

<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> † Currently at NOAA Pacific Marine Environmental Laboratory, Seattle, WA, USA.

<sup>12</sup> ‡ Corresponding author: eallan@uw.edu

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<sup>19</sup> *culvert*

20 **Abstract**

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

## 44 Introduction

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

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

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

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

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

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

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

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

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

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

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

## 142 Methods

### 143 Site and Species Selection

144 We selected sampling locations based loosely on an asymmetrical BACI (Before-After-Control-Impact) study  
145 design (Underwood 1992, 1994, Benedetti-Cecchi 2001) to measure the environmental impact of a culvert  
146 replacement using eDNA. We sampled four control creeks where construction was not occurring (Figure 1) at  
147 monthly intervals, both upstream and downstream of each creek's culvert. The two culverts in the treatment  
148 creek (Padden) were suspected to be partially impassable and thus were removed and replaced during the  
149 course of the study. The four control creeks ranged from preventing fish passage (Barnes and Chuckanut),  
150 partially passable (Squalicum), to allowing fish passage (Portage; see Appendix S1) (Washington Department  
151 of Fish and Wildlife 2019). These creeks were chosen due to their comparable size, flow, watersheds, and  
152 species presumed to be present to constrain as many ecological variables as possible.

153 The first culvert replacement (SR-11) in Padden Creek occurred over about two months and included the  
154 "de-watering" of the creek, removal of the existing culvert, installation of the new culvert, and then the  
155 "re-watering" of the creek from late August 2021 to early October 2021 (Appendix S1: Figure S4). The second  
156 culvert replacement (I-5) in Padden Creek was a much larger construction project, including daylighting the  
157 creek and building a bridge under a large, five-lane interstate. In-water work for the I-5 culvert replacement  
158 began in late June 2022 and was completed in September 2022. By sampling before, during, and after both  
159 construction events, we were then able to isolate the effect of the culvert replacement itself – controlling for  
160 temporal trends, background environmental variability, and sampling variability – using a linear mixed effects  
161 model of eDNA abundances across creeks, time points, sampling stations, and species.

162 Because salmonids are the primary species of management concern in these creeks, we focus the present  
163 analysis on the four salmonid species most common in our data: cutthroat trout (*Oncorhynchus clarkii*),  
164 coho salmon (*O. kisutch*), rainbow/steelhead trout (*O. mykiss*), and sockeye/kokanee salmon (*O. nerka*).  
165 Not all four salmonids are expected to be found in all five of the creeks sampled. As documented by WA  
166 Department of Fish and Wildlife SalmonScape (<http://apps.wdfw.wa.gov/salmonscape/map.html>), all creeks  
167 contain cutthroat trout, steelhead trout, and coho salmon. Barnes Creek is the only creek documented to  
168 have kokanee salmon (a freshwater sub-type of sockeye salmon). However, local spawner surveys conducted  
169 by the City of Bellingham from 2015-2020 in Padden Creek documented kokanee salmon, as well as the

170 other three species (City of Bellingham 2015). The four salmonid species in this study have different life  
171 histories and behaviors that would impact when fish (and therefore eDNA concentrations) occur in the creeks.  
172 Furthermore, three of the four species in this study have both freshwater resident and saltwater migrating  
173 behavior. For the fish exhibiting migratory behavior, the run timings vary for each species in the study area  
174 (see Discussion and Appendix S1: Figure S4). Therefore, our eDNA concentrations might reflect contributions  
175 from both migrating and non-migrating individuals at any given time point in the dataset.

176 **Water Sampling**

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

185 Over the course of the sampling, water discharge varied from very low to no flow in summer months to high  
186 flow in winter months (Figure 2). We account for this dilution by converting eDNA concentration [copies/ $\mu$ L]  
187 to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s] (Tillotson et al.  
188 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey (USGS) were  
189 used for Padden Creek (USGS Gauge 12201905), Chuckanut Creek (USGS Gauge 12201700), and Squalicum  
190 Creek (USGS Gauge 12204010; <https://maps.waterdata.usgs.gov/mapper/index.html>; U. S. Geological  
191 Survey (1994); Appendix S1: Figure S1). Over the course of sampling, the flow gauges at Chuckanut Creek  
192 and Squalicum Creek became inoperable after a major flooding event. To find discharge rates for Chuckanut  
193 and Squalicum Creeks, five years of historical data (2015-2020) were used to generate a monthly averaged  
194 correction factor based on Padden Creek (Appendix S1, Appendix S1: Figure S3). No discharge data was  
195 available for Portage Creek or Barnes Creek. Based on field sampling conditions, the discharge from Padden  
196 Creek was used as a proxy for both Portage and Barnes as they are in similarly sized watershed areas and  
197 land-cover characteristics.

198 **DNA Extraction, Amplification, Sequencing**

199 All molecular work prior to sequencing was performed at the University of Washington. Details of the  
200 molecular work can be found in Appendix S1. Briefly, DNA was extracted off filters using a QiaShredder  
201 column (Qiagen, USA) and the DNeasy Blood and Tissue Kit (Qiagen, USA) with an overnight incubation  
202 (Appendix S1, Thomas et al. (2019)). Extracts were stored at -20°C until PCR amplification within 2 months  
203 of extraction.

204 For metabarcoding, we targeted a ~186 bp hypervariable region of the mitochondrial DNA 12S rRNA gene for  
205 PCR amplification (MiFish; Miya et al. 2015), but using modified primer sequences as given in Praebel and  
206 Wangensteen (unpublished; via personal communication). The primer sequences, final reaction recipe, and  
207 cycling conditions can be found in Appendix S1. Each month of samples was amplified on a single plate with  
208 the addition of a no template control (NTC; molecular grade water in lieu of template) and a positive control  
209 (genomic DNA from kangaroo, a species not present in the environment). PCR products were visualized,  
210 size-selected, and diluted iteratively if inhibited. After cleaning, a second PCR amplification added unique  
211 indices to each sample using Nextera indices (Illumina, USA) to allow pooling multiple samples onto the same  
212 sequencing run (See Appendix S1 for details). Indexed PCR products were also size-selected and visualized  
213 before pooling for sequencing. Samples were randomized in 3-month blocks and each block split across 3  
214 sequencing runs to avoid run effects, for a total of 14 sequencing runs. The loading concentration of each  
215 library was 4-8 pM and 5-20% PhiX was included depending on the composition of the run. Sequencing  
216 was conducted using an Illumina MiSeq with v3 2x300 chemistry at the NOAA Northwest Fisheries Science  
217 Center and the University of Washington's Northwest Genomics Center.

218 Here, we used mock communities to determine the species-specific amplification efficiencies for each salmonid  
219 in the study. We constructed five communities with known proportions of starting DNA from different  
220 species (total DNA as measured by Qubit). The communities ranged from having a total of 12 to 20  
221 species, but six salmonid species were included in all five mock communities (Appendix S3: Table S2). We  
222 sequenced these communities using the same metabarcoding primers and thermocycling conditions above and  
223 then determined the species-specific amplification rates given the discrepancy between the known starting  
224 proportion and the proportion of reads after sequencing. The mock community data were then used to correct  
225 the sequencing reads from the environmental samples to estimate the starting DNA proportions of each  
226 species in environmental samples, which is the metric of interest (Figure 3, green boxes). This is the first  
227 application of the model to correct eDNA data from water samples with mock community data as described  
228 in Shelton et al. (2022) (see Appendix S2 for more information).

**229 Bioinformatics**

230 After sequencing, bioinformatic analyses were conducted in R (R Core Team 2017). A more detailed  
231 description of the bioinformatics pipeline is included in Appendix S1. Briefly, primer sequences were removed  
232 using *Cutadapt* (Version 1.18) (Martin 2011) before *dada2* (Callahan et al. 2016) trimmed, filtered, merged  
233 paired end reads, and generated amplicon sequence variants (ASVs). Taxonomic assignment was conducted  
234 via the *insect* package (Wilkinson et al. 2018) using a tree generated by the developers for the MiFish primers  
235 that was last updated in November 2018. Only species level assignments from *insect* were retained and ASVs  
236 not annotated or not annotated to species level were then checked against the NCBI nucleotide database  
237 using BLAST+ (Camacho et al. 2009). Query sequences that matched a single species at >95% identity  
238 were retained.

**239 Quantitative PCR and Inhibition Testing**

240 We quantified cutthroat trout (*O. clarkii*) DNA in each sample, targeting a 114 bp fragment of the cytochrome  
241 b gene with a qPCR assay (Duda et al. 2021). The primer/probe sequences, final recipe, and thermocycling  
242 conditions can be found in Appendix S1. Each DNA sample was run in triplicate and was checked for  
243 inhibition using the EXO-IPC assay (Applied Biosystems). The majority of environmental samples (60%)  
244 were inhibited and accordingly diluted for analysis. In 80% of inhibited samples, a 1:10 dilution or less  
245 remedied the inhibition, but some samples (11%) required dilution by a factor of up to 1000. Each plate  
246 included a 8-point standard curve created using synthetic DNA (gBlocks) ranging from 1 to 100,000 copies/ $\mu$ L  
247 and six no template controls (NTCs) were included on each plate with molecular grade water instead of  
248 template. All qPCRs were conducted on an Applied Biosystems StepOnePlus thermocycler.

249 All qPCR data was processed in R using Stan (Stan Development Team 2022), relating environmental  
250 samples to the standard curve via a linear model (Figure 3, blue boxes; Appendix S2: Figure S1). We  
251 amended the standard linear regression model to more realistically capture the behavior of qPCR observations,  
252 accommodating non-detections as a function of underlying DNA concentration, and letting the standard  
253 deviation vary with the mean (lower-concentration samples had more uncertainty). See McCall et al. (2014)  
254 and Shelton et al. (2019) for similar models; see Appendix S2 for full statistical details. Subsequent analysis  
255 corrected for sample-specific dilution if found inhibited and corrected for any variation in water-volume  
256 filtered during sample collection. Samples with standard deviations between technical replicates larger than  
257 1.5 Ct values were removed from analyses.

258 **Quantitative Metabarcoding**

259 The intercalibration of the mock community samples demonstrated the rank order of amplification efficiencies  
260 for salmonids (Appendix S1: Figure S10 and Appendix S1: Figure S11). Cutthroat trout (*O. clarkii*) and  
261 sockeye/kokanee salmon (*O. nerka*) had similar amplification efficiencies, both of which were higher than  
262 rainbow/steelhead trout (*O. mykiss*) and coho salmon (*O. kisutch*), which had the lowest amplification  
263 efficiency. Calibrated metabarcoding analysis yielded quantitative estimates of the proportions of species'  
264 DNA in environmental samples prior to PCR. We then converted these proportions into absolute abundances  
265 by expansion, using the qPCR results for our reference species, cutthroat trout (*O. clarkii*). We estimated the  
266 total amplifiable 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  
267 units of [DNA copies/uL] and then expanded species' proportions into absolute concentrations by multiplying  
268 these sample-specific total concentrations by individual species' proportions, such that for species  $j$  in sample  
269  $i$ ,  $C_{i,j} = C_{\text{amplifiable}_i} * \text{Proportion}_{i,j}$ . Here, we combine the modeled output of the qPCR model for cutthroat  
270 trout (Figure 3, dashed blue box) and modeled proportions of salmonid DNA from metabarcoding (Figure  
271 3, dashed green box). Although in the future this could be used as a joint model, here the precision of our  
272 modeled estimates were very high such that we used the mean of the posterior estimates from each model  
273 to move forward as input to the time series model (Figure 3, dashed purple box; see Appendix S2 for more  
274 details). Finally, due to the range of water discharge over the course of the year, we converted from DNA  
275 concentration [copies/L] to a mass flow rate [copies/s] after multiplying by the discharge of each creek [ $\text{m}^3/\text{s}$ ]  
276 (Figure 3, solid purple boxes).

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

278 We sampled four control creeks as context against which to compare the observations in Padden Creek,  
279 our treatment creek where the two culverts were being replaced. At a given station in a given creek, some  
280 DNA concentration exists for each species. For simplicity, we focus on a single species and a single station  
281 (downstream or upstream) for the moment. Our observations of the (log) DNA concentration in creek  $i$  at  
282 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,  
283 time points, species, or with environmental covariates of interest.

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

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

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

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

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

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

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

## Results

### Metabarcoding and Quantitative PCR

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

In the mock community samples, 98.7% of the ~5 million reads after quality filtering were annotated to species level. Importantly, the target salmonid ASVs in the mock communities were found in environmental samples, unambiguously linking the taxa in calibration samples with those in environmental samples. The most common salmonid species found in the environmental samples was cutthroat trout (*O. clarkii*), which was found in ~85% of samples, followed by coho salmon (*O. kisutch*) found in ~62% of samples, then rainbow/steelhead trout (*O. mykiss*) found in ~40% of samples, and finally sockeye/kokanee salmon (*O. 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 Appendix S1 and Appendix S2), we  
320 estimated the salmonid composition across time points, creeks, and stations (Figures 4 and 5). The culvert in  
321 one 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  
332 species (See Appendix S2). 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  
345 where culverts block the passage of some salmon but not others (Appendix S1: Figure S12). The maximum  
346 positive log-fold change (i.e., upstream having a higher mass flow rate) was 2.78 in Squalicum Creek for coho  
347 salmon (*O. kisutch*) in August 2021, while the maximum negative log-fold change (i.e., downstream having a

348 higher mass flow rate) was -1.11 found in Squalicum Creek for cutthroat trout (*O. clarkii*) in December 2021  
349 (Appendix S1: Figure S12). Of all species, creeks, and time points, 23 of the 151 observations were within a  
350 log-fold change of -0.1 to 0.1, which corresponds with eDNA mass flow rates upstream within 10% of mass  
351 flow rates downstream.

352 We also considered how the log-fold change in eDNA mass flow rates were impacted by the flow rate or  
353 discharge of the creek itself (Figure 9). We found that at months of the lowest flow (summer months), the  
354 log-fold changes between mass flow rates were the highest, while in winter months with highest discharge the  
355 log-fold changes were lower and downstream sites often had higher eDNA mass flow rates than upstream  
356 sites (Appendix S1: Figure S12).

### 357 Effects of Culvert Replacement

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

## 365 Discussion

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

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

375 A few other studies have used eDNA to measure environmental impacts in rivers and streams. Duda et al.  
376 (2021) used 11 species-specific qPCR assays to document the distribution of resident and migratory fish

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

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

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

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

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

412 For these four anadromous salmonids, the run timings for the migrating populations vary for each species in  
413 the study area (Bellingham, WA). Adult coastal cutthroat (*O. clarkii*) are documented to run throughout  
414 the entire year, whereas coho salmon (*O. kisutch*) run from September to December, sockeye salmon (*O.*  
415 *nerka*) run from October to December, and steelhead trout (*O. mykiss*) run from November to June. For  
416 migrating coho (*O. kisutch*) and steelhead trout (*O. mykiss*), juveniles may be present in the creeks year-round  
417 (Appendix S1: Figure S4). eDNA methods at present cannot distinguish adults versus juveniles from DNA  
418 found in a water sample.

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

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

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

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

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

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

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

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

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

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

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

## 501 Conclusion

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

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## 526 Conflict of Interest Statement

527 The authors declare there are no conflicts of interest.

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## 718 Figures

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

724 **Figure 2.** Discharge ( $\text{m}^3/\text{s}$ ) in Padden (USGS Gauge 12201905), Chuckanut (USGS Gauge 12201700),  
725 and Squalicum (USGS Gauge 12204010) Creeks over the course of sampling. Gauges at Chuckanut  
726 and Squalicum Creek went offline in November 2021 after a major storm event. Portage Creek and  
727 Barnes Creek did not have stream gauges. Circles designate the day of sampling. For Padden Creek,  
728 the nearest 15 minute interval of flow was used. For Chuckanut and Squalicum Creeks, the correction  
729 factor from five years of historical data from Padden Creek was used (see methods section and Appendix  
730 S1: Figure S2 and Appendix S1: Figure S3).

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

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

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

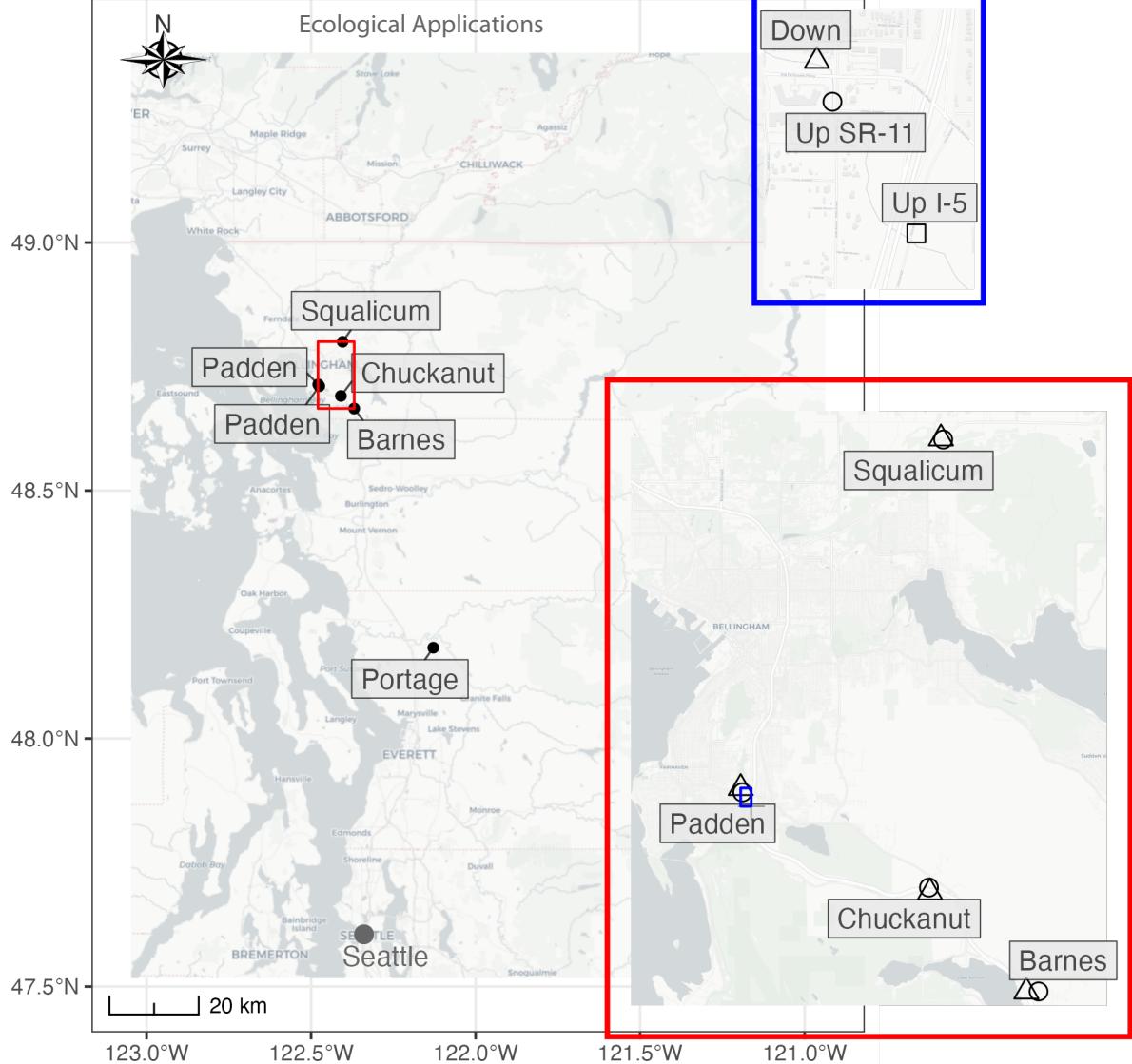
745 **Figure 6.** Absolute mass flow rate (log copies/s) of cutthroat trout (*O. clarkii*) as measured by qPCR  
746 after flow correction. Note that Barnes Creek and Chuckanut Creek were not sampled after February  
747 2022. Red crosses show the limit of detection for each species and time point, which changes with flow  
748 rate and total volume filtered per sample.

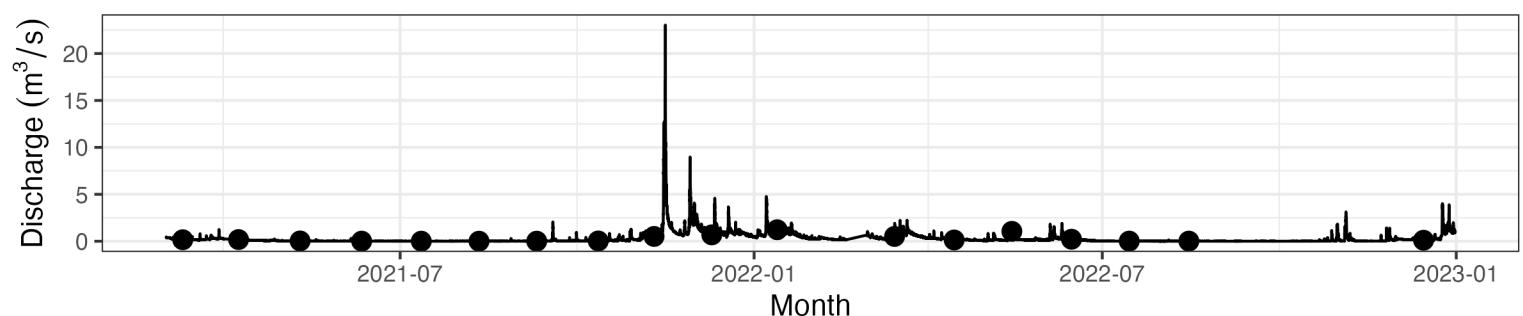
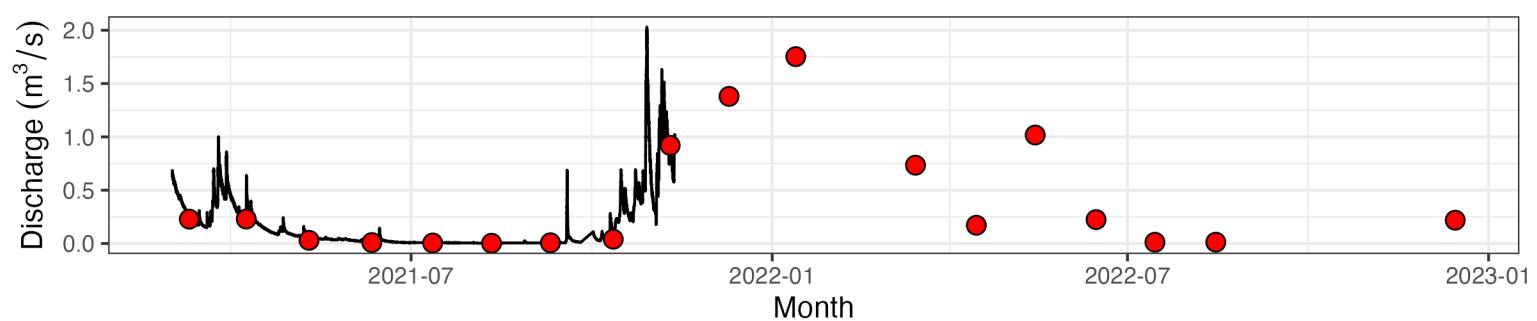
749 **Figure 7.** Trends in mass flow rate (log copies/s) for each of four salmonid species across creeks and  
750 across time as estimated by eDNA analysis. Points represent posterior means for the linear mixed  
751 effects model and error bars represent the 95% posterior confidence interval. Colors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

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

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

768 **Figure 10.** Effect of construction on log-fold change in eDNA mass flow rate upstream to downstream  
769 in Padden Creek. Yellow shading shows when construction started for each of the two culverts. Grey  
770 points show the corresponding log-fold changes in control creeks and black points show Padden Creek.  
771 Sockeye/kokanee salmon (*O. nerka*) was only found in Padden Creek so other creeks are not shown.  
772 Samples with very low eDNA mass flow rates (less than 150 copies/s) were removed before plotting to  
773 remove extreme proportional values due to large denominators.



**Padden Creek****Chuckanut Creek****Squalicum Creek**