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

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Abstract:	Environmental laws around the world require some version of an environmental impact assessment surrounding construction projects and other discrete instances of human development. Information requirements for these assessments vary by jurisdiction, but nearly all require an analysis of biological elements of ecosystems. Amplicon-sequencing - also called metabarcoding - of environmental DNA (eDNA) has made it possible to sample and amplify the genetic material of many species present in those environments, providing a tractable, powerful, and increasingly common way of doing environmental impact analysis for development projects. Here, we analyze a 18-month time-series of water samples taken before, during, and after two culvert removals in a salmonid-bearing freshwater stream. We also sampled multiple control

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

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Response to Reviewer Comments

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

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

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

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

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

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

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

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

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

More minor points are as follows:

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

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

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

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

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

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

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

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

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

This really is “though”. We are saying, the range of discharge was high throughout the whole year (0-23 m³/s), but on the days that we actually sampled, the maximum discharge was only 1.3 m³/s. Therefore, we did not change this. We did however, move this to the supplemental text in an effort to reduce the text in the methods section.

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

We have changed this accordingly.

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

We have italicized these.

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

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

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

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

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

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

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

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

We have updated the figure legend.

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

We have added titles and legends to the supplemental tables.

Reviewer #2 Comments to the Author:

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

General:

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

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

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

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

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

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

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

(2017; [https://urldefense.com/v3/_https://doi.org/10.1139/facets-2016-0058_";!K-Hz7m0Vt54!mN_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFB05N0T8WFa3cpODnOeu3iLbyjbUNnkTKHL3I4y_3zbDBAOqsQ\\$](https://urldefense.com/v3/_https://doi.org/10.1139/facets-2016-0058_)) argue that multiple years are needed to properly account for interannual variability when assessing impacts to fish populations. The authors of the current paper need to address or acknowledge the limitations of inference due to the relatively short time frame of the study.

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

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

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

Specific:

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

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

Line 222. Consider providing USGS stream gage station numbers (e.g., Chuckanut = 12201700). Also the citation for streamflow data by USGS is: USGS. (2022). USGS Water data for the nation. Retrieved from [<https://urldefense.com/v3/_https://waterdata.usgs.gov/nwis_>;](https://urldefense.com/v3/_https://waterdata.usgs.gov/nwis_) !!K-Hz7m0Vt54!mN_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFB05N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y_3zYcWRJOYw\$ <accessed on date>.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Figures:

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

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

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

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

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

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

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

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

¹ Quantifying Impacts of an Environmental Intervention Using Environmental
² DNA

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¹³ Manuscript type: Article

¹⁴ Open Research Statement: Data are already published and publicly available, with those publications properly
¹⁵ cited in this submission. The repository for code can be found at:

¹⁶ https://github.com/eandrusz/quantitative_salmon_resubmit.git

¹⁷ Keywords: *environmental DNA, quantitative metabarcoding, environmental impact assessments, salmon,*
¹⁸ *culvert*

19 **Abstract**

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

43 Introduction

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

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

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

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

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

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

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

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

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

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

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

141 **Methods**

142 **Site and Species Selection**

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

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

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

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

175 **Water Sampling**

176 From March 2021 to February 2022, all five creeks were sampled monthly (n=12). Monthly sampling continued
177 in Portage Creek, Padden Creek, and Squalicum Creek through August 2022, with one additional sampling
178 point in December 2022 (n=19). At each sampling station (upstream and downstream of a culvert) at each
179 creek, we collected three 2-liter water samples. Samples were collected using an eDNA Backpack (Smith
180 Root; Thomas et al. (2018)), a portable pumping-and-filtering device set to filter at 1 L/min at 82.7 kPa (12
181 psi). In some months, less than 2 L of water was filtered due to clogging. Water samples were filtered using
182 single-use inlet tubes through 5 μ m self-preserving filters (Smith Root, Vancouver, WA), which were then
183 dried and kept at room temperature until DNA extraction within 1 month of collection (Thomas et al. 2019).
184 Over the course of the sampling, water discharge varied from very low to no flow in summer months to high
185 flow in winter months (Figure 2). We account for this dilution by converting eDNA concentration [copies/ μ L]
186 to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s] (Tillotson et al.
187 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey (USGS) were
188 used for Padden Creek (USGS Gauge 12201905), Chuckanut Creek (USGS Gauge 12201700), and Squalicum
189 Creek (USGS Gauge 12204010; <https://maps.waterdata.usgs.gov/mapper/index.html>; U. S. Geological
190 Survey (1994); Appendix S1: Figure S1). Over the course of sampling, the flow gauges at Chuckanut Creek
191 and Squalicum Creek became inoperable after a major flooding event. To find discharge rates for Chuckanut
192 and Squalicum Creeks, five years of historical data (2015-2020) were used to generate a monthly averaged
193 correction factor based on Padden Creek (Appendix S1, Appendix S1: Figure S3). No discharge data was
194 available for Portage Creek or Barnes Creek. Based on field sampling conditions, the discharge from Padden
195 Creek was used as a proxy for both Portage and Barnes as they are in similarly sized watershed areas and
196 land-cover characteristics.

197 **DNA Extraction, Amplification, Sequencing**

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

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

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

228 **Bioinformatics**

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

238 **Quantitative PCR and Inhibition Testing**

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

248 All qPCR data was processed in R using Stan (Stan Development Team 2022), relating environmental
249 samples to the standard curve via a linear model (Figure 3, blue boxes; Appendix S2: Figure S13). We
250 amended the standard linear regression model to more realistically capture the behavior of qPCR observations,
251 accommodating non-detections as a function of underlying DNA concentration, and letting the standard
252 deviation vary with the mean (lower-concentration samples had more uncertainty). See McCall et al. (2014)
253 and Shelton et al. (2019) for similar models; see Appendix S2 for full statistical details. Subsequent analysis
254 corrected for sample-specific dilution if found inhibited and corrected for any variation in water-volume
255 filtered during sample collection. Samples with standard deviations between technical replicates larger than
256 1.5 Ct values were removed from analyses.

257 **Quantitative Metabarcoding**

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

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

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

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

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

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

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

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

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

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

301 Results

302 Metabarcoding and Quantitative PCR

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

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

317 50% of reads assigned to cutthroat trout.

318 After calibrating metabarcoding data using mock communities (See Appendix S1 and Appendix S2), we
319 estimated the salmonid composition across time points, creeks, and stations (Figures 4 and 5). The culvert in
320 one control creek (Barnes) appeared to be nearly a total barrier to salmonid passage, with salmonid eDNA
321 detected upstream of the culvert at only three time points, in contrast to being detected at every time point
322 in the downstream station of the same creek. The other four creeks had no such pattern associated with
323 the culverts, suggesting that fish passage may have been possible through the culverts, or that there were
324 resident populations upstream of the culverts.

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

329 We combined compositional information from metabarcoding with absolute concentrations from qPCR for
330 our reference species, cutthroat trout (*O. clarkii*), to estimate the total concentration of DNA for each
331 species (See Appendix S2). These quantitative data for all four target species were then used in the linear
332 mixed-effects model to assess salmonid trends over time (Figure 7).

333 Effects of Culverts

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

341 Summarizing over all species and the four creeks used in the time series model, the culvert effect was minimal
342 (Figure 8); the average log-fold change between upstream and downstream sites was not significantly different
343 from zero. Individual species' patterns were similar, indicating that there is not a species-specific effect
344 where culverts block the passage of some salmon but not others (Appendix S1: Figure S12). The maximum
345 positive log-fold change (i.e., upstream having a higher mass flow rate) was 2.78 in Squalicum Creek for coho
346 salmon (*O. kisutch*) in August 2021, while the maximum negative log-fold change (i.e., downstream having a

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

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

356 Effects of Culvert Replacement

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

364 Discussion

365 Environmental DNA can provide quantitative measurements of environmental impacts

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

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

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

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

401 Fish life histories and expected patterns

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

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

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

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

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

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

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

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

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

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

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

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

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

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

500 Conclusion

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

514 Conflict of Interest Statement

515 The authors declare there are no conflicts of interest.

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527 **Figures**

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

533 **Figure 2.** Discharge (m^3/s) in Padden (USGS Gauge 12201905), Chuckanut (USGS Gauge 12201700),
534 and Squalicum (USGS Gauge 12204010) Creeks over the course of sampling. Gauges at Chuckanut
535 and Squalicum Creek went offline in November 2021 after a major storm event. Portage Creek and
536 Barnes Creek did not have stream gauges. Circles designate the day of sampling. For Padden Creek,
537 the nearest 15 minute interval of flow was used. For Chuckanut and Squalicum Creeks, the correction
538 factor from five years of historical data from Padden Creek was used (see methods section and Appendix
539 S1: Figure S2 and Appendix S1: Figure S3).

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

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

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

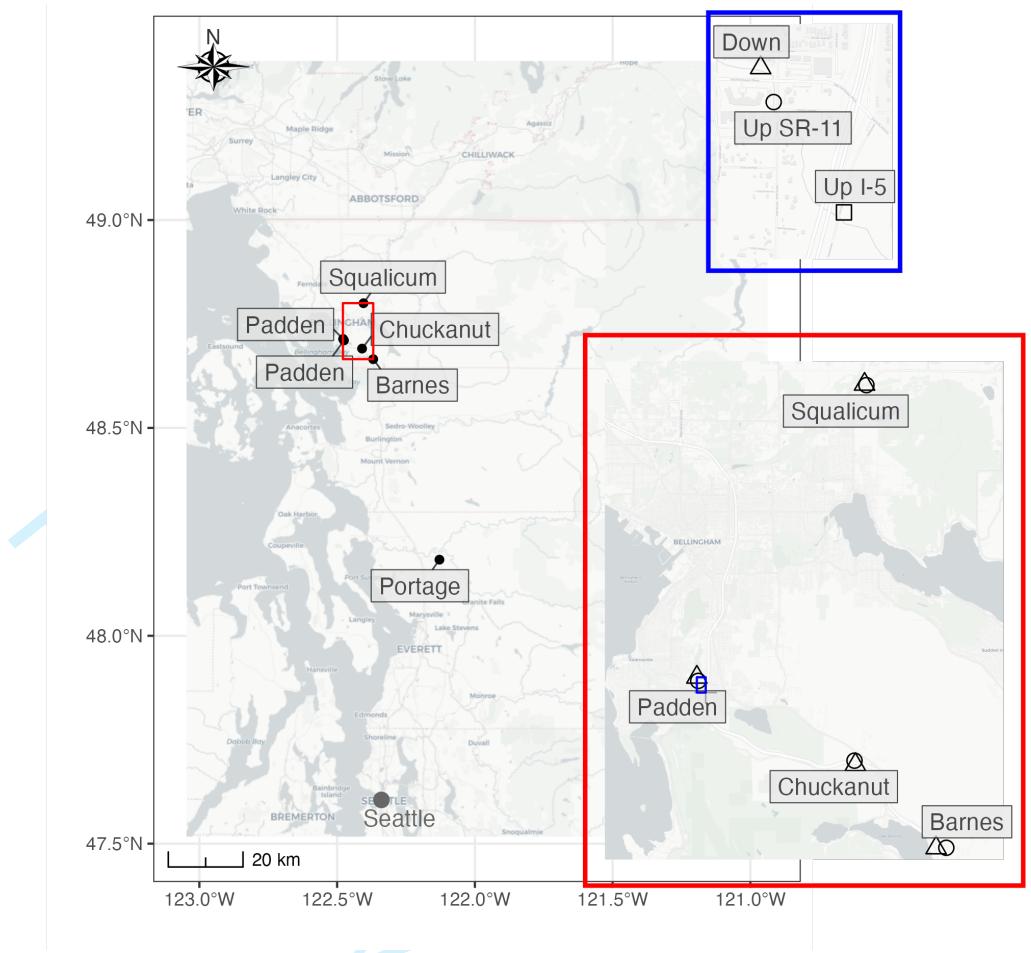
554 **Figure 6.** Absolute mass flow rate (log copies/s) of cutthroat trout (*O. clarkii*) as measured by qPCR
555 after flow correction. Note that Barnes Creek and Chuckanut Creek were not sampled after February
556 2022. Red crosses show the limit of detection for each species and time point, which changes with flow
557 rate and total volume filtered per sample.

558 **Figure 7.** Trends in mass flow rate (log copies/s) for each of four salmonid species across creeks and
559 across time as estimated by eDNA analysis. Points represent posterior means for the linear mixed
560 effects model and error bars represent the 95

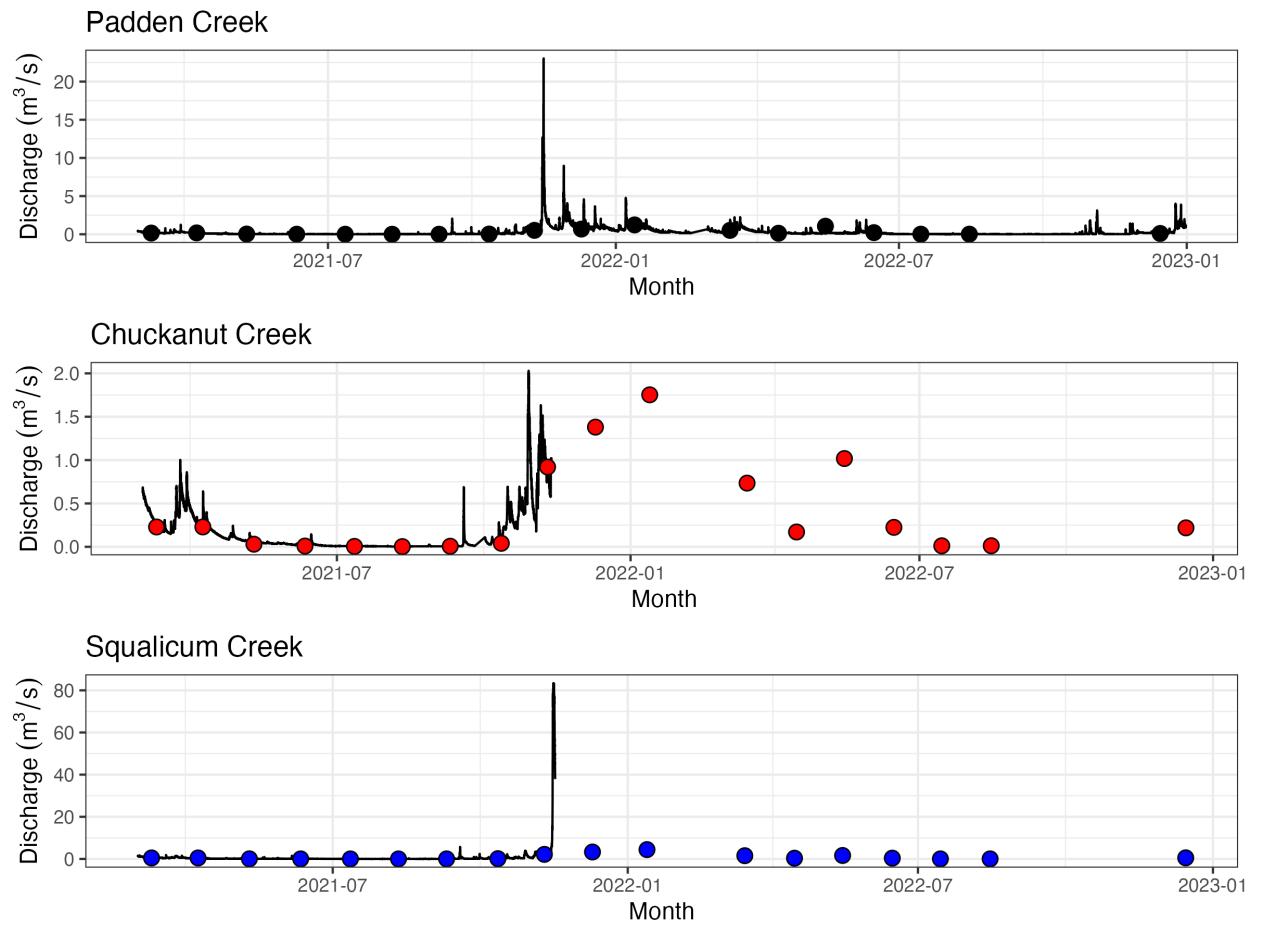
561 **Figure 8.** The effect of culvert on salmonid abundance summed across all species and creeks by time. The
562 y-axis shows the log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream,
563 normalized by upstream mass flow rate. The box boundaries correspond to the 25th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

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

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

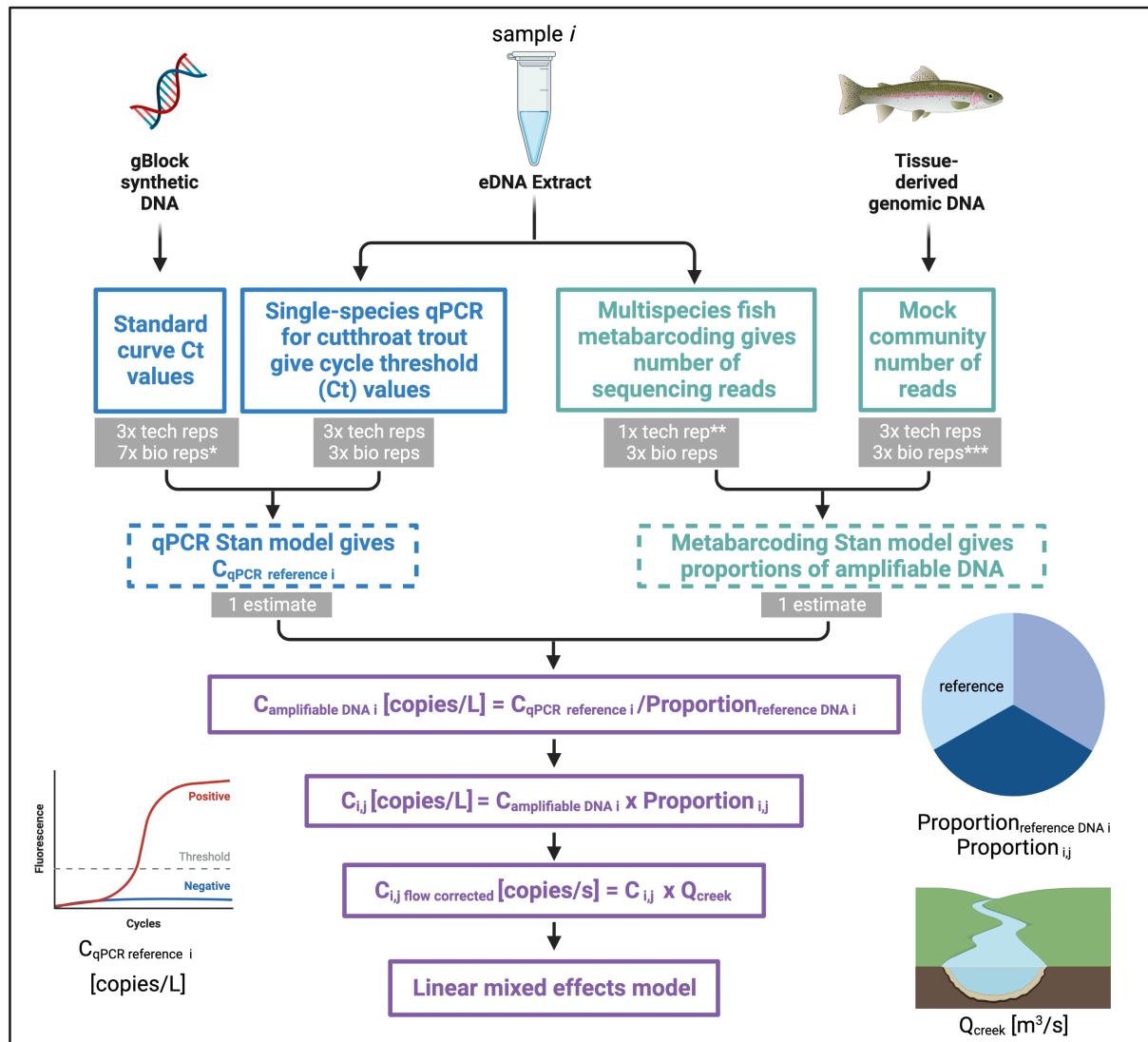


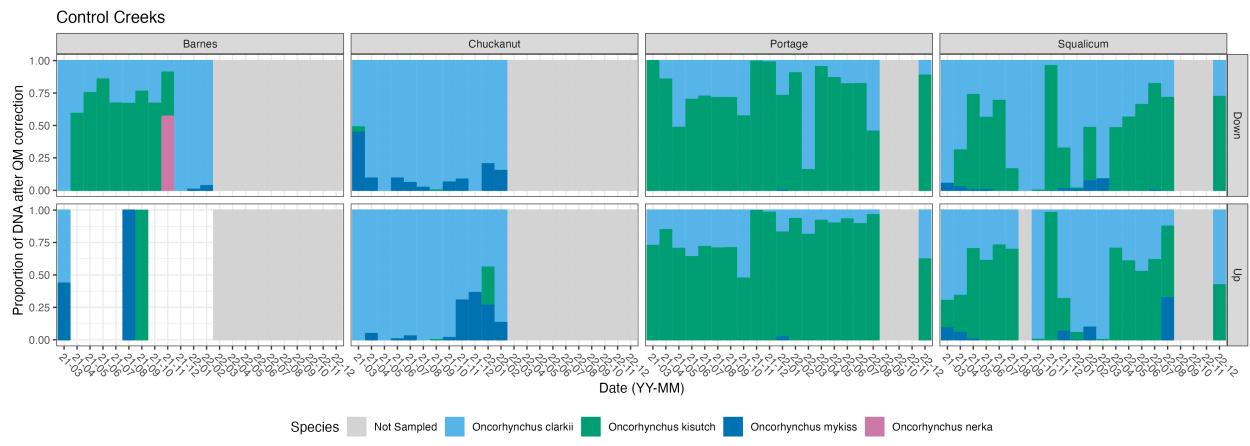
578 **Figure 1.**



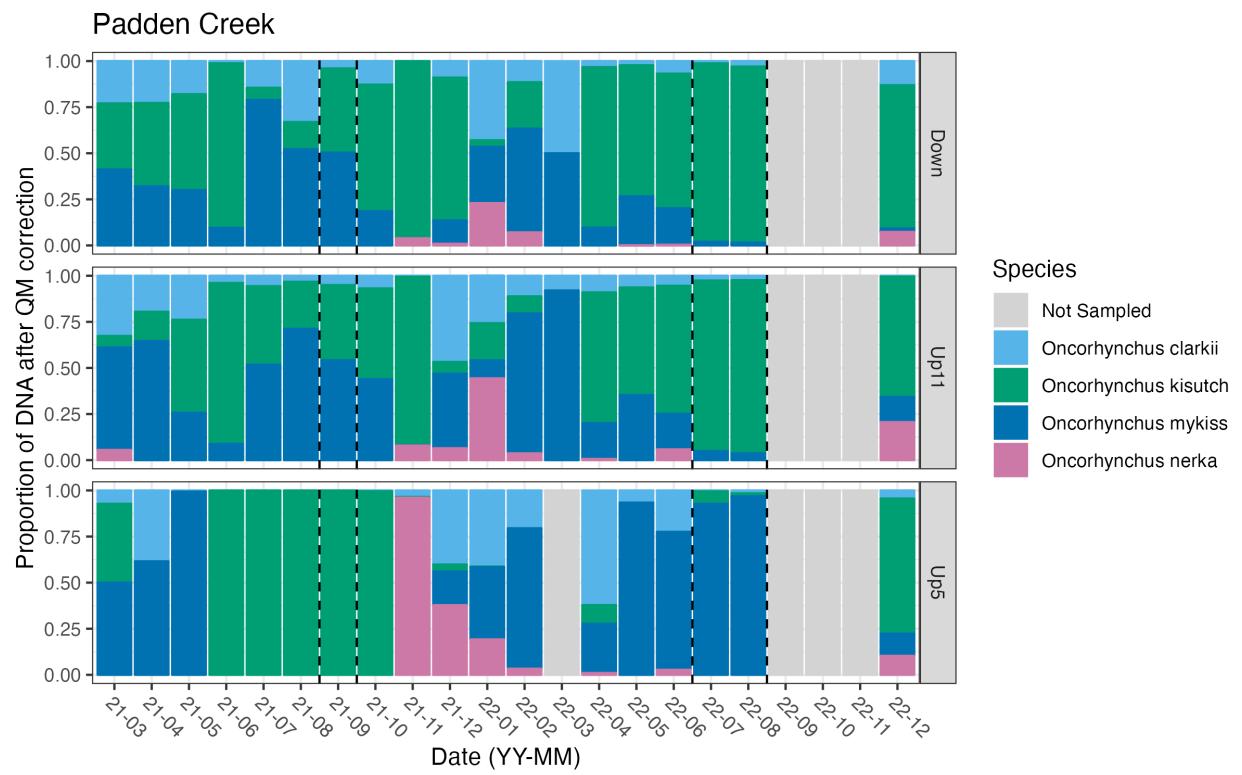
579 **Figure 2.**

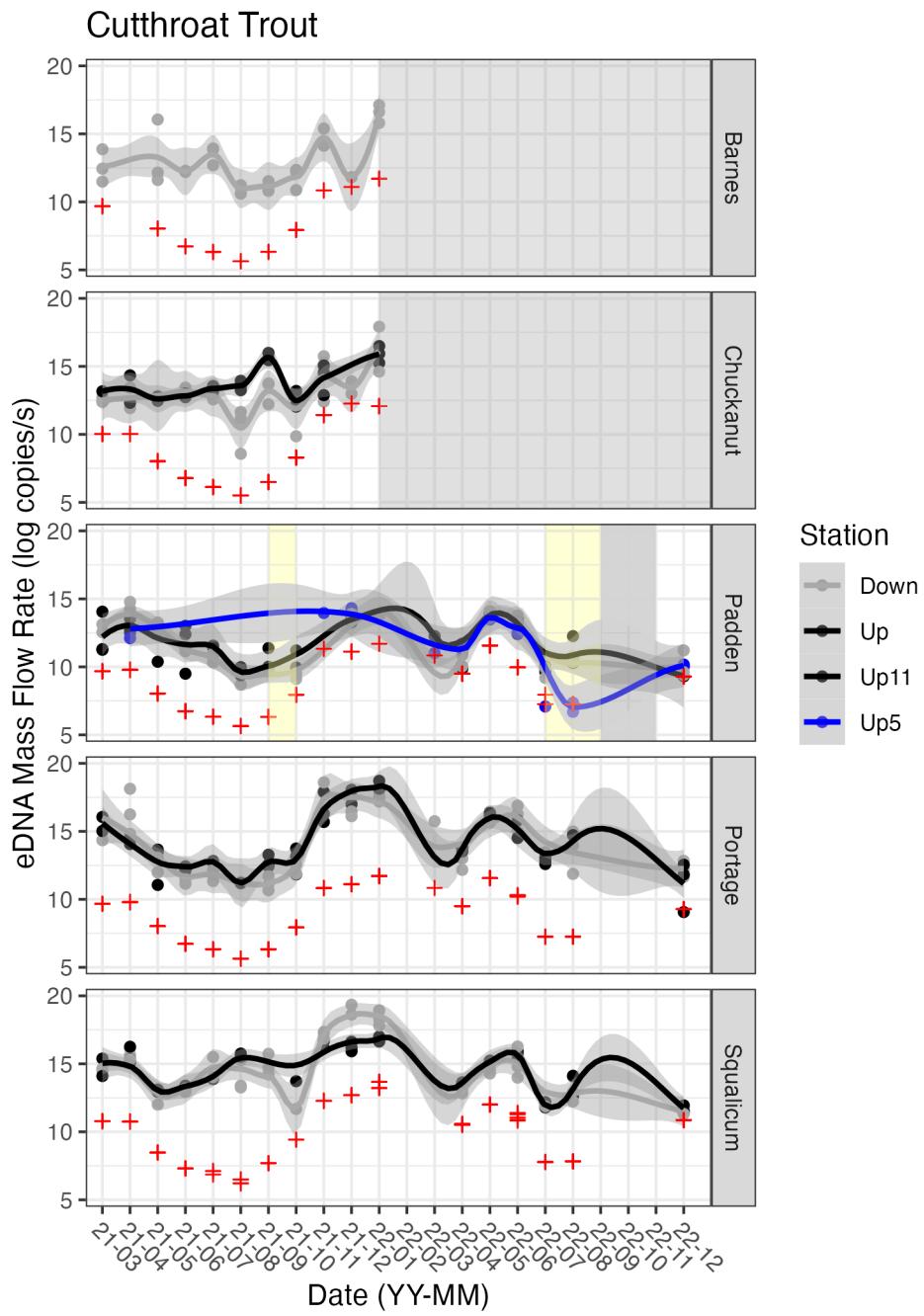
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580 **Figure 3.**

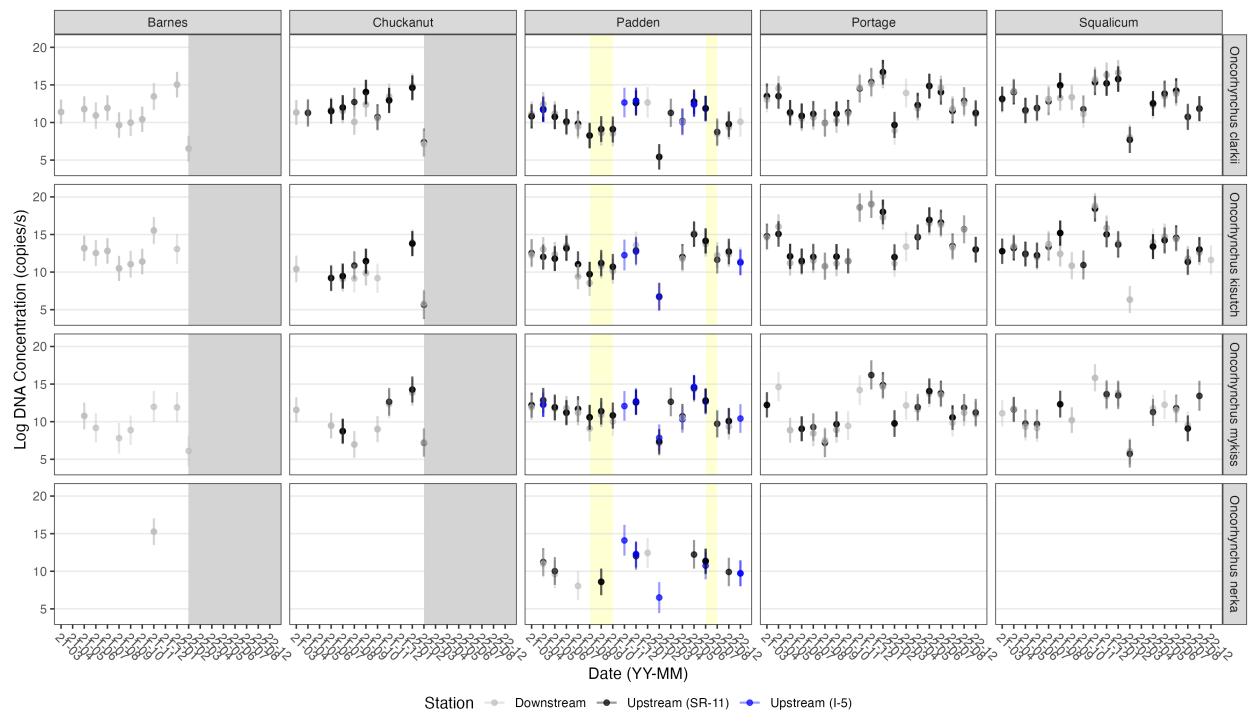


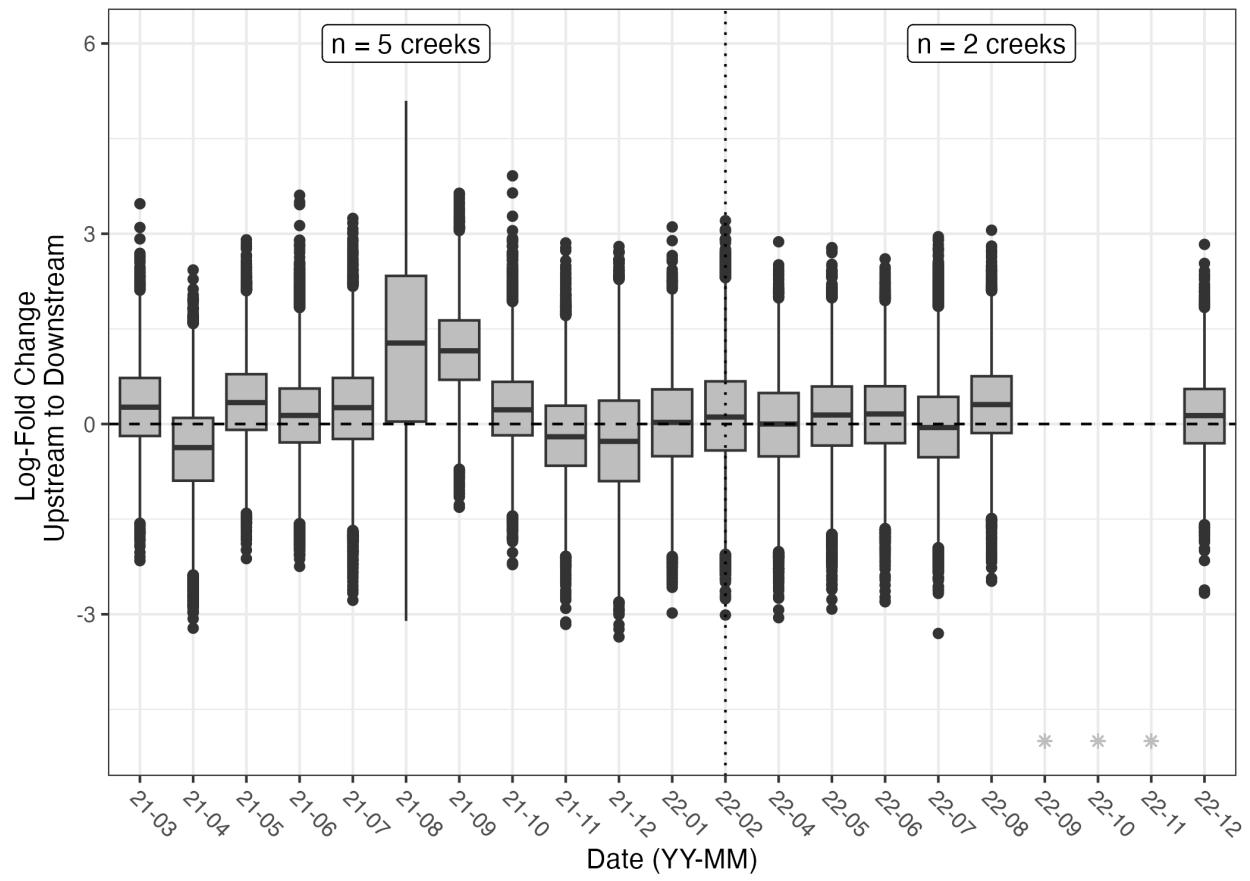
581 **Figure 4.**

582 **Figure 5.**



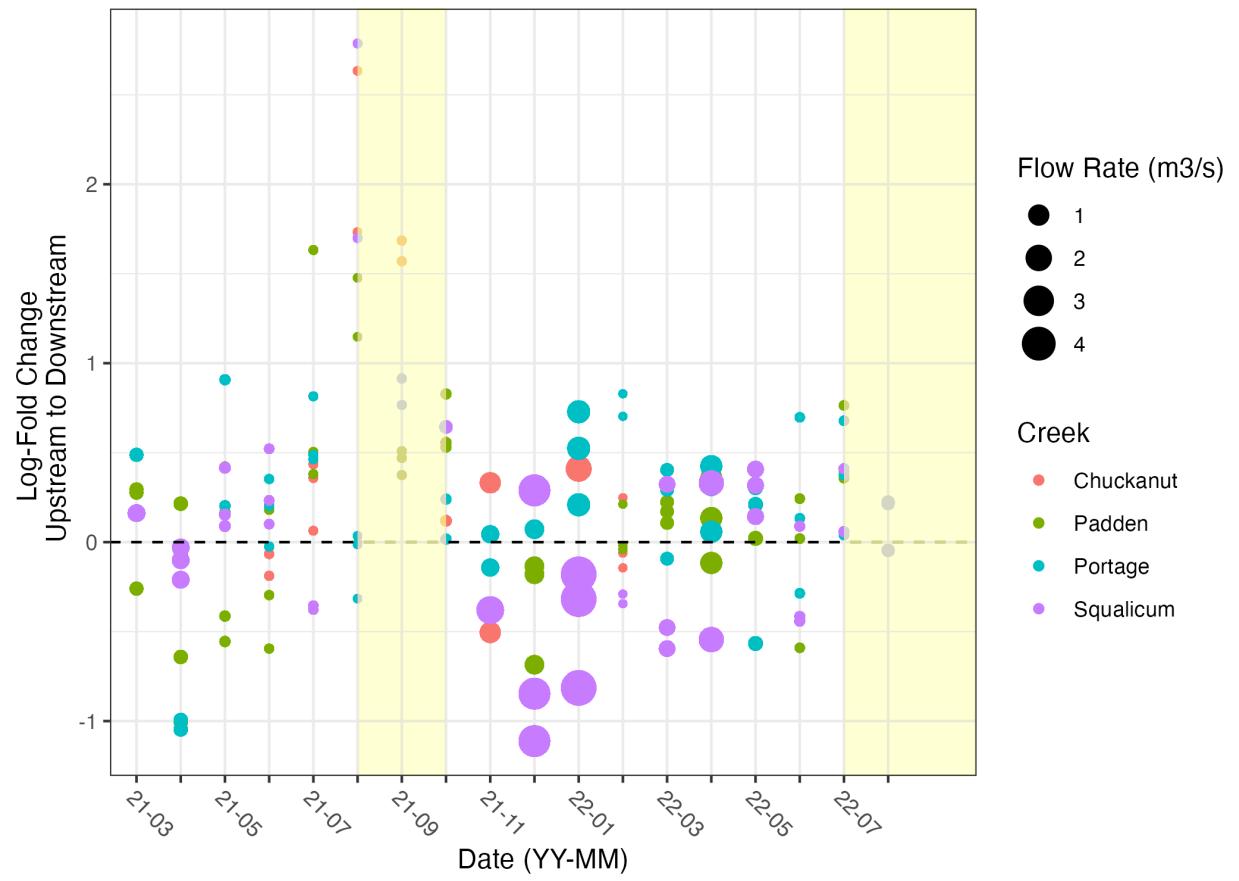
583 Figure 6.

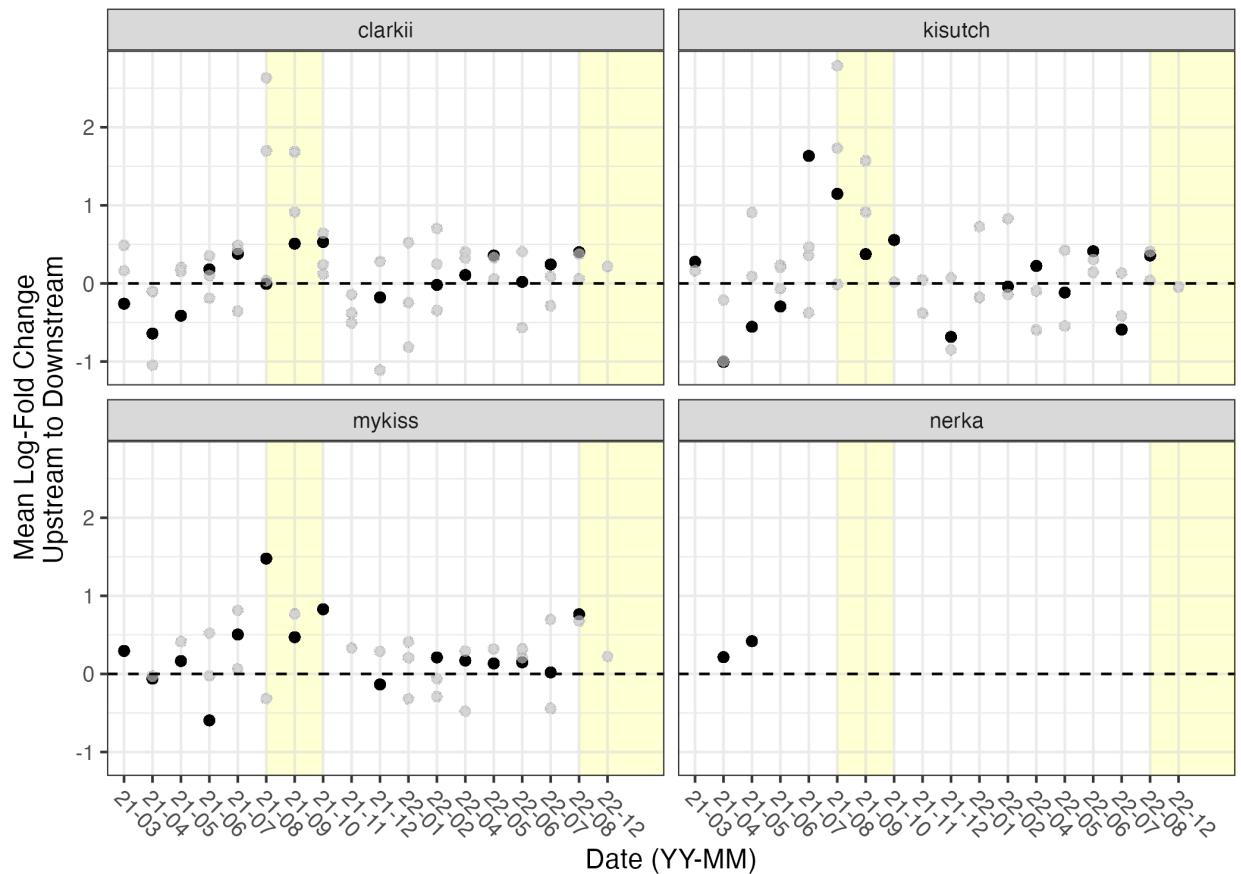
584 **Figure 7.**



585 Figure 8.

W Only

586 **Figure 9.**



587 Figure 10.

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

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5 2023

6 **Field Sampling**

7 **Site selection and study design**

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

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

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

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

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

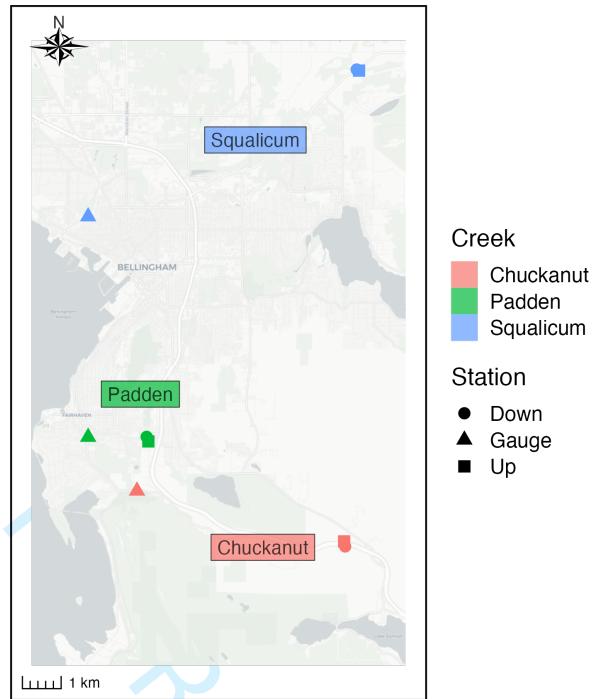


Figure S1. Location of flow gauges compared to sampling locations for Chuckanut, Padden, and Squalicum Creeks.

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

28 Due to the lack of flow data in Squalicum and Chuckanut Creeks from November 2021 to February 2022, we
29 used historical data from the three flow gauges to calculate the average discharge for each day of the year
30 from about 2015-2021 (Figure S2). We then used the value for the day of the year that we sampled in either
31 2021 or 2022 when the gauges were offline. For consistency, we also did this at Padden Creek despite the
32 gauge there being online for the entire sampling period.

33 We compared the different ways one could use flow data to correct the eDNA concentrations. We included
34 (1) the value from the closest time point from the gauge to the time point sampling, (2) the average flow
35 on the day of sampling from the gauge, (3) the monthly average for the month of sampling from the gauge,
36 and (4) the correction factor approach. For (4), the values for Padden Creek represent the same as (1) for
37 Padden Creek, the value from the closest time point in the gauge to the time point of sampling. The values
38 for Chuckanut and Squalicum Creek are based on the the correction factor from Padden Creek. First, five
39 years of historical data (2015-2020) were used to find monthly averages for flow rates for each creek. Because

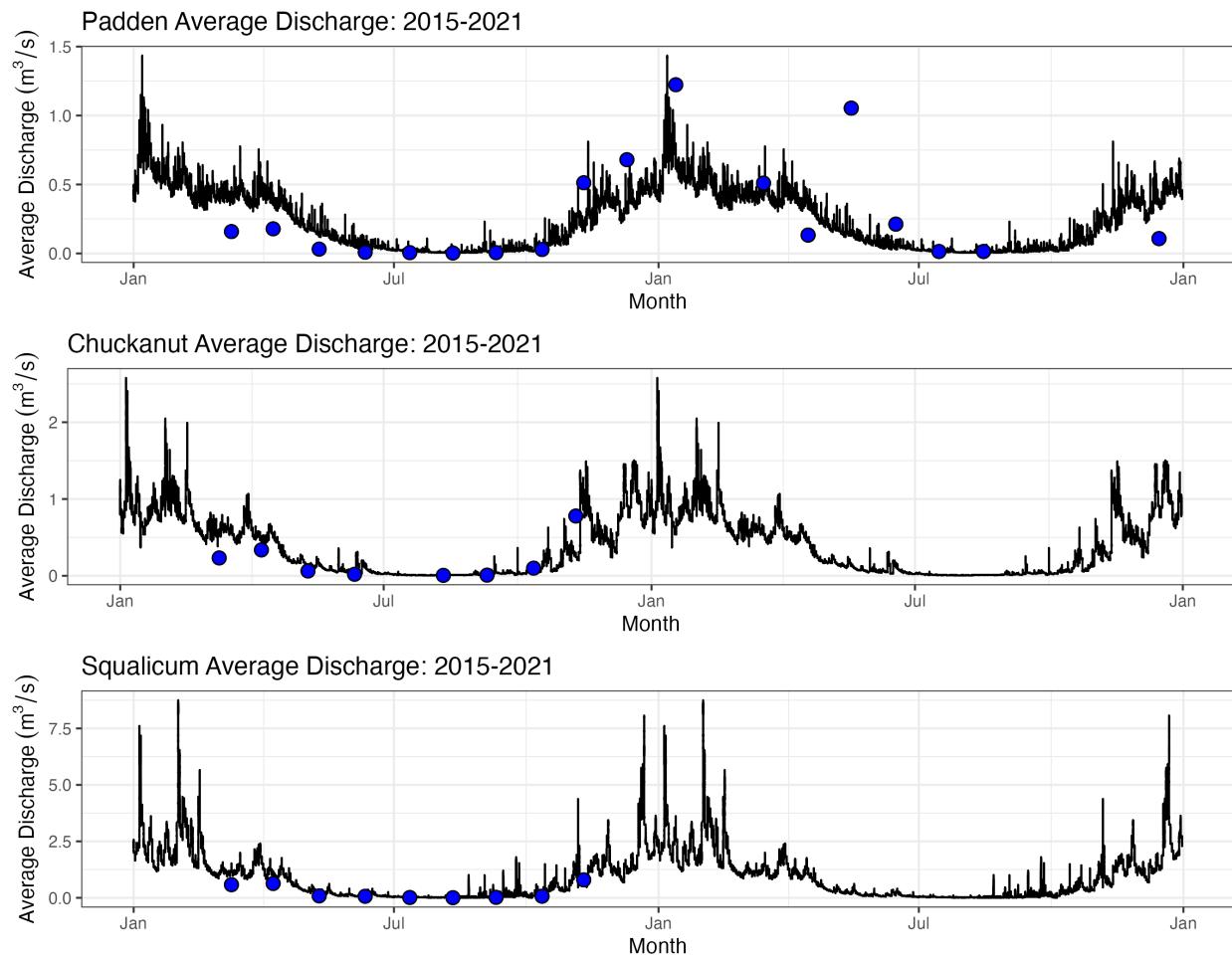


Figure S2. Daily average discharge from 2015-2020 in creeks from USGS gauges. Blue dots show the discharge time of sampling during the course of this study (2021-2022) for time points where gauges were online (note the missing data points after December through February in Chuckanut and Squalicum Creeks).

40 the gauges in Squalicum and Chuckanut Creeks stopped metering in 2021, we solved for the ratio of the
 41 monthly average of each of those creeks to Padden Creek. Then, we used the closest values from Padden
 42 Creek (1) and multiplied by the monthly correction factor from the 5 years of historical data to find a value
 43 for Squalicum and Chuckanut Creeks to use for the year of sampling (2021-2022). For all three creeks, we
 44 demonstrate the relatively small changes in discharge depending on which way flow data were used (Figure
 45 S3). Though in the course of sampling, the discharge in Padden Creek ranged from no metered flow to 23
 46 m³/s, the discharge on the dates of sampling only reached a maximum of 1.3 m³/s. For sites with no metered
 47 flow, half of the minimum verified discharge of the flow gauge was used (0.0014 m³/s).

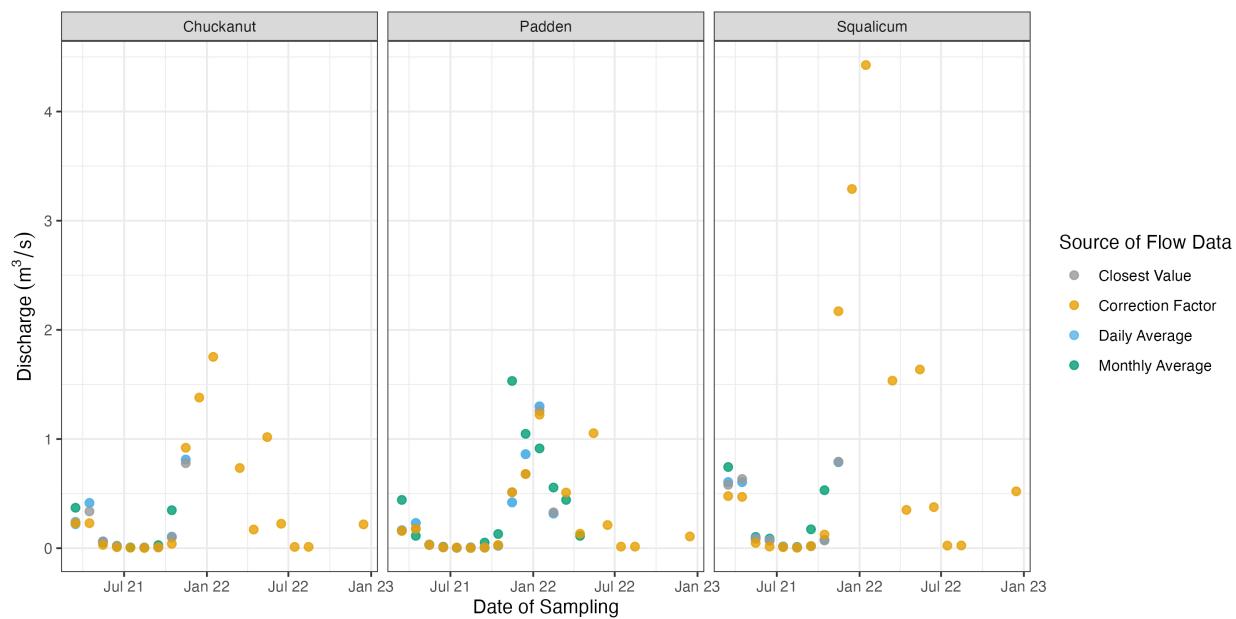


Figure S3. Comparison of different ways flow data can be used to correct eDNA concentrations. In the main text, the correction factor is used. Note that for Padden Creek, the “Correction Factor” method is the same as the “Closest Value” method. Also note that for Chuckanut and Squalicum Creeks, no data exist for closest value, daily average, or monthly average after November 2021 when the gauges went offline.

48 Construction and Fish Exclusion

49 Fish were excluded from Padden Creek on August 30th, 2021 in preparation for the stream to be diverted
 50 on September 9th, 2021 and the diversion was removed October 7th, 2021. Water sampling occurred on
 51 September 10th, 2021, the day after the diversion, and on October 12, 2021, just 5 days after reconnecting
 52 the stream (Figure S4).

53 **Stocking in Lake Padden**

54 Padden Lake has historically been stocked with hatchery fish by the Washington Department of Fish and
 55 Wildlife (Figure S4). Rainbow trout (*O. mykiss*) and occasionally cutthroat trout (*O. clarkii*) and kokanee
 56 salmon (*O. nerka*) are stocked in Lake Padden, approximately 1.5 km upstream of the sampling sites. During
 57 the course of the study, rainbow trout were stocked in April 2021 and April 2022, kokanee salmon were
 58 stocked in May 2021 and October 2022, and cutthroat trout were stocked in November 2022 (Figure S4).
 59 However, despite the stocking of 30,000 kokanee salmon in May in Lake Padden, *O. nerka* was not detected
 60 by metabarcoding in May 2021 or at any point in 2021 until November (see main text Results). Importantly,
 61 this suggests that Lake Padden is far enough upstream that the eDNA signal at the sampling sites by the
 62 culvert is not a result of stocking the lake 1.5 km upstream (see main text Discussion for more information).

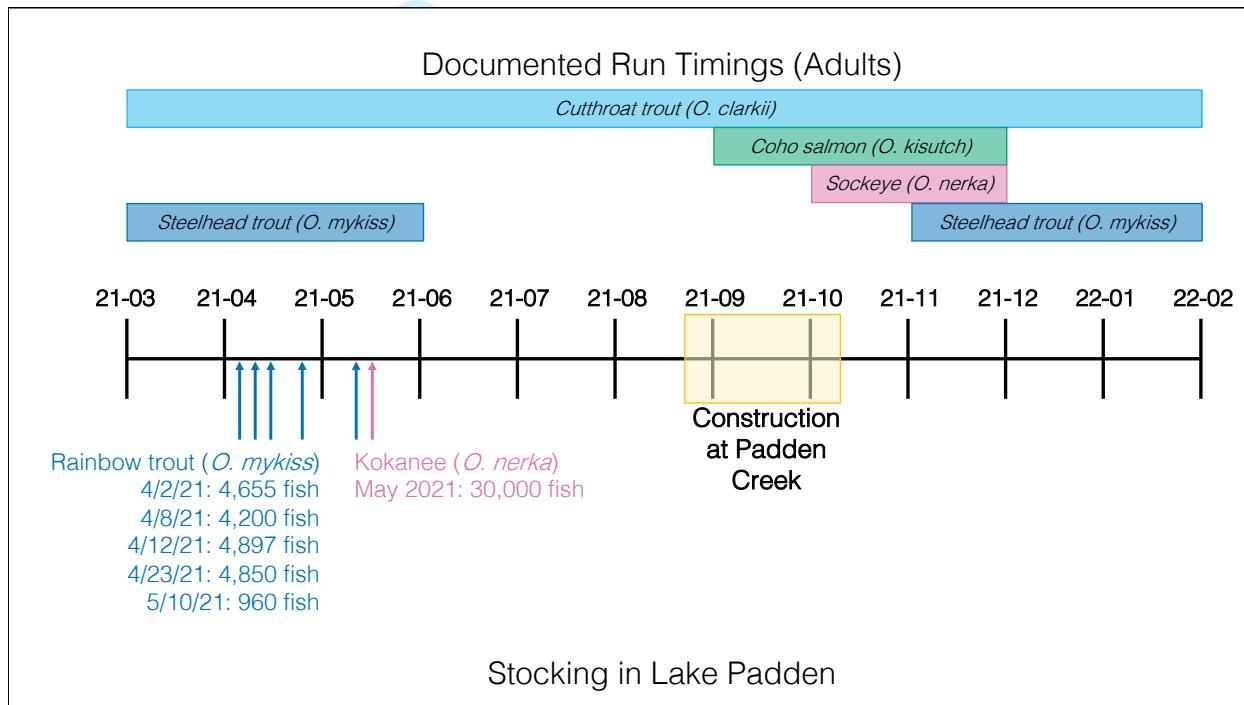


Figure S4. Timeline of runs for migrating species, stocking of Lake Padden, and construction at the intervention site (Padden Creek). Note, the dates of kokanee stocking do not include days, just the month and year. For plotting purposes, they are shown as the 15th.

63 **Water Sampling**

64 Water samples were collected using Smith Root's eDNA Backpack (Thomas et al. 2018), a portable pumping-
 65 and-filtering setup set to filter at 1 L/min at 82.7 kPa (12 psi). For most months, a trident sampler was used
 66 to collect all 3 biological replicates at the exact same time, for a total sampling time of about 5 minutes.
 67 Otherwise, the three replicates were collected consecutively, for a total sampling time of about 15 minutes.

68 Downstream sites were always sampled before upstream sites to ensure no potential DNA was introduced
69 into the stream before sampling. In some samples, less than 2 L of water was filtered due to clogging (mean
70 = 1.95 L).

71 Laboratory Processing

72 DNA Extraction, Amplification, Sequencing

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

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

89 DNA Extractions

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

98 PCR Amplification

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

109 Each month of samples was amplified on a single plate with the addition of a no template control (NTC;
110 molecular grade water in lieu of template) and a positive control (genomic DNA from kangaroo). After PCR
111 amplification, PCR products were visualized on a 1-2% gel. If no band was present for a given sample, a
112 new amplification was attempted with extracts diluted 1:10 iteratively until a band was detected. PCR
113 products were size-selected and cleaned using MagBind Beads (Omega Biotek, USA) at a sample:beads ratio
114 of 1.2. Bead-cleaned PCR products were eluted in 30 μ L of molecular grade water and quantified via Qubit
115 (Invitrogen, USA).

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

124 Indexed PCR products were also size-selected and purified using MagBind Beads (Omega Biotek, USA) at a
125 sample:beads ratio of 0.8. Bead-cleaned PCR products were eluted in 30 μ L of molecular grade water and
126 quantified via Qubit. Indexed and bead-cleaned products were normalized before pooling into libraries, which
127 were subsequently quantified via Qubit and visualized on a Bioanalyzer (Agilent, USA) before sequencing.
128 Samples were randomized in 3-month blocks and each block split across 3 sequencing runs, for a total of

129 12 sequencing runs. The loading concentration of each library was 4-8 pM and 5-20% PhiX was included
130 depending on the composition of the run. Sequencing was conducted using an Illumina Miseq with v3 2x300
131 chemistry at the NOAA Northwest Fisheries Science Center and the University of Washington's Northwest
132 Genomics Center.

133 **Species Specific qPCR**

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

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

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

156 To check for inhibition, the cycle threshold (Ct) value determined for the EXO-IPC assay from the NTC
157 was compared to the Ct value for the EXO-IPC assay in each of the environmental samples. If the Ct value
158 was >0.5 Ct values from the mean Ct for the NTCs, the sample was deemed inhibited and diluted 1:10
159 and re-assayed until the Ct value fell within the accepted range. The majority of environmental samples

160 (60%) were inhibited and accordingly diluted for analysis. In 80% of inhibited samples, a 1:10 dilution or less
161 remedied the inhibition, but some samples (11%) required dilution by a factor of up to 1000.

162 Bioinformatics Processing

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

168 Quality Controls

169 Positive controls were included on each sequencing run to monitor for cross contamination that might have
170 occurred in the laboratory or due to “tag jumping”. With 13 MiSeq runs, we included one sample of kangaroo
171 tissue on each run and then measured how many reads of kangaroo were found in environmental samples and
172 how many reads of non-kangaroo were found in kangaroo samples (Figure S5).

173 We can also check to make sure that no reads assigning to kangaroo were in the environmental samples. We
174 only found kangaroo in two environmental samples, both of which were a very small number (and proportion)
175 of reads (2 and 136 reads found in samples with 40,425 and 28,725 reads respectively) (Figure S6).

176 Annotation

177 We first used a tree-based annotation method (insect package) and then followed up with a BLAST search for
178 all ASVs that were not annotated to species level by insect. The percent of reads annotated did not correlate
179 with sample read depth, creek, station, or month of sampling. Read depth across samples ranged from 1,011
180 to 311,879, with a mean of 79,709 and median of 75,967 reads (Figure S7). With a total of ~565 samples, 93%
181 of samples had >20,000 reads and 65% of samples had over 50,000 reads. There did not seem to be a pattern
182 with samples of low reads with creek or time. Additionally, of the low read depth samples (<20,000 reads, 40
183 samples), there was only one sample in which all three replicates were low (March 2022 Squalicum Upstream),
184 meaning that it is very unlikely that low read depth samples would have lead to changing ecological results.

185 A total of 81 unique species were identified in the environmental samples by the MiFish primers, including 25
186 fish, 25 mammals, 23 birds , and 8 amphibians (Figure S8 and S9; see also Supplemental Table 1). Of the 81
187 species, 17 only were found in a single environmental sample. The three most commonly found species were
188 coho salmon (*O. kisutch*), cutthroat trout (*O. clarkii*), and rainbow trout (*O. mykiss*).

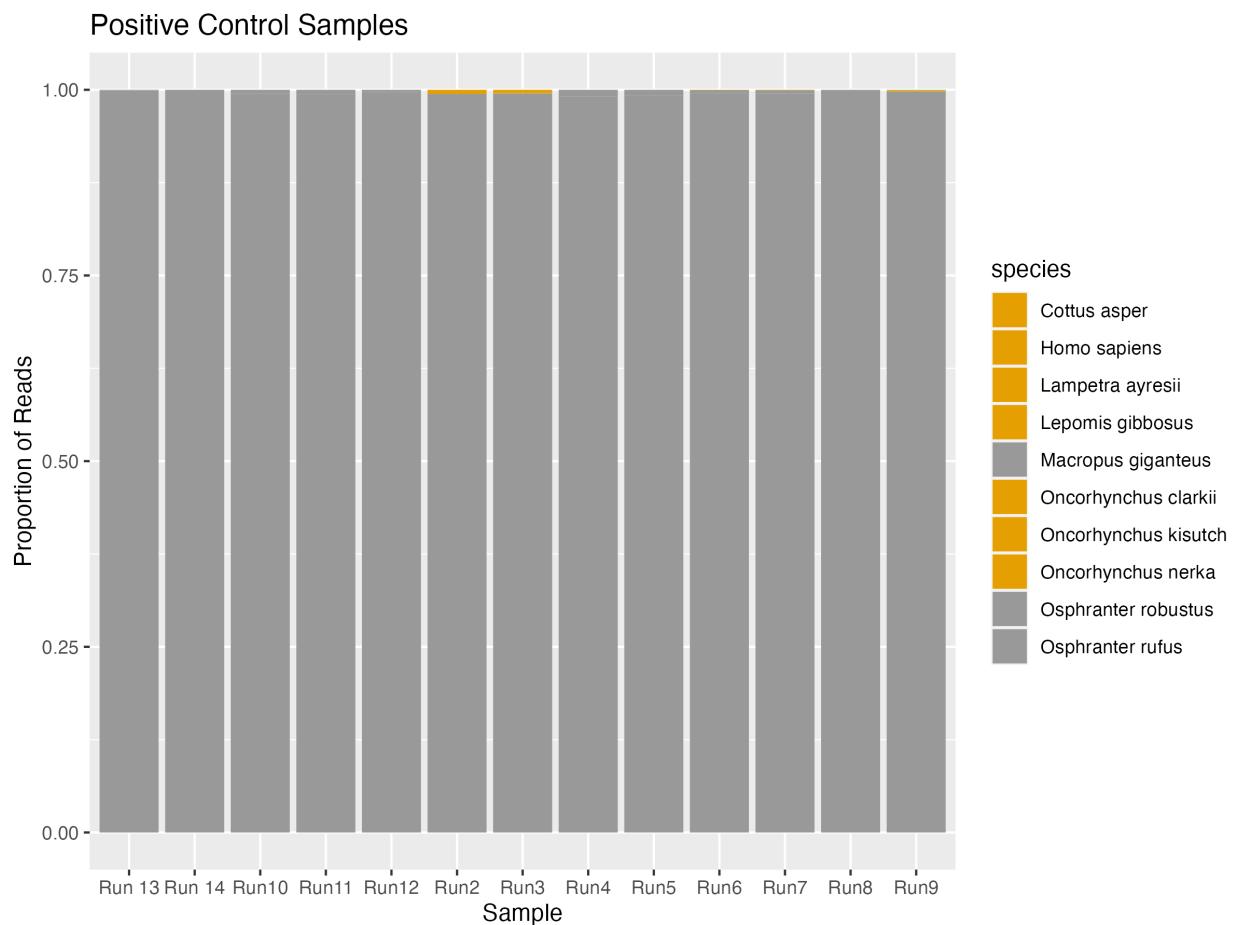


Figure S5. Proportion of annotated reads found in positive controls. Grey colors are the three species of kangaroo used for positive controls and are what should be in each sample. Orange species should not be in the positive controls and indicate low level contamination from environmental samples.

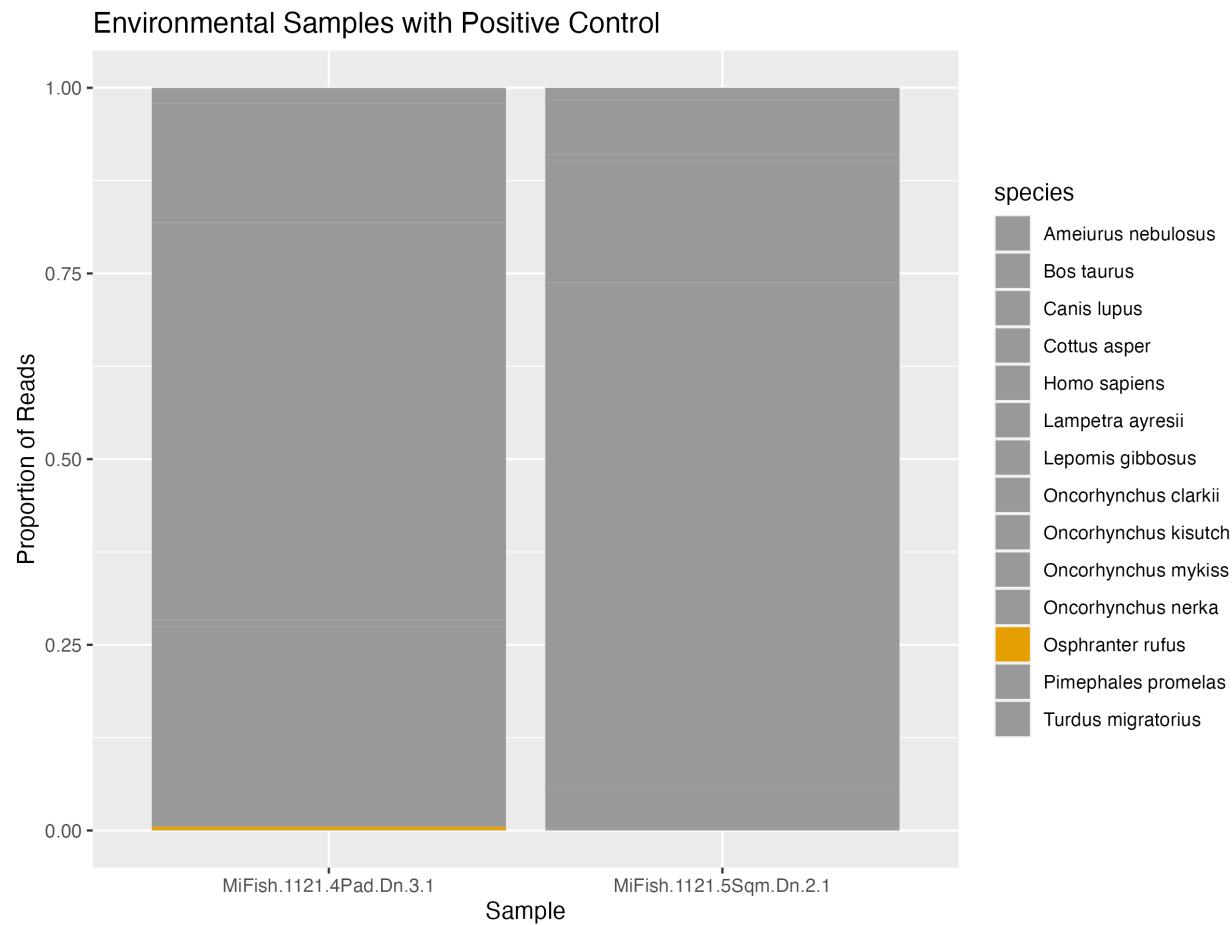


Figure S6. Proportion of annotated reads found in environmental samples with positive control. Grey colors are non-kangaroo reads and therefore are what should be in each sample. Orange species are kangaroo reads and therefore should not be in the environmental samples and indicate low level contamination from positive controls.

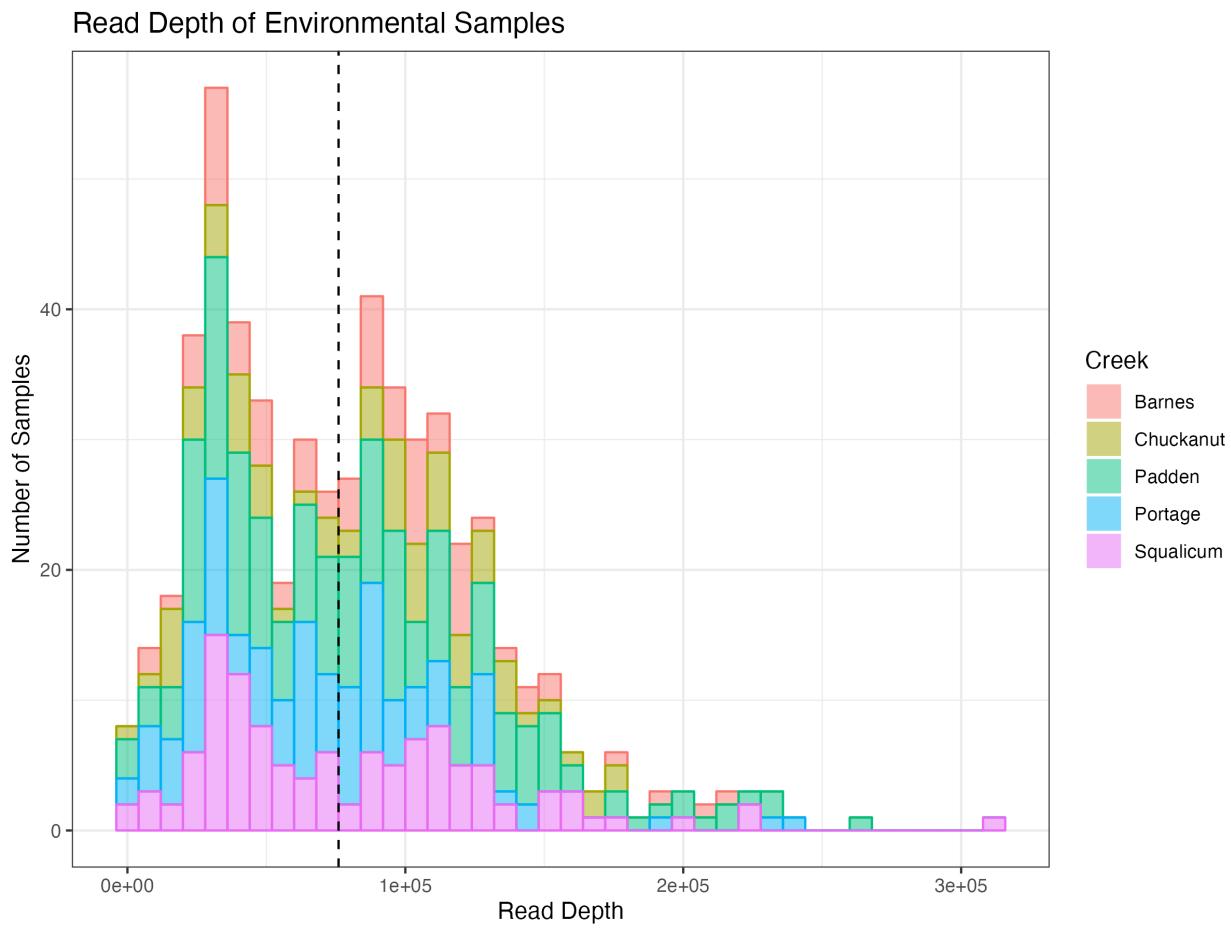


Figure S7. Read depth of samples colored by creek. Dashed line shows median read depth (87,698 reads).

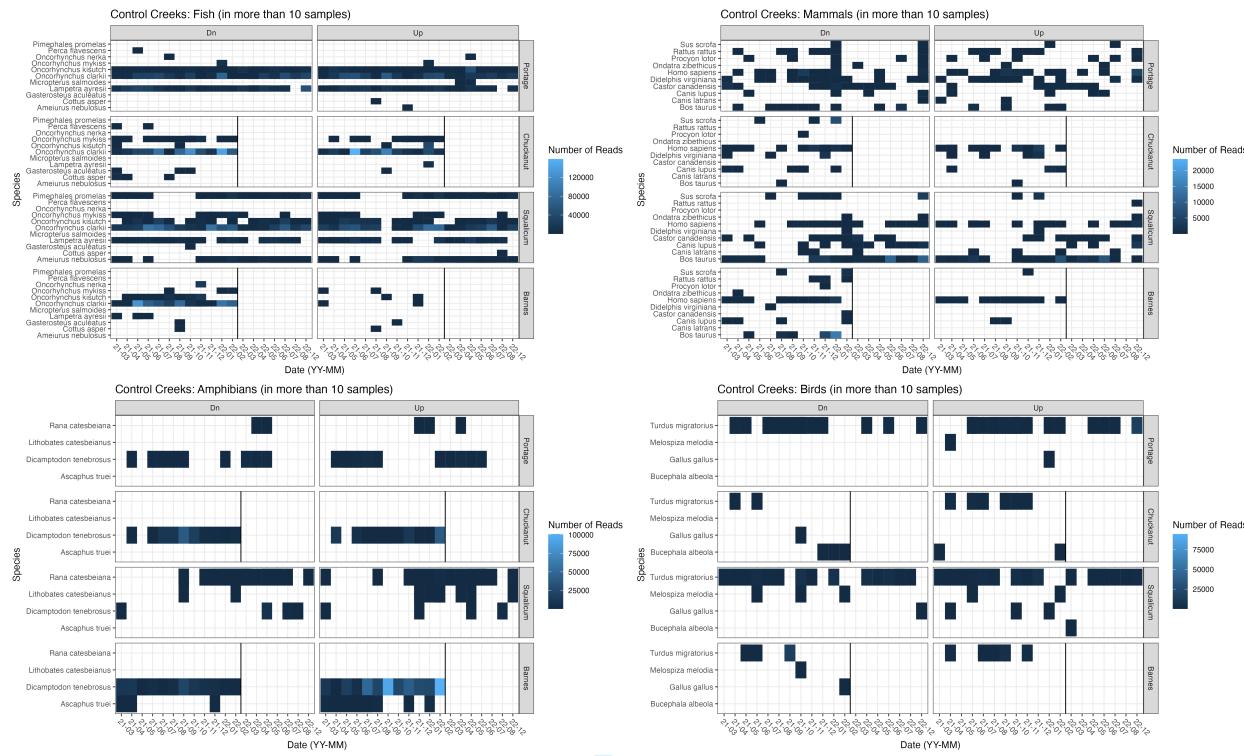


Figure S8. Heat map of all species found in control creeks in at least ten environmental samples.

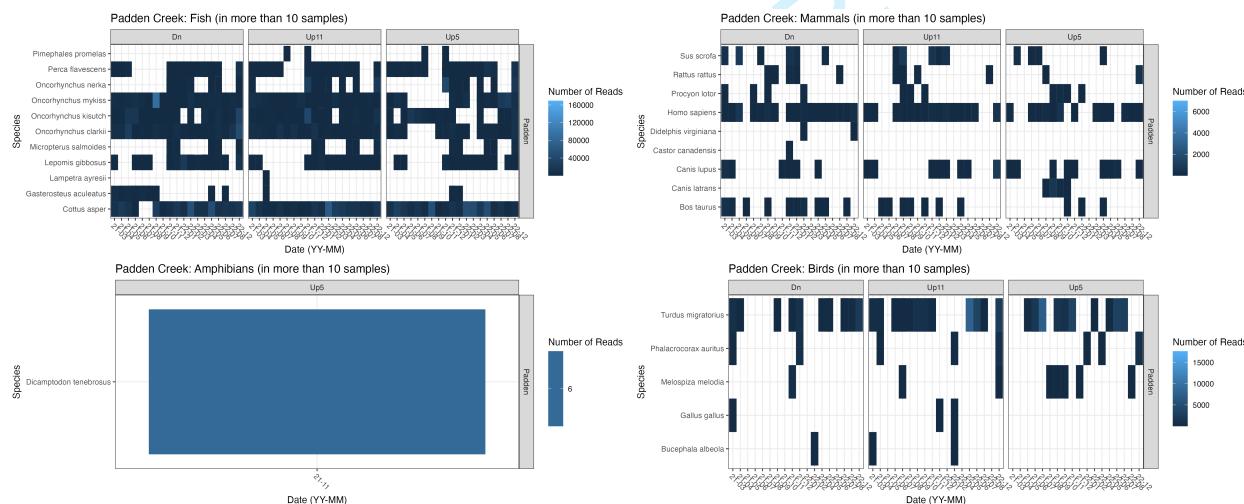


Figure S9. Heat map of all species found in Padden Creek in at least ten environmental samples.

¹⁸⁹ **Correcting metabarcoding data for amplification bias**

¹⁹⁰ Using our six mock communities (three different taxa compositions at two different proportions [even and
¹⁹¹ skewed]), we can first check how well the quantitative metabarcoding model corrects for amplification bias.
¹⁹² In one case, we consider the even mock communities as the mock community data and the skewed mock
¹⁹³ communities as unknown. We can then re-create what the model believes to be the original starting proportions
¹⁹⁴ of the skewed mock community given the proportions of reads found in the skewed mock communities and
¹⁹⁵ the proportion of DNA as compared to the proportion of reads found in the even mock communities. We can
¹⁹⁶ also do the same treating the skewed mock communities as known and even mock communities as unknown
¹⁹⁷ (Figure S10).

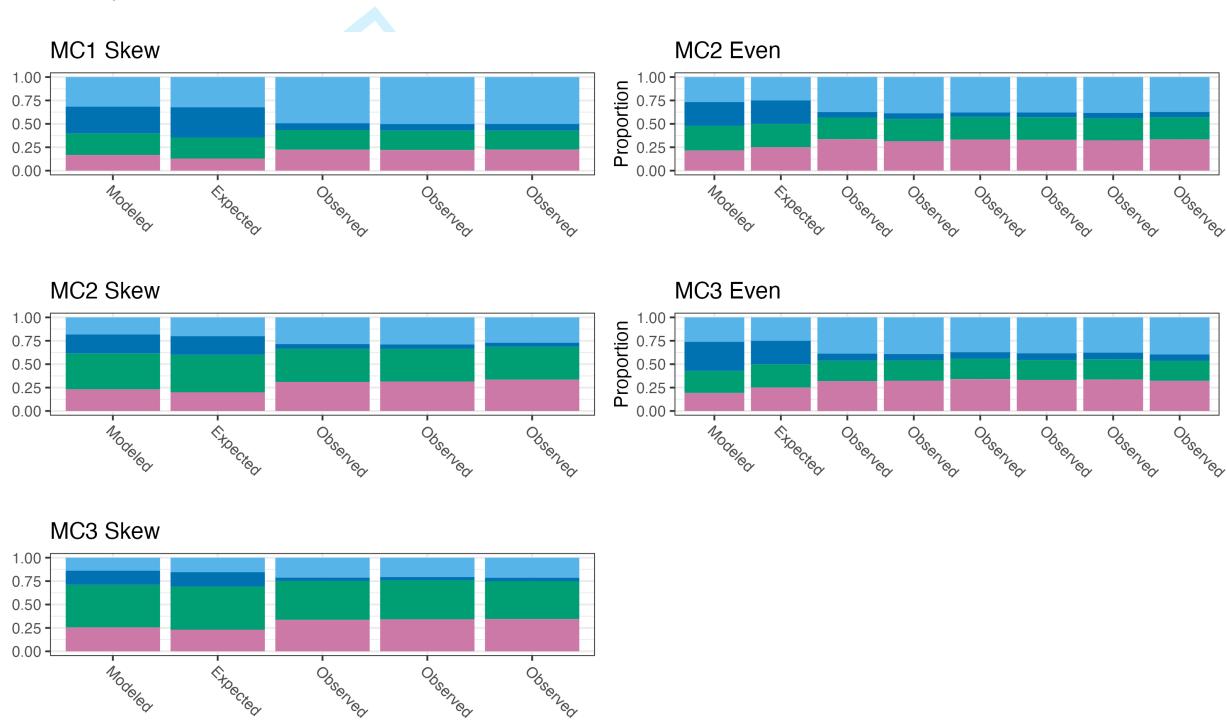


Figure S10. Intercalibration of mock communities used to correct environmental samples for amplification bias.

¹⁹⁸ We can also check how well the calibration is working by comparing the alpha values by using different
¹⁹⁹ subsets of mock community data as true and unknown (Figure S11). We can then use the mock communities
²⁰⁰ to correct the data from the MiSeq to account for the different alpha values. The corrected results are shown
²⁰¹ in the main text as Figure 4.

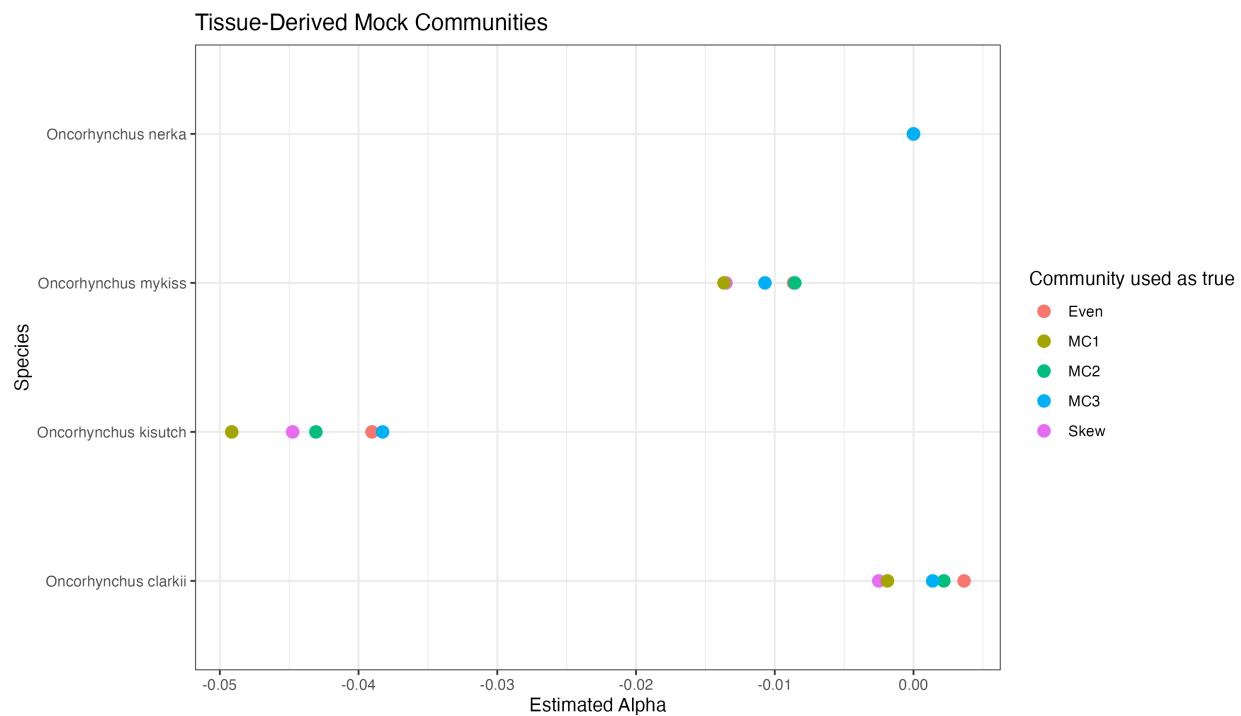


Figure S11. Estimated alpha values of salmonid species with different calibrations of the mock communities. Each color represents a different subset of mock community data treated as ‘true’ to calibrate the remainder of the mock community data.

202 Species-specific Effect of Culverts

203 In the main text, we show the effect of culverts averaged over creeks and species (Figure 8). Here, we show
 204 them separated by species and creek (Figure S12).

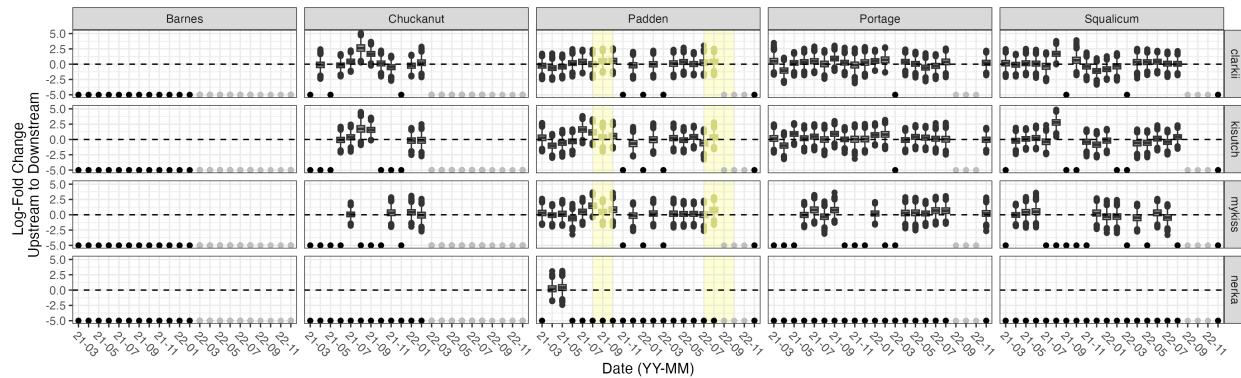


Figure S12. The effect of culvert on salmonid abundance separated by species and creeks across time. The y-axis shows the log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream, normalized by upstream mass flow rate. The box boundaries correspond to the 25th and 75th percentiles; the whiskers correspond to 1.5 times the interquartile range. Here, negative values imply that eDNA mass flow rates are higher downstream than upstream. Samples with very low eDNA mass flow rates (< 150 copies/s) were removed before plotting to remove extreme proportional values due to large denominators. Grey points indicate times when no samples were taken. Black points indicate times when samples were taken, but no target DNA was found in either upstream or downstream samples and therefore the log-fold change can not be calculated.

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²²³ with informatic sequence classification trees.

For Review Only

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

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For submission to: *Ecological Applications*

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

Calibration with a Mock Community

- See Shelton et al. (2022); McLaren et al. (2019); Silverman et al. (2021) for similar analyses.
- For ease of computation, we ran the metabarcoding-calibration model on data for each of our five creeks separately, using the same mock communities to calibrate each.
- Model Diagnostics: 3 chains, 1500 iterations, for all parameters, $\hat{R} \leq 1.02$

²⁴ qPCR Calibration

- ²⁵ See Shelton et al. (2019); McCall et al. (2014) for similar analyses.
- ²⁶ For all samples i , on qPCR plates j , we either observe ($z_{i,j} = 0$ or do not observe $z_{i,j} = 1$) amplification; we
²⁷ omit the subscripts i and j from the following description except where necessary for clarity. We assume an
²⁸ intercept of zero.
- ²⁹ We model the probability of detection $P(z = 1)$ as a linear function of concentration and slope parameter ϕ ,
³⁰ ($P(z = 1) = \theta = c\phi$), with a logit transform to constrain the inferred probability to between 0 and 1.
- ³¹ For those samples that amplify ($z = 1$), we model the observed Ct value (y) as a linear function of our
³² parameter of interest, the log-concentration of target-species DNA under analysis (c). We treat y as drawn
³³ from a normal distribution $y \sim N(\mu_{i,j}, \sigma_{i,j})$, where each triplicate sample on each qPCR plate has its
³⁴ own estimated mean and standard deviation. The means are estimated as a straightforward linear model,
³⁵ $\mu = \beta_{0,j} + \beta_{1,j}c$, but we allow the standard deviation to vary as a linear function of log-concentration so as
³⁶ to accurately capture decreasing precision with decreasing concentration: $\sigma = e^{\gamma_0 + \gamma_1 j c}$; we estimate these
³⁷ parameters as an exponent to constrain $\sigma > 0$.
- ³⁸ Samples with known concentrations (i.e., standards) were fit jointly with unknown samples (i.e., environmental
³⁹ samples); because qPCR plate identity was shared among all environmental samples and standards within a
⁴⁰ plate, this has the effect of applying plate-specific slope and intercept values for the standard curve to each of
⁴¹ the environmental samples on the plate (Figure S13).
- ⁴² We apply moderately informative priors that make use of background information in hand. For example,
⁴³ because qPCR standard curves of all kinds have slopes near -3, this slope becomes our background expectation
⁴⁴ as embodied in the prior on β_1 , but the standard deviation of that prior leaves plenty of room for this
⁴⁵ background to be overwhelmed by the observed data. The same logic applies to the intercept of the standard
⁴⁶ curve, which in qPCR (for any given species) generally falls near 39 cycles, an expectation that we formalize
⁴⁷ by having β_0 drawn from a normal distribution with $\mu = 39$ and $\sigma = 3$.
- ⁴⁸ Taken together with priors, the model is:

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

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

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

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

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

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

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

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

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

⁴⁹ Model Diagnostics: 3 chains, 2500 iterations, for all parameters, $\hat{R} \leq 1.002$.

⁵⁰ Expanding Proportions into Absolute Abundances

⁵¹ See Pont et al. (2022) and McLaren et al. (n.d.) (preprint) for examples of similar expansions.

⁵² As described in the main text, calibrated metabarcoding analysis yielded quantitative estimates of the
⁵³ proportions of species' DNA in environmental samples prior to PCR.

⁵⁴ We then converted these proportions into absolute abundances by expansion, in light of the qPCR results for
⁵⁵ our reference species *O. clarkii*. We estimated the total amplifiable salmonid DNA in environmental sample *i*
⁵⁶ as $DNA_{salmonid_i} = \frac{[qPCR_{reference_i}]}{Proportion_{reference_i}}$, and then expanded species' proportions into absolute concentrations
⁵⁷ by multiplying these sample-specific total concentrations by individual species' proportions, such that for
⁵⁸ species *j* in sample *i*, $DNA_{i,j} = DNA_{salmonid_i} * Proportion_{i,j}$.

⁵⁹ We transformed the resulting abundances to account for the creeks' flow-rates as described in the main text.

⁶⁰ Ideally, we would have fit a joint model that simultaneously estimated species proportions (metabarcoding),
⁶¹ absolute concentrations (qPCR), and developed the time-series trends for all species. As a practical
⁶² computational matter, we had to create these models individually, which entailed some loss of information
⁶³ about parameter variability and cross correlation. For the mixed-effects model describing trends over time
⁶⁴ (described below), we used the product of posterior means from the metabarcoding and the concentrations

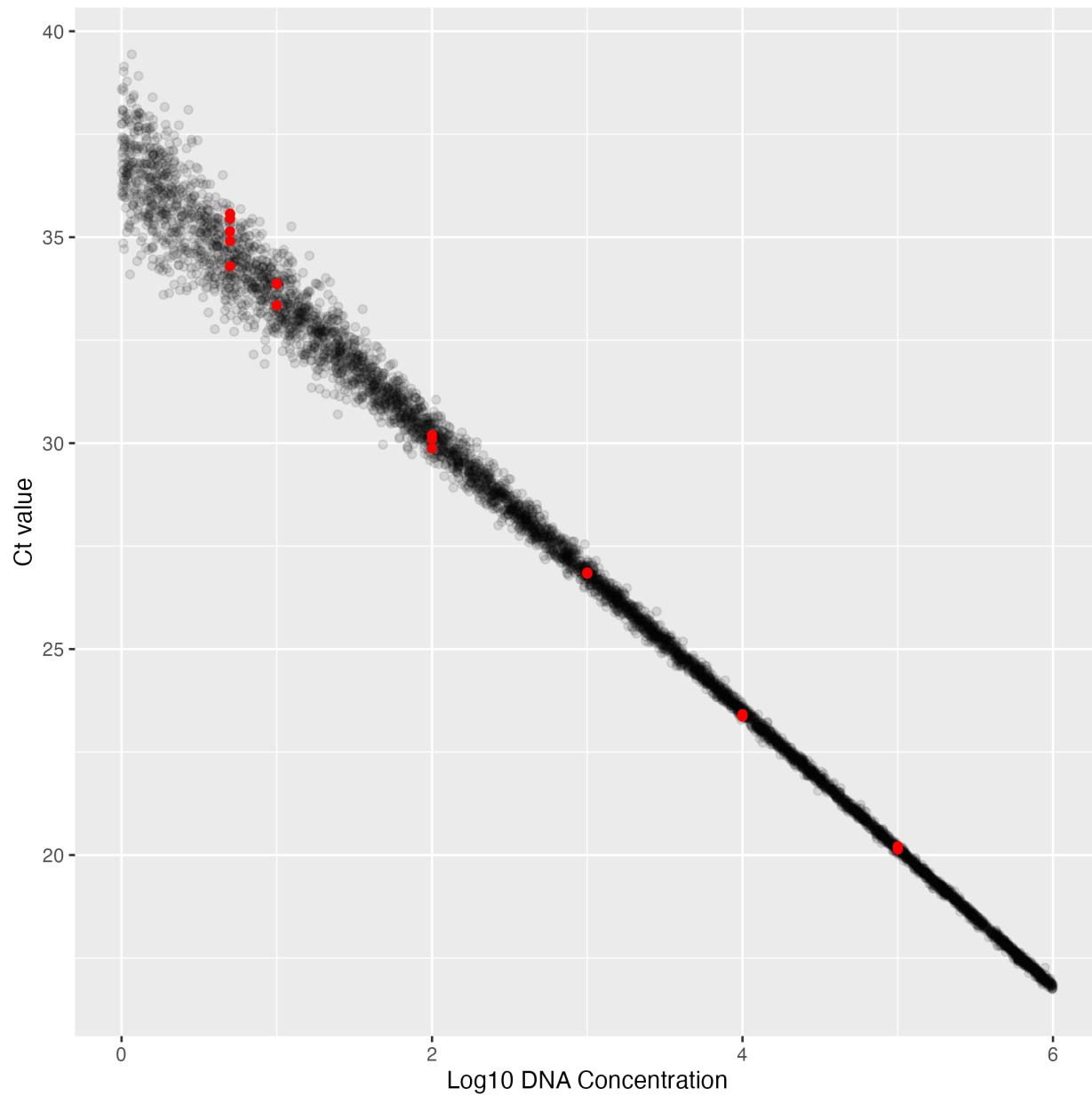


Figure S13. Example of 2500 samples from the joint posterior distribution of the model fit for a single representative qPCR plate. Red dots are standard-curve observations with known starting concentrations. The spread of black dots (posterior samples) indicates the shape of the calibration curve, with standard deviation increasing as concentration decreases.

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

69 Modeling Changes in Concentration over Time

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

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

73 More complex versions of the model may let σ vary across creeks, time points, species, or with environmental
74 covariates of interest.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

```
98     s(station, bs="re") + #random effect, station  
99     s(time_idx, by=creek, m=1, bs="cc")+ #main effect, creek  
100    s(creek, bs="re") + #random effect, creek  
101  )  
102 )
```

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

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

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

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

- 108 Ultimately, the three models yielded very similar results (Figure S14). The LME model proved simplest and
109 most flexible insofar as it could easily handle datasets with uneven sets of observations – for example, cases
110 in which a species was detected downstream of a barrier, but not upstream. We accordingly used the LME as
111 the model for the analysis given in the main manuscript.

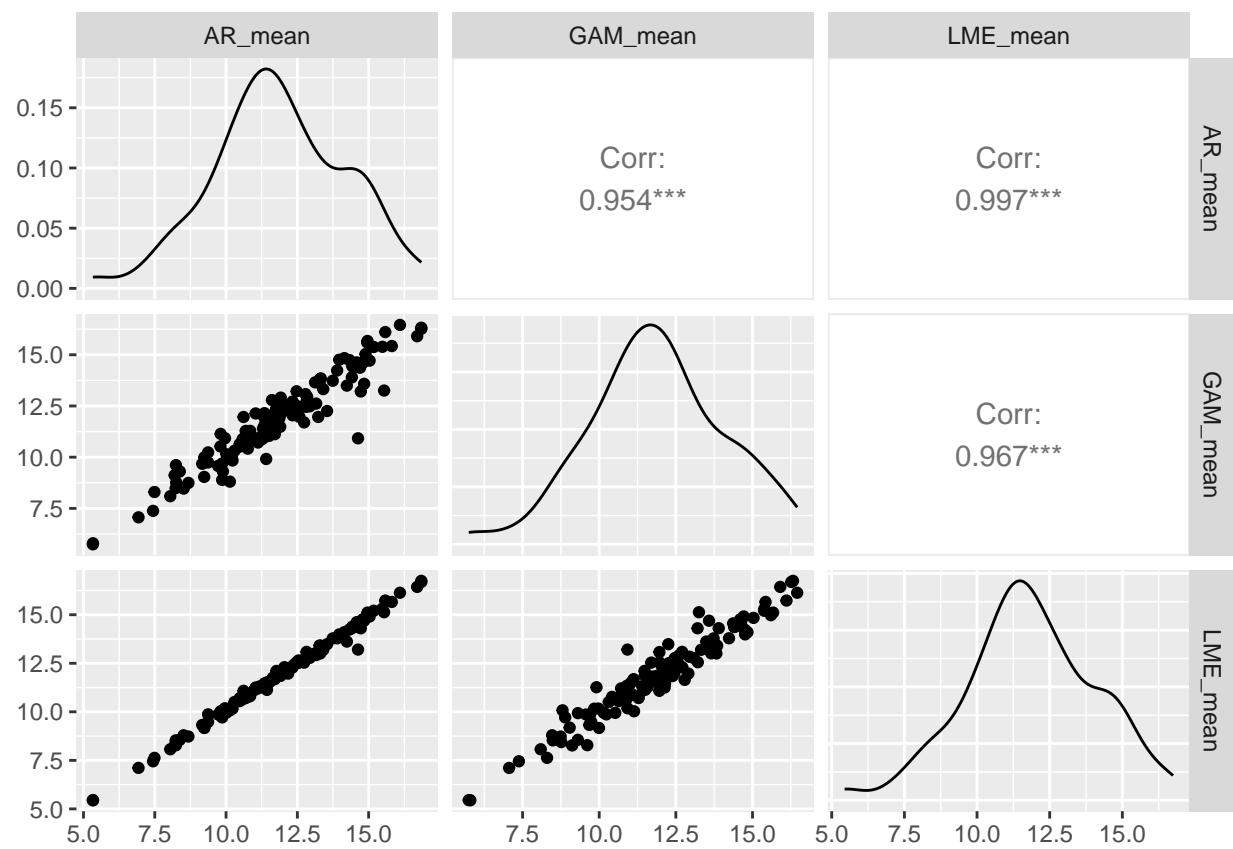


Figure S14. Comparison of three models (linear autoregressive, generalized additive model, and linear mixed effects model) shown for a subset of the data used in the main manuscript.

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

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

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

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

¹ Quantifying Impacts of an Environmental Intervention Using Environmental
² DNA

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¹⁵ repository). The repository for code can be found at: https://github.com/eandrusz/quantitative_salmon_re
¹⁶ submit.git

¹⁷ Keywords: environmental DNA, quantitative metabarcoding, environmental impact assessments, salmon,
¹⁸ culvert

19 **Abstract**

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

46 Introduction

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

169 **Methods**

170 **Site and Species Selection**

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

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

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

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

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

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

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

226 Water Sampling

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

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

239 Over the course of the year of sampling, water discharge varied from very low to no flow in summer months
240 to high flow in winter months (Figure 2). Thus, when considering eDNA concentrations at a sampling site,
241 we need to account for the large difference in water volume over the course of the year-long time series. In
242 other words, given the same number of fish and a constant eDNA shedding rate, we would expect to see
243 higher concentrations of DNA in summer months and lower concentrations in winter months due to dilution
244 of eDNA in higher water volumes just from the difference in flow. Other eDNA time series datasets also
245 correct for discharge to present eDNA data as a mass flow rate mass/time (Tillotson et al. 2018, Thalinger
246 et al. 2019). Here, we convert eDNA). We account for this dilution by converting eDNA concentration
247 [copies/ μ L] to an eDNA mass flow rate [copies/s] by multiplying eDNA concentrations by discharge [L/s] .
248 (Tillotson et al. 2018, Thalinger et al. 2019). Flow gauges maintained by the United States Geological Survey
249 (USGS) were used for Padden Creek (USGS Gauge 12201905), Chuckanut Creek (USGS Gauge 12201700),
250 and Squalicum Creek (USGS Gauge 12204010; <https://maps.waterdata.usgs.gov/mapper/index.html>;
251 U. S. Geological Survey (1994); Supplemental Figure 1). During the year Figure S1.1). Over the course
252 of sampling, the flow gauges at Chuckanut Creek and Squalicum Creek became inoperable after a major
253 flooding event. To find discharge rates for Chuckanut and Squalicum Creeks, five years of historical data
254 (2015-2020) were used to generate a daily monthly averaged correction factor based on Padden Creek .
255 For the year of sampling (2021-2022), the discharge rates used at Chuckanut and Squalicum Creeks were
256 estimated based on the correction factor from Padden Creek (Supplemental Figure 2(Supplemental Text 1,
257 Figure S1.3)). No discharge data was available for Portage Creek or Barnes Creek. Based on field sampling
258 conditions, the discharge from Padden Creek was used as a proxy for both Portage and Barnes as they
259 are in similarly sized watershed areas and land-cover characteristics. Though in the year of sampling, the
260 discharge in Padden Creek ranged from no metered flow to 23 m³/s, the discharge on the dates of sampling
261 only reached a maximum of 1.3 m³/s.

262 **DNA Extraction, Amplification, Sequencing**

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

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

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

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

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

312 Bioinformatics

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

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

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

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

338 Quantitative PCR and Inhibition Testing

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

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

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

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

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

374 Quantitative Metabarcoding

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

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

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

395 Estimating the Effects of Culvert Replacement and of Culverts Themselves

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

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

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

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

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

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

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

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

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

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

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

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

436 Results

437 Metabarcoding and Quantitative PCR

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

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

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

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

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

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

471 7).

472 **Effects of Culverts**

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

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

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

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

505 Effects of Culvert Replacement

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

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

522 Discussion

523 Environmental DNA can provide quantitative measurements of environmental impacts

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

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

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

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

560 Fish life histories and expected patterns

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

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

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

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

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

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

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

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

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

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

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

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

665 Not all culverts are barriers to salmonids

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

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

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

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

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

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

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

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

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

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

727 Conclusion

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

741 Conflict of Interest Statement

742 The authors declare there are no conflicts of interest.

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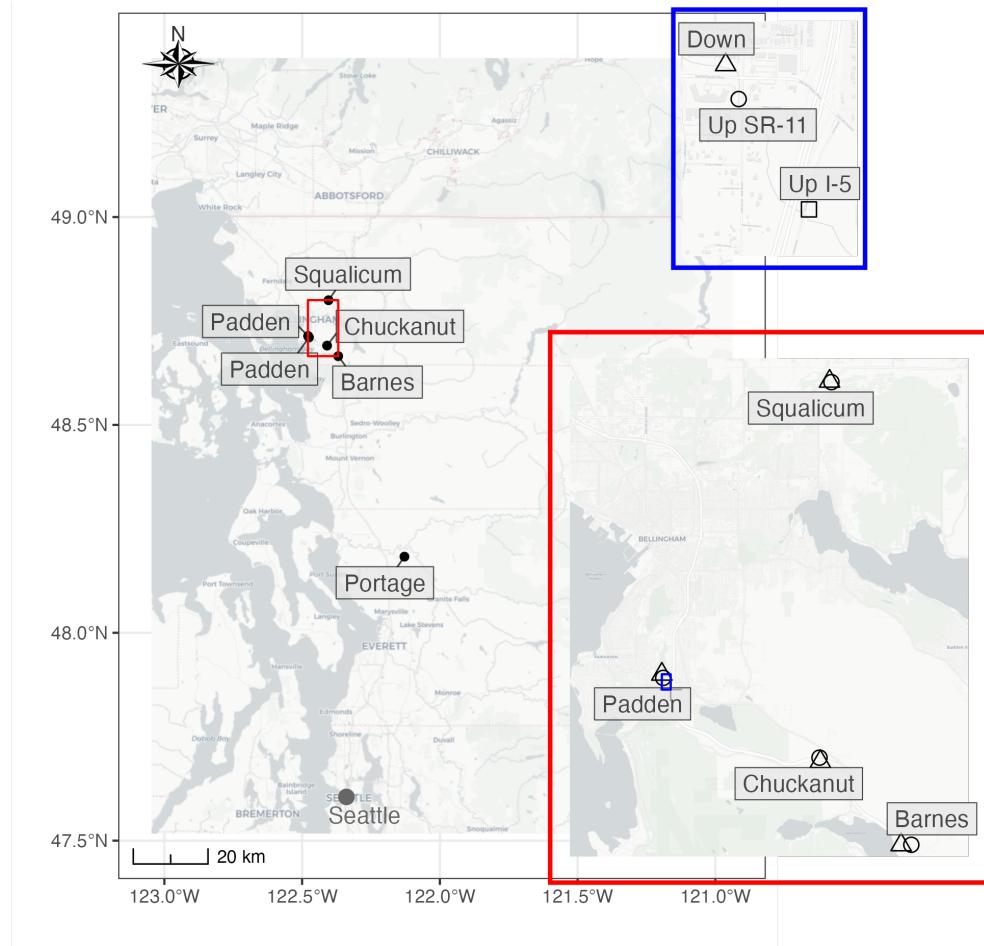
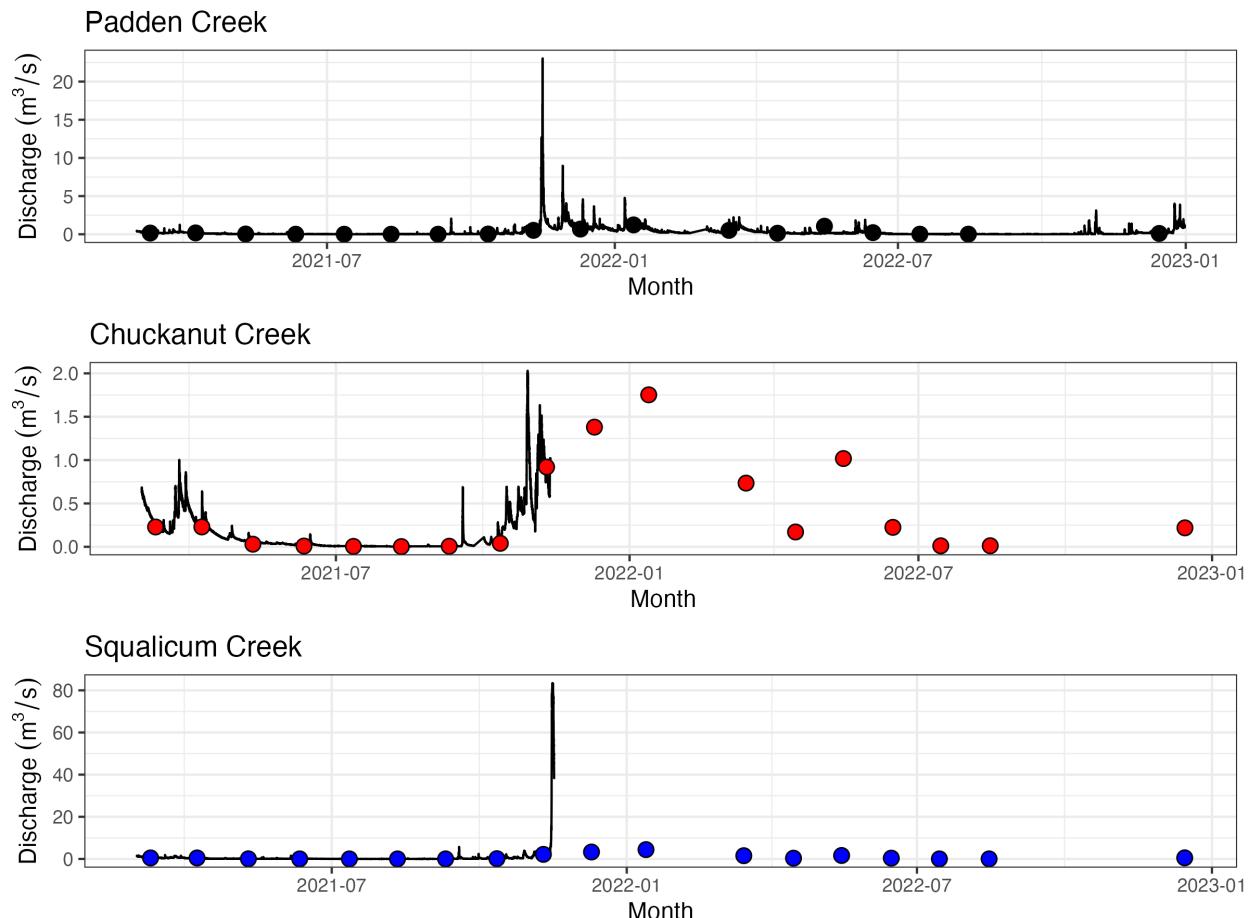


Figure 1: Map of sampling locations near Bellingham, Washington. In the red subset, triangles designate the downstream sampling location and circles designate the upstream sampling location. Padden Creek (the treatment creek where the two culverts were replaced) is shown in the blue subset where downstream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



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

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

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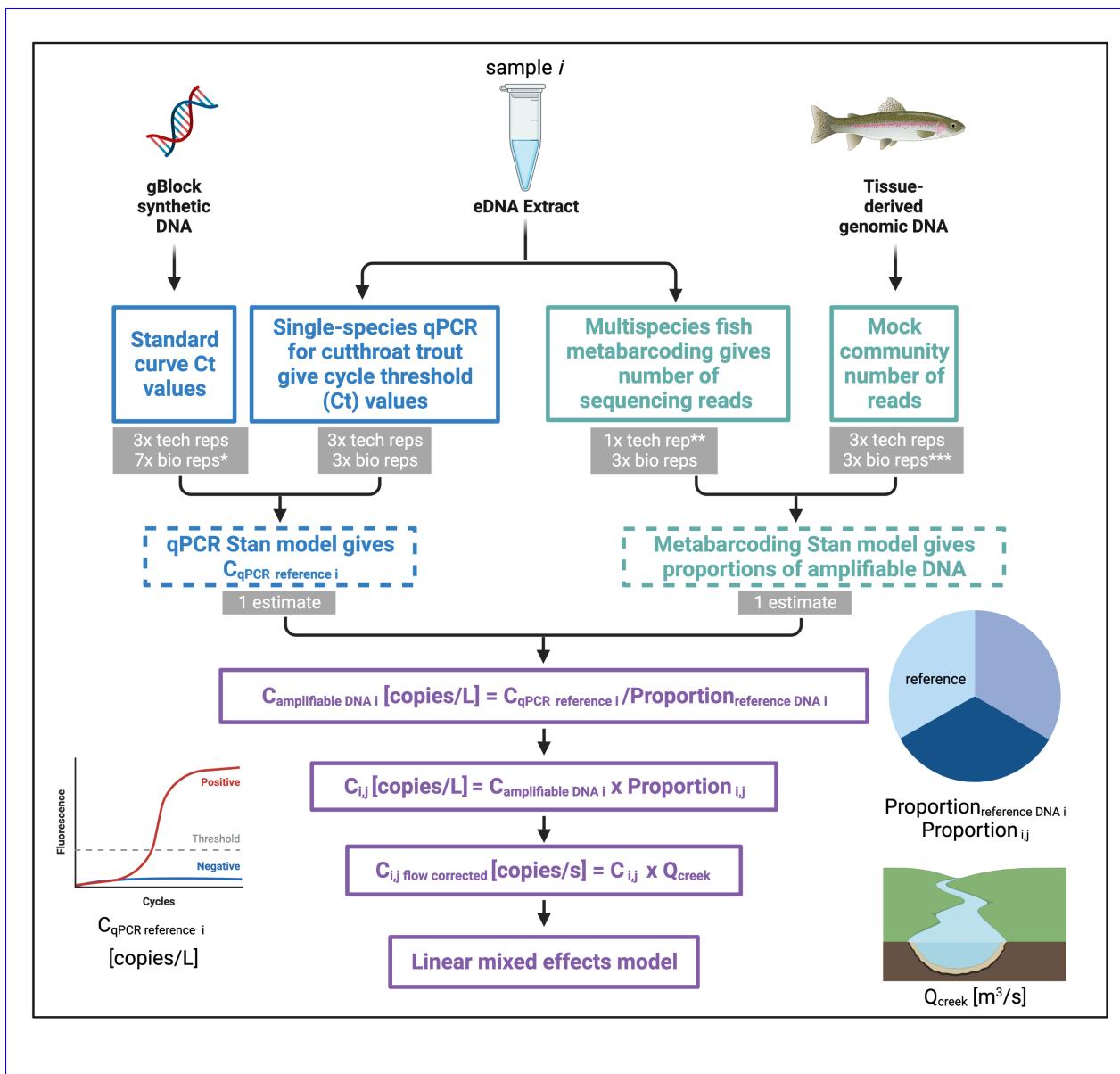


Figure 3: Conceptual figure of different datasets and models used for analyses. * indicates that here, biological replicates are different dilutions of the synthetic gBlock. ** indicates that for most samples, only one technical replicate was sequenced but for one sample per sampling month, three ~~technical~~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

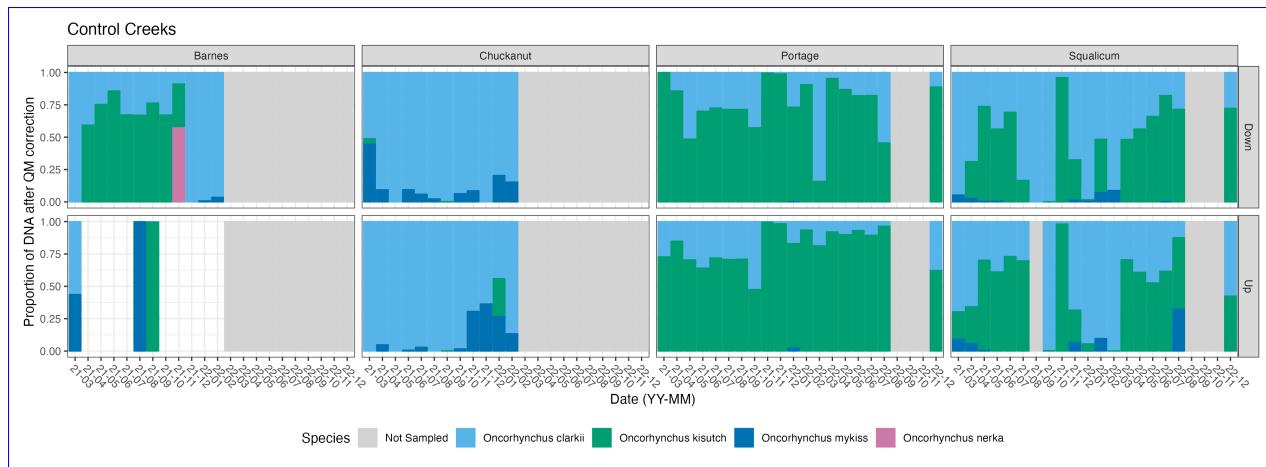


Figure 4: Compositions of salmonid DNA in control creeks as determined by metabarcoding after correction for amplification bias. Note Grey shading denotes time points that no sampling occurred were not sampled (Barnes and Chuckanut Creeks after March 2023 and Squalicum Creek in September 2021 at Squalicum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

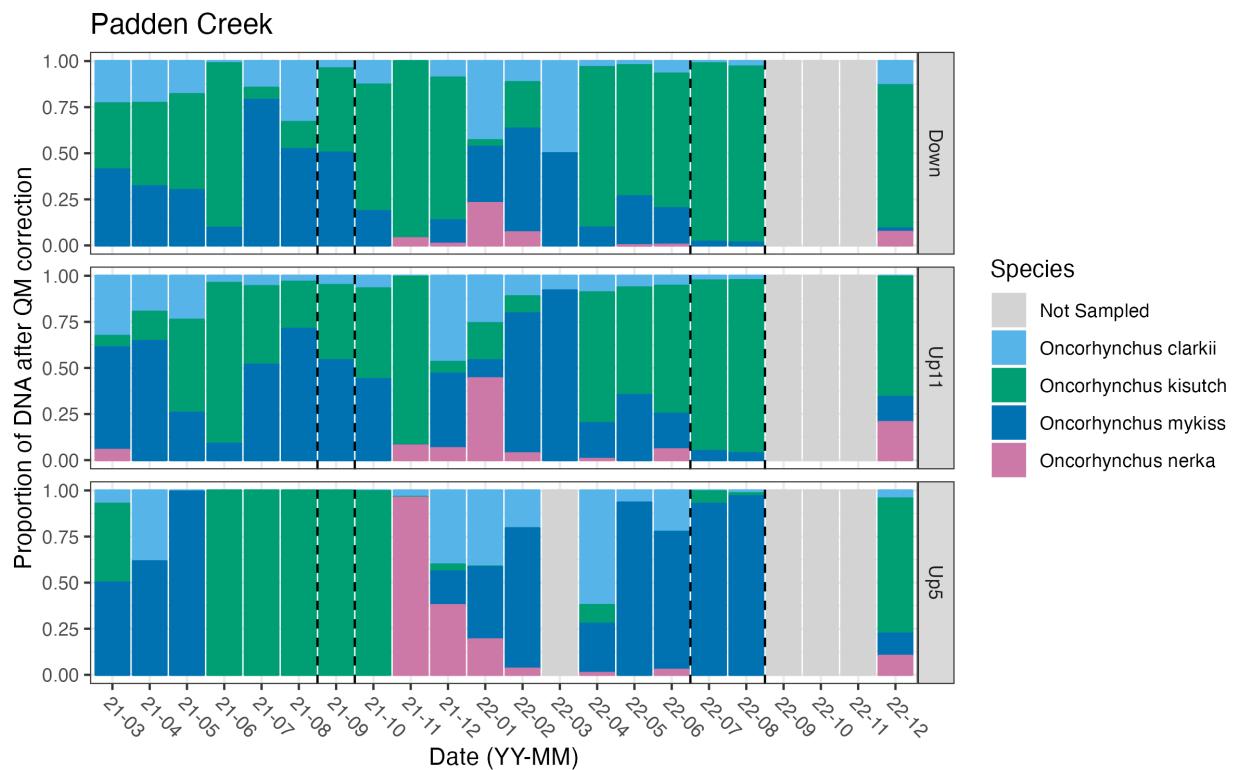


Figure 5: Compositions of salmonid DNA in Padden Creek as determined by metabarcoding after correction for amplification bias. The empty bar in the March 2023 in Up5 indicates that no salmonid DNA was found. The vertical dashed lines indicates the time period in which the culverts were replaced (SR-11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

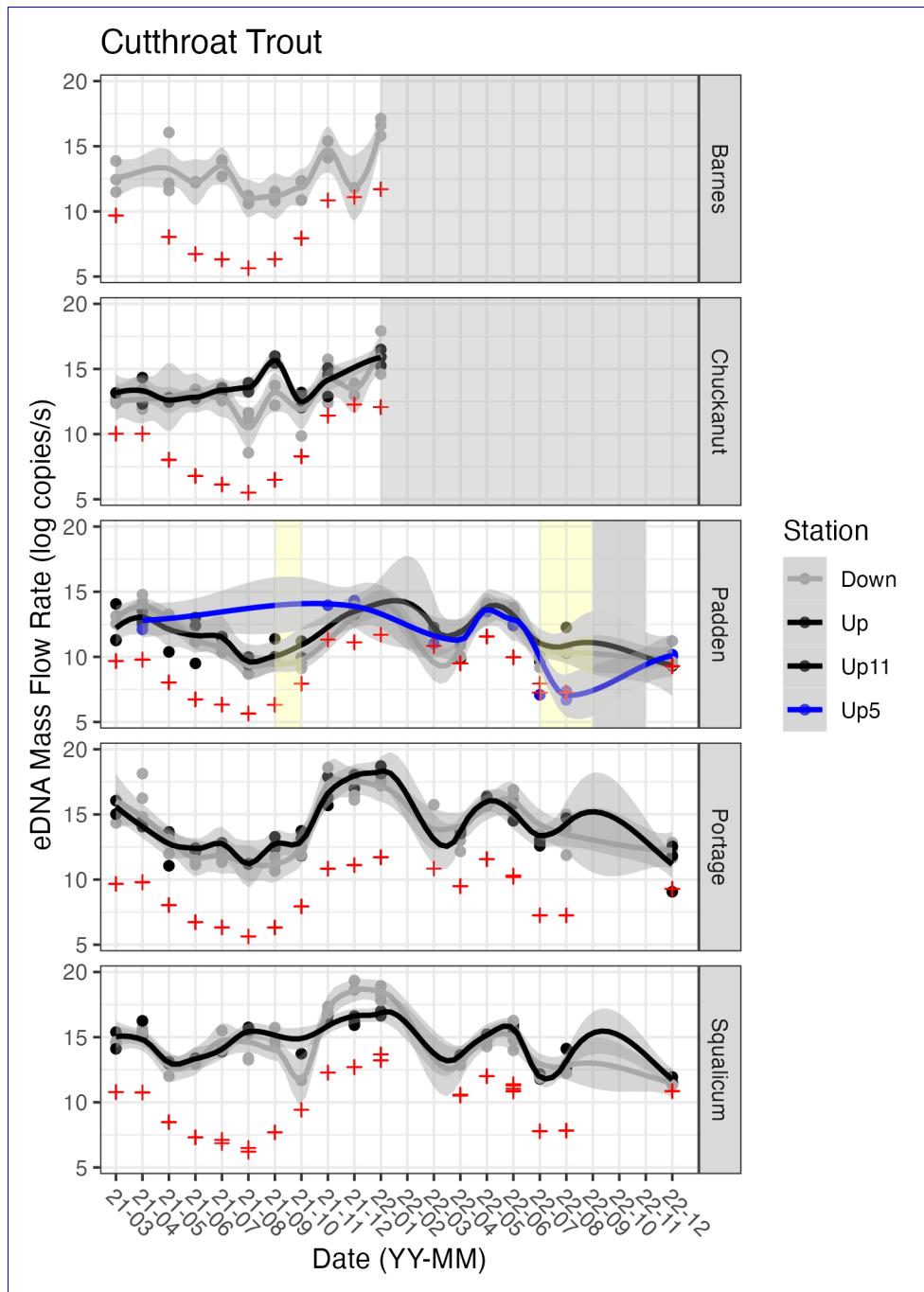


Figure 6: Absolute concentration-mass flow rate (log copies/L of water) of *O. clarkii* (cutthroat trout (*O. clarkii*)) as measured by qPCR before after flow correction. Note that Barnes Creek and Chuckanut Creek were not sampled after February 2022. Red crosses show the limit of detection for each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

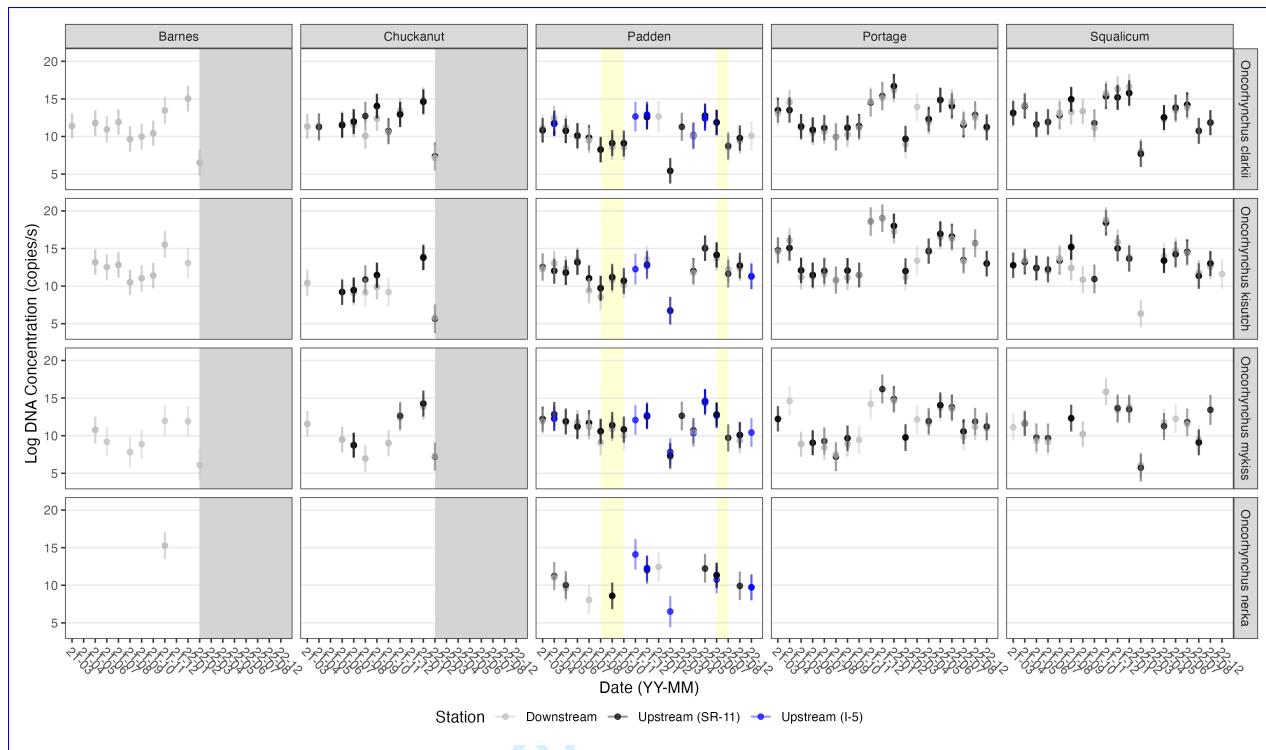


Figure 7: Trends across creeks and across time in mass flow rate (log copies/s) for each of four salmonid species across creeks and across time as estimated by eDNA analysis. Eight colored dots are Points represent posterior means derived by expanding the calibrated metabarcoding proportions as described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

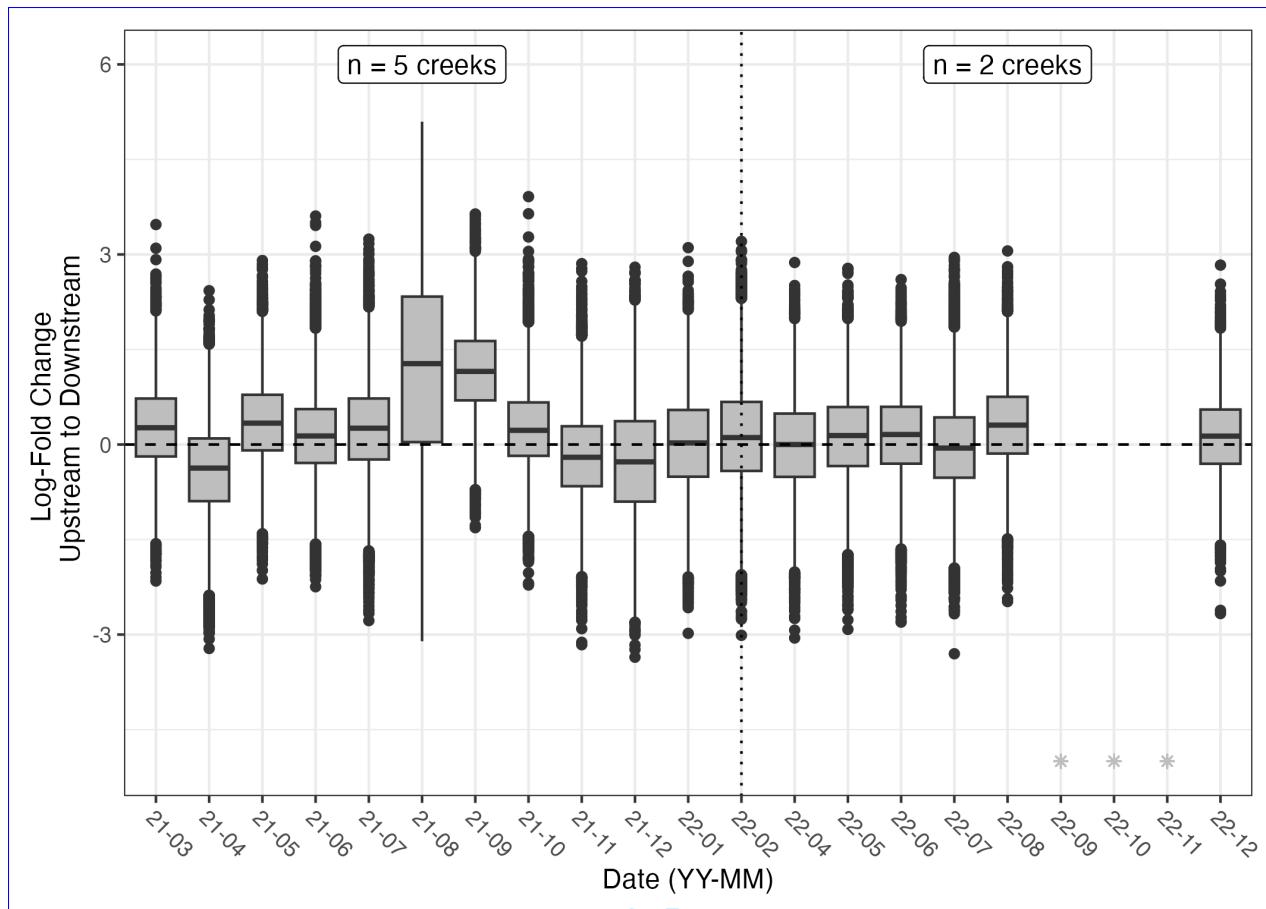


Figure 8: The effect of culvert on salmonid abundance summed across all species and creeks by time. The y-axis shows the difference log-fold change in eDNA mass flow rate (copies/s) between upstream and downstream concentrations, normalized by downstream concentration upstream mass flow rate. The box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

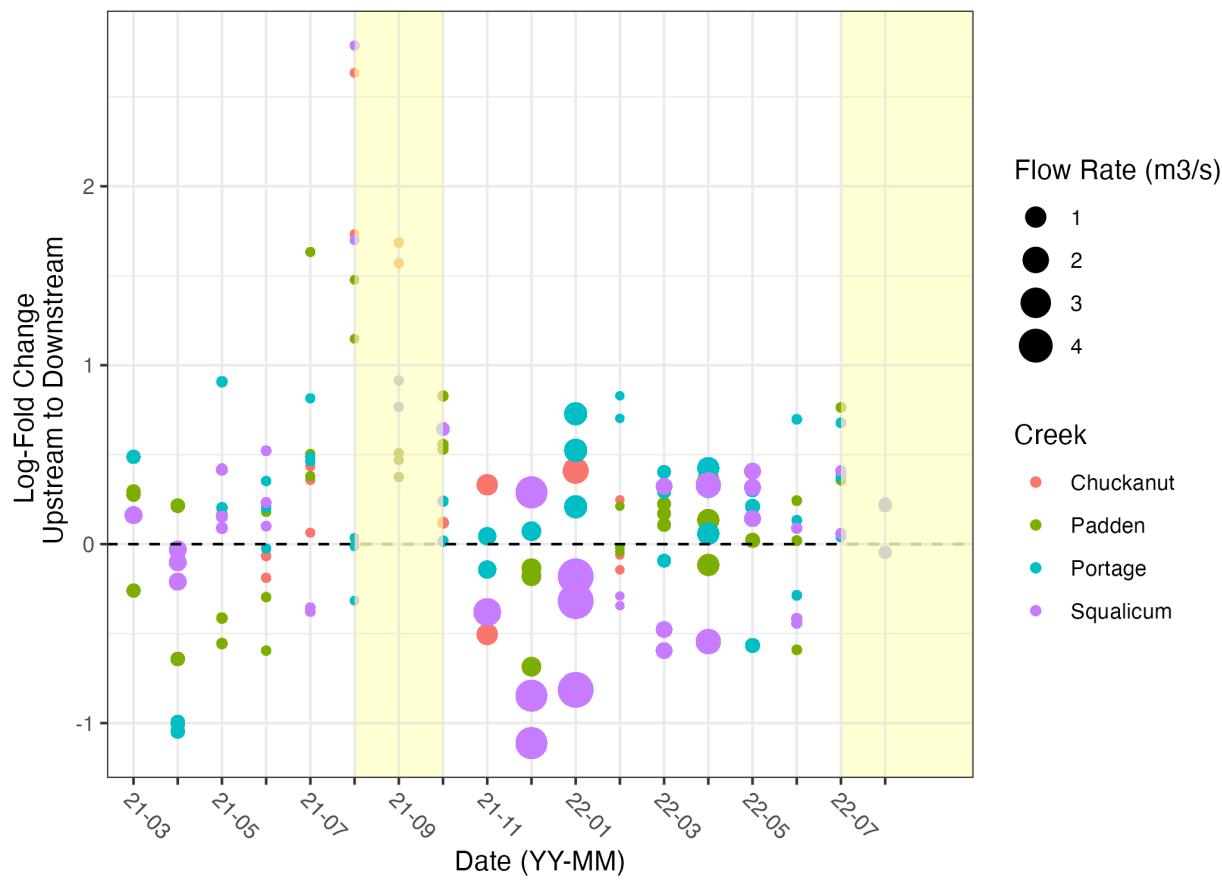


Figure 9: Log-fold change in eDNA mass flow rate over time. Size of circles corresponds to the discharge in each creek at each time point. Color of circles corresponds to each creek. Each creek and time point has up to four circles of the same color for the four salmonid species.

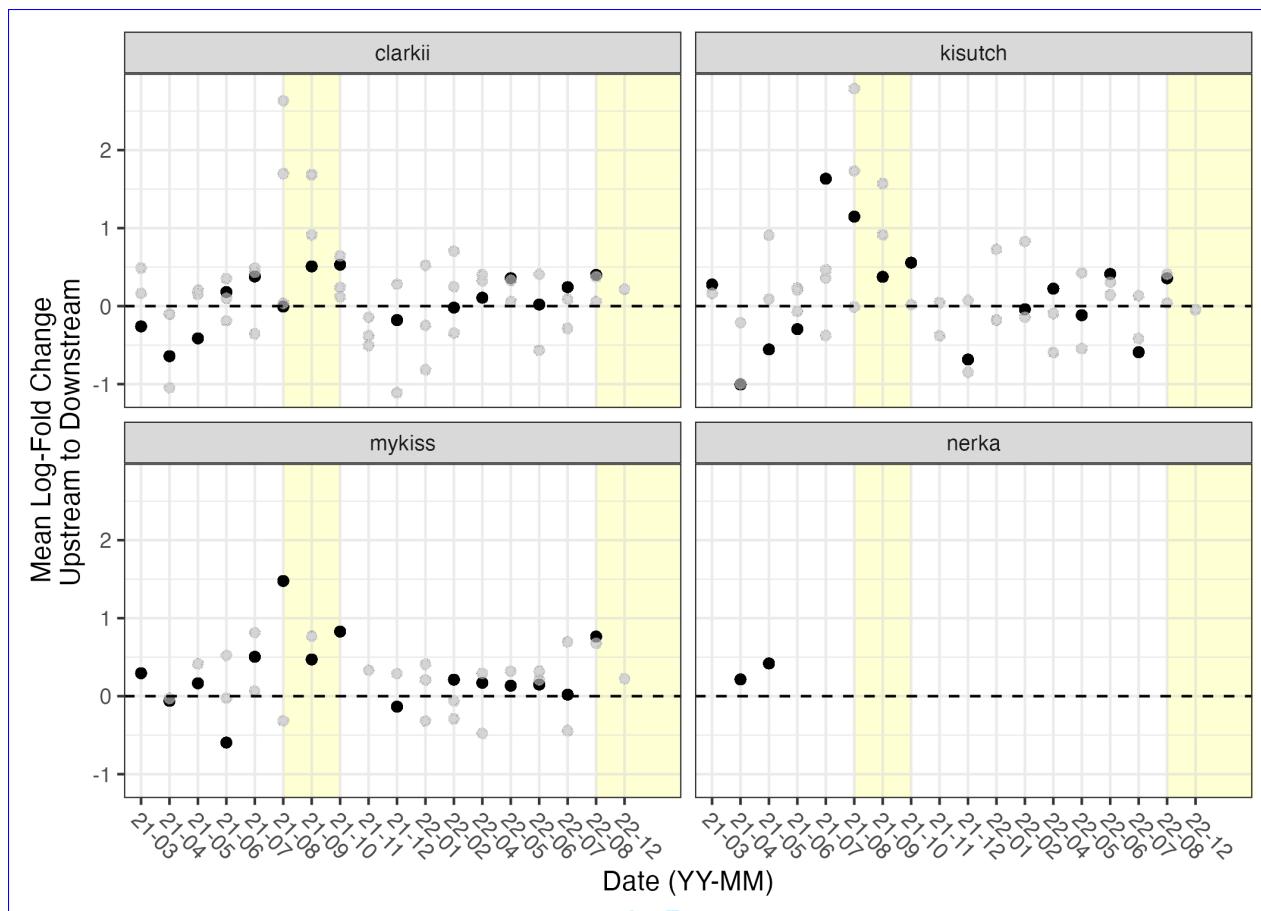


Figure 10: Effect of Construction on Salmonid DNA Concentrations Log-Fold Change in eDNA Flow Rate Upstream to Downstream in Padden Creek. Error bars show 95% confidence intervals of the normalized difference between upstream and downstream DNA concentrations. Grey-Yellow shading shows when construction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