Quantifying Impacts of an Environmental Intervention Using Environmental DNA

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For submission to: *Ecological Applications*

Manuscript type: Article

Open Research Statement: Data are provided as private-for-peer review (shared privately or publicly on a repository). The repository for code can be found at: https://github.com/eandrusz/quantitative_salmon_culverts.git

Keywords: environmental DNA, quantitative metabarcoding, environmental impact assessments, salmon, culvert

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 the biological elements of affected 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 12-month time-series of water samples taken before, during, and after a culvert removal project in a salmonid-bearing freshwater stream. We use an asymmetrical Before-After-Control-Impact (BACI) design with 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 hierarchical Bayesian time-series 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/steelhead trout (*O. mykiss*), and sockeye/kokanee salmon (*O. nerka*). After accounting for temporal variability common to the sampled creeks, we find only transient effects on these species during the several months after construction. 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.

Introduction

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

A key difficulty in conducting ecosystem assessments is that there is no one way to survey the world and just "see what is there." All methods of environmental sampling are biased as they capture a selective portion of the biodiversity present (Rubin et al. 2017). Net samples for fish, for example, fail to capture species too small or too large to be caught in the net. Environmental DNA (eDNA), however, comes as close to this goal as any method yet developed: a sample of water, soil, or even air, contains the genetic traces of many thousands of species, from microbes to whales. Sequencing eDNA is therefore a means of surveying many species in a consistent and scalable way (Taberlet et al. 2012, Thomsen and Willerslev 2015). Environmental assessments have begun to make use of eDNA for such work around the world (Muha et al. 2017, Duda et al. 2021, Klein et al. 2022, Maasri et al. 2022, Moss et al. 2022), but are not yet common practice. Surveying the world by eDNA has long been commonplace in microbial ecology (Ogram et al. 1987, Rondon et al. 2000, Turnbaugh et al. 2007) but has recently become popular for characterizing eukaryotic communities (Taberlet et al. 2012, Kelly et al. 2014, De Vargas et al. 2015, Port et al. 2015, Valentini et al. 2016, Stat et al. 2017). Techniques generally include an amplification step such as quantitative PCR, digital or digital-droplet PCR, or traditional PCR from mixed templates followed by high-throughput sequencing (Ruppert et al. 2019). This last technique is known as eDNA metabarcoding.

In a metabarcoding approach, broad-spectrum PCR primers identify hundreds or thousands of taxa across a very wide diversity of the tree of the life (e.g., Leray et al. (2013)), but nevertheless the absence of a taxon from a sequenced sample does not indicate the absence of that taxon from the environment but rather that the taxon failed to amplify (Shelton et al. 2016, Kelly et al. 2019, Buxton et al. 2021). In virtually all comparisons, metabarcoding recovers far more taxa than any other sampling method (Port et al. 2015, Kelly et al. 2017, Seymour et al. 2021). However, we expect results from metabarcoding to differ dramatically from non-PCR based sampling methods due to the fundamental differences in sampling genetic waste as opposed to whole organisms. Furthermore, eDNA analyses rely on several laboratory processes, including PCR amplification, all of which contribute to complicating the interpretation of results (see Shelton et al. (2016) and Kelly et al. (2019)). Specifically, PCR amplification is an exponential process for which the efficiency varies across species and primer set (Gloor et al. 2016). By understanding these differences, we can correct for taxon-specific biases to yield quantitative estimates of the community composition prior to PCR (McLaren et al. 2019, Shelton et al. 2022).

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

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

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

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

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

Here, we report the results of a year-long eDNA sampling effort before, during, and after a small construction project in our experimental creek, assessing the impact of that project on the salmonid species present. We do so using a combination of metabarcoding (12s mtDNA) and qPCR to yield estimates of the concentrations of DNA present at each time point, and we use parallel samples from an additional four control creeks to develop a causal analysis of changes in these concentrations. A clear opportunity for policy-relevant eDNA work is in using its power to survey many species at a time to improve the way we assess the impacts of human activities. Here, we demonstrate the utility of eDNA for such assessments.

Methods

Site and Species Selection

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

The intervention (i.e., culvert replacement) in Padden Creek occurred over about two months and included the "de-watering" of the creek, removal of the existing culvert, installation of the new culvert, and then the "re-watering" of the creek from late August 2021 to early October 2021 (Supplemental Text 1; Supplemental Figure 3). We were then able to quantify the effect of the culvert replacement itself – controlling for temporal trends, background environmental variability, and sampling variability – using a Bayesian time-series model to jointly model salmon eDNA abundances across creeks, time points, sampling stations, and species.

Because salmonids are the primary species of management concern in these creeks, we focus the present analysis on the four salmonid species most common in our data: cutthroat trout (*Oncorhynchus clarkii)*, coho salmon (*O. kisutch*), rainbow/steelhead trout (*O. mykiss*), and sockeye/kokanee salmon (*O. nerka*). Not all four salmonids are expected to be found in all five of the creeks sampled. As documented by WA Department of Fish and Wildlife SalmonScape (http://apps.wdfw.wa.gov/salmonscape/map.html), all creeks contain cutthroat trout, steelhead trout, and coho salmon. Barnes Creek is the only creek documented to have kokanee salmon (freshwater sub-type of sockeye salmon). However, local spawner surveys conducted by the City of Bellingham from 2015-2020 in Padden Creek documented kokanee salmon, as well as the other three species and importantly, several unknown species of live and dead fish and redds (nests dug by fish in gravel to deposit eggs) (City of Bellingham 2015). The four salmonid species in this study have different life histories and behaviors that would impact when fish (and therefore eDNA concentrations) occur in the creeks

. Furthermore, three of the four species in this study have both freshwater resident and saltwater migrating behavior. For the fish exhibiting migratory behavior , the run timings vary for each species in the study area (see Discussion and Supplemental Figure 3). Therefore, our eDNA concentrations might reflect contributions from both migrating and non-migrating individuals at any given time point in the dataset.

Water Sampling

We collected water samples monthly between March 2021 and February 2022. At each sampling station (N=2, upstream and downstream of a culvert) at each creek (N=5) in each month (N=12), we collected three 2-liter water samples, for a total of 360 water samples. Samples were collected using Smith Root's eDNA Backpack (Thomas et al. 2018), a portable pumping-and-filtering device set to filter at 1 L/min at 82.7 kPa (12 psi). In some months, less than 2 L of water was filtered due to clogging (min = 1.02 L, mean = 1.97 L, median = 2.01 L; see Supplemental Figure 4). Water samples were filtered using single-use inlet tubes through 5μm self-preserving filters (Smith Root, Vancouver, WA), which were then dried and kept at room temperature until DNA extraction within 1 month of collection (Thomas et al. 2019).

Over the course of the year of sampling, water discharge varied from very low to no flow in summer months to high flow in winter months (Figure **??**). Thus, we need to account for the large difference in water volume over the course of the year and thus dilution by converting eDNA concentration [copies/μL] to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s] (Tillotson et al. 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey (USGS) were used for Padden Creek (USGS Gage 12201905), Chuckanut Creek (USGS Gage 12201700), and Squalicum Creek (USGS Gage 12204010; https://maps.waterdata.usgs.gov/mapper/index.html; U. S. Geological Survey (1994); Supplemental Figure 1). During the year of sampling, the flow gauges at Chuckanut Creek and Squalicum Creek became inoperable after a major flooding event. To find discharge rates for Chuckanut and Squalicum Creeks, five years of historical data (2015-2020) were used to generate a monthly averaged correction factor based on Padden Creek. For the year of sampling (2021-2022), the discharge rates used at Chuckanut and Squalicum Creeks were estimated based on the correction factor from Padden Creek (Supplemental Figure 2). No discharge data was available for Portage Creek or Barnes Creek. Based on field sampling conditions, the discharge from Padden Creek was used as a proxy for both Portage and Barnes as they are in similarly sized watershed areas and land-cover characteristics. Though in the year of sampling, the discharge in Padden Creek ranged from no metered flow to 23 m^3/s, the discharge on the dates of sampling only reached a maximum of 1.3 m^3/s.

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 10,000 rainbow trout were stocked in April and May 2021 and 30,000 kokanee salmon were stocked in May 2021 (Supplemental Figure 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).

DNA Extraction, Amplification, Sequencing

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

For the metabarcoding approach, we targeted a ~186 bp hypervariable region of the mitochondrial DNA 12S rRNA gene for PCR amplification (MiFish; Miya et al. 2015), but using modified primer sequences as given in Praebel and Wangensteen (unpublished; via personal communication). The primer sequences, final reaction recipe, and cycling conditions can be found in Supplemental Text 1. Each month of samples was amplified on a single plate with the addition of a no template control (NTC; molecular grade water in lieu of template) and a positive control (genomic DNA from kangaroo). PCR products were visualized, size-selected, and diluted iteratively if inhibited. After cleaning, a second PCR amplification added unique indices to each sample using Nextera indices (Illumina, USA) to allow pooling multiple samples onto the same sequencing run (See Supplemental Text 1 for details). Indexed PCR products were also size-selected and visualized before pooling for sequencing. Samples were randomized in 3-month blocks and each block split across 3 sequencing runs, for a total of 12 sequencing runs. The loading concentration of each library was 4-8 pM and 5-20% PhiX was included depending on the composition of the run. Sequencing was conducted using an Illumina Miseq with v3 2x300 chemistry at the NOAA Northwest Fisheries Science Center and the University of Washington's Northwest Genomics Center.

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

Bioinformatics

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

Quantitative PCR and Inhibition Testing

We quantified cutthroat trout (*O. clarkii*) DNA in each sample, targeting a 114 bp fragment of the cytochrome b gene with a qPCR assay (Duda et al. 2021). The primer/probe sequences, final recipe, and thermocycling conditions can be found in Supplemental Text 1. Each DNA sample was run in triplicate and was checked for inhibition using the EXO-IPC assay. The majority of environmental samples (65%) were inhibited and accordingly diluted for analysis. In 75% of inhibited samples, a 1:10 dilution remedied the inhibition, but some samples required dilution by a factor of up to 1000 (Supplemental Figure 5). Each plate included a 8-point standard curve created using synthetic DNA (gBlocks) ranging from 1 to 100,000 copies/μL and six no template controls (NTCs) were included on each plate with molecular grade water instead of template. Plates were re-run if efficiency as determined by the standard curve was outside of the range of 90-110%. All qPCRs were conducted on an Applied Biosystems StepOnePlus thermocycler.

All qPCR data was processed in R using Stan (Stan Development Team 2022), relating environmental samples to the standard curve via a linear model (Figure 3, blue boxes). We amended the standard linear regression model to more realistically capture the behavior of qPCR observations, accommodating non-detections as a function of underlying DNA concentration, and letting the standard deviation vary with the mean (lower-concentration samples had more uncertainty). See McCall et al. (2014) and Shelton et al. (2019) for similar models; see Supplemental Text 2 for full statistical details. Subsequent analysis corrected for sample-specific dilution if found inhibited and corrected for any variation in water-volume filtered during sample collection.

Quantitative Metabarcoding

The intercalibration of the mock community samples demonstrated the rank order of amplification efficiencies for salmonids (Supplemental Figures 14 and 15). Cutthroat trout (*O. clarkii*) and sockeye/kokanee salmon (*O. nerka*) had similar amplification efficiencies, both of which were higher than rainbow/steelhead trout (*O. mykiss*) and coho salmon (*O. kisutch*), which had the lowest amplification efficiency. Calibrated metabarcoding analysis yielded quantitative estimates of the proportions of species' DNA in environmental samples prior to PCR. We then converted these proportions into absolute abundances by expansion, using the qPCR results for our reference species, *O. clarkii*. We estimated the total amplifiable salmonid DNA in environmental sample i as $\mathrm{C}_{\mathrm{amplifiable}_i} = \frac{\mathrm{C}_{\mathrm{qPCR\ reference}_i}}{\mathrm{Proportion}_{\mathrm{reference}_i}}$, where C has units of [DNA copies/uL] and then expanded species' proportions into absolute concentrations by multiplying these sample-specific total concentrations by individual species' proportions, such that for species j in sample i, $\mathrm{C}_{i,j} = \mathrm{C}_{\mathrm{amplifiable}_i} * \mathrm{Proportion}_{i,j}$. Here, we combine the modeled output of the qPCR model for cutthroat trout (Figure 3 dashed blue box) and modeled proportions of salmonid DNA from metabarcoding (Figure 3 dashed green box). Though in the future this could be used as a joint model, here the precision of our modeled estimates were very high such that we used the mean of the posterior estimates from each model to move forward as input to the time series model (Figure 3 dashed purple box; see Supplemental Text 2 for more details). Finally, due to the range of water discharge over the course of the year, we converted from DNA concentration [copies/L] to a mass flow rate [copies/s] after multiplying by the discharge of each creek [m^3/s] (Figure 3, solid purple boxes).

Estimating the Effects of Culvert Replacement and of Culverts Themselves

We sampled four control creeks as context against which to compare the observations in Padden Creek, our treatment creek where the culvert was being replaced. Recognizing that these observations are autocorrelated in time, we use an AR(1) autocorrelation model, implemented in Stan via R, to capture the observed temporal trends.

We observe the log-DNA concentration, Y, for a given species in a given sample as a random variable drawn from a normal distribution with mean μ and observation variance σ^2. For each species j, the expected log-DNA concentration μ at time t in creek i at station d is a linear function of the DNA concentration for the same creek/station at $t-1$.

$$Y_{i,j,t,d} \sim \mathcal{N}(\mu_{i,j,t,d},\, \sigma^2)$$
$$\mu_{i,j,t,d} = \alpha_{i,j,t} + \epsilon_{i,j,t,d} + \eta_{i,j,t,d}$$
$$\epsilon_{i,j,t,d} \sim \mathcal{N}(\beta_j \mu_{i,t-1,d},\, \phi^2)$$

Intercept α varies by time, creek, and species, capturing creek-level deviations from the previous time-step. The autoregression term ϵ is itself a random variable drawn from a normal distribution with expected value $\beta_j \mu_{i,t-1,d}$ and process variance ϕ^2, such that the species-specific slope term β_j estimates the degree of autocorrelation in log-DNA concentration between one time-step and the next. The model shares information across creeks and time-points via β_j.

Finally, η captures the difference in log-DNA concentration between upstream and downstream stations 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 a given creek at a given time. The effect of construction in our focal Padden Creek, then, is the change in η after construction versus prior to construction. We fit this model in a Bayesian framework using moderately informative priors on all parameters, and confirmed model convergence ($\hat{R} < 1.01$) across 3 chains and 3500 model iterations. See statistical supplement (Supplemental Text 2) for prior values, diagnostics, and full model details.

Results

Metabarcoding and Quantitative PCR

In total, sequencing runs generated ~42 million reads across all environmental samples (12 months x 2 stations x 5 creeks x 3 biological replicates = 360 filters) 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, ~33 million reads remained from ~21,000 amplicon sequence variants (ASVs) in the environmental samples, of which ~81% of reads were annotated to species level (per sample: mean = 78%, median = 88%, 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 ~68% 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 ~90% of samples, followed by coho salmon (*O. kisutch*) found in ~60% of samples, then rainbow/steelhead trout (*O. mykiss*) found in ~40% of samples, and finally sockeye/kokanee salmon (*O. nerka*) found in ~5% of samples. Not only was cutthroat trout (*O. clarkii*) found in the majority of environmental samples, but also ~63% of samples across all times, creeks, and stations had at least 50% of reads assigned to cutthroat trout.

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

All environmental samples were quantified for absolute concentrations of cutthroat trout DNA across 30 qPCR plates, resulting in 280 samples (~80%) with a positive detection in at least 1 of 3 technical replicates. The modeled output of cutthroat trout DNA concentrations, ranged from 10 copies/L to 1.4 x 10^6 copies/L, with a mean value of ~58,000 copies/L (Figure 5).

We combined compositional information from metabarcoding with absolute concentrations for our reference species, cutthroat trout (*O. clarkii*), from the qPCR to estimate the total concentration of DNA for each species (See Supplemental Text 2). The joint time-series model shared information across stations and creeks; consequently, data from one of the control creeks (Barnes) could not be included because of the nearly total absence of salmonids upstream of its culvert. However, data from the remaining creeks characterized trends in the other four target species well and could be modeled appropriately (Figure 6).

Effects of Culverts

Before considering the effect of construction, the difference in abundance trends between upstream and downstream stations (Figure 6) demonstrates that the culverts themselves have some effect, but not a large effect on the salmonid species surveyed. Therefore, these four creeks (which range in how they are classified in fish passability) do not seem to be blocking salmonid passage. A notable exception was Barnes Creek, which was not included in the time series model, as the culvert was so clearly a barrier as most time points had no salmonid DNA upstream and therefore models including Barnes do not converge as a result of the large fraction of sampling points with no observations of salmonids. (Figure 4).

Summarizing over all species and the four creeks used in the time series model, the culvert effect was minimal (Figure 7); the difference between upstream and downstream concentrations (normalized by upstream concentration) was on average only about 3%, indicating slightly higher concentrations upstream across creeks and species. Across species and times but within each creek, Chuckanut had the largest difference, followed by Portage, Padden, and Squalicum. In terms of species across time and creeks, rainbow trout (*O. mykiss*) saw the largest difference, followed by coho (*O. kisutch*), cutthroat (*O. clarkii*), and sockeye (*O. nerka*). Late summer / fall (August 2021 - October 2021) seemed to have the largest difference between upstream and downstream, which corresponds with when flows were at a minimum (i.e., August and September had lowest averge flows across creeks) and the connectivity between upstream and downstream was low (Figure 7). Individual species' patterns were similar, indicating that there is not a species-specific effect where culverts block the passage of some salmon but not others (Supplemental Figure 17). Across all species and time points, Squalicum Creek had the lowest mean percent difference in upstream and downstream salmonid DNA concentrations.

Effects of Culvert Replacement

By comparing the difference in upstream and downstream concentrations before and after construction in Padden Creek, we can assess how large of an impact the replacement had on salmonid species (Supplemental Figure 3). The effects of the culvert replacement operation appeared to have been transient and fairly minor for the four salmonid species surveyed. After the beginning of construction in September 2021 through the end of sampling in February 2022, we saw very minor fluctuations in the difference between upstream and downstream salmonid DNA concentrations, and did not see an increase in this difference due to the culvert removal (Figure 8, grey shading vs. no shading). The mean percent difference across all species prior to construction was 2.4% compared to 1.5% during and post-construction (Supplemental Table 2).

Discussion

Environmental DNA can provide quantitative measurements of environmental impacts

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

A few other studies have used eDNA to measure environmental impacts in rivers and streams. Duda et al. (2021) used 11 species-specific qPCR assays to document the distribution of resident and migratory fish after a large dam removal project (Elwha River near Port Angeles, Washington). No eDNA sampling was conducted before the dam removal, but the study provided a wealth of information about species returning after the dam removal, providing a very important dataset to use eDNA to monitor ecological changes due to human intervention. Similarly, Muha et al. (2017) sampled three locations upstream and three locations downstream before and after the removal of a weir that was thought to be a barrier to salmonid migrations. The authors only sampled once before and twice after the removal, spanning about a year, and used eDNA metabarcoding to look at the presence/absence of species detected. They found that in fact the before sample demonstrated that the weir was not preventing fish passage (similar to the results found in this study) and furthermore documented a slight increase in alpha diversity in the first time point after the barrier removal and then a return to a similar alpha diversity in the second time point after the removal (similar results found in this study using eDNA concentrations rather than diversity).

Importantly, our study demonstrates the value of combining a single qPCR assay with metabarcoding data to generate quantitative estimates of eDNA concentrations of many species without requiring n qPCR assays for n species of interest. 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). This can also be particularly helpful for taxa that don't have a previously published qPCR assay, but are detected using universal metabarcoding assays. Metabarcoding data alone only gives compositional data, which cannot be used in a time series to quantify environmental impacts because there is no information about absolute eDNA concentrations. However, by anchoring or grounding proportions using a single qPCR assay, the proportional data can be turned into quantitative data. The species for which to run the qPCR assay can be determined after the metabarcoding is completed; the most commonly found species with a robust qPCR assay should be used to glean the most information.

Fish life histories and expected patterns

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

Furthermore, three of the four species in this study have both freshwater resident and saltwater migrating behavior. Cutthroat trout (*O. clarkii*) encompasses both non-migrating, resident trout in the creeks and coastal run cutthroat that migrate into Padden Creek from saltwater (Bellingham Bay). Similarly, *O. nerka* includes both anadromous sockeye salmon and freshwater resident kokanee salmon and *O. mykiss* includes both anadromous steelhead trout and non-migrating rainbow trout. Using eDNA, we cannot distinguish between the migrating and non-migrating subspecies of *O. clarkii*, *O. nerka*, and *O. mykiss*. Therefore, our eDNA concentrations might reflect contributions from both migrating and non-migrating individuals at any given time point in the dataset.

Despite the mix of migrating and non-migrating populations and various run timings, our metabarcoding data demonstrate that in Padden Creek, there was a clear signal of sockeye/kokanee salmon (*O. nerka*) both upstream and downstream only in November 2021 - February 2022 (and only upstream in March 2021). This signal corresponds well with the documented run timing of October to December. In contrast, cutthroat trout (*O. clarkii*) and coho salmon (*O. kisutch*) were found nearly year-round in Padden Creek. The persistent signal from *O. clarkii* could be explained by resident cutthroat trout. However, *O. kisutch* does not have a resident subspecies and the run timing is only documented from September to December. This could potentially be due to juveniles maturing and residing in the creeks for 1-2 years after hatching while adults migrate into the creeks only during the run time to spawn. Visual surveys are conducted rarely and even if they were conducted, it might be difficult to identify juveniles to species level. Though *O. kisutch* eDNA was found year round, the highest concentrations were found near the expected run timing as expected and the life history of *O. kisutch* includes rearing year-round in freshwater. Finally, though the lowest concentrations on average, rainbow/steelhead trout (*O. mykiss*) was also found nearly year-round in Padden Creek, which could be contributions from migrating steelhead (November to June), juveniles maturing and migrating, or from resident rainbow trout. Though the *O. mykiss* signal is found year-round, the highest concentrations do seem to correspond with the steelhead run timing.

Decoupling of eDNA from fish abundance

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

Many previous papers have commented on the "ecology" of eDNA and the various processes that contribute to eDNA concentrations in environmental samples (e.g., shedding rates, decay rates, transport) (Barnes and Turner 2015). For example, higher concentrations of eDNA could be the result of a greater number (or biomass) of fish present, or increased shedding rates, or decreased decay. Many review papers document the nuances of interpreting eDNA data and we recommend reviewing them for a deeper understanding (see Andruszkiewicz Allan et al. (2020) for a review on shedding and decay rates and Harrison et al. (2019) for a review on transport). Certainly eDNA concentrations can arise from different scenarios and future work should continue to investigate how to tease apart the nuances of relating eDNA concentrations to fish abundance.

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

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

Accounting for flow with eDNA concentrations

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

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

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

Not all culverts are barriers to salmonids

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

Of the four creeks where salmonid DNA was consistently found, Chuckanut Creek had the largest discrepancies between DNA concentrations found below and above the barrier at each time point. The culvert in Chuckanut Creek is suspected to be a barrier to fish passage and the State of Washington's Department of Transportation is planning to replace it in the near future. The bridge at Portage Creek and the culvert at Squalicum Creek were more recently installed as compared to Padden, Chuckanut, and Barnes Creeks. They also were designated as only partially blocking fish passage, and here we find eDNA results suggest that they were in fact not major barriers to fish passage. Squalicum Creek had the lowest difference between upstream and downstream concentrations across all the surveyed creeks, which corresponds well with the classification that the culvert does not block fish passage. Also, Squalicum Creek is the only creek sampled that has baffles inside the culvert, which should help fish passage.

Here, we find that culverts designated as barriers were likely not blocking fish passage. Importantly, this demonstrates that collecting water samples for eDNA analysis might help to prioritize restoration of culverts suspected to be barriers to salmonids and provide a new method for post-restoration monitoring to confirm that the barrier has been corrected and allows for fish passage. Given the large amount of spending and effort required to replace culverts, this finding is important and emphasizes the potential for new tools for environmental impact assessments.

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

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

The construction timing did coincide with natural life history cycles for the salmon species. In the fall an influx of DNA would be expected not only from adults returning to spawn as they move through the system, but also from the presence of spawning material in the creek and decaying adults that die post reproduction. This may explain a portion of the changes in DNA concentrations found here as the construction timing coincided with run timings of the salmonids, however our time series model accounts for changes in season in attempt to isolate the effects of the culvert and construction. Regardless, the changes between upstream and downstream concentrations were very minor across time points and before and after construction.

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

Our findings here demonstrate that in addition to the value of using eDNA to select culverts to prioritize for replacement, sampling during and after construction can provide important information about the impacts (or lack of impacts) on salmonids. Here we found very minimal effects of both culverts in general and of construction during culvert replacement, but these findings are likely not universal and certainly projects need to monitor comprehensively and quantitatively in order to assess the passability of culverts and impacts of construction.

Conclusion

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

Conflict of Interest Statement

The authors declare there are no conflicts of interest.

Acknowledgements

This work was made possible by a grant from the National Philanthropic Trust to Ryan Kelly. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Tammy Schmidt and Susan Kanzler from Washington Department of Transportation for facilitating access to field sites and providing helpful feedback throughout the project. We also thank Dr. Jenna McLaughlin, Joe Duprey and Ally Im for help field sampling and Dr. Ramon Gallego, Dr. Kim Parsons, and the University of Washington's Northwest Genomics Center for sequencing support. Dr. Braeden Van Deynze, Dr. Sunny Jardine, and Dr. Julian Olden provided helpful insight into culverts and salmonid life histories. Thanks to Dr. Jameal Samhouri for reviewing the manuscript. A special thank you to Dr. Chris Sergeant for providing feedback throughout the project and improving the manuscript.

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List of Figures

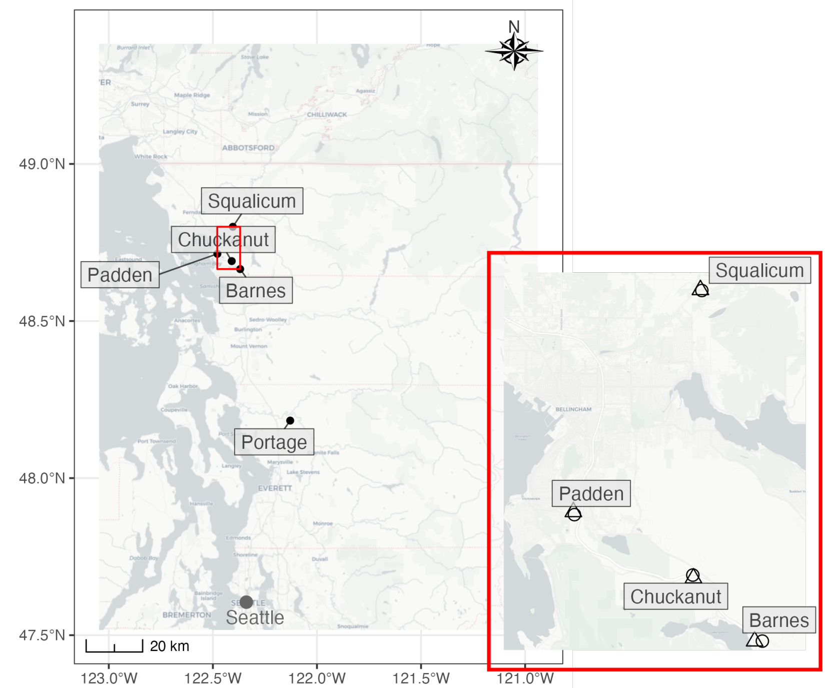

Figure 1: Map of sampling locations near Bellingham, Washington. In the subset, open triangles designate the downstream sampling location and open circles designate the upstream sampling location. Padden Creek is the treatment creek where the culvert was replaced.

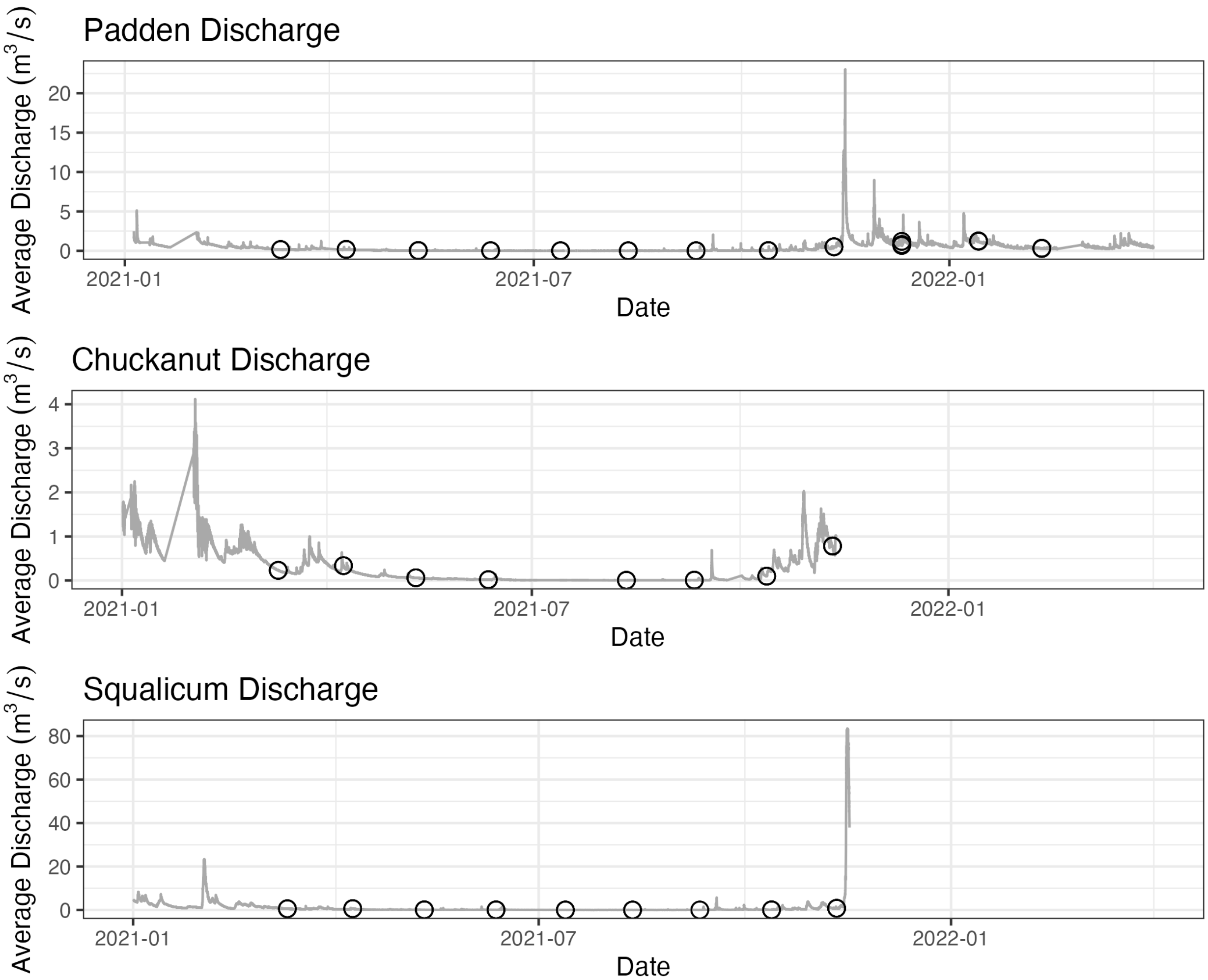

Figure 2: Discharge (m^3/s) in Padden (USGS Gage 12201905), Chuckanut (USGS Gage 12201700), and Squalicum (USGS Gage 12204010) Creeks over the course of sampling. Open circles show the days when sampling occurred. Gauges at Chuckanut and Squalicum Creek went offline in November 2021 after a major storm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

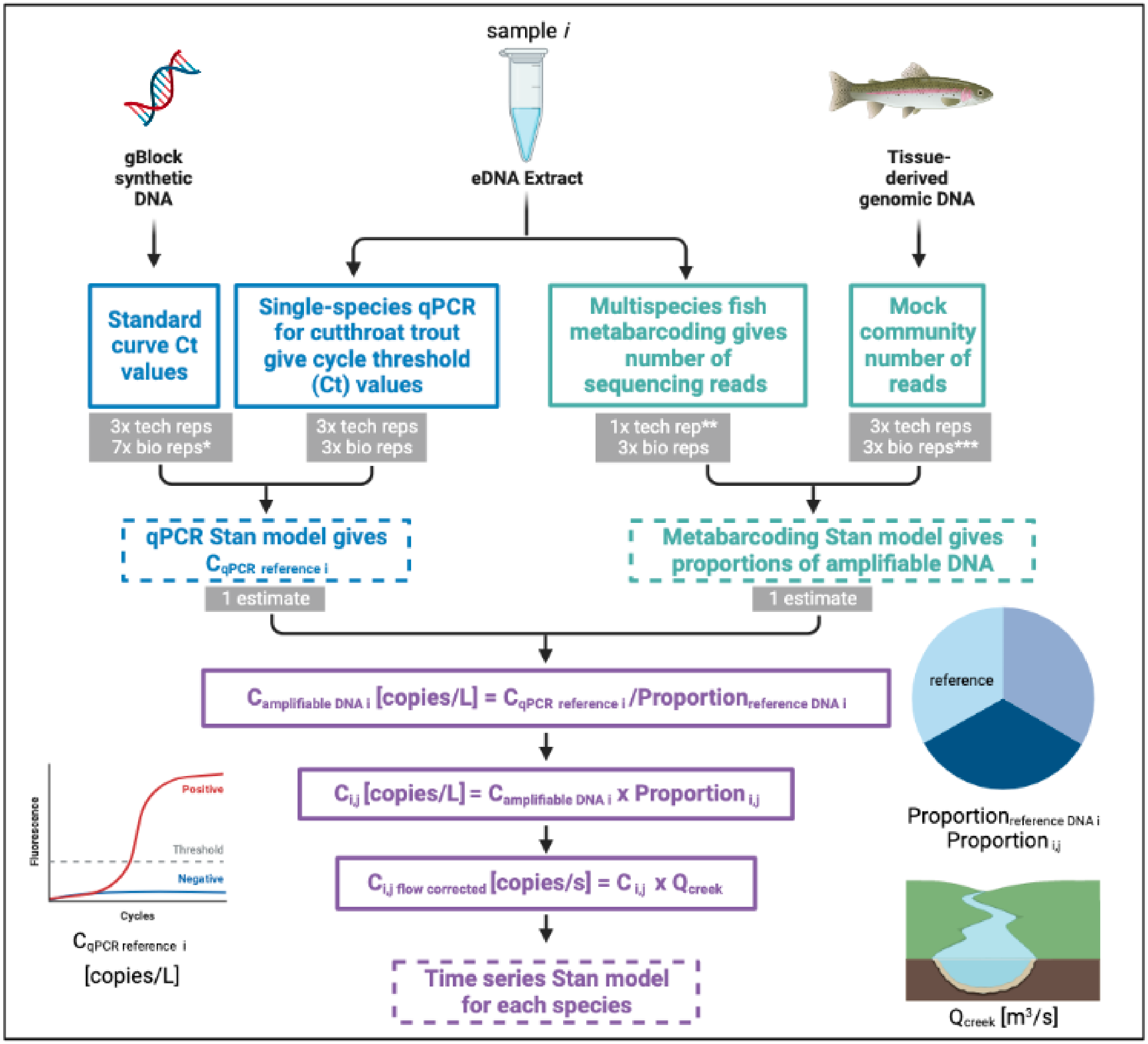

Figure 3: Conceptual figure of different datasets and models used for analyses. * indicates that here, biological replicates are different dilutions of the synthetic gBlock. ** indicates that for most samples, only one technical replicate was sequenced but for one sample per sampling month, three techincal 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.

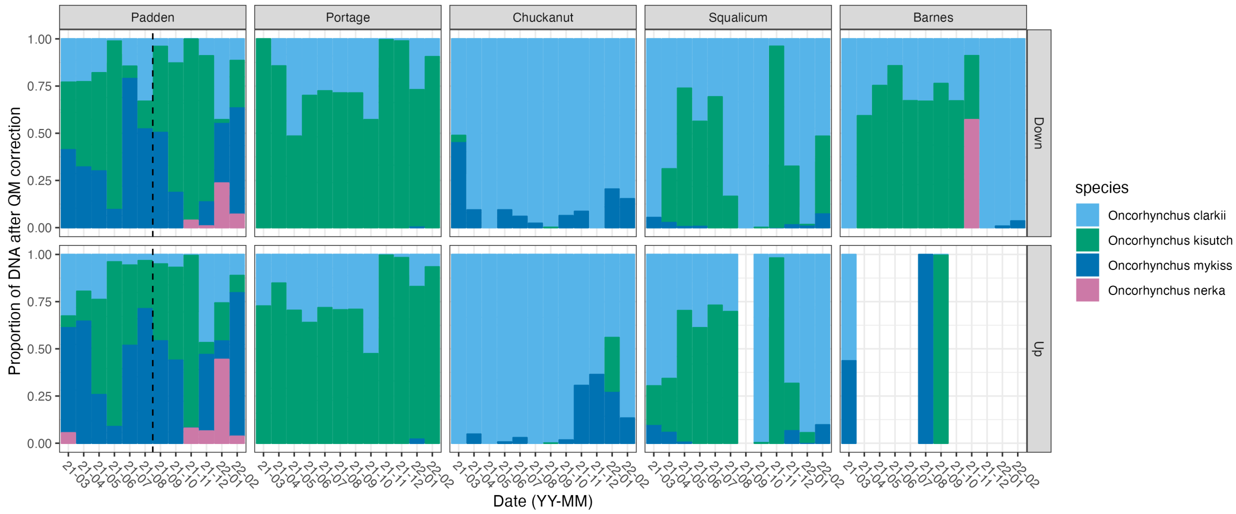

Figure 4: Compositions of salmonid DNA as determined by metabarcoding after correction for amplification bias. Note that no sampling occurred in September 2021 at Squalicum Creek because the creek was dry. The empty bars in the Barnes upstream sites indicate that no salmonid DNA was found at those time points. The vertical dashed line in Padden Creek indicates the time period in which the culvert was replaced.

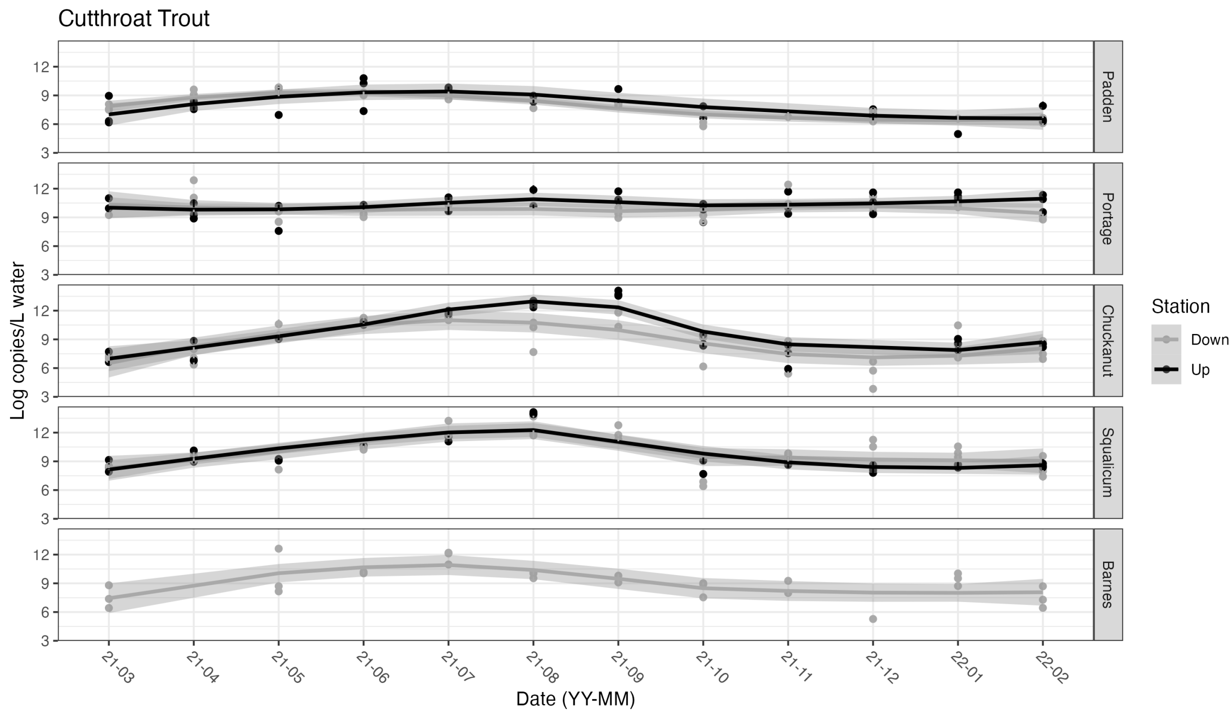

Figure 5: Absolute concentration (log copies/L of water) of cutthroat trout (*O. clarkii*) as measured by qPCR before flow correction.

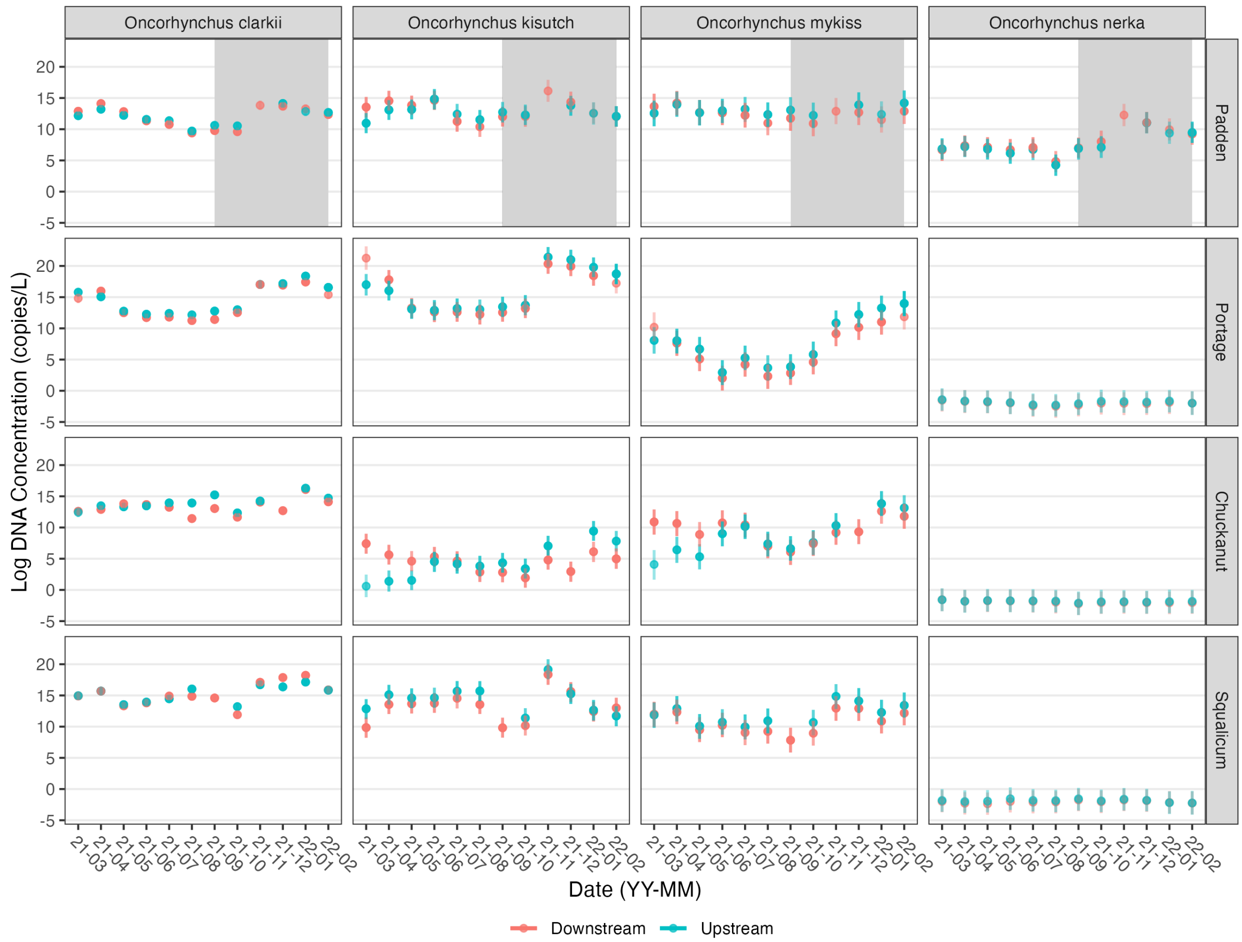

Figure 6: Trends across creeks and across time for each of four salmonid species as estimated by eDNA analysis. Points represent posterior means for the time-series model and error bars represent the 75% posterior confidence interval. Colors indicate station upstream or downstream of an under-road culvert. Grey shading indicates the time period in which the culvert in the treatment creek (Padden Creek) was replaced.

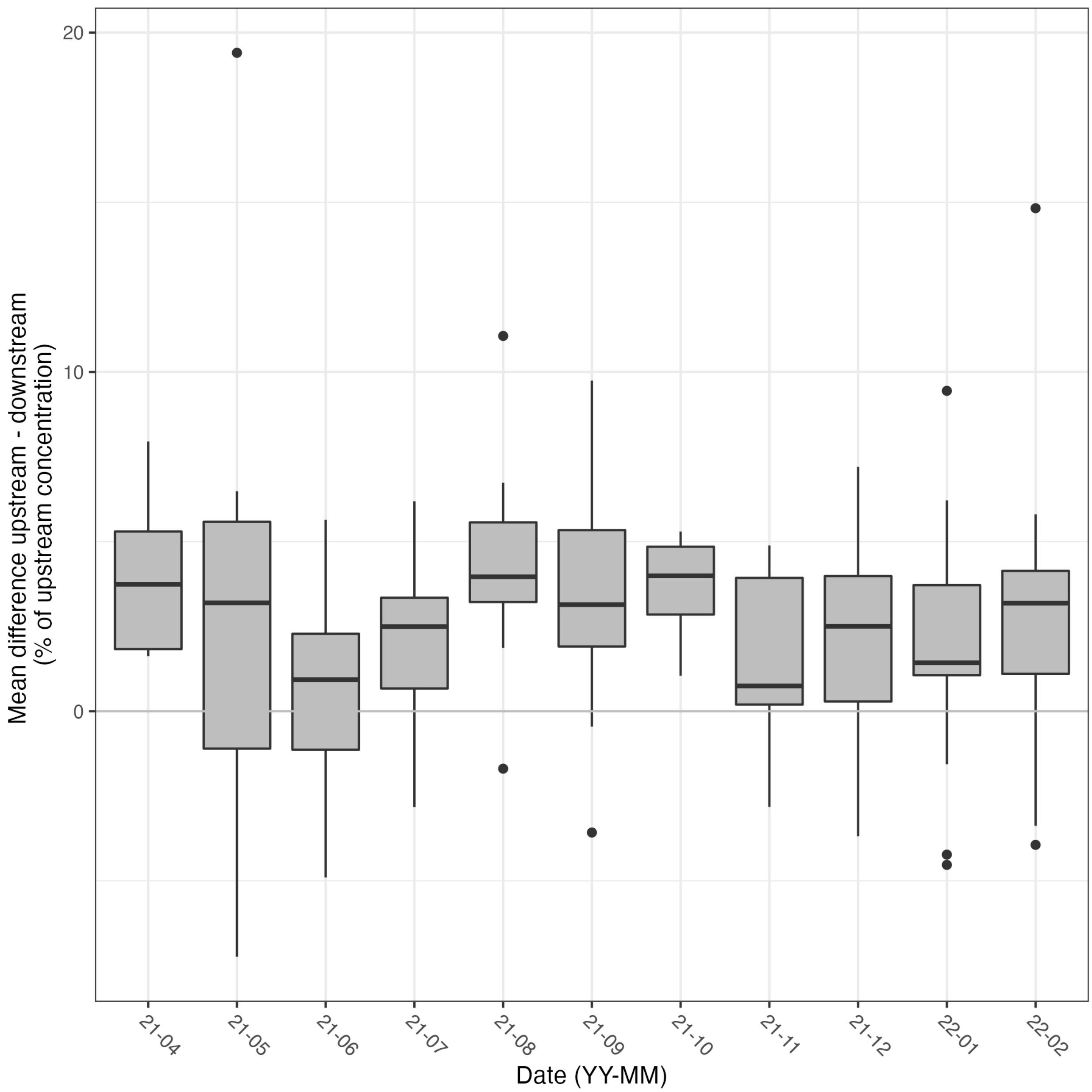

Figure 7: The effect of culvert on salmonid abundance summed across all species and creeks by time. The y-axis shows the difference between upstream and downstream concentrations, normalized by upstream concentration. The box boundaries correspond to the 25th and 75th percentiles; the whiskers correspond to 1.5 times the interquartile range. Here, negative values imply that eDNA concentrations 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.

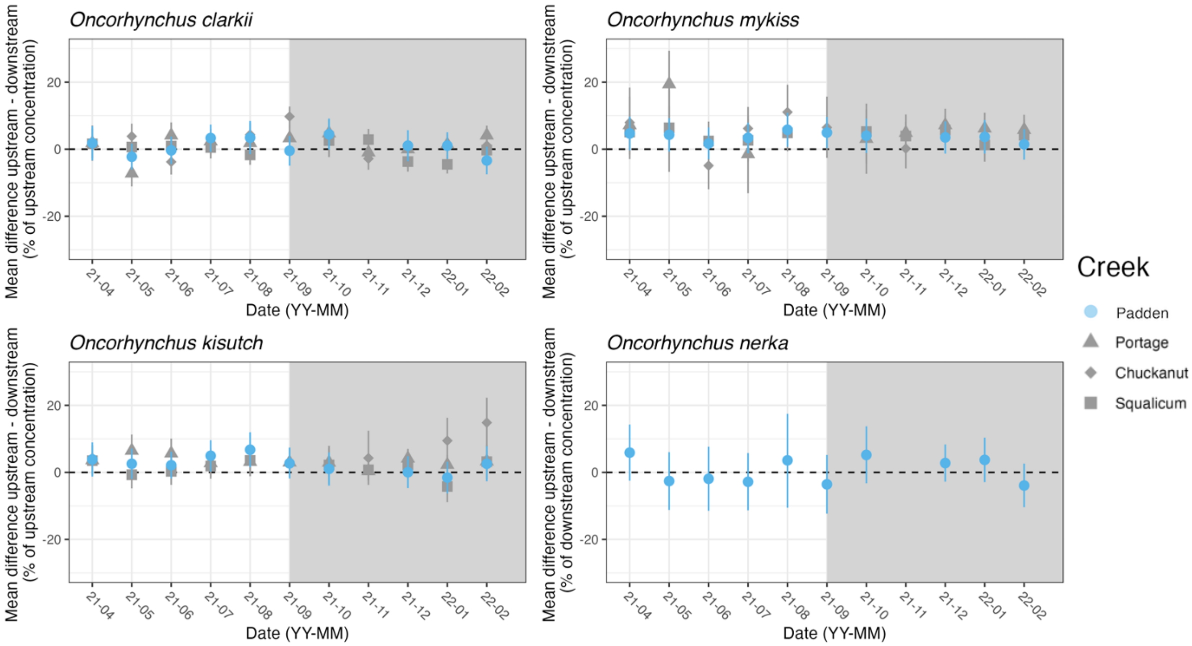

Figure 8: Effect of Construction on Salmonid DNA Concentrations in Padden Creek. Error bars show 75% confidence intervals of the normalized difference between upstream and downstream DNA concentrations. Grey shading shows when construction started through the end of sampling. Construction ended in early October 2022. 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 denomenators.