Monterey Bay Aquarium
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Effects of a Coho hatchery on downstream eDNA detection

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INTRODUCTION

Environmental DNA (eDNA) is becoming a popular tool for community assessment, population monitoring, and species detection. As a relatively new technique that has rapidly expanded, many protocols have been developed but their relative efficacy has not been evaluated in many cases. Here we assess the effects of using filters with two different pore sizes while also examining the influence of a Coho (*Oncorhynchus kisutch*) salmon hatchery on detection rates for water samples taken downstream.

Traditionally, population monitoring or new observations of invasive species involve technicians in the field manually counting organisms. For fish populations this may involve visual surveys while walking in a stream, electrofishing, capture in a seine, or fyke net. These surveys can be costly both as they require many hours in the field, and equipment that requires frequent replacement. Manual surveys can also fail to capture an accurate representation of the community because some species may be rare or hard to detect. Species may be rare because they are endangered, they may also suffer negative effects of being captured during sampling surveys. Many small fishes sustain injuries during sampling with nets and electrofishing gear that reduce their survival when released. Monitoring for the spread of invasive species can also be arduous, with repeated

sampling if sites looking for an organism you hope is not there. Utilization of eDNA techniques can circumvent some of the drawbacks of traditional sampling methods.

The general approach of collecting water to use for eDNA detection is widespread, however the specific methods used between studies can vary greatly. This includes differences in filtration method, filter material, filter pore size, volume of water filtered, DNA isolation method, and more. Some common variations include using filters with larger pores and filtering a larger volume of water for more turbid samples as filters with smaller pores can clog and fail to filter the entire sample volume. All these small differences in methods can influence the amount of eDNA recovered and the final results of experiments. In this study we test two different filtering methods with two filter pore sizes. In addition to assessing methodological variation, we also examined the influence of a fish hatchery rearing Coho salmon on levels of eDNA in the adjacent stream and assessed if New Zealand mud snails (*Potamopyrgus antipodarum*) are present in the Scott Creek watershed. We examined how detection methods that use eDNA influenced by other factors in the system, specifically an upstream fish hatchery and filtering method.

Question for this project: 1) Does the upstream hatchery impact eDNA levels at the downstream NOAA sampling fish weir and trap? 2) Does the Coho eDNA signal degrade over the 5km distance between the hatchery and weir? 3) Do different field sampling and water filtration methods capture similar concentrations of eDNA for Coho salmon, steelhead/rainbow trout (*Oncorhynchus mykiss*), and New Zealand mud snails? 4) Are New Zealand mud snails present in the watershed?

MATERIALS AND METHODS

SAMPLE COLLECTION

Water samples were collected on June 18th, 2019 from 8 sites in Scott Creek (Figure 1) starting with the most downstream site near the fish weir and trap managed by NOAA (site 1) and ending at the site adjacent to the fish hatchery (site 8) (Table). At each site three 1L samples and three 4L samples were collected and brought back to the MBARI lab in Moss

Landing, CA for filtering. The 1L samples were filtered on 0.45µm filters, and the 3L samples were filtered using 1.2µm filters. All filters were 47mm Nitrocellulose MF-Millipore™ MCE Membrane Filters (Millipore, Temecula, CA, USA). Additionally, at each site, three 3L samples were filtered using the Smith-Root eDNA backpack filtration system. For each set of samples, a control was filtered using distilled or milliq water. Water samples were stored on ice in coolers until they were filtered at MBARI and filters from the Smith-Root backpack sampler were stored on dry ice in the field. After sample filtration all filters were stored at -80°C.

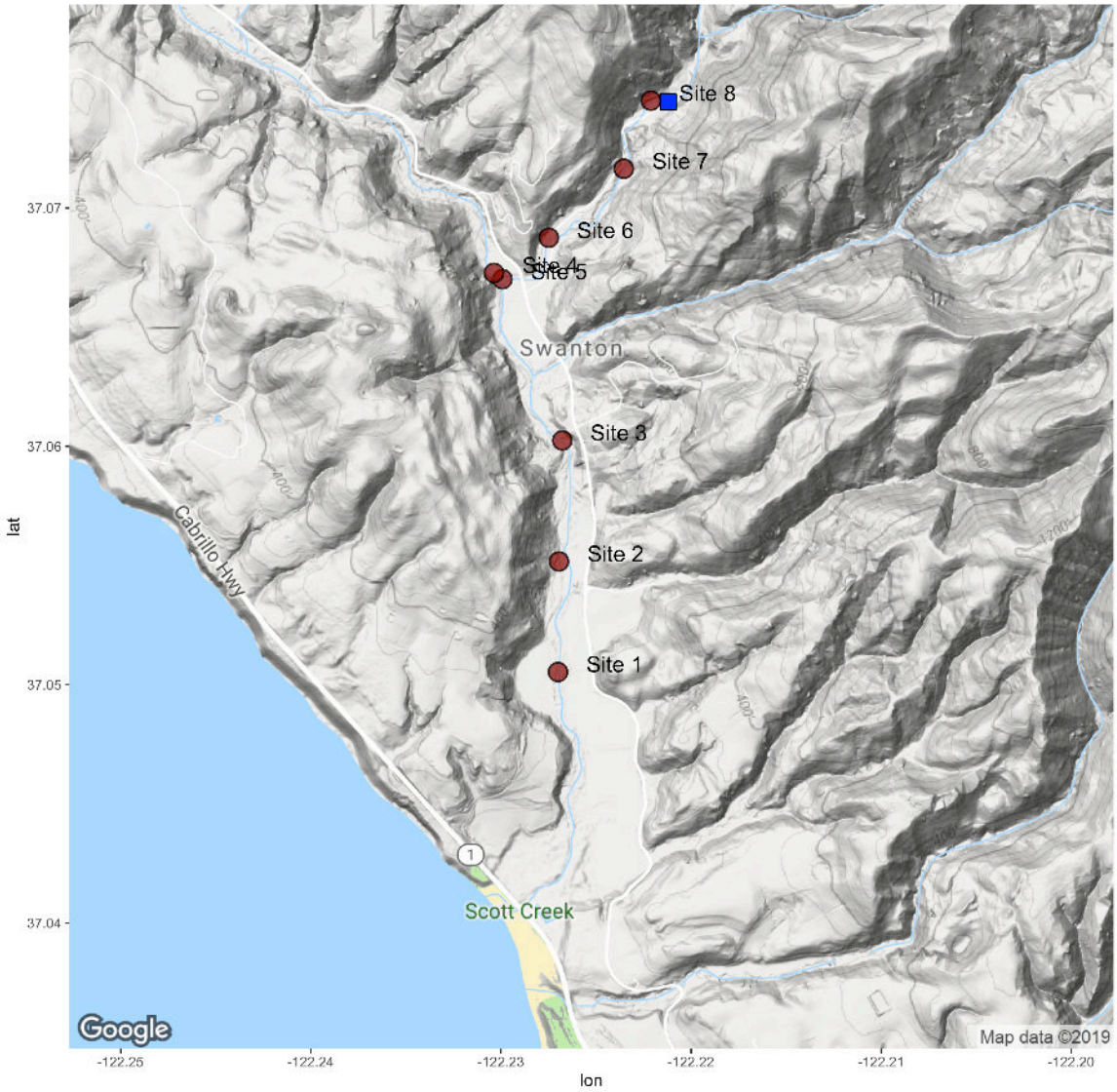


Figure 1 – Map of study the study area in Scott Creek. The red circles indicate sites where water samples were collected, and the blue square indicates the location of the Monterey Bay Salmon and Trout Project Hatchery that rears coho salmon. Sites 1-4 are on the main stem of Scott Creek and sites 5-8 are on Big Creek, a tributary of Scott Creek.

DNA ISOLATION

Filters were manually broken up with forceps or a scalpel blade in the extraction tube. 0.25g of 0.5mm glass beads, 0.25g of 0.1mm glass beads and 1 5mm glass bead were added to the extraction tube, and filters were subjected to two rounds of bead beating for 45 seconds followed by a 30 minute incubation at 56°C. Extractions were completed with the Qiagen DNeasy Blood and Tissue Kit, with a total extraction volume of 800µl. DNA concentrations were measured on a NanodropOne.

QUANTIFYING eDNA

To assess abundance of each organism at our sampling sites DNA was amplified using qPCR with species specific primers and probes (Table 1) in an optimized reaction master mix (Table 2). qPCR reactions were run in a ____ thermal cycler using StepOnePlus software to collect the amplification data. Thresholds to evaluate C_T values were set to 0.02 for *O. kisutch* and *O. mykiss*. Due to problems with optimization for the *P. antipodarum* assay these reactions were not quantified and the threshold was set to 0.16.

Table 1 – PCR reaction mixtures, optimized for each species.

Species (gene)	**Oligo**	**Sequence**	**T_{anneal}** (°C)	**Amplicon Length** (bp)	**Reference**
O. mykiss (NADH)	Forward	5'-AGTCTCTCCC TGTATATCGTC-3'	60	102	Wilcox 2015 PLOS ONE
	Reverse	5'-GATTTAGTTCATG AAGTTGCGTGAGTA-3'			
	Probe	6FAM-5'-CCAACAACTCT TTAACCATC-3' -MGBNFQ			
M. saxatilis (COI)	Forward	5'-TCCCCGAATGA ACAACATAAGTT-3'	60	63	Brandl 2015 Molecular Ecology Resources
	Reverse	5'-GAAGCTAGAAGG AGGAGGAAGGA -3'			
	Probe	6FAM-5'-TTGACTGC TTCCCCC-3'-MGBNFQ			
P. antipodarum (CYTB)	Forward	5'-TGTTTCAAGTG TGCTGGTTTAYA-3'	60	79	Goldberg 2013 Freshwater Science
	Reverse	5'-CAAATGGRGCT AGTTGATTCTTT-3'			

	Probe	6FAM-5'-CCTCGACCAAT ATGTAAAT3' -MGBNFQ

Table 2 – PCR reaction mixtures, optimized for each species.

Reagent	***O. kisutch* (µl)**	***O. mykiss* (µl)**	***P. antipodarum* (µl)**
Env. MMX (2x)	10.00	10.00	10.00
Forward Primer (100 uM)	0.08	0.06	0.08
Reverse Primer (100 uM)	0.08	0.12	0.08
Probe (100 uM)	0.05	0.05	0.05
Template	2.00	2.00	2.00
H20	7.79	7.77	7.79
Total Reaction Volume	20.00	20.00	20.00

ANALYSIS

All analyses were conducted in R. The concentration of target eDNA was calculated from the qPCR reaction concentration using Equation 1. ANOVA tests for differences in eDNA concentrations between species, sampling site, and sampling method were used for *O. kisutch* and *O. mykiss* data. A Kruskal-Wallis Rank Sum Test was used to assess differences in the *P. antipodarum* data due to assessing the data as presence/absence rather than a quantity.

(1) $[eDNA] = [eDNA_q](V_e)(V_l/V_t)(1/V_f)(d)$

Equation 1 – Where [eDNA] is the concentration of target eDNA per mL of filtered water sample; $[eDNA_q]$ is the concentration of eDNA in the qPCR reaction; V_e is the elution volume; V_l is the lysis volume; V_t is the transfer volume; V_f is the sample volume; and d is the dilution factor for the DNA sample prior to qPCR.

We used equation 2 to assess the decay of *O. kisutch* eDNA with distance from the hatchery source. To the determine the rate of decay we fit a linear model to these values.

(2) $attenuation = \ln\left(\frac{C_f}{C_i}\right)$

Equation 2 – Where C_f is the concentration of eDNA at the downstream site and C_i is the concentration of eDNA at the source (Site 8).

We checked the residuals of the data for normality visually and using the Shapiro-Wilk Normality Test. Due to our data having a non-normal distribution of residuals, we analyzed our data using non-parametric tests including the Kruskal-Wallis Rank Sum Test and Wilcoxon Rank Sum and Signed Rank Test.

RESULTS

We detected eDNA of *O. mykiss* at all study sites for all three sampling methods tested. For all three sampling methods, *O. kisutch* eDNA was detected at 7 of 8 sites, with no signal detected at site 4, the Scott Creek site just upstream of the confluence with Big Creek. eDNA of *P. antipodarum* was not detected at any site for any of the sampling methods. There was no detection of any amplification for the negative control samples. The mean copy number per mL across all samples ranged from 0.0 to 370.2 for *O. kisutch* (Figure 2), from 48.0 to 343.8 for *O. mykiss* (Figure 3), and from 0.0 to 0.0 for *P. antipodarum* (Figure 4).

The highest levels of *O. kisutch* eDNA were at the site adjacent to the hatchery (Site 8). There was an exponential decrease in the level of *O. kisutch* eDNA with distance from the hatchery (Figure 5). This decay occurred at a rate of 0.0008482/meter (0.08482%/meter?) according to Equation 2. No *O. kisutch* eDNA was detected at the site on Scott Creek just upstream of the confluence of Scott Creek and Big Creek (Site 4).

VARIATION FROM FILTER SIZE AND FILTERING METHOD

When comparing the DNA concentrations for all salmonid data a significant difference was detected between samples filtered with the smaller 0.45μm pore filter compared to the larger 1.2μm pore filters (Kruskal-Wallis chi-squared = 10.762, df = 2, p-value = 0.004602). When the data was separated by species, this difference was only detected for the *O. mykiss* samples (Kruskal-Wallis chi-squared = 27.077, df = 2, p-value = 1.319e-06). No difference in eDNA concentration was detected between the two filtering methods (Figure ?).

We found that *O. kisutch* eDNA could still be detected several kilometers downstream from the hatchery source.

Table 3 – Average detection levels for each sampling method in copies per mL of filtered water.

Species	Sample Method	Grand Mean	Min	Max
O. mykiss	0.45um vacuum	142.96	87.98	342.48
	1.2um vacuum	115.63	47.99	343.81
	1.2um backpack	97.33	48.86	262.31
O. kisutch	0.45um vacuum	88.65	0.00	370.16
	1.2um vacuum	52.11	0.00	192.23
	1.2um backpack	57.18	0.00	235.58

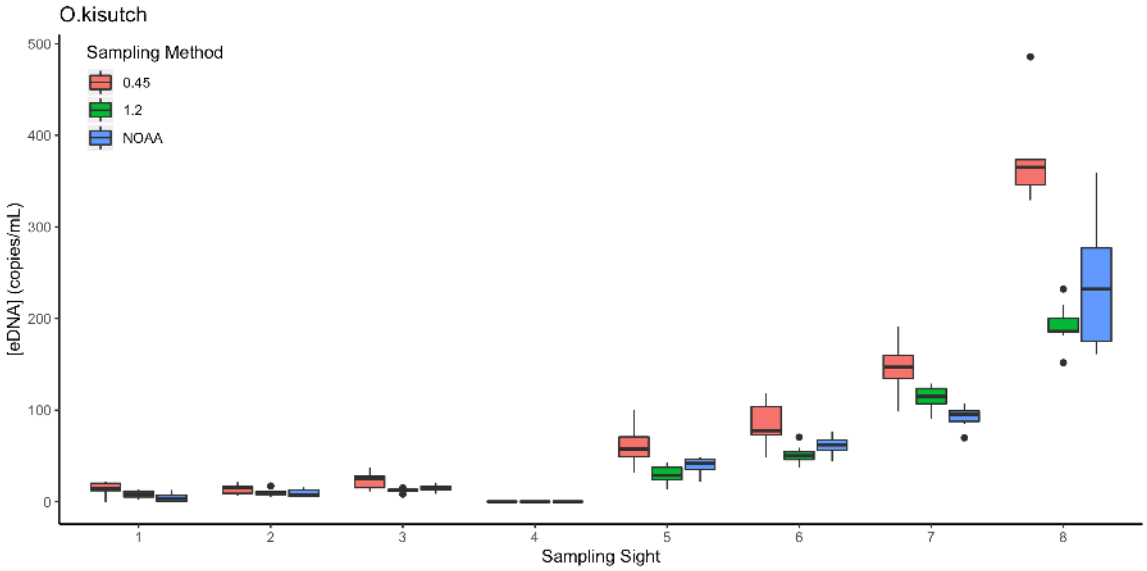


Figure 2 – Level of *O. kisutch* eDNA detected by site and filtering method. The median is shown as the bold band in the middle of the box, with the box representing the 2nd and 3rd quartiles of the data. Whiskers extend to the most extreme data values, but don't exceed 1.5 times the inter-quantile range.

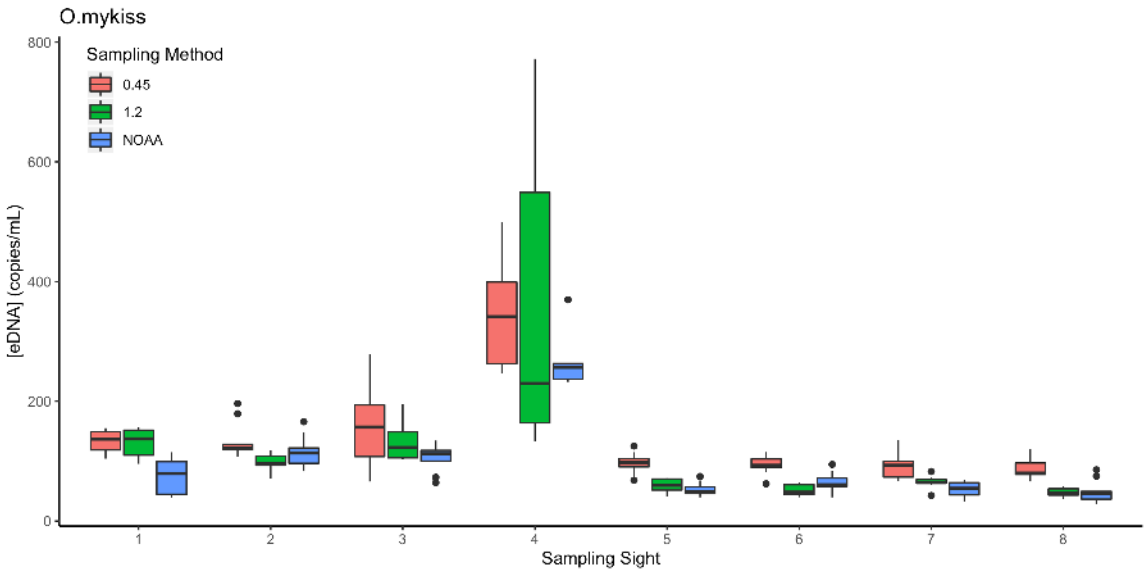


Figure 3 – Levels of *O. mykiss* eDNA detected by site and filtering method. The median is shown as the bold band in the middle of the box, with the box representing the 2nd and 3rd quartiles of the data. Whiskers extend to the most extreme data values, but don't exceed 1.5 times the inter-quantile range.

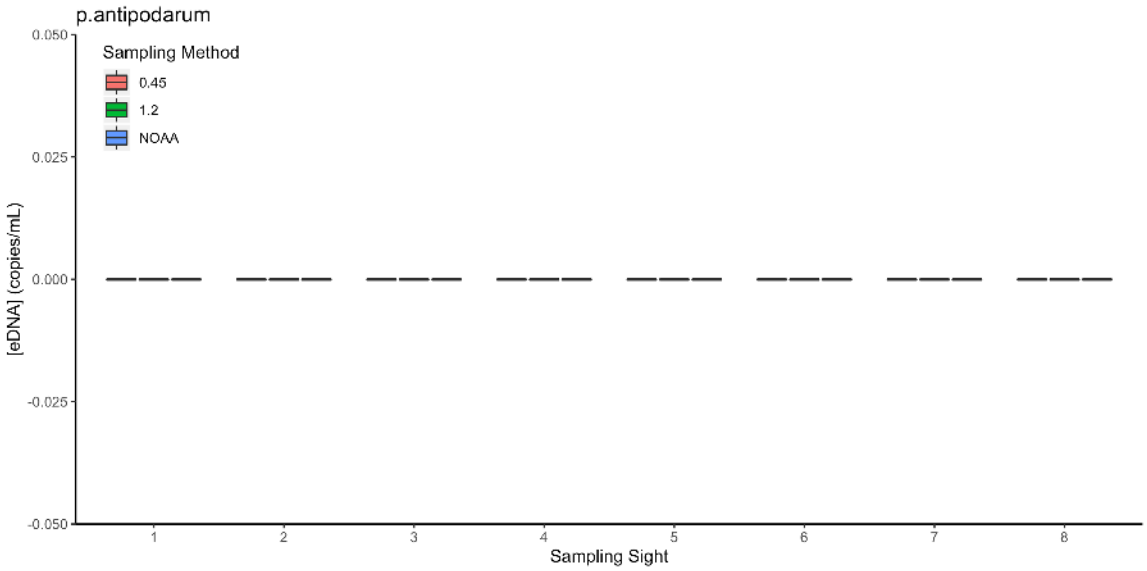


Figure 4 – Levels of *P. antipodarum* eDNA detected by site and filtering method. The median is shown as the bold band in the middle of the box, with the box representing the 2nd and 3rd quartiles of the data. Whiskers extend to the most extreme data values, but don't exceed 1.5 times the inter-quantile range.

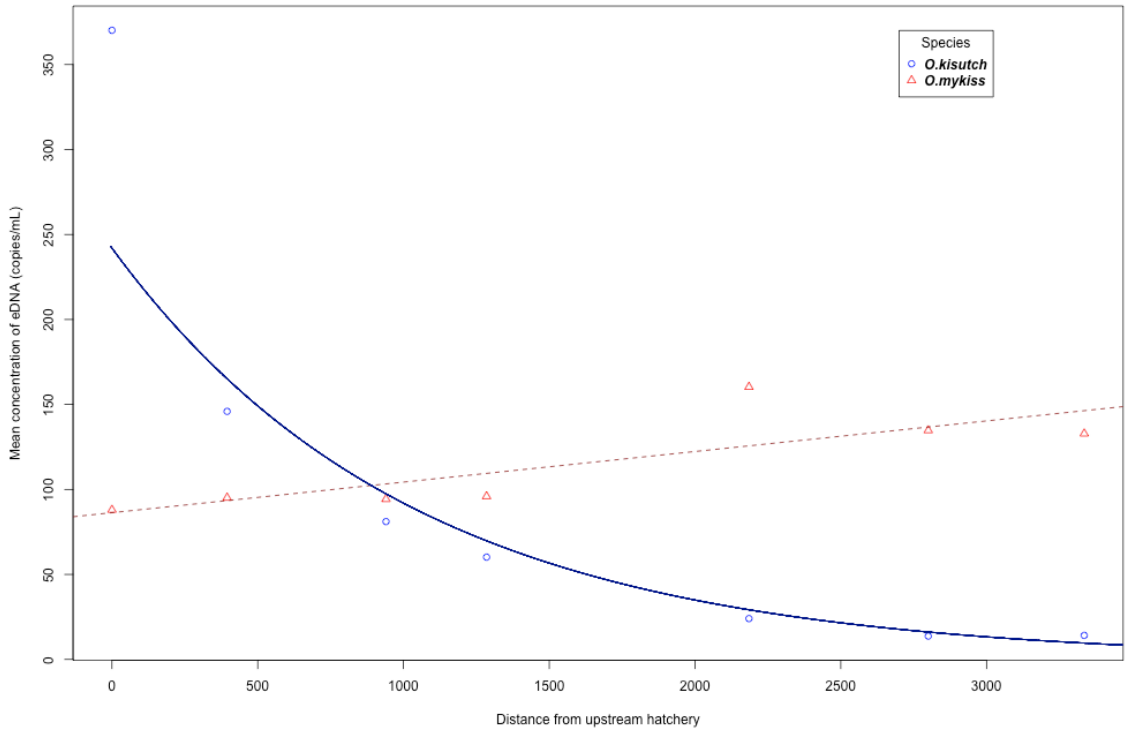


Figure 5 – Change in eDNA level with increasing distance downstream from the hatchery rearing Coho salmon.

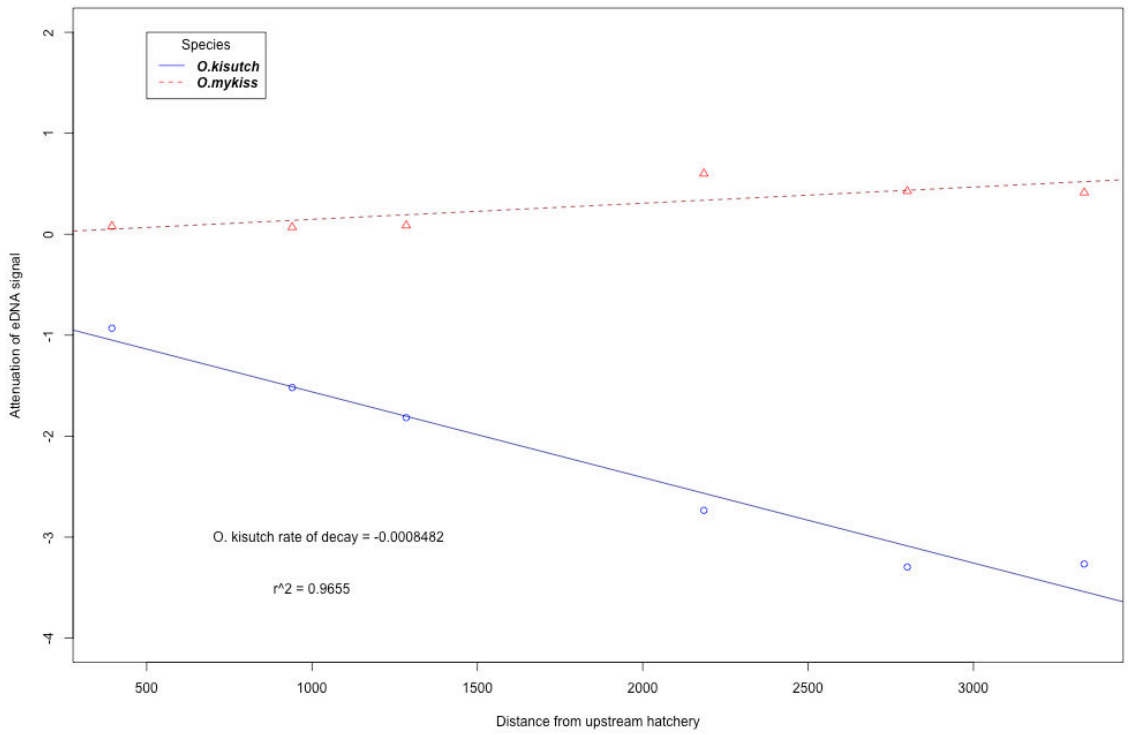


Figure 6 – Rate of decay of eDNA with increasing distance from the upstream hatchery rearing Coho salmon.

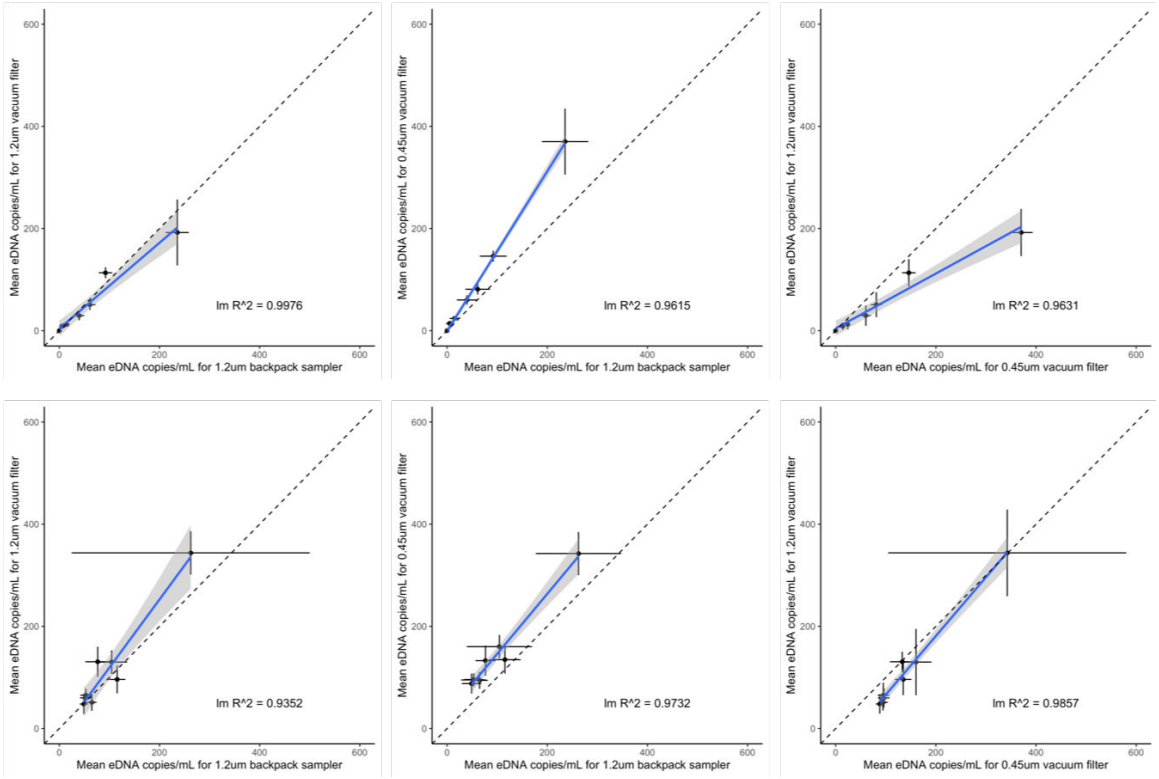
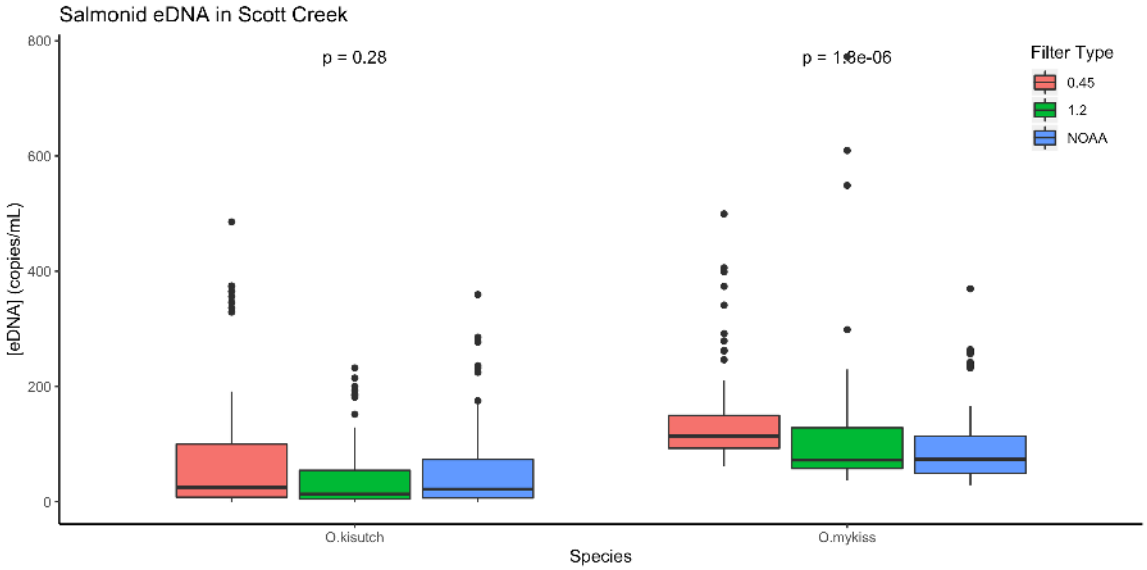


Figure 7 – Linear modes of pairwise comparison between sampling methods of measured eDNA levels for *O. kisutch* on the top row and *O. mykiss* on the bottom row. Error bars indicate standard deviation. The dashed line is the 1:1 line.



DISCUSSION

We found that a smaller pore sized filter recovers larger quantities of eDNA for *O. mykiss* we did not find the same pattern for *O. kisutch*. This could be due to several factors including the large relative difference in eDNA levels between sites for *O. kisutch,* relatively lower levels of eDNA for *O. kisutch* comparted to *O. mykiss*, differences in the efficacy of qPCR assays for different targets.

CONCLUSIONS/RECOMMENDATIONS

When designing studies that will utilize eDNA, scientists should carefully select the filtration method and filter pore size that will be used, and keep these sampling methods consistent across all samples. This may be of particular importance for long term studies where sampling materials have the possibility of changing over time.

Studies in freshwater systems should consider potential inputs of eDNA from external sources such as fish hatcheries. Furthermore, researchers should consider that any decay of

eDNA in the system of interest may vary over time due to other factors such as temperature and the amount of sunlight reaching the stream.

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