Canadian Journal of Fisheries and Aquatic Sciences

Research Article

The riverscape on a chip: high-throughput qPCR enables basin-wide fishery assessments

Joanna W. Elmore[a], **Taylor M. Wilcox**[a], **Michael K. Young**[a], **Steve M. Kopp**[b,c], **Kellie J. Carim**[d], **Daniel H. Mason**[a], **Thomas W. Franklin**[a], **and Michael K. Schwartz**[a]

[a]USDA Forest Service, Rocky Mountain Research Station, National Genomics Center for Wildlife and Fish Conservation, Missoula, MT, USA; [b]Trout Unlimited – North Sound Chapter, Bellingham, WA, USA; [c]Esri, Redlands, CA, USA; [d]USDA Forest Service, Rocky Mountain Research Station, Aldo Leopold Wilderness Research Institute, Missoula, MT, USA

Corresponding author: **Joanna W. Elmore** (email: joanna.elmore@usda.gov)

Abstract

To achieve reliable detection of environmental DNA (eDNA) from multiple species, a method which combines the specificity of single-taxon assays with the broad taxonomic coverage of metabarcoding is needed. High-throughput quantitative PCR (HT-qPCR) has emerged as one such method that holds particular promise for ecological applications. Here, we present the development and validation of a HT-qPCR biochip (i.e., biochip) protocol for a suite of western North American fishes with high cultural, economic, and ecological significance. Coupling this biochip with systematically distributed eDNA samples collected by citizen scientists, we evaluated the distribution of multiple species throughout a large river basin in the U.S. Pacific Northwest. Patterns of detection using the biochip were comparable to those using single-taxon qPCR (ST-qPCR; mean concordance = 0.917), despite the biochip using 90% less DNA per taxon and reducing costs by an estimated 40%. Comparisons between biochip results and distributions derived from conventional methods demonstrate that this approach not only offers more reliable and high-resolution distribution data for these species but also achieves this more efficiently than traditional sampling methods.

Key words: eDNA, environmental DNA, Pacific Northwest, biochip, lotic

1. Introduction

Analyzing environmental samples for organismal DNA is a powerful tool for species monitoring (Bohmann et al. 2014; Rees et al. 2014). Environmental DNA (eDNA) sampling boasts many advantages over other monitoring methods, including, but not limited to, the relative ease of sample collection (Jerde et al. 2011) and the potential for one sample to represent a comprehensive snapshot of the local biodiversity (Thomsen et al. 2012). Given the advantages of eDNA sampling and analysis, its applications have ranged from targeted approaches such as rare and invasive species detection, pathogen surveillance, and forensic science to broader approaches, e.g., biodiversity assessments and community analyses (Takahara et al. 2013; Kelly et al. 2014; Huver et al. 2015; Carim et al. 2016*a*; Hallam et al. 2021; Dass et al. 2022).

The various approaches to eDNA sample analysis, however, often involve a compromise between detection efficiency and taxonomic breadth. For example, methods designed to target a specific taxon, such as single-taxon quantitative PCR (ST-qPCR), can be remarkably sensitive and specific. Such methods often reliably detect target DNA at concentrations below 10 copies per reaction without amplification of non-target DNA (e.g., Wilcox et al. 2015; Klymus et al. 2020). This precision makes these approaches well-suited for the detection of rare species, a task in which they outperform multi-species methods (Harper et al. 2018; Wood et al. 2019). However, this sensitivity–specificity combination comes at a cost for questions involving multiple species. Performing iterative ST-qPCR analyses demands substantial laboratory resources and can rapidly deplete an environmental sample, thereby limiting the taxonomic scope of inference and precluding the ability to archive samples for future analyses (e.g., Dysthe et al. 2018*b*; Shaw et al. 2019).

In contrast to ST-qPCR, metabarcoding offers a much broader taxonomic scope. Metabarcoding methods, however, include a series of steps that can reduce the ability to detect individual target species. For example, a specific locus must be amplified across all target species, and those amplicons must be prepared for sequencing by indexing, sequenced on a high-throughput platform, properly demultiplexed, filtered bioinformatically, and assigned taxonomic identities (Coissac et al. 2012; Ruppert et al. 2019). Most parts of this process are susceptible to technical issues, such as primer bias and template competition, that can cause certain templates to be disproportionately amplified and sequenced, resulting in the under-representation or even nondetection of some taxa (Kelly et al. 2014; Hatzenbuhler et al. 2017). Additionally, generating taxonomic assignments from metabarcoding re-

sults is often more complex compared to single-taxon approaches. For example, taxonomic identifications from sequencing data can be hampered by incomplete or inaccurate DNA reference databases (e.g., Keck et al. 2023).

Often, managers require a middle ground, i.e., reliable detection of a featured suite of species for which an intermediate method that combines the specificity of single-taxon assays with the multi-species coverage of metabarcoding is needed. One such method is high-throughput qPCR (HT-qPCR), which was developed for microbial eDNA detection and has shown promise in macrobial eDNA applications (An et al. 2018; Zheng et al. 2018; Wilcox et al. 2020; Bernardi et al. 2021). The process of HT-qPCR involves simultaneously performing thousands of nanoliter-scale qPCR reactions, which allows for samples to be assayed against many different primer-probe sets with greatly reduced consumption of the sample and reagents. Moreover, leveraging the efficacy of multi-taxa eDNA analyses with ecologically informed and spatially comprehensive sample collection could provide definitive, large-scale assessments of the distributions of potentially sympatric species of conservation concern, such as rare indigenous taxa or introduced invasive species.

Here, we describe the development and optimization of a HT-qPCR biochip (hereafter, biochip) protocol for a widely distributed suite of freshwater fishes of cultural, economic, and conservation importance along North America's Pacific Rim. Next, we used the biochip to evaluate the distribution of these species throughout a large river basin in the U.S. Pacific Northwest based on eDNA sampling at systematically distributed points in potentially occupied habitat, conducted entirely by volunteer citizen scientists. We validated these results for a select set of species by comparing them to patterns of detection based on ST-qPCR protocols and species distribution maps based on conventional sampling. This approach provided a high-resolution description of the whole-basin distributions of these taxa, demonstrating that the combination of comprehensive eDNA sampling campaigns and reliable multi-species detection based on HT-qPCR biochips has the potential to revolutionize the assessment and monitoring of these freshwater species of conservation concern.

2. Materials and methods

We based the biochip on 10 ST-qPCR assays covering taxa of conservation concern in coastal western North America (Section 2.1). We optimized the biochip protocol (Section 2.2) with these assays and tested them for sensitivity (Section 2.3) and specificity (Section 2.4) on the platform. We then compared results from the biochip analyses to those from ST-qPCR (methods in Section 2.5) for a set of eDNA samples to benchmark biochip performance (Section 2.6). Finally, we used the biochip, supplemented by two ST-qPCR assays, to analyze eDNA samples from a large coastal river basin in the Pacific Northwest (Section 2.7).

2.1. Target species' qPCR assays

Our analyses incorporated 12 qPCR assays. All assays use a minor groove binding hydrolysis probe and are optimized for a 60 °C annealing and extension temperature, targeting either mitochondrial or multi-copy nuclear genes. Assays were either previously published (Wilcox et al. 2013, 2015; Carim et al. 2017; Dysthe et al. 2018*a*; Homel et al. 2021) or have their validation described here (Supplementary Material A), and methods which were used to evaluate assay performance can be found in these respective documents (Table 1).

Ten of these qPCR assays were included in the HT-qPCR biochip. These included one nonnative species (brook trout, *Salvelinus fontinalis*) and nine native taxa: bull trout (*Salvelinus confluentus*), rainbow trout (*Oncorhynchus mykiss*), coastal cutthroat trout (*Oncorhynchus clarkii*), sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), chum salmon (*Oncorhynchus keta*), pink salmon (*Oncorhynchus gorbuscha*), and Pacific lamprey (*Entosphenus tridentatus*; Table 1). In addition, two qPCR assays were used to determine occupancy status of Dolly Varden (*Salvelinus malma*) using ST-qPCR only. Because its distribution is limited in the U.S. portion of western North America, we chose not to include Dolly Varden in the biochip, which is intended to be broadly applicable across other systems in western North America. Due to the complex evolutionary relations between Dolly Varden and other chars (Dunham et al. 2008; Liu et al. 2023), existing assays for brook trout and bull trout can sometimes amplify the DNA of Dolly Varden. Consequently, we attempted to design an ST-qPCR assay specific to this species (Supplementary Material A), which we ran in concert with the biochip on each sample. The combined information from the biochip assays, the Dolly Varden assay, and a previously designed ST-qPCR mitochondrial assay for bull trout (Wilcox et al. 2013) enabled us to unambiguously determine the presence of bull trout and brook trout DNA from every sample. However, in some cases, the presence of Dolly Varden remained ambiguous when both bull trout and brook trout were also present (see Supplementary Material A). Similarly, we acknowledge that these assays cannot distinguish parental taxa from hybrid individuals. Although hybridization is common between cutthroat trout and rainbow trout and among the various species of char, populations composed solely, or even primarily, of hybridized individuals are rare (Baxter et al. 1997; DeHaan et al. 2010; McKelvey et al. 2016). Thus, we interpret a positive detection as evidence of the presence of the parental taxon. In addition, because stream-resident rainbow trout and anadromous steelhead share mitochondrial sequences, we treated all positive detections as evidence of the presence of rainbow trout.

2.2. Preparation of HT-qPCR biochips

A limitation of HT-qPCR is that target species detection may be reduced because of the small volume of DNA template in the reaction (reaction volume = 100 nL). To maximize sensitivity, we pre-amplified samples to enrich the amount of target DNA in a reaction—a common practice for HT-qPCR analysis, especially when working with noninvasive samples, that has been shown to have no major impacts on sensitivity, efficiency, and quantitative performance of qPCR (Ishii et al. 2013, 2014; Korenková et al. 2015; Malla et al. 2022; Hill et al. 2023; van der Pouw Kraan et al. 2024). Pre-amplification

Table 1. List of taxon-specific quantitative PCR assays used in this study.

Taxon	Oligo sequence (5′-3′)	Primer concentration (nmol/L)	Citation
Brook trout	F: CCACAGTGCTTCACCTTCTATTTCTA	900	Wilcox et al. (2013)
Salvelinus fontinalis	R: GCCAAGTAATATAGCTACAAAACCTAATAGATC	900	
	P: FAM-ACTCCGACGCTGACAA-NFQ-MGB	250	
Bull trout	F: TTCCTTTTGCCTAGGGTAGCG	600	Dysthe et al. (2018*a*)
Salvelinus confluentus	R: CGATACTCAACACGCTTCACAATT	900	
	P: FAM-CCACGGCCACACGG-NFQ-MGB	250	
Coastal cutthroat trout	F: AAATGGAAGCAACTGGCTACTCA	100	Supplementary Material A
Oncorhynchus clarkii	R: GGCGGTCTGTGCCATGC	600	
	P: FAM-CTCCTATACTTCTTCTAGCCTT-NFQ-MGB	250	
Chum salmon	F: CCGCTTTTTGTCTGAGCTGTACT	300	Homel et al. (2021)
Oncorhynchus keta	R: AATTTCGATCTGTGAGCAACATAGTAA	900	
	P: FAM-CTGTACTTCTACTATTATCACTCC-NFQ-MGB	250	
Chinook salmon	F: ATCAAACCCGAATGATACTTCTTATTC	900	Supplementary Material A
Oncorhynchus tshawytscha	R: TTGGAAGTGTGTAAGATAGGAACAACTATA	900	
	P: FAM-ATCTATTCCCAACAAACTA-NFQ-MGB	250	
Coho salmon	F: CTTATGGTTGTACCAATCTTRCACACC	600	Supplementary Material A
Oncorhynchus kisutch	R: AAGTATATCCGCCACCAAGGC	900	
	P: FAM-CACTAACCCAATTCCTATTCT-NFQ-MGB	250	
Pacific lamprey	F: GTACCACTCATACTTAGTGCCCCTG	600	Carim et al. (2017)
Entosphenus tridentatus	R: CTGTGCCAGCCCCTGCT	900	
	P: FAM-TTTGATTACTTCCACCCTCAC-NFQ-MGB	250	
Pink salmon	F1: CATACTTCTTAGTCTAACATCATTAGCCCTATT	900	Supplementary Material A
Oncorhynchus gorbuscha	F2: TTCTTGGTCTAACATCATTAGCCCTATT	900	
	R1: GGGGTGACTAGTGGGTTAGCG	900	
	R2: GGGTGACCAGTGGGTTAGCG	900	
	P: FAM-AACCTCTTAGGAGACCC-NFQ-MGB	250	
Sockeye salmon	F: TCTGCCCTTCTCCTTACGATTTT	900	Tillotson et al. (2018)*
Oncorhynchus nerka	R: AAGGGCGATTTCTAGGTCGAAC	900	
	P: FAM-CCATCCTGTTCCTCCT-NFQ-MGB	250	
Rainbow trout	F: AGTCTCTCCCTGTATATCGTC	300	Wilcox et al. (2015)
Oncorhynchus mykiss	R: GATTTAGTTCATGAAGTTGCGTGAGTA	600	
	P: FAM-CCAACAACTCTTTAACCATC-NFQ-MGB	250	
Dolly Varden	F: GCTGTGCTCCGAAAACCAAAG	300	Supplementary Material A
Salvelinus malma	R: TGCCCATAGGTGCGCCA	600	
	P: FAM-CCTAAGGGTTGCACCCGA-NFQ-MGB	250	

Note: All assays use optimized forward and reverse primer concentrations and FAM-labeled minor groove binding (MGB), nonfluorescent quencher (NFQ) probes at 250 μmol/L. The multiple primers in the pink salmon assay exist to account for an single-nucleotide polymorphism (SNP) within the targeted locus. The use of separate primers, instead of degenerate primers, allows for greater control over the Tm since the length of the oligos can be modified accordingly. These primers are pooled equimolar in the pink salmon assay.
*The sockeye salmon assay was originally described by Tillotson et al. (2018), but the assay was revalidated (Supplementary Material A).

reactions were composed of 22.5 μL TaqMan Environmental Master Mix 2.0 (2×; Thermo Fisher Scientific), 44 nmol/L of each species-specific assay primer and internal control assay primers (see below), 12 μL template, 1 μL internal positive control (IPC) template (see description below), and sufficient molecular-grade water to bring the total reaction volume to 45 μL.

Despite the availability of specialized PCR mixes optimized to minimize amplification bias, we used the aforementioned mixture because we found it to be superior to several commercial pre-amplification master mixes due to its robust resistance to the presence of PCR inhibitors, reflecting previous research on performance in ST-qPCR (Jane et al. 2015). Our reaction volumes were also larger than most other published pre-amplification protocols to accommodate the same template volume as used with our ST-qPCR analyses. Thermocycling was performed using previously optimized conditions (Supplementary Material D): 95 °C 10 min (95 °C 15 s, 60 °C 3 min) × 12 cycles, then held at 12 °C.

To simultaneously monitor for PCR inhibition and potential for contamination between samples during PCR set up, we designed a "tiled" IPC. This control is a paired set of two (types "A" and "B") qPCR assays and synthetic templates based on Deer et al. (2010; Table 2). We loaded 10 copies of the synthetic templates in each well of the 96-well pre-amplification plate, alternating the "A" and "B" types in a checkerboard, or "tiled" pattern. Both assays are then used for pre-amplification and final analysis of every well. Failure

Table 2. Oligos for paired, tiled internal positive control assays to monitor PCR amplification and potential cross-plate contamination.

Oligo	Sequence
F primer (A)	5′CTAACCTTCGTGATGAGCAATCG
R primer (A)	5′GATCAGCTACGTGAGGTCCTAC
Probe (A)	5′FAM-AGCTAGTCGATGCACTCCAGTCCTCCT-NFQ-MGB
Template (A)	5′CCTAACCTTCGTGATGAGCAATCGAACCATCTTAGCTAGTCGATGCACTCTAGCTAGTCGATGCACTCCAGTCCTCC TAGTCGACCTAACGTACGTGATGAGCAATCGATGCATCTTAGCTAGGGCATGCTTGGC-GATCGTCGTAGGACCTCACGTAGCTGATCG
F primer (B)	5′CTAACCTTCGTGATGAGCAATGC
R primer (B)	5′ GATCAGCTACGTGAGGTCCTTG
Probe (B)	5′FAM-AGCTAGTCGATGGACTGCTGTCGTCCA-NFQ-MGB
Template (B)	5′CCTAACCTTCGTGATGAGCAATGCAACCATCTTAGCTAGTCGATGCACTCTAGCTAGTCGATGGACTGCTGTCGTCC AAGTCGACCTAACGTACGTGATGAGCTTTCGATGCATCTTAGCTAGGGCATGCTTGGC-GATCGTCCAAGGACCTCACGTAGCTGATCG

to amplify for either assay indicated PCR inhibition or other amplification issues. Inhibition was also monitored by checking for Ct shifts ≥ 1 in the IPC assay when compared to positive controls. Amplification of assay "A" in a "B" well, or vice versa, indicated possible cross-well contamination.

Following pre-amplification, each sample underwent an exonuclease cleaning by adding 4.5 μL ExoSAP-IT and incubating at 37 °C for 30 min and then 80 °C (deactivation step) for 15 min. Each exonuclease-cleaned, pre-amplified sample was arranged on a 384-well plate in quadruplicate for analyses following the SmartChip MyDesign Gene Expression protocol (60 °C extension and annealing temperature) for a 12-assay/384-sample format (10 taxon-specific assays and both IPC assays). The sample plate was loaded with Takara SmartChip Probe qPCR Master Mix and an assay plate was loaded with 5 × assays for each taxon and the two IPC assays, along with ROX normalization dye as described in the manufacturer's protocol. These plates were shipped frozen to the Washington State University Laboratory of Biotechnology and Bioanalysis (Pullman, Washington, USA) for robotic loading into an aluminum HT-qPCR chip, amplification, and visualization. Setup for pre-amplification occurred in the same dedicated space as for ST-qPCR analysis. Exonuclease clean setup and plating for HT-qPCR analysis was in a different dedicated UV hood that was irradiated for 1 h prior to each use.

When analyzing environmental samples, we included multiple no-template control wells in each HT-qPCR analysis. The first plate of environmental samples incorporated one no-template control and all subsequent plates incorporated eight no-template controls (water in the place of template; $n = 7$ plates, 49 negative controls). The first plate included only one no-template control because our main goal was to assess assay sensitivity, specificity, and concordance on the HT-qPCR platform, so other sample types needed to accomplish this were prioritized. The eight no-template controls filled the last column on the 96-well plate that was pre-amplified and then plated in quadruplicate on the 384-well plate. Each no-template control was analyzed in quadruplicate resulting in 196 replicates tested against 10 taxon-specific assays (1960 control analyses total) over the course of the study. For each plate that included environmental samples, except those with tissue samples, we included two pooled positive controls (composed of synthetic genes covering the amplicon region at 10 copies/4 μL each taxa), which were analyzed in quadruplicate ($n = 5$ plates, 10 positive controls, 40 replicates per assay).

For each sample, we visually inspected the PCR results for amplification of each assay using WaferGen qPCR Software (version 2.8.33.0, Wafergen Biosystems, Inc.). We scored any replicate positive when there was amplification with an exponential phase at any point prior to 50 PCR cycles. Because there were occasional amplifications of no-template controls (see the "Results" section), we required amplification in at least two replicates for a given assay to denote a sample as positive.

2.3. Assay sensitivity on the HT-qPCR platform

To test assay sensitivity on the HT-qPCR platform, we tested the ability of the protocol to detect known-concentration samples with DNA contents similar to that of environmental samples used to detect rare fishes. We did this using synthetic genes covering the amplicon region for each of the 10 taxon-specific assays diluted to a concentration of 2.5 copies/μL, which is comparable to 10 copies per reaction in ST-qPCR analyses, the typical limit of detection for the assays in this study (Supplementary Material A). These positive samples for each species were pre-amplified in triplicate, with 12 μL per replicate. Each pre-amplification triplicate was then analyzed in quadruplicate on HT-qPCR, resulting in 12 analyses per known-concentration synthetic gene.

2.4. Assay specificity on the HT-qPCR platform

We tested assay specificity on the HT-qPCR platform with genomic DNA and synthetic DNA fragments of likely syntopic and closely related taxa: Arctic char (*Salvelinus alpinus*), brook trout, brown trout (*Salmo trutta*), bull trout, coastal cutthroat trout, Chinook salmon, chum salmon, coho salmon, Dolly Varden ($n = 3$ unknown haplotype/hybridization status), lake trout (*Salvelinus namaycush*), western brook lamprey (*Lampetra ayresii*; see Carim et al. 2023), pink salmon, rainbow trout, and sockeye salmon. Tissues were sourced from previous projects where collections adhered to appropriate sam-

pling permits or from fish harvested for human consumption. We extracted DNA from each sample using the QIAGEN DNEasy Blood and Tissue DNA Extraction Kit following the manufacturers protocol. Each pre-amplification reaction contained approximately 1.2 ng of genomic DNA (Qubit fluorometer; Broad Range Kit). Although we sought pieces of muscle tissue or thick pieces of fin tissue which we cleaned to remove surficial nontarget DNA, entirely avoiding low-level contamination was difficult (Rodgers 2017). Therefore, we supplemented our testing with synthetic gene fragments for each taxon that showed off-target amplification in the initial tissue specificity test ($n = 12$ taxa; Table S1.B). To do this cost-effectively, we pre-amplified and cleaned these synthetic gene fragments as in the HT-qPCR protocol, but then diluted products 1:100 and visualized them on an ST-qPCR platform. We found that HT-qPCR and ST-qPCR analyses of diluted, pre-amplified samples were highly concordant (100% concordance across > 50 samples; Supplementary Material D). Relevant reference sequences were not available for *Lampetra ayresii*, so this process was also conducted with a re-extraction of this tissue sample with additional cleaning steps. Also included in this ST-qPCR analysis were pre-amplification and qPCR no-template and positive controls, assessed in triplicate, for each assay.

2.5. Single-species qPCR analysis

The ST-qPCR analysis of environmental samples was conducted in triplicate reactions composed of 7.5 μL TaqMan Environmental Master Mix 2.0 (2×; Thermo Fisher Scientific), 0.75 μL 20 × primer/probe mix (probe at 250 μmol/L, primers at optimized concentrations; Table 1), 1.5 μL of 10 × IPC assay and 0.3 μL of 50 × IPC Template (TaqMan® Exogenous Internal Positive Control reagents; Life Technologies), and 4 μL template, with molecular-grade water added to create a reaction volume of 15 μL. The thermocycling conditions on a QuantStudio3 (ThermoFisher Scientific) were 95 °C 10 min, (95 °C 15 s, 60 °C 1 min) × 45 cycles. Each 96-well plate included triplicate positive controls and no-template controls for each assay. The setup for qPCR was done in an access-restricted space dedicated to environmental sample processing in a UV hood where all consumables and pipettes were irradiated for 1 h prior to setup. The presence of PCR inhibitors was assessed in each sample through the inclusion and analysis of an IPC assay (TaqMan Exogenous Internal Positive Control Kit; Life Technologies). If a sample showed evidence of PCR inhibition (≥1 Ct shift in the IPC assay relative to control samples), it was treated with a Zymo PCR Inhibitor Removal Kit (McKee et al. 2015) and re-analyzed. Amplification results were assessed visually. We considered any sample with exponential amplification in at least one qPCR replicate to be positive.

2.6. Comparing HT-qPCR and ST-qPCR

There were two sets of eDNA samples used in this study: one set of previously collected samples drawn from the eDNAtlas (described below) that was used to assess concordance between the two platforms and the other representing newly collected samples to inventory the Nooksack River basin (Section 2.7). Our species distribution descriptions focus on this new sampling effort only, but samples from both sets were used in concordance assessment.

We opportunistically selected archived environmental samples that had already been analyzed for at least one of the 10 target taxa with ST-qPCR (using methods described in Section 2.5 above) to assess concordance with HT-qPCR results. We selected 234 samples from the Aquatic eDNAtlas Project (Young et al. 2018), which includes an archive of tens of thousands of environmental samples collected using methods described in Carim et al. (2016*b*) and analyzed with ST-qPCR. Combined with a subset of samples later collected from the Nooksack River basin and analyzed using ST-qPCR ($n = 133$), this resulted in 367 samples with paired analyses for up to four of the 10 target taxa ($n = 696$ comparisons of ST-qPCR and HT-qPCR results, with 19–150 comparisons per taxon).

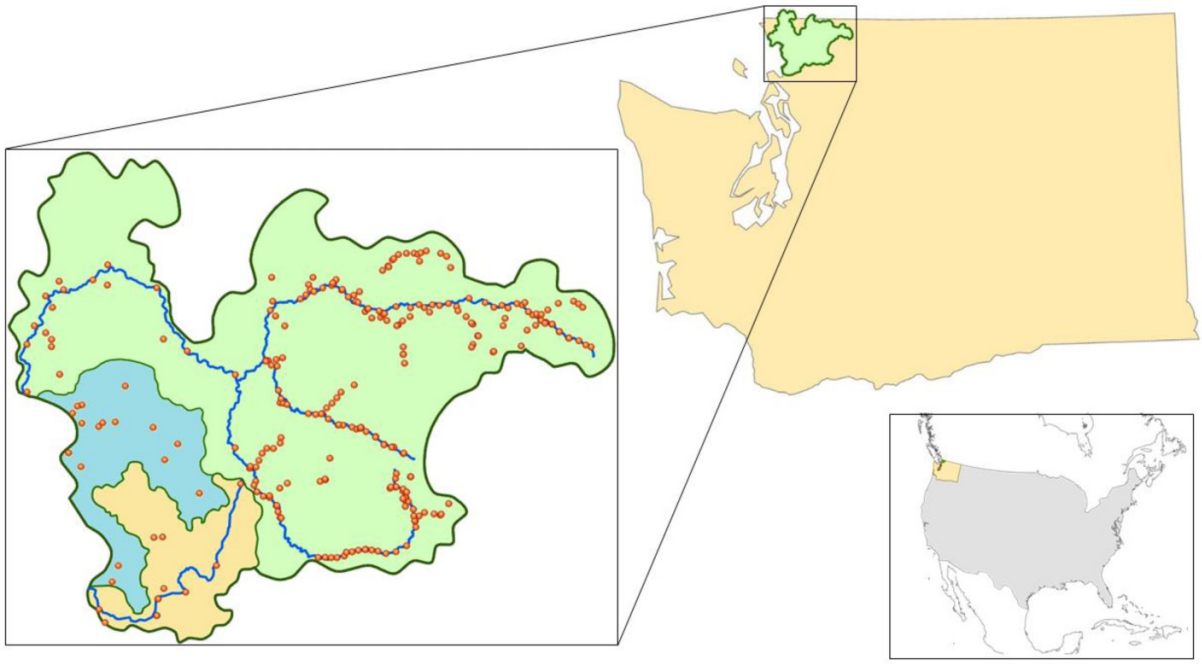
We calculated raw concordance (the proportion of results in agreement) and Cohen's kappa statistic, which accounts for unbalanced detection and nondetection data (Cohen 1960). We then bootstrapped 95% confidence intervals around raw concordance point estimates for each assay. We re-sampled the original sample size and re-calculated concordance 500 times for each assay, reporting the 2.5% and 97.5% quantiles. All statistical analyses were performed in R version 4.0.3 using the *irr* package (Gamer et al. 2012).

2.7. Field sampling and fish distribution comparison

We collected eDNA samples from across the Nooksack River basin, Samish River basin, and small costal drainages in northwestern Washington, USA, where all target taxa were known to be present in portions of the basin ($n = 269$; Fig. 1). For simplicity, we refer to this entire study area as the Nooksack River basin from here forward. Samples were collected August–October 2020, August–November 2021, and February–April and July–November 2022 by volunteers under the coordination of the North Sound Chapter of Trout Unlimited. Training in sample collection (following Carim et al. 2016*b*) was initially provided via webinar, then through a series of locally run workshops and one-on-one instruction, with maps and logistical guidance provided by SMK and equipment and site kits with single-use consumables provided by the National Genomics Center for Wildlife and Fish Conservation in Missoula, Montana, USA. Briefly, samples were collected using a peristaltic pump to filter water through a 1.5-micron-pore-size glass microfiber filter. Field crews attempted to filter 5 L at each site, but high turbidity from seasonally high flows or glacial runoff occasionally led to rapid filter clogging. At such sites, up to three samples were collected in attempts to filter a total of 5 L, but this goal was not always realized. Filters were placed in desiccant-filled plastic bags for field storage, and then frozen at −20 °C after returning from the field. Environmental DNA was extracted from filters following Franklin et al. (2019) and subsequently stored at −20 °C until analysis.

Sampling locations were selected using two approaches. In headwater areas, the sampling scheme was intended to re-

Fig. 1. Map of our study area with locations of environmental DNA samples (indicated by orange points) collected across the Nooksack River basin (green), Samish River basin (yellow), and adjacent coastal drainages (blue) in northwestern Washington, USA ($n = 269$). Blue lines show the mainstem of the Samish River and the mainstem and major tributaries of the Nooksack River. Map projections used are Albers Equal Area Conic NAD83 (locator map) and Lambert Conformal Conic—Washington State Plane North NAD 83 (watershed inset map). National and state boundaries and basemap provided by ArcGIS Online. Hydrologic features generalized from USGS National Hydrography Dataset.



solve the distribution of the three species of *Salvelinus* and followed that devised for the Rangewide Bull Trout eDNA Project (Young et al. 2017). Environmental DNA samples were collected at 1 km intervals throughout potential juvenile bull trout cold-water habitat patches identified using a juvenile bull trout environmental niche model (Isaak et al. 2015). Potential habitat patches typically consisted of contiguous stream segments in which individual reaches had mean August water temperatures $\leq$ 11 °C, summer flows $\geq$ 0.0057 $m^3 \cdot s^{-1}$, and stream reach gradients $\leq$ 15% (Isaak et al. 2015). Elsewhere in the basin, the sampling interval expanded (mean 11.52 km, range = 8.6–16.5 km), consistent with the greater species detection efficiencies often obtained in large-river sampling (Thalinger et al. 2021; Young et al. 2022). We attempted to reach all main-stem sites and all contiguous sites on selected tributaries, but private land or difficult terrain sometimes precluded access. Also, we rarely sampled warm, lower elevation tributaries that were unlikely to provide natal habitat for bull trout or Dolly Varden.

3. Results

3.1. ST-qPCR assay performance

Ten of the eleven ST-qPCR assays were both sensitive and specific. These 10 assays had a limit of detection (lowest concentration with > 95% amplification success) of 2 copies per reaction (coho salmon) or 10 copies per reaction (brook trout, bull trout, coastal cutthroat trout, chum salmon, Chinook salmon, Pacific lamprey, pink salmon, sockeye salmon, and rainbow trout) and avoided amplification of all closely related nontarget DNA tested during assay validation. The Dolly Varden assay also had a limit of detection of 10 copies per reaction but occasionally cross-amplified when exposed to DNA from certain strains of brook trout (Supplementary Material A). Nevertheless, subsequent analyses of select samples with the bull trout/Dolly Varden mitochondrial assay (Wilcox et al. 2013) largely enabled unambiguous inference about char species present in the Nooksack River basin. In only three cases (WA_022622_NOOR_94, WA_112221_NFNR_13, and WA_100120_NFNR_68; Supplemental Materials) did all char assays amplify, definitively indicating that bull trout and brook trout were present, but for which the presence of Dolly Varden was uncertain.

Considering all ST-qPCR analyses, no negative controls amplified and all positive controls did. Inhibition was detected in four of the 367 samples (Nooksack and additional samples from the eDNAtlas for biochip validation). These four samples were treated to remove PCR inhibitors with the Zymo PCR Inhibitor Removal Kit (McKee et al. 2015) and were reanalyzed for the target species.

3.2. Performance of HT-qPCR biochips

After modifying the protocol following the first biochip run, during which several cases of poor IPC performance were observed (see Supplementary Material D for revised

Table 3. Concordance between high-throughput quantitative PCR and single-taxon quantitative PCR for all 367 environmental samples analyzed on both platforms (696 comparisons overall).

Taxon	*N*	Concordance	Cohen's kappa
Brook trout *Salvelinus fontinalis*	129	0.938 (0.891–0.977)	0.837
Bull trout *Salvelinus confluentus*	149	0.953 (0.916–0.987)	0.844
Coastal cutthroat trout *Oncorhynchus clarkii*	52	0.942 (0.875–1.00)	0.862
Chinook salmon *Oncorhynchus tshawytscha*	32	0.906 (0.781–1.00)	0.811
Chum salmon *Oncorhynchus keta*	19	0.947 (0.842–1.00)	0.872
Coho salmon *Oncorhynchus kisutch*	54	0.833 (0.722–0.907)	0.664
Pacific lamprey *Entosphenus tridentatus*	141	0.965 (0.929–0.993)	0.868
Pink salmon *Oncorhynchus gorbuscha*	23	0.913 (0.783–1.00)	0.824
Sockeye salmon *Oncorhynchus nerka*	49	0.878 (0.776–0.959)	0.754
Rainbow trout *Oncorhynchus mykiss*	49	0.898 (0.816–0.959)	0.755

Note: Columns indicate the number of comparisons (N), raw concordance with bootstrapped 95% confidence intervals, and Cohen's kappa values.

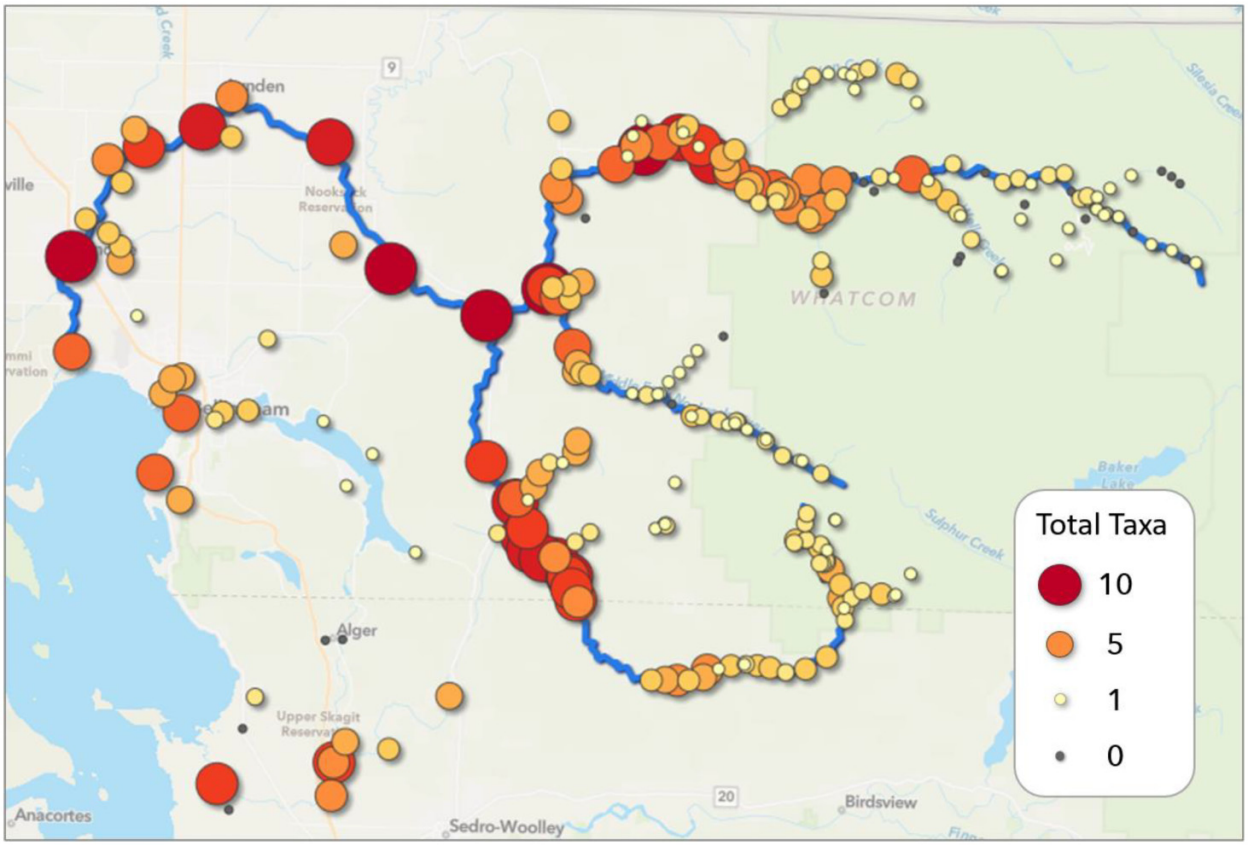
protocol), there were no issues with IPC amplification on subsequent plates ($n = 7$ plates; 100% of 4608 analyses producing the expected results). For the no-template controls, there was amplification in two controls on plate three (pink salmon and sockeye salmon), plate four (brook trout and pink salmon), and plate eight (coastal cutthroat trout and chinook salmon), and amplification in three controls on plate seven (coho salmon, chinook salmon, and rainbow trout; Table S1.D). All other control replicates were negative (40/49 controls and 1947/1960 of negative control analyses; >99.3%). When the detection threshold was applied and one-well amplifications in the no-template controls were filtered, 47/49 controls and 1958/1960 negative control analyses were considered negative (>99.8%). For the positive controls, 39/40 positive replicates amplified for the bull trout assay and 40/40 positive replicates amplified for all other assays on the biochip.

Assay sensitivity was similar to that observed in the ST-qPCR analyses. In HT-qPCR analyses, the coho salmon and bull trout assays resulted in amplification in 11/12 replicates of known concentration synthetic genes (91.7%), whereas the other eight assays resulted in 100% amplification across all 12 replicates. When testing specificity with tissue-derived DNA, we observed several cases of nontarget amplification ($n = 20$). The patterns of amplification, however, were unrelated to sequence similarity (Supplementary Material B) and subsequent testing with synthetic gene fragments and clean tissue extracts confirmed that the assays were specific against regional nontarget taxa. We attributed the apparent cross-amplification to low-level contamination of field-collected tissues with nontarget DNA (Rodgers 2017).

3.3. Comparing HT-qPCR and ST-qPCR results

For the 696 paired analyses, concordance was 0.833–0.965 per assay (mean, 0.917; 95% bootstrapped confidence intervals, 0.731–1.000; Table 3; Supplementary Material C). Concordance for coho salmon (mean, 0.833; 95% bootstrapped confidence interval, 0.731–0.926) was lower than that for all other taxa (next lowest concordance 0.878, sockeye salmon). The cause of comparatively low concordance for this assay is not known but could be due to relatively smaller sample size (see Supplementary Material A). Collectively, Cohen's kappa values (mean 0.809; range 0.664–0.872) indicated substantial agreement (0.61–0.80; three species) to near-perfect agreement (0.81–1.00; seven species; Cohen 1960). Looking across all assays, concordance was highest when ST-qPCR analyses had either zero or three wells of amplification (i.e., 0% or 100% amplification rate across replicates): a concordance of 0.9555 ($n = 652$), versus 0.558 for analyses with one or two wells of amplification ($n = 44$; 33% or 66% amplification rate). This is as expected; inconsistent amplification across ST-qPCR replicates is associated with very low DNA concentrations (i.e., below the assay limit of detection). Such inconsistency is not necessarily indicative of variable performance between methods; it may reflect low concentrations of target DNA in the environmental samples. Hence, the lack of perfect concordance can be indicative of genuine variation in the presence of tar-

Fig. 2. Environmental DNA samples collected across the Nooksack River basin, Samish River basin, and adjacent coastal drainages in northwestern Washington, USA (n = 269), represented by total number of taxa detected using quantitative PCR, ranging from 0 to 11. No samples had more than 10 taxa detected. Map projection used is Lambert Conformal Conic—Washington State Plane North NAD 83. Basemap provided by ArcGIS Online Community Basemap with primary contributions from Whatcom County, WA State Parks, and others.



get DNA in the reactions (Dysthe et al. 2018*a*; Lesperance et al. 2021).

3.4. Riverscape-scale sampling of the Nooksack River basin

The spatial extent of each species based on eDNA sampling (Fig. 2) were broadly similar to that based on traditional sampling and expert opinion, although eDNA sampling estimated less occupied habitat or less contiguous distributions for most species (Table 4). Traditional and eDNA sampling were concordant with respect to the presence of each species in all main-stem forks, except that eDNA sampling did not, and traditional sampling did, document chum salmon in the South Fork Nooksack River. A more consistent difference was that the two methods often differed substantially (>10 km) in the estimated occupied main-stem habitat by each species in the three main-stem forks. In the two focal basins previously known to contain Dolly Varden, but which lacked additional data on their extent, eDNA sampling refined the known distribution of that taxon, in general associated with the reduction of habitat thought to be occupied by bull trout. In Canyon Creek, only Dolly Varden, coastal cutthroat trout, and nonnative brook trout were detected upstream from a natural barrier thought to block anadromous fish migrations (U.S. Fish and Wildlife Service 2004). In the upper South Fork Nooksack River, Dolly Varden, bull trout, coastal cutthroat trout, and rainbow trout were broadly syntopic, with the first three erratically distributed in this stream network.

4. Discussion

4.1. Biochip performance

Overall, performance of the HT-qPCR biochip was comparable to that expected from ST-qPCR analyses (mean concordance = 0.917). The methods were highly concordant in nearly all analyses despite the HT-qPCR protocol using 90% less template DNA per taxon than ST-qPCR, which enabled more analyses to be run for each limited sample, enhancing the value of archived environmental samples. The efficiencies of scale also led to substantial reductions in cost; analyses of the 2690 species-site combinations on the biochip were estimated to cost < 40% of that to generate a similar dataset using ST-qPCR. Nevertheless, as with other assay-based tools that target taxa within large and diverse families, users should exercise caution and consider any region-specific caveats prior to applying this specific

Table 4. Comparison of the habitat occupied by 11 species in the main-stem forks of the Nooksack River based on environmental DNA (eDNA) sampling and traditional sampling.

	Occupied main-stem habitat (km)					
	North Fork		Middle Fork		South Fork	
Species	eDNA	Traditional	eDNA	Traditional	eDNA	Traditional
Bull trout	46.0	50.5[a]	13.2	28.7	63.0	63.6
Dolly Varden[b]	0	ND	0	ND	24.4	ND
Brook trout	44.4	ND	0	ND	0	ND
Rainbow trout[c]	70.3	46.0	28.7	12.5	63.6	63.2
Coastal cutthroat trout	35.1	65.5	28.7	28.7	63.6	62.6
Chinook salmon	46.0	46.0	7.3	12.5	42.4	51.6
Coho salmon	46.0	45.4	7.3	8.5	31.6	56.6
Chum salmon	22.5	43.5	7.3	8.1	0	32.4
Pink salmon	33.7	46.0	7.3	12.5	31.6	41.0
Sockeye salmon	46.0	43.4	0.8	8.1	44.6	51.6
Pacific lamprey	5.6	6.0	0.9	ND	46.9	12.7

Note: ND: no data. Note that temporal differences between seasonal and even-year habitat use by chum salmon and pink salmon likely resulted in reduced representation of these species in eDNA samples (See Section 4.2).
[a]Includes 4.5 km of habitat above Nooksack Falls unlikely to be occupied by bull trout.
[b]Traditional data did not discriminate between Dolly Varden and bull trout.
[c]Because eDNA assays cannot distinguish between rainbow trout and steelhead, traditional observations for summer steelhead, winter steelhead, and rainbow trout were combined.

biochip outside of the study region. Additionally, there are some issues unique to using the biochip that warrant further attention.

Previous studies have found that specific assays may sometimes perform poorly on an HT-qPCR platform. In Wilcox et al. (2020), using a different HT-qPCR system (OpenArray; ThermoFisher Scientific) the assay for westslope cutthroat trout (*Oncorhynchus lewisi*) performed poorly relative to other assays, and the reason was unclear, but could be related to an overlap between the hydrolysis probe and reverse primer in this assay (Wilcox et al. 2015). HT-qPCR results for the coho salmon assay in this study had the lowest concordance with single species qPCR across all assays on the biochip (concordance = 0.833). However, we do not believe that this was due to the coho assay's performance or compatibility with the HT-qPCR platform but instead because many of the samples selected for concordance testing for coho salmon had low amounts of target DNA, which likely caused stochasticity in detection across both platforms (see Supplementary Material A). Increasing the number of samples used for coho salmon concordance testing will likely improve concordance between the two platforms.

Another potential limitation of the biochip is the need for pre-amplification. Although pre-amplification is necessary to generate sufficient template, we expect that the risk of contamination is higher compared to ST-qPCR because of the handling of PCR products during the preparation of biochips; however, our observed rate of contamination was not high relative to that estimated in ST-qPCR-based eDNA projects (Smith and Goldberg 2020; Tingley et al. 2021; Buxton et al. 2022). We created and followed a protocol tailored to reducing contamination, yet still observed some level of false inference in our negative controls. False inference occurred in nine controls (0.58% of control analyses), although 78% of these amplifications were isolated to single-well amplifications. To address concerns related to pre-amplification and improve the reliability of results, we suggest replication not only at the analysis step but also spatially and temporally in the field.

Although concordance between HT-qPCR and ST-qPCR results were high overall, the majority of discordant HT-qPCR results (61%) were "false negatives", signifying instances where HT-qPCR did not, and ST-qPCR did, detect a taxon. Because the smaller reaction wells may reduce the likelihood that a target species DNA will be present in a reaction, exploring biochips with larger wells that could accommodate greater template volume holds promise for increasing sensitivity on the biochip.

Finally, the ability of HT-qPCR to produce accurate estimates of DNA concentrations from noninvasive samples, which are often used to estimate abundance or biomass in ST-qPCR applications (Takahara et al. 2012; Lacoursière-Roussel et al. 2016), has shown promising potential (Byappanahalli et al. 2015; Crane 2016; Dreier et al. 2021). In cases where accuracy of quantitative estimates from HT-qPCR has been benchmarked against ST-qPCR, significant correlations between the two suggest that HT-qPCR could be as accurate as ST-qPCR (Ishii et al. 2014; Hill et al. 2023). However, to our knowledge, quantitative estimates from the HT-qPCR platform used in this study have not been benchmarked against ST-qPCR, and accuracy of such estimates is likely to vary across HT-qPCR platforms, protocols, and samples analyzed. For example, Crane et al. (2018) found large discrepancies between HT-qPCR and ST-qPCR estimates when soil extracts were diluted 4-fold or less, i.e., the HT-qPCR platform and methods used were likely more sensitive to PCR inhibitors, which affected quantification accuracy. Future work should be directed toward assessing accuracy and precision of quantitative eDNA estimates on our specific platform to ensure its performance is comparable to ST-qPCR.

4.2. Riverscape patterns

We found that the estimates of fish habitat occupancy by both traditional methods and eDNA sampling were similar, despite the disparate levels of effort and expertise involved. Traditional estimates were based on over two decades of electrofishing, redd sampling, and in-stream trapping conducted by professional biologists. In contrast, the eDNA sampling campaign was conducted entirely by part-time volunteer citizen scientists working across a 2-year interval, in combination with a highly repeatable and potentially automatable laboratory approach. The potentially greater spatial precision and reduced labor costs of eDNA sampling campaigns, especially those that engage citizen scientists (e.g., Isaak et al. 2018; Young et al. 2022), combined with the efficiencies provided by the biochip suggest that near-real-time, whole-basin assessment and monitoring for suites of marquis taxa could readily become the norm.

Nevertheless, one of the advantages of the biochip—the ability to simultaneously assess presence for multiple species with high sensitivity and specificity—also demands greater rigor in sampling design or a willingness to compromise, because life history variation among members of the biochip species pool can lead to temporal variation in species detection and habitat occupancy. For example, we did not detect chum salmon in the South Fork Nooksack River, although the species has been documented using conventional sampling methods (Table 4). We believe that this is because sample timing did not coincide with the seasonal use of these habitats. Across the Nooksack River basin, this likely explains the lower eDNA-based estimates of habitat occupancy and more sporadic detections of chum salmon and pink salmon, both of which have life histories with relatively brief freshwater components (Waples et al. 2001). Previous work suggested that eDNA sampling for chum salmon would be most effective in late summer and when spawning peaks in late fall (Duda et al. 2021; Homel et al. 2021), a period when we collected few eDNA samples. For pink salmon, the year of sampling is also important. Stocks in most of western North America are dominated by odd-year runs (Ruggerone et al. 2023), whereas the bulk of our eDNA sampling was in 2020 and 2022. Further complicating this issue is that many portions of the Nooksack River basin exhibit high turbidity from glacial runoff during late summer baseflows, or from periodic spikes in flow associated with fall and winter frontal storms. Filter clogging from high turbidity can lead to reduced sample volumes and lower species detectability in places or at times otherwise regarded as optimal (Stoeckle et al. 2017; Williams et al. 2017; Holmes et al. 2024). Hence, a biochip-based eDNA sampling program can represent a compromise between maximizing across-species detectability with the feasibility of obtaining many environmental samples.

Substantial differences in the distribution of other species are more difficult to attribute to eDNA sampling issues. Distributions of coastal cutthroat trout and rainbow trout often differed by tens of kilometers between the two methods. Given that the eDNA-based detections of these species were often spatially consistent, and the assays exhibited high sensitivity and specificity, we suspect that the eDNA results are valid, and may have resulted from difficulties in distinguishing resident cutthroat trout from rainbow trout in traditional samples. That may also explain the discrepancy between methods in the estimated distribution of bull trout in the North Fork Nooksack River. The only char found by eDNA analyses above Nooksack Falls was introduced brook trout, which may have been misidentified as resident bull trout by traditional sampling. More problematic are the traditional sampling estimates of bull trout habitat occupancy in the Middle Fork Nooksack River. In previous decades, there were multiple observations of bull trout up to 16 km upstream from the City of Bellingham dam, which was removed in 2020 (U.S. Fish and Wildlife Service 2004), an area where environmental niche modeling (Isaak et al. 2015) suggested that high-quality natal habitat for bull trout was abundant (Nooksack sample map; https://www.fs.usda.gov/rm/boise/AWAE/projects/BullTrout_eDNA/SampleSites.html). The more recent eDNA sampling demonstrated that brook trout and Dolly Varden were absent from the entire Middle Fork basin, and that the upstream-most detection of bull trout was only 0.1 km upstream from the former dam site. These observations suggest a recent and marked decline in bull trout in this basin. Removal of the City of Bellingham dam, however, may enable recolonization of this watershed by bull trout, as well as the remaining anadromous species. Collectively, these observations emphasize how extensive, systematic eDNA sampling can refine estimates of occupied habitat for morphologically ambiguous taxa, as well as draw a distinction between habitats historically occupied (or believed to be, based on expert opinion), and current distributions.

Coupling the biochip with ST-qPCR assays also resolved the distribution of the three char species in the other two forks of the Nooksack River. In the South Fork, brook trout were restricted to a low-elevation tributary (Hutchinson Creek), bull trout were found throughout the main stem and portions of the headwaters, and Dolly Varden were largely restricted to headwater reaches, with periodic detections downstream in the main stem. We interpret these downstream detections as evidence of the presence of low densities of Dolly Varden, because most involved three-well, ST-qPCR amplifications, an index of eDNA abundance generally associated with nearby individuals (Robinson et al. 2019; Young et al. 2022). These three char species exhibited more overlap in the North Fork. Bull trout were common throughout the main-stem North Fork and its colder tributaries below Nooksack Falls but were absent above the barrier falls in Canyon Creek. In contrast, we detected Dolly Varden at every sampling site in Canyon Creek and its tributaries. We had unambiguous detections at only two other sites (Wells Creek) in the North Fork basin. Brook trout were the most ubiquitous char in the North Fork, including at many sites upstream from Nooksack Falls, in Wells Creek and Canyon Creek, and in many main-stem sites. Two of these main-stem sites (and one in the main-stem Nooksack River), were the only places where assays for all three species could not resolve the presence of Dolly Varden. Despite the potential for substantial overlap, the co-occurrence of these three species was rare in this river basin.

5. Conclusions

Previous studies have shown that basin-wide eDNA sampling datasets enable new inferences about species responses to habitat restoration (Duda et al. 2021), efficacy of reintroduction efforts (Riaz et al. 2020), determinants of species distribution (Isaak et al. 2022; Young et al. 2022), invasion dynamics (Winkowski et al. 2024), and species interactions (Wilcox et al. 2018). The biochip and sampling approach described here add to that list by demonstrating the feasibility of conducting standardized, cost-effective, whole-basin assessments of habitat occupancy of a suite of fishes that have been the focus of billions of dollars (US) of conservation spending (GSRO 2020; Jaeger and Scheuerell 2023) and for which billions more may be needed to achieve conservation goals (Bilby et al. 2024). We believe that a similar biochip-based approach, coupled with eDNA sampling that is systematically distributed, ecologically informed, and volunteer-driven, could be a valuable asset to these conservation efforts throughout western North America, and to similar assemblages of species of conservation concern throughout the globe.

Acknowledgements

We recognize the Indigenous people of the Nooksack River basin, including the Nooksack and Lummi people, who have stewarded of the land, water, and fish where this study took place since time immemorial. We acknowledge the contribution of local fisheries expertise of Ned Currence, Interim Natural Resources Director for the Nooksack Indian Tribe and Devin Flawd, Stock Assessment Manager for the Lummi Nation, with sampling design and review of results. Many sample sites did not fall on open public lands. The authors would like to acknowledge Whatcom Land Trust, Seattle City Light, City of Bellingham, Hampton Lumber, and other private landowners who granted access. The project would not have been possible without our 48 uncompensated field volunteers who dedicated many days and miles in support of fisheries conservation science. We particularly appreciate the students and recent graduates of Bellingham Technical College, Skagit Valley College, and Western Washington University who collected a large portion of the samples. Christopher Rees, Kevin McKelvey, Heidi Henderson, Caleb Dysthe, and Meredith Bartron contributed to assay development (see Supplementary Material A for details).

Article information

History dates

Received: 16 May 2024
Accepted: 28 August 2024
Accepted manuscript online: 9 September 2024
Version of record online: 29 November 2024

Notes

Select papers in this Collection are derived from the “Genes, genetics and genomics in fish conservation and fisheries” session from the 2024 meeting of the Society of Canadian Aquatic Sciences held February 21-24, 2024, in Fredericton, New Brunswick, Canada.

Copyright

Data availability

Data summaries and scripts for concordance testing are available at NationalGenomicsCenter/RiverscapeBiochip (github.com; DOI:10.5281/zenodo.13651524). Sampling results from the Nooksack River basin are available from the Aquatic eDNAtlas database (Young et al. 2018).

Author information

Author ORCIDs

Joanna W. Elmore https://orcid.org/0000-0002-2851-6290

Author contributions

Conceptualization: JWE, TMW, MKY, SMK, KJC, MKS
Data curation: JWE, TMW, MKY, SMK, KJC, DHM, TWF
Formal analysis: JWE, TMW, MKY, SMK, DHM, TWF
Funding acquisition: SMK, KJC, MKS
Investigation: JWE, TMW, MKY, SMK, KJC, DHM, TWF
Methodology: JWE, TMW, MKY, SMK, KJC, DHM, TWF
Project administration: TMW, MKY, SMK, KJC, MKS
Resources: MKY, SMK, MKS
Supervision: TMW, MKY, SMK, KJC, TWF, MKS
Validation: JWE, TMW, SMK, KJC
Visualization: JWE, TMW, MKY, SMK
Writing – original draft: JWE, TMW, MKY, SMK, KJC, DHM, TWF, MKS
Writing – review & editing: JWE, TMW, MKY, SMK, KJC, DHM, TWF, MKS

Competing interests

The authors declare there are no competing interests.

Funding information

In addition to USDA Forest Service Rocky Mountain Research Station support, project funding support was provided by Trout Unlimited, North Sound Chapter of Trout Unlimited, Washington State Chapter of Trout Unlimited, 4th Corner Fly Fishers, Orvis, and numerous individuals through a grass roots online fund raising campaign.

Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/cjfas-2024-0143.

References

An, X.-L., Su, J.-Q., Li, B., Ouyang, W.-Y., Zhao, Y., Chen, Q.-L., et al. 2018. Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR. Environ. Int. **117**: 146–153. doi:10.1016/j.envint.2018.05.011.

Baxter, J.S., Taylor, E.B., Devlin, R.H., Hagen, J., and McPhail, J.D. 1997. Evidence for natural hybridization between Dolly Varden (*Salvelinus malma*) and bull trout (*Salvelinus confluentus*) in a northcentral British Columbia watershed. Can. J. Fish. Aquat. Sci. **54**: 421–429. doi:10.1139/f96-289.

Bilby, R.E., Currens, K.P., Fresh, K.L., Booth, D.B., Fuerstenberg, R.R., and Lucchetti, G.L. 2024. Why aren't salmon responding to habitat restoration in the Pacific Northwest? Fisheries, **49**: 16–27. doi:10.1002/fsh.10991.

Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., et al. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends Ecol. Evol. **29**(6): 358–367. doi:10.1016/j.tree.2014.04.003.

Buxton, A., Diana, A., Matechou, E., Griffin, J., and Griffiths, R.A. 2022. Reliability of environmental DNA surveys to detect pond occupancy by newts at a national scale. Sci. Rep. **12**(1): 1295. doi:10.1038/s41598-022-05442-1. PMID: 35079132.

Byappanahalli, M., Nevers, M.B., Whitman, R.L., and Ishii, S. 2015. Application of a microfluidic quantitative polymerase chain reaction technique to monitor bacterial pathogens in beach water and complex environmental matrices. Environ. Sci. Technol. Lett. **12**: 347–351. doi:10.1021/acs.estlett.5b00251.

Carim, K.J., Dysthe, J.C., Young, M.K., McKelvey, K.S., and Schwartz, M.K. 2017. A noninvasive tool to assess the distribution of Pacific Lamprey (*Entosphenus tridentatus*) in the Columbia River Basin. PLoS One, **12**(1): e0169334. doi:10.1371/journal.pone.0169334. PMID: 28068358.

Carim, K.J., Dysthe, J.C.S., Young, M.K., McKelvey, K.S., and Schwartz, M.K. 2016a. An environmental DNA assay for detecting Arctic grayling in the upper Missouri River basin, North America. Conserv. Genet. Resour. **8**(3): 197–199. doi:10.1007/s12686-016-0531-1.

Carim, K.J., Larson, D.C., Helstab, J.M., Young, M.K., and Docker, M.F. 2023. A revised taxonomy and estimate of species diversity for western North American *Lampetra*. Environ. Biol. Fishes, **106**: 817–836. doi:10.1007/s10641-023-01397-y.

Carim, K.J., McKelvey, K.S., Young, M.K., Wilcox, T.M., and Schwartz, M.K. 2016b. A protocol for collecting environmental DNA samples from streams. Gen. Tech. Rep. RMRS-GTR-355. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO. p. 18.

Cohen, J. 1960. A coefficient of agreement for nominal scales. Educ. Psychol. Meas. **20**: 37–46. doi:10.1177/001316446002000104.

Coissac, E., Riaz, T., and Puillandre, N. 2012. Bioinformatic challenges for DNA metabarcoding of plants and animals. Mol. Ecol. **21**(8): 1834–1847. doi:10.1111/j.1365-294X.2012.05550.x.

Crane, S. 2016. Microfluidic qPCR for microbial ecotoxicology in soil: a pilot study. UNSW Sydney. Doctoral dissertation. doi: https://doi.org/10.26190/unsworks/19033.

Crane, S.L., Van Dorst, J., Hose, G.C., King, C.K., and Ferrari, B.C. 2018. Microfluidic qPCR enables high throughput quantification of microbial functional genes but requires strict curation of primers. Front. Environ. Sci. **6**: 145. doi:10.3389/fenvs.2018.00145.

Dass, A.M., Sherman, C.D.H., Nai, Y.H., Ellis, M.R., van Oorschot, R.A.H., and Durdle, A. 2022. Assessing the use of environmental DNA (eDNA) as a tool in the detection of human DNA in water. J. Forensic. Sci. **67**(6): 2299–2307. doi:10.1111/1556-4029.15124.

Deer, D.M., Lampel, K.A., and González-Escalona, N. 2010. A versatile internal control for use as DNA in real-time PCR and as RNA in real-time reverse transcription PCR assays. Lett. Appl. Microbiol. **50**(4): 366–372. doi:10.1111/j.1472-765X.2010.02804.x.

DeHaan, P.W., Schwabe, L.T., and Ardren, W.R. 2010. Spatial patterns of hybridization between bull trout, *Salvelinus confluentus*, and brook trout, *Salvelinus fontinalis* in an Oregon stream network. Conserv. Genet. **11**: 935–949. doi:10.1007/s10592-009-9937-6.

Di Bernardi, C., Wikenros, C., Hedmark, E., Boitani, L., Ciucci, P., Sand, H., and Åkesson, M. 2021. Multiple species-specific molecular markers using nanofluidic array as a tool to detect prey DNA from carnivore scats. Ecol. Evol. **11**: 11739–11748. doi:10.1002/ece3.7918.

Dreier, M., Berthoud, H., Shani, N., Wechsler, D., and Junier, P. 2021. Development of a high-throughput microfluidic qPCR system for the quantitative determination of quality-relevant bacteria in cheese. Front. Microbiol. **11**: 619166. doi:10.3389/fmicb.2020.619166. PMID: 33488561.

Duda, J.J., Hoy, M.S., Chase, D.M., Pess, G.R., Brenkman, S.J., McHenry, M.M., and Ostberg, C.O. 2021. Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam removal. Environ. DNA, **3**: 121–141. doi:10.1002/edn3.134.

Dunham, J., Baxter, C., Fausch, K., Fredenberg, W., Kitano, S., Koizumi, I., et al. 2008. Evolution, ecology, and conservation of Dolly Varden, white spotted char, and bull trout. Fisheries, **33**: 537–550. doi:10.1577/1548-8446-33.11.537.

Dysthe, J.C., Franklin, T.W., McKelvey, K.S., Young, M.K., and Schwartz, M.K. 2018a. An improved environmental DNA assay for bull trout (*Salvelinus confluentus*) based on the ribosomal internal transcribed spacer I. PLoS One, **13**(11): e0206851. doi:10.1371/journal.pone.0206851. PMID: 30399172.

Dysthe, J.C., Rodgers, T., Franklin, T.W., Carim, K.J., Young, M.K., McKelvey, K.S., et al. 2018b. Repurposing environmental DNA samples—detecting the western pearlshell (*Margaritifera falcata*) as a proof of concept. Ecol. Evol. **8**(5): 2659–2670. doi:10.1002/ece3.3898.

Franklin, T.W., Wilcox, T.M., McKelvey, K.S., Greaves, S.E., Dysthe, J.C., Young, M.K., et al. 2019. Repurposing environmental DNA samples to verify the distribution of Rocky Mountain tailed frogs in the Warm Springs Creek Basin, Montana. Northwest Sci. **93**(1): 85–92. doi:10.3955/046.093.0108.

Gamer, M., Lemon, J., Fellows, I., and Singh, P. 2012. Irr: various coefficients of interrater reliability and agreement. R package version 0.84.1. Available from https://CRAN.R-project.org/package=irr [accessed 15 May 2024].

GSRO (Governor's Salmon Recovery Office). 2020. State of salmon report. GSRO, Olympia, Washington. Available from https://stateofsalmon.wa.gov [accessed 29 April 2024].

Hallam, J., Clare, E.L., Jones, J.I., and Day, J.J. 2021. Biodiversity assessment across a dynamic riverine system: a comparison of eDNA metabarcoding versus traditional fish surveying methods. Environ. DNA, **3**(6): 1247–1266. doi:10.1002/edn3.241.

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., et al. 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). Ecol. Evol. **8**(12): 6330–6341. doi:10.1002/ece3.4013.

Hatzenbuhler, C., Kelly, J.R., Martinson, J., Okum, S., and Pilgrim, E. 2017. Sensitivity and accuracy of high-throughput metabarcoding methods for early detection of invasive fish species. Sci. Rep. **7**(1): Article 1. doi:10.1038/srep46393.

Hill, E.R., Chun, C.L., Hamilton, K., and Ishii, S. 2023. High-throughput microfluidic quantitative PCR platform for the simultaneous quantification of pathogens, fecal indicator bacteria, and microbial source tracking markers. ACS ES&T Water, **3**(8): 2647–2658. doi:10.1021/acsestwater.3c00169.

Holmes, A.E., Baerwald, M.R., Rodzen, J., Schreier, B.M., Mahardja, B., and Finger, A.J. 2024. Evaluating environmental DNA detection of a rare fish in turbid water using field and experimental approaches. PeerJ, **11**: e16453. doi:10.7717/peerj.16453.

Homel, K.M., Franklin, T.W., Carim, K.J., McKelvey, K.S., Dysthe, J.C., and Young, M.K. 2021. Detecting spawning of threatened chum salmon *oncorhynchus keta* over a large spatial extent using eDNA sampling: opportunities and considerations for monitoring recovery. Environ. DNA, **3**(3): 631–642. doi:10.1002/edn3.159.

Huver, J.R., Koprivnikar, J., Johnson, P.T.J., and Whyard, S. 2015. Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol. Appl. **25**(4): 991–1002. doi:10.1890/14-1530.1.

Isaak, D.J., Young, M.K., Horan, D.L., Nagel, D., Schwartz, M.K., and McKelvey, K.S. 2022. Do metapopulations and management matter for relict headwater bull trout populations in a warming climate? Ecol. Appl. **32**(5): p.e2594. doi:10.1002/eap.2594.

Isaak, D.J., Young, M.K., McConnell, C., Roper, B.B., Archer, E.K., Staab, B., et al. 2018. Crowd-sourced databases as essential elements for Forest Service partnerships and aquatic resource conservation. Fisheries, **43**: 423–430. doi:10.1002/fsh.10083.

Isaak, D.J., Young, M.K., Nagel, D.E., Horan, D.L., and Groce, M.C. 2015. The cold-water climate shield: delineating refugia for preserving salmonid fishes through the 21st century. Global Change Biol. **21**: 2540–2553. doi:10.1111/gcb.12879.

Ishii, S., Nakamura, T., Ozawa, S., Kobayashi, A., Sano, D., and Okabe, S. 2014. Water quality monitoring and risk assessment by simultaneous multipathogen quantification. Environ. Sci. Technol. **48**(9): 4744–4749. doi:10.1021/es500578s.

Ishii, S., Segawa, T., and Okabe, S. 2013. Simultaneous quantification of multiple food-and waterborne pathogens by use of microfluidic quantitative PCR. Appl. Environ. Microbiol. **79**(9): 2891–2898. doi:10.1128/AEM.00205-13.

Jaeger, W.K., and Scheuerell, M.D. 2023. Return(s) on investment: restoration spending in the Columbia River Basin and increased abundance of salmon and steelhead. PLoS One, **18**(7): e0289246. doi:10.1371/journal.pone.0289246.

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., et al. 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol. Ecol. Resour. **15**(1): 216–227. doi:10.1111/1755-0998.12285.

Jerde, C.L., Mahon, A.R., Chadderton, W.L., and Lodge, D.M. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conserv. Lett. **4**(2): 150–157. doi:10.1111/j.1755-263X.2010.00158.x.

Keck, F., Couton, M., and Altermatt, F. 2023. Navigating the seven challenges of taxonomic reference databases in metabarcoding analyses. Mol. Ecol. Resour. **23**(4): 742–755. doi:10.1111/1755-0998.13746.

Kelly, R.P., Port, J.A., Yamahara, K.M., and Crowder, L.B. 2014. Using environmental DNA to census marine fishes in a large mesocosm. PLoS One, **9**(1): e86175. doi:10.1371/journal.pone.0086175.

Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C., Hunter, M.E., et al. 2020. Reporting the limits of detection and quantification for environmental DNA assays. Environ. DNA, **2**: 271–282. doi:10.1002/edn3.29.

Korenková, V., Scott, J., Novosadová, V., Jindřichová, M., Langerová, L., Švec, D., et al. 2015. Pre-amplification in the context of high-throughput qPCR gene expression experiment. BMC Mol. Biol. **16**: 5. doi:10.1186/s12867-015-0033-9.

Lacoursière-Roussel, A., Rosabal, M., and Bernatchez, L. 2016. Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. Mol. Ecol. Resour. **16**: 1401–1414. doi:10.1111/1755-0998.12522.

Lesperance, M.L., Allison, M.J., Bergman, L.C., Hocking, M.D., and Helbing, C.C. 2021. A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environ. DNA, **3**: 970–981. doi:10.1002/edn3.220.

Liu, A., Geraldes, A., and Taylor, E.B. 2023. Development and testing of diagnostic molecular markers to differentiate among three species of *Salvelinus*: Arctic char, Dolly Varden, and Bull trout. Conserv. Genet. Resour. **15**: 83–96. doi:10.1007/s12686-023-01305-7.

Malla, B., Thakali, O., Shrestha, S., Segawa, T., Kitajima, M., and Haramoto, E. 2022. Application of a high-throughput quantitative PCR system for simultaneous monitoring of SARS-CoV-2 variants and other pathogenic viruses in wastewater. Sci. Total Environ. **853**. doi:10.1016/j.scitotenv.2022.158659.

McKee, A.M., Spear, S.F., and Pierson, T.W. 2015. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. Biol. Conserv. **183**: 70–76. doi:10.1016/j.biocon.2014.11.031.

McKelvey, K.S., Young, M.K., Wilcox, T.M., Bingham, D.M., Pilgrim, K.L., and Schwartz, M.K. 2016. Patterns of hybridization among cutthroat trout and rainbow trout in northern Rocky Mountain streams. Ecol. Evol. **6**: 688–706. doi:10.1002/ece3.1887.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., and Gough, K.C. 2014. REVIEW: the detection of aquatic animal species using environmental DNA—a review of eDNA as a survey tool in ecology. J. Appl. Ecol. **51**(5): 1450–1459. doi:10.1111/1365-2664.12306.

Riaz, M., Kuemmerlen, M., Wittwer, C., Cocchiararo, B., Khaliq, I., Pfenninger, M., and Nowak, C. 2020. Combining environmental DNA and species distribution modeling to evaluate reintroduction success of a freshwater fish. Ecol. Appl. **30**(2): e02034. doi:10.1002/eap.2034.

Robinson, A.T., Paroz, Y.M., Clement, M.J., Franklin, T.W., Dysthe, J.C., Young, M.K., et al. 2019. Environmental DNA sampling of small-bodied minnows: performance relative to location, species, and traditional sampling. North Am. J. Fish. Manage. **39**: 1073–1085. doi:10.1002/nafm.10344.

Rodgers, T. 2017. Proper fin-clip sample collection for molecular analyses in the age of eDNA. J. Fish Biol. **91**(5): 1265–1267. doi:10.1111/jfb.13485.

Ruggerone, G.T., Springer, A.M., van Vliet, G.B., Connors, B., Irvine, J.R., Shaul, L.D., et al. 2023. From diatoms to killer whales: impacts of pink salmon on North Pacific ecosystems. Mar. Ecol. Prog. Ser. **719**: 1–40. doi:10.3354/meps14402.

Ruppert, K.M., Kline, R.J., and Rahman, M.S. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: a systematic review in methods, monitoring, and applications of global eDNA. Global Ecol. Conserv. **17**: e00547. doi:10.1016/j.gecco.2019.e00547.

Shaw, J.L.A., Weyrich, L.S., Hallegraeff, G., and Cooper, A. 2019. Retrospective eDNA assessment of potentially harmful algae in historical ship ballast tank and marine port sediments. Mol. Ecol. **28**(10): 2476–2485. doi:10.1111/mec.15055.

Smith, M.M., and Goldberg, C.S. 2020. Occupancy in dynamic systems: accounting for multiple scales and false positives using environmental DNA to inform monitoring. Ecography, **43**: 376–386. doi:10.1111/ecog.04743.

Stoeckle, B.C., Beggel, S., Cerwenka, A.F., Motivans, E., Kuehn, R., and Geist, J. 2017. A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. PLoS One, **12**(12): e0189119. doi:10.1371/journal.pone.0189119.

Takahara, T., Minamoto, T., and Doi, H. 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLoS One, **8**(2): e56584. doi:10.1371/journal.pone.0056584.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., and Kawabata, Z.I. 2012. Estimation of fish biomass using environmental DNA. PLoS One, **7**(4): e35868. doi:10.1371/journal.pone.0035868.

Thalinger, B., Kirschner, D., Pütz, Y., Moritz, C., Schwarzenberger, R., Wanzenböck, J., and Traugott, M. 2021. Lateral and longitudinal fish environmental DNA distribution in dynamic riverine habitats. Environ. DNA, **3**: 305–318. doi:10.1002/edn3.171.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., et al. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. **21**(11): 2565–2573. doi:10.1111/j.1365-294X.2011.05418.x.

Tillotson, M.D., Kelly, R.P., Duda, J.J., Hoy, M., Kralj, J., and Quinn, T.P. 2018. Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. Biol. Conserv. **220**: 1–11. doi:10.1016/j.biocon.2018.01.030.

Tingley, R., Coleman, R., Gecse, N., van Rooyen, A., and Weeks, A. 2021. Accounting for false positive detections in occupancy studies based on environmental DNA: a case study of a threatened freshwater fish (*Galaxiella pusilla*). Environ. DNA, **3**: 388–397. doi:10.1002/edn3.124.

U.S. Fish and Wildlife Service. 2004. Draft recovery plan for the coastal-Puget Sound distinct population segment of bull trout (*Salvelinus confluentus*). Vol. 1. Region 1, U.S. Fish and Wildlife Service, Portland, Oregon. 409p.

van der Pouw Kraan, D., Graham, C.T., Kavanagh, F., and Mirimin, L. 2024. Development and validation of a DNA-based multi-species biomonitoring toolkit using a high-throughput qPCR platform: a case study of Irish shellfish species. Mol. Ecol. Resour. **24**: e13945. doi:10.1111/1755-0998.13945.

Waples, R.S., Gustafson, R.G., Weitkamp, L.A., Myers, J.M., Jjohnson, O.W., Busby, P.J., et al. 2001. Characterizing diversity in salmon from the Pacific Northwest. J. Fish Biol. **59**: 1–41. doi:10.1111/j.1095-8649.2001.tb01376.x.

Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., and Schwartz, M.K. 2015. The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *oncorhynchus*. PLoS One, **10**(11): e0142008. doi:10.1371/journal.pone.0142008.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Engkjer, C., Lance, R.F., Lahr, A., et al. 2020. Parallel, targeted analysis of environmental samples via high-throughput quantitative PCR. Environ. DNA, **2**: 544–553. doi:10.1002/edn3.80.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R., and Schwartz, M.K. 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS One, **8**(3): e59520. doi:10.1371/journal.pone.0059520.

Wilcox, T.M., Young, M.K., McKelvey, K.S., Isaak, D.J., Horan, D.L., and Schwartz, M.K. 2018. Fine-scale environmental DNA sampling reveals climate-mediated interactions between native and invasive trout species. Ecosphere, **9**(11): e02500. doi:10.1002/ecs2.2500.

Williams, K.E., Huyvaert, K.P., and Piaggio, A.J. 2017. Clearing muddied waters: capture of environmental DNA from turbid waters. PLoS One, **12**(7): e0179282. doi:10.1371/journal.pone.0179282.

Winkowski, J.J., Olden, J.D., and Brown, S. 2024. Integrating spatial stream network models and environmental DNA to estimate current and future distributions of nonnative Smallmouth bass. Trans. Am. Fish. Soc. **153**: 180–199. doi:10.1002/tafs.10454.

Wood, S.A., Pochon, X., Laroche, O., von Ammon, U., Adamson, J., and Zaiko, A. 2019. A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. Mol. Ecol. Resour. **19**(6): 1407–1419. doi:10.1111/1755-0998.13055.

Young, M.K., Isaak, D.J., McKelvey, K.S., Schwartz, M.K., Carim, K.J., Fredenberg, W., et al. 2017. Species occurrence data from the Range-Wide Bull Trout eDNA Project. Forest Service Research Data Archive, Fort Collins, CO. doi:10.2737/RDS-2017-0038.

Young, M.K., Isaak, D.J., Nagel, D., Horan, D.L., Carim, K.J., Franklin, T.W., et al. 2022. Broad-scale eDNA sampling for describing aquatic species distributions in running waters: Pacific lamprey *Entosphenus tridentatus* in the upper Snake River, USA. J. Fish Biol. **101**: 1312–1325. doi:10.1111/jfb.15202.

Young, M.K., Isaak, D.J., Schwartz, M.K., McKelvey, K.S., Nagel, D.E., Franklin, T.W., et al. 2018. Species occurrence data from the aquatic eDNAtlas database. Forest Service Research Data Archive, Fort Collins, CO. doi:10.2737/RDS-2018-0010.

Zheng, B., Zhu, Y., Sardans, J., Peñuelas, J., and Su, J. 2018. QMEC: a tool for high-throughput quantitative assessment of microbial functional potential in C, N, P, and S biogeochemical cycling. Sci. China Life Sci. **61**(12): 1451–1462. doi:10.1007/s11427-018-9364-7.