based on that alone, we do not expect that downstream  
469 accumulation of salmonid DNA is occurring to bias our results of whether fish can pass through these culverts.

470 **Not all culverts are barriers to salmonids**

471 By measuring DNA concentrations of salmonid species above and below culverts on a small spatial scale, we  
472 were able to determine how much of a barrier each culvert was to fish passage. Barnes Creek was clearly a  
473 very large barrier to fish passage as we only found salmonid DNA in three months of the twelve months of  
474 sampling, and those three months had very low concentration of salmonid DNA relative to the other creeks.  
475 Within the treatment creek (Padden Creek), the SR-11 culvert did not seem to be a large barrier, while the  
476 I-5 culvert clearly was a barrier, demonstrated by the difference in salmonid composition and eDNA mass  
477 flow rates over the course of sampling.

478 Here, we find instances where culverts designated as barriers were likely not blocking fish passage, while  
479 others (Padden I-5 and Barnes Creek) were barriers to fish passage. Importantly, this demonstrates that  
480 collecting water samples for eDNA analysis might help to prioritize restoration of culverts suspected to be  
481 barriers to salmonids and provide a new method for post-restoration monitoring to confirm that the barrier  
482 has been corrected and allows for fish passage. Given the large amount of spending and effort required to  
483 replace culverts, this finding is important and emphasizes the potential for new tools for environmental  
484 impact assessments. We note that our sampling occurred only over a short temporal scale and future work  
485 could monitor culverts for longer time periods, different species, and different environmental conditions.

486 **Salmonids can quickly recover from a short-term intervention in a creek**

487 The construction had remarkably minimal effects on salmonid DNA concentrations. The disruption of  
488 disconnecting the creek, demolition of the old culvert, installation of the new culvert, and the reconnecting  
489 of the creek during both culvert replacement events showed almost no change in the difference in eDNA  
490 concentrations between downstream and upstream sampling sites. The differences in the control creeks between  
491 upstream and downstream were often higher than the treatment creek. The post-construction sampling point  
492 of the I-5 culvert replacement (only one time point), does show that the composition of salmonid DNA after  
493 replacement is now very similar to the two downstream stations, whereas before construction compositions  
494 were very different (because the culvert was a barrier). However, we lack the quantitative analysis as the site  
495 upstream of SR-11 and downstream of I-5 had no quantifiable cutthroat DNA. More time points would help  
496 demonstrate the effect of the culvert replacement. Here we found that one culvert had very minimal effect on  
497 salmonid passage while the other culvert had a large effect on salmonid passage. We note that these findings  
498 are likely not universal and certainly projects need to monitor comprehensively and quantitatively in order to  
499 assess the passability of culverts and impacts of construction.

## 500 Conclusion

501 It is notoriously difficult to quantify the environmental impact of discrete human impacts on ecosystems  
502 and species. Surveying species and communities by eDNA provides an opportunity for monitoring before,  
503 during, and after impacts in a scaleable and cost-effective way. Here, we demonstrate that monthly eDNA  
504 sampling before, during, and after an intervention alongside control sites can quantify the environmental  
505 impact of replacing a culvert. We found that in our treatment creek and control sites, four of the six barriers  
506 did not prohibit salmonid passage. We found that of the two culvert replacements in the treatment creek,  
507 one was a barrier and one was not, but both had minimal impacts on the four salmonid species monitored  
508 over the course of construction. We also provide a framework in which compositional metabarcoding data  
509 can be linked with qPCR data to obtain quantitative estimates of eDNA concentrations of many species.  
510 This provides a practical way to utilize the large amount of information from metabarcoding data without  
511 needing a unique qPCR assay for every species of interest. Environmental DNA is moving into practice and  
512 this study demonstrates how eDNA can be broadly used for environmental impact assessments for a wide  
513 range of species and environments.

## 514 Conflict of Interest Statement

515 The authors declare there are no conflicts of interest.

## 516 Acknowledgements

517 This work was made possible by a grant from OceanKind to Ryan Kelly. The funders had no role in study  
518 design, data collection and analysis, decision to publish, or preparation of the manuscript. Figure 3 was  
519 created with BioRender.com. We thank Tammy Schmidt and Susan Kanzler from Washington Department  
520 of Transportation for facilitating access to field sites and providing helpful feedback throughout the project.  
521 We also thank Jenna McLaughlin, Joe Duprey and Ally Im for help field sampling and Dr. Ramon Gallego,  
522 Dr. Kim Parsons, and the University of Washington's Northwest Genomics Center for sequencing support.  
523 Dr. Braeden Van Deynze, Dr. Sunny Jardine, and Dr. Julian Olden provided helpful insight into culverts and  
524 salmonid life histories. We thank Katherine Pearson Maslenikov at the Burke Museum of Natural History  
525 and Culture for providing voucher specimens for Sanger sequencing. Thanks to Dr. Jameal Samhouri and  
526 Dr. Chris Sergeant for reviewing the manuscript.

## 527 References

- 528 Andruszkiewicz Allan, E., W. G. Zhang, A. Lavery, and A. Govindarajan. 2020. Environmental DNA  
529 shedding and decay rates from diverse animal forms and thermal regimes. *Environmental DNA*:edn3.141.
- 530 Barnes, M. A., and C. R. Turner. 2015. The ecology of environmental DNA and implications for conservation  
531 genetics. *Conservation Genetics* 17:117.
- 532 Benedetti-Cecchi, L. 2001. Beyond Baci: Optimization of Environmental Sampling Designs Through  
533 Monitoring and Simulation. *Ecological Applications* 11:783–799.
- 534 Buxton, A., E. Matechou, J. Griffin, A. Diana, and R. A. Griffiths. 2021. Optimising sampling and analysis  
535 protocols in environmental DNA studies. *Scientific Reports* 11:11637.
- 536 Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2:  
537 High resolution sample inference from illumina amplicon data. *Nature methods* 13:581–583.
- 538 Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009.  
539 BLAST+: Architecture and applications. *BMC Bioinformatics* 10:4219.
- 540 City of Bellingham. 2015. Urban spawner surveys.
- 541 Civade, R. I., T. Dejean, A. Valentini, N. Roset, J.-C. Raymond, A. Bonin, P. Taberlet, and D. Pont. 2016.  
542 Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in  
543 a natural freshwater system. *PLOS ONE* 11:e015736619.
- 544 De Vargas, C., S. Audie, N. Henry, J. Decelle, F. Mahe, R. Logares, E. Lara, C. Berney, N. Le Bescot, I.  
545 Probert, M. Carmichael, J. Poulain, S. Romac, S. Colin, J.-M. Aury, L. Bittner, S. Chaffron, M. Dunthorn,  
546 S. Engelen, O. Flegontova, L. Guidi, A. Horak, O. Jaillon, G. Lima-Mendez, J. Lukes, S. Malviya, R.  
547 Morard, M. Mulot, E. Scalco, R. Siano, F. Vincent, A. Zingone, C. Dimier, M. Picheral, S. Searson, S.  
548 Kandels-Lewis, T. O. coordinators, S. G. Acinas, P. Bork, C. Bowler, G. Gorsky, N. Grimsley, P. Hingamp,  
549 D. Iudicone, F. Not, H. Ogata, S. Pesant, J. Raes, M. E. Sieracki, S. Speich, L. Stemmann, S. Sunagawa,  
550 J. Weissenbach, P. Wincker, and E. Karsenti. 2015. Eukaryotic plankton diversity in the sunlit ocean.  
551 *Science* 348:112.
- 552 Duda, J. J., M. S. Hoy, D. M. Chase, G. R. Pess, S. J. Brenkman, M. M. McHenry, and C. O. Ostberg. 2021.  
553 Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam  
554 removal. *Environmental DNA* 3:121–141.
- 555 Frankiewicz, P., A. Radecki-Pawlik, A. Wałęga, M. Łapińska, and A. Wojtal-Frankiewicz. 2021. Small  
556 hydraulic structures, big environmental problems: is it possible to mitigate the negative impacts of culverts  
557 on stream biota? *Environmental Reviews* 29:510–528.
- 558 Gloor, G. B., J. M. Macklaim, M. Vu, and A. D. Fernandes. 2016. Compositional uncertainty should not be

- 559 ignored in high-throughput sequencing data analysis. *Austrian Journal of Statistics* 45:73–87.
- 560 Gold, Z., A. O. Shelton, H. R. Casendino, J. Duprey, R. Gallego, A. V. Cise, M. Fisher, A. J. Jensen, E.  
561 D’Agnese, E. A. Allan, A. Ramón-Laca, M. Garber-Yonts, M. Labare, K. M. Parsons, and R. P. Kelly.  
562 2023. Signal and noise in metabarcoding data. *PLOS ONE* 18:e0285674.
- 563 Harrison, J. B., J. M. Sunday, and S. M. Rogers. 2019. Predicting the fate of eDNA in the environment  
564 and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*  
565 286:20191409.
- 566 Hoshino, T., R. Nakao, H. Doi, and T. Minamoto. 2021. Simultaneous absolute quantification and sequencing  
567 of fish environmental DNA in a mesocosm by quantitative sequencing technique. *Scientific Reports*  
568 11:4372.
- 569 Jane, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. H. Lowe, B. H. Letcher, and  
570 A. R. Whiteley. 2014. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams.  
571 *Molecular Ecology Resources* 15:216227.
- 572 Jerde, C. L., B. P. Olds, A. J. Shogren, E. A. Andruszkiewicz, A. R. Mahon, D. Bolster, and J. L. Tank.  
573 2016. Influence of stream bottom substrate on retention and transport of vertebrate environmental DNA.  
574 *Environmental Science & Technology* 50:87708779.
- 575 Kelly, R. P., C. J. Closek, J. L. O’Donnell, J. E. Kralj, A. O. Shelton, and J. F. Samhouri. 2017. Genetic and  
576 manual survey methods yield different and complementary views of an ecosystem. *Frontiers in Marine  
577 Science* 3:73511.
- 578 Kelly, R. P., J. A. Port, K. M. Yamahara, and L. B. Crowder. 2014. Using environmental DNA to census  
579 marine fishes in a large mesocosm. *PLOS ONE* 9:e8617511.
- 580 Kelly, R. P., A. O. Shelton, and R. Gallego. 2019. Understanding PCR Processes to Draw Meaningful  
581 Conclusions from Environmental DNA Studies. *Scientific Reports* 9:12133.
- 582 Klein, S. G., N. R. Gerald, A. Anton, S. Schmidt-Roach, M. Ziegler, M. J. Cziesielski, C. Martin, N. Rädecker,  
583 T. L. Frölicher, P. J. Mumby, J. M. Pandolfi, D. J. Suggett, C. R. Voolstra, M. Aranda, and Carlos. M.  
584 Duarte. 2022. Projecting coral responses to intensifying marine heatwaves under ocean acidification.  
585 *Global Change Biology* 28:1753–1765.
- 586 Lackey, R. 2003. Pacific Northwest Salmon: Forecasting Their Status in 2100. *Reviews in Fisheries Science*  
587 11:35–88.
- 588 Leray, M., J. Y. Yang, C. P. Meyer, S. C. Mills, N. Agudelo, V. Ranwez, J. T. Boehm, and R. J. Machida. 2013.  
589 A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding  
590 metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology* 10:114.
- 591 Long, J. W., and F. K. Lake. 2018. Escaping social-ecological traps through tribal stewardship on national

- 592 forest lands in the pacific northwest, united states of america. *Ecology and Society* 23.
- 593 Maasri, A., S. C. Jähnig, M. C. Adamescu, R. Adrian, C. Baigun, D. J. Baird, A. Batista-Morales, N. Bonada,  
594 L. E. Brown, Q. Cai, J. V. Campos-Silva, V. Clausnitzer, T. Contreras-MacBeath, S. J. Cooke, T. Datry,  
595 G. Delacámarra, L. De Meester, K.-D. B. Dijkstra, V. T. Do, S. Domisch, D. Dudgeon, T. Erös, H. Freitag,  
596 J. Freyhof, J. Friedrich, M. Friedrichs-Manthey, J. Geist, M. O. Gessner, P. Goethals, M. Gollock, C.  
597 Gordon, H.-P. Grossart, G. Gulemvuga, P. E. Gutiérrez-Fonseca, P. Haase, D. Hering, H. J. Hahn, C. P.  
598 Hawkins, F. He, J. Heino, V. Hermoso, Z. Hogan, F. Höller, J. M. Jeschke, M. Jiang, R. K. Johnson, G.  
599 Kalinkat, B. K. Karimov, A. Kasangaki, I. A. Kimirei, B. Kohlmann, M. Kuemmerlen, J. J. Kuiper, B.  
600 Kupilas, S. D. Langhans, R. Lansdown, F. Leese, F. S. Magbanua, S. S. Matsuzaki, M. T. Monaghan,  
601 L. Mumladze, J. Muzon, P. A. Mvogo Ndongo, J. C. Nejstgaard, O. Nikitina, C. Ochs, O. N. Odume,  
602 J. J. Opperman, H. Patricio, S. U. Pauls, R. Raghavan, A. Ramírez, B. Rashni, V. Ross-Gillespie, M.  
603 J. Samways, R. B. Schäfer, A. Schmidt-Kloiber, O. Seehausen, D. N. Shah, S. Sharma, J. Soininen, N.  
604 Sommerwerk, J. D. Stockwell, F. Suhling, R. D. Tachamo Shah, R. E. Tharme, J. H. Thorp, D. Tickner,  
605 K. Tockner, J. D. Tonkin, M. Valle, J. Vitule, M. Volk, D. Wang, C. Wolter, and S. Worischka. 2022. A  
606 global agenda for advancing freshwater biodiversity research. *Ecology Letters* 25:255–263.
- 607 MacPherson, L. M., M. G. Sullivan, A. Lee Foote, and C. E. Stevens. 2012. Effects of Culverts on Stream  
608 Fish Assemblages in the Alberta Foothills. *North American Journal of Fisheries Management* 32:480–490.
- 609 Martin, C. J. B., B. J. Allen, and C. G. Lowe. 2012. Environmental impact assessment: Detecting changes in  
610 fish community structure in response to disturbance with an asymmetric multivariate BACI sampling  
611 design. *Bulletin, Southern California Academy of Sciences* 111:119–131.
- 612 Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMB-*  
613 *net.journal* 17:10.
- 614 Martinez, R. 2013. United states v. washington.
- 615 McCall, M. N., H. R. McMurray, H. Land, and A. Almudevar. 2014. On non-detects in qPCR data.  
616 *Bioinformatics* 30:2310–2316.
- 617 McLaren, M. R., A. D. Willis, and B. J. Callahan. 2019. Consistent and correctable bias in metagenomic  
618 sequencing experiments. *eLife* 8:e46923.
- 619 Morgan, R. K. 2012. Environmental impact assessment: The state of the art. *Impact Assessment and Project*  
620 *Appraisal* 30:5–14.
- 621 Moss, W. E., L. R. Harper, M. A. Davis, C. S. Goldberg, M. M. Smith, and P. T. J. Johnson. 2022.  
622 Navigating the trade-offs between environmental DNA and conventional field surveys for improved  
623 amphibian monitoring. *Ecosphere* 13:e3941.
- 624 Muha, T. P., M. Rodríguez-Rey, M. Rolla, and E. Tricarico. 2017. Using environmental DNA to improve

- 625 species distribution models for freshwater invaders. *Frontiers in Ecology and Evolution* 5:143957.
- 626 Nathan, L. R., A. A. Smith, A. B. Welsh, and J. C. Vokoun. 2018. Are culvert assessment scores an indicator  
627 of Brook Trout *Salvelinus fontinalis* population fragmentation? *Ecological Indicators* 84:208–217.
- 628 Ogram, A., G. Sayler, and T. Barkay. 1987. The extraction and purification of microbial DNA from sediments.  
629 *Journal of Microbiological Methods* 7:5766.
- 630 Ogren, S. A., and C. J. Huckins. 2015. Culvert replacements: improvement of stream biotic integrity?  
631 *Restoration Ecology* 23:821–828.
- 632 Pont, D., P. Meulenbroek, V. Bammer, T. Dejean, T. Erős, P. Jean, M. Lenhardt, C. Nagel, L. Pekarik,  
633 M. Schabuss, B. C. Stoeckle, E. Stoica, H. Zornig, A. Weigand, and A. Valentini. 2022. Quantitative  
634 monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR.  
635 *Molecular Ecology Resources* n/a.
- 636 Port, J. A., J. L. O'Donnell, O. C. Romero-Maraccini, P. R. Leary, S. Y. Litvin, K. J. Nickols, K. M.  
637 Yamahara, and R. P. Kelly. 2015. Assessing vertebrate biodiversity in a kelp forest ecosystem using  
638 environmental DNA. *Molecular Ecology* 25:527541.
- 639 Price, D. M., T. Quinn, and R. J. Barnard. 2010. Fish Passage Effectiveness of Recently Constructed Road  
640 Crossing Culverts in the Puget Sound Region of Washington State. *North American Journal of Fisheries  
641 Management* 30:1110–1125.
- 642 R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical  
643 Computing.
- 644 Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B.  
645 A. Lynch, I. A. MacNeil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman, and  
646 R. M. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional  
647 diversity of uncultured microorganisms. *Applied and Environmental Microbiology* 66:2541–2547.
- 648 Rubin, Z., G. M. Kondolf, and B. Rios-Touma. 2017. Evaluating Stream Restoration Projects: What Do We  
649 Learn from Monitoring? *Water* 9:174.
- 650 Ruppert, K. M., R. J. Kline, and M. S. Rahman. 2019. Past, present, and future perspectives of environmental  
651 DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global  
652 eDNA. *Global Ecology and Conservation* 17:e00547.
- 653 Schmidhauser, J. R. 1976. Struggles for cultural survival: The fishing rights of the treaty tribes of the Pacific  
654 Northwest. *Notre Dame Law* 52:30–40.
- 655 Seymour, M., F. K. Edwards, B. J. Cosby, I. Bista, P. M. Scarlett, F. L. Brailsford, H. C. Glanville, M. de  
656 Bruyn, G. R. Carvalho, and S. Creer. 2021. Environmental DNA provides higher resolution assessment of  
657 riverine biodiversity and ecosystem function via spatio-temporal nestedness and turnover partitioning.

- 658      Communications Biology 4:1–12.
- 659    Shelton, A. O., Z. J. Gold, A. J. Jensen, E. D’Agnese, E. Andruszkiewicz Allan, A. Van Cise, R. Gallego, A.  
660    Ramón-Laca, M. Garber-Yonts, K. Parsons, and R. P. Kelly. 2022. Toward quantitative metabarcoding.  
661    Ecology n/a:e3906.
- 662    Shelton, A. O., R. P. Kelly, J. L. O’Donnell, L. Park, P. Schwenke, C. Greene, R. A. Henderson, and E.  
663    M. Beamer. 2019. Environmental DNA provides quantitative estimates of a threatened salmon species.  
664    Biological Conservation 237:383–391.
- 665    Shelton, A. O., J. L. O’Donnell, J. F. Samhouri, N. C. Lowell, G. D. Williams, and R. P. Kelly. 2016. A  
666    framework for inferring biological communities from environmental DNA:115.
- 667    Shogren, A. J., J. L. Tank, E. A. Andruszkiewicz, B. P. Olds, C. L. Jerde, and D. Bolster. 2016. Modelling  
668    the transport of environmental DNA through a porous substrate using continuous flow-through column  
669    experiments. Journal of The Royal Society Interface 13:2016029011.
- 670    Silverman, J. D., R. J. Bloom, S. Jiang, H. K. Durand, E. Dallow, S. Mukherjee, and L. A. David. 2021.  
671    Measuring and mitigating PCR bias in microbiota datasets. PLoS Computational Biology 17:e1009113.
- 672    Stan Development Team. 2022. RStan: The r interface to stan.
- 673    Stat, M., M. J. Huggett, R. Bernasconi, J. D. DiBattista, T. E. Berry, S. J. Newman, E. S. Harvey, and M.  
674    Bunce. 2017. Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical  
675    marine environment. Scientific Reports:111.
- 676    Taberlet, P., E. Coissac, M. Hajibabaei, and L. H. Rieseberg. 2012. Environmental DNA. Molecular Ecology  
677    21:17891793.
- 678    Thalinger, B., E. Wolf, M. Traugott, and J. Wanzenböck. 2019. Monitoring spawning migrations of  
679    potamodromous fish species via eDNA. Scientific Reports 9:15388.
- 680    Thomas, A. C., J. Howard, P. L. Nguyen, T. A. Seimon, and C. S. Goldberg. 2018. ANDe <sup>TM</sup>: A fully  
681    integrated environmental DNA sampling system. Methods in Ecology and Evolution 9:13791385.
- 682    Thomas, A. C., P. L. Nguyen, J. Howard, and C. S. Goldberg. 2019. A self-preserving, partially biodegradable  
683    eDNA filter. Methods in Ecology and Evolution 10:1136–1141.
- 684    Thomsen, P. F., and E. Willerslev. 2015. Environmental DNA: An emerging tool in conservation for  
685    monitoring past and present biodiversity. Biological Conservation 183:418.
- 686    Tillotson, M. D., R. P. Kelly, J. J. Duda, M. Hoy, J. Kralj, and T. P. Quinn. 2018. Concentrations of  
687    environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales.  
688    Biological Conservation 220:111.
- 689    Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon. 2007. The  
690    Human Microbiome Project. Nature 449:804–810.

- 691 U. S. Geological Survey. 1994. USGS water data for the nation. Retrieved from [<https://waterdata.usgs.gov/nwis>](https://waterdata.usgs.gov/nwis)
- 692 <11/30/2022>.
- 693 Underwood, A. J. 1992. Beyond BACI: the detection of environmental impacts on populations in the real,
- 694 but variable, world. *Journal of Experimental Marine Biology and Ecology* 161:145–178.
- 695 Underwood, A. J. 1994. On Beyond BACI: Sampling Designs that Might Reliably Detect Environmental
- 696 Disturbances. *Ecological Applications* 4:3–15.
- 697 Valentini, A., P. Taberlet, C. Miaud, R. I. Civade, J. E. Herder, P. F. Thomsen, E. Bellemain, A. Besnard,
- 698 E. Coissac, F. Boyer, C. Gaboriaud, P. Jean, N. Poulet, N. Roset, G. H. Copp, P. Geniez, D. Pont, C.
- 699 Argillier, J.-M. Baudoin, T. Peroux, A. J. Crivelli, A. Olivier, M. Acqueberge, M. Le Brun, P. R. Moller, E.
- 700 Willerslev, and T. Dejean. 2016. Next-generation monitoring of aquatic biodiversity using environmental
- 701 DNA metabarcoding. *Molecular Ecology* 25:929942.
- 702 Washington Department of Fish and Wildlife. 2019. Fish passage inventory, assessment, and prioritization
- 703 manual.
- 704 Wellman, J., D. Combs, and S. B. Cook. 2000. Long-Term Impacts of Bridge and Culvert Construction
- 705 or Replacement on Fish Communities and Sediment Characteristics of Streams. *Journal of Freshwater*
- 706 *Ecology* 15:317–328.
- 707 Wilcox, T. M., K. S. McKelvey, M. K. Young, A. J. Sepulveda, B. B. Shepard, S. F. Jane, A. R. Whiteley, W.
- 708 H. Lowe, and M. K. Schwartz. 2016. Understanding environmental DNA detection probabilities: A case
- 709 study using a stream-dwelling char *salvelinus fontinalis*. *Biological Conservation* 194:209216.
- 710 Wilkinson, S. P., S. K. Davy, M. Bunce, and M. Stat. 2018. Taxonomic identification of environmental DNA
- 711 with informatic sequence classification trees.
- 712 Wood, D. M., A. B. Welsh, and J. Todd Petty. 2018. Genetic Assignment of Brook Trout Reveals Rapid
- 713 Success of Culvert Restoration in Headwater Streams. *North American Journal of Fisheries Management*
- 714 38:991–1003.
- 715 Yamanaka, H., and T. Minamoto. 2016. The use of environmental DNA of fishes as an efficient method of
- 716 determining habitat connectivity. *Ecological Indicators* 62:147153.

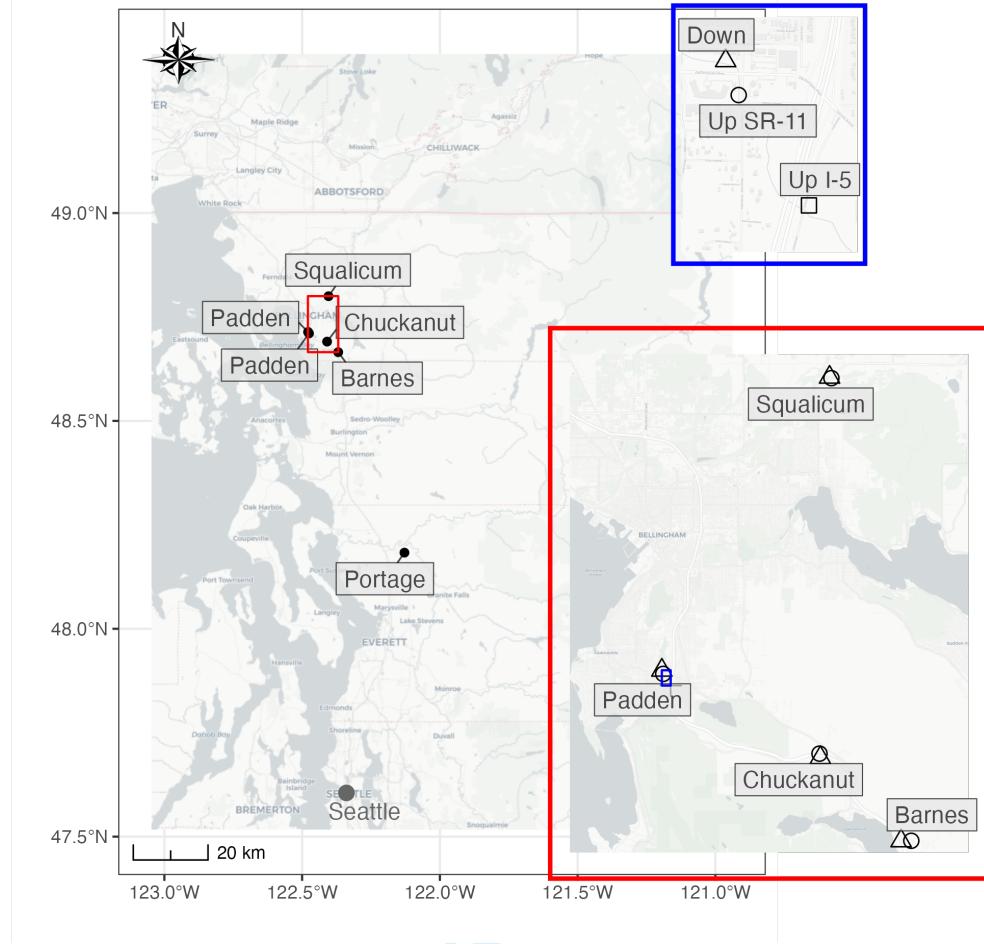


Figure 1: Map of sampling locations near Bellingham, Washington. In the red subset, triangles designate the downstream sampling location and circles designate the upstream sampling location. Padden Creek (the treatment creek where the two culverts were replaced) is shown in the blue subset where downstream is a triangle, upstream of the first culvert (SR-11) is a circle, and upstream of the second culvert (I-5) is a square.

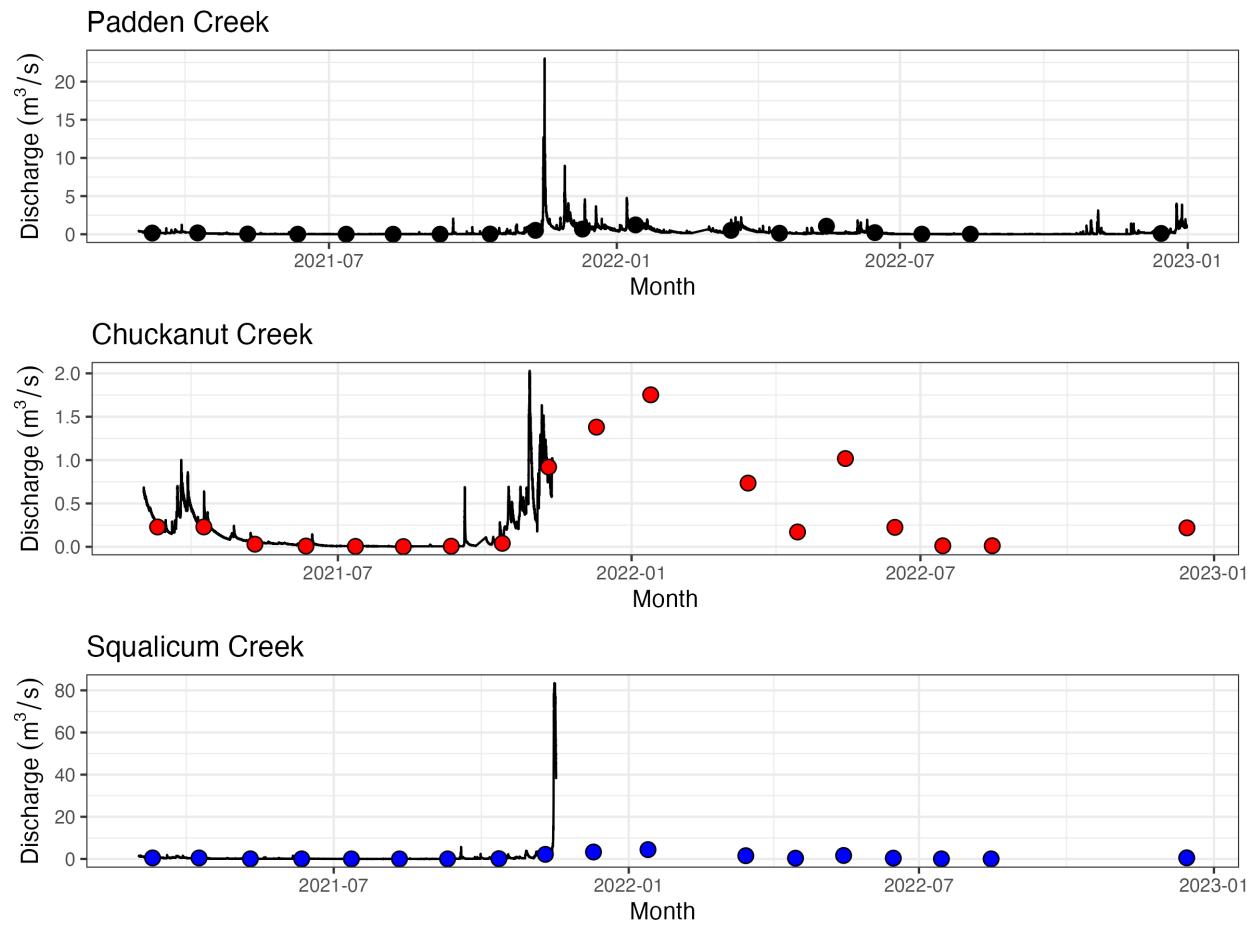


Figure 2: Discharge ( $\text{m}^3/\text{s}$ ) in Padden (USGS Gauge 12201905), Chuckanut (USGS Gauge 12201700), and Squalicum (USGS Gauge 12204010) Creeks over the course of sampling. Gauges at Chuckanut and Squalicum Creek went offline in November 2021 after a major storm event. Portage Creek and Barnes Creek did not have stream gauges. Circles designate the day of sampling. For Padden Creek, the nearest 15 minute interval of flow was used. For Chuckanut and Squalicum Creeks, the correction factor from five years of historical data from Padden Creek was used (see methods section and Supplemental Figures 2 and 3).

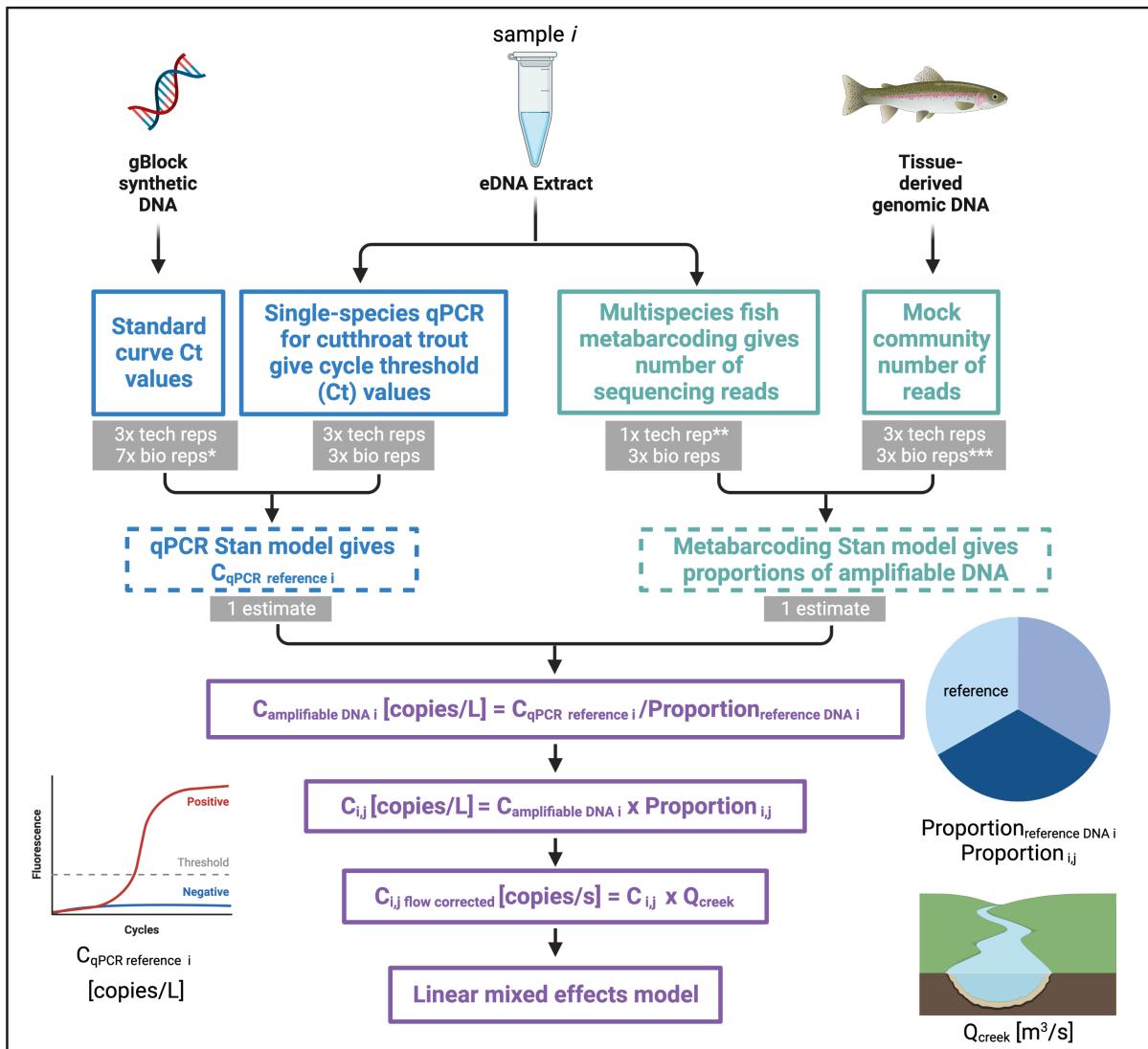


Figure 3: Conceptual figure of different datasets and models used for analyses. \* indicates that here, biological replicates are different dilutions of the synthetic gBlock. \*\* indicates that for most samples, only one technical replicate was sequenced but for one sample per sampling month, three technical replicates were sequenced to check for consistency across replicates. \*\*\* indicates that here, the three biological replicates indicate three different mock communities with varying species compositions, but all containing the four salmonids of interest. Created with BioRender.com.

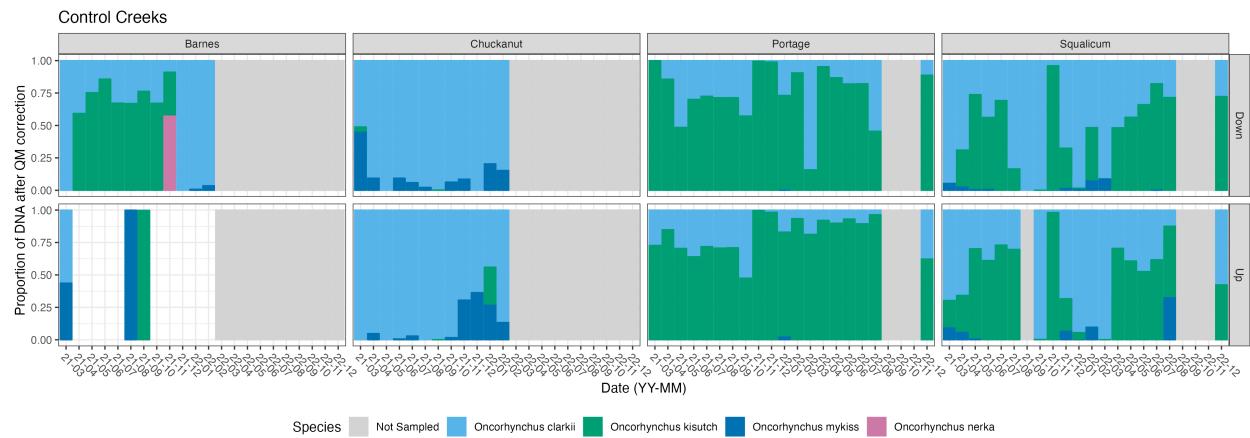


Figure 4: Compositions of salmonid DNA in control creeks as determined by metabarcoding after correction for amplification bias. Grey shading denotes time points that were not sampled (Barnes and Chuckanut Creeks after March 2023 and Squalicum Creek in September 2021 which was dry). The empty bars in the Barnes upstream sites indicate that no salmonid DNA was found at those time points.

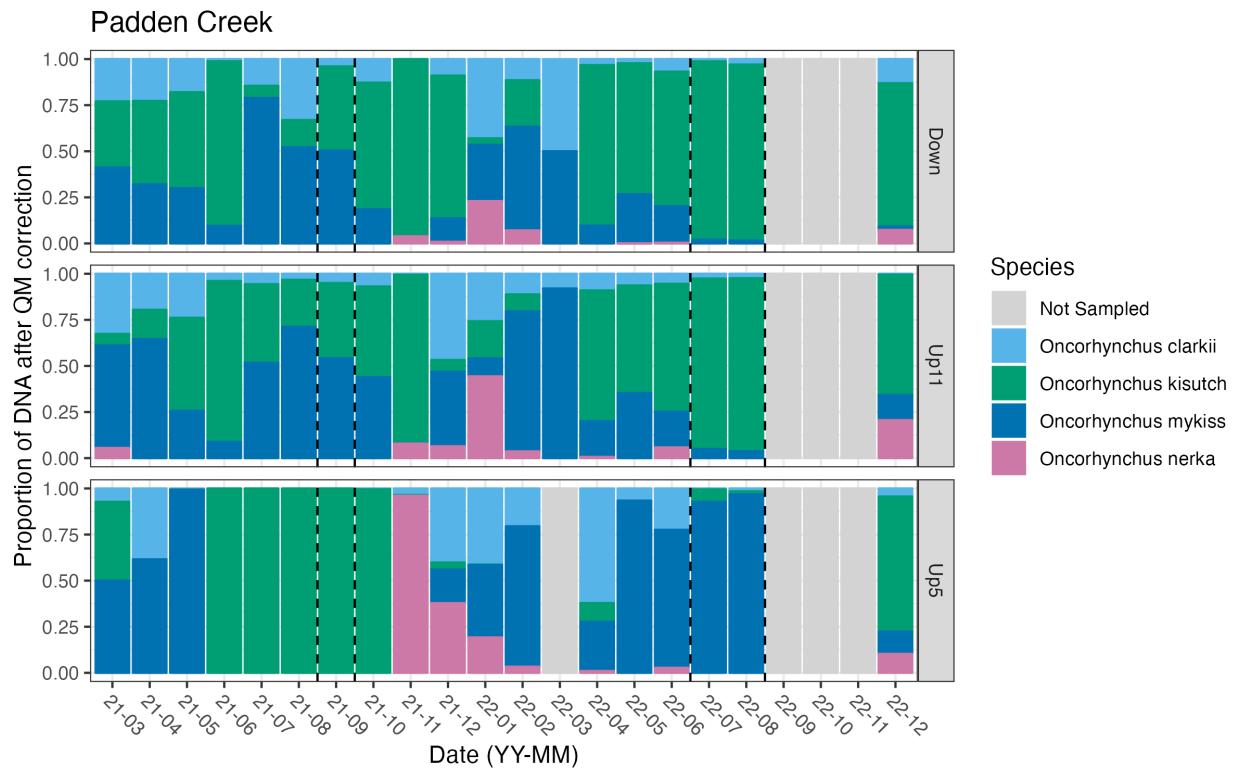


Figure 5: Compositions of salmonid DNA in Padden Creek as determined by metabarcoding after correction for amplification bias. The empty bar in the March 2023 in Up5 indicates that no salmonid DNA was found. The vertical dashed lines indicates the time period in which the culverts were replaced (SR-11 and I-5, respectively).

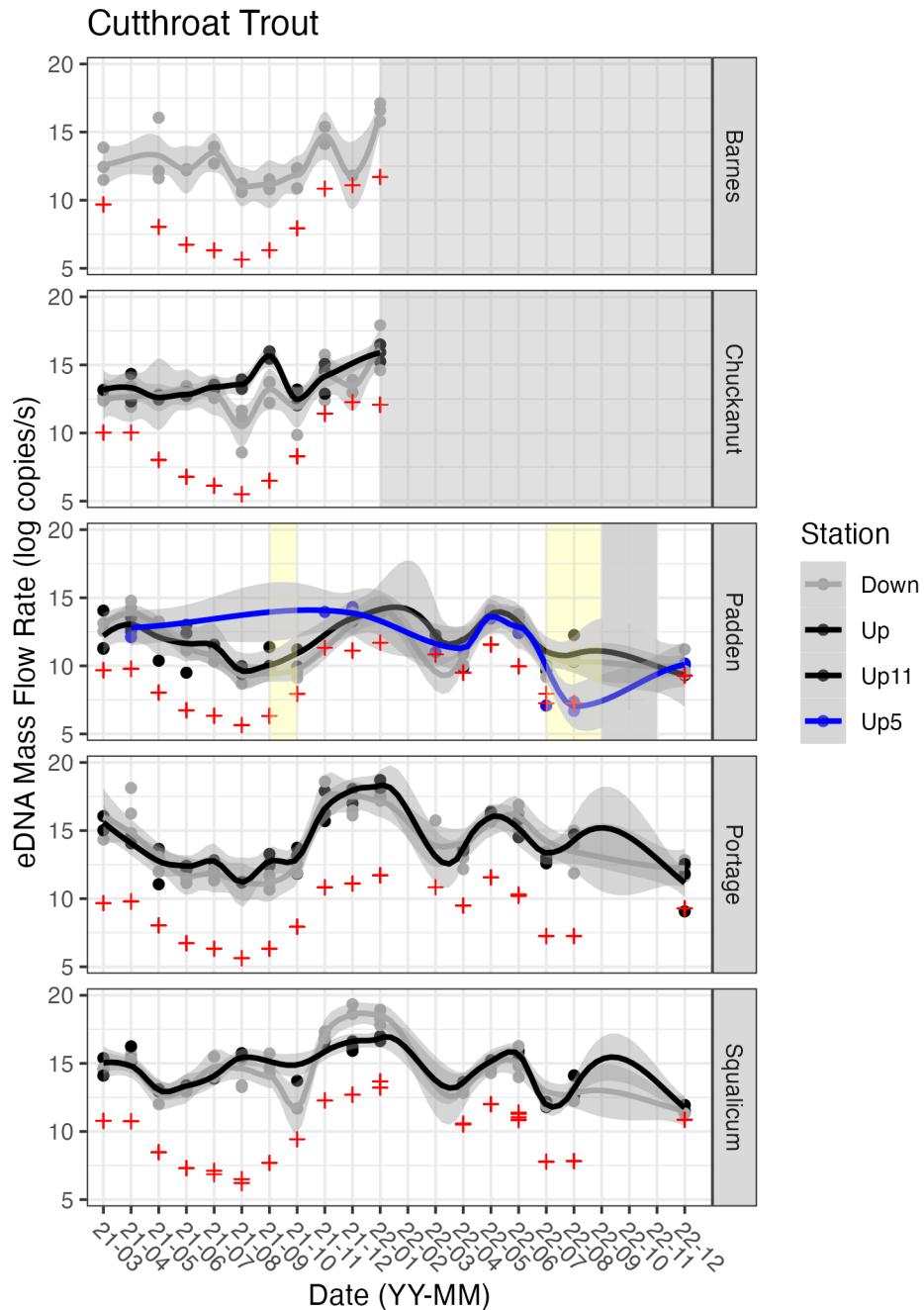


Figure 6: Absolute mass flow rate (log copies/s) of cutthroat trout (*O. clarkii*) as measured by qPCR after flow correction. Note that Barnes Creek and Chuckanut Creek were not sampled after February 2022. Red crosses show the limit of detection for each species and time point, which changes with flow rate and total volume filtered per sample.

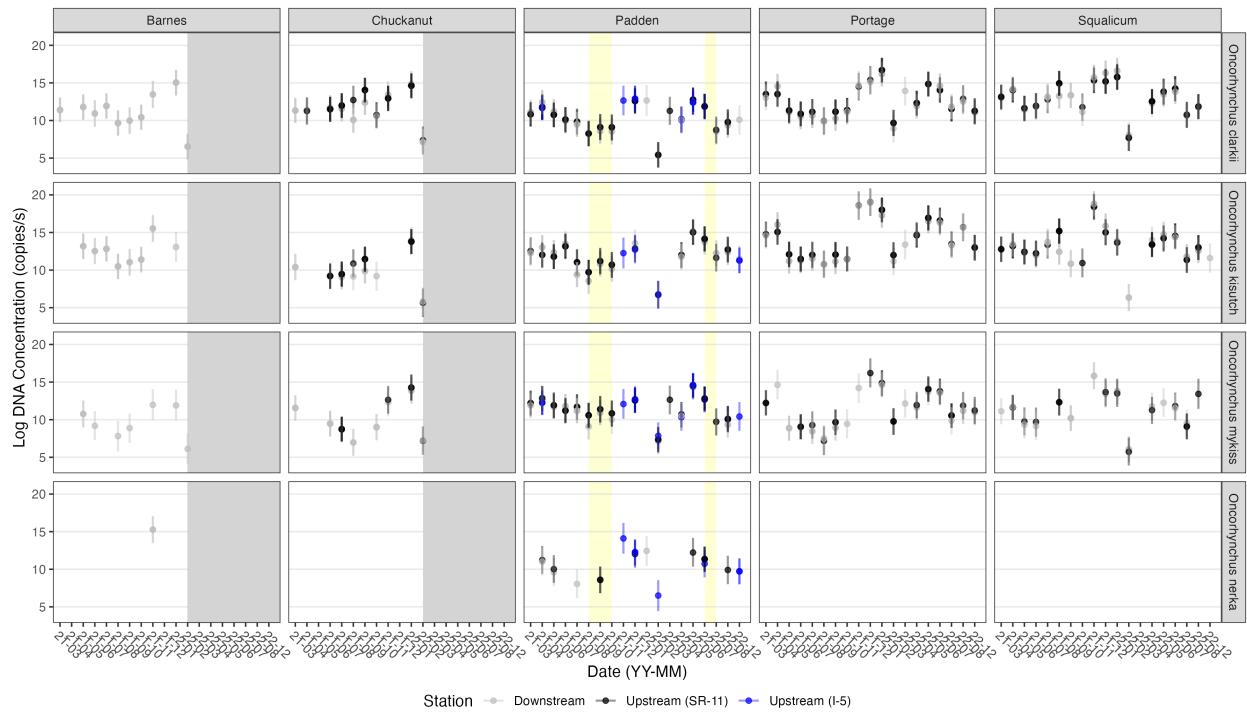


Figure 7: Trends in mass flow rate (log copies/s) for each of four salmonid species across creeks and across time as estimated by eDNA analysis. Points represent posterior means for the linear mixed effects model and error bars represent the 95% posterior confidence interval. Colors indicate station upstream (black) or downstream (grey) of the culvert. Padden has an additional sampling site upstream of the second culvert (I-5; blue). Yellow shading indicates the time period in which the culverts in the treatment creek (Padden Creek) were replaced. Grey shading indicates time points that were not sampled (Barnes and Chuckanut after February 2022). Time points with no data had no sequencing reads corresponding to that species or no quantifiable cutthroat DNA by qPCR.

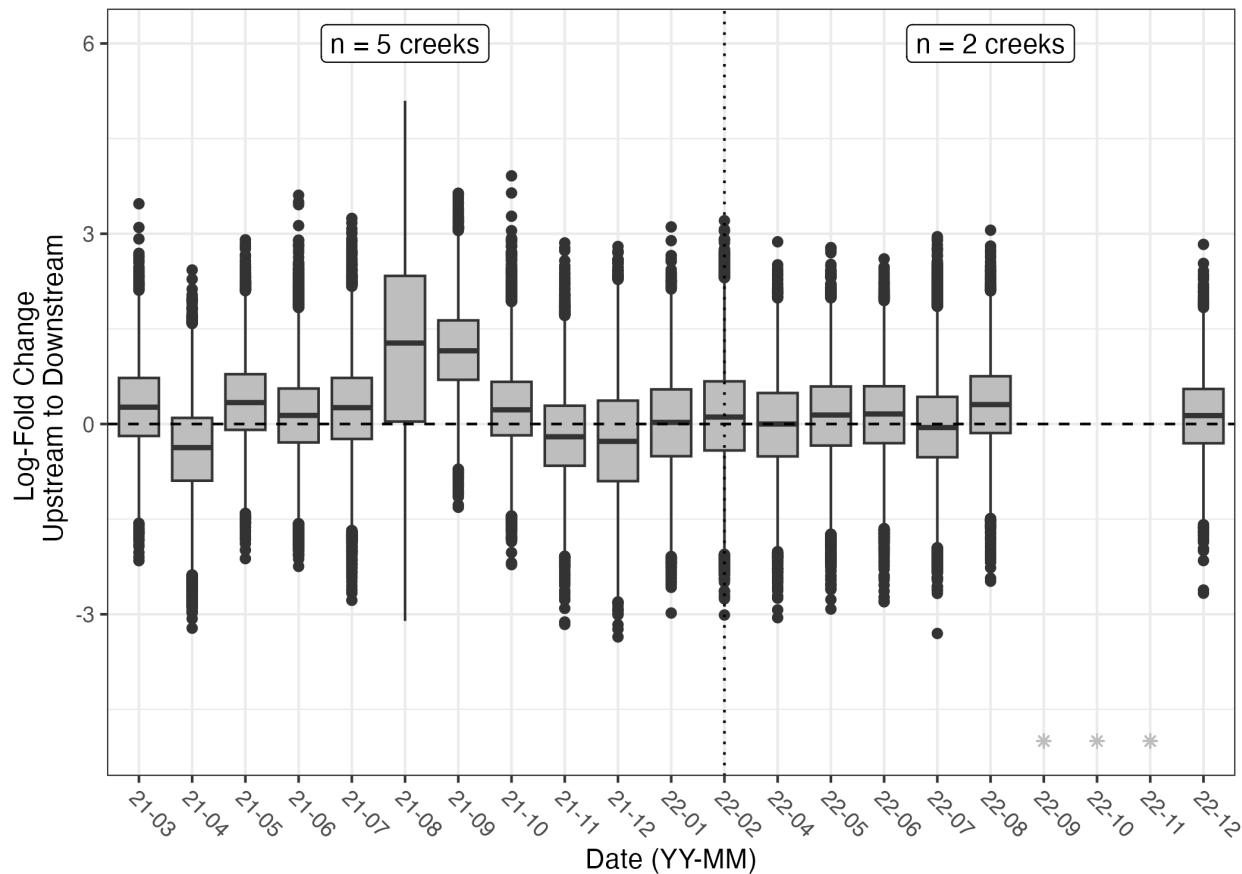


Figure 8: The effect of culvert on salmonid abundance summed across all species and creeks by time. The y-axis shows the log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream, normalized by upstream mass flow rate. The box boundaries correspond to the 25th and 75th percentiles; the whiskers correspond to 1.5 times the interquartile range. Here, negative values imply that eDNA mass flow rates are higher downstream than upstream. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators. Grey stars indicate times when no samples were taken.

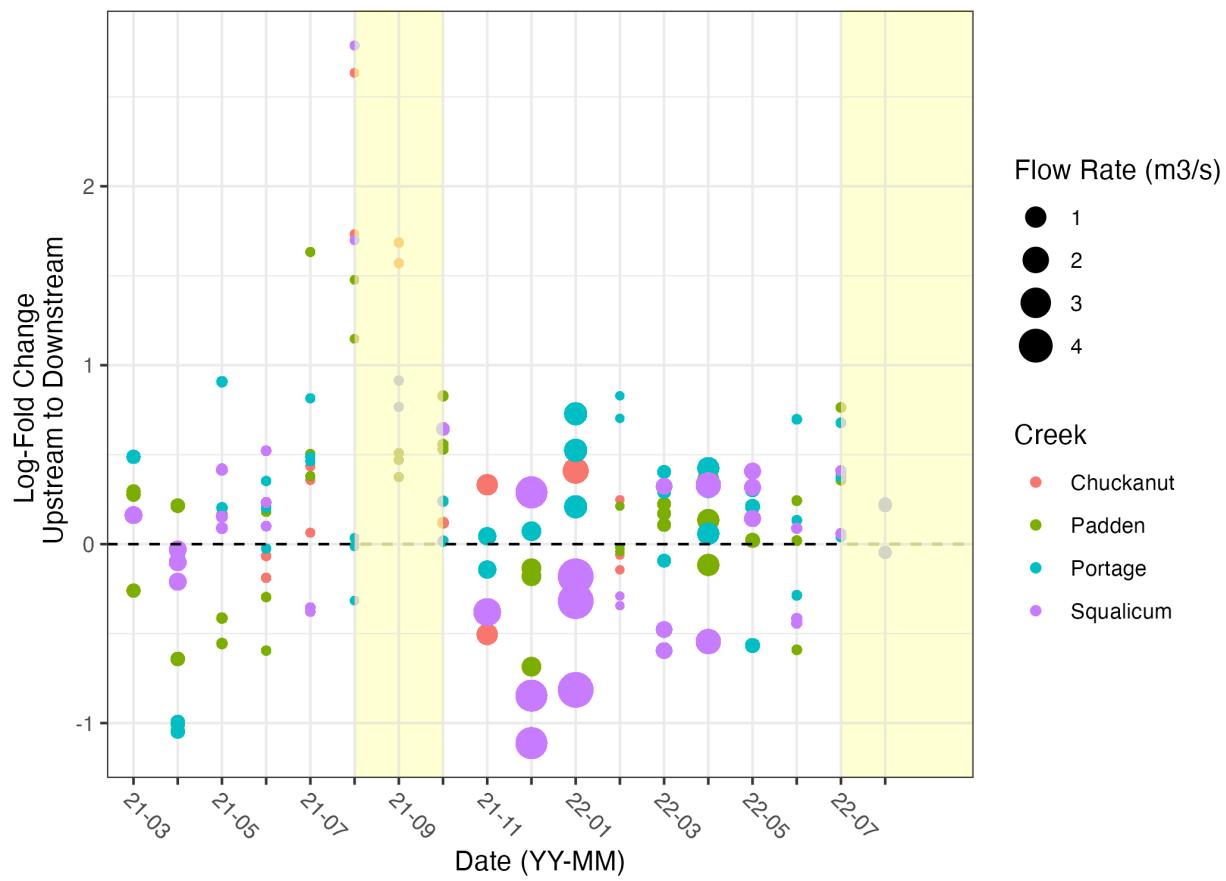


Figure 9: Log-fold change in eDNA mass flow rate over time. Size of circles corresponds to the discharge in each creek at each time point. Color of circles corresponds to each creek. Each creek and time point has up to four circles of the same color for the four salmonid species.

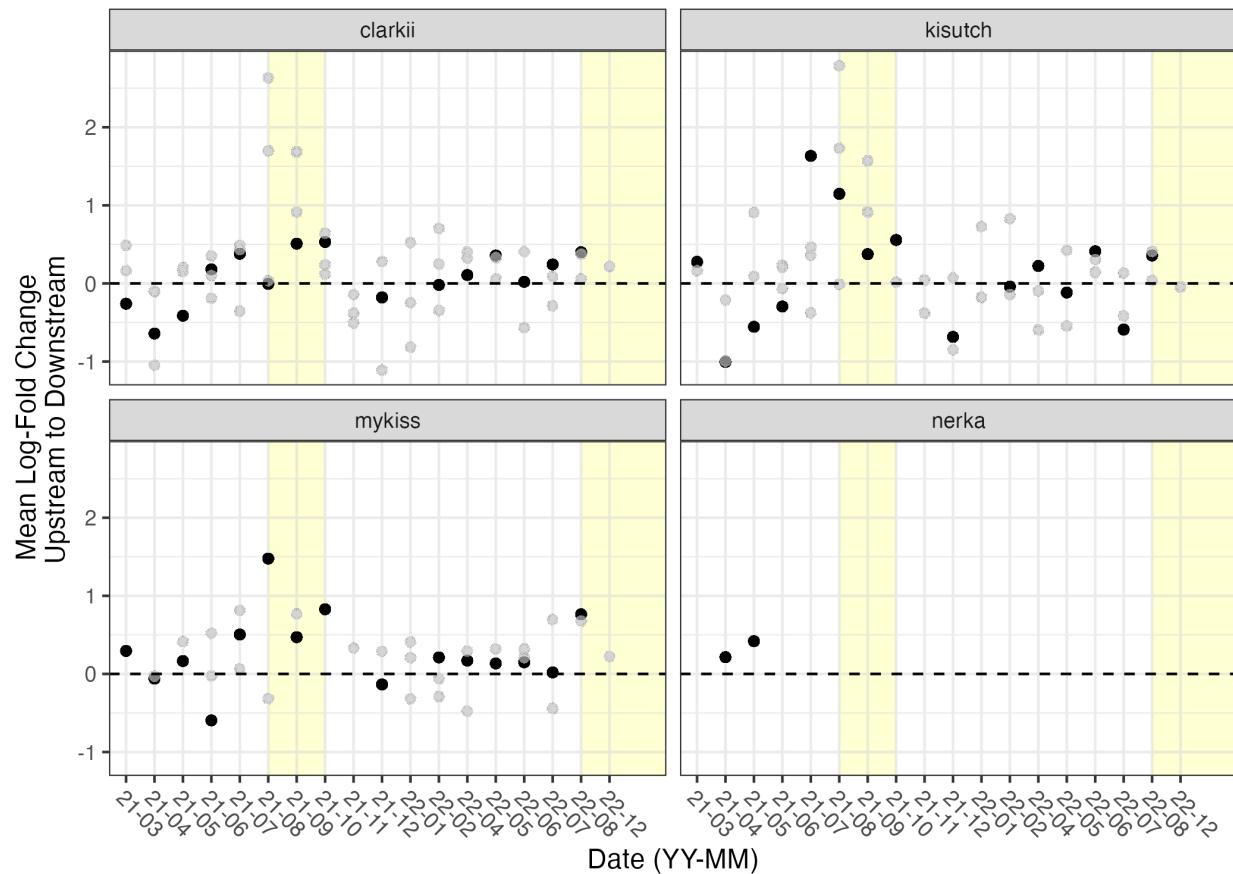


Figure 10: Effect of Construction on Log-Fold Change in eDNA Flow Rate Upstream to Downstream in Padden Creek. Yellow shading shows when construction started for each of the two culverts. Grey points show the corresponding log-fold changes in control creeks and black points show Padden Creek. Sockeye/kokanee salmon (*O. nerka*) was only found in Padden Creek so other creeks are not shown. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators.

<sup>1</sup> Quantifying Impacts of an Environmental Intervention Using Environmental  
<sup>2</sup> DNA

<sup>3</sup> Elizabeth Andruszkiewicz Allan<sup>1\*†</sup>, Ryan P. Kelly<sup>1\*</sup>, Erin R. D'Agnese<sup>1,3</sup>, Maya N. Garber-Yonts<sup>1</sup>, Megan  
<sup>4</sup> R. Shaffer<sup>1</sup>, Zachary J. Gold<sup>2</sup>, Andrew O. Shelton<sup>2</sup>

<sup>5</sup> <sup>1</sup> University of Washington, School of Marine and Environmental Affairs, 3737 Brooklyn Ave NE, Seattle,  
<sup>6</sup> WA 98105, U.S.A.

<sup>7</sup> <sup>2</sup> Conservation Biology Division, Northwest Fisheries Science Center, National Marine Fisheries Service,  
<sup>8</sup> National Oceanic and Atmospheric Administration, 2725 Montlake Blvd. E, Seattle, WA 98112, U.S.A.

<sup>9</sup> <sup>3</sup> Wild EcoHealth, Tacoma WA, 98465, USA

<sup>10</sup> \* Authors contributed equally to this work.

<sup>11</sup> † Corresponding author: eallan@uw.edu

<sup>12</sup> For submission to: *Ecological Applications*

<sup>13</sup> Manuscript type: Article

<sup>14</sup> Open Research Statement: Data are provided as private-for-peer review (shared privately or publicly on a  
<sup>15</sup> repository). The repository for code can be found at: [https://github.com/eandrusz/quantitative\\_salmon\\_re](https://github.com/eandrusz/quantitative_salmon_re)  
<sup>16</sup> submit.git

<sup>17</sup> Keywords: environmental DNA, quantitative metabarcoding, environmental impact assessments, salmon,  
<sup>18</sup> culvert

19 **Abstract**

20 Environmental laws around the world require some version of an environmental impact assessment surrounding  
21 construction projects and other discrete instances of human development. Information requirements for these  
22 assessments vary by jurisdiction, but nearly all require an analysis of ~~the~~ biological elements of ~~affected~~  
23 ecosystems. Amplicon-sequencing - also called metabarcoding - of environmental DNA (eDNA) has made  
24 it possible to sample and amplify the genetic material of many species present in those environments,  
25 providing a tractable, powerful, and increasingly common way of doing environmental impact analysis for  
26 development projects. Here, we analyze a ~~12-month–18-month~~ time-series of water samples taken before,  
27 during, and after ~~a culvert removal project~~ ~~two culvert removals~~ in a salmonid-bearing freshwater stream. We  
28 ~~use an asymmetrical Before-After-Control-Intervention (BACI) design with also sampled~~ multiple control  
29 streams to develop a robust background expectation against which to evaluate the impact of this discrete  
30 environmental intervention in the treatment stream. We generate calibrated, quantitative metabarcoding  
31 data from amplifying the 12s MiFish mtDNA locus and complementary species-specific quantitative PCR  
32 data to yield multi-species estimates of absolute eDNA concentrations across time, creeks, and sampling  
33 stations. We then use a ~~hierarchical Bayesian time-series~~ ~~linear mixed-effects~~ model to reveal patterns of  
34 eDNA concentrations over time, and to estimate the effects of the culvert removal on salmonids in the  
35 treatment creek. We focus our analysis on four common salmonid species: cutthroat trout (*Oncorhynchus*  
36 *clarkii*), coho salmon (*O. kisutch*), rainbow trout (*O. mykiss*), and sockeye salmon (*O. nerka*). ~~After~~  
37 ~~accounting for temporal variability common to the sampled creeks, we find~~ ~~We find that one culvert in the~~  
38 ~~treatment creek seemed to have no impact while the second culvert had a large impact on fish passage.~~ The  
39 ~~construction itself seemed to have~~ only transient effects on ~~these salmonid~~ species during the ~~several months~~  
40 ~~after construction~~ ~~two construction events~~. In the context of billions of dollars of court-mandated road culvert  
41 replacements taking place in Washington State, USA, our results suggest that culvert replacement can be  
42 conducted with only minimal impact of construction to key species of management concern. Furthermore,  
43 eDNA methods can be an effective and efficient approach for monitoring hundreds of culverts to prioritize  
44 culverts that are required to be replaced. More broadly, we demonstrate a rigorous, quantitative method for  
45 environmental impact reporting using eDNA that is widely applicable in environments worldwide.

## 46 Introduction

47 At present, it remains difficult to comprehensively measure the environmental impacts of discrete human  
48 activities, despite such assessment often being required by law. Within the United States, both state and  
49 federal laws often require a form of environmental-impact assessment for medium- to large-scale projects (i.e.,  
50 those that might have a significant impact on the environment) (Morgan 2012). Outside the US, many nations  
51 have their own versions of these same laws. Specifically when measuring impacts on aquatic ecosystems,  
52 assessments generally are based on literature reviews or field measurements of key species selected beforehand  
53 (Rubin et al. 2017). These traditional methods are often expensive, rely on just a few species, and are limited  
54 in spatial and temporal coverage (Martin et al. 2012). Moreover, they often lack pre-project monitoring and  
55 any or sufficient post-project monitoring, given that the goals of a development project normally focus on  
56 construction itself and funding is often extremely limited. For example, a recent literature review of stream  
57 restoration projects cited that more than half of projects evaluated (62%) had no pre-project monitoring and  
58 only sampled once per year (for before, during, and post-project sampling) (Rubin et al. 2017). Thus, current  
59 assessment efforts relying on traditional survey methods often fall short in documenting and quantifying  
60 environmental impacts.

61 A key difficulty in conducting ecosystem assessments is that there is no one way to survey the world and just  
62 “see what is there.” All methods of environmental sampling are biased, , in the sense that as they capture a  
63 selective portion of the biodiversity present (Rubin et al. 2017). Net samples for fish, for example, fail to  
64 capture species too small or too large to be caught in the net; ~~bacterial cultures capture only those species~~  
65 ~~that can be cultured on available media, and so forth~~. Environmental DNA (eDNA), however, comes as close  
66 to this goal as any method yet developed although not without bias (see below): a sample of water, soil, or  
67 even air, contains the genetic traces of many thousands of species, from microbes to whales. Sequencing  
68 eDNA is therefore a means of surveying many species in a consistent and scalable way (Taberlet et al. 2012,  
69 Thomsen and Willerslev 2015). Environmental assessments have begun to make use of eDNA for such work  
70 around the world (Muha et al. 2017, Duda et al. 2021, Klein et al. 2022, Maasri et al. 2022, Moss et  
71 al. 2022), but are not yet common practice. ~~Sampling water to collect eDNA before, during, and after a~~  
72 ~~development project would be a new and powerful method of assessing that project’s impacts on the local~~  
73 ~~biological communities, and could conceivably become the standard approach to conducting such impact~~  
74 ~~assessments (Hinz et al. 2022).~~

75 ~~Surveying the natural world by amplifying and sequencing DNA from environmental sources Surveying the~~  
76 ~~world by eDNA~~ has long been commonplace in microbial ecology (Ogram et al. 1987, Rondon et al. 2000,  
77 Turnbaugh et al. 2007) but has recently become popular for characterizing ~~ecological communities of~~

78 ~~eukaryotes eukaryotic communities~~ (Taberlet et al. 2012, Kelly et al. 2014, De Vargas et al. 2015, Port  
79 et al. 2015, Valentini et al. 2016, Stat et al. 2017). Techniques ~~that take advantage of such data may~~  
80 ~~include non-PCR-based methods such as hybridization, but~~ generally include an amplification step such as  
81 quantitative PCR, digital or digital-droplet PCR, or traditional PCR from mixed templates followed by  
82 high-throughput sequencing (Ruppert et al. 2019). This last technique is known as ~~metabarcoding, eDNA~~  
83 ~~amplion sequencing, or more generally, marker gene analysis~~eDNA metabarcoding.

84 In a metabarcoding approach, broad-spectrum PCR primers identify ~~many hundreds or thousands of~~ taxa  
85 across a very wide diversity of the tree of life (e.g., Leray et al. (2013)),~~but nevertheless~~. Nevertheless  
86 the absence of a taxon from a sequenced sample does not indicate the absence of that taxon from the  
87 environment ~~but rather that the taxon failed to amplify~~ (Shelton et al. 2016, Kelly et al. 2019, Buxton et  
88 al. 2021). ~~Instead, the unsampled species simply may not have been susceptible to that set of PCR primers,~~  
89 ~~and so failed to amplify.~~ The result is often a dataset that represents hundreds or thousands of taxa, but  
90 ~~these taxa are a fraction of a larger (and perhaps taxonomically broad) pool of species present~~ Gold et al.  
91 2023). In virtually all comparisons, metabarcoding recovers far more taxa ~~from an area~~ than any other  
92 sampling method (Port et al. 2015, Kelly et al. 2017, Seymour et al. 2021).

93 However, we expect results from metabarcoding to differ dramatically from non-PCR based sampling methods  
94 due to the fundamental differences in sampling ~~genetic waste residual genetic material~~ as opposed to whole  
95 organisms. Furthermore, eDNA analyses rely on several laboratory processes, including PCR amplification,  
96 all of which contribute to complicating the interpretation of results (see Shelton et al. (2016) and Kelly et  
97 al. (2019)). Specifically, PCR amplification is an exponential process for which the efficiency varies across  
98 species and primer set (Gloor et al. 2016). By understanding these ~~process~~ differences, we can correct for  
99 taxon-specific biases ~~in amplification efficieney~~ to yield quantitative estimates of the community composition  
100 prior to PCR (McLaren et al. 2019, Shelton et al. 2022). Other ways to conduct quantitative metabarcoding  
101 include using qSeq (Hoshino et al. 2021), a process in which a random tag is added to target sequences  
102 before PCR. However, if different species amplify at different rates during PCR, these quantifications would  
103 reflect not just the starting concentration but also the amplification efficiency.

104 After correcting for amplification biases, the resulting ~~metabarcoding~~ dataset is compositional, revealing the  
105 proportions of each species' DNA present in each sample, but importantly, contains no information about the  
106 absolute abundance of DNA present (Gloor et al. 2016, McLaren et al. 2019, Silverman et al. 2021, Shelton  
107 et al. 2022). We can tie these proportional estimates to absolute abundances using additional data such  
108 as a quantitative PCR (qPCR) assay for one of the taxa present. Thus, a single qPCR assay and a single  
109 metabarcoding assay can together provide quantitative estimates of many species as opposed to running as

110 many qPCR assays as species of interest –(see also (Pont et al. 2022)). Together, we can use these data  
111 to assess changes in eDNA concentrations of species over time, and due to environmental impacts, such as  
112 replacing a culvert under a road.

113 Because replacing culverts can require substantial intervention—for example, diverting the water from a creek  
114 segment and rebuilding the road with a redesigned culvert—they require environmental impact assessments.  
115 Furthermore, because these replacements occur serially according to a schedule, they present an attractive  
116 experimental design to use eDNA to assess environmental impacts.

117 As a result of a ruling in a federal court (Martinez 2013), Washington State is under a ~~court ordered~~ mandate  
118 to replace hundreds of culverts that allow water to pass under roads and highways, costing billions of dollars.  
119 Improperly designed culverts can lead to many negative consequences for fish, especially anadromous salmon,  
120 including habitat fragmentation, loss of accessibility to spawning and rearing habitat, and genetic isolation  
121 (Price et al. 2010, Frankiewicz et al. 2021). These impacts on salmonids furthermore violate the sovereign  
122 treaty rights of the region's indigenous tribes ~~to manage their people, land, and resources~~ (Schmidhauser  
123 1976a(Martinez 2013). Salmonid species are of cultural and economic importance to the indigenous peoples  
124 of the region, and without restoration of historic salmon-rearing habitat, the continued decline of salmonids  
125 can lead to not only ecological destruction, but the loss of cultural and economic viability for many indigenous  
126 tribes (Schmidhauser 1976b1976, Lackey 2003, Long and Lake 2018).

127 Presently, the prioritization process for replacing culverts preventing fish passage conducted by the Washington  
128 Department of Transportation is a protocol provided by the Washington Department of Fish and Wildlife,  
129 which includes factors such as the amount of habitat blocked by the barrier, the types of species blocked by  
130 the barrier, and estimated cost of repair, among other things (Washington Department of Fish and Wildlife  
131 2019a2019). However, data on fish presence upstream of barriers (i.e., culverts blocking fish passage) are rare  
132 and often not included in these assessments. Using eDNA as a proxy for fish presence could provide ~~another~~  
133 ~~important type of important~~ data for project prioritization and ~~increase efficiency in making prioritization~~  
134 ~~decisions have the potential to be more cost effective~~.

135 Once a culvert has been designated as in need of repair, the intention is to improve conditions for biota,  
136 including migrating fish, but ~~it might be that~~ the construction itself ~~has might have~~ a short-term negative  
137 effect ~~on fish and other organisms~~ before the longer-term improvements are realized. Specifically in ~~the ease~~  
138 ~~of culvert replacement~~culvert replacements, studies have cited the negative impacts of construction to include  
139 sediment accumulation, removal of vegetation, and blocking flow and stranding fish (Wellman et al. 2000,  
140 Washington Department of Fish and Wildlife 2019b2019). However, it is unclear how long these effects might

141 last and if the long-term benefits of the culvert replacement justify the short-term costs of the construction.  
142 These disruptions also underscore the importance of both properly assessing culverts to determine if they are  
143 blocking fish passage and monitoring after construction to ensure the replacement actually improved fish  
144 passage.

145 Many studies have attempted to quantify ~~if-when~~ culverts are barriers to fish passage and how effective  
146 culvert replacements are for fish passage, either by measuring physical parameters of the culvert and stream  
147 after replacement (Price et al. 2010), or by measuring biological ~~means~~parameters, including electrofishing  
148 (Ogren and Huckins 2015) or ~~utilizing~~ genetic differentiation from fish tissues (Wood et al. 2018, Nathan  
149 et al. 2018). ~~In some cases, culverts deemed blockages did not prove to block fish passage (MacPherson et~~  
150 al. (2012)~~found in a study of over 200 culverts, that for certain species, including rainbow trout (*O. mykiss*),~~  
151 ~~culverts were not blocking fish passage despite being deemed blockages. As for how effective replacements~~  
152 ~~are, while in others, blockages that were replaced were not found to improve fish passage (Price et al.~~  
153 ~~(2010) found in a study of ~75 culverts that, despite culvert replacement, about 30% of the new culverts~~  
154 ~~still remained blockages (by physical characterization), while or improve overall biotic integrity (Ogren and~~  
155 Huckins (2015)~~found in a more in-depth study of just three culverts that after biological sampling (i.e.,~~  
156 ~~electrofishing and macroinvertebrate surveys) 3–5 years after culvert replacements, the overall biotic integrity~~  
157 ~~was not improved.~~ Sampling water for eDNA analysis before, during, and post-restoration can provide  
158 valuable information on if the restoration is needed, how the restoration negatively impacts communities  
159 during construction, and if the restoration efforts did in fact correct the blockage.

160 Here, we report the results of ~~a year-long an approximately 18-month~~ eDNA sampling effort before, during,  
161 and after ~~a small construction project in our experimental~~the replacement of two culverts (one small and one  
162 large) ~~in a~~ creek, assessing the impact of ~~that project~~ these projects on the salmonid species present. We do  
163 so using a combination of metabarcoding (12s mtDNA) and qPCR to yield estimates of the concentrations of  
164 DNA present at each time point, and we use parallel samples from ~~an additional~~ four control creeks to develop  
165 a causal analysis of changes in these concentrations. A clear opportunity for policy-relevant eDNA work is in  
166 using its power to survey many species at a time to improve the way we assess the impacts of human activities.  
167 Here, we demonstrate the utility of eDNA for ~~such assessments~~ policy-relevant environmental assessments by  
168 surveying many species simultaneously and improving the way we assess the impacts of human activities.

169 **Methods**

170 **Site and Species Selection**

171 We used selected sampling locations based loosely on an asymmetrical BACI (Before-After-Control-Impact)  
172 study design (Underwood 1992, 1994, Benedetti-Cecchi 2001) to measure the environmental impact of a  
173 construction project replacing an under-road culvert culvert replacement using eDNA. We sampled four  
174 control creeks in addition to the treatment creek where the culvert was replaced where construction was not  
175 occurring (Figure 1) at monthly intervals, both upstream and downstream of each creek's culvert. The culvert  
176 two culverts in the treatment creek (Padden) was were suspected to be partially impassable and thus was were  
177 removed and replaced during the course of the study; one of the control creeks had a bridge, which allowed  
178 . The four control creeks ranged from preventing fish passage (Portage), one control creek had a culvert  
179 classified as having limited fish passability Barnes and Chuckanut, partially passable (Squalicum), and two  
180 control creeks had culverts classified as preventing to allowing fish passage (Barnes and Chuckanut Portage;  
181 see Supplemental Text 1) (Washington Department of Fish and Wildlife 2019a2019). These creeks were  
182 chosen due to their comparable size, flow, watersheds, and species presumed to be present to constrain as  
183 many ecological variables as possible.

184 The intervention (i.e., culvert replacement first culvert replacement (SR-11) in Padden Creek occurred over  
185 about two months and included the “de-watering” of the creek, removal of the existing culvert, installation  
186 of the new culvert, and then the “re-watering” of the creek from late August 2021 to early October 2021  
187 (Supplemental Figure 3). We Text 1; Figure S1.4). The second culvert replacement (I-5) in Padden Creek  
188 was a much larger construction project, including daylighting the creek and building a bridge under a large,  
189 five-lane interstate. In-water work for the I-5 culvert replacement began in late June 2022 and was completed  
190 in September 2022. By sampling before, during, and after both construction events, we were then able to  
191 quantify isolate the effect of the culvert replacement itself – controlling for temporal trends, background  
192 environmental variability, and sampling variability – using a Bayesian time-series model to jointly model  
193 salmon linear mixed effects model of eDNA abundances across creeks, time points, sampling stations, and  
194 species.

195 Because salmonids are the primary species of management concern in these creeks, we focus the present  
196 analysis on the four salmonid species most common in our data: cutthroat trout (*Oncorhynchus clarkii*), coho  
197 salmon (*O. kisutch*), rainbow/steelhead trout (*O. mykiss*), and sockeye/kokanee salmon (*O. nerka*). As further  
198 described below, we surveyed the salmonid DNA present in each creek via eDNA metabarcoding (targeting  
199 a region of the 12s mtDNA gene) and complementary quantitative PCR (qPCR; targeting a region of the

200 CytB gene) for a reference species (cutthroat trout, *O. clarkii*), which in combination yielded quantitative  
201 estimates for each fish species throughout the study area.

202 Not all four salmonids are expected to be found in all five of the creeks sampled. As documented by WA  
203 Department of Fish and Wildlife SalmonScape (<http://apps.wdfw.wa.gov/salmonscape/map.html>), all creeks  
204 contain cutthroat trout, steelhead trout, and coho salmon. Barnes Creek is the only creek documented to have  
205 kokanee salmon, ~~which are (a~~ freshwater sub-type of sockeye salmon~~that are not anadromous~~). However,  
206 local spawner surveys conducted by the City of Bellingham from 2015-2020 in Padden Creek documented  
207 kokanee salmon, as well as the other three species ~~and importantly, several unknown species of live and dead~~  
208 ~~fish and redds (nests dug by fish in gravel to deposit eggs)~~ ((City of Bellingham 2015).

209 The four salmonid species in this study have different life histories and behaviors that would impact when  
210 fish (and therefore eDNA concentrations) occur in the creeks. ~~For these four migratory salmonids, the run~~  
211 ~~timings vary for each species in the study area (Bellingham, WA). Adult coastal cutthroat (*O. clarkii*) are~~  
212 ~~documented to run throughout the entire year, whereas coho salmon (*O. kisutch*) run from September to~~  
213 ~~December, sockeye salmon (*O. nerka*) run from October to December, and steelhead trout (*O. mykiss*) run~~  
214 ~~from November to June. For migrating coho (*O. kisutch*) and steelhead trout (*O. mykiss*), juveniles may be~~  
215 ~~present in the creeks year-round (Supplemental Figure 3). Using eDNA methods, it cannot be determined~~  
216 ~~if the DNA found is sourced from adult or juvenile animals.~~

217 Furthermore, three of the four species in this study have both freshwater resident and saltwater migrating  
218 behavior. ~~Cutthroat trout (*O. clarkii*) encompasses both non-migrating, resident trout in the creeks and~~  
219 ~~coastal run cutthroat that migrate into Padden Creek from saltwater (Bellingham Bay). Similarly, *O. nerka*~~  
220 ~~includes both anadromous sockeye salmon and freshwater resident kokanee salmon and *O. mykiss* includes~~  
221 ~~both anadromous steelhead trout and non-migrating rainbow trout. Using eDNA, we cannot distinguish~~  
222 ~~between the migrating and non-migrating subspecies of *O. clarkii*, *O. nerka*, and *O. mykiss*. For the fish~~  
223 ~~exhibiting migratory behavior, the run timings vary for each species in the study area (see Discussion and~~  
224 ~~Figure S1.4).~~ Therefore, our eDNA concentrations might reflect contributions from both migrating and  
225 non-migrating individuals at any given time point in the dataset.

## 226 Water Sampling

227 We collected water samples monthly between From March 2021 and to February 2022 in each of five  
228 salmonid-bearing creeks in northwest Washington State, USA (Figure 1). We sampled each stream above  
229 and below under-road culverts., all five creeks were sampled monthly (n=12). Monthly sampling continued  
230 in Portage Creek, Padden Creek, and Squalicum Creek through August 2022, with one additional sampling

231 point in December 2022 (n=19). At each sampling station (N=2, upstream and downstream of a culvert) at  
232 each creek (N = 5) in each month (N = 12), we collected three 2-liter water samples, for a total of 360 water  
233 samples. Samples were collected using Smith Root's an eDNA Backpack (Smith Root; Thomas et al. (2018)),  
234 a portable pumping-and-filtering device set to filter at 1 L/min at 82.7 kPa (12 psi). In some months, less than  
235 2 L of water was filtered due to clogging(min = 1.02 L, mean = 1.97 L, median = 2.01 L; see Supplemental  
236 Figure 4). Water samples were filtered using single-use inlet tubes through 5 $\mu$ m self-preserving filters (Smith  
237 Root, Vancouver, WA), which were then dried and kept at room temperature until DNA extraction within 1  
238 month of collection (Thomas et al. 2019).

239 Over the course of the year of sampling, water discharge varied from very low to no flow in summer months  
240 to high flow in winter months (Figure 2). Thus, when considering eDNA concentrations at a sampling site,  
241 we need to account for the large difference in water volume over the course of the year-long time series. In  
242 other words, given the same number of fish and a constant eDNA shedding rate, we would expect to see  
243 higher concentrations of DNA in summer months and lower concentrations in winter months due to dilution  
244 of eDNA in higher water volumes just from the difference in flow. Other eDNA time series datasets also  
245 correct for discharge to present eDNA data as a mass flow rate mass/time (Tillotson et al. 2018, Thalinger  
246 et al. 2019). Here, we convert eDNA). We account for this dilution by converting eDNA concentration  
247 [copies/ $\mu$ L] to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s] .  
248 (Tillotson et al. 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey  
249 (USGS) were used for Padden Creek (USGS Gauge 12201905), Chuckanut Creek (USGS Gauge 12201700),  
250 and Squalicum Creek (USGS Gauge 12204010; <https://maps.waterdata.usgs.gov/mapper/index.html>;  
251 U. S. Geological Survey (1994); Supplemental Figure 1). During the year Figure S1.1). Over the course  
252 of sampling, the flow gauges at Chuckanut Creek and Squalicum Creek became inoperable after a major  
253 flooding event. To find discharge rates for Chuckanut and Squalicum Creeks, five years of historical data  
254 (2015-2020) were used to generate a daily monthly averaged correction factor based on Padden Creek .  
255 For the year of sampling (2021-2022), the discharge rates used at Chuckanut and Squalicum Creeks were  
256 estimated based on the correction factor from Padden Creek (Supplemental Figure 2(Supplemental Text 1,  
257 Figure S1.3)). No discharge data was available for Portage Creek or Barnes Creek. Based on field sampling  
258 conditions, the discharge from Padden Creek was used as a proxy for both Portage and Barnes as they  
259 are in similarly sized watershed areas and land-cover characteristics. Though in the year of sampling, the  
260 discharge in Padden Creek ranged from no metered flow to 23 m<sup>3</sup>/s, the discharge on the dates of sampling  
261 only reached a maximum of 1.3 m<sup>3</sup>/s.

262 **DNA Extraction, Amplification, Sequencing**

263 All molecular work prior to sequencing was performed at the University of Washington. ~~Bench-tops were~~  
264 ~~cleaned with 10% bleach for 10 minutes and then wiped with 70% ethanol. Molecular work was separated~~  
265 ~~onto pre- and post-PCR benches; all DNA extractions and PCR preparation was conducted on a bench~~  
266 ~~where no PCR product was handled. DNA was extracted from half of each filter.~~ Details of the molecular  
267 work can be found in Supplemental Text 1. Briefly, DNA was extracted off filters using a Qiashredder column  
268 (Qiagen, USA) and the DNEasy DNeasy Blood and Tissue Kit (Qiagen, USA) with an overnight incubation  
269 (Supplemental Text 1, Thomas et al. (2019)), ~~such that the effective filtering effort was 1 L/sample; the~~  
270 ~~remaining half of each filter was archived at -20°C.~~ Extracts were eluted in 100  $\mu$ L of molecular grade water,  
271 ~~quantified via Qubit (Invitrogen, USA) and stored at -20°C until PCR amplification within 2 months of~~  
272 extraction.

273 For ~~the metabarcoding approach~~metabarcoding, we targeted a ~186 bp hypervariable region of the mito-  
274 chondrial DNA 12S rRNA gene for PCR amplification (MiFish; Miya et al. 2015), but using modified  
275 primer sequences as given in Praebel and Wangensteen (unpublished; via personal communication)~~and~~  
276 ~~including the Illumina Nextera overhang sequences for subsequent indexing. The primers used were as~~  
277 ~~follows:~~ F 5' TGGTCGGCAGCGTCAGATGTGTATAAGAGACACGCGCTAAACTCGTGCCAGC 3',  
278 R 5' GTCCTCGTGGGCTCGGAGATGTGTATAAGAGACACCATAGTGGGCTATCTAATCCCAGTTG  
279 3': (*italics* indicate Nextera overhang). The final reaction recipe, and cycling  
280 conditions can be found in Supplemental Text 1. Each month of samples was amplified on a single plate  
281 with the addition of a no template control (NTC; molecular grade water in lieu of template) and a positive  
282 control (genomic DNA from kangaroo). After PCR amplification, PCR, ~~a species not present in the~~  
283 environment). PCR products were visualized ~~on a 1-2%~~ gel. If no band was present for a given sample, a  
284 new amplification was attempted with extracts diluted 1:10 iteratively until a band was detected. PCR  
285 ~~products were~~ size-selected and cleaned using MagBind Beads (Omega Biotek, USA) at a sample:beads  
286 ratio of 1:2. Bead-cleaned PCR products were eluted in 30  $\mu$ L of molecular grade water and quantified via  
287 Qubit (Invitrogen, USA).

288 An indexing PCR reaction added a unique index, and diluted iteratively if inhibited. After cleaning, a second  
289 PCR amplification added unique indices to each sample using Nextera indices (Illumina, USA) to allow pooling  
290 multiple samples onto the same sequencing run (See Supplemental Text 1 for details). Indexed PCR products  
291 were also size-selected and purified using MagBind Beads (Omega Biotek, USA) at a sample:beads ratio of  
292 0.8. Bead-cleaned PCR products were eluted in 30  $\mu$ L of molecular grade water and quantified via Qubit.  
293 Indexed and bead-cleaned products were normalized before pooling into libraries, which were subsequently

294 quantified via Qubit and visualized on a Bioanalyzer (Agilent, USA) before visualized before pooling for  
295 sequencing. Samples were randomized in 3-month blocks and each block split across 3 sequencing runs to  
296 avoid run effects, for a total of 12–14 sequencing runs. The loading concentration of each library was 4–8 pM  
297 and 5–20% PhiX was included depending on the composition of the run. Sequencing was conducted using an  
298 Illumina MiSeq with v3 2x300 chemistry at the NOAA Northwest Fisheries Science Center and the University  
299 of Washington's Northwest Genomics Center.

300 Here, we used mock communities to determine the species-specific amplification efficiencies for each salmonid  
301 in the study. Briefly, we We constructed five communities with known proportions of starting DNA from  
302 different species (total DNA as measured by Qubit). The communities ranged from having a total of 12 to 20  
303 species, but six salmonid species were included in all five mock communities to have more information on the  
304 amplification efficiencies of salmonids (Supplemental Table 32). We sequenced these communities using the  
305 same metabarcoding primers and thermocycling conditions above and then determined the species-specific  
306 amplification rates given the discrepancy between the known starting proportion and the proportion of  
307 reads after sequencing. These The mock community data were then used to correct the sequencing reads  
308 from the environmental samples to estimate the starting DNA proportions of each species in environmental  
309 samples, which is the metric of interest (Figure 3, green boxes). This is the first application of the model to  
310 correct eDNA data from water samples with mock community data as described in Shelton et al. (2022) (see  
311 Supplemental Text 2 for more information).

### 312 Bioinformatics

313 After sequencing, bioinformatic analyses were conducted in R (R Core Team 2017). A more detailed description  
314 of the bioinformatics pipeline is included in the supplement (Supplemental Text 1). Supplemental Text 1.  
315 Briefly, primer sequences were removed using Cutadapt (Cutadapt (Version 1.18) (Martin 2011) before dada2  
316 (dada2 (Callahan et al. 2016) trimmed, filtered, merged paired end reads, and generated amplicon sequence  
317 variants (ASVs). Taxonomic assignment was conducted via the insect (insect) package (Wilkinson et al. 2018)  
318 using a tree generated by the developers for the MiFish primers that was last updated in November 2018.  
319 Only species level assignments from insect (insect) were retained and ASVs not annotated or not annotated to  
320 species level were then checked against the NCBI nucleotide database using BLAST+ (Camacho et al. 2009).  
321 Query sequences that matched a single species at >95% identity were retained.

322 In total, sequencing runs generated 42 million reads across all environmental samples (12 months x 2  
323 stations x 5 creeks x 3 biological replicates = 360 filters) and 27 mock community samples (3 communities  
324 x 9 replicates 6 even, 3 skewed proportions) for calibration (see below). After quality filtering and merging

325 all runs, ~33 million reads remained from ~21,000 amplicon sequence variants (ASVs) in the environmental  
326 samples, of which ~81% of reads and ~2% of ASVs were annotated to species level (per sample: mean = 78%,  
327 median = 88%, min = 0%, max = 99.99% of reads annotated). We only focus on the metabarcoding data  
328 from four salmonids for the remainder of this paper. The four salmonids represent ~55% of all environmental  
329 reads and ~68% of the annotated reads found in environmental samples.

330 In the mock community samples, 98.7% of the ~5 million reads after quality filtering were annotated to  
331 species level. Importantly, the target salmonid ASVs in the mock communities were found in environmental  
332 samples, unambiguously linking the taxa in calibration samples with those in environmental samples. The  
333 most common salmonid species found in the environmental samples was *O. clarkii* (cutthroat trout), which  
334 was found in ~90% of samples, followed by *O. kisutch* found in ~60% of samples, then *O. mykiss* found  
335 in ~40% of samples, and finally *O. nerka* found in ~5% of samples. Not only was *O. clarkii* found in the  
336 majority of environmental samples, but also ~63% of samples across all times, creeks, and stations had at  
337 least 50% of reads assigned to *O. clarkii*.

### 338 Quantitative PCR and Inhibition Testing

339 We quantified cutthroat trout (*O. clarkii*) DNA in each sample, targeting a 114 bp fragment of the  
340 cytochrome b gene with a qPCR assay (Duda et al. 2021). The primer/probe sequences were: F  
341 5'-CCGCTACAGTCCTCACCTTCTA-3', R 5'-GATCTTTGTATGAGAAAGTAAGGATGGAA-3', P  
342 5'-6FAM-TGAGACACGGATCCAAC-MGB-NFQ-3'. The qPCR assay was multiplexed with TaqMan  
343 Exogenous Internal Positive Control Reagents (EXO-IPC) (Applied Biosystems, USA) to check for the  
344 presence of PCR inhibitors (Duda et al. 2021). The EXO-IPC mix includes the primers and probe for the  
345 EXO-IPC DNA, with the probe having a VIC reporter, allowing it to be multiplexed with the *O. clarkii*  
346 assay, which has a FAM reporter. Each DNA sample was run in triplicate; the final recipe, final recipe, and  
347 thermocycling conditions can be found in Supplemental Text 1. All qPCRs were conducted on an Applied  
348 Biosystems StepOnePlus thermocycler.

349 Each plate included a 8-point standard curve created using synthetic DNA (gBlocks) at the following  
350 concentrations: 100,000 copies/ $\mu$ L, 10,000 copies/ $\mu$ L, 1,000 copies/ $\mu$ L, 100 copies/ $\mu$ L, 10 copies/ $\mu$ L, 5  
351 copies/ $\mu$ L, 3 copies/ $\mu$ L, 1 copy/ $\mu$ L. Additionally, six no template controls (NTCs) were included on each  
352 plate: 3 with the IPC DNA mix and 3 with molecular grade water instead of template or IPC DNA mix.  
353 Plates were re-run if efficiency as determined by the standard curve was outside of the range of 90–110%.

354 To check for inhibition, the cycle threshold (Ct) value determined for the Each DNA sample was run in  
355 triplicate and was checked for inhibition using the EXO-IPC assay from the NTC was compared to the Ct

356 value for the EXO-IPC assay in each of the environmental samples. If the Ct value was >0.5 Ct values from  
357 the mean Ct for the NTCs, the sample was deemed inhibited and diluted 1:10 and re-assayed until the Ct  
358 value fell within the accepted range. (Applied Biosystems). The majority of environmental samples (65–60%)  
359 were inhibited and accordingly diluted for analysis. In 75–80% of inhibited samples, a 1:10 dilution or less  
360 remedied the inhibition, but some samples (11%) required dilution by a factor of up to 1000 (Supplemental  
361 Figure 5). Each plate included a 8-point standard curve created using synthetic DNA (gBlocks)  
362 ranging from 1 to 100,000 copies/μL and six no template controls (NTCs) were included on each plate  
363 with molecular grade water instead of template. All qPCRs were conducted on an Applied Biosystems  
364 StepOnePlus thermocycler.

365 All qPCR data was processed in R using Stan (Stan Development Team 2022), relating environmental samples  
366 to the standard curve via a linear model (Figure 3, blue boxes; Figure S2.1). We amended the standard  
367 linear regression model to more realistically capture the behavior of qPCR observations, accommodating  
368 non-detections as a function of underlying DNA concentration, and letting the standard deviation vary with  
369 the mean (lower-concentration samples had more uncertainty). See McCall et al. (2014) and Shelton et al.  
370 (2019) for similar models; see Supplemental Text 2 for full statistical details. Subsequent analysis corrected  
371 for sample-specific dilution if found inhibited and corrected for any variation in water-volume filtered during  
372 sample collection. Samples with standard deviations between technical replicates larger than 1.5 Ct values  
373 were removed from analyses.

#### 374 Quantitative Metabarcoding

375 The intercalibration of the mock community samples demonstrated the rank order of amplification  
376 efficiencies for salmonids (Supplemental Figures 14 and 15). Cutthroat trout (*O. clarkii*) and  
377 sockeye/kokanee salmon (*O. nerka*) had similar amplification efficiencies, both of which were  
378 higher than rainbow/steelhead trout (*O. mykiss*) and coho salmon (*O. kisutch*), which had the  
379 lowest amplification efficiency. Calibrated metabarcoding analysis yielded quantitative estimates of  
380 the proportions of species' DNA in environmental samples prior to PCR. We then converted these  
381 proportions into absolute abundances by expansion, using the qPCR results for our reference species,  
382 cutthroat trout (*O. clarkii*). We estimated the total amplifiable salmonid DNA in environmental  
383 sample  $i$  as  $\text{DNA}_{\text{salmonid}_i} = \frac{\text{qPCR}_{\text{reference}_i}}{\text{Proportion}_{\text{reference}_i}}$ ,  $C_{\text{amplifiable}_i} = \frac{C_{\text{qPCR reference}_i}}{\text{Proportion}_{\text{reference}_i}}$ , where  $C$  has units of [DNA  
384 copies/uL] and then expanded species' proportions into absolute concentrations by multiplying these  
385 sample-specific total concentrations by individual species' proportions, such that for species  $j$  in sample  
386  $i$ ,  $\text{DNA}_{i,j} = \text{DNA}_{\text{salmonid}_i} * \text{Proportion}_{i,j}$ . Here, we combine the modeled

387 output of the qPCR model for *O. clarkii* cutthroat trout (Figure 3 dashed blue box) and modeled proportions  
 388 of salmonid DNA from metabarcoding (Figure 3 dashed green box). ~~Though~~ Although in the future this  
 389 could be used as a joint model, here the precision of our modeled estimates were very high such that we used  
 390 the mean of the posterior estimates from each model to move forward as input to the time series model  
 391 (Figure 3 dashed purple box; see Supplemental Text 2 for more details).

392 Finally, due to the range of water discharge over the course of the year, we converted from DNA concentration  
 393 [copies/L] to a mass flow rate [copies/s] after multiplying by the discharge of each creek [ $m^3/s$ ] (Figure 3,  
 394 solid purple boxes).

### 395 Estimating the Effects of Culvert Replacement and of Culverts Themselves

396 ~~Consistent with the asymmetrical BACI study design, we generated data from our~~ We sampled four control  
 397 creeks as context against which to compare the observations in Padden Creek, our treatment creek where  
 398 the two culverts were being replaced. At a given station in a given creek, some DNA concentration exists  
 399 for each species. For simplicity, we focus on a single species and a single station (downstream or upstream)  
 400 for the moment. Our observations of the (log) DNA concentration in creek  $i$  at time  $t$  are distributed as  
 401  $Y_{i,t} \sim \mathcal{N}(\mu_{i,t}, \sigma^2)$ . More complex versions of the model may let  $\sigma$  vary across creeks, time points, species, or  
 402 with environmental covariates of interest. Recognizing that these observations are autocorrelated in time, we  
 403 use an AR(1) autocorrelation model, implemented in Stan via R, to capture the observed temporal trends.

404 We ~~observe the log-DNA concentration~~ are interested in how the DNA concentration changes over time, so  
 405 we assert that the expected value of DNA in a creek at time  $t$ ,  $Y$ , for a given species in a given sample as  
 406 a random variable drawn from a normal distribution with mean  $\mu$  and observation variance  $\sigma^2$ , depends  
 407 upon its time point, in some way. We considered three ways of modeling the salmonid eDNA data, each in  
 408 a Bayesian framework, but each treating non-independence among time points somewhat differently:

- 409 • A linear auto-regressive (AR(1)) model, written in `stan`. For each species ~~in each creek~~, the expected  
 410 ~~log-DNA concentration  $\mu$  at time  $t$  in creek  $i$  at station  $d$  concentration of eDNA of each month~~ is a  
 411 linear function of the ~~DNA concentration for the same creek/station at  $t-1$~~  expected value from the  
 412 previous month. Within a species, the monthly autoregressive parameters are shared across creeks.
- 413 • A generalized additive model (GAM), written in `brms` (which itself writes a `stan` model). For each  
 414 species in each creek, an independent set of spline (weighting) parameters describes the temporal trends  
 415 in expected eDNA concentration; the number of spline knots is shared across species and creeks.
- 416 • A linear mixed-effects (LME) model, written in `rstanarm`. For each species in each creek, sampling

417 month is treated as a random effect. Each species-creek-month effect is treated as an independent draw  
 418 from a common distribution.

$$Y_{i,t,d} \sim \mathcal{N}(\mu_{i,t,d}, \sigma^2)$$

$$\mu_{i,t,d} = \alpha_{i,t} + \epsilon_{i,t,d} + \eta_{i,t,d}$$

$$\epsilon_{i,t,d} \sim \mathcal{N}(\beta \mu_{i,t-1,d}, \phi^2)$$

419 Ultimately, the three models yielded very similar results (Figure S2.2), and the LME model proved simplest  
 420 and most flexible insofar as it could easily handle datasets with uneven sets of observations – for example,  
 421 cases in which a species was detected downstream of a barrier, but not upstream.

422 Intercept  $\alpha$  varies by time, creek, and species, capturing creek-level deviations from the previous time step.  
 423 The autoregression term  $\epsilon$  is itself a random variable drawn from a normal distribution with expected  
 424 value  $\beta \mu_{i,t-1,d}$  and process variance  $\phi^2$ , such that the species-specific slope term  $\beta$  estimates the degree of  
 425 autocorrelation in log-DNA concentration between one time-step and the next. The model shares information  
 426 across creeks and time-points via  $\beta$ . In R code using `rstanarm`, this model is coded as

427 Finally,  $\eta$  captures the difference in log-DNA concentration between upstream and downstream stations  
 428 within a creek; we set  $\eta_{d=1} = 0$  such that the value of  $\eta_{d=2}$  explicitly captures the effect of the culvert within  
 429 a given creek at a given time. The effect of construction in our focal Padden Creek, then, is the change in  $\eta$   
 430 after construction versus prior to construction. We fit this model in a Bayesian framework using moderately  
 431 informative priors on all parameters, and confirmed model convergence ( $\hat{R} < 1.01$ ) across 3 chains and 2500  
 432 model iterations. See statistical supplement (Supplemental Text 2) for prior values, diagnostics, and full  
 433 model details.

434 `stan_glmer(log(observed) ~ (1 + time_idx|creek:species) + (1|station:creek:species:time_idx))`

435 See Supplemental Text 2 for more details on the linear mixed-effects model.

## 436 Results

### 437 Metabarcoding and Quantitative PCR

438 In total, we generated ~52 million reads across all environmental samples and 27 mock community samples (3  
 439 communities x 9 replicates [6 even, 3 skewed proportions]) for calibration (see below). After quality-filtering

440 and merging all runs, ~45 million reads remained from ~24,000 amplicon sequence variants (ASVs) in the  
441 environmental samples, of which ~83% of reads were annotated to species level (per sample: mean = 82%,  
442 median = 93%, min = 0%, max = 99.99% of reads annotated). We only focus on the metabarcoding data  
443 from four salmonids for the remainder of this paper. The four salmonids represent ~54% of all reads and  
444 ~64% of the annotated reads found in environmental samples.

445 In the mock community samples, 98.7% of the ~5 million reads after quality filtering were annotated to  
446 species level. Importantly, the target salmonid ASVs in the mock communities were found in environmental  
447 samples, unambiguously linking the taxa in calibration samples with those in environmental samples. The  
448 most common salmonid species found in the environmental samples was cutthroat trout (*O. clarkii*), which  
449 was found in ~85% of samples, followed by coho salmon (*O. kisutch*) found in ~62% of samples, then  
450 rainbow/steelhead trout (*O. mykiss*) found in ~40% of samples, and finally sockeye/kokanee salmon (*O.  
451 nerka*) found in ~10% of samples. Over half of the samples across all times, creeks, and stations had at least  
452 50% of reads assigned to cutthroat trout.

453 After calibrating metabarcoding data using mock communities (See Supplemental Texts 1 and 2), we estimated  
454 the salmonid composition across time points, creeks, and stations (Figure ??Figures 4 and 5). The culvert in  
455 one control creek (Barnes) appeared to be nearly a total barrier to salmonid passage, with salmonid eDNA  
456 detected upstream of the culvert at only three time points, in contrast to being detected at every time point  
457 in the downstream station of the same creek. The other four creeks had no such pattern associated with  
458 the culverts, suggesting that fish passage may have been possible through the culverts, or that there were  
459 resident populations upstream of the culverts.

460 All environmental samples were quantified for absolute concentrations of cutthroat trout DNA across 30–32  
461 qPCR plates, resulting in 280 samples (~80–630 samples (~60%)) with a positive detection in at least 1 of 3  
462 technical replicates. The modeled output of cutthroat trout DNA concentrations, ranged from 10–50 copies/L  
463 to  $1.4 \times 10^6$  copies/L, with a mean value of ~58,000 copies/L (Figure 6).

464 We combined compositional information from metabarcoding with absolute concentrations from qPCR for  
465 our reference species, cutthroat trout (*O. clarkii*, from the qPCR), to estimate the total concentration of  
466 DNA for each species (See Supplemental Text 2). The joint time-series model shared information across  
467 stations and creeks; consequently, data from one of the control creeks (Barnes) could not be included because  
468 of the nearly total absence of salmonids upstream of its culvert. However, data from the remaining creeks  
469 characterized trends in the other. These quantitative data for all four target species well and could be modeled  
470 appropriately were then used in the linear mixed-effects model to assess salmonid trends over time (Figure

471 7).

472 **Effects of Culverts**

473 Before considering the effect of construction, the difference in abundance trends between upstream and  
474 downstream stations (Figure 7) demonstrates that the culverts themselves have some effect, but generally not  
475 a large effect on the salmonid species surveyed. Therefore, these four creeks (which include 3 culverts and  
476 one bridge) do not seem to be blocking salmonid passage. A notable exception was Barnes Creek,  
477 which was not included in the time series model, as the culvert was so clearly a barrier as most time points  
478 had no salmonid DNA upstream and therefore models including Barnes do not converge as a result of the  
479 large fraction of sampling points with no observations of salmonids. (Figure ??). Padden Creek upstream  
480 of I-5 also was more clearly a barrier to fish passage, while the culvert across SR-11 seemed to be less of a  
481 barrier to fish passage. In other cases, salmonid DNA is found upstream but not downstream, indicating  
482 that the culvert is likely not a barrier and there are resident individuals upstream of the culvert.

483 Summarizing over all species and the four creeks used in the time series model, the effect was largest  
484 during the dry periods of late summer / early fall (July to October), when flows were at a minimum (i.e.,  
485 September) and the connectivity culvert effect was minimal (Figure 8); the average log-fold change between  
486 upstream and downstream was low (Figure 8). Salmonid species DNA concentrations were higher upstream  
487 than downstream during this period, with mean upstream DNA concentrations only about 5% higher than  
488 downstream DNA concentrations. sites was not significantly different from zero. Individual species' patterns  
489 were similar, indicating that there is not a species-specific effect where culverts block the passage of  
490 some salmon but not others (Supplemental Figure 17). A notable exception is *O. kisutch* in Chuckanut  
491 Creek, which was overall much more variable where salmonid DNA concentrations were up to 30% higher  
492 upstream than downstream at certain time points and up to 44% higher downstream and upstream at others.  
493 Across all species (Figure S1.12). The maximum positive log-fold change (i.e., upstream having a higher mass  
494 flow rate) was 2.78 in Squalicum Creek for coho salmon (*O. kisutch*) in August 2021, while the maximum  
495 negative log-fold change (i.e., downstream having a higher mass flow rate) was -1.11 found in Squalicum  
496 Creek for cutthroat trout (*O. clarkii*) in December 2021 (Figure S1.12). Of all species, creeks, and time  
497 points, Squalicum Creek had the lowest mean percent difference in upstream and downstream salmonid DNA  
498 concentrations. 23 of the 151 observations were within a log-fold change of -0.1 to 0.1, which corresponds with  
499 eDNA mass flow rates upstream within 10% of mass flow rates downstream.

500 We also considered how the log-fold change in eDNA mass flow rates were impacted by the flow rate or  
501 discharge of the creek itself (Figure 9. We found that at months of the lowest flow (summer months), the

502 log-fold changes between mass flow rates were the highest, while in winter months with highest discharge  
503 the log-fold changes were lower and downstream sites often had higher eDNA mass flow rates than upstream  
504 sites (Figure S1.12).

## 505 Effects of Culvert Replacement

506 Fish were excluded from Padden Creek on August 30th, 2021 in preparation for the stream to be diverted  
507 on September 9th, 2021 and the diversion was removed October 7th, 2021. Water sampling occurred on  
508 September 10th, 2021, the day after the diversion, and on October 12, 2021, just 5 days after reconnecting  
509 the stream (Supplemental Figure 3). By comparing the difference in upstream and downstream concentrations  
510 mass flow rates before and after construction in Padden Creek, we can assess how large of an impact the  
511 replacement two culvert replacements had on salmonid species.

512 (Figure 10). The effects of the culvert replacement operation operations appeared to have been transient  
513 and fairly minor for the four salmonid species surveyed. After the beginning of construction in September  
514 2021 through the end of sampling in February 2022, we We saw very minor fluctuations in the difference  
515 between upstream and downstream salmonid DNA concentrations mass flow rates, and did not see an increase  
516 in this difference due to the culvert removal as the log-fold changes in Padden Creek were similar to those  
517 in the control creeks at the same time points (Figure 10, grey shading points vs. no shading). Overall, *O.*  
518 *elarkii* was the least impacted species of the construction while *O. nerka* was the most impacted species,  
519 likely due to the very low concentrations in the creek and the migration timing of *O. nerka* being during and  
520 post-restoration. The mean percent difference across all species prior to construction was 0.18% compared  
521 to 1.6% during and post-construction (Supplemental Table 2 black points in areas of yellow shading).

## 522 Discussion

### 523 Environmental DNA can provide quantitative measurements of environmental impacts

524 Here, we used both eDNA metabarcoding and a single species-specific qPCR assay to rigorously quantify  
525 both the effect of culverts and the impact of a culvert replacement on salmonids two culvert replacements  
526 on salmonids in the same creek. We observed a clear seasonal pattern in the DNA concentrations of four  
527 salmonid species detected in the study. The BACI sampling design and the time series model leveraged shared  
528 information across linear mixed effects model leveraged information across treatment and control creeks to  
529 integrate the change in eDNA concentrations due to time, whether a sample was collected below or above  
530 a barrier (i.e., culvert), and whether or not there was construction occurring. Thus, we could isolate the  
531 changes in eDNA concentrations as a result of the intervention (i.e., construction) while accounting for the

532 variance due to time and station (i.e., season and culvert).

533 A few other studies have used eDNA to measure environmental impacts in rivers and streams. Duda et al.  
534 (2021) used 11 species-specific qPCR assays to document the distribution of resident and migratory fish  
535 after a large dam removal ([Elwha Dam in project \(Elwha River near Port Angeles, Washington\)](#)). No eDNA  
536 sampling was conducted before the dam removal, but the study provided a wealth of information about species  
537 returning after the dam removal, providing a very important dataset to use eDNA to monitor ecological  
538 changes due to human intervention. Similarly, Muha et al. (2017) sampled three locations upstream and three  
539 locations downstream before and after the removal of a weir that was thought to be a barrier to salmonid  
540 migrations. The authors only sampled once before and twice after the removal, spanning about a year, and  
541 used eDNA metabarcoding to look at the presence/absence of species detected. They found that in fact the  
542 before sample demonstrated that the weir was not preventing fish passage (similar to the results found in  
543 this study) and furthermore documented a slight increase in alpha diversity in the first time point after the  
544 barrier removal and then a return to a similar alpha diversity in the second time point after the removal  
545 (similar results found in this study using eDNA concentrations rather than diversity). [Finally Yamanaka](#)  
546 [and Minamoto \(2016\) sampled along a river with three barriers, finding some fish able to cross barriers and](#)  
547 [some not, suggesting that the eDNA can indicated habitat connectivity for fishes across barriers.](#)

548 Importantly, our study demonstrates the value of combining a single qPCR assay with metabarcoding data to  
549 generate quantitative estimates of eDNA concentrations of many species without requiring ~~n-n~~ qPCR assays  
550 for ~~n-n~~ species of interest. Here, we ultimately only quantified the impacts of four species, but importantly,  
551 we did not know *a priori* how many species of interest there might be and we reduced our efforts two fold by  
552 only conducting two assays (one species-specific qPCR and one metabarcoding assay) as opposed to four  
553 assays (four species-specific qPCR assays). This can also be particularly helpful for taxa that don't have a  
554 previously published qPCR assay, but are detected using universal metabarcoding assays. Metabarcoding  
555 data alone only gives compositional data, which cannot be used in a time series to quantify environmental  
556 impacts because there is no information about absolute eDNA concentrations. However, by anchoring or  
557 grounding proportions using a single qPCR assay, the proportional data can be turned into quantitative data.  
558 The species for which to run the qPCR assay can be determined after the metabarcoding is completed; the  
559 most commonly found species with a robust qPCR assay should be used to glean the most information.

## 560 Fish life histories and expected patterns

561 [The four salmonid species in this study have different life histories and behaviors that would impact when fish](#)  
562 [\(and therefore eDNA concentrations\) occur in the creeks. Three of the four species in this study have both](#)

563 freshwater and anadromous populations. Cutthroat trout (*O. clarkii*) encompasses both non-migrating,  
564 resident trout in the creeks and coastal run cutthroat that migrate into Padden Creek from saltwater  
565 (Bellingham Bay). Similarly, *O. nerka* includes both anadromous sockeye salmon and freshwater resident  
566 kokanee salmon and *O. mykiss* includes both anadromous steelhead trout and non-migrating rainbow trout.  
567 Using eDNA, we cannot distinguish between the migrating and non-migrating subspecies of *O. clarkii*, *O.*  
568 *nerka*, and *O. mykiss*. Therefore, our eDNA concentrations might reflect contributions from both migrating  
569 and non-migrating individuals at any given time point in the dataset.

570 For these four anadromous salmonids, the run timings for the migrating populations vary for each species in  
571 the study area (Bellingham, WA). Adult coastal cutthroat (*O. clarkii*) are documented to run throughout the  
572 entire year, whereas coho salmon (*O. kisutch*) run from September to December, sockeye salmon (*O. nerka*)  
573 run from October to December, and steelhead trout (*O. mykiss*) run from November to June. For migrating  
574 coho (*O. kisutch*) and steelhead trout (*O. mykiss*), juveniles may be present in the creeks year-round (Figure  
575 S1.4). eDNA methods at present cannot distinguish adults versus juveniles from DNA found in a water  
576 sample.

577 Despite the mix of migrating and non-migrating populations and various run timings, our metabarcoding  
578 data demonstrate that in Padden Creek, there was a clear signal of *sockeye/kokanee salmon* (*O. nerka*)  
579 both upstream and downstream only in November 2021 - February 2022 (and only upstream in March  
580 2021).—and again in December 2022. This signal corresponds well with the documented run timing of  
581 October to December and the presence of out-migrating juveniles in early spring. In contrast, *cutthroat*  
582 *trout* (*O. clarkii* and) and *coho salmon* (*O. kisutch*) were found nearly year-round in Padden Creek. The  
583 persistent signal from *O. clarkii* could be explained by resident cutthroat trout. However, *O. kisutch* does  
584 not have a resident subspecies and the run timing is only documented from September to December. This  
585 could potentially be due to juveniles maturing and residing in the creeks for 1-2 years after hatching while  
586 adults migrate into the creeks only during the run time to spawn. Visual surveys are conducted rarely and  
587 even if they were conducted, it might be difficult to identify juveniles to species level (e.g., snorkel surveys,  
588 electrofishing, smolt traps) are conducted infrequently to determine adult and juvenile salmonid abundances.  
589 Though *O. kisutch* eDNA was found year round, the highest concentrations were found near the expected  
590 run timing as expected and the life history of *O. kisutch* includes rearing year-round in freshwater. Finally,  
591 though the lowest concentrations on average, *rainbow/steelhead trout* (*O. mykiss*) was also found nearly  
592 year-round in Padden Creek, which could be contributions from migrating steelhead (November to June),  
593 juveniles maturing and migrating, or from resident rainbow trout. Though the *O. mykiss* signal is found  
594 year-round, the highest concentrations do seem to correspond with the steelhead run timing.

595 **Interpreting eDNA with respect to fish abundance and flow**

596 By capturing residual eDNA from water samples, we are measuring a different signal than counting how  
597 many fish are in the creek at each time of sampling. We should not expect the eDNA concentration ~~for each~~  
598 ~~salmonid~~ to directly correlate to the number of fish in the creek at the time of sampling, ~~espeically as we~~  
599 ~~often did not visually see any fish when we took water samples~~. Shelton et al. (2019) ~~provides used~~ a paired  
600 eDNA sampling and seine netting analysis ~~demonstrating to demonstrate~~ that eDNA concentrations provide  
601 a smoothed biological signal over space and time. We acknowledge this smoothing effect and emphasize that  
602 in the context of using eDNA for environmental impact assessments, it is preferable to use a survey technique  
603 such as eDNA that integrates signal across a larger spatial and temporal scale.

604 Many previous papers have commented on the “ecology” of eDNA and the various processes that contribute  
605 to eDNA concentrations in environmental samples (e.g., shedding rates, decay rates, transport) (Barnes and  
606 Turner 2015). For example, higher concentrations of eDNA could be the result of a greater number (or biomass)  
607 of fish present, or increased shedding rates, or decreased decay. Many review papers document the nuances of  
608 interpreting eDNA data and we recommend reviewing them for a deeper understanding (see Andruszkiewicz  
609 Allan et al. (2020) for a review on shedding and decay rates and Harrison et al. (2019) for a review  
610 on transport). Other studies have also documented the relative importance of eDNA transport in streams.  
611 Most notably, Tillotson et al. (2018) measured eDNA at four sites with similar discharge rates to the creeks  
612 in this study and specifically addressed spatial and temporal resolutions, finding that eDNA concentrations  
613 reflect short time- (and therefore length-) scales by comparing peaks in eDNA concentrations to counts of  
614 salmon and accumulation by measuring both upstream and downstream sites. The authors found that the  
615 sampling site furthest downstream did not accumulate eDNA and that two tributaries feeding into a main  
616 channel were additive (Tillotson et al. 2018). For more general models and empirical data documenting  
617 transport distances in streams, see Wilcox et al. (2016), Jane et al. (2014), Jerde et al. (2016), Shogren et  
618 al. (2016), and Civade et al. (2016). Certainly eDNA concentrations can arise from different scenarios and  
619 future work should continue to investigate how to tease apart the nuances of relating eDNA concentrations  
620 to fish abundance.

621 In this study, to assess the impact of a culvert on fish passage, we compare eDNA concentrations upstream  
622 and downstream at the same time point in a given creek. The distance between the upstream and downstream  
623 sampling was minimal (~60-300 m, average distance of ~150 m). Therefore, we assume that the small  
624 differences in spatial and temporal scale between sampling locations is minimal such that the impacts of  
625 these various processes will affect the downstream and upstream concentrations equally.

626 For assessing the impact of construction, we needed to account for differences within the same creek over  
627 time (i.e., before and after construction). Because the sampling occurred over a whole year, transport and  
628 persistence times may have varied. However, the time series model uses information from the control creeks  
629 to understand seasonal trends in eDNA concentrations without needing to link eDNA concentrations to fish  
630 abundance. The impact of construction in Padden Creek can be understood by comparing the measured  
631 eDNA concentration during the time of construction to the expected eDNA concentration in the absence of  
632 construction by using information shared from the four other creeks that are not undergoing construction.  
633 However, we did correct eDNA concentrations mass/volume by discharge volume/time and use a mass flow  
634 rate mass/time for the time series model (see below) given the wide range of discharge over the course of the  
635 year.

636 Though eDNA can move downstream with water flow, here, we were measuring if culverts were barriers to  
637 fish moving upstream, as we were focused on the impact of culverts on migratory salmon. In our case, we  
638 were comparing if downstream stations had higher DNA concentrations than upstream stations as a result  
639 of fish being unable to get upstream. This is of course complicated as a result of non-migratory fish, which  
640 may be up or downstream and not attempting to pass through the culverts. However, the limited spatial  
641 scale between upstream and downstream is such that we can assume the transport would affect upstream  
642 and downstream locations in the same way. That is, in the upstream station, some amount of eDNA is  
643 coming from upstream of that location into the sampling station and leaving at the same time — in the same  
644 way that eDNA would be both entering and exiting the downstream station. Therefore, the relative change  
645 between upstream and downstream stations should be the same in terms of eDNA transport. Additionally,  
646 at almost every single time point for all creeks and species, the upstream DNA concentration is higher than  
647 the downstream DNA concentration. Based on that alone, we do not expect that downstream accumulation  
648 of salmonid DNA is occurring to bias our results of whether fish can pass through these culverts.

649 Other studies have documented the relative importance of eDNA transport in streams. Most notably,  
650 Tillotson et al. (2018) measured eDNA at four sites with similar discharge rates to the creeks in this  
651 study and specifically addressed spatial and temporal resolutions, finding that eDNA concentrations reflect  
652 short time (and therefore length) scales by comparing peaks in eDNA concentrations to counts of salmon  
653 and accumulation by measuring both upstream and downstream sites. The authors found that the sampling  
654 site furthest downstream did not accumulate eDNA and that two tributaries feeding into a main channel  
655 were additive (Tillotson et al. 2018). For more general models and empirical data documenting transport  
656 distances in streams, see Wilcox et al. (2016), Jane et al. (2014), Jerde et al. (2016), Shogren et al. (2016),  
657 and Civade et al. (2016).

658 Finally, it should be noted that Lake Padden, about 1.5 km upstream from the sampling sites, was stocked  
659 with cutthroat trout in January 2021, rainbow trout in April and May 2021, and kokanee salmon in May  
660 2021. Given that no sequencing reads in the metabarcoding data are found for *O. nerka* in May or June after  
661 stocking in May, the potential transport of eDNA downstream from Lake Padden to the location of eDNA  
662 sampling is expected to be negligible. Given the transport distances documented in the literature and flow  
663 rates in Lake Padden, we do not expect the stocking in Lake Padden to affect eDNA concentrations at the  
664 sampling locations.

#### 665 Not all culverts are barriers to salmonids

666 By measuring DNA concentrations of salmonid species above and below culverts on a small spatial scale,  
667 we were able to determine how much of a barrier each culvert was (or was not) to fish passage. We found  
668 by measuring eDNA concentrations that four of the five creeks sampled did not seem to be major barriers  
669 to fish passage. The only creek that was determined to be a Barnes Creek was clearly a very large barrier  
670 to fish passage was Barnes Creek, as we only found salmonid DNA in three months of the twelve months  
671 of sampling, and those three months had very low concentration of salmonid DNA relative to the other  
672 creeks. We note that our sampling occurred only over a single year and future work should monitor culverts  
673 for longer time periods, different species, and different environmental conditions.

674 Of the four creeks where salmonid DNA was consistently found, Chuckanut Creek had the largest  
675 discrepancies between DNA concentrations found below and above the barrier at each time point. The  
676 culvert in Chuckanut Creek is suspected Within the treatment creek (Padden Creek), the SR-11 culvert  
677 did not seem to be a barrier to fish passage and the State of Washington's Department of Transportation  
678 is planning to replace it in the near future. The bridge at Portage Creek and the culvert at Squalicum  
679 Creek were more recently installed as compared to Padden, Chuckanut, and Barnes Creeks. They also were  
680 designated as only partially blocking fish passage, and here we find eDNA results suggest that they were in  
681 fact not major barriers to fish passage. Squalicum Creek had the lowest difference between upstream and  
682 downstream concentrations across all the surveyed creeks, which corresponds well with the classification  
683 that the culvert does not block fish passage. Also, Squalicum Creek is the only creek sampled that has  
684 baffles inside the culvert, which should help fish passage. large barrier, while the I-5 culvert clearly was a  
685 barrier, demonstrated by the difference in salmonid composition and eDNA mass flow rates over the course  
686 of sampling.

687 Here, we find that instances where culverts designated as barriers were likely not blocking fish passage, while  
688 others (Padden I-5 and Barnes Creek) were barriers to fish passage. Importantly, this demonstrates that

689 collecting water samples for eDNA analysis might help to prioritize restoration of culverts suspected to be  
690 barriers to salmonids and provide a new method for post-restoration monitoring to confirm that the barrier  
691 has been corrected and allows for fish passage. Given the large amount of spending and effort required to  
692 replace culverts, this finding is important and emphasizes the potential for new tools for environmental  
693 impact assessments. We note that our sampling occurred only over a short temporal scale and future work  
694 could monitor culverts for longer time periods, different species, and different environmental conditions.

695 **Salmonids can quickly recover from a short-term intervention in a creek**

696 The impact of the construction itself on salmonid species demonstrated construction had remarkably minimal  
697 effects on salmonid DNA concentrations. The disruption of disconnecting Padden Creek in late August the  
698 creek, demolition of the old culvert, installation of the new culvert, and the reconnecting of the creek in  
699 early October 2021 during both culvert replacement events showed almost no change in the difference in  
700 eDNA concentrations between downstream and upstream sampling sites. The differences in the control creeks  
701 between upstream and downstream were often higher than the treatment creek.

702 The construction timing did coincide with natural life history cycles for the salmon species. In the fall an  
703 influx of DNA would be expected not only from adults returning to spawn as they move through the system,  
704 but also from the presence of spawning material in the creek and decaying adults that die post reproduction.  
705 This may explain a portion of the changes in DNA concentrations found here as the construction timing  
706 coincided with run timings of the salmonids, however our time series model accounts for changes in season  
707 in attempt to isolate the effects of the culvert and construction. Regardless, the changes between upstream  
708 and downstream concentrations were very minor across time points and before and after construction. The  
709 post-construction sampling point of the I-5 culvert replacement (only one time point), does show that the  
710 composition of salmonid DNA after replacement is now very similar to the two downstream stations, whereas  
711 before construction compositions were very different (because the culvert was a barrier).

712 This pattern of minimal disruption and quick recovery was consistent for all four species of salmonids, but  
713 the more abundant species seemed to have a dampened effect (i.e., less overall change) compared to the rarer  
714 species (i.e., *O. clarkii* was the least impacted and *O. nerka* was the most impacted). This also corresponds  
715 to species with different life histories and behaviors, and it might be that our most commonly and abundant  
716 species, *O. clarkii*, was more robust to the intervention because it displays both freshwater resident and  
717 saltwater migrating behaviors.

718 Our findings here demonstrate that in addition to the value of using eDNA to select culverts to prioritize for  
719 replacement, sampling during and after construction can provide important information about the impacts

720 (or lack of impacts) on salmonids However, we lack the quantitative analysis as the site upstream of SR-11 and  
721 downstream of I-5 had no quantifiable cutthroat DNA. More time points would help demonstrate the effect  
722 of the culvert replacement. Here we found very minimal effects of both culverts in general and construction,  
723 but that one culvert had very minimal effect on salmonid passage while the other culvert had a large effect  
724 on salmonid passage. We note that these findings are likely not universal and certainly projects need to  
725 monitor comprehensively and quantitatively in order to assess the passability of culverts and impacts of  
726 construction.

## 727 Conclusion

728 It is notoriously difficult to quantify the environmental impact of discrete human impacts on ecosystems and  
729 species. Surveying species and communities by eDNA provides an opportunity for monitoring before, during,  
730 and after impacts in a scaleable and cost-effective way. Here, we demonstrate that monthly eDNA sampling  
731 before, during, and after an intervention alongside control sites for one year can quantify the environmental  
732 impact of replacing a road culvert. We found that in our treatment creek and control sites, four of the  
733 five six barriers did not prohibit salmonid passage and that the culvert replacement. We found that of the  
734 two culvert replacements in the treatment creek, one was a barrier and one was not, but both had minimal  
735 impacts on the four salmonid species monitored over the course of construction. We also provide a framework  
736 in which compositional metabarcoding data can be linked with qPCR data to obtain quantitative estimates of  
737 eDNA concentrations of many species. This provides a practical way to utilize the large amount of information  
738 from metabarcoding data without needing a unique qPCR assay for every species of interest. Environmental  
739 DNA is moving into practice and this study demonstrates how eDNA can be broadly used for environmental  
740 impact assessments for a wide range of species and environments.

## 741 Conflict of Interest Statement

742 The authors declare there are no conflicts of interest.

## 743 Acknowledgements

744 This work was made possible by a grant from the National Philanthropic Trust OceanKind to Ryan Kelly.  
745 The funders had no role in study design, data collection and analysis, decision to publish, or preparation of  
746 the manuscript. Figure 3 was created with BioRender.com. We thank Tammy Schmidt and Susan Kanzler  
747 from Washington Department of Transportation for facilitating access to field sites and providing helpful  
748 feedback throughout the project. We also thank Dr. Jenna McLaughlin, Joe Duprey and Ally Im for help

749 field sampling and Dr. Ramon Gallego, Dr. Kim Parsons, and the University of Washington's Northwest  
750 Genomics Center for sequencing support. Dr. Braeden Van Deynze, Dr. Sunny Jardine, and Dr. Julian Olden  
751 provided helpful insight into culverts and salmonid life histories. We thank Katherine Pearson Maslenikov at  
752 the Burke Museum of Natural History and Culture for providing voucher specimens for Sanger sequencing.  
753 Thanks to Dr. Jameal Samhouri ~~for reviewing the manuscript. A special thank you to and~~ Dr. Chris Sergeant  
754 for ~~providing feedback throughout the project and improving the reviewing the~~ manuscript.

755 **References**

- 756 Andruszkiewicz Allan, E., W. G. Zhang, A. Lavery, and A. Govindarajan. 2020. Environmental DNA  
757 shedding and decay rates from diverse animal forms and thermal regimes. Environmental DNA:edn3.141.  
758 Barnes, M. A., and C. R. Turner. 2015. The ecology of environmental DNA and implications for conservation  
759 genetics. Conservation Genetics 17:117.
- 760 Benedetti-Cecchi, L. 2001. Beyond Baci: Optimization of Environmental Sampling Designs Through  
761 Monitoring and Simulation. Ecological Applications 11:783–799.
- 762 Buxton, A., E. Matechou, J. Griffin, A. Diana, and R. A. Griffiths. 2021. Optimising sampling and analysis  
763 protocols in environmental DNA studies. Scientific Reports 11:11637.
- 764 Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2:  
765 High resolution sample inference from illumina amplicon data. Nature methods 13:581–583.
- 766 Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009.  
767 BLAST+: Architecture and applications. BMC Bioinformatics 10:4219.
- 768 City of Bellingham. 2015. Urban spawner surveys.
- 769 Civade, R. I., T. Dejean, A. Valentini, N. Roset, J.-C. Raymond, A. Bonin, P. Taberlet, and D. Pont. 2016.  
770 Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in  
771 a natural freshwater system. PLOS ONE 11:e015736619.
- 772 De Vargas, C., S. Audie, N. Henry, J. Decelle, F. Mahe, R. Logares, E. Lara, C. Berney, N. Le Bescot, I.  
773 Probert, M. Carmichael, J. Poulain, S. Romac, S. Colin, J.-M. Aury, L. Bittner, S. Chaffron, M. Dunthorn,  
774 S. Engelen, O. Flegontova, L. Guidi, A. Horak, O. Jaillon, G. Lima-Mendez, J. Lukes, S. Malviya, R.  
775 Morard, M. Mulot, E. Scalco, R. Siano, F. Vincent, A. Zingone, C. Dimier, M. Picheral, S. Seaton, S.  
776 Kandels-Lewis, T. O. coordinators, S. G. Acinas, P. Bork, C. Bowler, G. Gorsky, N. Grimsley, P. Hingamp,  
777 D. Iudicone, F. Not, H. Ogata, S. Pesant, J. Raes, M. E. Sieracki, S. Speich, L. Stemmann, S. Sunagawa,  
778 J. Weissenbach, P. Wincker, and E. Karsenti. 2015. Eukaryotic plankton diversity in the sunlit ocean.  
779 Science 348:112.
- 780 Duda, J. J., M. S. Hoy, D. M. Chase, G. R. Pess, S. J. Brenkman, M. M. McHenry, and C. O. Ostberg. 2021.

- 781 Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam  
782 removal. *Environmental DNA* 3:121–141.
- 783 ~~Fish, W. D. of, and Wildlife. 2019a. Fish passage inventory, assessment, and prioritization manual.~~
- 784 ~~pre~~  
~~Fish, W. D. of, and Wildlife. 2019b. Fish passage inventory, assessment, and prioritization manual.~~
- 785 Frankiewicz, P., A. Radecki-Pawlik, A. Wałęga, M. Łapińska, and A. Wojtal-Frankiewicz. 2021. Small  
786 hydraulic structures, big environmental problems: is it possible to mitigate the negative impacts of culverts  
787 on stream biota? *Environmental Reviews* 29:510–528.
- 788 Gloor, G. B., J. M. Macklaim, M. Vu, and A. D. Fernandes. 2016. Compositional uncertainty should not be  
789 ignored in high-throughput sequencing data analysis. *Austrian Journal of Statistics* 45:73–87.
- 790 Gold, Z., A. O. Shelton, H. R. Casendino, J. Duprey, R. Gallego, A. V. Cise, M. Fisher, A. J. Jensen, E.  
791 D'Agnese, E. A. Allan, A. Ramón-Laca, M. Garber-Yonts, M. Labare, K. M. Parsons, and R. P. Kelly.  
792 2023. *Signal and noise in metabarcoding data*. *PLOS ONE* 18:e0285674.
- 793 Harrison, J. B., J. M. Sunday, and S. M. Rogers. 2019. Predicting the fate of eDNA in the environment  
pre  
794 and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*  
795 286:20191409.
- 796 Hinz, S., J. Coston-Guarini, M. Marnane, and J. M. Guarini. 2022. Evaluating eDNA for Use within Marine  
797 Environmental Impact Assessments. *Journal of Marine Science and Engineering* 10:375.
- 798 Hoshino, T., R. Nakao, H. Doi, and T. Minamoto. 2021. Simultaneous absolute quantification and sequencing of fish  
799 environmental DNA in a mesocosm by quantitative sequencing technique. *Scientific Reports* 11:4372.
- 800 Jane, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. H. Lowe, B. H. Letcher, and  
801 A. R. Whiteley. 2014. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams.  
802 *Molecular Ecology Resources* 15:216227.
- 803 Jerde, C. L., B. P. Olds, A. J. Shogren, E. A. Andruszkiewicz, A. R. Mahon, D. Bolster, and J. L. Tank.  
804 2016. Influence of stream bottom substrate on retention and transport of vertebrate environmental DNA.  
805 *Environmental Science & Technology* 50:87708779.
- 806 Kelly, R. P., C. J. Closek, J. L. O'Donnell, J. E. Kralj, A. O. Shelton, and J. F. Samhouri. 2017. Genetic and  
807 manual survey methods yield different and complementary views of an ecosystem. *Frontiers in Marine  
808 Science* 3:73511.
- 809 Kelly, R. P., J. A. Port, K. M. Yamahara, and L. B. Crowder. 2014. Using environmental DNA to census  
810 marine fishes in a large mesocosm. *PLOS ONE* 9:e8617511.
- 811 Kelly, R. P., A. O. Shelton, and R. Gallego. 2019. Understanding PCR Processes to Draw Meaningful  
812 Conclusions from Environmental DNA Studies. *Scientific Reports* 9:12133.

- 813 Klein, S. G., N. R. Gerald, A. Anton, S. Schmidt-Roach, M. Ziegler, M. J. Cziesielski, C. Martin, N. Rädecker,  
814 T. L. Frölicher, P. J. Mumby, J. M. Pandolfi, D. J. Suggett, C. R. Voolstra, M. Aranda, and Carlos. M.  
815 Duarte. 2022. Projecting coral responses to intensifying marine heatwaves under ocean acidification.  
816 *Global Change Biology* 28:1753–1765.
- 817 Lackey, R. 2003. Pacific Northwest Salmon: Forecasting Their Status in 2100. *Reviews in Fisheries Science*  
818 11:35–88.
- 819 Leray, M., J. Y. Yang, C. P. Meyer, S. C. Mills, N. Agudelo, V. Ranwez, J. T. Boehm, and R. J. Machida. 2013.  
820 A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding  
821 metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology* 10:114.
- 822 Long, J. W., and F. K. Lake. 2018. Escaping social-ecological traps through tribal stewardship on national  
823 forest lands in the pacific northwest, united states of america. *Ecology and Society* 23.
- 824 Maasri, A., S. C. Jähnig, M. C. Adamescu, R. Adrian, C. Baigun, D. J. Baird, A. Batista-Morales, N. Bonada,  
825 L. E. Brown, Q. Cai, J. V. Campos-Silva, V. Clausnitzer, T. Contreras-MacBeath, S. J. Cooke, T. Datry,  
826 G. Delacámarra, L. De Meester, K.-D. B. Dijkstra, V. T. Do, S. Domisch, D. Dudgeon, T. Erös, H. Freitag,  
827 J. Freyhof, J. Friedrich, M. Friedrichs-Manthey, J. Geist, M. O. Gessner, P. Goethals, M. Gollock, C.  
828 Gordon, H.-P. Grossart, G. Gulemvuga, P. E. Gutiérrez-Fonseca, P. Haase, D. Hering, H. J. Hahn, C. P.  
829 Hawkins, F. He, J. Heino, V. Hermoso, Z. Hogan, F. Höller, J. M. Jeschke, M. Jiang, R. K. Johnson, G.  
830 Kalinkat, B. K. Karimov, A. Kasangaki, I. A. Kimirei, B. Kohlmann, M. Kuemmerlen, J. J. Kuiper, B.  
831 Kupilas, S. D. Langhans, R. Lansdown, F. Leese, F. S. Magbanua, S. S. Matsuzaki, M. T. Monaghan,  
832 L. Mumladze, J. Muzon, P. A. Mvogo Ndongo, J. C. Nejstgaard, O. Nikitina, C. Ochs, O. N. Odume,  
833 J. J. Opperman, H. Patricio, S. U. Pauls, R. Raghavan, A. Ramírez, B. Rashni, V. Ross-Gillespie, M.  
834 J. Samways, R. B. Schäfer, A. Schmidt-Kloiber, O. Seehausen, D. N. Shah, S. Sharma, J. Soininen, N.  
835 Sommerwerk, J. D. Stockwell, F. Suhling, R. D. Tachamo Shah, R. E. Tharme, J. H. Thorp, D. Tickner,  
836 K. Tockner, J. D. Tonkin, M. Valle, J. Vitule, M. Volk, D. Wang, C. Wolter, and S. Worischka. 2022. A  
837 global agenda for advancing freshwater biodiversity research. *Ecology Letters* 25:255–263.
- 838 MacPherson, L. M., M. G. Sullivan, A. Lee Foote, and C. E. Stevens. 2012. Effects of Culverts on Stream  
839 Fish Assemblages in the Alberta Foothills. *North American Journal of Fisheries Management* 32:480–490.
- 840 Martin, C. J. B., B. J. Allen, and C. G. Lowe. 2012. Environmental impact assessment: Detecting changes in  
841 fish community structure in response to disturbance with an asymmetric multivariate BACI sampling  
842 design. *Bulletin, Southern California Academy of Sciences* 111:119–131.
- 843 Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMB-*  
844 *net.journal* 17:10.
- 845 Martinez, R. 2013. United states v. washington.

- 846 McCall, M. N., H. R. McMurray, H. Land, and A. Almudevar. 2014. On non-detects in qPCR data.  
847 Bioinformatics 30:2310–2316.
- 848 McLaren, M. R., A. D. Willis, and B. J. Callahan. 2019. Consistent and correctable bias in metagenomic  
849 sequencing experiments. eLife 8:e46923.
- 850 Morgan, R. K. 2012. Environmental impact assessment: The state of the art. Impact Assessment and Project  
851 Appraisal 30:5–14.
- 852 Moss, W. E., L. R. Harper, M. A. Davis, C. S. Goldberg, M. M. Smith, and P. T. J. Johnson. 2022.  
853 Navigating the trade-offs between environmental DNA and conventional field surveys for improved  
854 amphibian monitoring. Ecosphere 13:e3941.
- 855 Muha, T. P., M. Rodríguez-Rey, M. Rolla, and E. Tricarico. 2017. Using environmental DNA to improve  
856 species distribution models for freshwater invaders. Frontiers in Ecology and Evolution 5:143957.
- 857 Nathan, L. R., A. A. Smith, A. B. Welsh, and J. C. Vokoun. 2018. Are culvert assessment scores an indicator  
858 of Brook Trout *Salvelinus fontinalis* population fragmentation? Ecological Indicators 84:208–217.
- 859 Ogram, A., G. Sayler, and T. Barkay. 1987. The extraction and purification of microbial DNA from sediments.  
860 Journal of Microbiological Methods 7:5766.
- 861 Ogren, S. A., and C. J. Huckins. 2015. Culvert replacements: improvement of stream biotic integrity?  
862 Restoration Ecology 23:821–828.
- 863 Pont, D., P. Meulenbroek, V. Bammer, T. Dejean, T. Erős, P. Jean, M. Lenhardt, C. Nagel, L. Pekarik,  
864 M. Schabuss, B. C. Stoeckle, E. Stoica, H. Zornig, A. Weigand, and A. Valentini. 2022. Quantitative  
865 monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR.  
866 Molecular Ecology Resources n/a.
- 867 Port, J. A., J. L. O'Donnell, O. C. Romero-Maraccini, P. R. Leary, S. Y. Litvin, K. J. Nickols, K. M.  
868 pre Yamahara, and R. P. Kelly. 2015. Assessing vertebrate biodiversity in a kelp forest ecosystem using  
869 environmental DNA. Molecular Ecology 25:527541.
- 870 Price, D. M., T. Quinn, and R. J. Barnard. 2010. Fish Passage Effectiveness of Recently Constructed Road  
871 Crossing Culverts in the Puget Sound Region of Washington State. North American Journal of Fisheries  
872 Management 30:1110–1125.
- 873 R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical  
874 Computing.
- 875 Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B.  
876 A. Lynch, I. A. MacNeil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman, and  
877 R. M. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional

- 878 diversity of uncultured microorganisms. *Applied and Environmental Microbiology* 66:2541–2547.
- 879 Rubin, Z., G. M. Kondolf, and B. Rios-Touma. 2017. Evaluating Stream Restoration Projects: What Do We  
880 Learn from Monitoring? *Water* 9:174.
- 881 Ruppert, K. M., R. J. Kline, and M. S. Rahman. 2019. Past, present, and future perspectives of environmental  
882 DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global  
883 eDNA. *Global Ecology and Conservation* 17:e00547.
- 884 Schmidhauser, J. R. 1976a. ~~Struggles for cultural survival: The fishing rights of the treaty tribes of the  
Pacific Northwest. *Notre Dame Law* 52:30–40.~~
- 885 pre
- 886 Schmidhauser, J. R. 1976b. ~~Struggles for cultural survival: The fishing rights of the treaty tribes of the  
Pacific Northwest. *Notre Dame Law* 52:30–40.~~
- 887 Seymour, M., F. K. Edwards, B. J. Cosby, I. Bista, P. M. Scarlett, F. L. Brailsford, H. C. Glanville, M. de  
888 Bruyn, G. R. Carvalho, and S. Creer. 2021. Environmental DNA provides higher resolution assessment of  
889 riverine biodiversity and ecosystem function via spatio-temporal nestedness and turnover partitioning.  
890 Communications Biology 4:1–12.
- 891 Shelton, A. O., Z. J. Gold, A. J. Jensen, E. D'Agnese, E. Andruszkiewicz Allan, A. Van Cise, R. Gallego, A.  
892 Ramón-Laca, M. Garber-Yonts, K. Parsons, and R. P. Kelly. 2022. Toward quantitative metabarcoding.  
893 Ecology n/a:e3906.
- 894 Shelton, A. O., R. P. Kelly, J. L. O'Donnell, L. Park, P. Schwenke, C. Greene, R. A. Henderson, and E.  
895 M. Beamer. 2019. Environmental DNA provides quantitative estimates of a threatened salmon species.  
896 Biological Conservation 237:383–391.
- 897 Shelton, A. O., J. L. O'Donnell, J. F. Samhouri, N. C. Lowell, G. D. Williams, and R. P. Kelly. 2016. A  
898 framework for inferring biological communities from environmental DNA:115.
- 899 Shogren, A. J., J. L. Tank, E. A. Andruszkiewicz, B. P. Olds, C. L. Jerde, and D. Bolster. 2016. Modelling  
900 the transport of environmental DNA through a porous substrate using continuous flow-through column  
901 experiments. *Journal of The Royal Society Interface* 13:2016029011.
- 902 Silverman, J. D., R. J. Bloom, S. Jiang, H. K. Durand, E. Dallow, S. Mukherjee, and L. A. David. 2021.  
903 Measuring and mitigating PCR bias in microbiota datasets. *PLoS Computational Biology* 17:e1009113.
- 904 Stan Development Team. 2022. RStan: The r interface to stan.
- 905 Stat, M., M. J. Huggett, R. Bernasconi, J. D. DiBattista, T. E. Berry, S. J. Newman, E. S. Harvey, and M.  
906 Bunce. 2017. Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical  
907 marine environment. *Scientific Reports*:111.
- 908 Taberlet, P., E. Coissac, M. Hajibabaei, and L. H. Rieseberg. 2012. Environmental DNA. *Molecular Ecology*

- 910 21:17891793.
- 911 Thalinger, B., E. Wolf, M. Traugott, and J. Wanzenböck. 2019. Monitoring spawning migrations of  
912 potamodromous fish species via eDNA. *Scientific Reports* 9:15388.
- 913 Thomas, A. C., J. Howard, P. L. Nguyen, T. A. Seimon, and C. S. Goldberg. 2018. ANDe™: A fully  
914 integrated environmental DNA sampling system. *Methods in Ecology and Evolution* 9:13791385.
- 915 Thomas, A. C., P. L. Nguyen, J. Howard, and C. S. Goldberg. 2019. A self-preserving, partially biodegradable  
916 eDNA filter. *Methods in Ecology and Evolution* 10:1136–1141.
- 917 Thomsen, P. F., and E. Willerslev. 2015. Environmental DNA: An emerging tool in conservation for  
918 monitoring past and present biodiversity. *Biological Conservation* 183:418.
- 919 Tillotson, M. D., R. P. Kelly, J. J. Duda, M. Hoy, J. Kralj, and T. P. Quinn. 2018. Concentrations of  
920 environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales.  
921 *Biological Conservation* 220:111.
- 922 Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon. 2007. The  
923 Human Microbiome Project. *Nature* 449:804–810.
- 924 U. S. Geological Survey. 1994. USGS water data for the nation. Retrieved from [<https://waterdata.usgs.gov/nwis>](https://waterdata.usgs.gov/nwis)  
925 <11/30/2022>.
- 926 Underwood, A. J. 1992. Beyond BACI: the detection of environmental impacts on populations in the real,  
927 but variable, world. *Journal of Experimental Marine Biology and Ecology* 161:145–178.
- 928 Underwood, A. J. 1994. On Beyond BACI: Sampling Designs that Might Reliably Detect Environmental  
929 Disturbances. *Ecological Applications* 4:3–15.
- 930 Valentini, A., P. Taberlet, C. Miaud, R. l. Civade, J. E. Herder, P. F. Thomsen, E. Bellemain, A. Besnard,  
931 E. Coissac, F. Boyer, C. Gaboriaud, P. Jean, N. Poulet, N. Roset, G. H. Copp, P. Geniez, D. Pont, C.  
932 Argillier, J.-M. Baudoin, T. Peroux, A. J. Crivelli, A. Olivier, M. Acqueberge, M. Le Brun, P. R. Moller, E.  
933 Willerslev, and T. Dejean. 2016. Next-generation monitoring of aquatic biodiversity using environmental  
934 DNA metabarcoding. *Molecular Ecology* 25:929942.
- 935 Washington Department of Fish and Wildlife. 2019. Fish passage inventory, assessment, and prioritization  
936 manual.
- 937 Wellman, J., D. Combs, and S. B. Cook. 2000. Long-Term Impacts of Bridge and Culvert Construction  
pre  
938 or Replacement on Fish Communities and Sediment Characteristics of Streams. *Journal of Freshwater  
939 Ecology* 15:317–328.
- 940 Wilcox, T. M., K. S. McKelvey, M. K. Young, A. J. Sepulveda, B. B. Shepard, S. F. Jane, A. R. Whiteley, W.  
941 H. Lowe, and M. K. Schwartz. 2016. Understanding environmental DNA detection probabilities: A case

- study using a stream-dwelling char *salvelinus fontinalis*. *Biological Conservation* 194:209216.
- Wilkinson, S. P., S. K. Davy, M. Bunce, and M. Stat. 2018. Taxonomic identification of environmental DNA with informatic sequence classification trees.
- Wood, D. M., A. B. Welsh, and J. Todd Petty. 2018. Genetic Assignment of Brook Trout Reveals Rapid Success of Culvert Restoration in Headwater Streams. *North American Journal of Fisheries Management* 38:991–1003.
- Yamanaka, H., and T. Minamoto. 2016. The use of environmental DNA of fishes as an efficient method of determining habitat connectivity. *Ecological Indicators* 62:147153.

For Review Only

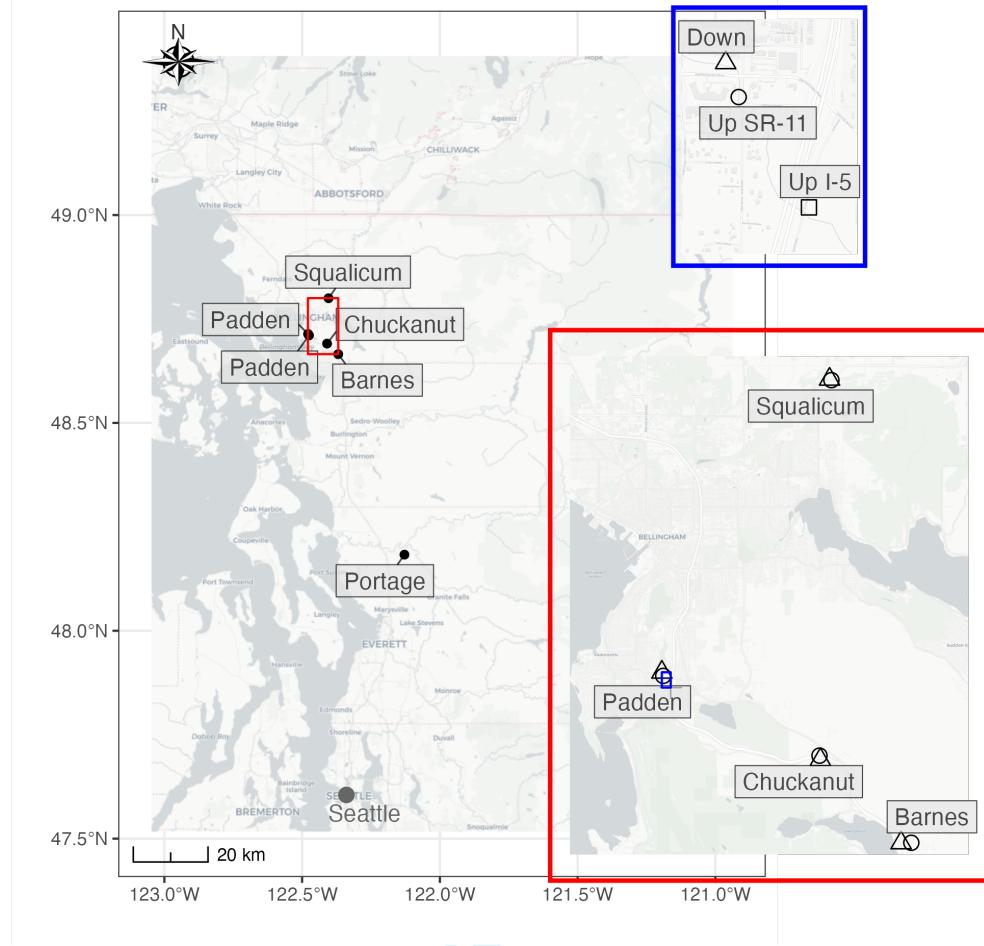
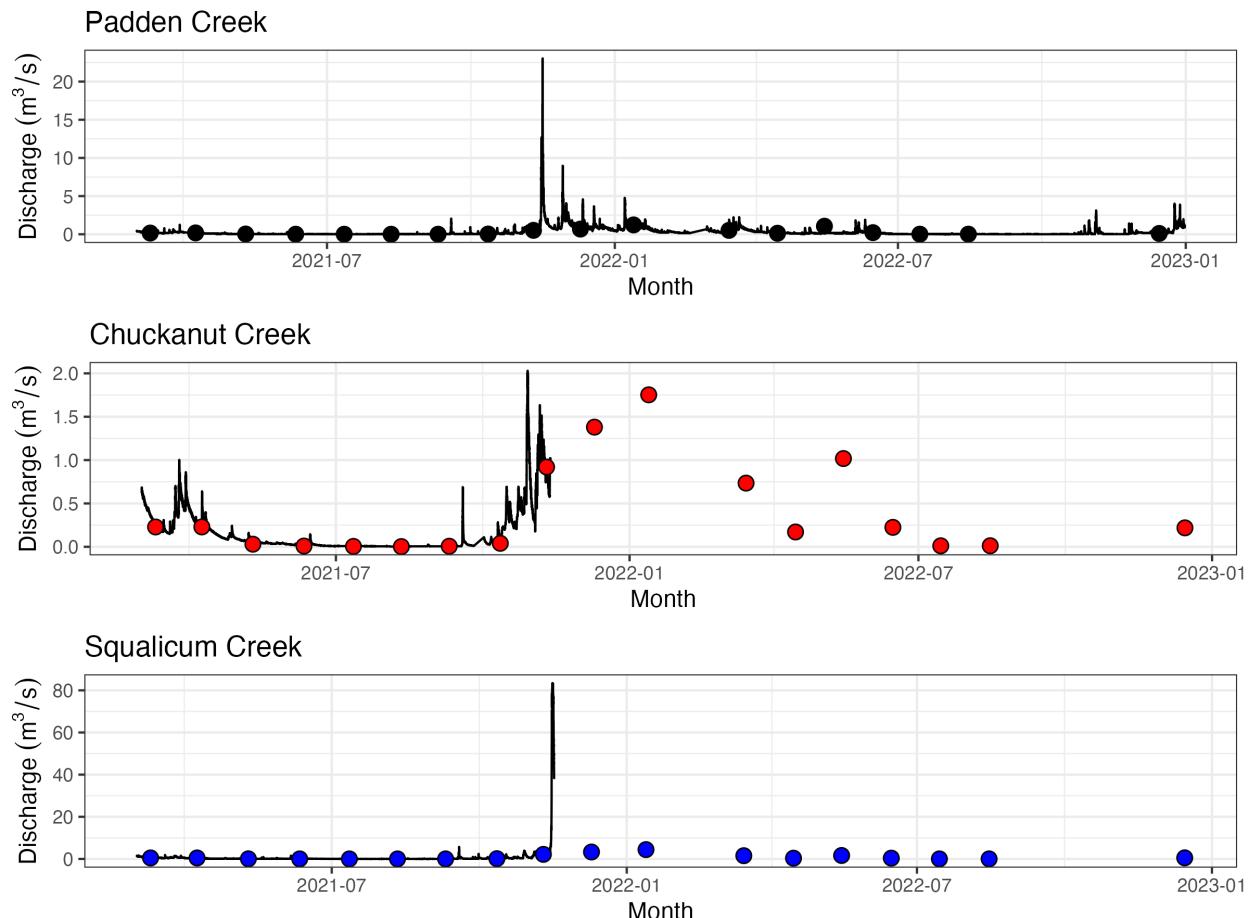


Figure 1: Map of sampling locations near Bellingham, Washington. In the red subset, triangles designate the downstream sampling location and circles designate the upstream sampling location. Padden Creek (the treatment creek where the two culverts were replaced) is shown in the blue subset where downstream is a triangle, upstream of the first culvert (SR-11) is a circle, and upstream of the second culvert (I-5) is a square.



Additionally, in the creek of interest, Padden Creek, rainbow trout (*O. mykiss*) were stocked in Lake Padden, approximately 1.5 km upstream of the sampling sites. Occasionally, cutthroat trout (*O. clarkii*) and kokanee salmon (*O. nerka*) have been stocked in the past as well. During the course of the study, a total of 10 Circles designate the day of sampling. For Padden Creek, 000 rainbow trout were stocked in April and May 2021—the nearest 15 minute interval of flow was used. For Chuckanut and 30 Squalicum Creeks, 000 kokanee salmon were stocked in May 2021 (Supplemental Figure the correction factor from five years of historical data from Padden Creek was used (see methods section and Supplemental Figures 2 and 3)). Despite the stocking of 30,000 kokanee salmon in May in Lake Padden, *O. nerka* was only detected by metabarcoding in March 2021, August 2021, and then November 2021 through February 2022 (see results below). Importantly, this suggests that Lake Padden is far enough upstream that the eDNA signal at the sampling sites by the culvert is not a result of stocking the lake 1.5 km upstream (see discussion for more information). ).

Additionally, in the creek of interest, Padden Creek, rainbow trout (*O. mykiss*) were stocked in Lake Padden, approximately 1.5 km upstream of the sampling sites. Occasionally, cutthroat trout (*O. clarkii*) and kokanee salmon (*O. nerka*) have been stocked in the past as well. During the course of the study, a total of 10 Circles designate the day of sampling. For Padden Creek, 000 rainbow trout were stocked in April and May 2021—the nearest 15 minute interval of flow was used. For Chuckanut and 30 Squalicum Creeks, 000 kokanee salmon were stocked in May 2021 (Supplemental Figure the correction factor from five years of historical data from Padden Creek was used (see methods section and Supplemental Figures 2 and 3)). Despite the stocking of 30,000 kokanee salmon in May in Lake Padden, *O. nerka* was only detected by metabarcoding in March 2021, August 2021, and then November 2021 through February 2022 (see results below). Importantly, this suggests that Lake Padden is far enough upstream that the eDNA signal at the sampling sites by the culvert is not a result of stocking the lake 1.5 km upstream (see discussion for more information). ).

Figure 2: Discharge ( $\text{m}^3/\text{s}$ ) in Padden (USGS Gauge 12201905), Chuckanut (USGS Gauge 12201700), and Squalicum (USGS Gauge 12204010) Creeks over the course of sampling. Open circles show the days when sampling occurred. Gauges at Chuckanut and Squalicum Creek went offline in November 2021 after a major storm event. Portage Creek and Barnes Creek did not have stream gauges.

Additionally, in the creek of interest, Padden Creek, rainbow trout (*O. mykiss*) were stocked in Lake Padden, approximately 1.5 km upstream of the sampling sites. Occasionally, cutthroat trout (*O. clarkii*) and kokanee salmon (*O. nerka*) have been stocked in the past as well. During the course of the study, a total of 10 Circles

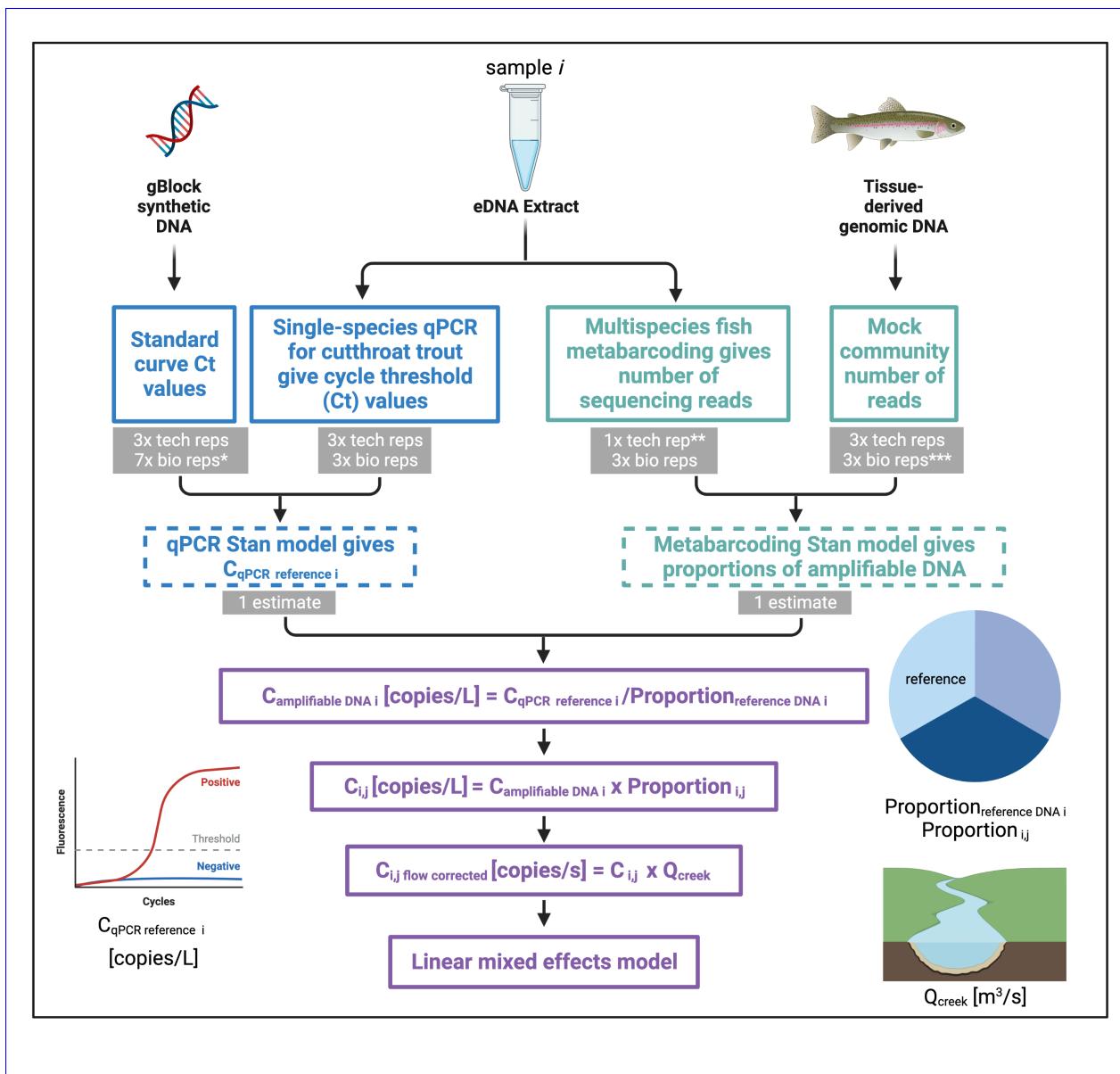


Figure 3: Conceptual figure of different datasets and models used for analyses. \* indicates that here, biological replicates are different dilutions of the synthetic gBlock. \*\* indicates that for most samples, only one technical replicate was sequenced but for one sample per sampling month, three ~~technical~~ technical replicates were sequenced to check for consistency across replicates. \*\*\* indicates that here, the three biological replicates indicate three different mock communities with varying species compositions, but all containing the four salmonids of interest. [Created with BioRender.com](#).

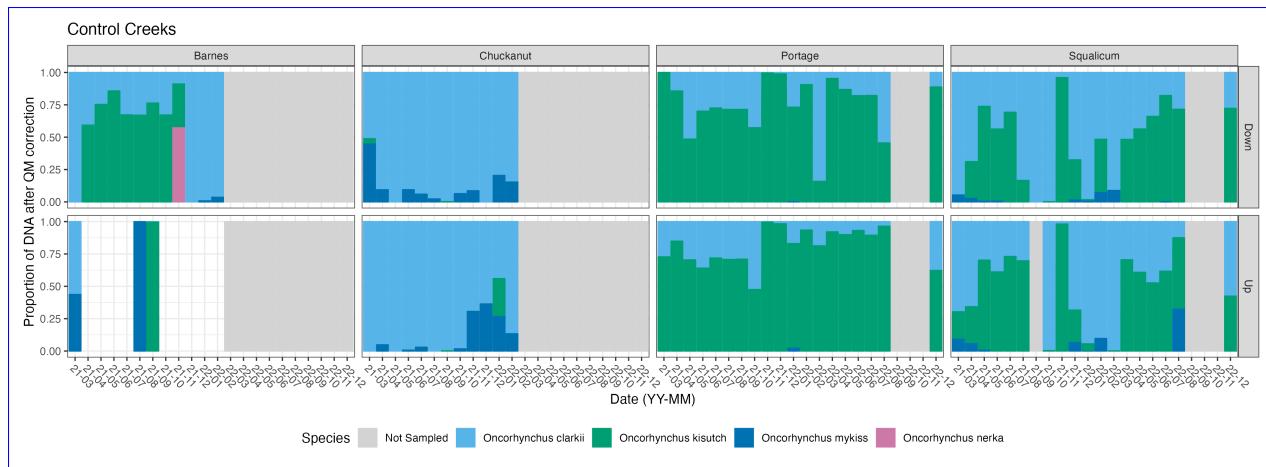


Figure 4: Compositions of salmonid DNA in control creeks as determined by metabarcoding after correction for amplification bias. Note Grey shading denotes time points that no sampling occurred were not sampled (Barnes and Chuckanut Creeks after March 2023 and Squalicum Creek in September 2021 at Squalicum Creek because the creek which was dry). The empty bars in the Barnes upstream sites indicate that no salmonid DNA was found at those time points.

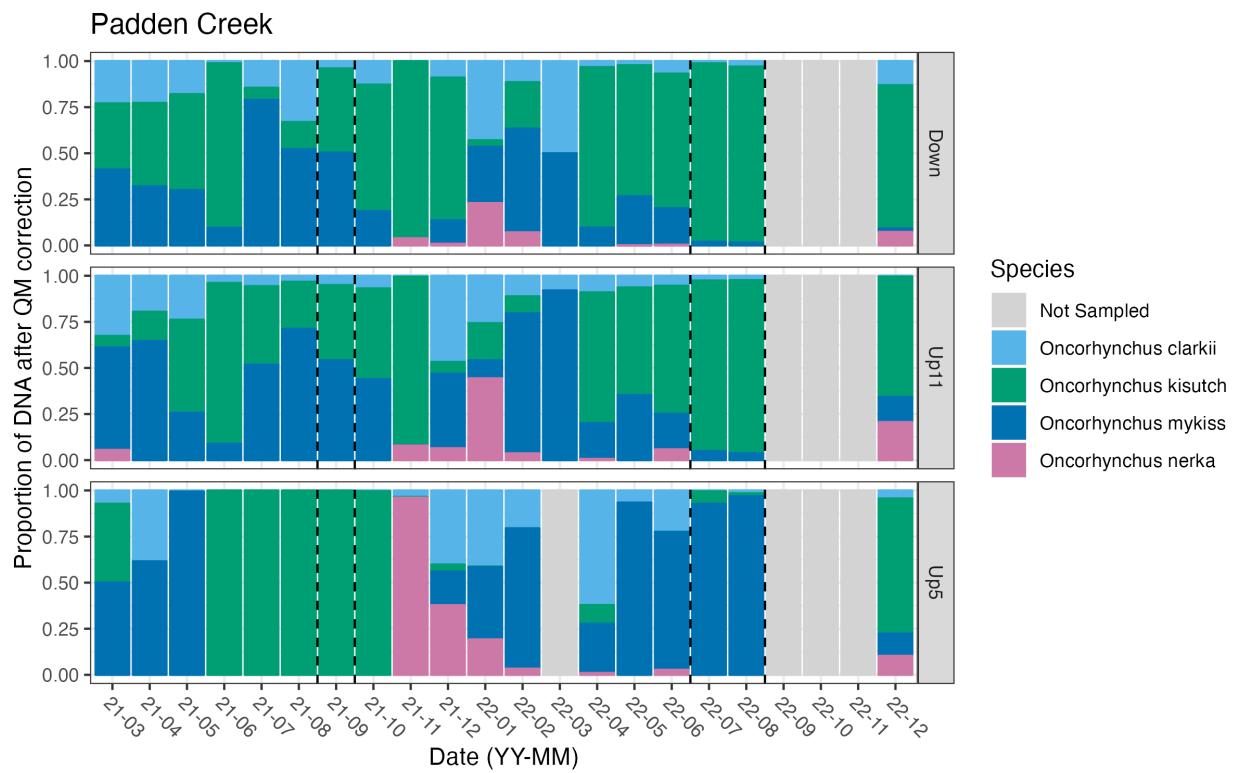


Figure 5: Compositions of salmonid DNA in Padden Creek as determined by metabarcoding after correction for amplification bias. The empty bar in the March 2023 in Up5 indicates that no salmonid DNA was found. The vertical dashed lines indicates the time period in which the culverts were replaced (SR-11 and I-5, respectively).

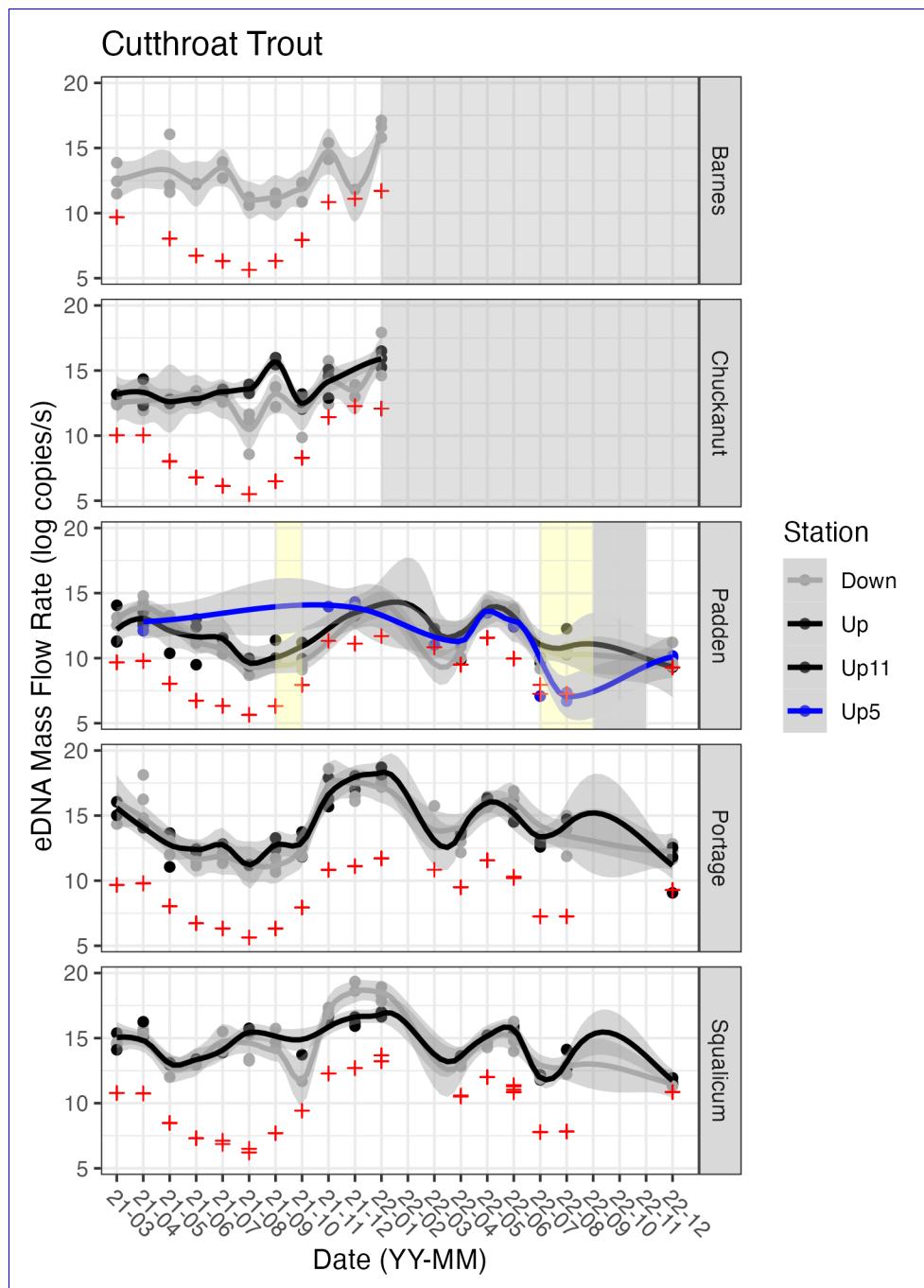


Figure 6: Absolute concentration-mass flow rate (log copies/L of water) of *O. clarkii* (cutthroat trout (*O. clarkii*)) as measured by qPCR before-after flow correction. Note that Barnes Creek and Chuckanut Creek were not sampled after February 2022. Red crosses show the limit of detection for each species and time point, which changes with flow rate and total volume filtered per sample.

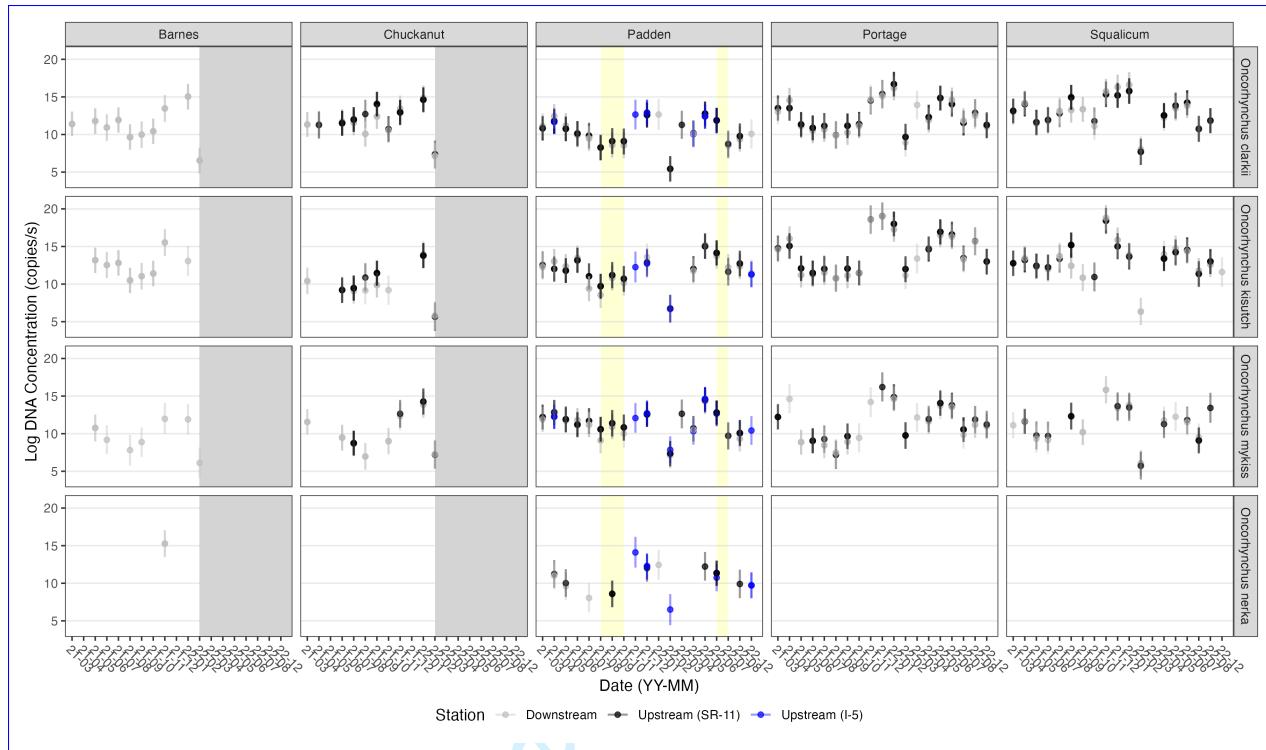


Figure 7: Trends across creeks and across time in mass flow rate (log copies/s) for each of four salmonid species across creeks and across time as estimated by eDNA analysis. Eight colored dots are Points represent posterior means derived by expanding the calibrated metabarcoding proportions as described in the main text; darker-colored dots are posterior means for the time-series linear mixed effects model of and error bars represent the same 95% posterior confidence interval. Colors indicate station upstream (black) or downstream (grey) of the culvert. Padden has an under-road additional sampling site upstream of the second culvert (I-5; blue). 75% and 95% posterior CI plotted for each time point. Grey-Yellow shading indicates the time period in which the culvert culverts in the treatment creek (Padden Creek) was/were replaced. Grey shading indicates time points that were not sampled (Barnes and Chuckanut after February 2022). Time points with no data had no sequencing reads corresponding to that species or no quantifiable cutthroat DNA by qPCR.

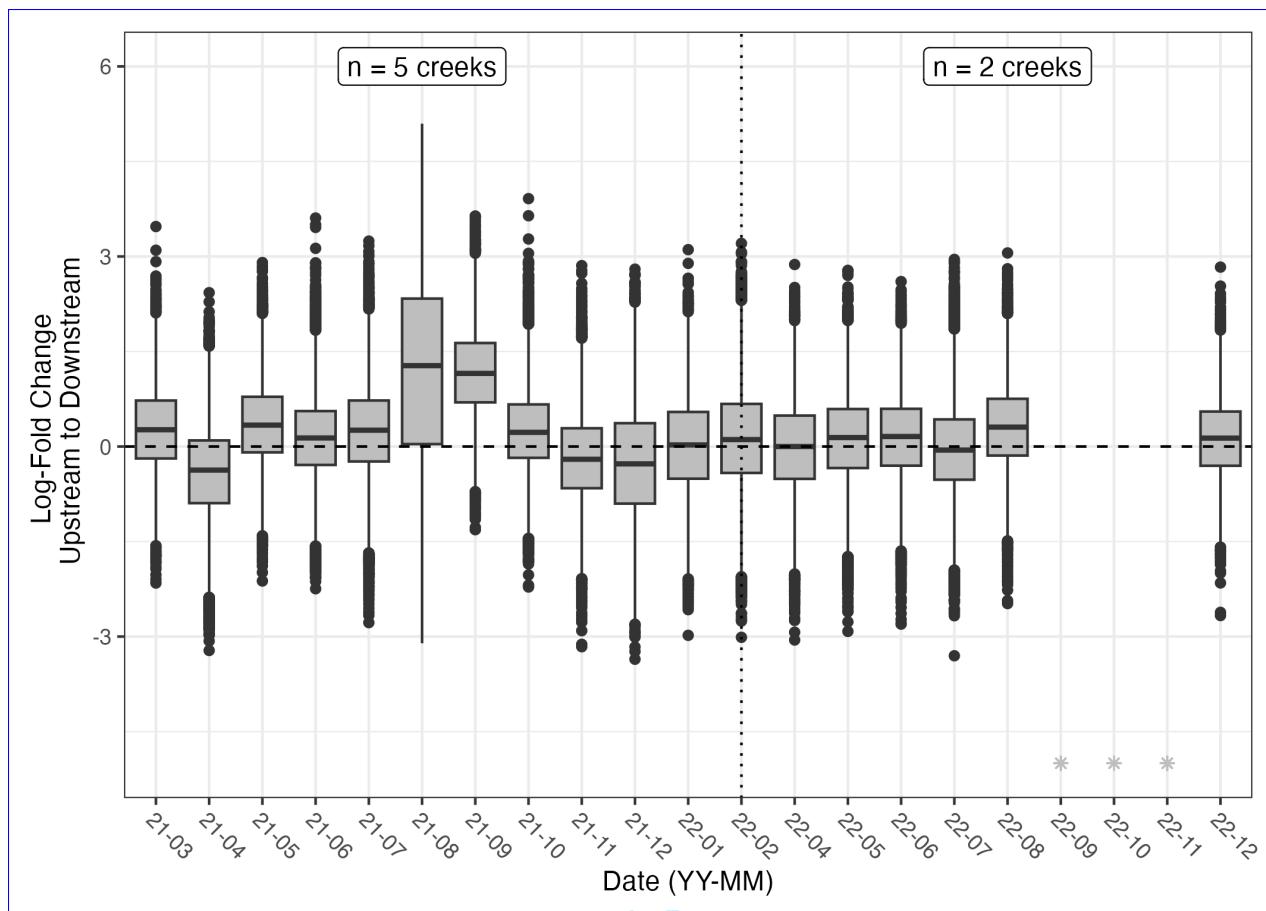


Figure 8: The effect of culvert on salmonid abundance summed across all species and creeks by time. The y-axis shows the difference-log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream concentrations, normalized by downstream-concentrationupstream mass flow rate. The box boundaries correspond to the 25th and 75th percentiles; the whiskers correspond to 1.5 times the interquartile range. Here, negative values imply that eDNA concentrations-mass flow rates are higher downstream than upstream. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators. Grey stars indicate times when no samples were taken.

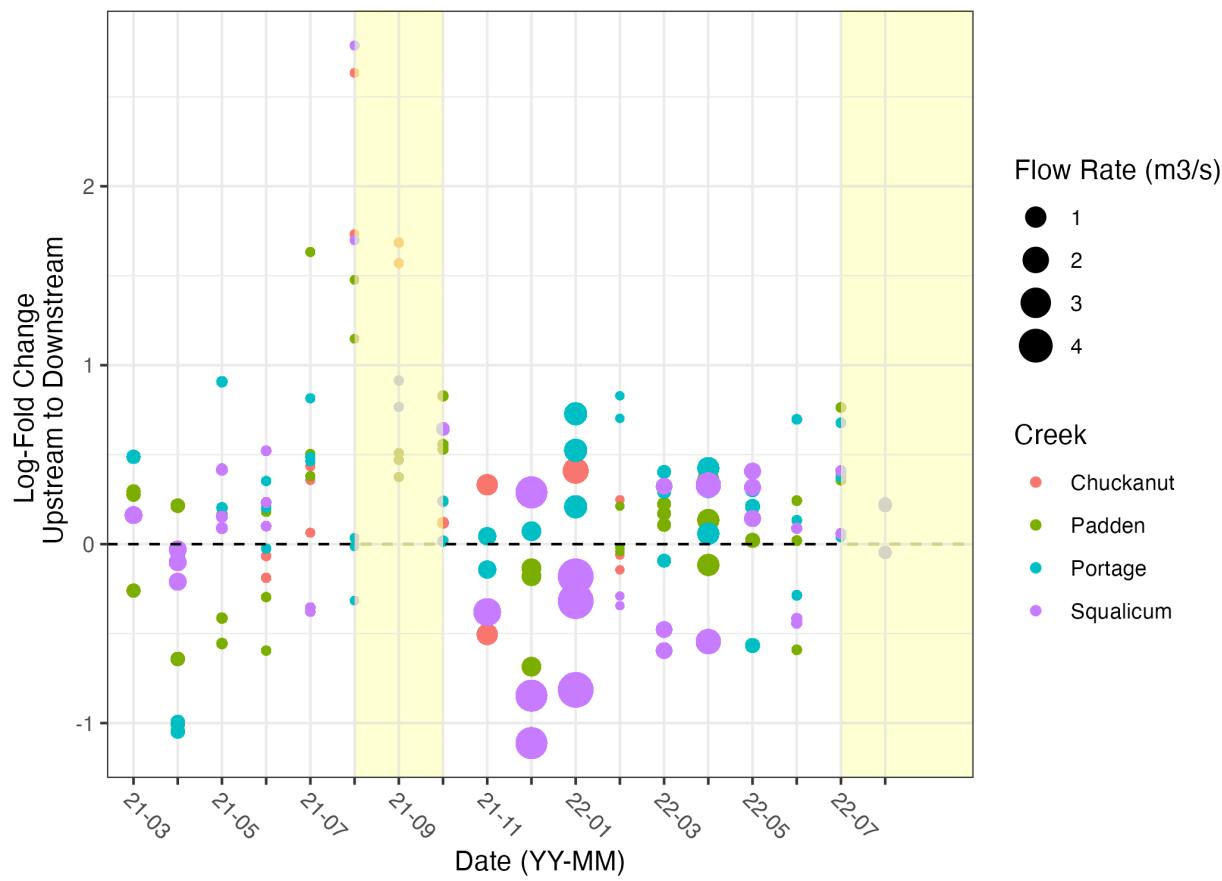


Figure 9: Log-fold change in eDNA mass flow rate over time. Size of circles corresponds to the discharge in each creek at each time point. Color of circles corresponds to each creek. Each creek and time point has up to four circles of the same color for the four salmonid species.

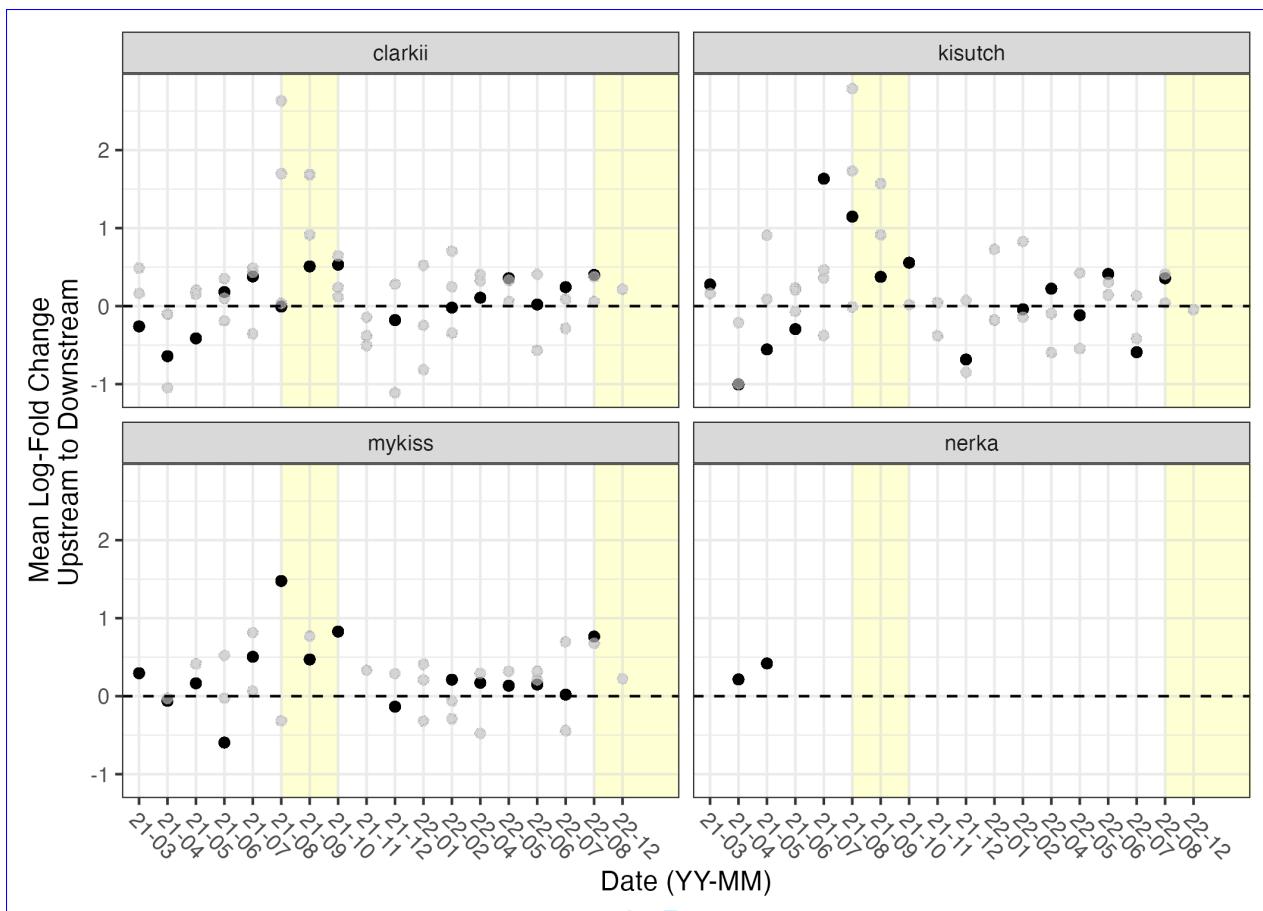


Figure 10: Effect of Construction on Salmonid DNA Concentrations Log-Fold Change in eDNA Flow Rate Upstream to Downstream in Padden Creek. Error bars show 95% confidence intervals of the normalized difference between upstream and downstream DNA concentrations. Grey-Yellow shading shows when construction started through the end for each of sampling the two culverts. Construction ended Grey points show the corresponding log-fold changes in early October 2022. Control creeks and black points show Padden Creek. Sockeye/kokanee salmon (*O. nerka*) was only found in Padden Creek so other creeks are not shown. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators.

1      Quantifying Impacts of an Environmental Intervention Using  
2      Environmental DNA: Supplemental Text 1

3      Elizabeth Andruszkiewicz Allan,      Ryan P. Kelly,      Erin D'Agnese,  
4      Maya Garber-Yonts,      Megan Shaffer,      Zachary Gold,      Andrew O. Shelton

5      2023

6      **Field Sampling**

7      **Site selection and study design**

8      There were two culverts in the treatment creek (Padden) that were suspected to be partially impassible and  
9      thus was removed and replaced during the course of the study; one of the control creeks had a bridge, which  
10     allowed fish passage (Portage), one control creek had a culvert classified as having limited fish passability  
11     (Squalicum), and two control creeks had culverts classified as preventing fish passage (Barnes and Chuckanut)  
12     (Washington Department of Fish and Wildlife 2019). These creeks were chosen due to their comparable size,  
13     flow, watersheds, and species presumed to be present to constrain as many ecological variables as possible.

14     **Distance between sites and flow variability at sites**

15     The average distance between upstream and downstream sampling within a creek was about 160 m; the  
16     largest distance between downstream and upstream sampling was at Barnes Creek, which was approximately  
17     330 m, whereas the shortest distance between sampling was at Squalicum Creek at approximately 66 m.

18     Over the course of the year, flow within each creek varied. USGS flow gauges were located in three of the five  
19     creeks, relatively nearby to the sampling locations (Figure S1.1). The closest gauge to sampling locations was  
20     Padden Creek (~1.5 km); the gauge at Chuckanut Creek was ~5.5 km and the gauge at Squalicum Creek was  
21     ~7.9 km away (calculated using the Haversine distance in R).

22     The flow meters at Squalicum Creek and Chuckanut Creek were offline from November 2021 for the remainder  
23     of the sampling period. The highest discharge seen during the course of the study from January to November

<sup>24</sup> 2021 occurred in November 2021 at Squalicum Creek. The mean discharge in each creek was: 0.42 m<sup>3</sup>/s in  
<sup>25</sup> Padden, 0.29 m<sup>3</sup>/s in Chuckanut, and 1.14 m<sup>3</sup>/s in Squalicum Creek. The lowest discharge registered by the  
<sup>26</sup> flow meters is 0.0028 m<sup>3</sup>/s, which occurred 8.5%, 1.6%, and 0.78% of the time in Padden, Chuckanut, and  
<sup>27</sup> Squalicum, respectively.

<sup>28</sup> Due to the lack of flow data in Squalicum and Chuckanut Creeks from November 2021 to February 2022, we  
<sup>29</sup> used historical data from the three flow gauges to calculate the average discharge for each day of the year  
<sup>30</sup> from about 2015-2021 (Figure S1.2). We then used the value for the day of the year that we sampled in either  
<sup>31</sup> 2021 or 2022 when the gauges were offline. For consistency, we also did this at Padden Creek despite the  
<sup>32</sup> gauge there being online for the entire sampling period.

<sup>33</sup> We compared the different ways one could use flow data to correct the eDNA concentrations. We included  
<sup>34</sup> (1) the value from the closest time point from the gauge to the time point sampling, (2) the average flow  
<sup>35</sup> on the day of sampling from the gauge, (3) the monthly average for the month of sampling from the gauge,  
<sup>36</sup> and (4) the correction factor approach. For (4), the values for Padden Creek represent the same as (1) for  
<sup>37</sup> Padden Creek, the value from the closest time point in the gauge to the time point of sampling. The values  
<sup>38</sup> for Chuckanut and Squalicum Creek are based on the the correction factor from Padden Creek. First, five  
<sup>39</sup> years of historical data (2015-2020) were used to find monthly averages for flow rates for each creek. Because  
<sup>40</sup> the gauges in Squalicum and Chuckanut Creeks stopped metering in 2021, we solved for the ratio of the  
<sup>41</sup> monthly average of each of those creeks to Padden Creek. Then, we used the closest values from Padden  
<sup>42</sup> Creek (1) and multiplied by the monthly correction factor from the 5 years of historical data to find a value  
<sup>43</sup> for Squalicum and Chuckanut Creeks to use for the year of sampling (2021-2022). For all three creeks, we  
<sup>44</sup> demonstrate the relatively small changes in discharge depending on which way flow data were used (Figure  
<sup>45</sup> S1.3). Though in the course of sampling, the discharge in Padden Creek ranged from no metered flow to  
<sup>46</sup> 23 m<sup>3</sup>/s, the discharge on the dates of sampling only reached a maximum of 1.3 m<sup>3</sup>/s. For sites with no  
<sup>47</sup> metered flow, half of the minimum verified discharge of the flow gauge was used (0.0014 m<sup>3</sup>/s).

#### <sup>48</sup> Construction and Fish Exclusion

<sup>49</sup> Fish were excluded from Padden Creek on August 30th, 2021 in preparation for the stream to be diverted  
<sup>50</sup> on September 9th, 2021 and the diversion was removed October 7th, 2021. Water sampling occurred on  
<sup>51</sup> September 10th, 2021, the day after the diversion, and on October 12, 2021, just 5 days after reconnecting  
<sup>52</sup> the stream (Figure S1.4).

53 **Stocking in Lake Padden**

54 Padden Lake has historically been stocked with hatchery fish by the Washington Department of Fish and  
55 Wildlife (Figure S1.4). Rainbow trout (*O. mykiss*) and occasionally cutthroat trout (*O. clarkii*) and kokanee  
56 salmon (*O. nerka*) are stocked in Lake Padden, approximately 1.5 km upstream of the sampling sites. During  
57 the course of the study, rainbow trout were stocked in April 2021 and April 2022, kokanee salmon were  
58 stocked in May 2021 and October 2022, and cutthroat trout were stocked in November 2022 (Figure S1.4).  
59 However, despite the stocking of 30,000 kokanee salmon in May in Lake Padden, *O. nerka* was not detected  
60 by metabarcoding in May 2021 or at any point in 2021 until November (see main text Results). Importantly,  
61 this suggests that Lake Padden is far enough upstream that the eDNA signal at the sampling sites by the  
62 culvert is not a result of stocking the lake 1.5 km upstream (see main text Discussion for more information).

63 **Water Sampling**

64 Water samples were collected using Smith Root's eDNA Backpack (Thomas et al. 2018), a portable pumping-  
65 and-filtering setup set to filter at 1 L/min at 82.7 kPa (12 psi). For most months, a trident sampler was used  
66 to collect all 3 biological replicates at the exact same time, for a total sampling time of about 5 minutes.  
67 Otherwise, the three replicates were collected consecutively, for a total sampling time of about 15 minutes.  
68 Downstream sites were always sampled before upstream sites to ensure no potential DNA was introduced  
69 into the stream before sampling. In some samples, less than 2 L of water was filtered due to clogging (mean  
70 = 1.95 L).

71 **Laboratory Processing**

72 **DNA Extraction, Amplification, Sequencing**

73 Here, we used mock communities to determine the species-specific amplification efficiencies for each salmonid  
74 in the study. Briefly, we constructed five communities with known proportions of starting DNA from different  
75 species (total DNA as measured by Qubit). The communities ranged from having a total of 12 to 20  
76 species, but six salmonid species were included in all five mock communities to have more information on the  
77 amplification efficiencies of salmonids (Supplemental Table 2). We sequenced these communities using the  
78 same metabarcoding primers and thermocycling conditions above and then determined the species-specific  
79 amplification rates given the discrepancy between the known starting proportion and the proportion of  
80 reads after sequencing. The mock community data were then used to correct the sequencing reads from the  
81 environmental samples to estimate the starting DNA proportions of each species in environmental samples,  
82 which is the metric of interest (Figure 3, green boxes). This is the first application of the model to correct

83 eDNA data from water samples with mock community data as described in Shelton et al. (2022) (see  
84 Supplemental Text 2 for more information).

85 All molecular work prior to sequencing was performed at the University of Washington. Bench-tops were  
86 cleaned with 10% bleach for 10 minutes and then wiped with 70% ethanol. Molecular work was separated  
87 onto pre- and post-PCR benches; all DNA extractions and PCR preparation was conducted on a bench where  
88 no PCR product was handled.

### 89 **DNA Extractions**

90 We followed a protocol developed for extracting DNA off the self-preserving Smith Root filters (Thomas et  
91 al. 2019). Filters were removed from their housing with sterile tweezers and cut in half using sterile razor  
92 blades. One half was archived and the other half was used for extraction. DNA was extracted from half of  
93 each filter using a Qiashredder column (Qiagen, USA) and the DNeasy Blood and Tissue Kit (Qiagen, USA)  
94 with an overnight incubation (Thomas et al. (2019)), such that the effective filtering effort was 1 L/sample;  
95 the remaining half of each filter was archived at -20°C. Extracts were eluted in 100 µL of molecular grade  
96 water, quantified via Qubit (Invitrogen, USA) and stored at -20°C until PCR amplification within 2 months  
97 of extraction.

### 98 **PCR Amplification**

99 For the metabarcoding approach, we targeted a ~186 bp hypervariable region of the mitochondrial DNA 12S  
100 rRNA gene for PCR amplification (MiFish; Miya et al. 2015), but using modified primer sequences as given  
101 in Praebel and Wangensteen (unpublished; via personal communication) and including the Illumina Nextera  
102 overhang sequences for subsequent indexing. The primers used were as follows: F 5' *TCGTCGGCAGCGTCA-*  
103 *GATGTGTATAAGAGACAGGCCGGTAAACTCGTGCCAGC* 3', R 5' *GTCTCGTGGGCTCGGAGAT-*  
104 *GTGTATAAGAGACAGCATAGTGGGTATCTAATCCCAGTTG* 3' (*italics* indicate Nextera overhang).  
105 PCR reactions included 10 µL of 5X Platinum ii Buffer, 0.4 µL of Platinum ii Taq, 1.25 µL of 8 mM dNTPS,  
106 1.25 µL of 10 µM F primer, 1.25 µL of 10 µM R primer, 5 µL of template, and 30.85 µL of molecular grade  
107 water, for a total reaction volume of 50 µL. Cycling conditions were as follows: 95°C for 2 min, 35 cycles of  
108 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by a final extension of 72°C for 5 min.

109 Each month of samples was amplified on a single plate with the addition of a no template control (NTC;  
110 molecular grade water in lieu of template) and a positive control (genomic DNA from kangaroo). After PCR  
111 amplification, PCR products were visualized on a 1-2% gel. If no band was present for a given sample, a  
112 new amplification was attempted with extracts diluted 1:10 iteratively until a band was detected. PCR

113 products were size-selected and cleaned using MagBind Beads (Omega Biotek, USA) at a sample:beads ratio  
114 of 1.2. Bead-cleaned PCR products were eluted in 30  $\mu$ L of molecular grade water and quantified via Qubit  
115 (Invitrogen, USA).

116 An indexing PCR reaction added a unique index to each sample using Nextera indices (Illumina, USA) to  
117 allow pooling multiple samples onto the same sequencing run. For indexing, 10 ng of PCR product was used  
118 as template in a final volume of 11.25  $\mu$ L. For samples with concentrations less than 0.88 ng/ $\mu$ L, 11.25  $\mu$ L  
119 was added despite being less than 10 ng of amplicon. Each sample received a unique index; Nextera index  
120 sets A and B were used to avoid using the same index for more than one sample on a single sequencing run.  
121 The PCR reaction included the 11.25  $\mu$ L of template, 12.5  $\mu$ L of Kapa HiFi MMX (Roche, USA), and 1.25  
122  $\mu$ L of indexed primer. Cycling conditions were as follows: 95°C for 5 min, 8 cycles of 98°C for 20 sec, 56°C  
123 for 30 sec, 72°C for 3 min, and a final extension of 72°C for 5 min.

124 Indexed PCR products were also size-selected and purified using MagBind Beads (Omega Biotek, USA) at a  
125 sample:beads ratio of 0.8. Bead-cleaned PCR products were eluted in 30  $\mu$ L of molecular grade water and  
126 quantified via Qubit. Indexed and bead-cleaned products were normalized before pooling into libraries, which  
127 were subsequently quantified via Qubit and visualized on a Bioanalyzer (Agilent, USA) before sequencing.  
128 Samples were randomized in 3-month blocks and each block split across 3 sequencing runs, for a total of  
129 12 sequencing runs. The loading concentration of each library was 4-8 pM and 5-20% PhiX was included  
130 depending on the composition of the run. Sequencing was conducted using an Illumina MiSeq with v3 2x300  
131 chemistry at the NOAA Northwest Fisheries Science Center and the University of Washington's Northwest  
132 Genomics Center.

### 133 Species Specific qPCR

134 We quantified cutthroat trout (*O. clarkii*) DNA in each sample, targeting a 114 bp fragment of the  
135 cytochrome b gene with a qPCR assay (Duda et al. 2021). The primer/probe sequences were: F 5'  
136 CCGCTACAGTCCTCACCTTCTA 3', R 5' GATCTTGATGAGAAGTAAGGATGGAA 3', P 5' 6FAM-  
137 TGAGACAGGATCCAAC-MGB-NFQ 3'. The qPCR assay was multiplexed with TaqMan Exogenous Internal  
138 Positive Control Reagents (EXO-IPC) (Applied Biosystems, USA) to check for the presence of PCR inhibitors  
139 (Duda et al. 2021). Each DNA sample was run in triplicate using Gene Expression Mastermix (ThermoFisher,  
140 USA), a final concentration of 0.375  $\mu$ M F primer, 0.375  $\mu$ M R primer, and 0.105  $\mu$ M probe, as well as 1X  
141 EXO-IPC mix, 1X EXO-IPC DNA, 3.5  $\mu$ L of template for a final reaction volume of 12  $\mu$ L. The EXO-IPC  
142 mix includes the primers and probe for the EXI-IPC DNA, with the probe having a VIC reporter, allowing it  
143 to be multiplexed with the *O. clarkii* assay, which has a FAM reporter. All qPCRs were conducted on an

<sup>144</sup> Applied Biosystems StepOnePlus thermocycler.

<sup>145</sup> Thermocycling was as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 60°C  
<sup>146</sup> for 1 min. The cycle threshold (Ct) value determined for the EXO-IPC assay from the NTC was compared  
<sup>147</sup> to the Ct value for the EXO-IPC assay in each of the environmental samples. If the Ct value was >0.5 Ct  
<sup>148</sup> values from the mean Ct for the NTCs, the sample was deemed inhibited and diluted 1:10 and re-assayed  
<sup>149</sup> until the Ct value fell within the accepted range. After converting Ct values to DNA concentrations using  
<sup>150</sup> the standard curve (see below), the concentration was multiplied by the dilution factor.

<sup>151</sup> Each plate included a 8-point standard curve created using synthetic DNA (gBlocks) at the following  
<sup>152</sup> concentrations: 100,000 copies/µL, 10,000 copies/µL, 1,000 copies/µL, 100 copies/µL, 10 copies/µL, 5  
<sup>153</sup> copies/µL, 3 copies/µL, 1 copy/µL Additionally, six no template controls (NTCs) were included on each  
<sup>154</sup> plate: 3 with the IPC DNA mix and 3 with molecular grade water instead of template or IPC DNA mix.  
<sup>155</sup> Plates were re-run if efficiency as determined by the standard curve was outside of the range of 90-110%.

<sup>156</sup> To check for inhibition, the cycle threshold (Ct) value determined for the EXO-IPC assay from the NTC  
<sup>157</sup> was compared to the Ct value for the EXO-IPC assay in each of the environmental samples. If the Ct value  
<sup>158</sup> was >0.5 Ct values from the mean Ct for the NTCs, the sample was deemed inhibited and diluted 1:10  
<sup>159</sup> and re-assayed until the Ct value fell within the accepted range. The majority of environmental samples  
<sup>160</sup> (60%) were inhibited and accordingly diluted for analysis. In 80% of inhibited samples, a 1:10 dilution or less  
<sup>161</sup> remedied the inhibition, but some samples (11%) required dilution by a factor of up to 1000.

## <sup>162</sup> Bioinformatics Processing

<sup>163</sup> Primers were removed with cutadapt (Martin 2011) and then reads were de-noised, filtered, merged, and  
<sup>164</sup> ASVs were generated using dada2 (Callahan et al. 2016). For each MiSeq run, the trimming lengths were  
<sup>165</sup> determined by visually assessing the quality score plots. After ASVs were generated, taxonomy was assigned  
<sup>166</sup> using the “classify” function in the insect package in R using the classifier published by the authors of the  
<sup>167</sup> package (Wilkinson et al. 2018).

## <sup>168</sup> Quality Controls

<sup>169</sup> Positive controls were included on each sequencing run to monitor for cross contamination that might have  
<sup>170</sup> occurred in the laboratory or due to “tag jumping”. With 13 MiSeq runs, we included one sample of kangaroo  
<sup>171</sup> tissue on each run and then measured how many reads of kangaroo were found in environmental samples and  
<sup>172</sup> how many reads of non-kangaroo were found in kangaroo samples (Figure S1.5).

173 We can also check to make sure that no reads assigning to kangaroo were in the environmental samples. We  
174 only found kangaroo in two environmental samples, both of which were a very small number (and proportion)  
175 of reads (2 and 136 reads found in samples with 40,425 and 28,725 reads respectively) (Figure S1.6).

176 **Annotation**

177 We first used a tree-based annotation method (insect package) and then followed up with a BLAST search  
178 for all ASVs that were not annotated to species level by insect. The percent of reads annotated did not  
179 correlate with sample read depth, creek, station, or month of sampling. Read depth across samples ranged  
180 from 1,011 to 311,879, with a mean of 79,709 and median of 75,967 reads (Figure S1.7). With a total of  
181 ~565 samples, 93% of samples had >20,000 reads and 65% of samples had over 50,000 reads. There did  
182 not seem to be a pattern with samples of low reads with creek or time. Additionally, of the low read depth  
183 samples (<20,000 reads, 40 samples), there was only one sample in which all three replicates were low (March  
184 2022 Squalicum Upstream), meaning that it is very unlikely that low read depth samples would have lead to  
185 changing ecological results.

186 A total of 81 unique species were identified in the environmental samples by the MiFish primers, including 25  
187 fish, 25 mammals, 23 birds , and 8 amphibians (Figure S1.8 and S1.9; see also Supplemental Table 1). Of the  
188 81 species, 17 only were found in a single environmental sample. The three most commonly found species  
189 were coho salmon (*O. kisutch*), cutthroat trout (*O. clarkii*), and rainbow trout (*O. mykiss*).

190 **Correcting metabarcoding data for amplification bias**

191 Using our six mock communities (three different taxa compositions at two different proportions [even and  
192 skewed]), we can first check how well the quantitative metabarcoding model corrects for amplification bias.  
193 In one case, we consider the even mock communities as the mock community data and the skewed mock  
194 communities as unknown. We can then re-create what the model believes to be the original starting proportions  
195 of the skewed mock community given the proportions of reads found in the skewed mock communities and  
196 the proportion of DNA as compared to the proportion of reads found in the even mock communities. We can  
197 also do the same treating the skewed mock communities as known and even mock communities as unknown  
198 (Figure S1.10).

199 We can also check how well the calibration is working by comparing the alpha values by using different subsets  
200 of mock community data as true and unknown (Figure S1.11). We can then use the mock communities to  
201 correct the data from the MiSeq to account for the different alpha values. The corrected results are shown in  
202 the main text as Figure 4.

203 **Species-specific Effect of Culverts**

204 In the main text, we show the effect of culverts averaged over creeks and species (Figure 8). Here, we show  
205 them separated by species and creek (Figure S1.12).

206 **References**

- 207 Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2:  
208 High resolution sample inference from illumina amplicon data. *Nature methods* 13:581–583.
- 209 Duda, J. J., M. S. Hoy, D. M. Chase, G. R. Pess, S. J. Brenkman, M. M. McHenry, and C. O. Ostberg. 2021.  
210 Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam  
211 removal. *Environmental DNA* 3:121–141.
- 212 Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMB-*  
213 *net.journal* 17:10.
- 214 Shelton, A. O., Z. J. Gold, A. J. Jensen, E. D'Agnese, E. Andruszkiewicz Allan, A. Van Cise, R. Gallego, A.  
215 Ramón-Laca, M. Garber-Yonts, K. Parsons, and R. P. Kelly. 2022. Toward quantitative metabarcoding.  
216 *Ecology* n/a:e3906.
- 217 Thomas, A. C., J. Howard, P. L. Nguyen, T. A. Seimon, and C. S. Goldberg. 2018. ANDe™: A fully  
218 integrated environmental DNA sampling system. *Methods in Ecology and Evolution* 9:13791385.
- 219 Thomas, A. C., P. L. Nguyen, J. Howard, and C. S. Goldberg. 2019. A self-preserving, partially biodegradable  
220 eDNA filter. *Methods in Ecology and Evolution* 10:1136–1141.
- 221 Washington Department of Fish and Wildlife. 2019. Fish passage inventory, assessment, and prioritization  
222 manual.
- 223 Wilkinson, S. P., S. K. Davy, M. Bunce, and M. Stat. 2018. Taxonomic identification of environmental DNA  
224 with informatic sequence classification trees.

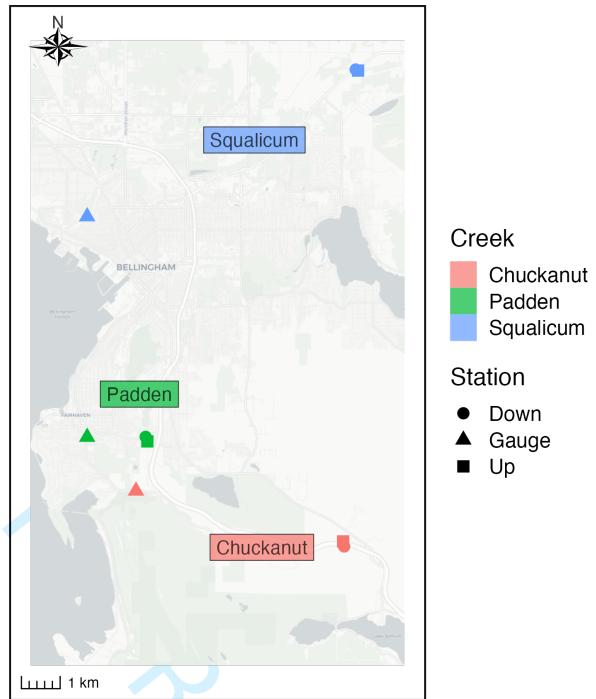


Figure S1.1. Location of flow gauges compared to sampling locations for Chuckanut, Padden, and Squalicum Creeks.

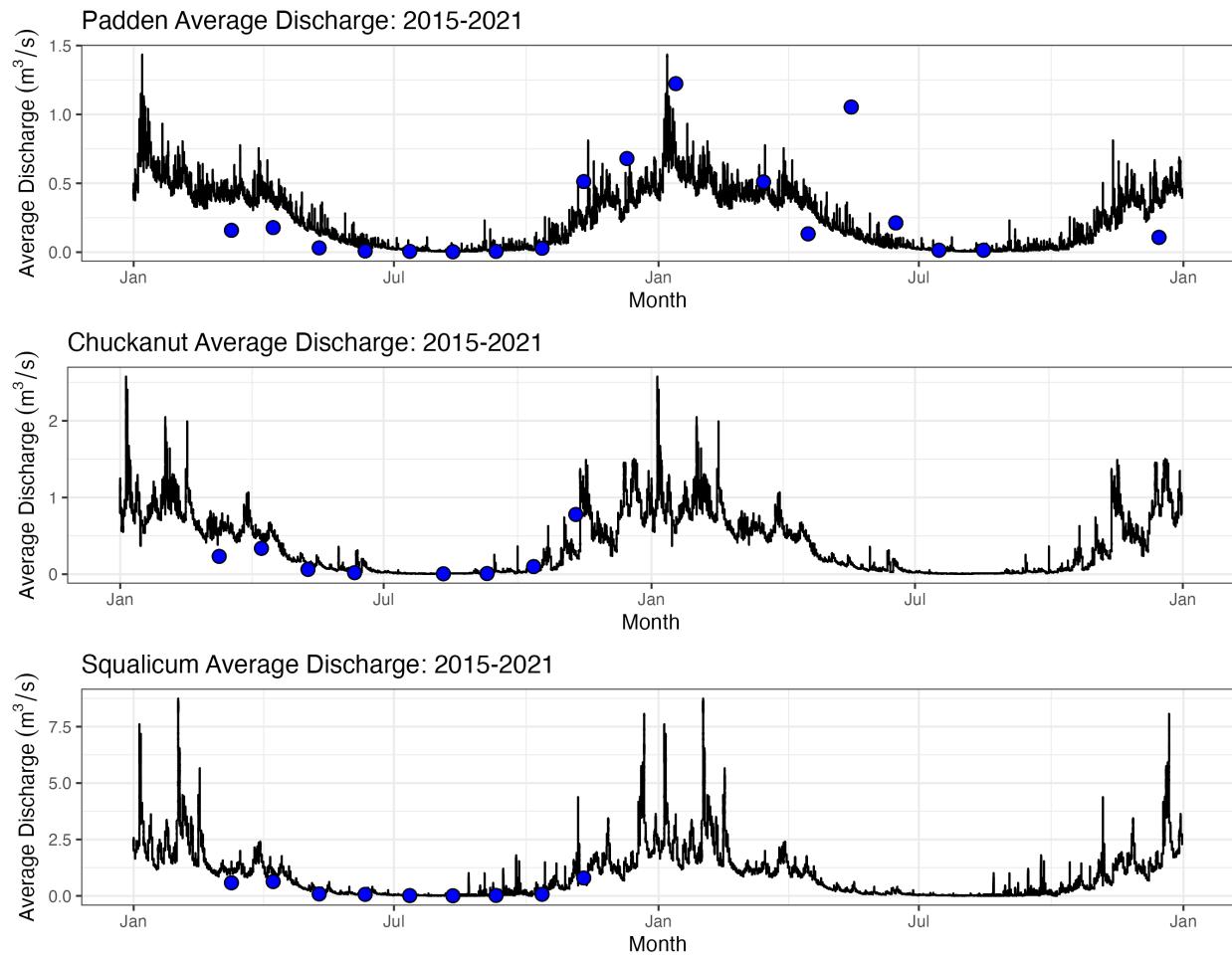


Figure S1.2. Daily average discharge from 2015-2020 in creeks from USGS gauges. Blue dots show the discharge time of sampling during the course of this study (2021-2022) for time points where gauges were online (note the missing data points after December through February in Chuckanut and Squalicum Creeks).

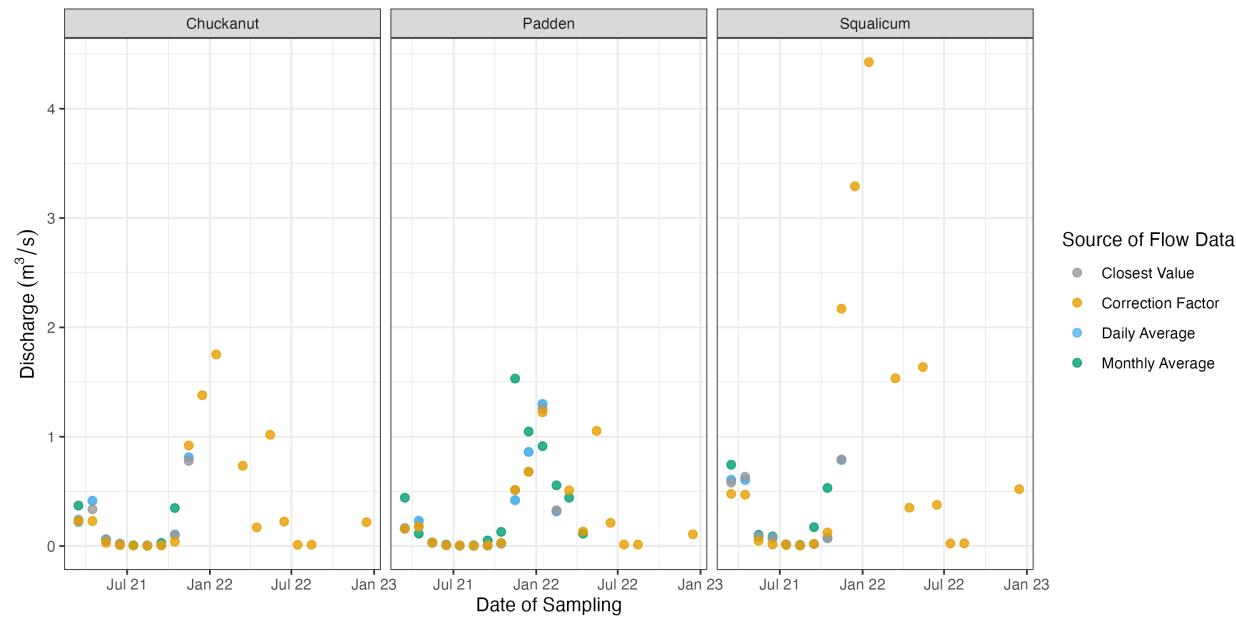


Figure S1.3. Comparison of different ways flow data can be used to correct eDNA concentrations. In the main text, the correction factor is used. Note that for Padden Creek, the “Correction Factor” method is the same as the “Closest Value” method. Also note that for Chuckanut and Squalicum Creeks, no data exist for closest value, daily average, or monthly average after November 2021 when the gauges went offline.

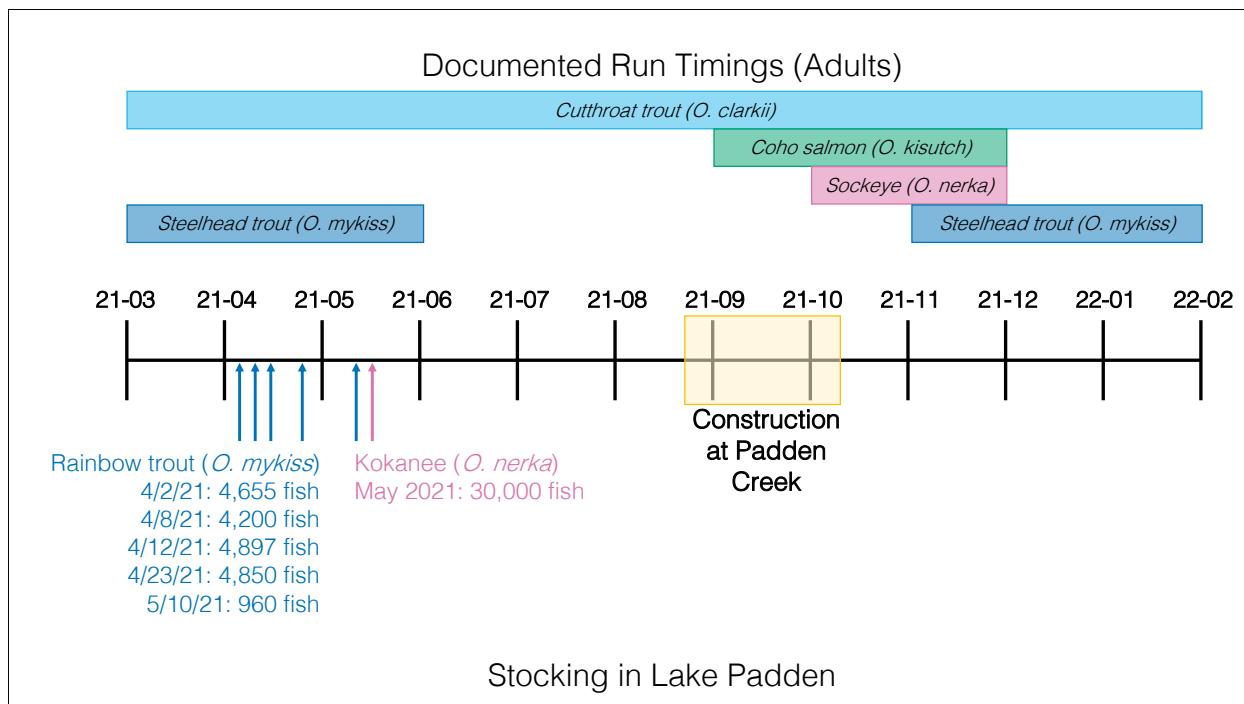


Figure S1.4. Timeline of runs for migrating species, stocking of Lake Padden, and construction at the intervention site (Padden Creek). Note, the dates of kokanee stocking do not include days, just the month and year. For plotting purposes, they are shown as the 15th.

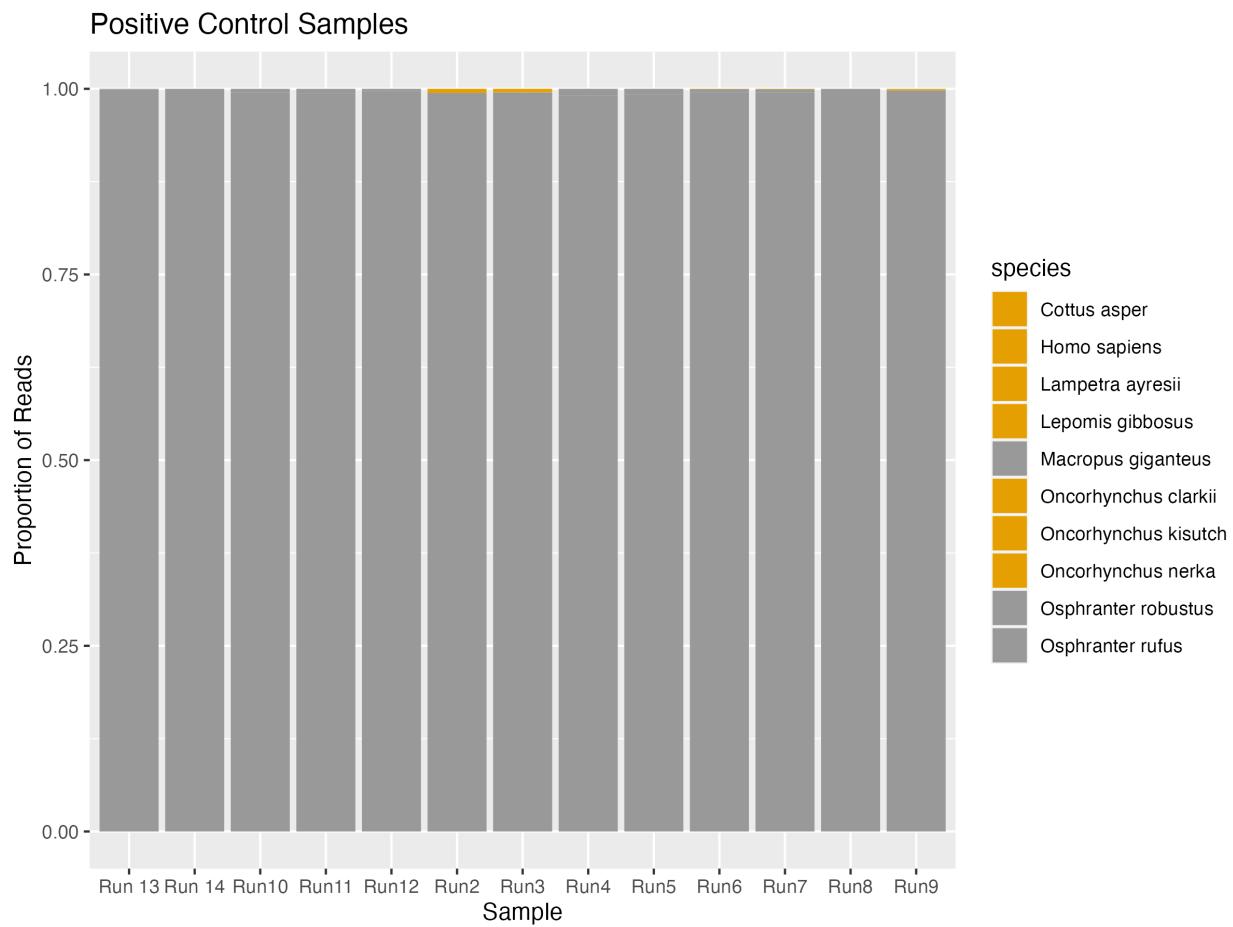


Figure S1.5. Proportion of annotated reads found in positive controls. Grey colors are the three species of kangaroo used for positive controls and are what should be in each sample. Orange species should not be in the positive controls and indicate low level contamination from environmental samples.

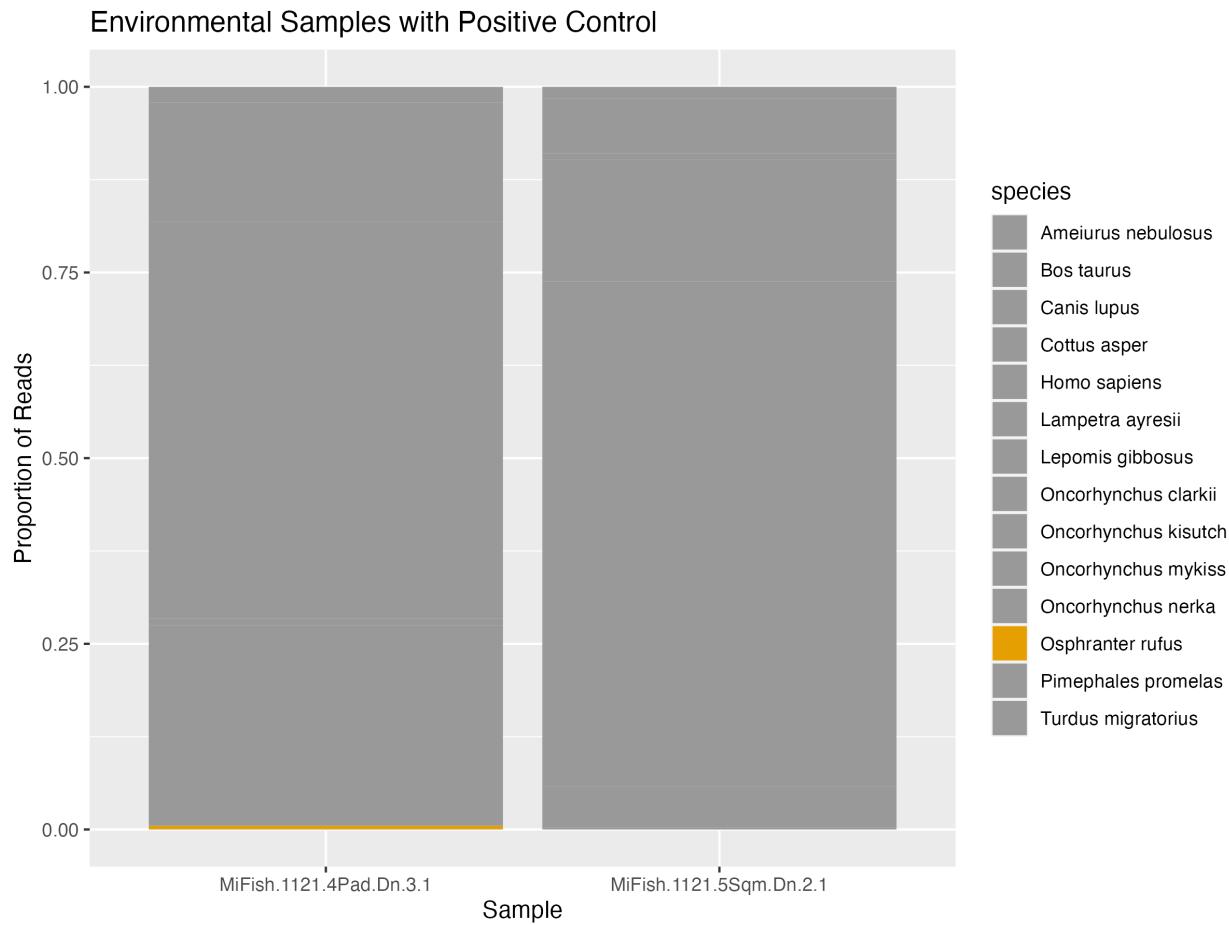


Figure S1.6. Proportion of annotated reads found in environmental samples with positive control. Grey colors are non-kangaroo reads and therefore are what should be in each sample. Orange species are kangaroo reads and therefore should not be in the environmental samples and indicate low level contamination from positive controls.

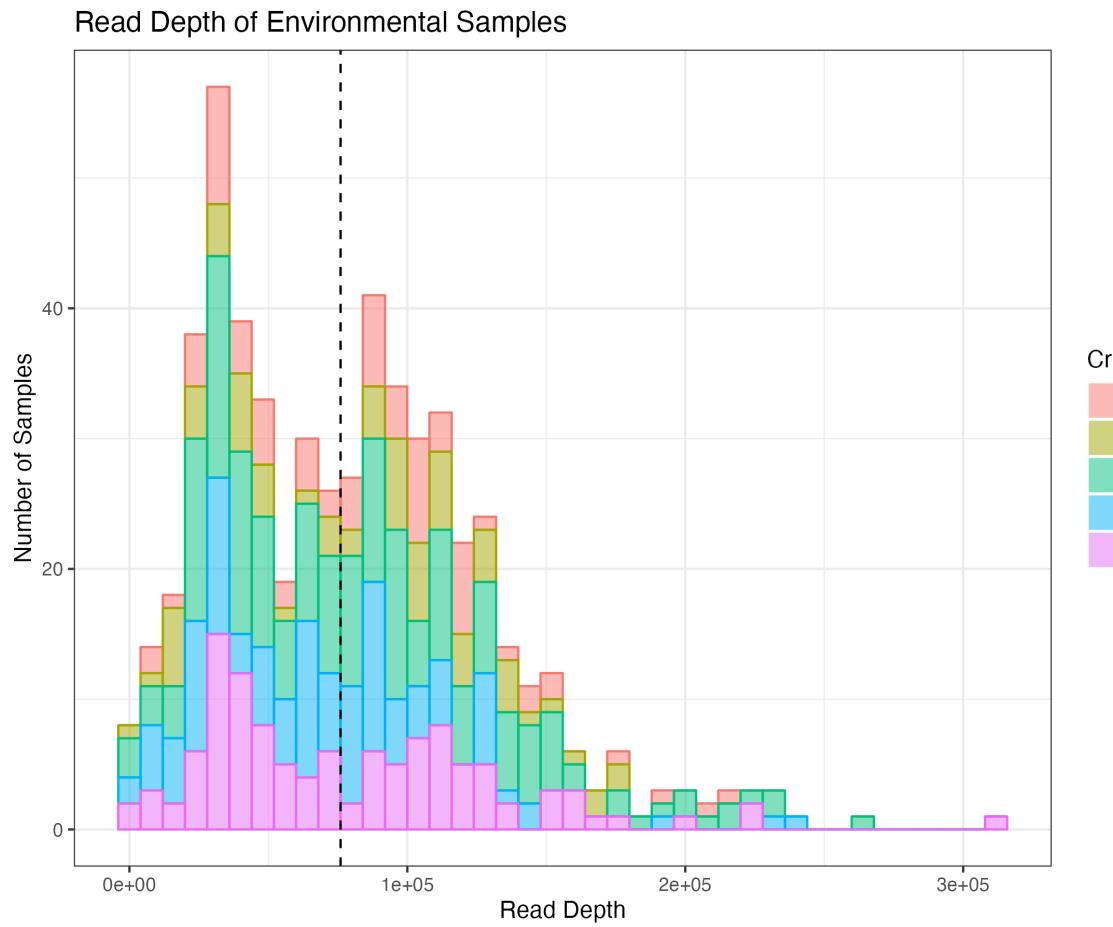


Figure S1.7. Read depth of samples colored by creek. Dashed line shows median read depth (87,698 reads).

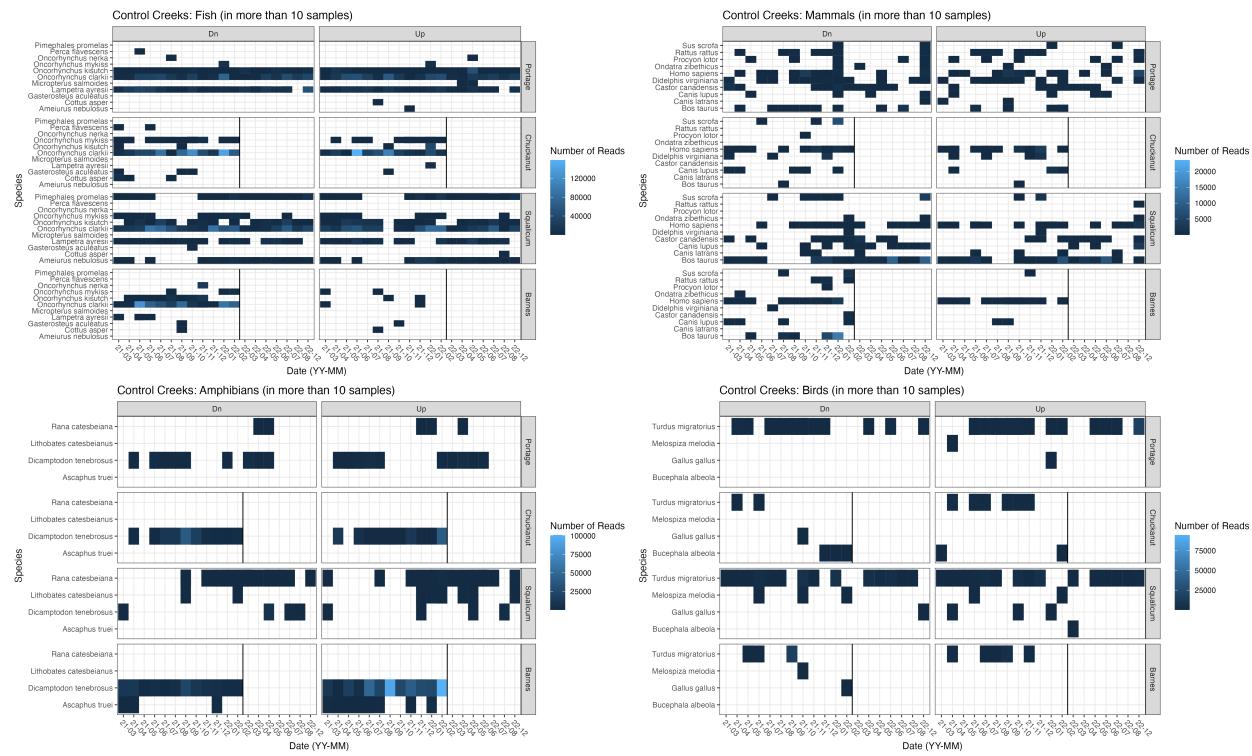


Figure S1.8. Heat map of all species found in control creeks in at least ten environmental samples.

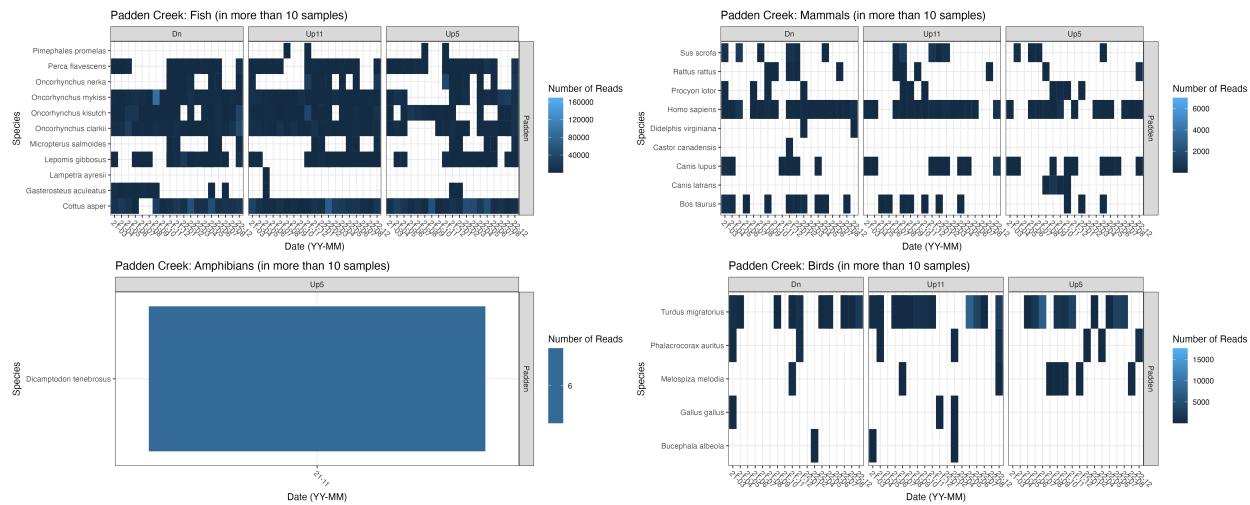


Figure S1.9. Heat map of all species found in Padden Creek in at least ten environmental samples.

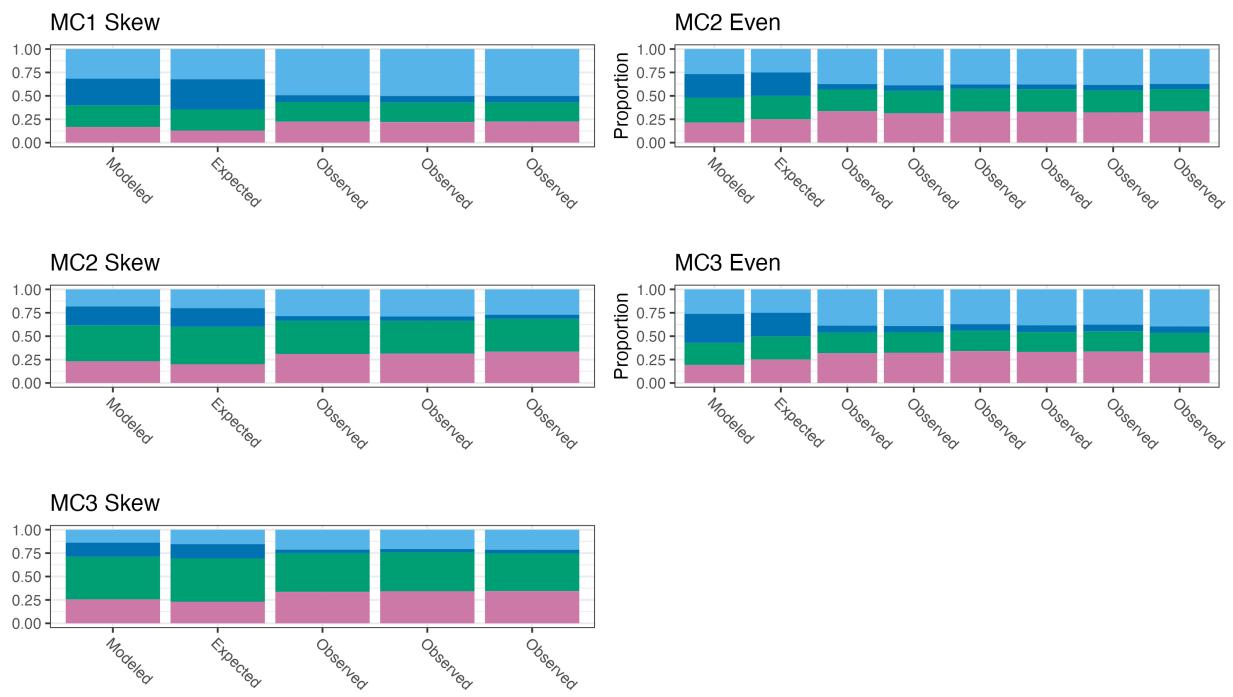


Figure S1.10. Intercalibration of mock communities used to correct environmental samples for amplification bias.

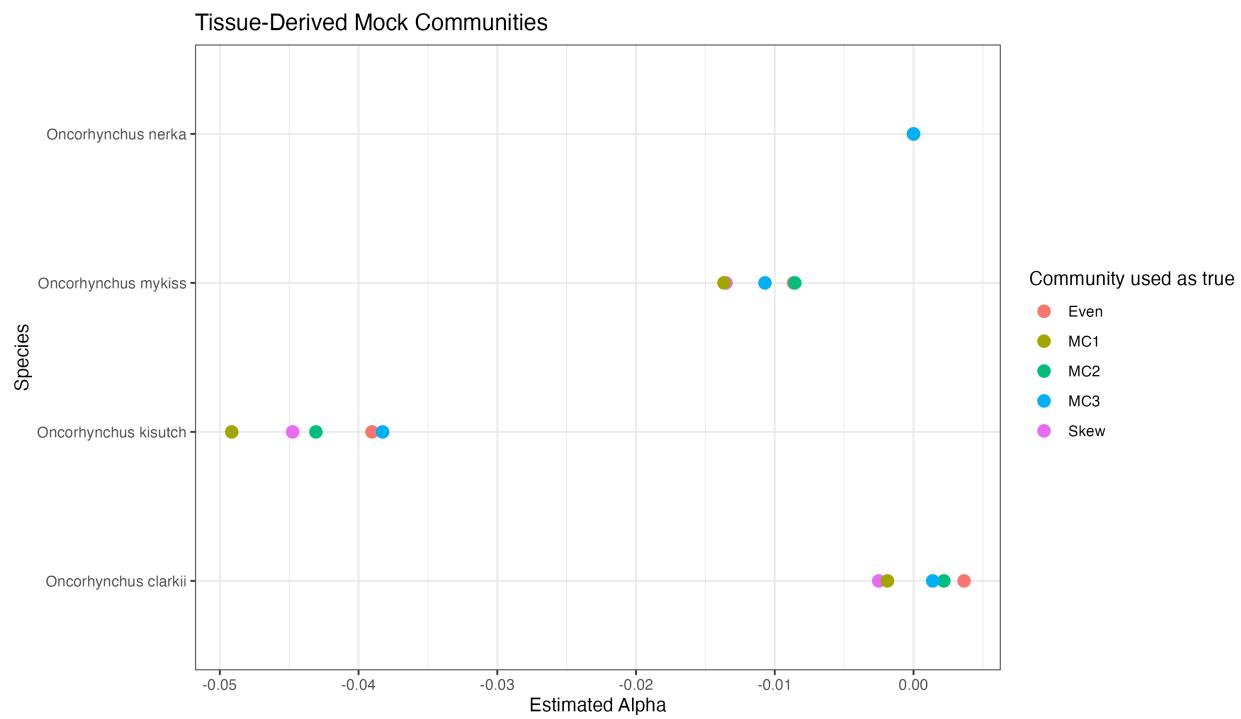


Figure S1.11. Estimated alpha values of salmonid species with different calibrations of the mock communities. Each color represents a different subset of mock community data treated as ‘true’ to calibrate the remainder of the mock community data.

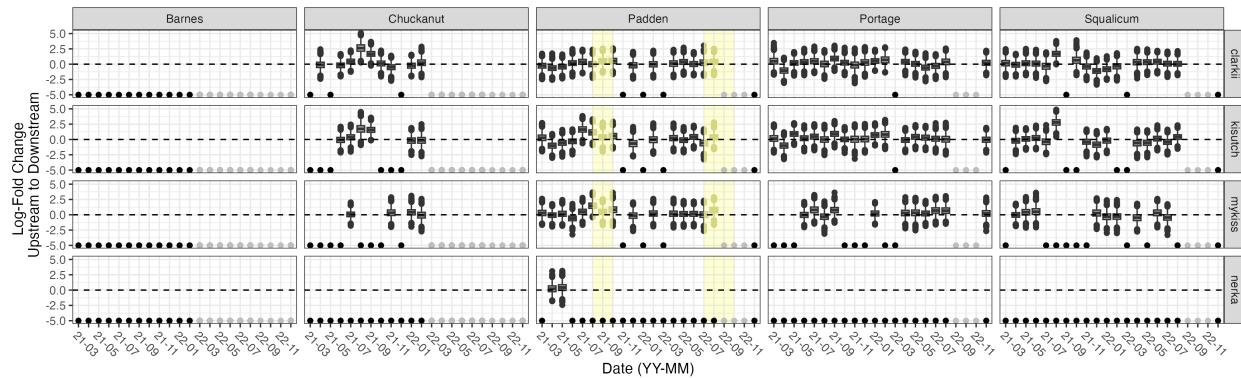


Figure S1.12. The effect of culvert on salmonid abundance separated by species and creeks across time. The y-axis shows the log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream, normalized by upstream mass flow rate. The box boundaries correspond to the 25th and 75th percentiles; the whiskers correspond to 1.5 times the interquartile range. Here, negative values imply that eDNA mass flow rates are higher downstream than upstream. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators. Grey points indicate times when no samples were taken. Black points indicate times when samples were taken, but no target DNA was found in either upstream or downstream samples and therefore the log-fold change can not be calculated.

1 Quantifying Impacts of an Environmental Intervention Using  
2 Environmental DNA: Supplemental Text 2

3 Elizabeth Andruszkiewicz Allan, Ryan P. Kelly, Erin D'Agnese,  
4 Maya Garber-Yonts, Megan Shaffer, Zachary Gold, Andrew O. Shelton

5 2023

6 Our analysis depends upon a set of quantitative models, each linking our observations of metabarcoding  
7 reads or qPCR cycle-threshold values to an underlying concentration of target-species DNA in water samples.  
8 In summary, we (1) use a mock community with a known composition to calibrate our environmental  
9 metabarcoding data as described in (Shelton et al. 2022). The result is a set of estimated proportions of DNA  
10 from each species in each sample. We then (2) relate qPCR cycle-threshold values for a reference species  
11 (here, cutthroat trout (*O. clarkii*)) from the same set of samples to a standard curve to yield quantitative  
12 estimates of the concentration of our reference species in each sample. We (3) use these absolute estimates of  
13 DNA concentration to expand the metabarcoding-derived proportion data into a complete set of quantitative  
14 estimates of DNA concentrations for each species in each sample. We account for the variable water-flow-rates  
15 of the sampled creeks by converting these concentrations from units of copies/L into units of copies/s, given an  
16 flow rate in L/s. Finally, we (4) construct a model describing changes in these species-specific concentrations  
17 over time. We give the statistical details of these steps below.

18 **Calibration with a Mock Community**

19 See Shelton et al. (2022); McLaren et al. (2019); Silverman et al. (2021) for similar analyses.

20 For ease of computation, we ran the metabarcoding-calibration model on data for each of our five creeks  
21 separately, using the same mock communities to calibrate each.

22 Model Diagnostics: 3 chains, 1500 iterations, for all parameters,  $\hat{R} \leq 1.02$

## 23 qPCR Calibration

- 24 See Shelton et al. (2019); McCall et al. (2014) for similar analyses.
- 25 For all samples  $i$ , on qPCR plates  $j$ , we either observe ( $z_{i,j} = 0$  or do not observe  $z_{i,j} = 1$ ) amplification; we  
26 omit the subscripts  $i$  and  $j$  from the following description except where necessary for clarity. We assume an  
27 intercept of zero.
- 28 We model the probability of detection  $P(z = 1)$  as a linear function of concentration and slope parameter  $\phi$ ,  
29 ( $P(z = 1) = \theta = c\phi$ ), with a logit transform to constrain the inferred probability to between 0 and 1.
- 30 For those samples that amplify ( $z = 1$ ), we model the observed Ct value ( $y$ ) as a linear function of our  
31 parameter of interest, the log-concentration of target-species DNA under analysis ( $c$ ). We treat  $y$  as drawn  
32 from a normal distribution  $y \sim N(\mu_{i,j}, \sigma_{i,j})$ , where each triplicate sample on each qPCR plate has its  
33 own estimated mean and standard deviation. The means are estimated as a straightforward linear model,  
34  $\mu = \beta_{0,j} + \beta_{1,j}c$ , but we allow the standard deviation to vary as a linear function of log-concentration so as  
35 to accurately capture decreasing precision with decreasing concentration:  $\sigma = e^{\gamma_0 + \gamma_1 j c}$ ; we estimate these  
36 parameters as an exponent to constrain  $\sigma > 0$ .
- 37 Samples with known concentrations (i.e., standards) were fit jointly with unknown samples (i.e., environmental  
38 samples); because qPCR plate identity was shared among all environmental samples and standards within a  
39 plate, this has the effect of applying plate-specific slope and intercept values for the standard curve to each of  
40 the environmental samples on the plate (Figure S2.1).
- 41 We apply moderately informative priors that make use of background information in hand. For example,  
42 because qPCR standard curves of all kinds have slopes near -3, this slope becomes our background expectation  
43 as embodied in the prior on  $\beta_1$ , but the standard deviation of that prior leaves plenty of room for this  
44 background to be overwhelmed by the observed data. The same logic applies to the intercept of the standard  
45 curve, which in qPCR (for any given species) generally falls near 39 cycles, an expectation that we formalize  
46 by having  $\beta_0$  drawn from a normal distribution with  $\mu = 39$  and  $\sigma = 3$ .
- 47 Taken together with priors, the model is:

$$z_{i,j} \sim Bernoulli(\theta_{i,j})$$

$$\theta_{i,j} = logit^{-1}(\phi c_{i,j})$$

$$y_{i,j} \sim Normal(\mu_{i,j}, \sigma_{i,j}) \text{ if } z_{i,j} = 1$$

$$\mu_{i,j} = \beta_{0,j} + \beta_{1,j} c_{i,j}$$

$$\sigma_{i,j} = e^{\gamma_0 + \gamma_{1,j} c_{i,j}}$$

$$\beta_0 \sim normal(39, 3)$$

$$\beta_1 \sim normal(-3, 1)$$

$$\gamma_1 \sim normal(0, 5)$$

$$\gamma_0 \sim normal(-2, 1)$$

<sup>48</sup> Model Diagnostics: 3 chains, 2500 iterations, for all parameters,  $\hat{R} \leq 1.002$ .

#### <sup>49</sup> Expanding Proportions into Absolute Abundances

<sup>50</sup> See Pont et al. (2022) and McLaren et al. (n.d.) (preprint) for examples of similar expansions.

<sup>51</sup> As described in the main text, calibrated metabarcoding analysis yielded quantitative estimates of the  
<sup>52</sup> proportions of species' DNA in environmental samples prior to PCR.

<sup>53</sup> We then converted these proportions into absolute abundances by expansion, in light of the qPCR results for  
<sup>54</sup> our reference species *O. clarkii*. We estimated the total amplifiable salmonid DNA in environmental sample *i*  
<sup>55</sup> as  $DNA_{salmonid_i} = \frac{[qPCR_{reference_i}]}{Proportion_{reference_i}}$ , and then expanded species' proportions into absolute concentrations  
<sup>56</sup> by multiplying these sample-specific total concentrations by individual species' proportions, such that for  
<sup>57</sup> species *j* in sample *i*,  $DNA_{i,j} = DNA_{salmonid_i} * Proportion_{i,j}$ .

<sup>58</sup> We transformed the resulting abundances to account for the creeks' flow-rates as described in the main text.

<sup>59</sup> Ideally, we would have fit a joint model that simultaneously estimated species proportions (metabarcoding),  
<sup>60</sup> absolute concentrations (qPCR), and developed the time-series trends for all species. As a practical  
<sup>61</sup> computational matter, we had to create these models individually, which entailed some loss of information  
<sup>62</sup> about parameter variability and cross correlation. For the mixed-effects model describing trends over time  
<sup>63</sup> (described below), we used the product of posterior means from the metabarcoding and the concentrations

64 of the qPCR model as observations, rather than being able to use the full posteriors for each input to the  
65 model. We deemed this acceptable because our metabarcoding proportions were quite precisely estimated:  
66 for example, in our focal Padden Creek, the coefficient of variation for estimated proportions of our reference  
67 species (*O. clarkii*) ranged from 0.008 to 0.25.

## 68 Modeling Changes in Concentration over Time

69 At a given station in a given creek, some DNA concentration exists for each species. For simplicity, we focus  
70 on a single species and a single station (downstream or upstream) for the moment.

71 Our observations of the (log) DNA concentration in creek  $i$  at time  $t$  are distributed as  $Y_{i,t} \sim \mathcal{N}(\mu_{i,t}, \sigma^2)$ .

72 More complex versions of the model may let  $\sigma$  vary across creeks, time points, species, or with environmental  
73 covariates of interest.

74 We are interested in how the DNA concentration changes over time, so we model the expected value of DNA  
75 in a creek at time  $t$ ,  $\mu_{i,t}$ .

76 We considered three ways of modeling the salmonid eDNA data, each in a Bayesian framework, but each  
77 treating non-independence among time points somewhat differently:

- 78 • A linear auto-regressive (AR(1)) model, written in `stan`. For each species in each creek, the expected  
79 concentration of eDNA of each month is a linear function of the expected value from the previous  
80 month. Within a species, the monthly autoregressive parameters are shared across creeks. For each  
81 species  $j$  – the subscript for which we omit here for clarity – we have a single overall model of the  
82 change in eDNA concentration among species, creeks ( $i$ ), timepoints ( $t$ ), and stations ( $d$ ).

$$Y_{i,t,d} \sim \mathcal{N}(\mu_{i,t,d}, \sigma_{\text{obs}}^2)$$

$$\mu_{i,t,d} = \alpha_{i,t} + \epsilon_{i,t,d} + \eta_{i,t,d}$$

$$\epsilon_{i,t,d} \sim \mathcal{N}(\beta \mu_{i,t-1,d}, \phi^2)$$

$$\alpha_{i,t} \sim \mathcal{N}(\mu_\alpha, \sigma_\alpha)$$

$$\beta \sim \mathcal{U}(-1, 1)$$

$$\sigma_{\text{obs}} \sim \text{gamma}(1, 2)$$

$$\sigma_\alpha \sim \text{gamma}(1, 2)$$

$$\eta \sim \mathcal{N}(\mu_\eta, 1)$$

$$\phi \sim \text{gamma}(1, 2)$$

$$\mu_\alpha \sim \mathcal{N}(\mathbf{0}, \mathbf{10})$$

$$\mu_\alpha \sim \mathcal{N}(\mathbf{0}, \mathbf{5})$$

- 83     • A generalized additive model (GAM), written in `brms` (which itself writes a `stan` model). For each  
 84 species in each creek, an independent set of spline (weighting) parameters describes the temporal trends  
 85 in expected eDNA concentration; the number of spline knots is shared across species and creeks. We  
 86 follow (Pedersen et al. 2019) to create a hierarchical GAM in which the expected value for each species  
 87 in each creek at each time point is a spline function of time, time-by-creek, and time-by-station, with  
 88 random effects for creek and station. Here, time-by-creek and time-by-station splines are centered,  
 89 requiring additional fixed-effect terms for station and creek. Because no information is shared across  
 90 species in this model, we fit the model each species independently.

$$\mu_{idt} = \beta_0 + s(t) + s_d(t) + s_i(t) + s(d) + s(i)$$

- 91 In R code using `brms`, this model is coded as

```
92 brm(  
93   bf(  
94     log(observed) ~  
95       s(time_idx, bs="cc") +  
96       s(time_idx, by=station, m=1, bs="cc")+  #main effect, station
```

```

97     s(station, bs="re") + #random effect, station
98     s(time_idx, by=creek, m=1, bs="cc")+ #main effect, creek
99     s(creek, bs="re") + #random effect, creek
100   )
101 )

```

- A linear mixed-effects (LME) model, written in `rstanarm`. For each species in each creek, time (i.e., sampling month) is treated as a random effect. Each species-creek-month effect is treated as an independent draw from a common distribution.

$$\mu_{ijdt} = \beta_0 + \beta_{1_{ij}} + Month * \beta_{2_{ij}} + \beta_{3_{ijdt}}$$

- 105 In R code using `rstanarm`, this model is coded as

```
106 stan_glmer(log(observed) ~ (1 + time_idx|creek:species) + (1|station:creek:species:time_idx))
```

- 107 Ultimately, the three models yielded very similar results (Figure S2.2)). The LME model proved simplest  
 108 and most flexible insofar as it could easily handle datasets with uneven sets of observations – for example,  
 109 cases in which a species was detected downstream of a barrier, but not upstream. We accordingly used the  
 110 LME as the model for the analysis given in the main manuscript.

## 111 References

- 112 McCall, M. N., H. R. McMurray, H. Land, and A. Almudevar. 2014. On non-detects in qPCR data.  
 113 Bioinformatics 30:2310–2316.
- 114 McLaren, M. R., J. T. Nearing, A. D. Willis, K. G. Lloyd, and B. J. Callahan. (n.d.). Implications of  
 115 taxonomic bias for microbial differential-abundance analysis.
- 116 McLaren, M. R., A. D. Willis, and B. J. Callahan. 2019. Consistent and correctable bias in metagenomic  
 117 sequencing experiments. eLife 8:e46923.
- 118 Pedersen, E. J., D. L. Miller, G. L. Simpson, and N. Ross. 2019. Hierarchical generalized additive models in  
 119 ecology: an introduction with mgcv. PeerJ 7:e6876.
- 120 Pont, D., P. Meulenbroek, V. Bammer, T. Dejean, T. Erős, P. Jean, M. Lenhardt, C. Nagel, L. Pekarik,  
 121 M. Schabuss, B. C. Stoeckle, E. Stoica, H. Zornig, A. Weigand, and A. Valentini. 2022. Quantitative  
 122 monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR.  
 123 Molecular Ecology Resources n/a.
- 124 Shelton, A. O., Z. J. Gold, A. J. Jensen, E. D'Agnese, E. Andruszkiewicz Allan, A. Van Cise, R. Gallego, A.

- 125 Ramón-Laca, M. Garber-Yonts, K. Parsons, and R. P. Kelly. 2022. Toward quantitative metabarcoding.
- 126 Ecology n/a:e3906.
- 127 Shelton, A. O., R. P. Kelly, J. L. O'Donnell, L. Park, P. Schwenke, C. Greene, R. A. Henderson, and E.
- 128 M. Beamer. 2019. Environmental DNA provides quantitative estimates of a threatened salmon species.
- 129 Biological Conservation 237:383–391.
- 130 Silverman, J. D., R. J. Bloom, S. Jiang, H. K. Durand, E. Dallow, S. Mukherjee, and L. A. David. 2021.
- 131 Measuring and mitigating PCR bias in microbiota datasets. PLoS Computational Biology 17:e1009113.

For Review Only

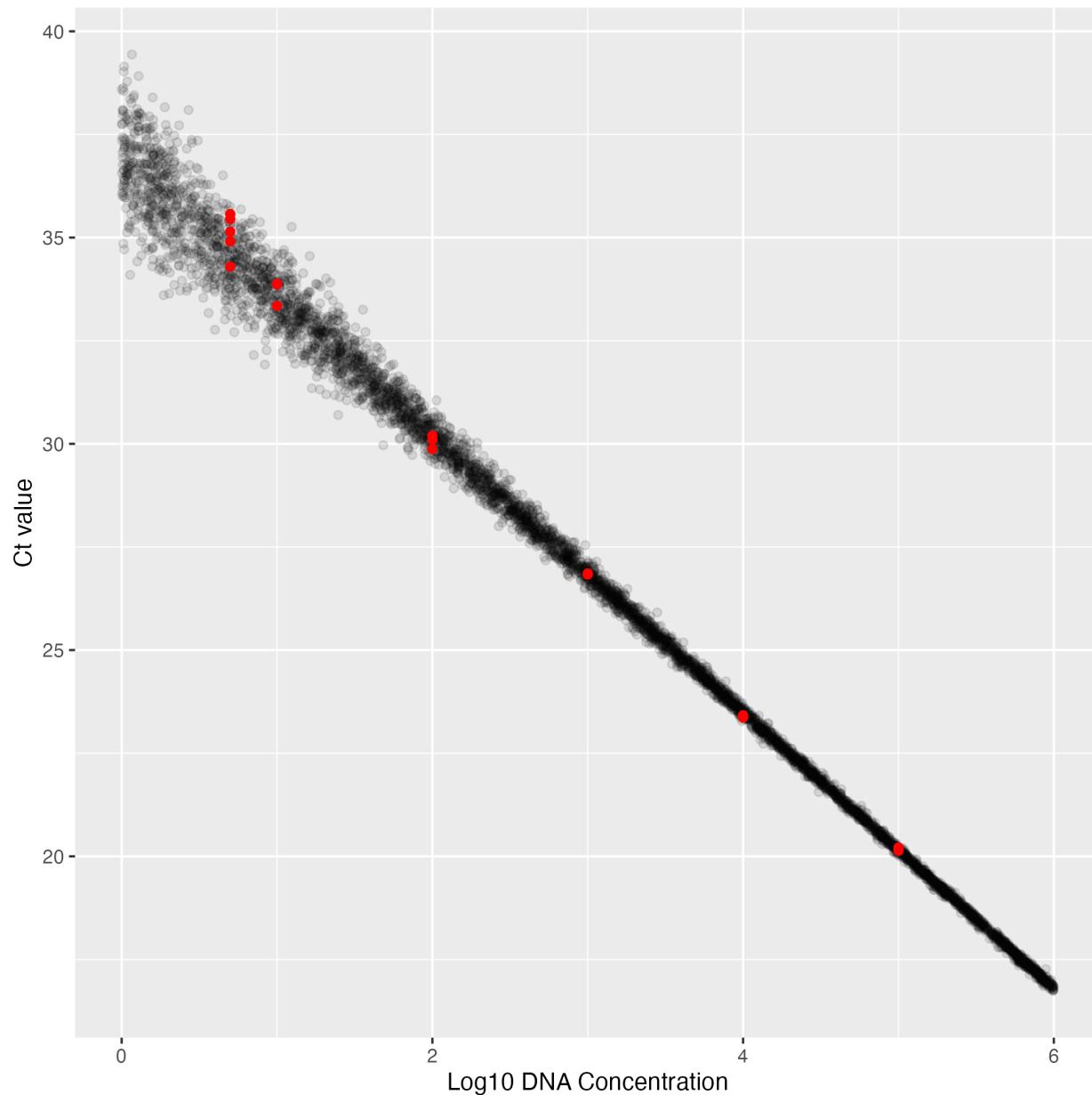


Figure S2.1. Example of 2500 samples from the joint posterior distribution of the model fit for a single representative qPCR plate. Red dots are standard-curve observations with known starting concentrations. The spread of black dots (posterior samples) indicates the shape of the calibration curve, with standard deviation increasing as concentration decreases.

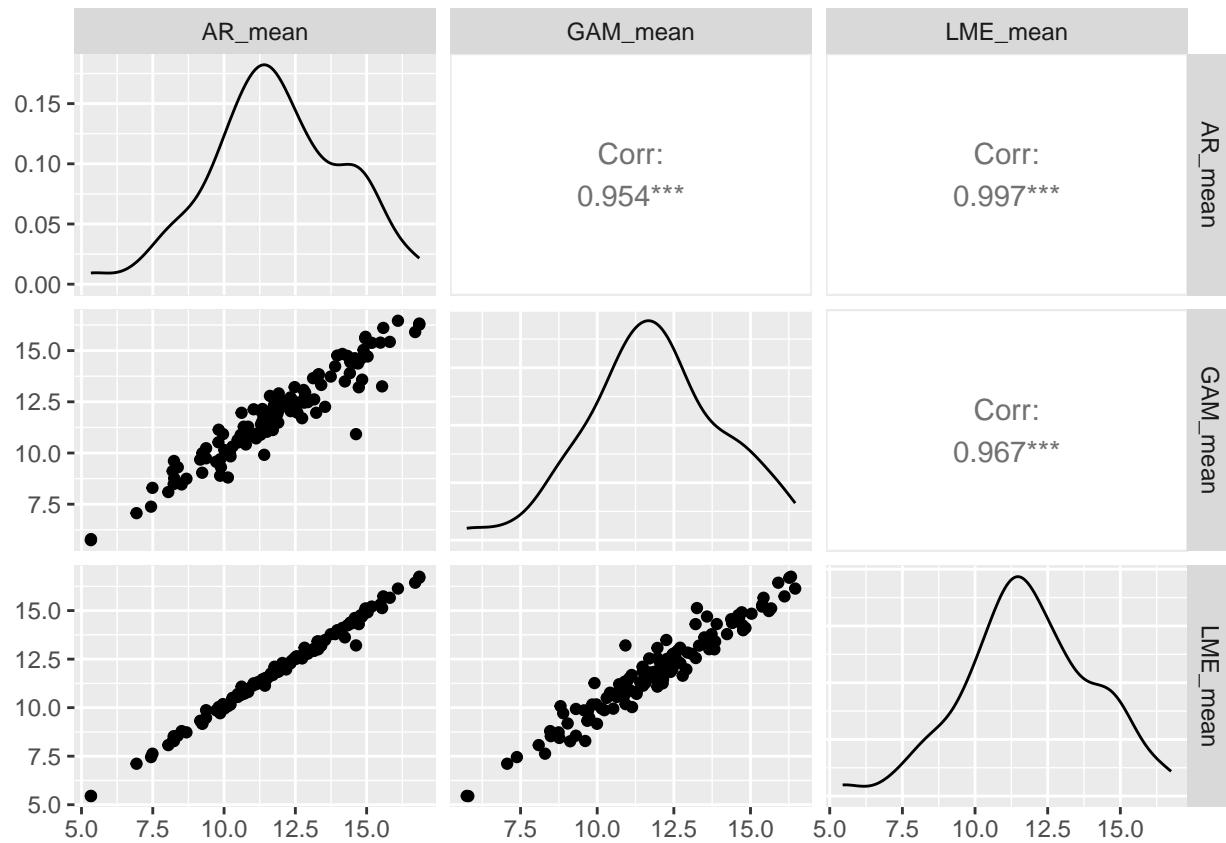


Figure S2.2. Comparison of three models (linear autoregressive, generalized additive model, and linear mixed effects model) shown for a subset of the data used in the main manuscript.

**Title:** Quantifying Impacts of an Environmental Intervention Using Environmental DNA  
**Authors:** Elizabeth Andruszkiewicz Allan  
Ryan P. Kelly  
Erin D'Agnese  
Maya Garber-Yonts  
Megan Shaffer  
Zachary Gold  
Andrew O. Shelton  
**Journal Name:** Ecological Applications

**Table S1.** Complete list of species found over the course of the study in all environmental samples.

**Table S2.** Compositions of species in the mock communities used to correct metabarcoding data for relative proportions.

<b>Amphibians</b>	<b>Birds</b>	<b>Fish</b>	<b>Mammals</b>
<i>Anaxyrus boreas</i>	<i>Aix sponsa</i>	<i>Alburnus alburnus</i>	<i>Aplodontia rufa</i>
<i>Ascaphus truei</i>	<i>Ardea herodias</i>	<i>Ameiurus nebulosus</i>	<i>Bos taurus</i>
<i>Dicamptodon tenebrosus</i>	<i>Bombycilla cedrorum</i>	<i>Barbatula barbatula</i>	<i>Canis latrans</i>
<i>Ensatina eschscholtzii</i>	<i>Branta canadensis</i>	<i>Cottus asper</i>	<i>Canis lupus</i>
<i>Lithobates catesbeianus</i>	<i>Bucephala albeola</i>	<i>Cottus marginatus</i>	<i>Capra hircus</i>
<i>Lithobates clamitans</i>	<i>Cathartes aura</i>	<i>Cottus pollux</i>	<i>Castor canadensis</i>
<i>Rana catesbeiana</i>	<i>Cinclus mexicanus</i>	<i>Entosphenus tridentatus</i>	<i>Cervus elaphus</i>
<i>Taricha granulosa</i>	<i>Colaptes auratus</i>	<i>Gasterosteus aculeatus</i>	<i>Didelphis virginiana</i>
	<i>Corvus corax</i>	<i>Gobio gobio</i>	<i>Equus caballus</i>
	<i>Fulica americana</i>	<i>Lampetra ayresii</i>	<i>Felis catus</i>
	<i>Gallus gallus</i>	<i>Lepomis gibbosus</i>	<i>Homo sapiens</i>
	<i>Junco hyemalis</i>	<i>Micropterus salmoides</i>	<i>Lynx rufus</i>
	<i>Melospiza melodia</i>	<i>Oncorhynchus clarkii</i>	<i>Myotis keenii</i>
	<i>Phalacrocorax auritus</i>	<i>Oncorhynchus gorbuscha</i>	<i>Myotis lucifugus alascensis</i>
	<i>Pipilo maculatus</i>	<i>Oncorhynchus keta</i>	<i>Neovison vison</i>
	<i>Piranga ludoviciana</i>	<i>Oncorhynchus kisutch</i>	<i>Neurotrichus gibbsii</i>
	<i>Poecile atricapillus</i>	<i>Oncorhynchus mykiss</i>	<i>Ondatra zibethicus</i>
	<i>Regulus calendula</i>	<i>Oncorhynchus nerka</i>	<i>Ospryanter rufus</i>
	<i>Regulus satrapa</i>	<i>Oncorhynchus tshawytscha</i>	<i>Procyon lotor</i>
	<i>Sturnus vulgaris</i>	<i>Perca flavescens</i>	<i>Rattus norvegicus</i>
	<i>Tachycineta bicolor</i>	<i>Pimephales promelas</i>	<i>Rattus rattus</i>
	<i>Tachycineta thalassina</i>	<i>Prosopium williamsoni</i>	<i>Rattus sp. NH 2147</i>
	<i>Turdus migratorius</i>	<i>Salmo trutta</i>	<i>Scapanus orarius</i>
		<i>Sardinops sagax</i>	<i>Sus scrofa</i>
		<i>Spirinchus thaleichthys</i>	<i>Ursus americanus</i>

Species	Percent Composition in Each Community				
	MC1-Skew	MC2-Even	MC2-Skew	MC3-Even	MC3-Skew
<i>Oncorhynchus clarkii</i>	9.5%	5.6%	4.5%	5%	3.6%
<i>Oncorhynchus kisutch</i>	9.5%	5.6%	4.5%	5%	3.6%
<i>Oncorhynchus tschawtscha</i>	3.8%	5.6%	9.0%	5%	7.1%
<i>Oncorhynchus nerka</i>	3.8%	5.6%	4.5%	5%	5.3%
<i>Oncorhynchus mykiss</i>	6.7%	5.6%	9.0%	5%	10.6%
<i>Oncorhynchus gorbuscha</i>	19.1%	5.6%	22.4%	5%	17.7%
<i>Salmo trutta</i>	1.9%	0%	0%	0%	0.0%
<i>Salvelinus malma</i>	1.9%	0%	0%	5%	1.8%
<i>Entosphenus tridentatus</i>	7.6%	5.6%	13.5%	0%	0%
<i>Lampetra ayresii</i>	19.1%	0%	0%	5%	7.1%
<i>Cottus asper</i>	8.6%	5.6%	3.4%	0%	0%
<i>Cottus marginatus</i>	8.6%	0%	0%	5%	7.1%
<i>Salvelinus confluentus</i>	0%	5.6%	3.6%	5%	0.7%
<i>Castor canadensis</i>	0%	5.6%	6.7%	0%	0%
<i>Turdus migratorius</i>	0%	5.6%	1.8%	0%	0%
<i>Ameiurus nebulosus</i>	0%	5.6%	3.4%	0%	0%
<i>Gasterosteus aculeatus</i>	0%	5.6%	1.1%	0%	0%
<i>Taricha granulosa</i>	0%	5.6%	6.7%	0%	0%
<i>Ondatra zibethicus</i>	0%	5.6%	0.9%	0%	0%
<i>Felis catus</i>	0%	5.6%	0.9%	0%	0%
<i>Rana pretiosa</i>	0%	5.6%	3.4%	0%	0%
<i>Ascaphus truei</i>	0%	5.6%	0.9%	0%	0%
<i>Novumbra hubbsi</i>	0%	0%	0%	5%	2.7%
<i>Procyon lotor</i>	0%	0%	0%	5%	2.7%
<i>Rana catesbeiana</i>	0%	0%	0%	5%	8.9%
<i>Micropterus salmoides</i>	0%	0%	0%	5%	7.1%
<i>Bos taurus</i>	0%	0%	0%	5%	0.9%
<i>Didelphis virginiana</i>	0%	0%	0%	5%	0.9%
<i>Neurotrichus gibbsii</i>	0%	0%	0%	5%	0.9%
<i>Neovison vison</i>	0%	0%	0%	5%	0.9%
<i>Anaxyrus boreas</i>	0%	0%	0%	5%	3.6%
<i>Ardea herodias</i>	0%	0%	0%	5%	7.1%

## Response to Reviewer Comments

We want to thank both reviewers and the editor for the thoughtful comments on the manuscript. In response to the comments, and given the time during the review process, we are happy to resubmit the paper with an additional 7 months of data, including a second culvert replacement. We believe the addition of the new data strengthens the analyses and importantly, though we found a minimal effect of the first culvert (in the original submission), we found that the second culvert was in fact a blockage and therefore the inclusion of these data strengthen the message of the manuscript. Additionally, we have moved from using the auto-regressive time series model to a linear mixed effect model. We hope the reviewers and editors find the new version of this manuscript to satisfy the original comments.

*I have reviewed the manuscript by Allan et al. The authors tried to quantify the impact of environmental alteration (a culvert removal) on salmonid fish by using eDNA analysis. Quantitative PCR and metabarcoding was applied and the results showed that eDNA methods can be an effective and efficient approach to monitor the impacts. Overall, the manuscript is well-written, and the quality of the experiments is high. However, the organization of the paper is not excellent and needs to be reconstructed.*

We thank the reviewer for their comments and taking the time to improve the organization of the paper.

*First, Introduction is a bit long (12 paragraphs!) and not well constructed. Please consider reconstruction of Introduction to five to six paragraphs. Also, some previous studies reported the methods for quantitative metabarcoding, such as adding known concentration of standard DNA to the samples and using random sequence tags. I suggest authors to cite the literature and compare pros and cons with the method used in this manuscript.*

We have shortened and restructured the introduction. We note that there is quite a lot to cover from environmental impact assessments, eDNA (including metabarcoding, relative abundance, quantification), culverts, and how eDNA has been used to monitor culvert replacements in the past. We found it hard to add more information of the other ways to conduct quantitative metabarcoding while not adding significantly to the length of the paper. We did add one sentence in the introduction.

*Second, the Methods are overly descriptive. For example, "Site and Species Selection" consists of six paragraphs, much of which should be written in the Introduction. It could be shortened to 3-4 paragraphs and the volume could be halved. Also, the Results section contains information that should be written in the Methods section. For example, information on Line 420-425 should appear in Methods.*

We have moved some of the text in the Methods to the supplemental and have rearranged things between the Results and Methods. We did not move anything in the methods to the introduction given the reviewers previous comment that the introduction is too long as well. We again note the large volume of background required to provide important and relevant context for this study.

*Finally, Discussion should also be reconstructed. I think the sections "Decoupling of eDNA from fish abundance" and "Accounting for flow with eDNA concentrations" should be in one section, "Appropriateness of methods used" etc. Also, the volume of discussion on this should be reduced.*

We have shortened the discussion and combined the sections that the reviewer has suggested.

*More minor points are as follows:*

*Throughout the manuscript: Scientific names and common names are mixed and confusing, so please unify them into one after indicating both at the beginning.*

We thank the reviewer for the suggestion to unify common and scientific names. We have gone throughout the manuscript and unified so that when referring to a species, it is common name and then scientific name in parentheses. At some points, we just use the scientific name as described in the text there are different haplotypes of species that we cannot distinguish genetically with eDNA metabarcoding. Therefore, there are a few select points in the text where we just use scientific names.

*L 76: From my understanding, quantitative PCR includes realtime PCR and digital PCR. Also, there are non-droplet digital PCR. So, this should be "... such as realtime PCR, digital PCR, or traditional PCR..."*

We thank the reviewer for this suggestion but have chosen to keep the original wording to be as explicit as possible. As we expect the audience of *Ecological Applications* to have varying levels of molecular expertise, we think that explicitly listing both quantitative PCR and digital droplet PCR rather than “realtime PCR” is helpful for readers.

*L 209 (Sup Fig 4): I think this information is presented by a supplemental table, not figure.*

We have plotted this as a supplemental figure, not as a table, for a quick visual reference of which time points and which creeks did not filter the full 2 L.

*L 228: The effectiveness of the correction with "correction factor" should be demonstrated by comparison with available measured data.*

We have added a supplemental figure where we compare the different ways of using flow data for correct the eDNA concentrations. For the two creeks where the flow gauges went offline in the middle of the study, the effectiveness of the correction factor is demonstrated in the first half of the time series. For the second half when there is no gauge data, we can only show the flow rate as calculated by the correction factor.

*L 231: "Though" should be "Through".*

This really is “though”. We are saying, the range of discharge was high throughout the whole year (0-23 m<sup>3</sup>/s), but on the days that we actually sampled, the maximum discharge was only 1.3 m<sup>3</sup>/s. Therefore, we did not change this. We did however, move this to the supplemental text in an effort to reduce the text in the methods section.

*L 248 : "DNEasy" should be "DNeasy".*

We have changed this accordingly.

*L 293 and else: Name of the program is better to be Italicized.*

We have italicized these.

*L 302: Only ~2% of ASVs were annotated to species level, it is too low. What is the reason for this?*

We understand that this number seems very low. But in the same sentence, we note that though only 2% of ASVs are annotated to the species level, those ASVs represents 81% of reads. In other words, we ended up with very many ASVs with just a few reads. From our processing, we also know that a large percentage of these ASVs (90%) with just a few reads were longer than 200 bp (our target is ~170 bp). We expect that these are off-target amplification of the 16S gene representing bacteria, which has been documented by other researchers as well. In the text, we have removed the 2% of ASVs and kept that 81% of reads are annotated to species level. Note that other studies have also used MiFish primers and annotated 80% of reads to species level (Kumar et al. 2022; <https://doi.org/10.1371/journal.pone.0266720>)

Furthermore, because we have mock community data for the four salmonids used in the analysis, we are confident that we are capturing species-level annotations for those salmonids because we find the same exact ASVs in the mock community as the environmental samples.

*L 350: Terminology should be unified between text and Figure 3.*

We thank the reviewer for this suggestion and have updated the terminology in both the main text and Figure 3 to be more clear and ensured that they are unified now.

*Reference: Fish, W. D. and Wildlife 2019ab seems odd.*

We have corrected the citation to be “Washington Department of Fish and Wildlife”.

*Fig 1. The legend of Fig.1 is insufficient. Please provide the meaning of triangles and circles. Also, indicate which is the treatment creek.*

We have updated the figure legend.

*Supplementary tables: Please provide titles and legends for Supplemental Tables.*

We have added titles and legends to the supplemental tables.

---

*Reviewer #2 Comments to the Author:*

*Review of Andruszkiewica Allan et al., “Quantifying impacts of an environmental intervention using environmental DNA”*

*General:*

*This is a well written paper that provides interesting results on a topic that could be very valuable in assessing the effects of culvert replacement on stream communities. Given minor/moderate revision, the paper should be accepted and will provide value to the growing eDNA literature as the science moves from research to practice. The authors use existing eDNA techniques that have proven successful for other comparable studies. The results focus mostly on short-term effects of the construction itself (i.e., construction effects that could possibly occur to impact downstream communities) instead of longer-term upstream effects after fish passage was returned. The authors make a confident claim that the technique could be widely used in assessing the (presumably short-term) impacts of culvert replacement projects from the actual construction/engineering impacts of “working in the wet”, but also presumably to the longer term intended impact of restoring connectivity for migratory fish populations. Given this goal, I have two concerns with the existing study that the authors should address in their revisions.*

We thank the reviewer for the positive feedback on the manuscript.

*-One of the strengths of BACI approaches is that it allows comparisons of a main factor (the impact, in this case culvert replacement) on a variable of interest (fish community diversity/eDNA abundance) while accounting for natural differences due to some other factor(s). Without getting into the thick weeds of past controversies of BACI approach (i.e., Hurlbert v. Stewart-Oaten; Underwood), two issues seem to be at play in the current paper. (1) The differences in the streams in the control group, in terms of things that might affect any upstream-downstream differences (e.g., passable, maybe passable) and (2) the duration of the before/after monitoring.*

*I think that the issue in #1 is accounted for in the time series modeling approach used, but the authors should provide some additional explanation in their description of the time-series modeling approach.*

We thank the reviewer for the careful consideration of the BACI approach and its strengths and pitfalls. We would like to emphasize and added text to clarify that we loosely based sample design on a BACI approach, but really the analysis relies on the linear mixed effects model (formerly the time series model; see more below), and the control creeks add data to the model but we aren't relying on BACI analysis. In the linear mixed effects model, each species in each creek, time (i.e., sampling month) is treated as a random effect – and each species-creek-month effect is treated as an independent draw from a common distribution. The linear mixed effect model is a simpler way to analyze the data and with the additional months of sampling and the

additional culvert, it is more appropriate than the previous time series model (in part due to the two culverts being located on the same treatment creek at different points in time). Please see more information in the Statistical Supplement on how we chose this model and a comparison of three models (an autoregressive model, a generalized additive model, and the linear mixed effects model).

*Issue #2 (the duration of the before and after monitoring), however, is a concern, especially in the context of how confident the authors frame the utility of eDNA metabarcoding for estimating environmental impacts of culvert replacement (e.g., lines 436 – 444, but especially lines 602-603). Smokorowski and Randall*

(2017; [https://urldefense.com/v3/\\_https://doi.org/10.1139/facets-2016-0058\\_";!!K-Hz7m0Vt54!mN\\_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFB05N0T8WFa3cpODnOeu3iLbyjbUNnkTKHL3I4y\\_3zbDBAOqsQ\\$](https://urldefense.com/v3/_https://doi.org/10.1139/facets-2016-0058_)) argue that multiple years are needed to properly account for interannual variability when assessing impacts to fish populations. The authors of the current paper need to address or acknowledge the limitations of inference due to the relatively short time frame of the study.

We thank the reviewer for the additional reference and for acknowledging the need for the manuscript to emphasize the short duration of this particular study. The duration of the after monitoring of the first culvert (SR-11) and the before monitoring of the second culvert (I-5) are now much longer with the extended time series. The duration of the before monitoring for the first culvert (SR-11) is unchanged and short, though we do not believe there was much of a barrier beforehand and we can't add more time points. The duration of the after monitoring of the second culvert (I-5) is only one time point and we do acknowledge this is much too short, but we obviously note that more monitoring should be conducted to measure the effect of the culvert replacement.

*-Another factor worth mentioning is that this study is of a single stream where culvert replacement has been conducted. I'm assuming that a population of culvert replacements was not available for the authors during the conduct of the study...but some mention of the limitations of replication in the discussion seems warranted. There are very few citations of culvert replacement results, outside of a few in the introduction. How do the results of the current paper compare to previous studies of culvert replacement and is there any additional context that they might provide for making policy recommendations (i.e., using eDNA metabarcoding as a methodology to assess culvert replacement impacts)?*

We certainly would have liked to have many culverts in many streams. The new data added does have two culverts, but on the same stream. Now we find that one culvert was a barrier and one was not. We have added more text in the discussion to highlight the limitation of replication.

*Specific:*

*Line 26-27. Is the use of “intervention” here a typo? BACI designs are universally referred to as Before-After-Control-Impact. While your “environmental intervention” is a specific type of impact, it still is an impact and I don’t think we need to muddy the water by calling it something else. Suggest change to Before-After-Control-Impact.*

We thank the reviewer for catching this typo but we also have removed this sentence from the abstract.

*Line 222. Consider providing USGS stream gage station numbers (e.g., Chuckanut = 12201700). Also the citation for streamflow data by USGS is: USGS. (2022). USGS Water data for the nation. Retrieved from [<https://urldefense.com/v3/\\_https://waterdata.usgs.gov/nwis\\_>;](https://urldefense.com/v3/_https://waterdata.usgs.gov/nwis_)!!K-Hz7m0Vt54!mN\_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFB05N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y\_3zYcWRJOYw\$ <accessed on date>.*

We have added the gauge numbers and updated the citations for the USGS streamflow data.

*Line 314; 347-357-Quantitative PCR testing. It is unclear (in the main text) why you didn't do qPCR on all four salmon species given that qPCR primers exist for all 4 species. Because of life history differences, the fact that not all streams contain all species, and seasonal differences, variable PCR efficiencies among the assays, could there be an effect of using C\_cutthroat DNA as a surrogate for the other 3 species concentrations from the metabarcoding? Was using just cutthroat qPCR along just a matter of cost? Would using all 4 species, or selecting a different species as the reference (e.g., rainbow trout) appreciably change your results? Including a bit more description of this topic in the main text would help others considering the approach assess why and how to pick a reference species for such an analysis. Although covered in part in the discussion, a bit more exploration of this topic could be helpful.*

We thank the reviewer for this question! In fact, we could have done 4 qPCR assays as opposed to metabarcoding and using a single qPCR assay to expand from proportions to quantities. Here, we are trying to not only answer the question about culverts and salmonid passage, but also demonstrate the ability to use a single qPCR assay in combination with metabarcoding results for many species. It is true that in this case -- with only four species of interest, which all have published qPCR assays – quantifying all four using qPCR would be appropriate. However, in other studies with either many species (>6 say) or species where qPCR assays are not developed, this is a very cost and time effective approach. Additionally, we did not know how many species of interest we were going to have *before* we ran our samples. We might have had 20 species of interest from the metabarcoding data, at which point qPCR assays would have been unmanageable.

As for the question on selecting a different species as the reference, the main difference is that we would lose information on many samples – or would have to use multiple assays to get from proportions to absolute concentrations. In our case, we chose cutthroat trout because it was found in almost every single environmental sample. If we had used rainbow trout, there were several samples where rainbow trout were not found in the metabarcoding results and therefore we could not obtain quantitative information about cutthroat and coho in those samples.

In theory, using quantitative PCR for all species in a sample to obtain the total quantifiable DNA in a sample should give the same results regardless of which the reference species was. We suspect this will not work when there are very, very low percentages of a species in the metabarcoding results, resulting in very small denominators when expanding. However, that is

beyond the scope of this work. Here, we hoped to demonstrate this technique, while also answering an ecologically relevant question about culverts and culvert replacement.

We did not add any text because we feel as though this existing text in the discussion addresses the reviewers question directly: “Here, we ultimately only quantified the impacts of four species, but importantly, we did not know *a priori* how many species of interest there might be and we reduced our efforts two fold by only conducting two assays (one species-specific qPCR and one metabarcoding assay) as opposed to four assays (four species-specific qPCR assays).”

*Line 404-407. This seems a strange case. If the culvert is indeed a total barrier and there are no resident populations upstream, then you should get something close to the patterns you describe. But what accounts for those positive detections in 25% of the sampling months. Both clarkii and mykiss could be resident upstream, but if so then you would expect to see them in other months as well. If a “partial barrier” then you wouldn’t necessarily expect detections during the lowest flow time period in August/September. I think it appropriate to exclude Barnes because of the wonky results upstream, but what could be some explanations? Contamination, exogenous sources of eDNA?*

We acknowledge the reviewers concern that in three months we do see some salmonid DNA upstream of the culvert. However, this plot is proportions of salmonid eDNA on the y-axis, so you lose the resolution of quantification (this is before we have paired the compositional metabarcoding data with the qPCR model). So, in the three months that salmonid DNA is found upstream, it is a total of 3,262 reads in March, 1,902 reads in August, and 13 reads in September – as compared to an average number of salmonid reads of 63,375 in all the months of downstream samples.

We can offer that contamination could possibly account for the very small reads found in the upstream samples, but based on our positive controls monitoring for cross-contamination, we do not believe this to be true. In our monitoring of positive control (kangaroo) reads in environmental samples and environmental reads in positive control samples, we found this only occurred on two of the 13 MiSeq runs with total reads of 202 (min: 2, max: 136) on one run and total reads of 22 (min: 4, max: 10) on the other run. The Barnes upstream samples with 13, 1,902, and 3,262 reads were not on either of those runs with low levels of cross-contamination. The 13 reads of coho in September could possibly be contamination, possibly low-level cross contamination between another sample on that plate. Other sources of exogenous eDNA would be movement by predators. This sample ultimately does not move on in analysis because there was no quantifiable cutthroat DNA in the sample to move it from proportion space to DNA concentration.

If there were other samples with very low level contamination (i.e., 10s of reads) during metabarcoding, the difference in either proportion space or absolute abundance should be very, very small. For example, with an average read depth of nearly 80,000 reads per sample (and 46,000 salmonid reads per sample), 10 reads is on the order of .01%. Furthermore, proportions get multiplied by qPCR quantities, so again, the difference in say 46,000 reads and 46,010 reads when multiplying to get out of proportion space and into absolute space should be very small.

We have no evidence of contamination in the qPCR data as all no template controls (NTCs) were assigned a Ct value.

*Line 447: Elwha not Elwah. Also, suggest changing to "...after a large dam removal project (Elwha River near Port Angeles, Washington) since there were two dams removed as part of this project.*

We thank the reviewer for catching this typo. We have changed this sentence accordingly.

*Line 477 to 480: I'd say that it is likely to be the case that they are overwintering juveniles. Also, with both snorkel and electrofishing surveys it is possible to identify young of year individuals to species.*

We thank the reviewer for the comment and have changed the sentence to say that it is possible to identify year of young individuals to species level, but kept that these visual surveys are conducted infrequently as we have had much contact with the City of Bellingham and their data are limited for visual surveys. To our knowledge, there are no snorkel surveys or electrofishing surveys, just spawner surveys. They also have used smolt traps in the past, but only have data for one year (2018), which found coho, cutthroat, and rainbow, and unknown *Onchorynchus spp.* (<https://cob.org/wp-content/uploads/2018-PaddenCrk-Smolt-Trap-Summary-Full.pdf>).

*Line 568-573. "...we find that culverts designated as barriers were likely not blocking fish passage." I can't understand how you can make this statement when all of the species except Coho can have both anadromous and resident forms. Those resident forms could have been present upstream and downstream of the culvert before one was ever installed, and then the remained as two separate populations upstream and downstream. Using eDNA (or any other method) to show that there are fish upstream of the barrier does not by itself demonstrate the blocking of fish passage. Obligate anadromous species like Pink, Chum, Chinook salmon would have been much better species to use for addressing the question because if a culvert is indeed a barrier, then you shouldn't find any signal upstream of total barriers. In the case where species can be both anadromous and resident, other methods (e.g. radio telemetry, pit tagging) would have to be used to show that fish are migrating past the barrier.*

We thank the reviewer for pointing out the challenges associated with designating barriers when a species has both a resident and migratory form. Unfortunately, we did not have signals from pink, chum, and chinook salmon in these creeks for information on obligate anadromous species. We would have loved to have other methods like pit tagging or telemetry data to supplement this study but unfortunately we did not.

However, we do note that with the additional data on the second culvert, there is clearly a difference in species compositions above/below the second culvert (I-5) and there is clearly very little difference in species compositions above/below the first culvert (SR-11) in Padden Creek. Given the extremely small spatial scale over which these two culverts span, it seems unlikely that the resident populations could explain the differences (and lack of difference).

*Line 596-97. "...Here we found very minimal effects of both culverts in general and construction,..." Something seems to be missing from this sentence.*

This sentence no longer exists with the addition of new data and also the suggestion from the first reviewer to shorten the Discussion.

*Figures:*

*Figure 2. Cite source as USGS gage data somewhere in figure or in caption.*

We have added the gage numbers in the caption of Figure 2.

*Figure 4. An indication of when the intervention occurred on Padden Creek would be helpful on this graph.*

We have added a black dashed line indicating the time of construction in Padden Creek and have edited the caption to reflect this.

*Figure 6. It is really hard to distinguish between the light and dark colored symbols because of the overlapping in many of the time periods.*

This figure does not exist anymore but we have taken the reviewers comment into consideration for the new figures and hope that it is easier to distinguish.

*Figure 8. Unclear why you've included the other creeks in a graph about Padden...the overlapping just serves to wash out the view of symbols (clearly shown in the O. nerka graph, which is labeled incorrectly – should be blue symbol not gray).*

Similarly, this figure does not exist anymore but have a similar version of the new figure. We kept the non-treatment creeks plotted in grey to demonstrate the effect of culvert over time on creeks where construction did not happen. It gives a reference point for whether culverts have a temporal impact in the absence of construction. We think it is helpful to visualize the impact of the culverts in the control creeks over time as compared to the impact of the culvert in Padden both before and after construction. We did take the reviewer's comment into consideration and hope it is clear why we kept the control creeks in.