**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 re-submit 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 culver 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\_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFBo5N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y\_3zbDBAOqsQ$*](https://urldefense.com/v3/__https:/doi.org/10.1139/facets-2016-0058__;!!K-Hz7m0Vt54!mN_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFBo5N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y_3zbDBAOqsQ$)*) 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\_\_;!!K-Hz7m0Vt54!mN\_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFBo5N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y\_3zYcWRJOYw$*](https://urldefense.com/v3/__https:/waterdata.usgs.gov/nwis__;!!K-Hz7m0Vt54!mN_SEOLYNXNnOr6TlJ186HeixWIasV3g5veTzOUBFBo5N0T8WFa3cpODnOeu3iLbvjbUNnkTKHL3I4y_3zYcWRJOYw$)*<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%. Futhermore, 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.