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Modernizing fish surveys: evaluation how reliable environmental DNA sampling is in monitoring fish populations

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Modernizing Fish Surveys: Evaluating how Reliable Environmental DNA Sampling is in Monitoring Fish Populations

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Chapter 1

Comparing eDNA Results to Electrofishing in Lotic Systems

Introduction

Fisheries managers and researchers regularly monitor freshwater systems to assess species richness and abundance and to gather biological data using traditional methods such as electrofishing, netting (seining, gillnets, etc.), and trapping (weirs, minnow traps, etc.) (Murphy and Willis 1996). To increase the odds of detecting a species that is in low abundance, researchers typically have to increase sampling efforts (McDonald 2004), but this is not always successful. With the discovery of environmental DNA (eDNA) in the 1980s and its subsequent application on microbial communities (1980-1990s) and then macroorganisms (2000s), alternative methods have opened up for monitoring aquatic species (Taberlet et al. 2018).

eDNA is a mixture of exogenous DNA that is shed from organisms via their skin cells, mucus, feces, and gametes into their environment (Lacoursière-Roussel and Deiner 2021) and, therefore, represents all of the species present in a system. Only a water sample is needed to collect eDNA from an aquatic system. The collection is done without handling the species of interest or negatively affecting the environment (Goldberg and Strickler 2017; Kasai et al. 2020). The minimal sampling equipment and effort make eDNA analysis cost-effective (Evans et al. 2017b) and extremely flexible, allowing for a sampling setup to be moved between waterbodies with ease (Olds et al. 2016).

eDNA analyses initially targeted individual species (barcoding) using species-specific PCR primers and a polymerase chain reaction (PCR) or quantitative polymerase chain reaction (qPCR). This has been used to target specific species' DNA and answer questions involving presence/absence, distributions, and more recently, abundance (Thomsen et al. 2012; Takahara et al. 2013; Penaluna et al. 2021). With the advancement of next-generation sequencing methods,

eDNA has expanded from identifying singular species to identifying whole communities (metabarcoding) with a single sample collection (Taberlet et al. 2018).

Metabarcoding targets mitochondrial DNA regions that exhibit high interspecific variability but low intraspecific variability (Cristescu 2014) in order to target multiple species in a community. There are no standardized PCR primers for metabarcoding fish assemblages, but recent studies have identified PCR primers that target regions of mitochondrial DNA for various species groups which show promising results (Evans et al. 2016; Zhang et al. 2020). In addition, numerous studies have shown the value of metabarcoding to assess fish species richness in aquatic communities (Hänfling et al. 2016; Evans et al. 2017a; Deiner et al. 2017; Li et al. 2018).

When compared to traditional methods, eDNA detection has been shown to be more sensitive at detecting hard to identify species and low-abundance species (Evans et al. 2016; Olds et al. 2016). What is considered a "detection" when working with eDNA can vary, however, where traditional methods are more straightforward. Depending on the study design, a single sample and PCR primer detection can be considered a positive read; whereas, other studies may require a higher stringency which can alter the results (Olds et al. 2016; Evans et al. 2017a). Assessing different stringencies and identifying protocols to standardize positive eDNA detections in varying environments is needed.

The characteristics of the eDNA molecule itself need to be considered to infer results as well. In caged fish studies, eDNA was shown to act like fine particulate matter and produce a plume of eDNA downstream of an organism (Laporte et al. 2020). The plume showed low lateral dispersion immediately downstream of an organism with it slowly spreading in the stream as it moved downstream, typically collecting more on one bank than another (Laporte et al. 2020;

Thalinger et al. 2021a). Assessing where specific species are detected across the stream channel using multiple water samples can be critical to the overall eDNA results.

While suspended in the water column, eDNA is exposed to environmental factors that can affect its persistence and, in turn, species detection. Degradation of eDNA is driven by time, pH (Strickler et al. 2015; Seymour et al. 2018), UV-B exposure (Strickler et al. 2015), and temperature (Strickler et al. 2015; Kasai et al. 2020). Lab experiments indicate that the persistence of eDNA in the water column varies by species, but typically eDNA persists for less than a month making it indicative of current species in the waterbody (Dejean et al. 2011; Pilliod et al. 2014). Extreme environmental factors can speed up the degradation process and decrease the persistence time of eDNA in the water which can shorten the window of detection.

In lotic waters, higher discharge rates can transport eDNA further downstream which determines where eDNA is detected versus where the fish are in the environment (Deiner and Altermatt 2014; Li et al. 2018). Transport distances can vary widely depending on discharge, target species, and degradation of the eDNA, but initial studies indicate that eDNA can travel anywhere from 5 meters to several kilometers downstream of the source (Pilliod et al. 2014; Deiner and Altermatt 2014). Therefore, transport of eDNA can dramatically affect the results of species detections in lotic waters and should be investigated further.

Most studies that have looked at environmental factors and the persistence of eDNA have been based in a lab and/or have targeted a singular species. With singular species detection using a highly specific PCR primer, the results may produce a higher rate of detection than what metabarcoding in a natural environment may detect (Shaw et al. 2016). Further research is needed to understand how environmental factors influence eDNA detection with metabarcoding

and how to develop sampling plans for specific waterbodies with this information in mind (Seymour et al. 2018).

Studying eDNA from streams which may experience large shifts in temperature, pH, and discharge due to natural seasonal changes and agricultural operations are necessary to assess reliability of eDNA metabarcoding. By comparing electrofishing data to that of eDNA metabarcoding in multiple streams with varying environmental characteristics, and while assessing PCR primer specificity, we can further the understanding of eDNA metabarcoding results. This will help to reproduce sampling methods in the future allowing for eDNA metabarcoding to be applied more widely.

The objectives in this study were threefold: 1) Compare the detection probability of various fish species using eDNA metabarcoding and electrofishing, 2) Assess the variation of eDNA detection sensitivity and the transport distance of eDNA with varying seasonal environmental factors including pH, temperature, and discharge rate, and 3) Evaluate how the number of PCR primers used and the number of detections required to confirm a positive read varies the results for eDNA detections. I hypothesized that eDNA would detect the same number of species or more species when compared to electrofishing data with some seasonal variation where eDNA detections would be lower in summer compared to fall. Using multiple PCR primers will increase the number of species detected overall but requiring a species to be detected in multiple samples or by multiple PCR primers to be considered a positive detection will decrease the number of species detected.

Overview of Study

Sampling was conducted twice per stream: once in summer (June and August) and once in fall (October) after the streams had been allowed to cool with fall weather. During both sampling periods, each stream was electrofished and three eDNA samples were collected and

filtered on the same day. All animal handling protocols were approved by EWU's Institutional Animal Care and Use Committee and collecting permits were obtained from both the Washington Department of Fish and Wildlife (permit # 20-051) Idaho Department of Fish and Game (permit # F-04-03-21) before field sampling.

Methods

Study Sites

Sampling was conducted in four streams with two located in Spokane County, one in Whitman County and one in Bonner County: Latah Creek, California Creek, Rock Creek, and North Fork Grouse Creek. Three of the streams were selected for sampling due to their history of high temperatures (Latah and California Creek) or their location in exposed scablands where high temperatures may be encountered (Rock Creek). The fourth stream (North Fork Grouse) was chosen due to its location within forested land and its low likelihood of experiencing extreme shifts in temperature between seasons.

Latah Creek is a large stream that begins in Benewah County, Idaho, and flows northwest for 69.5 miles until it drains into the Spokane River in Spokane County (Scholz et al. 2014). Latah Creek drainage is mostly comprised of agricultural land with 58% of the total 431,000 acres being agricultural land (Lee 2005). The development of the land has resulted in changes to the watershed that include increased nutrients and bacteria, changing pH levels, lowered dissolved oxygen, erosion, reduced shade, and increased temperatures (Joy 2008; N.A. 2019). Latah Creek exhibits high water temperatures in the summer which regularly exceed Washington's water quality standard of 18° Celsius (C) (Schultz 2019) and is the site for many restoration efforts (Marten and Thorson 2018).

A small tributary of Latah Creek, California Creek originates near Mica Peak southeast of Spokane and travels northwest until it meets Latah Creek. California Creek's watershed consists of approximately 55% agricultural, 23% forested, 19% shrub/steppe, and 2% developed land (Joy et al. 2009). Although California Creek has a relatively healthy population of Redband Trout *(Oncorhynchus mykiss)*, cool groundwater inputs, and some unspoiled forested habitat, it still displays increased temperatures during the summer months (Schultz 2019). Spokane Riverkeeper indicated that California Creek had seven-day average temperatures of 19.67° C in 2019, which is higher than the Washington water quality standard (Schultz 2019).

Rock Creek begins in Turnbull National Wildlife Refuge (TNWR) at Pine Lakes and flows southwest for 52 miles through the Palouse's channeled scablands, including the Bureau of Land Management's (BLM) Escure Ranch, until it empties into the Palouse River (Cook and Gilmore 2004). Along its length, it gains water through springs and tributaries. Historical data on water temperatures in this area could not be located; however, within Escure Ranch, Rock Creek has few shaded areas and is mostly exposed to the elements which increases the likelihood of extreme shifts in temperature during the summer months.

North Fork Grouse Creek is a small stream located in Bonner County, Idaho. North Fork Grouse Creek originates on Grouse Mountain within Kaniksu National Forest where it resides for most of its length until it empties into Grouse Creek near Sandpoint, Idaho. North Fork Grouse Creek is a popular fishing destination for the native cold-water species, Westslope Cutthroat Trout (*Oncorhynchus clarkii lewisi*) ("North Fork Grouse Creek Fishing near Sandpoint, Idaho | HookandBullet.com" n.d.).

eDNA Water Collection

eDNA samples were collected immediately before starting each electrofishing sampling and prior to block nets being installed (Penaluna et al. 2021). Three samples of 250 mL of water

were collected from the downstream end of each electrofishing sampling reach (Olds et al. 2016; Evans et al. 2017a; Li et al. 2018) for each season, with a total of 6 samples being collected from each creek. During each sampling event, one water sample was collected near the right and left banks, and one in the center of the stream to increase the likelihood of detecting all species in the community. Prior to collecting the sample, a sterile 250 mL Nalgene bottle was rinsed 3 additional times in the stream water to ensure no bleach residue remained from decontamination procedures, and the remnants were poured onshore where it would not run back into the stream (Goldberg and Strickler 2017). Each water sample was then collected just under the surface of the water (Evans et al. 2017a; Thalinger et al. 2021a) while I stood at least three feet downstream in moving water to minimize contamination. In addition to the three samples collected from each stream, one Nalgene bottle filled with 250 mL of distilled (DI) water acted as a negative control (Olds et al. 2016; Li et al. 2018). The bottles were then closed, wiped down with a 10% bleach solution (Deiner and Altermatt 2014; Olds et al. 2016; Evans et al. 2017a; Li et al. 2018) and placed in individual Ziploc bags within a cooler (Goldberg et al. 2016) for transport back to the EWU fisheries lab.

Electrofishing

At each creek, one sampling transect of 100 meters was selected for electrofishing based on access to the water. This same 100-m section was sampled twice at each creek: once during summer (June-August) and again in the fall (October) of 2021. Prior to the start of electrofishing, both the downstream and the upstream end of the transect was blocked with stop nets to prevent fish from escaping the area (Civade et al. 2016). Electrofishing was started from the downstream end and proceeded to the upstream end. One person operated the backpack electrofishing unit (Smith Root model LR-20B) and at least one person netted fish (Reid and Haxton 2017). After each 10-meter section of electrofishing, fish were identified to species or the lowest taxonomic

grouping as possible, quantified, and then placed in a mesh container that was placed in the stream. Multiple mesh containers were placed at intervals along the sampling transect to prevent crowding in each container, to allow convenient access once fish were identified, and to be out of the electric field o the electrofishing unit when the next section was started. Once the sampling of the 100 meters transect was completed, fish were released from the mesh containers and the block nets removed.

Environmental Sampling

After collecting water samples and prior to the start of electrofishing, coordinates were recorded at the downstream end of the sampling transect using a handheld global positioning system (GPS) unit (Garmin). Water temperature, pH, wetted width, depth, and velocity were then measured. Temperature and pH were measured using a multi-parameter instrument (YSI model 556) while standing in the middle of the stream. Ten depths and flow velocity measurements were collected evenly across each stream to calculate discharge (Latah visit 1 included only 3 measurements while Latah visit 2 included 9 measurements; Marsh-McBirney Flo-Mate model 2000).

eDNA Filtering

Water samples were filtered on the same day as field collection (Evans et al. 2017a), typically within 7 hours of sample collection. All water samples were filtered in a laminar flow hood located in a room separate from the molecular preparation laboratory to reduce the risk of contamination. Each sample was vacuum filtered onto a one-time use 47 mm, 1.2 μm pore size cellulose membrane filter (EMD Millipore) (Lacoursière-Roussel et al. 2016; Deiner et al. 2018), using a pre sterilized reusable filter setup (Millipore), and the house-plumbed vacuum system. Filters were removed from the filter funnel using sterilized forceps, loosely folded and placed in individual 2 mL vials, 75% filled with molecular-grade ethanol. Preserved filters were

then stored at -20°C until DNA extraction (Laramie et al. 2015; Goldberg and Strickler 2017; Duda et al. 2021; Penaluna et al. 2021).

DNA Extraction

DNA was extracted from whole filters, including field controls, by following a modified animal tissues protocol using the DNeasy Blood and Tissue Kit (Qiagen) (Mächler et al. 2019; Bessey et al. 2020; Penaluna et al. 2021). Lysis was extended to 48 hours at 55°C , and the final DNA was eluted to 100 uL using the elution buffer in the kit (Mansfeldt et al. 2019; Penaluna et al. 2021). Field controls were extracted separately from stream samples to isolate any field contamination from lab contamination (Li et al. 2018). With all extraction batches, a negative extraction control was included of just extraction reagents to isolate any potential contamination during extraction. With all stream samples, a positive control containing 60 ng of non-local fish DNA (15 ng each of Pacific Cod (*Gadus macrocephalus),* Nile Tilapia *(Oreochromis niloticus),* Pacific Halibut *(Hippoglossus stenolepis),* and Atlantic Bluefin Tuna (*Thunnus thynnus)*) was also included (Evans et al. 2016; Olds et al. 2016; Li et al. 2018). Each DNA extraction was then cleaned using One Step PCR Inhibitor removal kit (Zymo) in order to remove any potential PCR inhibitors (Evans et al. 2017a; Li et al. 2018; Mächler et al. 2019).

Decontamination Procedures

All equipment that came in direct contact with eDNA samples (bottles, tweezers, filter apparatus, etc.), was sterilized with a 50% bleach solution for at least 1 minute and rinsed with distilled water three times between samples and streams (Goldberg et al. 2016; Penaluna et al. 2021). All other supplies (aside from pipettes) and laboratory surfaces, were cleaned and disinfected with a 10% bleach solution, and rinsed thoroughly (when applicable) and allowed to dry between samples (Deiner et al. 2015a; Olds et al. 2016; Goldberg et al. 2016; Evans et al. 2017a; Goldberg and Strickler 2017; Li et al. 2018). Pipettes were wiped down with DNA Away

(Thermo Scientific) and allowed to dry before starting any molecular work with samples (Evans et al. 2016).

PCR Primer Testing

To test the PCR primers and thermocycler protocols as outlined in Evans et al. (2016) prior to applying them on unknown samples, all three metabarcoding PCR primers were used to amplify known tissue samples from species that made up a positive control: Pacific Cod (*Gadus macrocephalus),* Nile Tilapia *(Oreochromis niloticus),* Pacific Halibut *(Hippoglossus stenolepis),* and Atlantic Bluefin Tuna (*Thunnus thynnus).* Due to higher concentrations of DNA from the extracted tissue samples, the following recipe was used per 20 uL PCR reaction: 10 uL Amplitaq Gold 360 Master Mix, 1 uL Forward PCR Primer, 1 uL Reverse PCR Primer, 6 uL molecular-grade water, and 2 uL DNA. To verify proper amplification, products were run through a 2% agarose gel and visualized using Smart Glow Stain (Accuris Instruments) on a UV platform prior to starting molecular work with eDNA samples.

DNA Amplification, Illumina Library Preparation, and Sequencing

All samples including field and lab controls were PCR amplified using the 2-step Illumina 16S Metagenomic Sequencing Library Preparation and three separate PCR primers that targeted three mitochondrial gene regions (Table 1): Actinopterygii 16S (L1865/H2195, Ac16s), Amphibia 12S (L909/H1155, Am12s), and Cytochrome B (L14735/H15149c, CytB) (Illumina n.d.; Burgener and Hübner 1998; Evans et al. 2016).

Table 1: PCR Primer sets used for amplifying target regions from eDNA.

Primer Name	Target Gene	Forward Primer	Reverse Primer	Amplicon Length (bp)	Annealing Temperatures (AT1, AT2, AT3)
L14735/ H15149c	Cyt B	AAAAACCACCGTTGTTATTCAACTA	GCCCCTCAGAATGATATTTGTCCTCA	413	60°C, 58°C, 55°C
Am12S	12s	AGCCACCGCGGTTATACG	CAAGTCCTTTGGGTTTTAAGC	241	65°C, 62°C, 60°C
Ac16S	16s	CCTTTTGCATCATGATTTAGC	CAGGTGGCTGCTTTTAGGC	330	63°C, 60°C, 58°C

Table 2: Thermocycler conditions for all metabarcoding PCR primers. Annealing temperatures are indicated by AT1, AT2, and AT3.

Thermocycler conditions		
Step	**Temperature (°C)**	**Time**
1	*98*	2 min
2	*98*	10 sec
3	*AT1*	20 sec
4	*72*	30 sec
5	*repeat steps 2-4 nine times, 10 cycles total*	
6	*98*	10 sec
7	*AT2*	20 sec
8	*72*	30 sec
9	*repeat steps 6-8 nine times, 10 cycles total*	
10	*98*	10 sec
11	*AT3*	20 sec
12	*72*	30 sec
13	*repeat steps 10-12 twenty-nine times, 30 cycles total*	
14	*72*	10 min
15	*4*	Hold

The following recipe was used per 30uL reaction per PCR primer in PCR tubes with individually attached lids: 15 uL Amplitaq Gold 360 Master Mix, 1.5 uL Forward PCR Primer, 1.5 uL Reverse PCR Primer, and 12 uL DNA. The thermocycler settings for the first PCR reaction used a cycle of three annealing temperatures for each PCR primer (Table 2). For each batch of samples per PCR primer run, a single PCR negative control was added using DNA-free water in place of DNA.

To verify proper amplification, after the first PCR, products were run through a 2% agarose gel and visualized (Evans et al. 2016, 2017a; Li et al. 2018) using Smart Glow Stain (Accuris Instruments) on a UV platform. After verification, DNA was quantified using a Qubit 4 Flourometer (Invitrogen). The three separate amplicon products per sample were then pooled at differing amounts to ensure even PCR amplification during the second PCR step where barcodes were attached (Li et al. 2018). Due to low concentrations in the samples, the target of pooling Ac16S = 3.75ng; Am12S = 2.5ng; CytB = 18.75 ng was not reached except in one sample (CA Creek). All PCR products were kept in ratio to each other based on these targets while pooling with a final volume of 30 uL pooled PCR product before PCR cleanup. PCR cleanup was modified from the Illumina 16S protocol and was completed using the QIAquick PCR Purification Kit (Qiagen) per manufacturer's instructions for all stream samples. Due to a shortage of the QIAquick spin columns, all control PCR products were cleaned with a similar PCR cleanup product: Omega Biotek EZNA Kit (Omega).

For the final PCR step, unique barcodes were added for every sample collected during each sampling period (summer and fall). The following PCR recipe was used: 25 uL Amplitaq Gold 360 Master Mix, 5 uL each Forward and Reverse Nextera Adapters (Illumina Nextera XT Index Kit), and 15 uL PCR 1 product. All controls that exhibited a positive band on the gel were

sent to be sequenced along with the samples (Nguyen et al. 2015). All samples were sent to the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute for paired-end sequencing on Illumina Miseq using a 600 cycle (v3) kit.

BLAST Alignment Search

All three PCR primer sequences were used in a Primer-BLAST search (NIH) which included all bony fish (*Actinopterygii*) and amphibians (*Amphibia*) with the expected amplicon lengths within a wider range than expected. Ranges included in the search were 250-450 base pairs (Ac16S), 200-350 base pairs (Am12S), and 350-750 base pairs (CytB). The results were then compared to the species that were confirmed via electrofishing in each stream.

Data Analyses

All data analyses were performed in R 4.2.1. All variables were visualized with histograms and had a Shapiro-Wilk test performed to check the distribution. Data were considered normally distributed with the Shapiro-Wilk test ($p > 0.05$). To compare environmental factors between seasons, a paired t-test was used for each variable: temperature, pH, and discharge. In addition, a paired t-test was used to compare total amplifications per PCR primer between seasons. Due to the lack of sequencing data, eDNA results could not be compared to electrofishing results.

Results

Environmental Variables

All environmental variables are summarized in table 3. Discharge and pH did not differ significantly between fall and summer (Discharge: paired t test, $p = 0.3752$; Figure 1; pH: paired t test, $p = 0.06653$; Figure 3). Water temperature was the only environmental variable to vary significantly with the temperature being 8.56 degrees lower, on average, during fall compared to summer (paired t test, $p = 0.0452$; Figure 2).

Electrofishing Sampling

A total of 14 unique fish and amphibian species were identified across all streams with an additional grouping for *Cottidae* species which could not be identified to species in the field (Table 4). The species richness per electrofishing period did not differ significantly between seasons (paired t test, $p = 0.6638$, Figure 4). Abundance of all fish and amphibian species combined per transect did not differ significantly between seasons (paired t test, $p = 0.6773$, Figure 5).

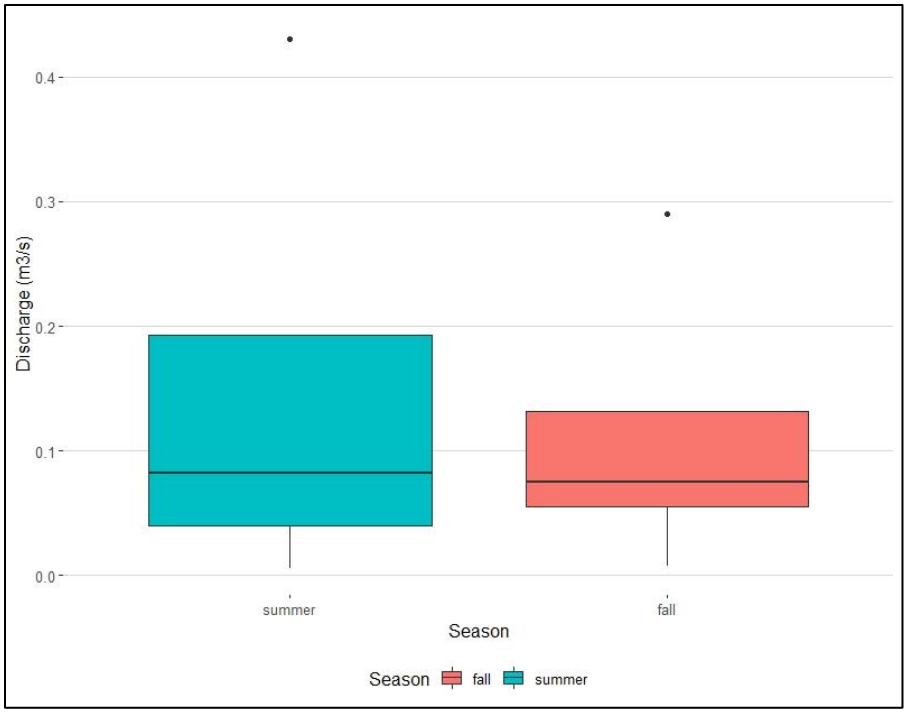

Figure 1: Difference in discharge across all four creeks by season of sampling (summer and fall 2021). Black horizontal bars represent median values and boxes give 25 and 75 percentiles.

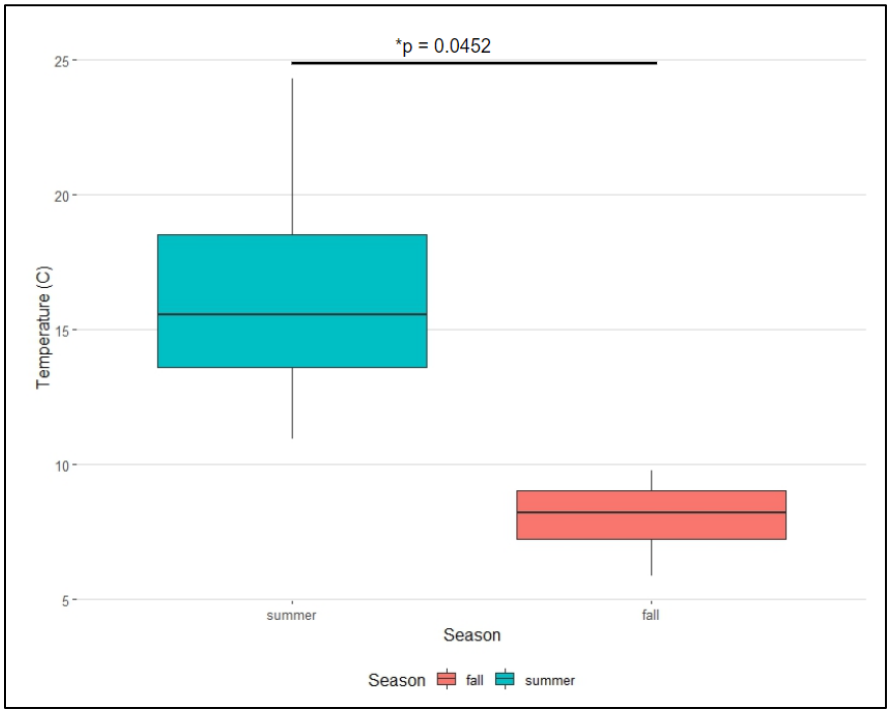

Figure 2: Difference in water temperature across all four creeks by season of sampling (summer and fall 2021). Black horizontal bars represent median values and boxes give 25 and 75 percentiles.

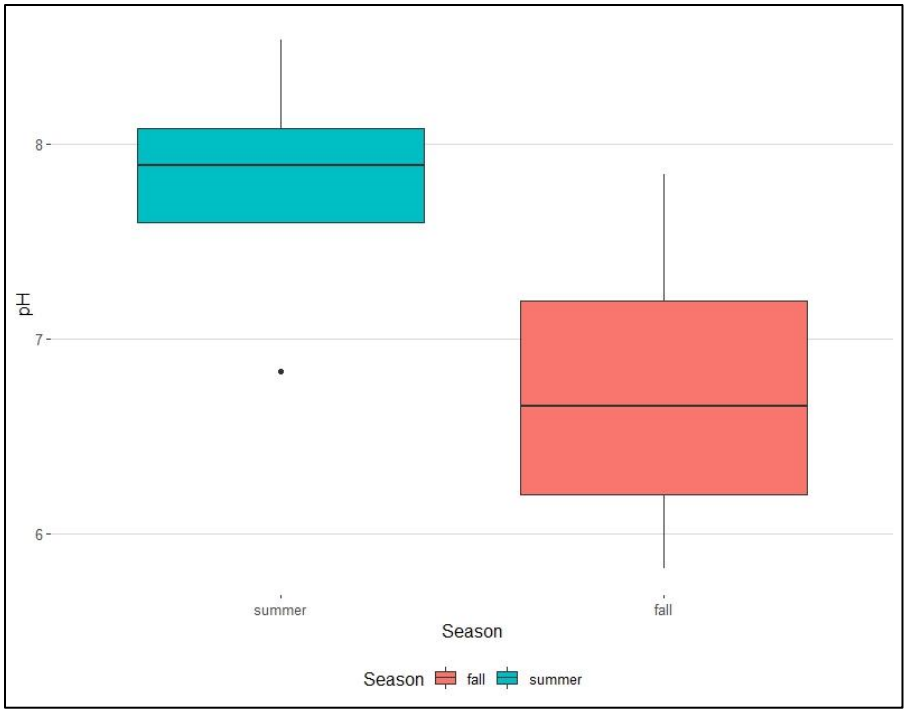

Figure 3: Difference in pH across all four creeks by season of sampling (summer and fall 2021). Black horizontal bars represent median values and boxes give 25 and 75 percentiles.

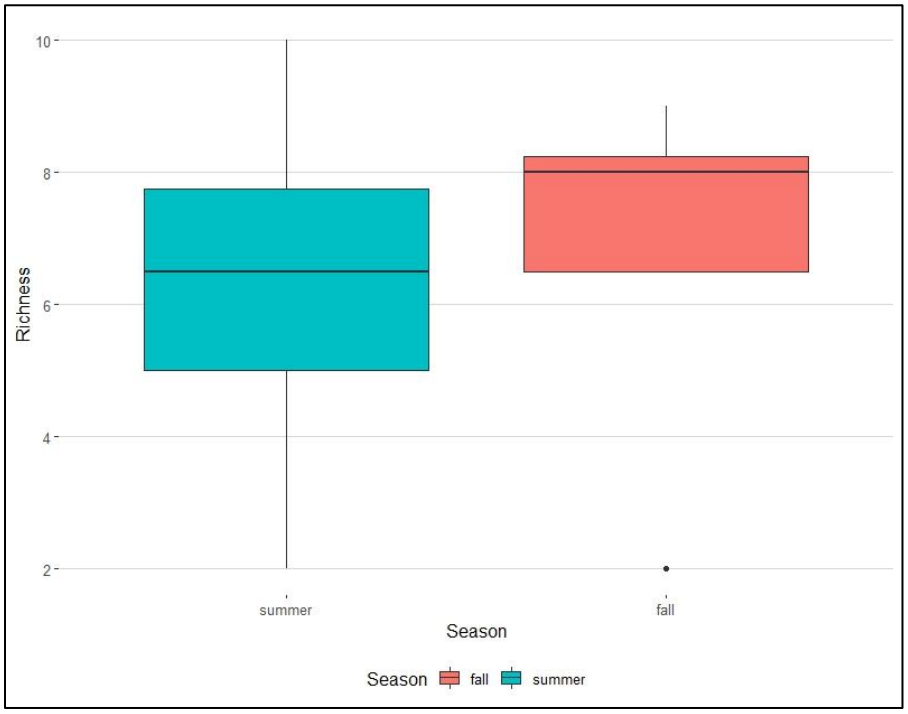

Figure 4: Difference in species richness across all four creeks by season of sampling (summer and fall 2021). Black horizontal bars represent median values and boxes give 25 and 75 percentiles.

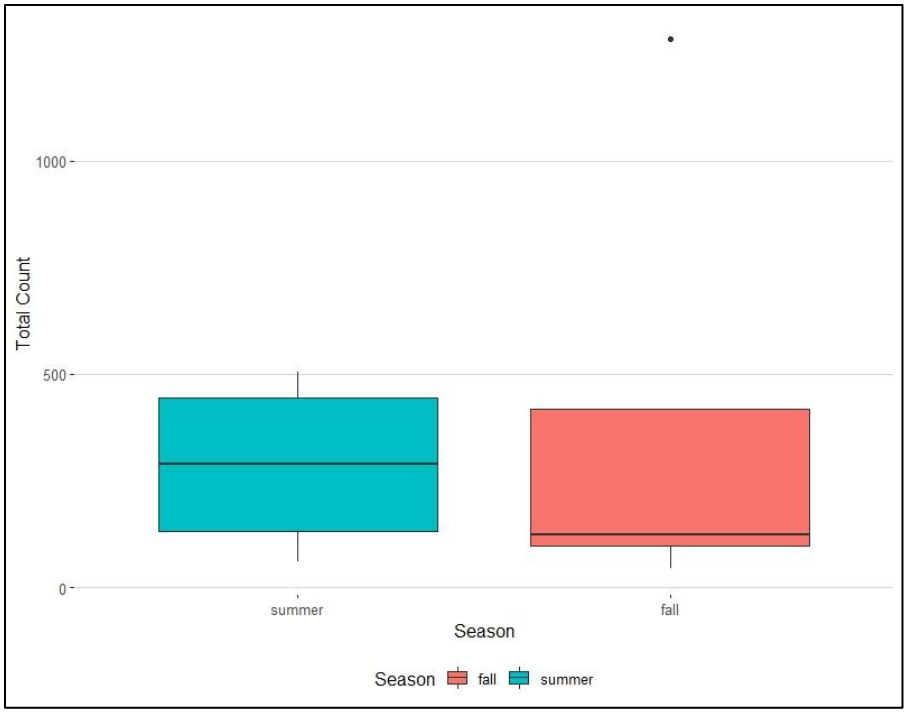

Figure 5: Difference in species total abundance across all four creeks by season of sampling (summer and fall 2021). Black horizontal bars represent median values and boxes give 25 and 75 percentiles.

PCR Primer Testing

All DNA samples were successfully amplified using the PCR primers and thermocycler programs presented in Evans et al. (2016). All four positive control samples were successfully amplified with Ac16s and Am12s with an approximate size of 400 bp and 350 bp, respectively. This size range is within the expected size of the amplicon with the Illumina adapter attached. Three of the four samples were amplified with the CytB PCR primer with Atlantic Bluefin Tuna being the exception. The size of the CytB amplicons were approximately 650 bp which was outside the range of the expected size of ~480 with the Illumina adapter. Due to the consistency of the size of the CytB amplicons, the PCR primer was considered stable and used for the rest of the experiment.

Table 3: Environmental parameters recorded for both visits to all four creeks.

Creek	Season (2021)	Sampling Month (2021)	GPS Coordinates	Discharge (m3/s)	Wetted Width (m)	Temperature (C)	pH
California Creek	Summer	August	47 31.011 N, 117 19.609 W	0.005586	3.5	14.5	7.93
California Creek	Fall	October	47 31.011 N, 117 19.609 W	0.007339	3.58	5.86	6.33
Latah Creek	Summer	June	47 23.801 N, 117 15.972 W	0.114004	3.23	24.3	8.53
Latah Creek	Fall	October	47 23.801 N, 117 15.972 W	0.071681	4.02	8.76	6.98
North Fork Grouse Creek	Summer	August	48 29.972 N, 116 20.569 W	0.050985	4.42	10.93	6.83
North Fork Grouse Creek	Fall	October	48 29.972 N, 116 20.569 W	0.078449	4.85	7.68	5.82
Rock Creek	Summer	August	47 00.850 N, 117 56.678 W	0.430114	6.42	16.6	7.85
Rock Creek	Fall	October	47 00.850 N, 117 56.678 W	0.290125	5.85	9.79	7.84

Table 4: Total abundance by species per visit to each creek based on electrofishing. Counts are for total abundance per 100 meters transect. Visit 1 is summer 2021 and visit 2 is fall 2021.

Creek	Visit	Bridgelip Sucker	Brook Stickleback	Brook Trout	Brown Trout	American Bullfrog	Chiselmouth	Cottidae spp.	Fathead Minnow	Frog Unidentified	Largescale Sucker	Longnose Dace	Northern Pikeminnow	Rainbow Trout	Redside Shiner	Speckled Dace	Unknown spp.	Westslope Cutthroat Trout
California	1	11						31					4	56	46	7		
California	2	15		1			1	11					5	52	19	10	2	
Latah	1	65				4	94	81			8	2	28		113	28		
Latah	2	32					34	12		1	6		15		14	16		
NF Grouse	1							26										35
NF Grouse	2							19										26
Rock	1				15			70				57		6	15	328	15	
Rock	2		10		14			159	2			1		3	66	1029		

eDNA Controls

All field controls, extraction controls, and PCR controls showed no bands using all three PCR primers when run on a 2% agarose gel. The positive control displayed a band for all three PCR primers.

Amplification

Amplifications, as indicated on the gel, are shown in Table 5 per creek by PCR primer and season. Amplifications are listed out of the 3 replicates collected. In general, summer samples showed more amplifications for all PCR primers than the fall samples with Ac16S having the highest number of bands across all samples. There were no significant differences, however, between seasonal amplifications within PCR primer groups (paired t tests, CytB = 0.391; Ac16S $p = 0.1411$; Am12S $p = 0.1817$).

Sequencing

After submission to the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute, initial testing by the laboratory determined that the concentrations were insufficient to be run on the Illumina Miseq resulting in no sequencing data. DNA for samples was depleted during PCR and library preparation preventing subsequent submissions for sequencing.

BLAST Results

Primer-BLAST results indicated that of the 14 identified species collected via electrofishing, 11 would be amplified by at least one of the three metabarcoding PCR primers used (Table 6). Multiple *Cottidae* species matched to all three PCR primers but without species identification, it could not be determined if the specific sculpin species in the four creeks would have been amplified with any of the three PCR primers. The only 3 species that were not returned within the length range were Fathead Minnow, Chiselmouth, and American Bullfrog.

Table 5: Number of amplifications per PCR primer out of three replicates. Results are provided per creek and season combination.

	Summer			Fall		
Creek	**Ac16S**	**Am12S**	**CytB**	**Ac16S**	**Am12S**	**CytB**
California Creek	3	2	2	0	2	0
Latah Creek	2	1	0	1	0	0
NF Grouse Creek	0	1	0	0	0	0
Rock Creek	1	0	0	0	0	0

Table 6: Expected amplification for species verified through electrofishing according to the BLAST Primer search and PCR primer sequences. Asterisks denote species that were detected during only one of the visits. Question marks indicate that some *Cottidae* species would amplify using the indicated PCR primer, but species could not be identified in field to compare.

	Electrofishing				**Expected Primer Amplification**		
Species	**California Creek**	**Latah Creek**	**NF Grouse Creek**	**Rock Creek**	**Ac16S**	**Am12S**	**CytB**
Bridgelip Sucker *(Catostomus columbianus)*	X	X			X	X	
Brook Stickleback *(Culaea inconstans)*				X*		X	
Brook Trout *(Salvelinus fontinalis)*	X*				X	X	X
Brown Trout *(Salmo trutta)*				X	X	X	X
American Bullfrog *(Lithobates catesbeianus)*		X					
Chiselmouth *(Acrocheilus alutaceus)*	X	X					
Cottidae species	X	X	X	X	?	?	?
Fathead Minnow *(Pimephales promelas)*				X*			
Largescale Sucker *(Catostomus macrocheilus)*		X			X	X	
Longnose Dace *(Rhinichthys cataractae)*		X		X	X		
Northern Pikeminnow *(Ptychocheilus oregonensis)*	X	X			X		
Rainbow Trout *(Oncorhynchus mykiss)*	X			X	X	X	X
Redside Shiner *(Richardsonius balteatus)*	X	X		X	X		
Speckled Dace *(Rhinichthys osculus)*	X	X		X	X		
Westslope Cutthroat Trout *(Oncorhynchus clarkii lewisi)*			X		X	X	X

Discussion

In this study, I sought to assess how electrofishing and eDNA metabarcoding results compared when sampling within the same streams over two seasons. Furthermore, I planned to use varying definitions of what constitutes an eDNA detection to determine how species detections may change with changing definitions. After completing all field and molecular work, it was determined that the library concentrations were too low to sequence using the Illumina Miseq platform, so I was unable to compare eDNA results to electrofishing results.

Electrofishing efforts were successful in all 4 streams during both summer and fall. I was able to capture 14 unique species across all streams. One downfall of the electrofishing capture method is that *Cottidae* species were caught in 3 of the 4 streams but could not be identified to species in the field. To positively identify *Cottidae* species, individuals would have had to be collected and analyzed in the laboratory, or a fin clip collected from each individual for DNA sequencing. Due to the quantity of *Cottidae* individuals collected, this species grouping was left as-is and the individuals were not identified to a lower taxonomic level. This would have been an excellent test using the eDNA metabarcoding results to determine the species of sculpin in a less invasive manner, however, the eDNA sampling methods need to be evaluated further to ensure successful sampling in the future.

While all environmental parameters (water temperature, pH, discharge) varied slightly between summer and fall, the only significant change was in temperature with cooler water temperatures exhibited during fall sampling. Based on successful amplifications over all samples with all three PCR primers, there was no significant difference between the amplifications by season which indicate that environmental factors were unlikely the cause of the low eDNA concentrations in this study.

During communications with Dr. Yiyuan Li, the lead author of Li et al. (2018) which utilized the same metabarcoding PCR primers as this study, he provided some additional details on how they pooled their samples for indexing after the first PCR step (Y. Li, personal communication, March 18, 2022). Although they aimed to reach the amounts laid out in the methods of Ac16S = 3.75ng; Am12S = 2.5ng; CytB = 18.75 ng, if they did not have enough of any one of the PCR primer products, they included all the sample they had. This altered the ratios of the PCR primer products in the second PCR and was different to what we did in this study. When pooling for the second PCR, if we did not have enough DNA to meet the required amount, we reduced all PCR primer products per sample to keep them in ratio to each other to prevent bias during PCR 2. This effectively decreased the overall DNA concentration that was pooled to index for Illumina sequencing. This likely influenced the overall concentration issue; however, almost all of the post-PCR 1 primer products were too low to meet the required amounts to pool which indicated issues with low concentrations earlier in the workflow.

Aquatic eDNA is typically in low concentrations and potentially degraded which requires optimal sampling and protocols to extract sufficient DNA to sequence (Hunter et al. 2019). All eDNA studies utilize multiple steps in analyzing eDNA which can significantly affect results including DNA concentrations. The water volume collected (Bessey et al. 2020), filter material and pore size (Deiner et al. 2018; Jo et al. 2020), and extraction method (Hinlo et al. 2017; Deiner et al. 2018) can all impact eDNA results.

A combination of cellulose nitrate filters with the Qiagen DNEasy Blood and Tissue kit for extraction was used in this study and was indicated in previous studies to outperform other combinations for overall DNA recovery (Hinlo et al. 2017; Deiner et al. 2018). The filter pore size used for this study was 1.2 μm which is considered a “moderate” pore size. Previous studies

indicate that most of eDNA particles are larger than 1 μm (Turner et al. 2014; Piggott 2016) due to capturing whole cells or DNA that is bound to particulate matter. In addition, larger pore sizes can increase the capture of longer DNA fragments (Jo et al. 2020) which may be less degraded and better amplified in downstream molecular methods. Overall, it's likely that the filtration and extraction methods used in this study were sufficient and were not the main contributing factor to the low concentrations that prevented sequencing.

Within this study, 250 mL of water was collected for each sample which is on the lower end of water volumes collected across eDNA studies (Li et al. 2018; Bessey et al. 2020). Lower water volumes filtered have been shown to yield less DNA (Bessey et al. 2020) which affect all downstream analyses in the metabarcoding process. Within this study, the water volume collected appears to be the main factor that affected the inability to sequence the samples; however, further studies need to be completed to verify this.

With the extreme variation in methodology within eDNA studies, pilot studies should be conducted within any ecosystem or with a specific species to determine the best approach for that scenario (Deiner et al. 2015b). Water volumes should be maximized, molecular protocols verified, and PCR primers tested to ensure that the combination of steps is adequate for the target organism(s). Overall, further studies are needed within the four streams from this study to better analyze the cause of low DNA concentrations and determine what sample volumes would be sufficient to analyze the eDNA.

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Chapter 2

Evaluating the Relationship Between Water Volume Sampled and eDNA Concentration

Introduction

eDNA is a useful molecule to noninvasively analyze and manage fish populations. It is being used globally in studies and the field is growing exponentially due to eDNA's flexibility. Analyzing eDNA does have its challenges, however, which include the wide variety of methods and protocols that are used in its study (Goldberg et al. 2016). Each study that is conducted needs to consider the environment being studied and the target taxa which can alter the protocol across numerous studies.

One of the key considerations in an eDNA study is the low concentrations of DNA that will be present in samples which require optimal strategies to reduce false negative results (Hunter et al. 2019). While the environmental factors involved can degrade eDNA within the study system (Barnes and Turner n.d.; Strickler et al. 2015; Seymour et al. 2018; Kasai et al. 2020), the sample volume and laboratory protocols can be even more critical to the results (Alberdi et al. 2018; Bessey et al. 2020).

In aquatic systems, a water sample for eDNA extraction can vary from 250 milliliters up to several liters depending on the environment (Bedwell and Goldberg 2020; Bessey et al. 2020) which can significantly affect the starting DNA for downstream analysis and lead to false negatives. During my study outlined in chapter 1, I collected 250 milliliters of water from creeks in eastern Washington and Idaho. This volume of water from these creeks using the indicated protocols was insufficient to provide enough DNA to sequence using Illumina Miseq and resulted in inconsistent bands during gel electrophoresis. This indicated that the initial sample may have been too low in volume for these circumstances.

In addition, the combination of filtration and extraction has been shown to dramatically affect the concentration of extracted DNA (Deiner et al. 2018) and should be optimized. In previous studies a cellulose filter combined with the DNEasy Blood and Tissue Kit (Qiagen) was an optimal pairing and generally had one of the highest DNA concentrations post-extraction (Hinlo et al. 2017; Deiner et al. 2018) which is what was used in the extraction process for the study outlined in chapter 1. One detail that is not mentioned in most studies is whether the filter is left whole or cut during lysis which may be a critical detail to eDNA analysis. Cutting the filter may allow less DNA to remain trapped within the folds of the filter and increase the total DNA concentration of a sample.

During the study for chapter 1, I discovered that all creek samples amplified differently across all PCR primers with California Creek having the most amplifications and NF Grouse having the least. Using these amplifications as a proxy for concentrations, I sampled both creeks to get a range of water volumes that would be sufficient to detect fish species within these creeks and similar creeks using the chosen PCR primers. To assess how to optimize DNA concentrations from samples within these creeks, and similar local environments, I isolated two steps of the methodology used: water volume and filter treatment (whole vs cut for extraction). I hypothesized that increasing the water volume sampled would increase DNA concentration in each sample which would then amplify more frequently using all three PCR primers, and that cutting the filter prior to lysis will increase extracted DNA allowing for better downstream analyses.

Methods

Study Sites

Sampling was conducted in two streams, one located in Spokane County and one in Bonner County: California Creek and North Fork Grouse Creek. Both creeks were previously sampled in summer and fall of 2021 and the sampling locations were the same as those sampled in chapter 1.

eDNA collection

All water samples were collected in August 2022 and filtered on the same day of collection. Two replicates of four volumes were collected from each stream: 250 mL, 500 mL, 1L, and 2L for a total of 8 samples from each stream. Prior to collecting the sample, each sterile Nalgene bottle was rinsed 3 times in the stream water to ensure no bleach residue remained from decontamination procedures, and the remnant was poured onshore where it would not run back into the stream (Goldberg and Strickler 2017). Each water sample was then collected just under the surface of the water (Evans et al. 2017a; Thalinger et al. 2021a) in the thalweg while standing at least three feet downstream in moving water to minimize contamination. In addition to the eight samples collected from each stream, two Nalgene bottles filled with 250 mL of distilled (DI) water acted as negative field controls (Olds et al. 2016; Li et al. 2018). After sample collection, bottles were then closed, wiped down with a 10% bleach solution (Deiner and Altermatt 2014; Olds et al. 2016; Evans et al. 2017a; Li et al. 2018) and placed in individual Ziploc bags within a cooler (Goldberg et al. 2016) for transport back to the EWU fisheries lab.

Environmental Sampling

After collecting water samples, coordinates were recorded at the downstream end of the sampling transect using a handheld global positioning system (GPS) unit (Garmin). Water

temperature, pH, wetted width, depth, and velocity were then measured. Temperature and pH were measured using a multi-parameter instrument (YSI model 556) while standing in the middle of the stream. Ten depths and flow velocity measurements were collected evenly across each stream to calculate discharge (Marsh-McBirney Flo-Mate model 2000).

eDNA Filtering

Water samples were filtered on the same day as field collection (Evans et al. 2017a) in a laminar flow hood located in a room separate from the molecular preparation laboratory to reduce the risk of contamination. Each sample was vacuum filtered onto a one-time use 47 mm, 1.2 μm pore size cellulose membrane filter (EMD Millipore) (Lacoursière-Roussel et al. 2016; Deiner et al. 2018) using a pre sterilized reusable filter setup (Advantec filter funnel, 1L Nalgene flask), and the house-plumbed vacuum system. Filters were removed from the filter funnel using sterilized forceps, loosely folded and placed in individual 2 mL vials, 75% filled with molecular-grade ethanol. Preserved filters were then stored at -20°C until DNA extraction took place (Laramie et al. 2015; Goldberg and Strickler 2017; Duda et al. 2021; Penaluna et al. 2021).

All equipment that came in direct contact with eDNA samples (bottles, tweezers, filter apparatus, etc.), was sterilized with a 50% bleach solution for at least 1 minute and rinsed with distilled water between samples and streams (Goldberg et al. 2016; Penaluna et al. 2021). All other supplies (aside from pipettes) and laboratory surfaces, were cleaned and disinfected with a 10% bleach solution, and rinsed thoroughly (when applicable) and allowed to dry between samples (Deiner et al. 2015a; Olds et al. 2016; Goldberg et al. 2016; Evans et al. 2017a; Goldberg and Strickler 2017; Li et al. 2018). Pipettes were wiped down with DNA Away (Thermo Scientific) and allowed to dry before starting any molecular work with samples (Evans et al. 2016).

DNA Extraction

DNA was extracted from whole and cut filters, including field controls, by following a modified animal tissues protocol using the DNeasy Blood and Tissue Kit (Qiagen) (Mächler et al. 2019; Bessey et al. 2020; Penaluna et al. 2021). One replicate of each volume, and one of the field controls was randomly selected to have the filter cut before extraction. This was completed by using sterilized and autoclaved forceps and scissors along with an autoclaved piece of foil for each filter. The filter was cut using the scissors and placed in the Eppendorf tube that was then used for lysis. All other filters were left whole and transferred to a new lysis tube. Lysis was extended to 48 hours at 55°C, and the final DNA was eluted to 100 uL (Mansfeldt et al. 2019; Penaluna et al. 2021). Field controls were extracted separately from stream samples to isolate any field contamination from lab contamination (Li et al. 2018). With extraction batches, 3 extraction controls were used to isolate any potential contamination: 1 with a sterile whole filter and reagents, 1 with a sterile cut filter with reagents, and 1 with reagents only. After extraction, all samples were quantified using a Qubit 4 Flourometer (Invitrogen).

Amplification

All samples were amplified using the three PCR primers used in chapter 1: Actinopterygii 16S (L1865/H2195, Ac16s), Amphibia 12S (L909/H1155, Am12s), and Cytochrome B (L14735/H15149c, CytB) (Illumina n.d.; Burgener and Hübner 1998; Evans et al. 2016) and the step-down thermocycler settings. The following recipe was used per 20uL reaction per PCR primer in PCR tubes with individually attached lids: 10 uL Amplitaq Gold 360 Master Mix, 6 uL DNA-Free water, 1 uL Forward PCR Primer, 1 uL Reverse PCR Primer, and 2 uL DNA. For each batch of samples per PCR primer run, a single PCR negative control was added using DNA-free water in place of DNA.

To verify proper amplification, products were run through a 2% agarose gel and visualized (Evans et al. 2016, 2017a; Li et al. 2018) using Smart Glow Stain (Accuris Instruments) on a UV platform. No pattern in amplifications across samples was seen, and the extraction control that contained the cut filter showed contamination with the Ac16S PCR primer, so these samples were not analyzed further. All other samples (whole filter extractions) were further cleaned using One Step PCR Inhibitor removal kit (Zymo) in order to remove any potential PCR inhibitors (Evans et al. 2017a; Li et al. 2018; Mächler et al. 2019).

The cleaned samples were amplified once more using all three PCR primers and more DNA in the PCR recipe: 10 uL Amplitaq Gold 360 Master Mix, 1 uL Forward PCR Primer, 1 uL Reverse PCR Primer, and 8 uL DNA. The PCR products were run through a 2% gel and visualized to analyze potential patterns in amplification across volumes of water collected.

Data Analyses

All data analyses were conducted with R 4.2.1. DNA concentrations were visualized in a histogram and a Shapiro-Wilk test performed which indicated a non-normal distribution ($p<0.05$). To determine if cutting the filter during extraction increased DNA concentrations, a paired Wilcoxon test was conducted.

With the non-normal distribution, transformation was needed for a linear model. DNA concentrations were log10 transformed to fit the assumption of normality. A linear model was then fit to the data, and an analysis of variance (ANOVA) run to assess effects of increasing water volume on DNA concentration. Finally, the amplification success indicated by positive bands on the gel were qualitatively analyzed to determine whether there was an optimal volume to ensure amplification success.

Results

All field controls from California creek indicated no DNA via Qubit, but both the whole and cut filter samples from NF Grouse indicated small amounts of DNA shown in Table 7 (0.0075 ng/uL for the whole filter and 0.0068 ng/uL for the cut filter). All extraction controls measured at 0 ng/uL of DNA.

The average concentration of samples extracted with a whole filter was 0.0942 ng/uL and the average concentration of samples with a cut filter was 0.0522 ng/uL. These concentrations were not significantly different from each other ($p = 0.9057$). Due to this, all concentrations were combined to assess the relationship with volume and DNA concentration. DNA concentrations showed a positive correlation with water volume (linear model, $p < 0.05$, $r^2 = 0.8219$; Table 8) with each creek having a different slope (California slope=0.000140, NF Grouse slope=0.00000540; Figure 6 and Figure 7).

Prior to Zymo Onestep treatment, amplifications as indicated by a positive band on the gel were inconsistent across PCR primers from amplification using 2 uL of DNA in the PCR recipe (Table 7) with the cut filter extraction control indicating contamination along with the field control from California creek where the filter was cut. Due to this contamination and the inability to determine the cause through sequencing, cut filter samples were removed from further analysis. After Zymo Onestep treatment of just whole filter samples, CytB and Ac16S samples indicated no amplifications for any sample volume for either creek. Am12S samples showed consistent bands after collection of 500 mL in California Creek and 1 L in NF Grouse Creek using 8 uL of DNA in the PCR recipe.

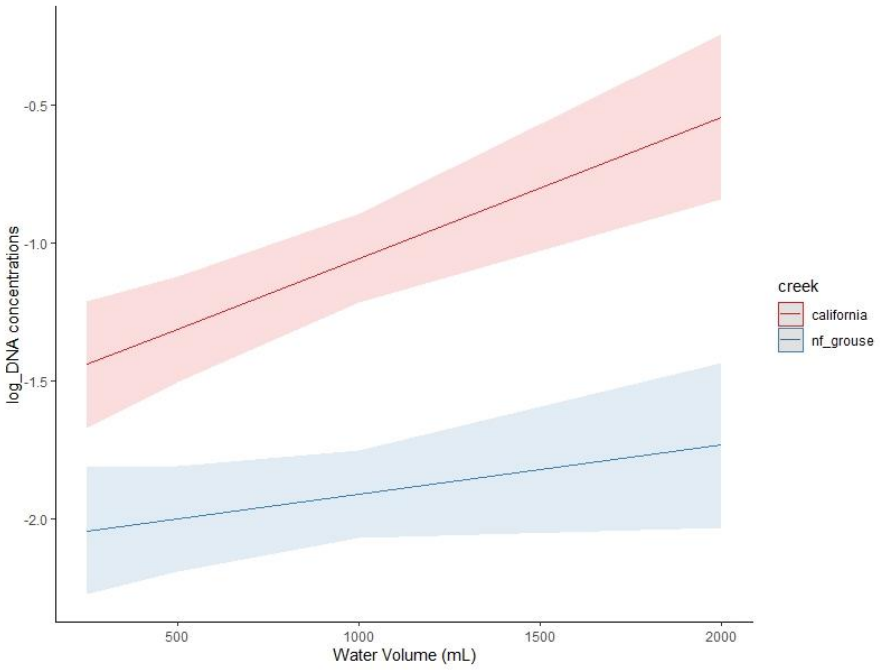

Figure 6: Relationship between water volume sampled and log-transformed DNA concentrations for California creek and North Fork Grouse creek samples collected August 2022. Shading indicates the 95% confidence interval.

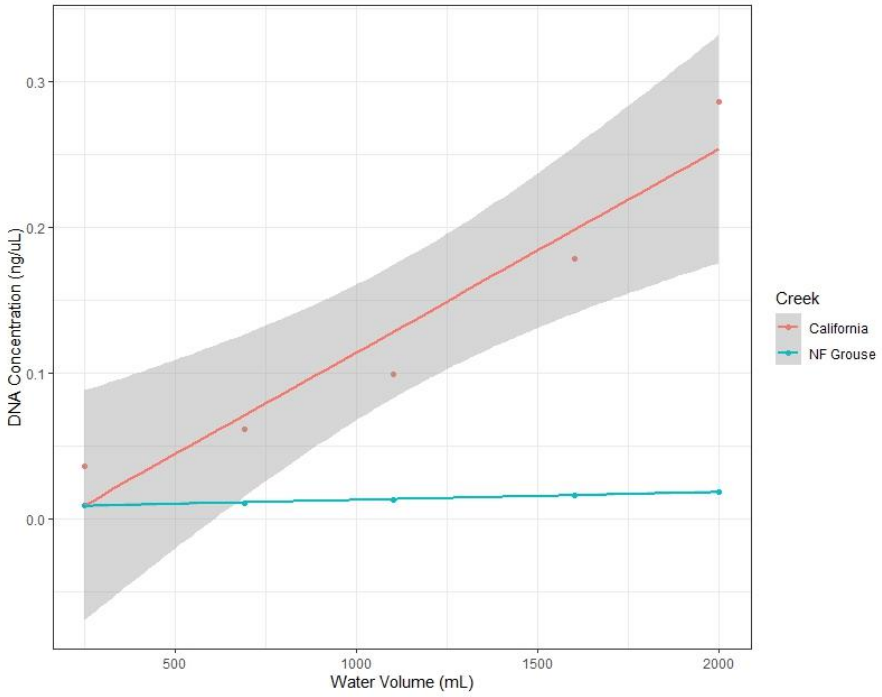

Figure 7: Relationship between water volume sampled and DNA concentrations derived from linear model and log-transformed concentrations for California creek and North Fork Grouse creek samples collected August 2022. Shading indicates the 95% confidence interval.

Table 7: DNA concentration and amplification results by sample combination of creek, water volume, and filter treatment.

Creek	Sample Volume (mL)	Filter Treatment	DNA Concentration (ng/uL)	Amplification? Pre-Zymo, 2 uL DNA			Amplification? Post-Zymo, 8 uL DNA		
				Ac16S	CytB	Am12S	Ac16S	CytB	Am12S
California Creek	250	cut	0.0268						
California Creek	500	cut	0.0624	X		X			
California Creek	1000	cut	0.0764						
California Creek	2000	cut	0.202		X	X			
California Creek- Field Control	250	cut	0	X					
NF Grouse	250	cut	0.0077						
NF Grouse	500	cut	0.0125						
NF Grouse	1000	cut	0.0122	X		X			
NF Grouse	2000	cut	0.0175			X			
NF Grouse- Field Control	250	cut	0.0068						
Extraction Control	NA	cut	0	X					
Extraction Control	NA	NA	0						
California Creek	250	whole	0.0204			X			
California Creek	500	whole	0.0412		X	X			X
California Creek	1000	whole	0.424						X
California Creek	2000	whole	0.217	X		X			X
California Creek- Field Control	250	whole	0						
NF Grouse	250	whole	0.0075						
NF Grouse	500	whole	0.0107						
NF Grouse	1000	whole	0.0152	X		X			X
NF Grouse	2000	whole	0.0173						X
NF Grouse- Field Control	250	whole	0.0075						
Extraction Control	NA	whole	0						

Table 8: Results of a linear model (lm) showing the relationship between water volume sampled and DNA concentration with creeks as an interaction variable.

Source of Variations	Sum of Squares	Df	F Value	Pr (>F)
(Intercept)	6.6477	1	125.4117	0.000000104***
volume	0.9418	1	17.767	0.001199**
creek	0.3645	1	6.8756	0.022297*
volume: creek	0.2014	1	3.7992	0.075037
Residuals	0.6361	12		

Discussion

The goal of this study was threefold: assess how DNA concentrations vary with increasing water volume sampled, whether this affects amplification using the chosen metabarcoding PCR primers and assess whether cutting the filter during lysis would influence the extracted DNA concentration.

My results indicated that there is a linear relationship between the volume of water sampled and the DNA extracted; however, the relationship varied by stream. NF Grouse creek DNA concentrations increased at a much slower rate per unit of volume than the California creek samples did. As evidenced by 2021 electrofishing data, California Creek has both a higher species richness and abundance of species; whereas, NF Grouse only has two known species and a low abundance of both which would impact the overall DNA concentrations (Chapter 1). In addition, California Creek is known to have high levels of coliform bacteria (Joy 2008) which would contribute to the total DNA concentrations collected.

When comparing samples within each creek for each volume, cutting the filter did not significantly change the DNA concentration. The whole filter samples had slightly more DNA in them; however, this is likely due to natural variation as indicated by the insignificant results. Once the samples were each amplified using the three metabarcoding PCR primers, one critical difference between the whole filter and cut filter samples was the contamination indicated in the controls. Two controls where the filters were cut displayed a band for the Ac16S PCR primer indicating fish DNA was present. None of the whole filter controls indicated DNA presence on the gel. No significant differences in the DNA concentrations between the two groups indicates that taking the extra step to cut the filter is unnecessary and can be an additional source of contamination to avoid.

The amplification of all samples using 2 uL of DNA showed inconsistent amplification across sample volumes and PCR primers with no visible pattern seen. With the low amount of DNA added to the PCR reaction and the potential of inhibitors preventing amplification (Jane et al. 2015; Goldberg et al. 2016), whole filter samples were cleaned and re-run with 8 uL of DNA per PCR reaction. This created an obvious pattern within Am12S samples over 500 uL for California Creek and 1 L for NF Grouse being sufficient for consistent amplification, but this was not the case for either the Ac16S or the CytB samples. There were no bands indicating amplification in any of the sample volumes for either creek with the Ac16S or CytB PCR primer. This is likely due to the sensitivity of the Am12S PCR primer as indicated in previous studies by the high number of reads from this PCR primer when compared to the other two PCR primers (Evans et al. 2016; Olds et al. 2016; Li et al. 2018). This may also be partially due to the Am12S amplicon size, which is ~100 bp shorter than Ac16S, and ~200 bp shorter than the CytB amplicons. eDNA from these streams are likely partially degraded which may limit the size of the target DNA preventing the longer amplicons from amplifying properly (Piggott 2016).

With the target species being mainly fish in these creeks, and BLAST results indicating that their DNA should be amplified by all PCR primers (Chapter 1), these results raise some questions on why the Ac16S and CytB PCR primers are not amplifying consistently despite the DNA concentrations seen. Although 250 mL of water is on the lower end of water volume collected, more studies have had successful eDNA results using 1-2 L of water (Pilliod et al. 2013; Penaluna et al. 2021; Wood et al. 2021). The results in this study are solely based on DNA concentrations and the presence/absence of bands on a gel with no positive control to verify the PCR cycle. The eDNA came from a natural system which includes other non-target DNA such as that from bacteria or fungi which may not amplify using all PCR primers. In addition, using

bands on the gel as evidence of amplification may be problematic since some sequencing data may indicate species presence even when a band is not present (Nguyen et al. 2015; Li et al. 2018) especially when working with low concentration samples.

PCR inhibitors naturally occurring in the water samples may have played a larger role than anticipated in this study. In many eDNA studies, samples are preemptively treated post-extraction for PCR inhibitors using a spin column inhibitor remover kit (Evans et al. 2016; Olds et al. 2016; Li et al. 2018) which was used on these samples and the samples from chapter 1. Studies have indicated this spin column treated DNA had the least amount of DNA removed during treatment while still removing inhibitors when compared to other methods such as sample dilution (McKee et al. 2015) allowing for increased amplification downstream. There does appear to be a seasonal component to the total PCR inhibitors in the environment, and samples, with higher levels of PCR inhibitors seen during fall after more plant matter has fallen into the water (Jane et al. 2015). The samples taken for this study were collected in late August which may have impacted the amount of PCR inhibitors in the samples. To test whether PCR was inhibited even with the spin column treatment, treating samples with a simple dilution in addition to the spin column treatment would be beneficial.

The sampling location of all water samples was in the thalweg just under the surface of the water. Due to the unique hydrodynamics and the species that are present, this may not be the best sampling method to ensure optimal target DNA collection. eDNA can accumulate on one side of the stream over the other (Laporte et al. 2020; Thalinger et al. 2021a) or settle into the sediment instead of floating downstream (Turner et al. 2014; Nevers et al. 2020). In addition, with bottom-dwelling species like *Cottidae*, their eDNA may reflect the individuals position in

the water column for a distance downstream (Thalinger et al. 2021b), which would not be fully accounted for with a surface-level sample collection.

Overall, my results indicate a positive relationship of DNA concentration extracted to water volume sampled, but the identity of the DNA is unknown, and the amplifications were inconsistent across PCR primers indicating further issues. Furthermore, my results are inconclusive on what sample volume is ideal to utilize the three chosen metabarcoding PCR primers in these local creeks. More studies assessing the role of PCR inhibitors on amplification along with the concentration of target species eDNA in each sample, its variability, and the relationship to amplification using gel electrophoresis is needed.

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Chapter 3

Positive Correlation of Brook Stickleback (*Culaea inconstans*) Biomass to eDNA Concentration and Detection Using Species-Specific Primer

Introduction

eDNA studies have indicated the value of eDNA analysis and its ability to outperform standard methods (Olds et al. 2016; Evans et al. 2017b; Penaluna et al. 2021); however, the methods used across studies vary tremendously. eDNA results are heavily influenced by the protocols used (Pilliod et al. 2013; Deiner et al. 2018), the species targeted (Barnes et al. 2014), and the environmental conditions present in the study system (Strickler et al. 2015). In chapter 1, previously used eDNA metabarcoding methods (Olds et al. 2016; Li et al. 2018) were applied to similar lotic systems without success due to low DNA concentrations. While environmental factors have a major impact on the eDNA molecules once they are shed into the environment, the species themselves can significantly impact the eDNA entering that environment (Thalinger et al. 2021b). Due to the difference in species present within each system, understanding how their metabolic rates, and species-specific shedding rates affect DNA concentration and eDNA results is crucial to apply these eDNA methods.

One species detected during electrofishing in at least one creek from the study outlined in chapter 1 was Brook Stickleback *(Culaea inconstans).* Brook Stickleback are a small non-native and invasive species in Eastern Washington (Scholz et al. 2003). In the United States, they are native to the area east of the Rocky Mountains and are thought to have been introduced illegally in Montana, and spread west from there (Scholz et al. 2003). Since their introduction, Brook Stickleback have expanded into the previously fishless waterbodies at Turnbull National Wildlife Refuge (TNWR) where they are impacting the ecology of these systems and the waterfowl that depend on them (Walston and Hall 2015). Monitoring the spread of Brook Stickleback is critical to management efforts, but traditional methods can be difficult to utilize. eDNA monitoring is

an option to monitor Brook Stickleback occupancy at TNWR; however, to apply this method, more research is needed on the species-specific shedding of eDNA from these fish.

The aim of this study was to determine how fish biomass affects DNA concentrations extracted from a water sample, how the DNA concentration varies with filter pore sizes, and to test species-specific eDNA primers for Brook Stickleback. By using species-specific eDNA primers, a positive band on the gel would indicate the species that was being amplified using PCR alone with non-target species not amplifying at all. This study used Brook Stickleback captured from TNWR in a controlled aquaria setting to isolate species-specific eDNA traits from environmental impacts. My hypotheses were that high biomass aquaria would result in higher DNA concentrations than the low biomass aquaria, and that 0.65 μm pore size would increase the DNA yield from water samples over the 1.2 μm pore size but not significantly.

Methods

The experiment was conducted within controlled conditions in aquaria using Brook Stickleback *(Culaea inconstans).* Brook Stickleback were used due to their small size, ease of capture, and their presence as an invasive species in local waterbodies. Brook Stickleback were present in at least one of the creeks sampled from chapter 1, and are spreading throughout the previously fishless ponds on Turnbull National Wildlife Refuge (TNWR; Scholz et al. 2003; Walston and Hall 2015). Being able to detect Brook Stickleback presence via eDNA in new waterbodies can increase management options on the refuge.

The Brook Stickleback used in this experiment were collected from Middle Pine Pond on the TNWR by using minnow traps that had been set for ~24 hours. The fish were transported back to the EWU fisheries laboratory inside a large cooler filled with pond water and portable

aerator. Fish were transferred to a holding tank filled with dechlorinated water upon arrival and left to acclimate for 24 hours before the experiment would begin. From collection on, the fish were held at room temperature and not fed to reduce the impact that excess food and feces would have on eDNA measurements (Maruyama et al. 2014). The light schedule was intended to be 12:12 light and dark cycle; however, due to access to this room, the light cycle varied across the experiment. This study was performed in compliance with and approval by TNWR (collection permit # 13560-22-23), Washington Department of Fish and Wildlife (WDFW; transport permit # 8981-06-30-22), and EWU's Institutional Animal Care and Use Committee (IACUC).

On the day of the start of the experiment, fifteen 7-gallon aquaria and their lids were cleaned, sterilized with a 10% bleach solution for 10 minutes (Maruyama et al. 2014; Kasai et al. 2020) and then fully rinsed with tap water followed by dechlorinated water. Aquaria were then filled with 5 gallons of dechlorinated water. An air pump with air stone was added to each tank to oxygenate and circulate the water without filtering out DNA (Maruyama et al. 2014). There were 6 replicates of two treatment groups: low biomass (1 fish) and high biomass (10 fish). In addition, there were 3 tanks that acted as controls (no fish, just dechlorinated water, and an air pump). The fifteen aquaria with lids were arranged on a 4 -shelf rack and were assigned randomly to one of the groups.

After the tanks were filled and the air pumps were operating, but prior to introducing fish, 3 controls were collected. A 1 L water sample was collected from a randomly chosen tank (Thalinger et al. 2021b), one 1 L sample was collected from the dechlorinated water source, and one 1 L sample was filled with distilled water. Nalgene bottles treated with 50% bleach for >1 minute and rinsed with distilled water six times were used to collect samples (Goldberg et al.

2016). All samples were then processed using the same methods outlined below to act as an initial negative control.

Fish were randomly chosen from the holding tank and assigned to one of the experimental tanks. Wetted weight and length were collected for each fish before placing them in their assigned tank where they remained throughout the 7-day experiment which spanned October 21st-October 28th , 2022. Only fish that were 50 mm or longer were used to improve survival over the experiment and reduce size variation within the experimental groups. Fish were monitored daily along with water temperature and pH. On the seventh day of the experiment, after water sample collection, fish were gently removed from their tanks and euthanized using a >250 mg/L of MS-222.

Water Sampling

Water samples were collected from each tank using a new 1 L sterile Whirl Pak bag (Whirl-Pak) for each tank. The bags were then closed, wiped down with a 10% bleach solution (Deiner and Altermatt 2014; Olds et al. 2016; Evans et al. 2017a; Li et al. 2018) and placed in a bin to be transported to the filtering room. One Whirl Pak bag was filled with distilled water to act as a control during transportation and filtration. Once in the filtering room, all Whirl Pak bags were rinsed with distilled water prior to filtering.

eDNA Filtration

All water samples were filtered in a laminar flow hood located in a room separate from the molecular preparation laboratory to reduce the risk of contamination. To isolate how pore size may change extracted DNA concentrations, each sample was vacuum filtered onto a one-time use 47 mm, 1.2 μm pore size cellulose membrane filter (Lacoursière-Roussel et al. 2016;

Deiner et al. 2018) and a 0.65 μm pore size cellulose membrane filter (EMD Millipore) using a pre sterilized reusable filter setup (Advantec filter funnel, 1L Nalgene flask), and the house-plumbed vacuum system. Filters were removed from the filter funnel using sterilized forceps, loosely folded and placed in individual 2 mL vials, 75% filled with molecular-grade ethanol. Preserved filters were then stored at -20°C until DNA extraction took place (Laramie et al. 2015; Goldberg and Strickler 2017; Duda et al. 2021; Penaluna et al. 2021).

All equipment that came in direct contact with eDNA samples (bottles, tweezers, filter apparatus, etc.), was sterilized with a 50% bleach solution for at least 1 minute and rinsed with distilled water between samples and streams (Goldberg et al. 2016; Penaluna et al. 2021). All other supplies (aside from pipettes) and laboratory surfaces were cleaned and disinfected with a 10% bleach solution, rinsed thoroughly (when applicable), and allowed to dry between samples (Deiner et al. 2015a; Olds et al. 2016; Goldberg et al. 2016; Evans et al. 2017a; Goldberg and Strickler 2017; Li et al. 2018). Pipettes were wiped down with DNA Away (Thermo Scientific) and allowed to dry before starting any molecular work with samples (Evans et al. 2016).

DNA Extraction

DNA was extracted from whole, including controls, by following a modified animal tissues protocol using the DNeasy Blood and Tissue Kit (Qiagen) (Mächler et al. 2019; Bessey et al. 2020; Penaluna et al. 2021). Lysis was extended to 48 hours at 55°C , and the final DNA was eluted to 100 uL (Mansfeldt et al. 2019; Penaluna et al. 2021). Pre experimental controls were extracted separately from stream samples to isolate any aquaria contamination from lab contamination. With each extraction batch, an extraction control was used to isolate any contamination within this step. After extraction, all samples were quantified using a Qubit 4 Flourometer (Invitrogen).

PCR Primer Design and Testing

Two PCR primers were designed to target Brook Stickleback mitochondrial DNA in two regions: 16S and cytochrome oxidase 1 (CO1). Reference sequences were downloaded from GenBank. Due to Brook Stickleback being commonly found with Pumpkinseed (*Lepomis gibbosus)*, the two species sequences were aligned to identify areas where they differed by 5 or more base pairs in the annealing region (Farley et al. 2018). The modified Primer 3 within Geneious was then used to design the PCR primers that would amplify a 200-300 base pair amplicon of both regions.

The PCR primer outputs were then tested in silico against other common species in the area followed by a BLAST Primer (NIH) search to ensure species specificity. Both searches returned results indicating that they would amplify Brook Stickleback along with only one other species that occurs in the Pacific Northwest within the expected amplicon length. The 16S PCR primer indicated it would amplify Threespine Stickleback (*Gasterosteus aculeatus)*, and the CO1 PCR primer indicated it may amplify Prickly Sculpin (*Cottus asper*) in the expected amplicon range. While both species do occur in Washington, they are isolated to the area west of the Cascades and are not known to be present in eastern Washington.

The two chosen PCR primers (Table 9) were then tested on tissue samples of fish that occur in eastern Washington including Brook Stickleback *(Culaea inconstans),* Pumpkinseed (*Lepomis gibbosus),* Black Crappie (*Pomoxis nigromaculatus*), Yellow Perch (*Perca flavescens*), two *Cottidae* species, Rainbow Trout (*Oncorhynchus mykiss*), Brown Trout (*Salmo trutta*), and Longnose Dace (*Rhinichthys cataractae*). Thermocycling parameters are outlined in table 10 and

Table 9: PCR Primer sets used for amplifying target regions from eDNA of Brook Stickleback.

Primer Name	Target Gene	Forward Primer	Reverse Primer	Amplicon Length (bp)	Annealing Temperatures °C (AT)
BS-16S	16S	TTCTGACCAAAAAGATCCGGCA	CGTACCATAGGTATTGGCCTCC	282	52°C
BS-CO1	CO1	CTAGCTTCCTCAGGGGTCGA	GGGGAGGGAAAGGAGGAGTA	263	57°C

Table 10: Thermocycler conditions for all Brook Stickleback PCR primers. Annealing temperatures are indicated by AT.

Thermocycler conditions		
Step	**Temperature (°C)**	**Time**
1	94	2 min
2	94	30 sec
3	AT	30 sec
4	72	30 sec
5	repeat steps 2-4 thirty-four times, 35 cycles total	
14	72	10 min
15	4	Hold

the following recipe was used per 20 uL PCR reaction: 10 uL Amplitaq Gold 360 Master Mix, 1 uL Forward PCR Primer, 1 uL Reverse PCR Primer, 6 uL molecular-grade water, and 2 uL DNA. The annealing temperatures were started at 5 ° C lower than the melting temperature for each PCR primer and increased in 1° - 2° C increments in each subsequential run to test PCR primer specificity. Due to the CO1 PCR primer amplifying non-target species, only the 16S PCR primer was used going forward.

Amplification and Sequencing

All samples, including controls, were amplified using the Brook Stickleback specific 16S PCR primer. The following recipe was used per 20uL reaction per PCR primer in PCR tubes with individually attached lids: 10 uL Amplitaq Gold 360 Master Mix, 1 uL Forward PCR Primer, 1 uL Reverse PCR Primer, and 8 uL DNA. For each batch of samples per primer run, a single PCR negative control was added using DNA-free water in place of DNA.

To verify proper amplification, products were run through a 2% agarose gel and visualized (Evans et al. 2016, 2017a; Li et al. 2018) using Smart Glow Stain (Accuris Instruments) on a UV platform. To verify PCR primer specificity, four randomly chosen samples from positive samples were then sent to Genewiz for sequencing.

Data Analyses

All data analyses were performed in R 4.2.1. PCR primer amplifications were both assessed qualitatively for positive bands on the gel for target species only at the expected length. All variables were visualized with histograms and the distribution tested with a Shapiro-Wilk test. All variables indicated a non-normal distribution. Temperatures, and weights did not appear normally distributed after attempting transformation of the data, so Wilcoxon tests were performed to assess differences between treatment groups for these two variables. Fish lengths

were normally distributed after log10 transformation. A t test was performed to compared average fish lengths between treatment groups. DNA concentrations were normally distributed after log10 transformation. A paired t test was performed to compare the total DNA concentration extracted from the 1.2 μm filter to the 0.65 μm filter. Finally, to determine the relationship between total fish biomass and total fish length, and log10 transformed 0.65 μm DNA concentrations (total DNA concentration per sample extracted), a linear model was performed. Due to total fish length and total fish biomass being highly correlated, the model including both as predictor variables confounded the results and was redundant. Log-transformed total fish biomass was used as the only predictor variable in the final linear model.

Results

PCR Primer specificity

During PCR primer testing on tissue samples, the CO1 PCR primer amplified Brook Stickleback along with Pumpkinseed, Black Crappie, and Brown Trout at the expected amplicon length for all tested annealing temperatures. Due to this, the CO1 PCR primer was not used to amplify DNA from water samples from the aquaria. The 16S PCR primer amplified only Brook Stickleback at the expected amplicon length, and it was used to amplify the aquaria samples. The amplicon sequences were verified using PCR sequencing which matched the GenBank reference sequences for Brook Stickleback.

Controls

All controls indicated no DNA present as measured by Qubit 4 Flourometer (Invitrogen) except the pre-experimental tank control which indicated that minute amounts of DNA were present (0.0106 ng/uL) and the 3 experimental tank controls (0.0142 ng/uL, 0.0160 ng/uL and

0.0200 ng/uL). No controls were amplified using the Brook Stickleback 16S PCR primer, and no bands were seen on the gel for any control.

Treatment Groups

pH stayed consistent across all tanks for the entire 7-day experiment. Average temperature over the 7-day experiment was not significantly different between the low and high biomass treatment groups (Wilcoxon test, $p = 0.4493$). The average fish also did not differ significantly between the low and high biomass treatment groups (Wilcoxon test, $p = 0.0682$). The average length of fish did differ significantly between the low and high biomass treatment groups with the low biomass average fish length being 62 mm and the high biomass average fish length being 56.74 (t test, $p = 0.01589$).

DNA concentrations

The DNA extracted from the 0.65 μm filter was significantly higher than DNA extracted from the 1.2 μm filter (paired t test, $p = 0.006423$). The average DNA concentration was approximately 0.22 ng/μL higher in the high biomass samples and 0.023 ng/μL higher in the low biomass samples for DNA extracted from the 0.65 μm filters when compared to the DNA extracted from the 1.2 μm filters (Table 11).

Biomass and DNA Concentration

The linear model with total fish biomass as the predictor to total DNA concentration extracted from particles over 0.65 μm, indicated the total fish biomass did significantly impact the DNA concentration (ANOVA, $p < 0.05$, $r^2 = 0.71$; Table 12 and Figure 8).

Table 11: Sample data by treatment group with concentrations and amplification indicated.

Treatment Group	Total Biomass (g)	Total Length (g)	DNA Concentration (1.2 um; ng/uL)	DNA Concentration (0.65 um; ng/uL)	Amplified?
high biomass	15	595	36.4	36.8	X
high biomass	19	589	2.7	2.7758	X
high biomass	13	560	0.732	0.851	X
high biomass	12	541	0.414	0.4846	X
high biomass	13	552	2.78	3.192	X
low biomass	2	63	0.0298	0.0556	X
low biomass	2	60	0.104	0.1392	
low biomass	2	65	0.0518	0.0518	X
low biomass	2	63	0.186	0.2052	X
low biomass	1	56	0.0634	0.094	
low biomass	2	65	0.0916	0.1162	X

Table 12: Results of a linear model (lm) showing the relationship between total fish biomass and DNA concentration.

Source of Variations	Sum of Squares	Df	F Value	Pr (>F)
Total Biomass	5.9055	1	25.699	0.0006724***
Residuals	2.0681	9		

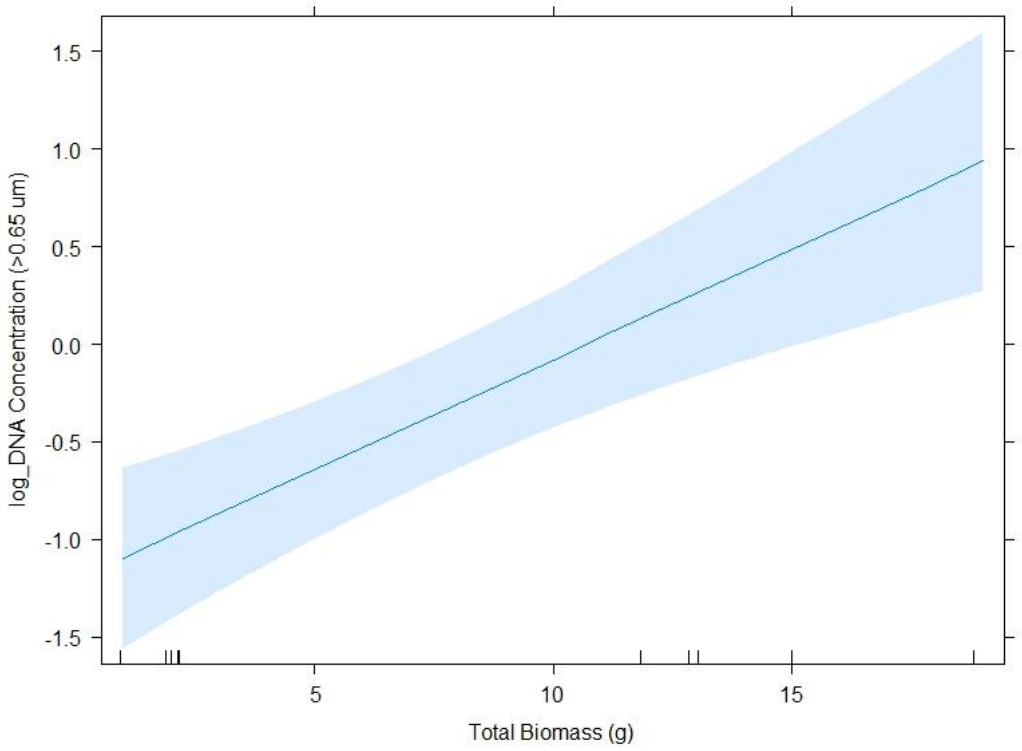

Figure 8: Relationship between total fish biomass (g) and log-transformed DNA concentrations (>0.65 um). Shading indicates the 95% confidence interval.

Discussion

This study's goal was to evaluate the relationship between fish biomass and eDNA concentrations extracted from water samples for Brook Stickleback in controlled aquaria, to determine how these concentrations differed with filter pore size, and to assess the application of two new species-specific PCR primers. Overall, my findings indicate that a pore size of 0.65 μm captures significantly more eDNA particles than a pore size of 1.2 μm and support a linear relationship between total biomass of Brook Stickleback and the total eDNA concentrations.

The highest concentration of eDNA was captured by the 1.2 μm filters, but the smaller pore size of 0.65 μm filters significantly increased the DNA yield from the water samples. This is supported by other studies with fish species where the most eDNA particles are 1-10 μm in size (Turner et al. 2014) with smaller particles (< 1 μm) accounting for up to 25% of eDNA captured (Wilcox et al. 2015). In aquaria, these smaller eDNA molecules may be more stable; however, in a natural system, these are likely to degrade more quickly (Woodruff 2015) and have a lesser chance of being captured during sampling efforts. In addition, the turbidity of the environment and the size of the PCR primer target would also need to be considered when choosing a filter size for a natural system.

While the 0.65 μm filters captured more DNA, the 16S PCR primer amplified all aquaria samples from the 1.2 μm filters where Brook Stickleback were present except two samples from the low biomass treatment group. These two samples did not have the lowest DNA concentrations which indicate that this may be due to a PCR issue which may have been resolved with running replicates of the samples through PCR. Since we did not amplify the 0.65 μm filter samples, it is unknown whether a smaller filter alone may have increased the chance of amplification. One improvement in this study, or any eDNA study, would be to increase the PCR

replicates for every sample to both improve the chance of amplification, and decrease the chance of PCR bias in the case of metabarcoding (Alberdi et al. 2018).

During testing of the PCR primers designed for this study, the CO1 PCR primer amplified non-target species regardless of annealing temperatures tested which indicated that it is not ideal for detecting Brook Stickleback in a natural system. The 16S PCR primer, however, amplified only the target species which makes it a great candidate for detecting Brook Stickleback. Since newly invaded waterbodies would have low densities of Brook Stickleback, this 16S PCR primer should be tested further in a natural system using multiple water samples, and multiple PCR replicates to verify successful application for management purposes.

Finally, DNA concentrations showed a clear positive linear relationship with Brook Stickleback biomass. More fish biomass equated to more eDNA collected which is supported by other studies with different species (Pilliod et al. 2013; Klymus et al. 2015). This relationship has pushed the eDNA field into the realm of assessing fish abundance through eDNA alone (Lacoursière-Roussel et al. 2016; Sansom and Sassoubre 2017). The caveat in this study, however, is eDNA is heterogeneous in the water column with some samples containing much higher concentrations of eDNA such as the high biomass treatment group in this study (36.8 ng/uL; Table 11) which contained ~76 times more eDNA than the lowest concentration in the high biomass treatment group. The high biomass aquarium with the highest eDNA concentration contained one fish that showed lethargic behavior towards the end of the experiment with its caudal fin showing damage on day 6. It's likely that during water collection, a piece of fin was collected I the water sample which increased the DNA concentration substantially. These scenarios occur in a natural system which can significantly impact eDNA samples and results. In addition, the environmental factors of a natural system affect eDNA in a way that makes it less

predictable than in a controlled environment as evidenced by studies indicating the extreme heterogeneity of eDNA particle distribution in the water column (Pilliod et al. 2013; Lacoursière-Roussel et al. 2016; Bessey et al. 2020), and the lower concentration of eDNA in a natural environment (Klymus et al. 2015). These results support the need for multiple sample replicates from any one system to better assess species distribution, and especially species abundance.

Overall, this study is an important step in understanding how eDNA concentrations relate to the biomass of Brook Stickleback and, in turn, how that affects detection using a 16S targeted species-specific PCR primer. These results are promising for management efforts in monitoring the invasive species through eDNA, but further research in a natural environment is needed to optimize this approach and ensure consistent and accurate results.

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Appendices

Appendix A

Brook Stickleback 16S PCR Sequencing Data

High Biomass 1:

>AC3-BS16SF_D07.ab1
NNNNNNNNCNAGTTACCCTAGGGANAACAGCGCAATCCTCTTTTAGAGCCCATATCGACAAGAGGGTTTACGACC
TCGAT
GTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATCT
GAGT
TCAGACCGGAGTAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAG
GCCAA
NNNNTATGGTACGA

Low Biomass 1:

>AC7-BS16SF_E07.ab1
NNNNNNNANNNNNNNNNNNNNNNTTNNTANANNCNNNNCCNCTTTTNAGCCCTTATCTTCAACAGGGTTTACTAC
CTCCATGATGGATCAGGACTCCTATTGGTGCCGCCGCTATTAAGGGTTCGTTTGTTCNCTATTAAAGTCCTACTTGA
TCTGAGTTCANACCGGAATAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGNACGAANGGACCGAGAA
GAGGAGGCCAAT
ACCTATGGAACGATTGATGTANATAAATANATGTAAATAAATAAGGGTAACTATTTGAATCTGCCTGACAACTTGT
TTAC
GTCATGCATTTNTATTTTTTTTTTTCTTCAATGAACTCTTATTGTTTTTCATGTTTTTACTGACTGCCCCCTNACCAAT
C
TCCTTCAAGTCNATGTTAACCCATAGNCCTCTGACTCCNACTCAAACAATNTGGGACTCTGCTGNNAGGGGAGATT
ACCT
G

High Biomass 2:

>AC8-BS16SF_F07.ab1
NNNNNNNNCNNGTTACCCTAGGGNTNNAGCGCAATCCTCTTTTAGAGCCCATATCGACAAGAGGGTTTACGACCT
CGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTG
ATCTGAGTT
CAGACCGGAGTAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAGG
CCANN
NNNTATGGTACGA

Low Biomass 2:

>AC14-BS16SF_G07.ab1
NNNNNNNNNNNNNNNNNNNNNNCNNTTNNTANNNNGCTACNTCCTCTTTTAGAGCCCTTATCTTCNTAAGGGTTT
ACGAC
CTCNATGTAGGATCAAGACATCCTAATGGGGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACN
TGAT
CTGAGTTCNGACCGGAGCAATTCNGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAG
AGGA
GGCCAATACCTATGGTACGACCAATTGTTTACAGCCTGTGAAGGTGATGTATTTTTATCAACANGACTCCTTTANG
AAGT
CCATGCCAAACTGACTGTTTTTACNCAAATCTCCTTAAGGTCAATGCCTACNATAANACAGTGACCCCTTGTCAGC
AAAC
TGTGACTCTCTNTAAANGNNANATGACCTGNNNCNCNNANNTTANNNNN

Appendix B

Brook Stickleback 16S PCR Sequencing BLAST Returns

High Biomass 1:

>AC3-BS16SF_D07.ab1

```
Query: None Query ID: lcl|Query_29101 Length: 254


>Culaea inconstans mitochondrial DNA, complete genome
Sequence ID: AB445125.1 Length: 16465
Range 1: 2454 to 2696

Score:425 bits(230), Expect:1e-114,
Identities:237/243(98%),  Gaps:0/243(0%), Strand: Plus/Plus

Query  11    AGTTACCCTAGGGANAACAGCGCAATCCTCTTTTAGAGCCCATATCGACAAGAGGGTTTA  70
             ||||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  2454  AGTTACCCTAGGGATAACAGCGCAATCCTCTTTTAGAGTCCATATCGACAAGAGGGTTTA  2513

Query  71    CGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGT  130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2514  CGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGT  2573

Query  131   TCAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTA  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2574  TCAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTA  2633

Query  191   TCTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAGGCCAANNNNTATGGT  250
             ||||||||||||||||||||||||||||||||||||||||||||||||||    ||||||
Sbjct  2634  TCTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAGGCCAATACCTATGGT  2693

Query  251   ACG  253
             |||
Sbjct  2694  ACG  2696
```

Low Biomass 1:

>AC7-BS16SF_E07.ab1

```
Query: None Query ID: lcl|Query_63913 Length: 481


>Culaea inconstans mitochondrial DNA, complete genome
Sequence ID: AB445125.1 Length: 16465
Range 1: 2480 to 2696

Score:281 bits(152), Expect:6e-71,
Identities:194/217(89%),  Gaps:3/217(1%), Strand: Plus/Plus

Query  39    CCNCTTTT-NAGCCCTTATCTTCAACAGGGTTTACTACCTCCATGATGGATCAGGAC-TC  96
             || |||||  || || ||||  ||| ||||||||| ||||| ||| |||||||||||| ||
Sbjct  2480  CCTCTTTTAGAGTCCATATCGACAAGAGGGTTTACGACCTCGATGTTGGATCAGGACATC  2539

Query  97    CTATTGGTGCCGCCGCTATTAAGGGTTCGTTTGTTC-NCTATTAAAGTCCTACTTGATCT  155
             ||| |||||| ||||||||||||||||||||||||  | |||||||||||| ||||||
Sbjct  2540  CTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATCT  2599

Query  156   GAGTTCANACCGGAATAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGNACG  215
             ||||||| |||||| |||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  2600  GAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTACG  2659

Query  216   AANGGACCGAGAAGAGGAGGCCAATACCTATGGAACG  252
             || |||||||||||||||||||||||||||| |||
Sbjct  2660  AAAGGACCGAGAAGAGGAGGCCAATACCTATGGTACG  2696
```

High Biomass 2:

>AC8-BS16SF_F07.ab1

```
Query: None Query ID: lcl|Query_17145 Length: 253


>Culaea inconstans mitochondrial DNA, complete genome
Sequence ID: AB445125.1 Length: 16465
Range 1: 2455 to 2682

Score:398 bits(215), Expect:3e-106,
Identities:223/228(98%),  Gaps:1/228(0%), Strand: Plus/Plus

Query  12    GTTACCCTAGGGNT-NNAGCGCAATCCTCTTTTAGAGCCCATATCGACAAGAGGGTTTAC  70
             ||||||||||||| |   |||||||||||||||||| ||||||||||||||||||||||
Sbjct  2455  GTTACCCTAGGGATAACAGCGCAATCCTCTTTTAGAGTCCATATCGACAAGAGGGTTTAC  2514

Query  71    GACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTT  130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2515  GACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTT  2574

Query  131   CAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTAT  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2575  CAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTAT  2634

Query  191   CTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAGGCCA  238
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2635  CTATGAAGTGCTCTTCTCTAGTACGAAAGGACCGAGAAGAGGAGGCCA  2682
```

Low Biomass 2:

>AC14-BS16SF_G07.ab1

```
Query: None Query ID: lcl|Query_9085 Length: 448


>Culaea inconstans mitochondrial DNA, complete genome
Sequence ID: AB445125.1 Length: 16465
Range 1: 2479 to 2696

Score:324 bits(175), Expect:9e-84,
Identities:202/218(93%),  Gaps:0/218(0%), Strand: Plus/Plus

Query  42    TCCTCTTTTAGAGCCCTTATCTTCNTAAGGGTTTACGACCTCNATGTAGGATCAAGACAT  101
             |||||||||||||  ||  ||||  |   ||||||||||||||| |||| ||||||  |||||
Sbjct  2479  TCCTCTTTTAGAGTCCATATCGACAAGAGGGTTTACGACCTCGATGTTGGATCAGGACAT  2538

Query  102   CCTAATGGGGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACNTGATC  161
             ||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||  |||||
Sbjct  2539  CCTAATGGTGCAGCCGCTATTAAGGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATC  2598

Query  162   TGAGTTCNGACCGGAGCAATTCNGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTAC  221
             |||||||  ||||||||  |||  |  ||||||||||||||||||||||||||||||||||||
Sbjct  2599  TGAGTTCAGACCGGAGTAATCCAGGTCAGTTTCTATCTATGAAGTGCTCTTCTCTAGTAC  2658

Query  222   GAAAGGACCGAGAAGAGGAGGCCAATACCTATGGTACG  259
             |||||||||||||||||||||||||||||||||||||
Sbjct  2659  GAAAGGACCGAGAAGAGGAGGCCAATACCTATGGTACG  2696
```

Vita

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Undergraduate Schools Attended: Spokane Community College,
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