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Name of Candidate: Aiman Raza xm40857@umbc.edu

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Thesis and Abstract Approved:

DocuSigned by:
Tamra Mendelson
87C6E4D57DEF4A1...

Tamra Mendelson

tamram@umbc.edu

Professor

Biological Sciences

4/29/2024 | 4:56 PM EDT

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CURRICULUM VITAE

Name:	Aiman Raza
Degree and date to be conferred:	M.S., 2024
Secondary education:	Al Rahmah School, Windsor Mill, MD August 2015 – June 2018
Collegiate institutions attended:	University of Maryland, Baltimore County Bachelor of Science (Biological Sciences, Minor: Environmental Science) August 2018 – May 2022 University of Maryland, Baltimore County Master of Science (Biological Sciences) August 2022 – May 2024
Major:	Biological Sciences
Professional Publications:	Moyer, M. J., E. M. Rose, D. A. Moreland, **A. Raza**, S. M. Brown, A. L. Scarselletta, B. Lohr, K. J. Odom, and K. E. Omland. 2022. Female song is structurally different from male song in Orchard Orioles, a temperate-breeding songbird with delayed plumage maturation. *Journal of Field Ornithology* 93(1):3. [*Accepted*] Anchondo, Z. B., A. M. Tracy, **A. Raza**, K. Meckler, M. Ogburn. 2024. Reefs in no-take reserves host more oysters, macroparasites, and macrofauna than harvested reefs across an estuarine salinity gradient. *Marine Ecology Press Series*
Professional positions held:	Graduate Research Assistant (August 2022 – May 2024) University of Maryland, Baltimore County Baltimore, MD Undergraduate Research Assistant (August 2019 – May 2022) University of Maryland, Baltimore County Baltimore, MD

Supplemental Instruction Peer Assisted Study Session Leader
(June 2021 – May 2022)
University of Maryland, Baltimore County
Academic Success Center
Baltimore, MD

Summer Research Intern
(June 2020 – August 2020)
Smithsonian Environmental Research Center
Edgewater, MD

Summer Research Intern
(June 2019 – August 2019)
Institute of Marine and Environmental Technology
Baltimore, MD

ABSTRACT

Title of Document:	USING ENVIRONMENTAL DNA TO STUDY BROOK TROUT POPULATIONS IN THE HEADWATERS OF THE CHESAPEAKE BAY
	Aiman Raza, M.S., 2024
Directed By:	Professor Tamra Mendelson, Biological Sciences

Brook Trout (*Salvelinus fontinalis*), a cold-freshwater species native to eastern North America, are bioindicators of cold, clean water. Recent restoration efforts require improved monitoring methods to assess progress toward conservation goals. Environmental DNA (eDNA) is a potentially rapid, cost-effective method to detect Brook Trout presence from water samples. However, eDNA dynamics in aquatic ecosystems are not well understood for this species. We assessed how water temperature, distance from an eDNA source, and filter pore size affect Brook Trout eDNA concentrations. We found significantly higher Brook Trout eDNA concentration at 20 ℃ compared to 10 ℃ in a lab experiment. We were unable to obtain quantifiable eDNA concentration in a natural stream setting, likely due to insufficient water volume collection or filter material. This study highlights the importance of optimized eDNA collection methods for accurate and effective species detection to identify priority restoration streams for Brook Trout conservation.

USING ENVIRONMENTAL DNA TO STUDY BROOK TROUT POPULATIONS IN THE HEADWATERS OF THE CHESAPEAKE BAY

By

Aiman Raza

Thesis submitted to the Faculty of the Graduate School of the
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I would like to extend my gratitude to my ICARE research team and all my various collaborators. This was a shining example of an interdisciplinary, collaborative research project, and I could not have accomplished it alone. I want to give a big thank you to my amazing faculty advisor, Tamra Mendelson, for supporting and encouraging me through the highs and lows of graduate school. I would like to thank my partner mentors, Aaron Aunins, Cheryl Morrison, Nathaniel Hitt, and Stephen Faulker, for sharing their expertise and making my time at Leetown pleasant and welcoming. Special thanks to Karmann Kessler, Karli Rogers, Zack Kelly, and Colleen Young for all the help in the lab and field. I want to thank my community stakeholder, Theaux Le Gardeur, for his enthusiastic attitude and creative ideas throughout the research process. I also want to thank Evan Childress with the National Park Service for his time and for being a wonderful collaborator. Thank you to Mark Staley from MD DNR for providing information and resources essential to the project, and thank you to Bob Hilderbrand for the guidance and insight.

Lastly, I want to extend a big thank you to my awesome ICARE cohort and DART lab members for all the encouragement and support over the past two years. And thank you to my family and friends for being there for me at every step of my journey.

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Introduction

Brook Trout (*Salvelinus fontinalis*) are a culturally, economically, and ecologically valuable freshwater species in the United States (Hudy et al., 2008). Native to eastern North America, this species is part of the char genus in the Salmonidae family. Their remarkable coloration, vermillion underbellies contrasted against dark-green marbled bodies, have also made them popular among anglers. Brook Trout are the state fish of nine U.S. states: Michigan, New Hampshire, New Jersey, New York, North Carolina, Pennsylvania, Vermont, Virginia, and West Virginia. Brook Trout are highly sensitive to water temperature and pollution, making them important bioindicators of cold, clean water (Chadwick et al., 2015). Their presence in small headwater streams has been a hallmark of pristine water quality and excellent stream habitat for centuries. Unfortunately, human development, pollution, and climate change have extirpated Brook Trout populations from over 71% of their native range (Hudy et al., 2008). These human-driven environmental issues are also placing pressure on the already dwindling populations in watersheds across eastern North America.

The Chesapeake Bay watershed, over 64,000 square miles and home to 18 million people, is a national treasure with a rich biodiversity (Chesapeake Bay Program). The watershed has been the focus of intense conservation work by a multitude of organizations to preserve the health of this important ecosystem. This effort also includes protecting the Bay's only native trout species, Brook Trout. The Chesapeake Bay Program (CBP) Watershed Agreement aims to increase Brook Trout populations by 8% by 2025. Many different organizations across the watershed are

developing and implementing significant restoration efforts to achieve this goal. With over 100,000 streams, rivers, and tributaries feeding into the Chesapeake Bay, monitoring Brook Trout populations across the watershed can be cost- and labor-intensive. In addition, considering the size of the watershed, there is a significant need for better monitoring methods to assess progress towards this goal.

Recent breakthroughs in biological monitoring techniques have ushered in a new era of species conservation methods. One such method has been environmental DNA, commonly referred to as eDNA. eDNA is genetic material left in the environment from an organism that can be collected and analyzed to identify the presence of a species (Thomsen & Willerslev, 2015). This technique has revolutionized the study of wild ecosystems, as it does not rely on the physical capture or observation of the species of interest. eDNA is also more cost-effective and less labor-intensive than traditional monitoring methods. Scientists have used eDNA for many purposes across various taxa (Thomsen & Willerslev, 2015). It has been especially useful for detecting rare or cryptic populations, such as early invasive species detection or endangered populations (Piaggio et al., 2014). eDNA is a promising research tool that can supplement traditional monitoring methods like electrofishing. Electrofishing involves sending mild electric shocks through a section of stream to temporarily stun fish to the surface for recording, which causes stress among aquatic organisms (Nolan et al., 2022). eDNA is a less invasive and cheaper method of monitoring fish populations as it only requires collecting a sample of water. Researchers can predict Brook Trout presence and ground truth electrofishing estimates using eDNA (Baldigo et al., 2016; Nolan et al., 2022). However, eDNA is a

relatively new technique, and there are gaps in our understanding of eDNA for identifying species presence. Brook Trout conservation work would greatly benefit from having eDNA as a reliable tool for assessing progress toward restoration goals. This thesis research is an important step in improving our understanding of eDNA dynamics and collection methods for Brook Trout conservation efforts.

This research project was funded by the Interdisciplinary Consortium for Applied Research in the Environment (ICARE) program at the University of Maryland, Baltimore County (UMBC). The ICARE program at UMBC is funded through a National Science Foundation Research Training (NRT) Grant (NSF-DGE-1922579). The program has a dual mission of increasing the diversity of the environmental workforce and conducting community-engaged research in and around the Baltimore Harbor. Each ICARE student has a research team composed of a faculty mentor at UMBC, a non-academic scientist, and a non-scientist community stakeholder. This team allows for an interdisciplinary, multi-faceted approach to developing and executing an applied research project with practical implications for addressing local environmental issues. My ICARE research team for this project consisted of UMBC faculty mentor Tamra Mendelson, U.S. Geological Survey Eastern Ecological Science Center (USGS EESC) scientists Aaron Aunins, Cheryl Morrison, Nathaniel Hitt, and Stephen Faulker, and the Gunpowder Riverkeeper Theaux Le Gardeur. This unique team of academics, government scientists, and community organizations assisted me in developing a master's thesis project that utilized eDNA for Brook Trout conservation in the headwaters of the Chesapeake Bay.

My partner mentors at the USGS EESC provided invaluable guidance on lab techniques and Brook Trout biology. The USGS is a member of the CBP and is obligated to conduct research that helps accomplish program goals, such as the Brook Trout conservation work. The USGS EESC has a genetic laboratory facility in Leetown, WV, conducting cutting-edge research in fish health and genomics. The facility aims to increase research efforts on advancing molecular and genetic methods for advanced species monitoring and conservation, motivating them to join the ICARE Brook Trout eDNA project. All lab work for this project was conducted at the USGS EESC.

Another integral collaboration was with the Gunpowder Riverkeeper, Theaux Le Gardeur. The Gunpower RIVERKEEPER® is a grassroots, advocacy-based, 501(c)(3) nonprofit membership organization charged with protecting, conserving, and restoring the Gunpowder watershed. The Gunpowder Riverkeeper advocates for the health of the Gunpowder watershed by enforcing compliance with environmental laws. This partnership brought a unique community partnership that directly connected this ICARE Brook Trout eDNA project with the Gunpowder watershed. I worked with Theaux to identify Brook Trout streams in the upper Gunpowder Falls watershed to sample for eDNA analysis (see Chapter 3). Working with the Riverkeeper also allowed me to connect directly with the Gunpowder watershed community.

The upper Gunpowder Falls watershed is one of the last remaining Brook Trout strongholds in Maryland. Brook Trout were once common throughout the northwest of the state, but human encroachment on their stream habitats has led to

their extirpation across 62% of their historic range (MD DNR). The three remaining wild Brook Trout populations in the state are in upper Gunpowder Falls watershed in northern Baltimore County, northern Frederick County, and Garret County in the far west. The upper Gunpowder Falls watershed is home to the second-largest MD population, encompassing about 25.2% of the total MD population. These three areas are considered priority Brook Trout restoration watershed by the Maryland Department of Natural Resources (Coldwater Resources Mapping Tool, MD DNR). The relative lack of human development, pollution, and competition with invasive trout species has made these watersheds an excellent Brook Trout habitat. The Gunpowder watershed is not only a critical Brook Trout habitat but also a major drinking water source. The tributaries of the watershed feed into the Prettyboy Reservoir, which supplies about 61% of the drinking water supply of the Baltimore metropolitan area including Baltimore City, Baltimore County, and Anne Arundel County. The presence of Brook Trout in these tributaries is a good sign of water quality and stream habitat, considering the status of Brook Trout as a bioindicator. However, without appropriate conservation efforts, these populations still face extirpation. That is why the Gunpowder Riverkeeper served as the community stakeholder for my ICARE project.

A core principle of the ICARE program is to conduct engaged research in and around Baltimore. This project was not directly community-engaged because we did not receive input on our research goals from community members. However, we did consult with community leaders to focus our sampling efforts on streams of interest. For example, we consulted with the Gunpowder Riverkeeper and MD DNR managers

to identify streams they would be interested in sampling with eDNA (See Chapter 3). We coproduced our research goals with other academic and government entities, such as MD DNR, the National Park Service, and the University of Maryland Center for Environmental Science.

The MD DNR assisted in identifying streams with known Brook Trout occupancy in the Gunpowder watershed for the filter pore size comparison study (see Chapter 3). The MD DNR is a state government agency committed to protecting natural resources and public lands. Managers at the agency are interested in utilizing eDNA to identify streams with Brook Trout occupancy. The agency conducts annual electrofishing surveys across the state and has information on current and historic Brook Trout occupancy. This project is an essential step in optimizing eDNA collection methods for managers to study Brook Trout populations around the state.

We also collected samples in Shenandoah National Park with the help of Evan Childress, a fish biologist at the National Park Service (NPS). We connected with Childress through one of my partner mentors, Nathaniel Hitt, who collaborated with NPS scientists for Brook Trout occupancy modeling work. In the Rappahannock watershed, Shenandoah National Park provides a critical Brook Trout habitat and also conducted a citizen-science Brook Trout eDNA study in 100+ streams across the park. To compare our results, we coordinated sampling sites in Shenandoah that overlapped with their previous Brook Trout study.

Lastly, we consulted with Robert Hilderbrand at the University of Maryland Center for Environmental Science (UMCES) Appalachian Laboratory in Frostburg, MD. Hilderbrand has experience using the Smith-Root eDNA sampling method for

Brook Trout research in MD, including an experiment assessing the effect of distance on eDNA detection by implementing a Brook Trout cage experiment similar to one of our experiments (see Chapter 2). Our experiments had similar results, so we plan to jointly publish them in a publication.

Although this research project was not focused on community engagement, I did engage with the community through outreach activities. On September 28th, 2023, the Gunpowder Riverkeeper invited me to give a talk at the Sparks Bank Nature Center in Sparks Glencoe, MD. The talk, "Forest to Faucet: Safeguarding Clean Drinking Water through Brook Trout Science and Conservation," taught community members about the importance of Brook Trout as a bioindicator and the connection of the Gunpowder to their drinking water supply. Before the talk, I encouraged audience members to complete a survey to assess their prior knowledge about Brook Trout and eDNA. I revealed the answers throughout the talk and, at the end, encouraged the audience to retake the survey to test their new knowledge. The survey is accessible online and through the Nature Center. This outreach experience was an informal and fun way to engage with community members who did not have a science background. The talk also encouraged people to take an interest in the Brook Trout in their neighborhood streams and get involved with volunteer opportunities with the Gunpowder RIVERKEEPER®.

There are a variety of applications for this project for influencing future Brook Trout eDNA studies. Identifying these vulnerable populations can influence where government agencies distribute funding for Brook Trout conservation efforts. This work can recommend best practices for eDNA field collection methods for managers

at MD DNR. We can also compare our findings from our Shenandoah sites with those obtained from the Shenandoah Brook Trout citizen science project. This project also utilized the Smith-Root eDNA sampler, a user-friendly method that non-specialists can employ. The extracted DNA from the eDNA samples we collected can also be archived and assayed for other species of interest in the future.

In summary, eDNA is an efficient method to monitor Brook Trout populations in the headwaters of the Chesapeake Bay. My master's thesis research project improves our understanding of how factors like temperature and collection methods influence eDNA concentration. I accomplished this Brook Trout eDNA project through collaborations with various government and community entities, including the USGS EESC and Gunpowder Riverkeeper. Aligning with the ICARE master's program goals, this project addressed the research needs of optimizing eDNA methods while emphasizing the importance of Brook Trout as bioindicators of clean water.

Chapter 1: Water temperature affects the concentration of Brook Trout (*Salvelinus fontinalis*) eDNA

Abstract

Brook Trout (*Salvelinus fontinalis*) are a cold-freshwater species native to eastern North America and serve as important bioindicators of cold, clean water. Recent conservation efforts require more cost- and labor-effective methods to identify Brook Trout presence in their headwater stream habitats. Environmental DNA (eDNA) is a potential cost- and labor-effective means for species detection. However, the effect of environmental factors such as water temperature on eDNA dynamics is not well understood in this species. We conducted an experiment in an indoor recirculating tank where we exposed Brook Trout to two temperature treatments of 10 ℃ and 20 ℃ and compared the eDNA concentration obtained at each. We found significantly higher Brook Trout eDNA concentration at 20 ℃ compared to 10 ℃. This could be due to increased shedding from thermal stress and increased metabolism at higher temperatures. This provides evidence that temperature is an important factor to consider when detecting Brook Trout presence from eDNA samples.

Introduction

Effective conservation strategies rely on accurate and reliable methods for species detection to protect threatened populations. Environmental DNA (eDNA) is a relatively new method of species detection that is increasingly used for biomonitoring and is revolutionizing how we study and assess biodiversity in aquatic ecosystems

(Bohmann et al., 2014; Thomsen & Willerslev, 2015). eDNA is the genetic material left behind in the environment from an organism in the form of skin cells, scales, hair, mucus, feces, or gametes (Ficetola et al., 2008; Sassoubre et al., 2016). This genetic material can be present in water, soil, sediment, and ice samples (Strickland & Roberts, 2019). eDNA may be bound to other particulate matter in the water or may be extracellular fragments (Turner et al., 2014). eDNA can be collected from environmental samples and amplified with quantitative polymerase chain reaction (qPCR) or droplet digital PCR (ddPCR) using species-specific primers to detect the number of copies of eDNA from one or a few species with high sensitivity (Strickland & Roberts, 2019). Alternatively, metabarcoding is used to assess whole communities with primers that amplify DNA from a group or organisms (Strickland & Roberts, 2019). Thus, eDNA is a relatively non-invasive method of species detection as it does not rely on capturing, harming, or even sighting the target species (Lodge et al., 2012). Researchers have utilized eDNA to detect the presence of a wide variety of taxa, including mammals (Foote et al., 2012), amphibians (Pilliod et al., 2014), insects (Thomsen et al., 2012), reptiles (Piaggio et al., 2014), and many types of fish (Karlsson et al., 2022; Klymus et al., 2015; Nevers et al., 2018; Nolan et al., 2022; Plough et al., 2018; Strickland & Roberts, 2019; Wood et al., 2021). The wide breadth of application has made eDNA a promising tool for studying a variety of ecosystems. eDNA can be a valuable tool in informing the management of threatened aquatic populations, such as Brook Trout (*Salvelinus fontinalis*). (Baldigo et al., 2016; Nolan et al., 2022; Thomsen & Willerslev, 2015).

Brook Trout is a cold-freshwater salmonid native to eastern North America (Mitchill, 1814). Their native range is from the Hudson Bay and Newfoundland in Canada through the Appalachian mountain range down to Georgia (Haxton et al., 2020). Brook trout have experienced a drastic population decline due to human development and increases in stream temperatures accelerated by climate change (Chadwick et al., 2015). These fish rely on pristine stream habitats and clean water from groundwater upwelling zones for spawning, making them important bioindicators of cold, clean water (Haxton et al., 2020). The optimal growth temperature for Brook Trout is between 13 and 16 ℃, with temperatures above 20 ℃ initiating physiological stress responses (Chadwick et al., 2015). Natural resource managers are working to protect and restore Brook Trout habitat, highlighting the need for accurate population monitoring (Nolan et al., 2022). In other parts of the world, Brook Trout have been introduced as an invasive species, further supporting the need for accurate detection of this species (Dunham et al., 2002). Traditionally, researchers have used invasive methods such as electrofishing to study the size and distribution of fish populations (Plough et al., 2018). These methods have cost, labor, and spatial limitations, which increase the risk of imprecise or incomplete data (Nolan et al., 2022). Due to its invasive nature, the electrofishing process can also induce stress, injuries, and mortality in stream organisms (Nolan et al., 2022). eDNA is an emerging tool that can serve as an efficient, non-invasive, and cost-effective method of studying Brook Trout, as it only requires the collection of a water sample.

Despite the promising future of eDNA as an accurate method to detect species presence and perhaps even abundance, eDNA dynamics in aquatic systems (e.g.,

state, transport, persistence, and fate) are still poorly understood. Many factors determine the detectability and concentration of eDNA in the environment. Abiotic factors such as temperature, pH, and ultraviolet (UV) radiation interact with DNA in the environment and influence degradation rates (Jo et al., 2019; Robson et al., 2016). In addition, eDNA concentration is influenced by shedding rates, with more sloughing or shedding leading to higher accumulation of DNA in water (Strickler et al., 2015).

One major factor that might affect eDNA shed rate is temperature. Temperature is one of the most important abiotic factors that affect physiological processes in ectotherms (Beitinger et al., 2000; Somero, 2005). Prior research in lab settings on different fish species has demonstrated mixed results on the relationship between temperature and eDNA shedding (Jo et al., 2019; Klymus et al., 2015; Robson et al., 2016; Takahara et al., 2012). Some studies on carp have found no significant relationship between eDNA shedding and temperature (Klymus et al., 2015; Takahara et al., 2012), whereas other studies have found significantly higher eDNA concentrations at higher temperatures in different fish species (Jo et al., 2019; Robson et al., 2016). Klymus et al. (2015) found no relationship between temperature and eDNA shed rate in two Bighead Carp species (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*). Takahara et al. (2012) also found no relationship between eDNA shedding and the lower temperature range of Common Carp (*Cyprinus carpio* L.). However, Robson et al. (2016) found significantly higher eDNA concentrations at higher temperatures in Mozambique Tilapia (*Oreochromis mossambicus*), a tropic species. Jo et al. (2018) also found a higher eDNA

concentration at higher water temperatures in the Japanese Jack Mackerel (*Trachurus japonicus*). These studies were all conducted in a lab setting but used different fish species with various physiologies and habitats. The relationship between temperature and eDNA shedding in Brook Trout, a cold-dwelling freshwater species, has not yet been explored.

eDNA concentrations may positively correlate with higher temperatures due to increased metabolism and stress. Factors like increased metabolic activity at higher temperatures might increase shedding and eDNA concentrations (Barton, 2002; Wendelaar Bonga, 1997). Cellular stress responses to higher temperatures may increase oxygen consumption, which might translate to passing more water through the gills. Brook Trout generally show faster ventilation rates, which is an index of oxygen consumption, at 20 ℃ compared to lower temperatures (Hitt et al., 2017). The increased gill beat rate could increase shedding, especially near the thermal threshold limits of an organism (Mackey et al., 2021; White et al., 2019). For a temperature-sensitive species like Brook Trout, which exhibit signs of cellular stress responses at temperatures above 20 ℃, temperature likely significantly impacts shedding (White et al., 2019). However, higher temperatures also tend to increase eDNA degradation, which may offset the amount of intact eDNA in the water (Jo et al., 2019). We need to improve our understanding of how temperature affects eDNA shedding at increased temperatures so that eDNA can be used as a practical tool to quantify eDNA levels (Robson et al., 2016). Additionally, as climate change increases stream temperatures worldwide, cold-dwelling fish species will be severely impacted,

increasing the need for accurate monitoring and management strategies (White et al., 2019).

The main objective of this study was to determine the effect of water temperature on Brook Trout eDNA concentration. We exposed Brook Trout to two temperature treatments of 10 ℃ and 20 ℃ in an indoor recirculating tank. The concentration of eDNA obtained at each temperature was compared using a previously described qPCR assay named *BRK2*, specific for a segment of the mitochondrial cytochrome b gene in Brook Trout (Wilcox et al., 2013). We predicted that we would obtain higher eDNA concentrations at 20 ℃ than 10 ℃ because 20 ℃ is near the thermal tolerance limit of Brook Trout, and the increased stress may lead to higher metabolic activity and shedding of eDNA.

Methods

Study Organisms and Experimental Set-up

We conducted the study in an indoor stream laboratory at the U.S. Geological Survey's Eastern Ecological Science Center in Leetown, West Virginia (hereafter referred to as USGS EESC). Trials were run from January 4th, 2023, to February 6th, 2023. We used 15 adult Brook Trout provided by the West Virginia Department of Natural Resources (WV-DNR) state hatchery in Petersburg, West Virginia. Fish were certified disease-free by the WV-DNR. Before housing and experimental trials, we collected fish length and weight and surgically implanted a Passive Integrated Transponder (PIT) tag under each individual's left pectoral fin for identification. We anesthetized the fish before handling for length, weight, and PIT tag implantation using buffered 100 mg l^{-1} MS-222. We housed the fish in five groups of five of

approximately equal biomass in indoor flow-through tanks at an ambient stream temperature of 13℃. Fish were fed commercial pellet food (Floating Zeigler Silver Game Fish Food) at a rate of around 1% of total fish biomass per day.

We conducted experimental trials in recirculating artificial streams separate from the holding tanks. These stream tanks consisted of four 7.6 m stream channels, each containing two riffles and three pools (Figure 1). The four streams were divided into pairs, each sharing a sump, heating, cooling, and filtration system. Fish could swim freely between pools in the stream channels but could not move between channels (White et al., 2019). We used one channel in the pair as an acclimation chamber and another as an experimental chamber. We used the acclimation chamber to acclimate fish to the exposure temperature and the experimental chamber to collect water samples for eDNA analyses.

Temperature Adjustment

We subjected each group to a 10 ℃ and 20 ℃ temperature treatment. Treatment sequences were randomized among the three groups to account for the dependency of temperature effects. We subjected the first and third groups to 10 ℃ followed by 20 ℃, and the second group to 20 ℃ followed by 10 ℃. Prior to placing the fish in the acclimation chamber, we adjusted the water temperature to the ambient stream temperature of the holding tanks (13℃) using a combination of controlled heating (Aqua Logic 12 kW in-line heater; www.aqualogicinc.com) and cooling (Aqua Logic 17.4 kW chiller barrel) (White et al., 2019). To confirm exposure temperatures, we placed a temperature logger in each pool to record the temperature at 15-minute intervals (Onset ProV2). In addition, we verified uniform temperature

throughout the streams using a handheld YSI probe before placing the fish in the chambers.

For each trial, we quickly netted five fish from the holding tank and placed them in the pool farthest from the sump in the acclimation chamber. We adjusted the temperature of both the acclimation and experimental chambers to the target temperature, either 10 ℃ or 20 ℃, at a rate of approximately 1 ℃ per hour. Before moving the fish from the acclimation chamber to the experimental chamber, we turned off the UV light filter in the experimental chamber to prevent accelerated eDNA degradation. Before placing the fish in, we collected a blank eDNA water sample from the sump of the experimental chamber to ensure the absence of Brook Trout eDNA (referred to as "tank blanks"). Using a net, we individually moved each fish from the acclimation chamber to the experimental chamber in the pool furthest from the sump. After one hour, we collected eDNA samples from the sump of the experimental channel. After sample collection, we moved the fish back to the acclimation chamber from the experimental chamber and adjusted the temperature back to 13 ℃ at a rate of approximately 1 ℃ per hour. Once the fish were adjusted to ambient stream temperature in the acclimation chamber, we moved them back to the holding tanks using a net.

eDNA Sample Collection

We filtered water samples using a Smith-Root eDNA backpack sampler according to the manufacturer's instructions (Thomas et al., 2018). Precisely one hour after placing the fish in the experimental chamber, we collected triplicate water samples using 47 mm 1.2-micron polyethersulfone (PES) Smith-Root self-preserving

filters. We collected samples from the sump of the experimental chamber. We filtered 1 L of water simultaneously through three filters using the Smith root trident attachment (SKU: 11203), each collecting approximately 333 mL per filter. Once samples were collected, we placed the filters in their original foil packaging and stored them at room temperature until DNA extraction (Thomas et al., 2019). DNA was extracted from filters within a week of collection.

Cleaning and Disinfecting

We drained and disinfected the artificial streams between trials to prevent eDNA carryover contamination. Once the fish were removed from the experimental chamber, we siphoned out the water from the pools using hoses and used a Shop Vac to drain the remaining water. We sprayed down the tank surfaces with a 5% bleach solution. After 5 minutes, we rinsed the bleach solution with fresh water and vacuumed the excess water from the pools. We disinfected the pipes by passing 1 L of a 5% bleach solution through the pump system and vacuumed the remaining bleach solution. We refilled the entire tank with fresh spring water, and the recirculating system was turned back on, ready for the subsequent trial.

DNA Extraction and Quantitative PCR

All filter eDNA extractions were performed in a UV-sanitized laminar flow hood in a lab space with no PCR product handling to minimize the chance of contamination. We first removed the filters from their filter housing using sterile tweezers, using freshly gloved hands, and taking care not to touch or drop the filter. We extracted the DNA from each whole filter using a Qiagen DNeasy PowerWater DNA extraction kit according to the manufacturer's protocol. This commercially

available kit is specifically for processing water-derived DNA samples and uses a silica column that captures DNA while contaminants are washed through the column (Jane et al., 2015). Extraction blanks were performed with each set of extractions. All DNA samples were eluted using 100 µL of elution buffer and frozen at -20 ℃ until qPCR analysis.

We used a TaqMan© qPCR assay that targets the Brook Trout mitochondrial cytochrome b region, specifically the *BRK2* assay developed by Wilcox et al. (Wilcox et al., 2013). The primer sequences were F-5' CCA CAG TGC TTC ACC TTC TAT TTC TA and R-5' GCC AAG TAA TAT AGC TAC AAA ACC TAA TAG ATC. The FAM-labeled probe sequence was (5-ACTCCGACGCTGACAA-3). We created a standard curve of seven dilution points from a gBlock® synthetic fragment of the region amplified by the primers. Dilution points ranged from 69,734.6 to 4.46 copies/µL (Table 1).

We ran all qPCR reactions on a Qiagen Rotor-Gene Q Real-Time qPCR Cycler with a final reaction volume of 20 µL. Each reaction included 10 µL of PrimeTime® Gene Expression Master Mix, 3.6 µL of F+R primer mix (stock solution of 5 µM F and 5 µM R primers), 1 µL of FAM-labeled probe (5 µM stock solution), 3.4 µL of PCR-grade water, and 2 µL of template DNA. Each qPCR run included one positive control of Brook Trout genomic DNA and two negative no-template controls (NTCs) where PCR-grade water was added instead of the template. All standard curve points were set up in triplicate, and all eDNA samples and extraction blanks were in quadruplicate (technical replicates). qPCR was performed with the following conditions: 10 min at 95°C, 45 cycles of 15 sec at 95°C, and 1 min at 60°C. All qPCR

set-up stages were carried out in a laminar flow UV hood in a lab separate from the eDNA extraction area. We wiped down all surfaces with a 10% bleach solution before and after each set-up and sanitized the hood interior with a 15-minute UV exposure.

The eDNA concentrations of each sample replicate were calculated based on comparing the C_t values to those of the standard curve through relative quantification (Table 1). C_t is the PCR cycle number at which the sample fluorescence is discerned from the background to show a detectable amount of target DNA (Figure 5). C_t is inversely related to the initial DNA copy number; the lower the C_t value, the higher the amount of starting DNA in the sample (Wilcox et al., 2013). The C_t values were calculated using the default settings of the Rotor-Gene software.

The discrete limit of detection (LOD) of the *BRK2* assay, which is the lowest standard tested with >94% positive detections, was previously reported as 10 copies/reaction (Klymus et al., 2020). The discrete limit of quantification (LOQ) of the *BRK2* assay, the lowest concentration that can be accurately quantified, was 50 copies/reaction (Klymus et al., 2020). Ideally, LOD and LOQ should be determined empirically when using different instrumentation or different reagents, such as qPCR Master Mix. The qPCR Master Mix and instrument used by Klymus et al. (2020) differed from what was used in the current study. However, we routinely obtained consistent amplifications of our standard curve points of 4.46 copies/µL, suggesting our LOD and LOQ were at least as sensitive as reported in Klymus et al. (2020).

The copies/µL reported in this study are the copies of the template per µL of the qPCR reaction. To convert the copies/µL values per reaction in this manuscript into copies in the original filtered sample, the following conversion is required:

$$\mu = \left(\frac{(\alpha * \beta)}{\sigma}\right) * \varepsilon$$

where (μ) is the number of eDNA copies in the original water volume sample, (α) is the total qPCR reaction volume, (β) is the reported copies/µL, (σ) is the eDNA sample volume used in the qPCR reaction, and (ε) is the total eDNA sample eluate volume from the DNA extraction.

Statistical Analysis

All statistical analyses and graphical visualizations were performed using R version 4.2.3. We used a mixed-effects model (function *lme*) to test the hypothesis that eDNA concentration is significantly different at 10 ℃ and 20 ℃. We repeatedly measured each group of fish at different temperature treatments. The nested structure of the mixed-effects model accounts for the dependency of the temperature treatment sequence. The experimental units are the groups of fish. This mixed effect model has a fixed effect of temperature treatment and a random effect of the independent group of fish. These effects are expressed in the following model: $y_i = \mu + \alpha_g + b_i + \varepsilon_i$ where eDNA concentration from each group (y_i) depends on global intercept (μ), temperature treatment (α_g), group of fish (b_i), and residual error (ε_i). eDNA concentrations from qPCR replicates were averaged across filters. The null hypothesis is that there is no difference in mean eDNA concentration collected at 10 ℃ and 20 ℃. The alternative hypothesis is that there is a difference in mean eDNA

concentration collected at 10 ℃ and 20 ℃. The model was run with a significance level of $\alpha = 0.05$.

Results

We subjected three unique groups of fish, with five fish per group, to a 10 ℃ and 20 ℃ temperature treatment (six trials in total). At each trial, we collected eDNA samples through three filters simultaneously. The results indicate a significantly higher eDNA concentration obtained at 20 ℃ than 10 ℃ (Repeated Measures ANOVA, $p = 0.0019$). All qPCR standard curves had R and R^2 values around 0.99 and efficiencies near 1.00, indicating assay reliability. There was no detectable Brook Trout eDNA in all blank samples (tank blank, extraction blank, or NTCs), indicating no eDNA contamination between trials.

The mean Brook Trout eDNA concentration across all trials was 122.5 copies/µL at 20 ℃ and 58.2 copies/µL at 10 ℃ (Figure 2). We developed a mixed-effects model with temperature treatment as a fixed effect and group as a random effect. This test showed a significantly higher eDNA concentration collected at 20 ℃ compared to 10 ℃, even when accounting for variance in the group of fish.

Within each group of fish, we obtained a higher eDNA concentration at 20 ℃ than 10 ℃ (Figure 3). However, trials 3 and 4 had a higher level of eDNA concentration than the other trials, even at 10 ℃. Trial 1 had a mean eDNA concentration of 14.5 copies/µL, Trial 2 had a mean of 104.5 copies/µL, Trial 3 had a mean of 158.3 copies/µL, Trial 4 had a mean of 117.0 copies/µL, Trial 5 had a mean of 43.1 copies/µL, and Trial 6 had a mean of 104.6 copies/µL. However, the repeated

measures ANOVA test showed that the sequence of group trials did not significantly affect eDNA concentration.

Discussion

The objective of the study was to determine the effect of water temperature on Brook Trout eDNA concentration. We successfully compared eDNA concentrations at two temperatures within the natural thermal limits of the Brook Trout in an artificial recirculating stream tank system. On average, we collected significantly higher Brook Trout eDNA concentrations at 20 ℃ than at 10 ℃ (Figure 2). Although the second group of fish had overall higher eDNA concentrations at both temperatures, the sequence of group trials was not significant (Figure 3). The results support the prediction that higher water temperature results in higher eDNA concentrations. None of the tank, extraction, or NTC blank samples had any measurable amounts of eDNA concentration, indicating that the disinfection method employed between temperature treatment trials successfully eliminated Brook Trout DNA from the experimental chambers. This result shows that warmer temperatures may significantly affect the concentration of Brook Trout eDNA.

Previous lab studies that have tested the effect of temperature on the eDNA shedding of fishes have found mixed results, with some studies finding that temperature does not affect eDNA concentration (Klymus et al., 2015; Takahara et al., 2012). Klymus et al. (2015) studied two Bighead Carp species (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*) at three temperatures: 19, 25, and 31 ℃. They found no relationship between water temperature and shedding rate. Another study on Common Carp (*Cyprinus carpio* L.) by Takahara et

al. (2012) tested the lower range of temperatures in which carp can survive (7, 15, and 25 ℃) and found no relationship between water temperature and eDNA shedding. The absence of a correlation between eDNA concentration and temperature for some species could be due to those species having a wider thermal tolerance range. However, Robson et al. (2016) found significantly higher eDNA concentrations at higher temperatures in a tropical aquatic system. Using Mozambique Tilapia (*Oreochromis mossambicus*), they collected eDNA samples at 23, 29, and 35 ℃ and found that eDNA levels in the 35 ℃ tanks were significantly higher than the other two temperatures (Robson et al., 2016). This study was one of the first to find a positive relationship between temperature and eDNA shedding and accumulation rates. Jo et al. (2018) found that eDNA shedding rates increased at higher water temperatures in the Japanese Jack Mackerel (*Trachurus japonicus*). They tested four water temperatures (13, 18, 23, and 28 ℃) and three fish biomasses and found that eDNA shedding rates per treatment and biomass tended to increase at higher temperatures (Jo et al., 2019). Our study provides further evidence that temperature plays a significant role in accurately detecting and quantifying eDNA for practical applications.

The increased eDNA concentration at higher temperatures is likely due to increased shedding, which could be explained by higher stress and increased metabolic rate at higher temperatures (Jo et al., 2019; Mackey et al., 2021; Millidine et al., 2008). The optimal temperature for Brook Trout growth and survival is around 15 ℃, with temperatures at or above 20 ℃ triggering elevated cortisol levels and stress-related physiological changes (Mackey et al., 2021). The upper threshold limit

for Brook Trout habitat is 21 – 23.5 ℃ when the mortality risk is high (Mackey et al., 2021). In the study, we exposed fish to a maximum of 20 ℃ for an hour before taking the eDNA sample. We then slowly adjusted the temperature to the ambient stream temperature of around 13 ℃. The thermal tolerance of a species influences the rate at which a stress response occurs. Since Brook Trout are particularly sensitive to water temperature, stress may play an important role in increased shedding at higher temperatures (Nevers et al., 2018). High stress generally reduces swimming activity but increases gill ventilation rates (Hitt et al., 2017). Stress responses include increased shedding of larger-sized DNA particles through mucus and scales (Sassoubre et al., 2016). After exposure to stressful stimuli, cortisol levels could remain elevated for up to 24 hours after exposure (Mackey et al., 2021). Although cortisol levels were not measured here, stress could explain why the second group of fish (Figure 3, Trials 3 and 4) had much higher overall levels of eDNA concentration. The second group was exposed to the thermally stressful 20 ℃ before 10 ℃ and may not have had enough time to recover from elevated cortisol levels before the next trial. Groups 1 and 3, who were exposed to the 10 ℃ before 20 ℃, had lower but similar eDNA concentrations at both temperatures (Figure 3).

The increased stress could also be linked to increased metabolism at higher temperatures. Water temperature is vital for fish growth and metabolism, and metabolic requirements generally increase with temperature (Clarke & Johnston, 1999; Norin & Clark, 2016). As the temperature approaches the lethal limit and cortisol levels rise, an organism expends more energy resources, such as glucose, to maintain homeostasis (Mackey et al., 2021). This process increases tissue metabolic

rates at higher temperatures (Barton, 2002; Wendelaar Bonga, 1997). Although eDNA is known to degrade faster in higher temperatures (Strickler et al., 2015), the experiment results show that high temperatures still significantly increase shedding. However, it should be noted that samples were collected only one hour after exposing the fish to the target temperature. Previous studies measuring eDNA degradation collected samples up to six days later (Jo et al., 2019; Robson et al., 2016; Takahara et al., 2012). One hour might not have been enough time for significant eDNA degradation.

Although we successfully conducted this experiment in an indoor recirculating tank, it may be challenging to translate this to a natural stream environment. The experimental chamber recirculated water during the trials. DNA was allowed to accumulate and recirculate with the water current for the hour the fish were in the experimental chamber. In addition, we switched off the experimental chamber's UV light filter when fish were present. These conditions are different from a natural stream setting. In a natural stream, water continuously flows downstream, and many environmental factors such as UV radiation, water chemistry, microbes, and enzymes degrade eDNA (Jo et al., 2019; Strickler et al., 2015; Thomsen & Willerslev, 2015; Turner et al., 2014). We did not account for these additional environmental factors in an artificial tank setting. Therefore, temperature might not be the most crucial factor that influences eDNA shedding in the environment in a natural stream. Despite this, our experimental results provide a basis for future field-based investigations to consider water temperature.

The results provide evidence of the importance of water temperature in using eDNA for species detection. The study shows that the eDNA shedding rate in Brook Trout is significantly higher at warmer temperatures near the thermal tolerance threshold. As eDNA techniques improve and are used to estimate the abundance of wild populations, it might be essential to consider temperature to quantify eDNA levels accurately (Robson et al., 2016). Depending on the water temperature, eDNA concentration may not accurately predict abundance if fish shed more eDNA at warmer temperatures. Samples taken from the wild at different temperatures are not necessarily comparable, mainly if DNA copy numbers are used to estimate abundance. The results suggest increased chances of Brook Trout eDNA detection at warmer temperatures. Managers wanting to incorporate this technology into tracking and management efforts might consider sampling in warmer months to have a better chance of detecting low population numbers.

It is crucial to test eDNA dynamics in various settings to better understand the effect of temperature on eDNA detection in aquatic environments. A possible follow-up experiment would be to repeat this experiment in a flow-through tank system instead of a recirculating one to better replicate a natural stream setting. Another experiment could repeat the same experiment while measuring physiological responses in the fish. We did not measure physiological and cellular variables in the fish, but that could be valuable in determining the mechanisms of fish stress responses and metabolic rates to better understand shedding. Visual measurements of opercular ventilatory beat rate using a camera could be an accurate, cheap, and non-invasive method to measure energy expenditure in fish to estimate metabolic rate

(Hitt et al., 2017; Millidine et al., 2008). In addition, cortisol levels could be measured for the experiment to estimate fish stress levels at different temperatures (Mackey et al., 2021). Although much progress has been made in the field of eDNA, many new questions about eDNA dynamics continue to be revealed. This study showed that Brook Trout shed more at higher temperatures near their thermal tolerance limit, which could help inform managers to better implement eDNA in conserving this valuable trout species.

Chapter 2: Effect of distance on Brook Trout (*Salvelinus fontinalis*) eDNA concentration

Executive Summary

We conducted a cage experiment using Brook Trout (*Salvelinus fontinalis*) in a natural stream to test the effect of distance on environmental DNA (eDNA) concentration. On March 22nd, 2023, we placed three Brook Trout in a cage in Hopewell Run, a troutless stream in Leetown, WV. After 24 hours, we collected eDNA samples at 1, 10, 50, 100, and 2,000 m downstream of the cage and 5 m upstream. We used the Smith-Root eDNA backpack sampler and filtered triplicate 1L water samples (~333 mL per filter) through 1.2-micron Smith-Root self-preserving filters. We repeated this experiment on June 8th, 2023. DNA was extracted from the filters, and Brook Trout eDNA concentration was measured using quantitative PCR (qPCR). Despite brook trout presence in the stream, no quantifiable Brook Trout eDNA was detected in the samples. We discuss the role of factors such as fish biomass and water sample volume on the lack of Brook Trout eDNA detections in Hopewell Run.

Introduction

Environmental DNA (eDNA) is a relatively new and promising technique for species detection in freshwater ecosystems (Thomsen & Willerslev, 2015). Researchers have been able to use eDNA to detect fish species presence or absence with surprising accuracy (Baldigo et al., 2016; Nolan et al., 2022; Plough et al., 2018; Strickland & Roberts, 2019; Wood et al., 2021). Managers can use eDNA as a

potentially cost- and labor-effective method of monitoring species of interest. eDNA can be especially useful for studying rare or cryptic species that would be otherwise difficult to capture, or may be harmed using traditional methods (Thomsen & Willerslev, 2015). eDNA can be used for single-species identification using quantitative PCR (qPCR), droplet digital PCR (ddPCR), or for multi-species assays using metabarcoding (Thomsen & Willerslev, 2015). eDNA can also track progress toward conservation goals for threatened species, such as Brook Trout (*Salvelinus fontinalis*).

Brook Trout are a freshwater salmonid species native to eastern North America. These fish can only survive in cold, clean water, making them essential bioindicators of water quality (Haxton et al., 2020). Their populations have drastically declined since the European colonization of the Americas due to human development, pollution, and climate change (Hudy et al., 2008). eDNA can help monitor Brook Trout populations and identify priority streams for protection and restoration. For example, eDNA has been used to detect Brook Trout in streams where their occupancy was previously unknown (Nolan et al., 2022). Brook Trout inhabit smaller-order headwater streams that are usually difficult to survey with electrofishing but may be more feasible for eDNA sampling (Nolan et al., 2022).

Numerous studies have successfully used eDNA to predict Brook Trout presence in natural stream settings (Baldigo et al., 2016; Nolan et al., 2022). Beyond presence/absence detection, a major goal of eDNA practitioners is to estimate organism abundance, but there are many factors to consider, such as the distance over which eDNA remains detectable in a stream and the relationship between fish

biomass and amount of eDNA shed. Jane et al. (2015) conducted cage experiments using Brook Trout at a low- and high-flow stream and found that the relationship between eDNA concentration and the downstream distance from the source depended primarily on stream discharge. In the low-flow stream, eDNA concentration linearly decreased as the distance from the cage increased. In contrast, the high-flow stream had relatively stable levels of eDNA at all distances (Jane et al., 2015). The differing eDNA levels at different flows suggest we cannot use eDNA concentration as a direct measure of organism presence or abundance. eDNA plume dynamics are also different at different distances from a source of DNA (Wood et al., 2021). eDNA tends to be concentrated near the center of the stream close to the DNA source and spreads out towards the banks at further distances (Wood et al., 2021). eDNA detection remains complex and depends upon many factors, including biomass, distance, and flow.

The main objective of this study was to determine the effect of distance on Brook Trout eDNA detection and concentration to provide information for fisheries managers who may want to employ eDNA surveys for assessing Brook Trout occupancy. We conducted a cage experiment, placing three Brook Trout in a cage in a troutless stream and comparing eDNA concentrations at various distances. We collected eDNA samples at 1, 10, 50, 100, and 2,000 m downstream of the cage and 5 m upstream. We quantified Brook Trout eDNA concentration using a previously described qPCR assay for Brook Trout. We conducted the trials on March 22nd and June 8th, 2023.

We predicted lower Brook Trout eDNA concentrations at further distances from the cage due to dilution of eDNA and less accumulation at further distances. This expectation would be consistent with the findings of Jane et al. (2015), who found a relationship between distance and eDNA concentration at lower flows, with decreased Brook Trout eDNA concentration at further distances. We also predicted higher eDNA concentrations in June versus March because of a previous experiment in which we measured higher eDNA concentrations at a warmer temperature (see Chapter 1).

Methods

Study Organisms

The study took place at the U.S. Geological Survey Eastern Ecological Science Center (USGS EESC) in Leetown, WV. We used adult Brook Trout provided by the West Virginia Department of Natural Resources (WV-DNR) state hatchery in Petersburg, WV. Fish were certified disease-free by the WV-DNR. Prior to housing and experimental trials, fish length and weight were collected, and we surgically implanted a Passive Integrated Transponder (PIT) tag under the left pectoral fin of each individual for unique identification. We anesthetized the fish before handling for length, weight, and PIT tag implantation using buffered 100 mg l−1 MS-222. Prior to cage experiments in the natural stream, fish were housed in indoor flow-through tanks at an ambient stream temperature of 13℃ and fed commercial pellet food (Floating Zeigler Silver Game Fish Food) at a rate of approximately 1% of total fish biomass per day. Water was supplied to the flow-through tanks from Hopewell Run.

Experimental Setup

We conducted the cage experiments in Hopewell Run (39.355214, -77.933428), a karst stream in Leetown, WV, near the USGS EESC, and a tributary of the Potomac River watershed. Hopewell Run has no known wild Brook Trout population. However, it occasionally receives Rainbow Trout escapees from a nearby effluent pond that receives wastewater from the neighboring U.S. Department of Agriculture facility that drains into Hopewell Run. For cage placement, we selected a pool deep enough to submerge a wire metal cage (106.68 cm long, 78.74 cm wide, 30.48 cm tall) and secured the cage by driving a steel rebar into the streambed at each corner. We measured the distance from the cage to eDNA sampling locations by wading through the stream with an open reel tape measure. We marked the sampling locations using blue flags to ensure sampling took place in the exact location across trials and confirmed approximate distances using a laser rangefinder. The 2000 m site was not physically measured, but its distance was confirmed using a mapping tool and marked by a bridge that crossed over the stream. We conducted the first trial from March 20th – 22nd, 2023, and the second from June 7th – 9th, 2023. The temperature of Hopewell Run typically matches the groundwater-inflow temperature of the Brook Trout holding tanks in the stream lab where fish were held, so temperature acclimation prior to placement in the stream was unnecessary. To avoid thermal shock, laboratory and field temperatures were confirmed prior to placement to ensure fish were not moved between waters with more than one-degree difference.

Before placing Brook Trout in the stream, we collected an eDNA water sample to serve as a baseline measure of Brook Trout eDNA, which should be zero copies detected. We selected three Brook Trout from the stream lab and transported

them in an aerated cooler to Hopewell Run (a distance of approximately 322 m), where they were placed in the cage using a net. The cage was secured from the top using zip ties. Fish were briefly monitored for 10 minutes to ensure they were uninjured from handling. After 24 hours, we collected eDNA samples at 1, 10, 50, 100, and 2000 m downstream from the cage and 5 m upstream of the cage (Figure 4), starting with the most downstream site and working upstream to avoid upsetting sediment. After eDNA sample collection, we removed the fish from the cage and transported them back to the stream lab in an aerated cooler. Stream discharge data was obtained from a nearby USGS gauge (Hopewell Run at Leetown, WV – 01616425).

Sample Collection

We used the Smith-Root eDNA sampler backpack to collect eDNA samples at each sample site approximately 24 hours after placing the fish in the cage. We filtered triplicate water samples using 1.2-micron Smith-Root self-preserving filters (made of polyethersulfone (PES), 47 mm in diameter). We filtered 1 L of water simultaneously through three filters, each filtered approximately 333 mL, using the Smith-Root trident attachment (SKU: 11203). Once samples were collected, we placed the filters back in their original foil packaging and stored them at room temperature until DNA extraction no more than four days later (Thomas et al., 2019).

DNA Extraction and qPCR

We extracted DNA and ran qPCR analyses at the USGS EESC at the Leetown Research Laboratory in Leetown, WV. All filter eDNA extractions were performed in a UV-sanitized laminar flow hood in a lab space with no PCR product handling to

minimize the chances of contamination. We removed the filters from the filter housing using sterile tweezers and gloved hands to avoid any contact with the filter membrane. DNA was extracted from each whole filter using the Qiagen DNeasy PowerWater DNA extraction kit according to the manufacturer's protocol. This commercially available kit is specifically designed for processing water-derived DNA samples and uses a silica column that captures DNA while contaminants are washed through the column (Jane et al., 2015). All DNA samples were eluted using 100 µL of elution buffer and frozen at -20 ℃ until qPCR analysis.

We used a TaqMan© qPCR assay that targets the Brook Trout mitochondrial cytochrome b region, specifically the *BRK2* assay developed by Wilcox et al. (Wilcox et al., 2013). The primer sequences were (F-5' CCA CAG TGC TTC ACC TTC TAT TTC TA) and (R-5' GCC AAG TAA TAT AGC TAC AAA ACC TAA TAG ATC). The FAM-labeled probe sequence was (5-ACTCCGACGCTGACAA-3). A TaqMan Exogenous Internal Positive Control consisting of a separate HEX-labeled probe, primers, and complementary template was included in each qPCR reaction to test for inhibition (ThermoFisher Scientific Catalog Number: 4308321). A difference of 1 – 2 in the C_t values compared to the no template controls would indicate the presence of inhibitors (Hartman et al., 2005). For quantification of brook trout eDNA, we created a standard curve of seven dilution points from a gBlock® synthetic fragment of the mitochondrial cytochrome b region amplified by the primers. Dilution points ranged from 69,734.6 to 4.46 copies/µL (Table 1).

All qPCR reactions were run on a Qiagen Rotor-Gene Q real-time qPCR cycler with a final reaction volume of 20 µL. Each reaction included 10 µL of

PrimeTime® Gene Expression Master Mix, 3.6 µL of F+R primer mix (stock solution of 5 uM F and 5 uM R primers), 1 µL of FAM-labeled probe (5 uM stock solution), 1 µL of PCR-grade water, 2 µL of 10X Eco IPC mix, 0.4 µL of 50X Exo IPC DNA, and 2 µL of template DNA. Each qPCR run included one positive control consisting of Brook Trout genomic DNA from a random wild individual from North Carolina in an unrelated study and two negative no-template controls where PCR-grade water was added instead of the template. All standard curve points were set up in triplicate, and all eDNA samples and extraction blanks were in quadruplicate (technical replicates). qPCR was performed with the following conditions: 10 min at 95°C, 45 cycles of 15 sec at 95°C, and 1 min at 60°C. We carried out all stages of qPCR setup in a laminar flow UV hood in a lab separate from the eDNA extraction area. All surfaces were wiped down with a 10% bleach solution before and after each setup, and the hood interior was sanitized before and after uses with a 15-minute UV exposure.

The eDNA concentrations of each sample replicate were calculated based on comparing the C_t values from the standard curve (Table 1). C_t is the PCR cycle number at which the sample fluorescence is discerned from the background to show a detectable amount of target DNA (Figure 5). C_t is inversely related to the initial DNA copy number; the lower the C_t value, the higher the amount of starting DNA in the sample (Wilcox et al., 2013). The C_t values were calculated using the default settings of the Rotor-Gene software.

The discrete limit of detection (LOD) of the *BRK2* assay, which is the lowest standard tested with 95% or greater positive detections, was previously reported as 10 copies/reaction (Klymus et al., 2020). The discrete limit of quantification (LOQ) of

the *BRK2* assay, the lowest concentration that can be accurately quantified, was 50 copies/reaction (Klymus et al., 2020). Ideally, LOD and LOQ should be determined empirically when using different instrumentation or different reagents, such as qPCR Master Mix. The qPCR Master Mix and instrument used by Klymus et al. (2020) differed from what was used in the current study. However, we routinely obtained consistent amplifications of our standard curve points of 4.46 copies/µL, suggesting our LOD and LOQ were at least as sensitive as reported in Klymus et al. (2020). The copies/µL reported in this study are the copies of template per µL of the qPCR reaction. To convert the copies/µL values per reaction in this manuscript into copies in the original filtered sample, the following conversion is required:

$$\mu = \left(\frac{(\alpha * \beta)}{\sigma}\right) * \varepsilon$$

where (μ) is the number eDNA copies in the original water volume sample, (α) is the total qPCR reaction volume, (β) is the reported copies/µL, (σ) is the eDNA sample volume used in the qPCR reaction, and (ε) is the total eDNA sample eluate volume from the DNA extractions.

Results

All qPCR standard curves had R and R^2 values around 0.99 and efficiencies near 1.00, indicating assay reliability. Most samples collected downstream from the caged fish yielded little to no detectable eDNA at any distance (Table 2). Many downstream samples had no amplification, indicated by 0 copies/µL (Table 2). For the samples that did amplify, many of them yielded too few copy numbers to quantify and were below the lowest point of the standard curve (Figure 6). In addition, most of

the samples that amplified were not consistent between replicates. All field blanks taken before fish were placed in the stream were confirmed to be free of Brook Trout eDNA, though this result should be interpreted with caution given that no positive detections were obtained below the caged brook trout (Table 2). There was no evidence of PCR inhibition in any of our samples.

The water temperature was approximately 11.8 ℃ during March sampling and 15.5 ℃ in June sampling. The average Hopewell Run discharge was 0.15 m^3/s at the start of March sampling and 0.08 m^3/s at the start of June sampling.

Discussion

This study aimed to determine the effect of distance from caged Brook Trout on eDNA concentration in a natural stream setting. The qPCR analysis yielded few copies of amplified Brook Trout DNA in the samples at all distances (Table 2). The results were similarly inconclusive in both the March and June trials. We expected eDNA concentrations to be highest directly downstream of the cage and decrease as distance increased. However, we obtained low copy numbers across all distances, even 1 m directly downstream of the caged fish. This result indicates that our selected pore size filter and filtered water volume were insufficient to quantify eDNA of the Brook Trout we placed into Hopewell Run.

We expected to collect sufficient eDNA concentrations because previous studies have been able to detect Brook Trout in natural stream settings (Baldigo et al., 2016; Jane et al., 2015). Jane et al. (2015) conducted a cage experiment in which they placed five Brook Trout in two troutless streams and collected eDNA 24 hours later.

They measured nine distances downstream of the cage, between 27.5 m and 239.5 m. We modeled our cage experiment on the study by Jane et al. (2015). They found detectable levels of eDNA at all measured distances, with stream discharge significantly affecting eDNA concentration. They also observed a positive relationship between biomass and eDNA concentration and interactions between distance, flow, and biomass in each stream (Jane et al., 2015). However, Jane et al. (2015) collected 6 L of water filtered through 1.5-micron glass fiber filters with a peristaltic pump, whereas we collected ~333 mL per filter and used a 1.2-micron PES filter. It is possible that the volume we filtered was not sufficient to concentrate Brook Trout eDNA from Hopewell Run.

Another difference between our methods and those of Jane et al. (2015) is the Brook Trout qPCR assay used. They used the *BRK1* assay described in Wilcox et al. (2013), and we used the *BRK2* assay from the same paper (Wilcox et al., 2013). The *BRK1* assay maximizes probe sequence mismatches between Brook Trout and non-Brook Trout salmonid species, while *BRK2* maximizes primer sequence mismatches (Wilcox et al., 2013). Wilcox et al. (2013) compared the two assays and found that the *BRK2* assay was more specific to Brook Trout than the *BRK1* assay. This indicates that the *BRK2* assay that we used should have been more efficient at amplifying Brook Trout eDNA, but it is possible that it was not specific enough for the fish we used. Our Brook Trout were from the east coast of the U.S., and those analyzed in Wilcox et al. (2013) were from the west coast. Geographically distant populations of stream-dwelling fishes such as brook trout could possibly have a polymorphism in the primer or probe binding region that influences assay sensitivity

(Wilcox, Carim, et al., 2015)). However, this assay was successfully used with the same hatchery trout in a previous indoor tank study we conducted (see Chapter 1). Therefore, it is unlikely that the qPCR assay was the reason for low Brook Trout detection in the natural stream setting.

Baldigo et al. (2016) also successfully quantified Brook Trout eDNA in a natural stream setting. They aimed to improve sampling methods and determine the accuracy of presence/absence predictions based on the density of wild Brook Trout populations in 40 streams in the Adirondack Mountains (Baldigo et al., 2016). They found that their eDNA results correctly predicted the presence and confirmed the absence of Brook Trout in 85 – 92.5% of study sites (Baldigo et al., 2016). They also found that their eDNA samples explained 44% of the variability in population densities and 24% of the variability in biomass. Baldigo et al. (2016) used the same collection method as Jane et al. (2015), a sample volume of 6 L of water per filter, and filtered it through 1.5-micron glass fiber filters with a hand pump. These two studies used eDNA to detect Brook Trout presence and density, even at low target organism densities (Baldigo et al., 2016; Jane et al., 2015). This lends additional support to the reason for differences in our eDNA results that may be due to differences in collection methods.

There are multiple reasons why our experiment may not have yielded sufficient Brook Trout eDNA concentrations. One possible explanation is that Brook Trout biomass was below detectable levels. We collected eDNA from three caged fish, but Jane et al. (2015) collected eDNA from five caged fish. There is likely to be

a higher abundance of Brook Trout in streams with known Brook Trout occupancy; however, it would be essential to know the lower limits of Brook Trout eDNA detection in streams with low densities for management purposes. Robert Hilderbrand at the University of Maryland Center for Environmental Science Appalachian Laboratory in Frostburg, Maryland, obtained similar results from a Brook Trout cage experiment they conducted in 2022 (unpublished data). Hilderbrand et al. used one, five, and ten caged fish and used the Smith-Root eDNA backpack sampler and collected 1 – 2 L per filter with the 1.2-micron PES self-preserving filters. However, they failed to detect Brook Trout eDNA at any distance downstream of the cage, even with ten fish using the *BRK2* qPCR assay. The densities that they tested may be too low to detect using qPCR.

The spatial distribution of eDNA is also not uniform throughout streams, with eDNA plumes observed to be more concentrated midstream near a source and dispersing towards the banks at further distances (Wood et al., 2021). Wood et al. (2021) conducted a cage experiment with juvenile Atlantic Salmon (*Salmo salar*) abundances ranging from 3-36 individuals and found that eDNA plume dynamics corresponded with that observation. Their study challenged the previously held idea that streams are well-mixed and cause gradual loss in eDNA downstream from a source (Jane et al., 2015; Wood et al., 2021). This observation of eDNA plume dynamics might also explain the non-repeatable positive eDNA detections among qPCR replicates in our samples (Table 2). The lack of thorough mixing near the cage might explain the patchy eDNA detections at closer distances. The eDNA particles

may have become more thoroughly and uniformly mixed further downstream, but concentrations might have been too low to detect at further distances.

Another explanation for the low eDNA concentrations is insufficient sample volume collection. Jane et al. and Baldigo et al. collected 6 L of water per filter, whereas we collected about 333 mL per filter. Although this sample volume was sufficient to capture enough eDNA in a previous recirculating indoor tank study (see Chapter 1), a higher water volume might be required for DNA collection in the field. Baldigo et al. (2016) suggested that higher volumes of water be collected at lower densities, recommending filtering 12 L of water to detect low eDNA concentrations accurately (Baldigo et al., 2016). A higher filtered water volume may allow more eDNA to accumulate on the filter and increase the chances of species detection in the field where eDNA concentration may be low (Minamoto et al., 2016). However, turbidity limits how much water can be filtered through a single filter until clogging with sediment. In Hopewell Run, the maximum water volume we could collect through a single filter before clogging ranged from 0.82 – 1.24 L. One solution is to use a larger pore size to collect higher water volumes in turbid water (Thomas et al., 2018). Smith-Root manufactures three pore sizes of self-preserving filters: 0.45, 1.2, and 5 microns. A larger pore size, such as 5 microns, may allow a higher water volume to be collected before clogging, which could be better in high-turbidity streams such as Hopewell Run.

Fish eDNA fragments have been demonstrated to exist between 1 – 10 microns, suggesting filter materials and pore size should be chosen to capture eDNA

in this size range (Turner et al., 2014). However, filter materials do not all perform the same, and there are tradeoffs between different filter materials and storage methods. For example, Smith-Root self-preserving PES filters may be superior in preserving eDNA compared to standard ethanol storage methods (Thomas et al., 2019). The self-preserving filters also prevent contamination by limiting necessary contact with the filter membrane and can be stored at room temperature for up to six months (Thomas et al., 2019). Another study found that cellulose nitrate outperformed PES in eDNA yield. (Majaneva et al., 2018). Glass fiber filters were used by both Jane et al. (2015) and Baldigo et al. (2016). When tested against other filter membrane materials, one study found that glass fiber filters outperformed other materials for the passive collection of amphibian eDNA (Chen et al., 2022). Glass fiber filters capture more eDNA fragments in the filter matrix as opposed to polycarbonate filters, which capture large fragments on the filter surface (Eichmiller et al., 2016). However, glass fiber filters must be removed from the filter housing and frozen after filtration, increasing the chances of contamination through additional handling. Various filter materials, therefore, present tradeoffs between ease of handling, preservation methods, and eDNA capture efficiency.

We used a different water filtration method from previous Brook Trout eDNA studies. The pumping method may vary in efficiency (e.g. pressure driven vs vacuum filtration), and the effect of different filtration methods on eDNA collection efficiency is unclear. Jane et al. (2015) used a peristaltic pump and vacuum filtration, while Baldigo et al. (2016) used a hand pump and vacuum filtration. The Smith-Root eDNA backpack sampler uses a diaphragm pump and vacuum filtration, automating the

collection process by regulating the filtration rate and pressure across the filter (Thomas et al., 2018). Too high of filter pressure (e.g., when trying to obtain a target volume filtered in turbid water, and the last small remaining volume left takes many minutes longer than the initial three-quarters of water filtered) is hypothesized to reduce retention of eDNA on the filter membrane, though the exact mechanism of why this happens is unclear (Thomas et al., 2018). The Smith-Root sampler allows for more efficient and replicable eDNA collection through precise measurement of the volume filtered and measurement of the pressure applied to the filter (Thomas et al., 2018). Therefore, it is not likely that the Smith-Root filtration method was the reason for our low Brook Trout eDNA collection since all methods used vacuum filtration.

Our study reveals the many challenges in using eDNA to detect low Brook Trout densities in a natural stream setting. Although researchers have successfully used eDNA to estimate Brook Trout presence and observed some instances of significant correlation with abundance, collection methods are obviously not interchangeable (Baldigo et al., 2016; Jane et al., 2015; Nolan et al., 2022). One of the primary hypotheses for the low detection in our study is the volume of water we filtered in each sample. Higher water volumes should be filtered in the future; however, this can be a challenge in turbid waters like we encountered in Hopewell Run, where there is a risk of filter clogging even at low volumes. A workaround is to use a larger pore size to allow more water to be filtered and delay filter clogging. Future research should focus on identifying the effect of filter pore size on collecting sufficiently high volumes of water in a natural stream setting. Using a larger filter

pore size may be a way to increase collection volume to increase eDNA collection. As we improve our understanding of eDNA methods in stream applications, we aim to be able to use eDNA to estimate the presence and, eventually, abundance of wild Brook Trout populations accurately.

Chapter 3: Effect of filter pore size on Brook Trout eDNA detection in streams with known Brook Trout occupancy

Executive Summary

This study aimed to compare the effect of filter pore size on Brook Trout (*Salvelinus fontinalis*) environmental DNA (eDNA) concentration in natural streams with known Brook Trout occupancy. On September 22nd, 2023, we sampled three streams in Shenandoah National Park in the Rappahannock watershed, VA. On September 27th and October 12th, 2023, we sampled three streams in the Gunpowder Falls watershed, MD. The Smith-Root eDNA backpack sampler was used to collect triplicate 3 L water samples using two different filter pore sizes (1.2 and 5 microns) at each stream. We also measured water turbidity at each site using a transparent turbidity tube with a Secchi disk. DNA was extracted from the filters, and Brook Trout eDNA concentration was measured using the *BRK2* TaqMan quantitative PCR (qPCR) assay. Hogcamp Branch in Shenandoah National Park was the only site to yield quantifiable eDNA, and there was no significant difference between eDNA concentrations obtained with the 1.2- or 5-micron filters. However, the sample size was too low to consider this a robust result, necessitating further studies. This study highlights the importance of optimizing sampling methods to avoid false negatives.

Introduction

Recent advances in biological monitoring techniques have ushered in a new era of species conservation strategies. Environmental DNA (eDNA) is a relatively new and promising technique for species detection in freshwater ecosystems.

Researchers use eDNA to detect fish species presence or absence with surprising accuracy (Baldigo et al., 2016; Nolan et al., 2022; Plough et al., 2018; Strickland & Roberts, 2019; Wood et al., 2021). Managers can use eDNA as a potentially cost- and labor-effective method of monitoring species of interest. eDNA can be especially useful for studying rare or cryptic species that would otherwise be difficult to capture or may be harmed using traditional methods (Thomsen & Willerslev, 2015). eDNA can be used to detect single species using quantitative PCR (qPCR), droplet digital PCR (ddPCR), or multi-species identification using metabarcoding (Thomsen & Willerslev, 2015). eDNA can also track progress toward conservation goals for threatened species, such as Brook Trout (*Salvelinus fontinalis*).

Brook Trout are a cold-dwelling freshwater species native to eastern North America. This species can only survive in cold, clean water, making them essential bioindicators of water quality (Haxton et al., 2020). Their populations have drastically declined since the European colonization of the Americas due to human development, pollution, and climate change (Hudy et al., 2008). Brook Trout inhabit smaller-order headwater streams that can be difficult to survey with electrofishing (Nolan et al., 2022). Many streams remain unassessed for Brook Trout occupancy due to limitations of electrofishing, but eDNA can serve as a cost-effective method to identify Brook Trout presence. eDNA can help monitor Brook Trout populations in less accessible streams and identify priority streams for protection and restoration.

Numerous studies have successfully used eDNA to predict Brook Trout presence in natural stream settings (Baldigo et al., 2016; Jane et al., 2015; Nolan et al., 2022). Baldigo et al. (2016) concluded that eDNA is an effective tool for

estimating biomass and density, even at low population numbers. Compared to electrofishing data, their results correctly predicted the presence and confirmed the absence of Brook Trout in most of the 40 streams they sampled across the Adirondack region in upstate New York (Baldigo et al., 2016). Nolan et al. (2022) further provided technical validation for using eDNA in Brook Trout monitoring by comparing eDNA results to traditional survey methods. They were able to confirm presence using eDNA in most sites where Brook Trout were surveyed using electrofishing and detected Brook Trout in two streams where their occupancy was previously unknown (Nolan et al., 2022). Supplementing traditional Brook Trout surveys with eDNA analysis can improve our estimates of fish presence in streams. Optimizing eDNA sampling methods before widespread implementation and adoption is essential to maximize the chances of species detection and ensure inferences about presence and absence are most likely correct.

Selecting the appropriate filter pore size is essential for maximizing the amount of eDNA collected in one sampling event (Turner et al., 2014). Most fish eDNA particles exist in the aquatic environment as intracellular organelles and extracellular fragments, ranging between 1 – 10 μm in size (Turner et al., 2014; Wilcox, McKelvey, et al., 2015). Therefore, filter pore size can affect how much and what kind of eDNA fragments can be collected. Smaller pore sizes clog faster in higher turbidity water, which could lead to insufficient eDNA collection as a result of not enough water being filtered (Muha et al., 2019). A larger pore size is one way to collect more water at high turbidity, but it might not capture finer eDNA particles.

However, it might allow for higher water volume collection before clogging (Baldigo et al., 2016).

The main objective of this study was to determine the effect of filter pore size on Brook Trout eDNA concentration in streams with known Brook Trout occupancy. We aimed to test whether a larger pore size would allow us to capture higher eDNA concentrations, increasing the chances of accurate Brook Trout detection. Using the 1.2- and 5-micron Smith-Root self-preserving filters, we collected samples in the Gunpowder watershed in Maryland and the Rappahannock watershed in Virginia in September – October 2023. We collected eDNA samples using the Smith-Root eDNA backpack sampler from three streams with known Brook Trout occupancy from each watershed. We quantified Brook Trout eDNA concentrations using *BRK2* TaqMan qPCR assay (Wilcox et al., 2013). We also attempted to collect turbidity measurements using a transparent turbidity tube with a Secchi disk.

We hypothesized that the 5-micron filter would yield higher Brook Trout eDNA concentrations than the 1.2-micron filter by allowing larger particles to collect on the filter membrane and minimizing the shearing of DNA fragments (Wilcox, McKelvey, et al., 2015; Yates et al., 2021). The larger pore size will also minimize the chances of the filter clogging before the target sample volume collection. We expected the sites with higher eDNA concentrations to correspond with less turbid water.

Methods

Study Site Selection

We collected eDNA samples in streams with known Brook Trout occupancy in the Gunpowder Falls watershed, Maryland, and Rappahannock watershed, Virginia. The Maryland Department of Natural Resources (DNR), National Park Service (NPS), and the Gunpowder Riverkeeper assisted in identifying accessible streams with known Brook Trout occupancy. Streams had similar bedrock geologies within each watershed.

The sites in the Rappahannock River watershed within Shenandoah National Park were Mill Prong (38.49202, -78.42065), Laurel Prong (38.49065, -78.42087), and Hogcamp Branch (38.52275, -78.42057). Shenandoah National Park electrofishing surveys confirmed Brook Trout populations in Hogcamp Branch. Although Mill and Laurel Prong did not have recent electrofishing surveys, we observed Brook Trout fry near the sampling sites in both streams. Thus, we confirmed that all our sampling sites had Brook Trout present. All Shenandoah sites were sampled on September 22nd, 2023.

The sites in the Gunpowder River watershed were Walker Run (39.6914090, -76.7778250), Gunpowder mainstem near Silver Run (39.6841942, -76.7707722), and Bush Cabin Run (39.5987120, -76.7076270). Walker Run and Bush Cabin Run are smaller order streams. Electrofishing surveys indicated a Brook Trout population in Silver Run. Due to difficulties in reaching this site, water was sampled at the confluence of Silver Run and the Gunpowder mainstem. Every Gunpowder site had established Brook Trout populations according to DNR summer electrofishing surveys. Walker Run and the Gunpowder mainstem near Silver Run were sampled on September 27th, 2023, and Bush Cabin Run was sampled on October 12th, 2023.

Sample Collection

eDNA samples were collected using a Smith-Root eDNA sampler backpack to compare the 1.2- and 5-micron self-preserving Smith-Root filters. The Smith-Root sampler was equipped with the Trident attachment to enable simultaneous collection of triplicate water samples (SKU: 11203). The filters are made of polyethersulfone (PES) and are 47 mm in diameter. Before each day of sample collection, we filtered 2 L of distilled water through a 1.2-micron filter as a negative control blank at the stream site. At each of the six sites, we collected triplicate water samples using the 1.2-micron filters, filtering 3 L of water simultaneously through three filters, collecting approximately 9 L total. The same process was repeated with the 5-micron filters with the same volume filtered. After each day of sample collection, we filtered another 2 L of distilled water through a 1.2-micron filter as a negative control. Filters were placed back in the original foil packaging and stored at room temperature until DNA extraction within a month (Thomas et al., 2019). The Smith-Root eDNA sampler was disinfected between watersheds by filtering a 2% bleach solution through the tubing and rinsed with purified water.

Turbidity Measurements

We attempted to take turbidity measurements at each stream using a 100 cm long transparent turbidity tube with a small Secchi disk at the bottom (Eisco Scientific). The tube was filled with stream water, and a mechanism near the bottom was used to drain the tube slowly. The height at which the black and white pattern Secchi disk became visible was recorded as the Secchi depth of the water. A larger Secchi depth indicates the water is more transparent than a shallow Secchi depth,

which suggests high turbidity. We photographed the top of the tube looking vertically down at the disk.

DNA Extraction and qPCR

We extracted DNA from all filters and ran qPCR analysis at the USGS Eastern Ecological Science Center at the Leetown Research Laboratory in Leetown, WV. All filter extractions were performed in a UV-sanitized laminar flow hood in a lab space with no PCR product handling to minimize the chances of contamination. Filters were removed from the filter housing using sterile tweezers and gloved hands to avoid contact with the filter membrane. DNA was extracted using the Qiagen DNeasy PowerWater DNA extraction kit according to the manufacturer's protocol. This commercially available kit is specifically for processing water-derived DNA samples. This kit uses a silica column that captures DNA while contaminants are washed through the column (Jane et al., 2015). All DNA samples were eluted using 100 μL of elution buffer and frozen at -20 ℃ until qPCR analysis.

We used a TaqMan© qPCR assay that targets the Brook Trout mitochondrial cytochrome b region, specifically the *BRK2* assay developed by Wilcox et al. (Wilcox et al., 2013). The primer sequences were (F-5' CCA CAG TGC TTC ACC TTC TAT TTC TA) and (R-5' GCC AAG TAA TAT AGC TAC AAA ACC TAA TAG ATC). The FAM-labeled probe sequence was (5-ACTCCGACGCTGACAA-3). A TaqMan Exogenous Internal Positive Control consisting of a separate HEX-labeled probe, primers, and complementary template was included in each qPCR reaction to test for inhibition (ThermoFisher Scientific Catalog Number: 4308321). A difference of 1 – 2 in the C_t values compared to the no template controls would indicate the presence of

inhibitors (Hartman et al., 2005). For quantification of brook trout eDNA, we created a standard curve of seven dilution points from a gBlock® synthetic fragment of the mitochondrial cytochrome b region amplified by the primers. Dilution points ranged from 69,734.6 to 4.46 copies/μL (Table 1).

All qPCR reactions were run on a Qiagen Rotor-Gene Q real-time qPCR cycler with a final reaction volume of 20 μL. Each reaction included 10 μL of PrimeTime® Gene Expression Master Mix, 3.6 μL of F+R primer mix (stock solution of 5 μM F and 5 μM R primers), 1 μL of FAM-labeled probe (5 μM stock solution), 1 μL of PCR-grade water, 2 μL of 10X Eco IPC mix, 0.4 μL of 50X Exo IPC DNA, and 2 μL of template DNA. Each qPCR run included one positive control of brook trout genomic DNA and two negative no-template controls where PCR-grade water was added instead of the template. All standard curve points were set up in triplicate, and all eDNA samples and extraction blanks were in quadruplicate (technical replicates). qPCR was performed with the following conditions: 10 min at 95°C, 45 cycles of 15 sec at 95°C, and 1 min at 60°C. We carried out all stages of qPCR setup in a laminar flow UV hood in a lab separate from the eDNA extraction area. All surfaces were wiped down with a 10% bleach solution before and after each setup, and the hood interior was sanitized before and after uses with a 15-minute UV exposure.

The eDNA concentrations of each sample replicate were calculated based on comparing the C_t values to those of the standard curve through relative quantification (Table 1). C_t is the PCR cycle number at which the sample fluorescence is discerned from the background to show a detectable amount of target DNA (Figure 5). C_t is inversely related to the initial DNA copy number; the lower the C_t value, the higher

the amount of starting DNA in the sample (Wilcox et al., 2013). The C_t values were calculated using the default settings of the Rotor-Gene software.

The discrete limit of detection (LOD) of the *BRK2* assay, which is the lowest standard tested with 95% or greater positive detections, was previously reported as 10 copies/reaction (Klymus et al., 2020). The discrete limit of quantification (LOQ) of the *BRK2* assay, the lowest concentration that can be accurately quantified, was 50 copies/reaction (Klymus et al., 2020). Ideally, LOD and LOQ should be determined empirically when using different instrumentation or different reagents, such as qPCR Master Mix. The qPCR Master Mix and instrument used by Klymus et al. (2020) differed from what was used in the current study. However, we routinely obtained consistent amplifications of our standard curve points of 4.46 copies/µL, suggesting our LOD and LOQ were at least as sensitive as reported in Klymus et al. (2020). The copies/µL reported in this study are the copies of template per µL of the qPCR reaction. To convert the copies/µL values per reaction in this manuscript into copies in the original filtered sample, the following conversion is required:

$$\mu = \left(\frac{(\alpha * \beta)}{\sigma}\right) * \varepsilon$$

where (μ) is the number eDNA copies in the original water volume sample, (α) is the total qPCR reaction volume, (β) is the reported copies/µL, (σ) is the eDNA sample volume used in the qPCR reaction, and (ε) is the total eDNA sample eluate volume from the DNA extractions.

Statistical Analysis

All statistical analyses and graphical visualizations were performed using R version 4.2.3. Only values that were above the lowest dilution point of 4.46 copies/µL

on the qPCR standard curve were quantifiable. Only sites with a majority (more than 2/3rds) replicates above this threshold were considered for statistical analysis. The only site to fit these criteria was Hogcamp Branch in Shenandoah. A Wilcoxon rank sum test was used to test whether eDNA concentrations from different filter pore sizes were significantly different in Hogcamp Branch. Values of copies/µL per qPCR replicate were averaged across filters.

Results

All qPCR standard curves had R and R^2 values around 0.99 and efficiencies near 1.00, indicating assay reliability. There was no detectable Brook Trout eDNA in all negative control field blanks (distilled water) (Table 3). There was no evidence of PCR inhibition in any of the samples. The eDNA concentrations obtained at most sites yielded detections of eDNA below the lowest point of the standard curve and were thus not quantifiable, except for the third Shenandoah site: Hogcamp Branch (Table 3). There was no significant difference in Brook Trout eDNA concentration yielded from the 1.2- or 5-micron filters in Hogcamp Branch (Wilcoxon Rank Sum Test, $p = 0.1904$). However, this result is not robust due to the small sample size. Although our assay was more sensitive to detecting lower copy numbers than Klymus et al. (2020), we could not accurately quantify most of the samples within the dynamic range of the assay (i.e., within the points of the standard curve) due to low copy numbers of brook trout eDNA in the field samples.

We were able to obtain sufficient eDNA concentration to quantify from Hogcamp Branch, which was the third site sampled in Shenandoah. The mean eDNA concentration obtained with the 5-micron filters was higher than the 1.2-micron

filters, but there was no significant difference between the mean Brook Trout eDNA concentration between the two filter pore sizes (Figure 7). However, the sample size is too small to consider this a robust conclusion. The other two Shenandoah streams, Mill Prong and Laurel Prong had low detectable Brook Trout eDNA levels but were not high enough to quantify accurately as they amplified past the lowest point of the standard curve, and as such can only be noted as possible indications of presence of Brook Trout. In the Gunpowder River watershed, Walker Run had no detectable Brook Trout eDNA levels. The Gunpowder mainstem, near Silver Run, had low levels of detectable Brook Trout eDNA. However, just as for the two streams in the Rappahannock watershed, the concentration was not high enough to accurately quantify. The last Gunpowder site, Bush Cabin Run, had almost no detectable Brook Trout eDNA. Samples with no amplification are indicated by 0 copies/μL (Table 3). For the samples that did amplify, many of them yielded too few copy numbers to quantify and were below the lowest point of the standard curve (Figure 6).

We attempted to measure stream water turbidity at every eDNA collection site. The maximum height at which the Secchi disk was discernable from the top of the tube is a measurement for turbidity. However, the Secchi disk at the bottom was visible when the 100 cm long tube was completely filled, indicating a Secchi depth of >100 cm for all sites. The clear stream water made it impossible to quantitively compare the turbidity across streams using the turbidity tube (Figure 8).

Discussion

This study aimed to determine the effect of filter pore size on Brook Trout eDNA concentration in streams with known Brook Trout occupancy. We obtained

sufficient Brook Trout eDNA concentrations in the one Shenandoah site, Hogcamp Branch (Table 3). The qPCR analysis yielded low copies of amplified Brook Trout eDNA in the other two Shenandoah sites and almost no copies amplified in the Gunpowder watershed (Table 3). Also, we could not obtain comparable turbidity measurements because the water was too clear to measure using a transparent turbidity tube. We expected to obtain higher eDNA concentrations using the 5-micron compared to the 1.2-micron filter pore size. However, there was no significant difference between the concentrations from the 1.2- and 5-micron filters in Hogcamp Branch (Figure 7). Therefore, there is some indication that pore size does not affect eDNA concentration, but the sample size is too low to consider this a robust result. Further studies are necessary to ascertain the effect of filter pore size on eDNA concentration.

Previous studies successfully utilized eDNA sampling to detect Brook Trout presence (Baldigo et al., 2016; Nolan et al., 2022). Baldigo et al. (2016) found that eDNA samples correctly predicted Brook Trout presence and absence at 85 – 92.5% of the 40 sites in the sampling region in the Adirondack Mountains. They also found a relationship between eDNA concentration (in ng/µL) and Brook Trout population density and biomass (Baldigo et al., 2016). Based on their findings, they concluded that eDNA is an accurate tool to detect species presence and predict Brook Trout biomass and density (Baldigo et al., 2016). Another study by Nolan et al. (2022) successfully validated eDNA results with Brook Trout presence using conventional methods. They were able to accurately detect Brook Trout presence, which they later validated with electrofishing surveys (Nolan et al., 2022). Additionally, they detected

Brook Trout presence with eDNA in two sites with previously unknown occupations (Nolan et al., 2022). Although eDNA accurately predicted Brook Trout presence, they did not find a significant correlation between eDNA concentration and the number of fish caught (Nolan et al., 2022). Nolan et al. (2022) demonstrates the drawbacks of eDNA for predicting abundance at a restricted spatial scale due to finding no significant relationship between Brook Trout density and eDNA concentration. Some differences in the collection methods that these studies utilized might explain the low concentration of eDNA collection from our study.

One possible reason our samples did not yield sufficient Brook Trout eDNA is that 3 L is an insufficient water volume to filter. Baldigo et al. (2016) and Jane et al. (2015) filtered 6 L of water for Brook Trout eDNA collection. Muha et al. (2019) recommends collecting as much water as is feasible. They collected significantly higher eDNA concentrations with 2 L of water compared to lower volumes (Muha et al., 2019). Baldigo et al. (2016) recommended collecting 12 L of water for sufficient eDNA collection in turbid streams (Baldigo et al., 2016). However, the streams we collected water from were not excessively turbid, so water turbidity might not have been a significant factor (Figure 8). Nolan et al. only filtered 1 L of water, indicating that 1 L was a sufficient volume to collect Brook Trout eDNA (Nolan et al., 2022). Although Nolan et al. (2022) were able to detect quantifiable levels of Brook Trout eDNA from 1 L of water, Baldigo et al. (2016) required upwards of 6 L. It is important to note that the required water volume might depend on the density of Brook Trout in the stream. The difference in water volume collection suggests that a

higher water volume might allow for optimal eDNA collection, especially in turbid streams or streams with low Brook Trout density.

Another possible explanation for the low eDNA concentrations obtained in this study is inappropriate filter material. Jane et al. (2015) and Baldigo et al. (2016) both utilized glass fiber filters. Different filter materials have varying filter pore structures and may capture eDNA fragments with varying efficiency. In one study, glass fiber filters were shown to capture eDNA more efficiently using a passive collection method for amphibian eDNA (Chen et al., 2022). Another study found that glass fiber filters captured more particles within the filter matrix compared to polycarbonate filters which captured larger particles on the filter surface (Eichmiller et al., 2016). We used Smith-Root self-preserving filters made of polyethersulfone (PES). Thomas et al. (2019) found that the Smith-Root self-preserving filters stored eDNA better than traditional ethanol preservation methods. These filters have been shown to decrease the risk of contamination by reducing handling time (Thomas et al., 2019). Nolan et al. (2022) also used Smith-Root self-preserving filters and found higher eDNA capture efficiency than Halltech filters. They also found evidence that Smith-Root filters collected more inhibitors from the water (Nolan et al., 2022). Our internal positive controls found no evidence of inhibition in our samples. However, another study found that PES filters yielded lower eDNA than cellulose nitrate (Majaneva et al., 2018). Different filter materials have benefits and drawbacks, and perhaps using a different material may have allowed us to collect Brook Trout eDNA more efficiently.

Lastly, another explanation for the low Brook Trout eDNA copy numbers in our study is the filtration method. We used a Smith-Root eDNA sampler backpack, which automates water volume collection and flow rate while regulating pressure. Nolan et al. (2022) also utilized the Smith-Root eDNA sampler backpack for sample collection and collected sufficient Brook Trout eDNA. Baldigo et al. (2016) and Jane et al. (2015) used more traditional vacuum filtration methods; the former used a hand pump and the latter a peristaltic pump. Traditional methods rely on manual pressure control to prevent high pressures from shearing eDNA. In contrast, the Smith-Root allows users to control variables such as flow rate and maximum pressure, allowing for more replicable eDNA collection (Thomas et al., 2018). Across all vacuum filtration methods, water is passed through a filter membrane to capture eDNA. Therefore, it is unlikely that our filtration method was the reason for our low Brook Trout eDNA collection since all methods used vacuum filtration.

This study highlights the limitations of eDNA methods for Brook Trout detection in natural stream settings. The only site where relatively quantifiable concentrations of Brook Trout eDNA were collected was in the Hogcamp Branch in the Rappahannock watershed (Table 3). One possible explanation for the increased Brook Trout eDNA in Hogcamp Branch is that this stream was larger than the other Shenandoah streams and had a steady cold groundwater input. The steady cold-water source has historically allowed Hogcamp Branch to serve as an ideal Brook Trout habitat. The relatively low Brook Trout biomass in the Gunpowder watershed might explain the overall lower eDNA concentration compared to the Rappahannock watershed. Lower biomass limits the effectiveness of eDNA sampling, but optimizing

collection methods may allow for more efficient eDNA detection, even at low densities.

Using eDNA as a standard method for Brook Trout detection would benefit from understanding the effect of filter material and collection methods on efficient eDNA collection. Our study shows the limits of eDNA detection in wild stream settings and the risk of false negatives with unoptimized collection methods. For example, failing to detect Brook Trout when they are present could mistakenly exclude a stream from Tier III protections in MD. To avoid such errors, a higher water volume collection could allow for more efficient eDNA collection. A different filter material, such as glass fiber filters, may be more effective at capturing Brook Trout eDNA particles. A future follow-up experiment could compare glass fiber filters to PES filters and collect at least 6 L of water per filter. Sampling could be conducted in Hogcamp Branch since this site had relatively higher Brook Trout eDNA than other sites. As we improve our understanding of optimal eDNA collection methods and dynamics in natural streams, we can use eDNA to supplement traditional methods of detecting Brook Trout. Understanding Brook Trout occupancy in streams is critical for identifying streams for conservation and restoration.

Tables

Table 1. qPCR assay standard curve of known Brook Trout DNA concentrations, including negative and positive controls. C_t values, given concentration (copies/µL), and calculated concentrations (copies/µL) are displayed.

Type	**Ct**	**Given Conc (copies/µL)**	**Calc Conc (copies/µL)**
Trout Standard	22.72	69734.6	57094.37
Trout Standard	22.43	69734.6	69370.2
Trout Standard	22.28	69734.6	76580.97
Trout Standard	24.67	13946.92	15489.29
Trout Standard	24.83	13946.92	13883.57
Trout Standard	24.82	13946.92	14070.26
Trout Standard	27.19	2789.38	2862.51
Trout Standard	27.22	2789.38	2813.46
Trout Standard	27.31	2789.38	2648.58
Trout Standard	29.56	557.88	590.26
Trout Standard	29.5	557.88	610.95
Trout Standard	29.59	557.88	575.34
Trout Standard	32.61	111.58	76.42
Trout Standard	32.03	111.58	112.46
Trout Standard	31.74	111.58	137.07
Trout Standard	34.64	22.32	19.72
Trout Standard	34.31	22.32	24.59
Trout Standard	34.43	22.32	22.7
Trout Standard	36.79	4.46	4.67
Trout Standard	36.69	4.46	4.98
Trout Standard	37.07	4.46	3.88
Negative Control			
Negative Control			
Positive Control	29.83		492.67

Table 2. qPCR assay results for March and June cage experiments. Columns show sample ID, sample type (blank or sample), distance from the cage, collected date, and estimated concentrations per qPCR replicate (in copies/μL).

Sample ID	Type	Distance (m)	Collection Date	rep1	rep2	rep3	rep4
B1	Blank	0	3/21/2023	0	0	0	0
B2	Blank	0	3/21/2023	0	0	0	0
B3	Blank	0	3/21/2023	0	0	0	0
2000_1	Sample	2000	3/22/2023	0	0	0	0
2000_2	Sample	2000	3/22/2023	0	0	0	0
2000_3	Sample	2000	3/22/2023	0	0	0	0
100_1	Sample	100	3/22/2023	0	1.6	0	0
100_2	Sample	100	3/22/2023	0	3.6	0	0.9
100_3	Sample	100	3/22/2023	0	0	0.8	0
50_1	Sample	50	3/22/2023	0	0	0	0
50_2	Sample	50	3/22/2023	0	0	0	0
50_3	Sample	50	3/22/2023	0	0	0	0
10_1	Sample	10	3/22/2023	0	0	0	0
10_2	Sample	10	3/22/2023	0	0	0	0
10_3	Sample	10	3/22/2023	0	0	0	0
1_1	Sample	1	3/22/2023	5.5	9.4	7	0
1_2	Sample	1	3/22/2023	5.5	0	0	0
1_3	Sample	1	3/22/2023	0	3.6	0	0
5UP_1	Sample	5 (upstream)	3/22/2023	0	0	0	0
5UP_2	Sample	5 (upstream)	3/22/2023	0	0	0	0
5UP_3	Sample	5 (upstream)	3/22/2023	0	0	0	0
B1 J	Blank	0	6/8/2023	0	0	0	0
B2 J	Blank	0	6/8/2023	0	0	0	0
B3 J	Blank	0	6/8/2023	0	0	0	0
2000_1 J	Sample	2000	6/9/2023	0	0	0	0
2000_2 J	Sample	2000	6/9/2023	0	0	0	0
2000_3 J	Sample	2000	6/9/2023	0	0	0	0
100_1 J	Sample	100	6/9/2023	0	0	0	0
100_2 J	Sample	100	6/9/2023	0	0	1.2	0
100_3 J	Sample	100	6/9/2023	1.4	2.1	0	0
50_1 J	Sample	50	6/9/2023	0	0	0	0
50_2 J	Sample	50	6/9/2023	0	0	0.7	0
50_3 J	Sample	50	6/9/2023	0	0	0	0

10_1 J	Sample	10	6/9/2023	0	3	1.1	0
10_2 J	Sample	10	6/9/2023	0	0	1.4	1.8
10_3 J	Sample	10	6/9/2023	0.6	0.5	1.5	0
1_1 J	Sample	1	6/9/2023	0	0	0	0
1_2 J	Sample	1	6/9/2023	0	0	0	0
1_3 J	Sample	1	6/9/2023	0	0	0	0
5UP_1 J	Sample	5 (upstream)	6/9/2023	0	0	0	0
5UP_2 J	Sample	5 (upstream)	6/9/2023	0	0	0	0
5UP_3 J	Sample	5 (upstream)	6/9/2023	0	0	0	0

Table 3. Results of qPCR assays. Columns show sample ID, sample type (field blank or sample), location, stream site, filter pore size (in μm), collection date, and estimated concentrations per qPCR replicate (in copies/μL). Bolded samples were used for statistical analysis.

Sample ID	Type	Location	Stream	Pore Size	Date Collected	rep1	rep2	rep3	rep4
SB1	Field Blank	Shenandoah		1.2	9/22/2023	0	0	0	0
S1A1	Sample	Shenandoah	1 (Mill Prong)	1.2	9/22/2023	0	3.5	0	2.66
S1A2	Sample	Shenandoah	1 (Mill Prong)	1.2	9/22/2023	3.65	0	0	5.44
S1A3	Sample	Shenandoah	1 (Mill Prong)	1.2	9/22/2023	0	0	0	4.35
S1B1	Sample	Shenandoah	1 (Mill Prong)	5	9/22/2023	2.27	3.31	4	3.99
S1B2	Sample	Shenandoah	1 (Mill Prong)	5	9/22/2023	2.09	6.56	5.83	0
S1B3	Sample	Shenandoah	1 (Mill Prong)	5	9/22/2023	4.27	2.36	0	4.32
S2A1	Sample	Shenandoah	2 (Laurel Prong)	1.2	9/22/2023	5.26	0	0	0
S2A2	Sample	Shenandoah	2 (Laurel Prong)	1.2	9/22/2023	8.55	5.6	5.7	5.38
S2A3	Sample	Shenandoah	2 (Laurel Prong)	1.2	9/22/2023	10.7	5.28	2.72	0
S2B1	Sample	Shenandoah	2 (Laurel Prong)	5	9/22/2023	0	0	0	4.92
S2B2	Sample	Shenandoah	2 (Laurel Prong)	5	9/22/2023	0	5.64	5.8	8.27
S2B3	Sample	Shenandoah	2 (Laurel Prong)	5	9/22/2023	2.81	5.76	0	10.9
S3A1	**Sample**	**Shenandoah**	**3 (Hogcamp Branch)**	**1.2**	**9/22/2023**	**6.68**	**12.26**	**13.83**	**6.11**
S3A2	**Sample**	**Shenandoah**	**3 (Hogcamp Branch)**	**1.2**	**9/22/2023**	**0**	**3.61**	**2.36**	**2.24**
S3A3	**Sample**	**Shenandoah**	**3 (Hogcamp Branch)**	**1.2**	**9/22/2023**	**0**	**5.39**	**0**	**4.69**
S3B1	**Sample**	**Shenandoah**	**3 (Hogcamp Branch)**	**5**	**9/22/2023**	**17.82**	**4.75**	**5.49**	**9.26**
S3B2	**Sample**	**Shenandoah**	**3 (Hogcamp Branch)**	**5**	**9/22/2023**	**7.6**	**4.25**	**14.46**	**14.5**

S3B3	Sample	Shenandoah	3 (Hogcamp Branch)	5	9/22/2023	19.49	7.74	6.78	8.94
SB2	Field Blank	Shenandoah		1.2	9/22/2023	0	0	0	0
GB1	Field Blank	Gunpowder		1.2	9/27/2023	0	0	0	0
G1A1	Sample	Gunpowder	1 (Walker Run)	1.2	9/27/2023	0	0	0	0
G1A2	Sample	Gunpowder	1 (Walker Run)	1.2	9/27/2023	0	0	0	0
G1A3	Sample	Gunpowder	1 (Walker Run)	1.2	9/27/2023	0	0	0	0
G1B1	Sample	Gunpowder	1 (Walker Run)	5	9/27/2023	0	0	0	0
G1B2	Sample	Gunpowder	1 (Walker Run)	5	9/27/2023	0	0	0	0
G1B3	Sample	Gunpowder	1 (Walker Run)	5	9/27/2023	0	0	0	0
G2A1	Sample	Gunpowder	2 (Gunpowder Mainstem)	1.2	9/27/2023	0	9.49	0	0
G2A2	Sample	Gunpowder	2 (Gunpowder Mainstem)	1.2	9/27/2023	7.38	0	0	4.93
G2A3	Sample	Gunpowder	2 (Gunpowder Mainstem)	1.2	9/27/2023	6.24	4.67	0	0
G2B1	Sample	Gunpowder	2 (Gunpowder Mainstem)	5	9/27/2023	0	0	0	0
G2B2	Sample	Gunpowder	2 (Gunpowder Mainstem)	5	9/27/2023	4.03	0	5.38	0
G2B3	Sample	Gunpowder	2 (Gunpowder Mainstem)	5	9/27/2023	0	0	5.5	0
G3A1	Sample	Gunpowder	3 (Bush Cabin Run)	1.2	10/12/2023	0	0	0	0
G3A2	Sample	Gunpowder	3 (Bush Cabin Run)	1.2	10/12/2023	0	0	0	0
G3A3	Sample	Gunpowder	3 (Bush Cabin Run)	1.2	10/12/2023	0	0	0	3.72
G3B1	Sample	Gunpowder	3 (Bush Cabin Run)	5	10/12/2023	0	0	0	0
G3B2	Sample	Gunpowder	3 (Bush Cabin Run)	5	10/12/2023	0	0	0	0
G3B3	Sample	Gunpowder	3 (Bush Cabin Run)	5	10/12/2023	0	0	0	0
GB2	Field Blank	Gunpowder		1.2	10/12/2023	0	0	0	0

Figures

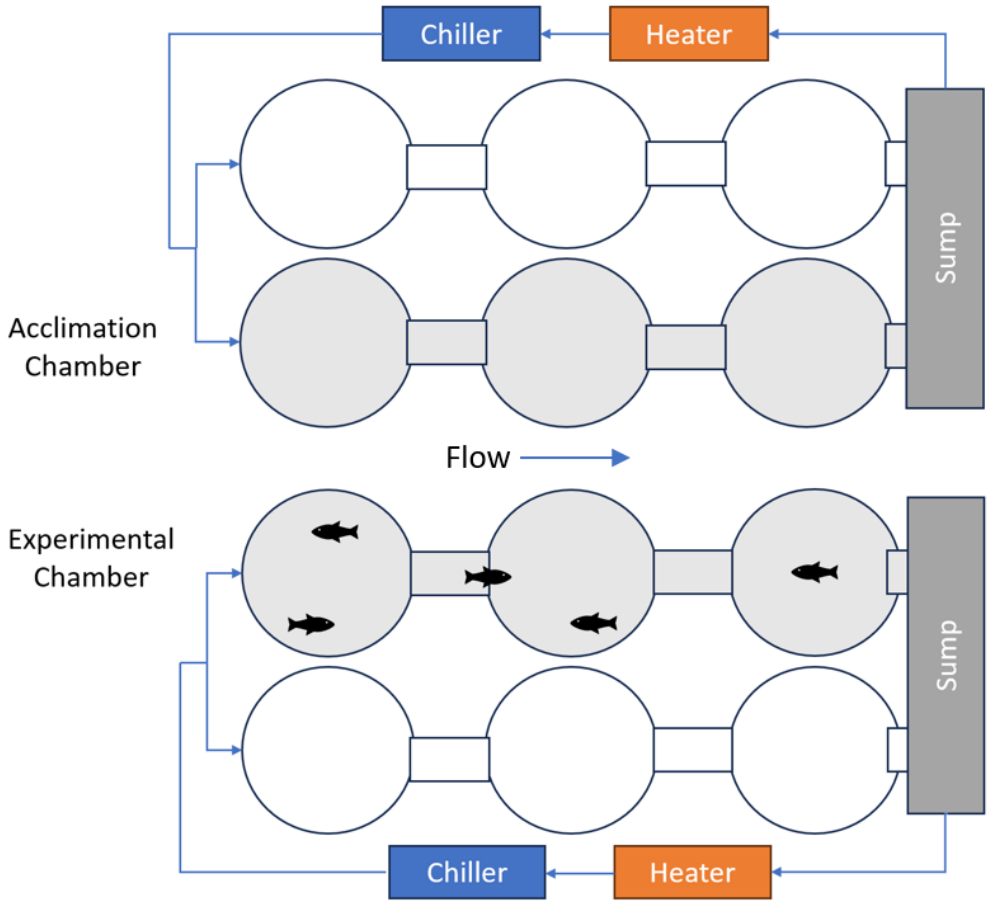

Figure 1. Diagram of experimental streams used to acclimate fish to target temperature (top system) and collect eDNA samples after one hour of exposure to target temperature (bottom system). Each artificial stream comprises two channels with three pools and two riffles, which fish can move freely between. The light gray channels are where we placed the fish. Chiller and heater units were used to regulate the temperature of each system.

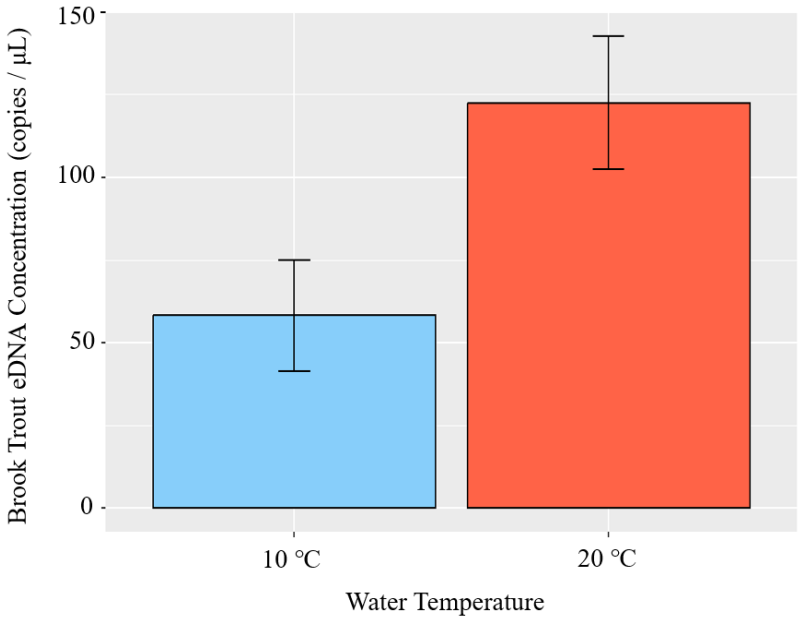

Figure 2. Bar plot displaying mean Brook Trout eDNA concentration (copies/µL) at 10 ℃ and 20 ℃ across all trials. Error bars indicate the standard error of the mean. The blue bar indicates 10 ℃, and the red bar indicates 20 ℃.

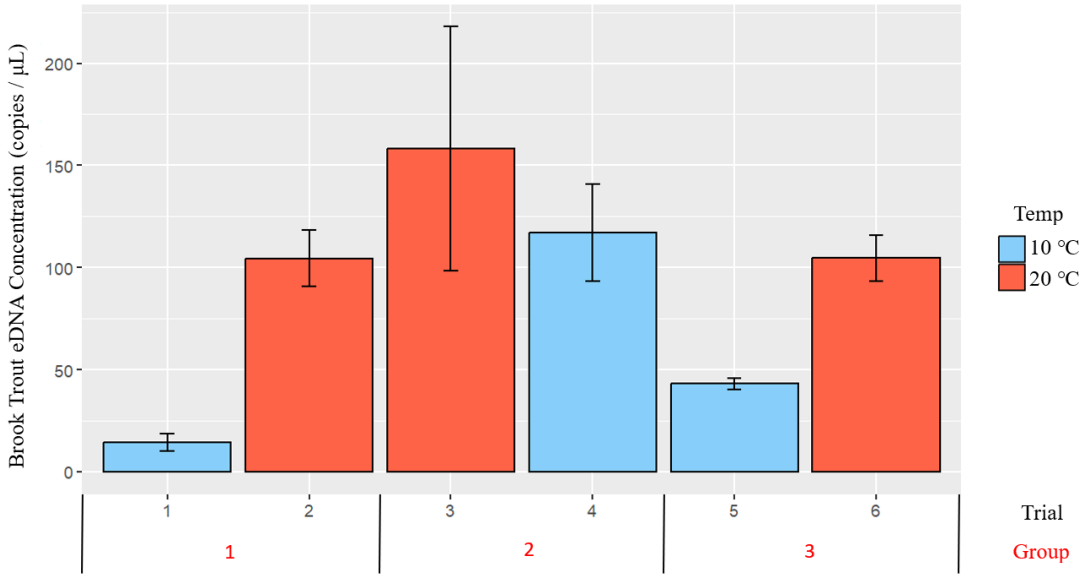

Figure 3. Mean Brook Trout eDNA concentration (copies/µL) across trials. Blue bars indicate 10 °C trials, and red bars indicate 20 °C trials. The trial represents a sampling event, and the group represents the three unique groups of fish. Trials 1 and 2 were conducted on the first group, trials 3 and 4 on the second group, and trials 5 and 6 on the third group. Error bars indicate the standard error of the mean.

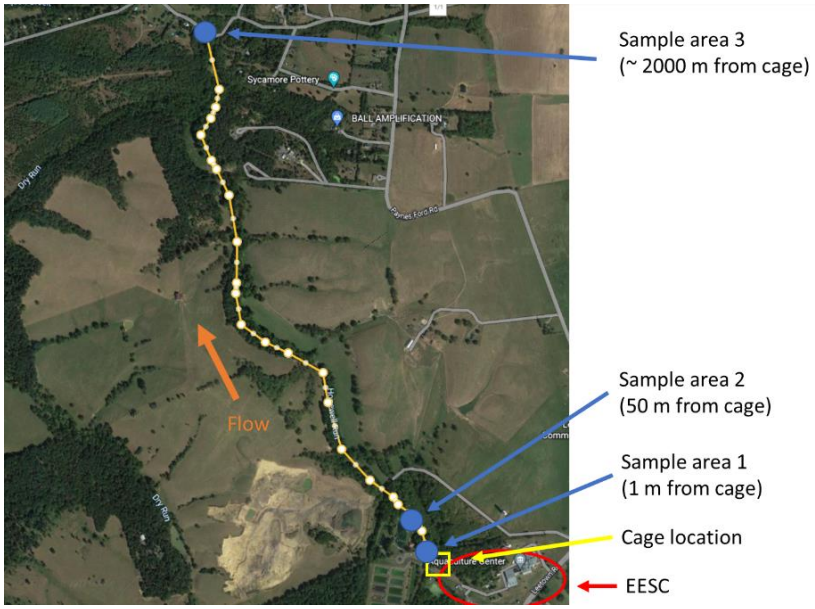

Figure 4. Map of study site setup in Hopewell Run, WV. Yellow square indicates cage location, and blue dots indicate sample locations at 1, 5, and 2,000 m from the cage. Orange arrow indicates the direction of stream flow.

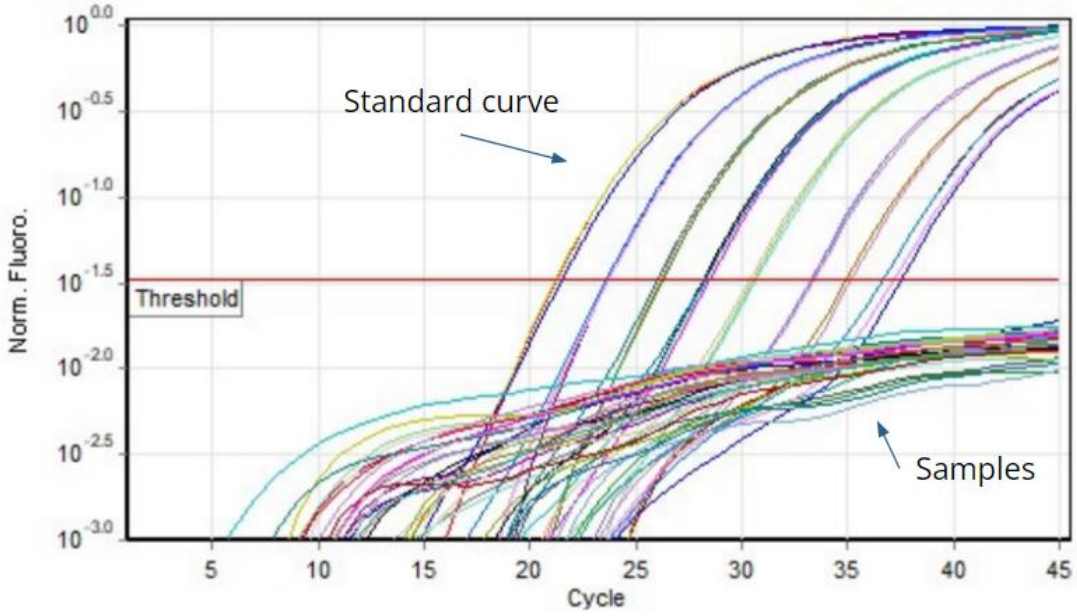

Figure 5. Example of quantitation results from Brook Trout qPCR assay. The x-axis indicates the PCR cycle number, and the y-axis indicates normalized fluorescence. Threshold denotes minimum detectable fluorescence limit.

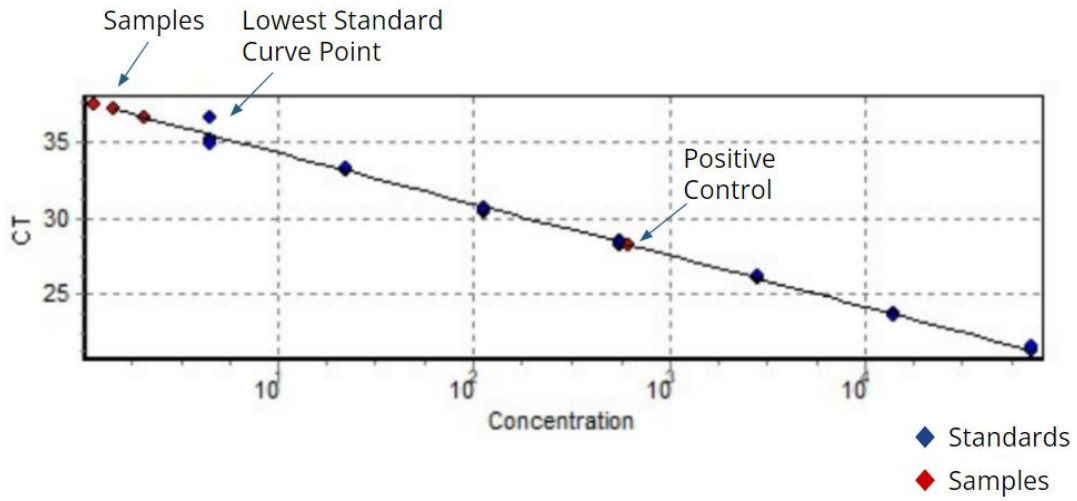

Figure 6. Example of Brook Trout qPCR assay with no quantifiable results in regression line format. The x-axis represents DNA concentration (copies/ μL), and the y-axis indicates the C_t value. Blue dots are the standard curve of known Brook Trout DNA concentrations, and red dots are collected eDNA samples.

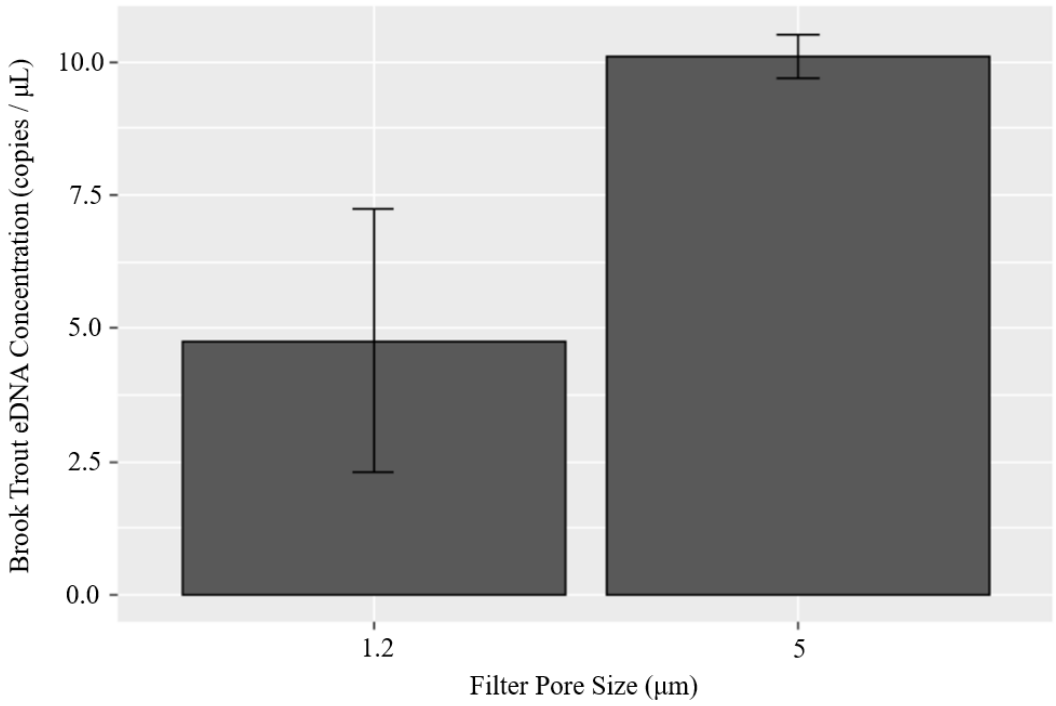

Figure 7. Mean Brook Trout eDNA concentration (copies/µL) obtained using 1.2- and 5-micron Smith-Root self-preserving filters at Hogcamp Branch, Shenandoah. Error bars indicate the standard error of the mean.

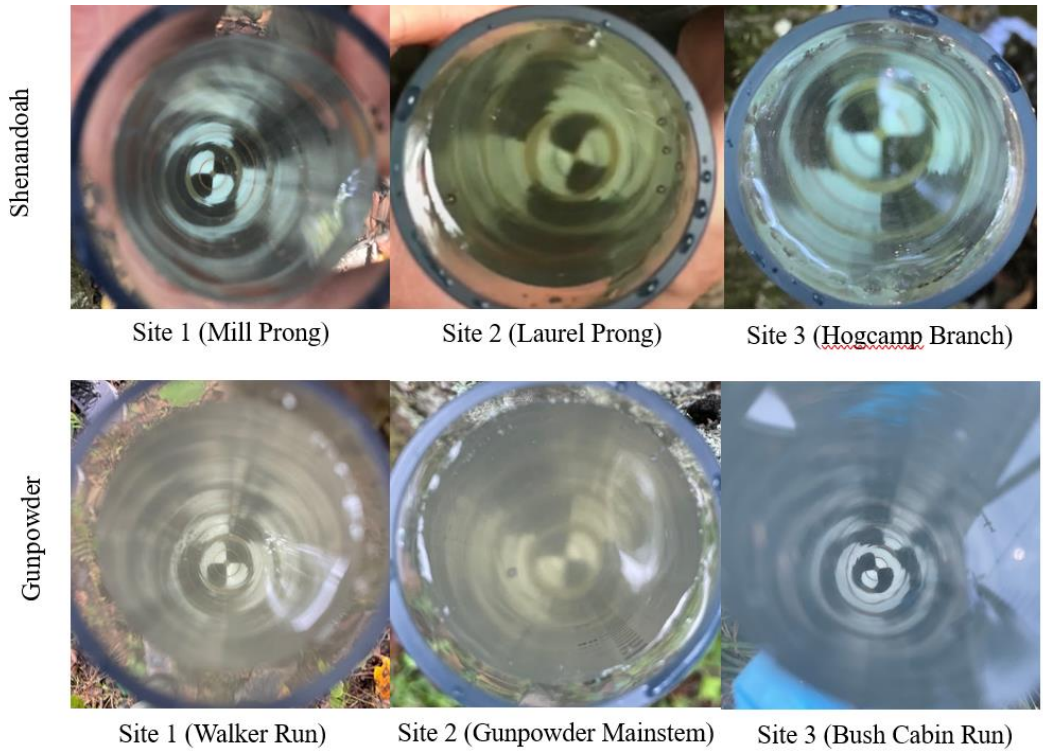

Figure 8. Top-view photographs of transparent turbidity tube with Secchi disk at Brook Trout eDNA collection sites. The top row of photos is from the three Shenandoah sites, and the bottom is from the three Gunpowder sites.

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