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Better boundaries: identifying the upper extent of fish distributions in forested streams using eDNA and electrofishing

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Citation: Penaluna, B. E., J. M. Allen, I. Arismendi, T. Levi, T. S. Garcia, and J. K. Walter. 2021. Better boundaries: identifying the upper extent of fish distributions in forested streams using eDNA and electrofishing. Ecosphere 12(1): e03332. 10.1002/ecs2.3332

Abstract. The management of species that occur in low densities is a conservation concern worldwide across taxa with consequences for managers and policymakers. The distribution boundary at the upper extent of fish in North America receives extra attention because stream reaches with fish are managed differently and often have more protections than fishless reaches. Here, we examine the relative reliability of water environmental DNA (eDNA), polymerase chain reaction (PCR)-amplified for Coastal Cutthroat Trout (*Oncorhynchus clarkii clarkii*) to detect the upper extent of fish across streams as a potential management tool compared to standard electrofishing methods. We provide estimates of fish detection probabilities from eDNA analyses, and probabilities of detection for both eDNA field samples and quantitative PCR (qPCR) given covariates of habitat characteristics and fish densities from electrofishing. We present a primer and probe based on the cytochrome oxidase I gene using qPCR to detect trout DNA across water samples from 60 forested streams in the Pacific Northwest, USA using high-resolution spatial sampling. In 28% of streams, the upper extent of fish matches between methods. In over half of the streams, Coastal Cutthroat Trout eDNA was detected above the electrofishing last-fish boundary. Although some detections could be attributed to false-positive errors, eDNA results extend the upstream, leading edge of fish by 50–250 m from the electrofishing boundary. In 20% of the streams, detections of last-fish occurred higher in the stream network with electrofishing rather than eDNA, but generally by only 50 m. Modeled results revealed that the occurrence of trout eDNA was higher in wider-stream locations and that eDNA detections occurred at lower electrofishing densities (<5 trout per 50 linear m). We also showed that three replicate eDNA samples were sufficient to capture trout eDNA when eDNA was present. Although eDNA constitutes an effective addition to approaches to delimit the upper extent of fish, its effectiveness depends on previous knowledge of the last-fish boundary to apprise where to start sampling and targeting fish species anticipated to be last-fish. We present evidence that eDNA is a valuable tool in investigating fish distributions taking its place alongside traditional high-effort catch–release tools.

Key words: Coastal Cutthroat Trout; Cutthroat Trout; electrofishing; end-of-fish; environmental DNA; fish distributions; HJ Andrews Experimental Forest; last-fish; streams; upper extent of fish; upper fish boundary.

Received 10 April 2020; revised 26 June 2020; accepted 10 July 2020; final version received 20 October 2020. Corresponding Editor: Ryan A. Martin.

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Introduction

Delimiting geographic distributional boundaries of species is fundamental for conservation and management decision-making. Most studies that focus on distributional boundaries evaluate range changes for species across broad spatial extents, including continental (birds, Jetz et al. 2012), latitudinal (multi-taxon, Parmesan and Yohe 2003), and elevational (butterflies, Forister et al. 2010) gradients. Delimiting the edges of populations at local scales, however, is less well understood, especially when species are found in low densities or are rare. For example, the upper extent of fish distributions in stream networks receives significant attention in North America, especially in western US and Canada, because reaches with fish are both managed differently and often have more protections from forest harvest practices, including wider riparian buffers than fishless reaches (Blinn and Kilgore 2001, Lee et al. 2004). Considerable efforts are made each year by managers to delineate the upper extent of fish in stream networks to inform management decisions, and this task is made difficult by detection errors in surveys.

Electrofishing is currently the most widely used approach to delineate the upstream extent of fish in streams because it allows for the detection of fish in real-time (Table 1). At its best, standard backpack electrofishing is most efficient at capturing larger fish in shallower water with simple stream-habitat conditions, including very clear water (Price and Peterson 2010). Electrofishing estimates of stream-living trout are generally negatively biased and sampling efficiency decreases with stream size and complexity of habitats (*Salvelinus confluentus* and *Oncorhynchus clarkii lewisi*, Peterson et al. 2004; *Oncorhynchus mykiss*, Rosenberger and Dunham 2005). Trout have higher capture probabilities than other fishes, such as those with coarse scales (cyprinids) or without swim bladders (freshwater sculpins, *Cottus* spp.). However, electrofishing can be time-consuming, labor-intensive, can harm fishes, and requires fish-take permits and animal care and use permission (Evans et al. 2017), and sampling efficiency is low when fish abundances are low (Rosenberger and Dunham 2005). Consequently, questions remain about the most effective means of locating fish at their upper extent given the myriad of challenges in detecting them in stream reaches with low and patchy fish densities (Torgersen et al. 2004, Chelgren and Dunham 2015).

A potential alternative method for delineating species distributions, environmental DNA (eDNA) analyses, detects the presence of target species by measuring target DNA that is left behind in the environment. It is an indirect sampling method that does not harm animals and requires fewer permissions (Goldberg et al. 2016, Evans and Lamberti 2018). Environmental DNA can detect Killer Whales *Orcinus orca* in seawater (Baker et al. 2018), carnivores in snow (Franklin et al. 2019), and fish in freshwater (Jerde et al. 2011, Lacoursière-Roussel et al. 2016). Specifically, eDNA has been shown to be

Table 1. Comparison of environmental DNA (eDNA) vs. electrofishing approaches to delimit upper extent of fish occurrences in streams.

Characteristic of method	eDNA	Electrofishing
Detects presence of fish	**Yes**	**Yes**
Detects absence of fish†	No	No
Estimates relative abundance of fish	**Yes**	**Yes**
Can archive fish as museum voucher	No	**Yes**
Can archive eDNA sample	**Yes**	No
Obtains data on length, mass, or fish characteristics	No	**Yes**
Obtains genetic data‡	**Yes**	**Yes**
Effectively samples complex habitat and wetlands	**Yes**	Depends
Allows for sampling year-round	With safe access	In wadeable waters
Can directly harm fish	**No**	Yes
Need state/federal scientific take permit	**No**	Yes
Could offer data instantaneously§	**Yes**	**Yes**
Identifies exact time and place of fish	No	**Yes**
Potential for false positives¶	Yes	**No**
Potential for false negatives	Yes	Yes

Note: Boldface denotes positive characteristics of method.

† Absence of fish can be assumed following extensive sampling at a site with either approach.

‡ Genetic data can be obtained from eDNA samples if they are sequenced in addition to quantitative PCR analysis,

§ Technology is rapidly developing, such as hand-held products by Biomeme, Inc, Philadelphia, PA, USA.

¶ Electrofishing could produce false positives if there are issues with field identification of target species, such as distinguishing between young-of-year *Oncorhynchus mykiss* and *Oncorhynchus clarkii clarkia*.

comparable to, or more sensitive at, detecting fish than electrofishing in streams (Wilcox et al. 2016, Baldigo et al. 2017, Evans et al. 2017, Ostberg et al. 2019), particularly when species are low in abundance (Dejean et al. 2012, Pilliod et al. 2013, Sigsgaard et al. 2015, Itakura et al. 2019). Although eDNA has been used to delimit fish distribution boundaries (Jerde et al. 2011, McKelvey et al. 2016, Carim et al. 2019), it has yet to be evaluated empirically within the context of forest management by identifying the transition from fish to no fish in a stream network (Coble et al. 2019). Further, despite the recent expansion of eDNA approaches into monitoring programs around the globe, there remain issues with detections of false-positive and false-negative errors (Roussel et al. 2015, Guillera-Arroita et al. 2017). For example, it is not always clear how to translate positive eDNA detections into actual living trout (or eggs) in the stream, which is an optimal management outcome, vs. detection failure or true absence (Darling and Mahon 2011, Jerde et al. 2011, Wilson et al. 2014).

Our objective was to understand the relative reliability of eDNA as a management tool to detect the upper extent of fish. Distributional boundaries of fish are characterized by low fish abundance and eDNA may be particularly adept in low abundance detection. Large gaps remain in our understanding of eDNA to detect species when they are low in numbers given habitat characteristics. To do so, we compare the upper distribution boundary of Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* in 60 forested streams of the Pacific Northwest, USA, by comparing eDNA to the gold standard approach of electrofishing. Both approaches have imperfect detection and resulting bias, and consequently, it cannot be expected that both methods would completely agree with each other when compared side-by-side (Darling 2019). We evaluate estimates of fish detection probabilities of eDNA while simultaneously considering probabilities of detection in both eDNA field samples and quantitative polymerase chain reaction (qPCR) replicates from the eDNA samples given covariates of habitat characteristics and fish densities from electrofishing. We predict that eDNA will detect fish further upstream than electrofishing across streams because of its acknowledged strength at identifying species when they occur at low abundances and because headwater streams have physical habitat complexity where fish may be more able to hide (and not be detected) from electrofishing. Ultimately, our results aim to inform managers and decision-makers about the relative reliability of sampling approaches to identify quality data and to delimit the upper extent of fish distributions.

Methods

Study streams and study design

In western North America, Coastal Cutthroat Trout *O. clarkii clarkii* are the fish generally found the highest in their stream networks and are, therefore, the central focus when considering the upper extent of fish in streams. They are spring spawners, and likely they are stream-living trout because of how high they are located in the stream network. All sampling was conducted during spring to early summer (March–June) to coincide with the recognized sampling window for evaluating the end-of-fish boundary under Forest Practices Rules for each state. The March–June sampling window is considered the optimal time period to sample for last-fish because fish may be at their uppermost extent, and water flows begin to drop making it easier to see and capture fish as well as begin to offer field crews safer access to streams.

We sampled 60 streams throughout western Washington and Oregon in 2017 and 2018. Streams occurred on a range of landownerships, including lands designated as federal (U.S. Forest Service, Bureau of Land Management), state (Oregon Department of Forestry), and private (multiple ownerships; Fig. 1). In 2017, we used Coastal Cutthroat Trout distribution information from the randomly selected populations in Torgersen et al. (2004) and Gresswell et al. (2006) to select 29 streams on federal (n = 19), state (n = 2), and private lands belonging to Silver Butte Timber Land (n = 1), Juniper Companies (Lone Rock Timber, n = 1), Lewis and Clark Timber (n = 2), and Weyerhaeuser Company (n = 4). In 2018, we increased our sampling efforts on private lands to 31 streams using company information, including additional streams on lands of Hancock Forest Management (n = 9), Port Blakely (n = 11), and Weyerhaeuser Company (n = 11).

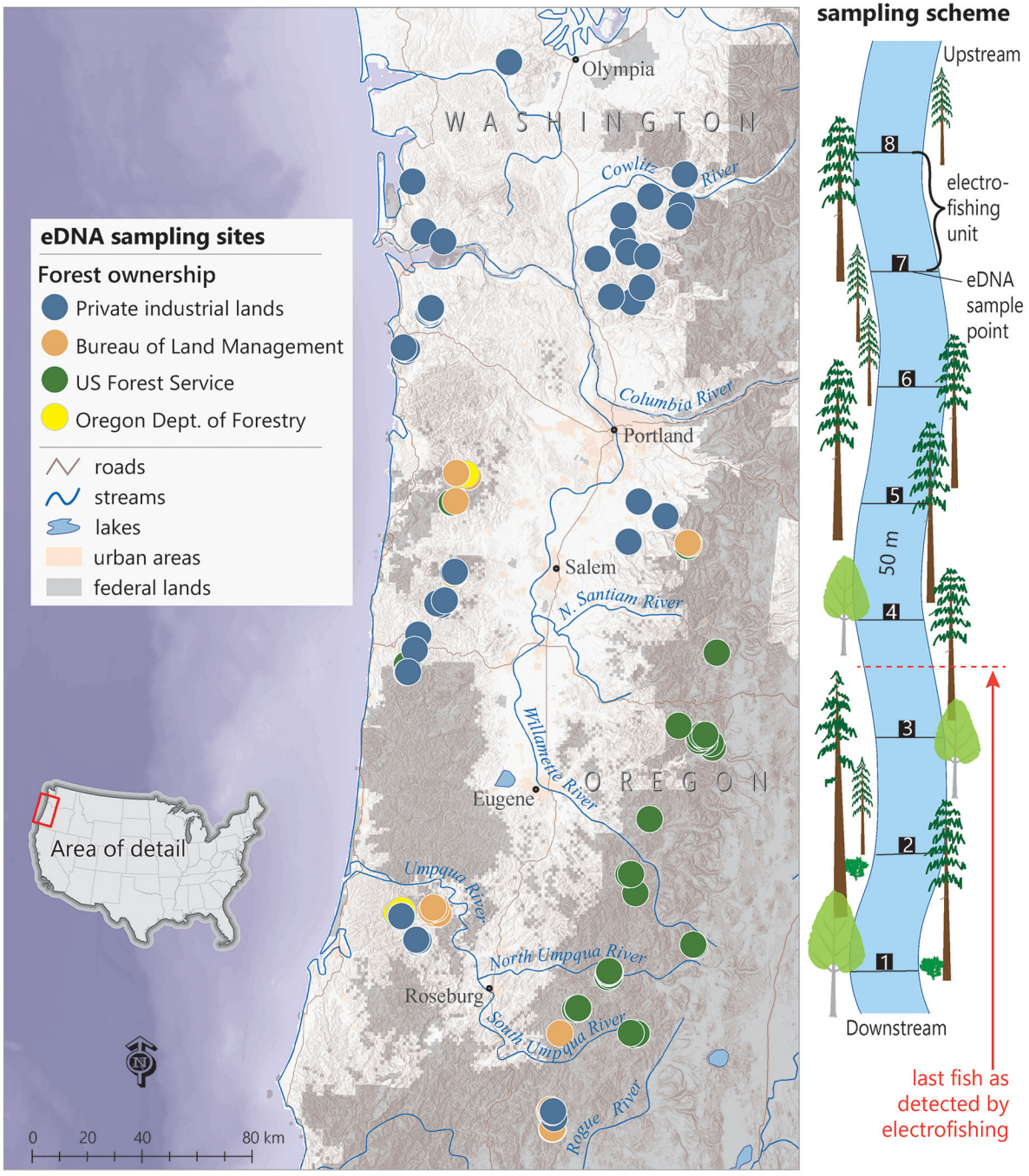

Fig. 1. Map of 60 streams in Washington and Oregon, USA of mixed ownership, including federal land in collaboration with U.S. Forest Service and Bureau of Land Management, state land managed by Oregon Department of Forestry, and various private landowners. Sampling schematic shows an environmental DNA (eDNA) sampling point at the bottom of each of the eight electrofishing units for each stream.

The starting point for each survey was 175 m further downstream from the last upstream boundary noted in prior surveys (Torgersen et al. 2004, Gresswell et al. 2006, information from private owners). We collected eDNA samples immediately in advance of electrofishing to decrease contamination risk due to field crews working in streams. For each stream, we collected water samples for eDNA analyses from stream water every 50 m at discrete sampling sites moving in an upstream direction (Fig. 1). We designed the sampling so that the current electrofishing last-fish boundary fell between sites 3 and 4, except for six streams where field logistics made it difficult to keep to this protocol. Consequently, we collected water from 12 to 20 sites on a stream and adjusted the sample reach based on electrofishing resulting in sample processing from the eight sites appropriate for our design. Accordingly, three sites (150 m) were generally downstream of previously reported last-fish occurrences from electrofishing and five sites (250 m) were upstream. Sample spacing of 50 m provided additional point information on the detection probabilities of fish above and below where fish were noted during continuous electrofishing.

eDNA sampling

We collected 1-L water samples in triplicate from the thalweg at each of the eight sites and pumped sample water through 0.45-μm single-use cellulose nitrate filters (Sterlitech, Kent, Washington, USA) using a vacuum pump (pumping time range: 4–137 min, average = 9.3 min). Water was collected with either a 1-L Nalgene bottle or Whirlpak bag and held in the stream to remain cool for 1–3 h while sampling was completed. After electrofishing efforts identified the current last-fish, we discarded samples if they fell outside the 8-site design (Fig. 1) and filtered water on-site. Filters were loosely rolled and stored on ice in separate 5-mL vials during transport and were frozen at −20°C within 6 h of collection. Filters were stored at −20°C until DNA extraction. We washed bottles and tweezers with a 50% bleach solution followed by a triple deionized water rinse before use at another stream (Goldberg et al. 2016).

We extracted DNA from each filter using a modified protocol of the Qiagen DNeasy Blood and Tissue kit (Levi et al. 2019). We added 1.0-mm zirconia–silica beads to the initial lysis buffer followed by a 15-min vortex step to loosen the DNA from the filters. Incubation in lysis buffer was increased to 48 h. After incubation, we transferred 300 μL of the lysed product to a new 1.7-mL microcentrifuge tube. Thereafter, we followed the manufacturer's protocol for isolation of tissue. DNA was eluted in a total volume of 100 μL. All DNA extractions and polymerase chain reaction (PCR) setup were done inside a clean laboratory space with a separate hepa-filtered and UV-irradiated PCR cabinet (Air Science LLC, Fort Meyers, Florida, USA) in a laboratory where no PCR products or other sources of high-concentration DNA are allowed. We used low-retention filter pipette tips for eDNA laboratory processing and qPCR assay to decrease the potential for contamination and we used control blanks for each set of extractions to detect any contamination carried out during the extraction process.

There are currently no consistent criteria for determining what is considered a positive detection for eDNA (Goldberg et al. 2016). We consider detection of trout DNA in a sample as a positive signal from a single replicate out of nine possible replicates (3 field replicates × 3 qPCR or technical replicates), but also recognize that a single positive sample provides weak evidence of species presence relative to consistent positive samples across replicates over time (Jerde et al. 2011).

eDNA quantitative PCR and development of Coastal Cutthroat Trout primer

We developed a species-specific assay for Coastal Cutthroat Trout that targets the cytochrome oxidase I (COI) of the mitochondrial genome (Appendix S1: Table S1). Using reference sequences obtained from GenBank, we created an alignment of Coastal Cutthroat Trout and sympatric *Oncorhynchus* spp. that occur in the study area, including Rainbow Trout/steelhead *O. mykiss*, Coho Salmon *O. kisutch*, Westslope Cutthroat Trout *O. clarkii lewisi*, and Chinook Salmon *O. tshawytscha* (Appendix S1: Table S2). We developed primers in a 171-bp region of the COI gene specific to Coastal Cutthroat Trout, ensuring enough mismatches among congeners to ensure amplification would only occur in the

target species (Appendix S1: Table S3). To ensure this experimentally, we obtained tissue samples from the field of known individuals and ran through the qPCR assay. Extracted tissues samples were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and diluted to 1 ng/μL. Serial dilutions were performed in a 1:10 ratio from 1 to 10^{-4} ng/μL. Each reaction contained 10-μL Environmental Master mix 2.0 (Thermo Fisher Scientific), 900-nmol/L forward primer, 900-nmol/L reverse primer, 250-nmol/L hydrolysis probe labeled with 6-FAM at the 5′ end and a minor groove binding non-fluorescent quencher (MGB-NFQ), 1 μL of DNA, and sterile water to bring the volume to 20 μL. Polymerase chain reaction cycling conditions included 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. All eDNA filter samples were then run through the assay using these conditions.

Each sample was run in triplicate qPCR reactions. Polymerase chain reaction was performed using the ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems, Foster City, California, USA). Each 20-μL qPCR reaction contained 6 μL of DNA template, 10-μL Environmental Master Mix 2.0 (Thermo Fisher Scientific), 900 nmol/L of both forward and reverse primers, 250 nmol/L of the TaqMan MGB probe, and sterile water. Additionally, each plate contained a four-point standard curve using DNA obtained from Coastal Cutthroat Trout tissue. Extracted tissue was quantified using a Qubit Fluorometer (Thermo Fisher Scientific) and diluted from 10^{-1} to 10^{-4} ng/μL. Polymerase chain reaction cycling conditions involved an initial denaturation step of 10 min at 95°C to activate the HotStart Taq DNA polymerase, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. All reaction plates contained a negative control of water and extraction blanks.

Electrofishing sampling and physical habitat surveys

After eDNA water samples were collected at each stream, we electrofished to delimit last-fish using a spatially continuous, single-pass backpack electrofishing approach similar to that described by Torgersen et al. (2004) and validated by Bateman et al. (2005), but we sampled all accessible stream habitat. We optimized electrofishing effectiveness so that units were set at the minimum needed to achieve satisfactory electrofishing in each stream (Reynolds and Harlan 2011). Detections with electrofishing were made visually by identifying individual fish to the lowest taxonomic unit as possible, which was generally species. We electrofished 29 streams in 2017 to understand presence of fish, which is representative of surveys generally used to determine the occurrence of last-fish, but in 2018 relative abundance was also estimated in an additional 31 streams. We also characterized stream size for each 50-m site, including wetted-width (m) and depth (m). For each stream, we noted potential physical barriers (e.g., waterfalls) to fish movement (Appendix S2: Fig. S1 and Fig. S2).

Data analysis

We compared the proportion of agreement between the detection of fish by eDNA and electrofishing across streams and sites. We displayed information for all results across streams and sites, including all field and qPCR replicates, to reveal the variability in eDNA results, especially because we are near the lower detection limits of the focal species at the upper extent of their distribution.

To account for imperfect detection owing to eDNA being heterogeneously distributed in water, we used occupancy models to estimate detection probabilities (Hunter et al. 2015). We chose a Bayesian, three-level occupancy model eDNAoccupancy in R that uses Markov Chain Monte Carlo (MCMC) methods of maximum-likelihood to estimate model parameters (i.e., $\Psi(.)\theta(.)p(.)$) and include covariates (Dorazio and Erickson 2018). Accordingly, we can estimate fish detection probabilities while also estimating the conditional probability of detecting trout DNA that may be present in a field sample or qPCR replicate. The three levels of sampling included aspects of the nested sampling design innate in eDNA sampling of location (stream × site), field sample, and qPCR replicate (equations in Table 2). In the model, Ψ is the probability that eDNA is present at a location, θ is the conditional probability that eDNA occurred in a replicate field sample given that it occurred at the location

Table 2. Parameter estimates (posterior mean ± standard error) and model-selection criteria (PPLC and WAIC) for each candidate occupancy model of Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* environmental DNA detections in streams in Washington and Oregon, USA.

Model	Occupancy in location (Ψ)	Occupancy in sample (θ)	Detection in replicate (*p*)	PPLC	WAIC
Ψ(.), θ(.), *p*(.)	0.53 (0.46, 0.59)	0.78 (0.73, 0.83)	0.89 (0.86, 0.91)	190.176	0.3673
Ψ(depth + width), θ (trout), *p*(all fishes)	α_0 = 0.469 (±0.013) α_1 = −0.153 (±0.006) α_2 = 0.593 (±0.012)	β_0 = 0.930 (±0.004) β_1 = 2.331 (±0.025)	δ_0 = 1.102 (±0.001) δ_1 = 0.191 (±0.001)	222.008	0.4153
Ψ(depth), θ(trout + all fishes), *p*(all fishes)	α_0 = 0.649 (±0.016) α_1 = 0.092 (±0.004)	β_0 = 1.031 (±0.006) β_1 = 1.986 (±0.028) β_2 = 1.863 (±0.017)	δ_0 = 1.101 (±0.001) δ_1 = 0.191 (±0.001)	222.756	0.4158
Ψ(width), θ(trout + all fishes), *p*(all fishes)	α_0 = 0.635 (±0.011) α_1 = 0.504 (±0.004)	β_0 = 1.046 (±0.006) β_1 = 1.892 (±0.021) β_2 = 1.918 (±0.018)	δ_0 = 1.102 (±0.001) δ_1 = 0.191 (±0.001)	222.320	0.4158
Ψ(width), θ(trout), *p*(all fishes)	α_0 = 0.359 (±0.007) α_1 = 0.410 (±0.004)	β_0 = 0.928 (±0.004) β_1 = 2.169 (±0.022)	δ_0 = 1.104 (±0.001) δ_1 = 0.192 (±0.001)	221.471	0.4167
Ψ(.), θ(trout), *p*(all fishes)	0.63 (0.51, 0.79)	β_0 = 0.938 (±0.004) β_1 = 2.231 (±0.029)	δ_0 = 1.104 (±0.001) δ_1 = 0.194 (±0.001)	221.540	0.4172
Ψ(width + depth), θ (trout + all fishes), *p*(all fishes)	α_0 = 0.801 (±0.011) α_1 = 0.851 (±0.010) α_2 = −0.267 (±0.006)	β_0 = 1.034 (±0.006) β_1 = 1.936 (±0.017) β_2 = 1.971 (±0.019)	δ_0 = 1.102 (±0.001) δ_1 = 0.189 (±0.001)	222.940	0.4174
Ψ(depth), θ(trout), *p*(all fishes)	α_0 = 0.329 (±0.008) α_1 = 0.122 (±0.002)	β_0 = 0.936 (±0.004) β_1 = 2.184 (±0.026)	δ_0 = 1.104 (±0.001) δ_1 = 0.193 (±0.001)	221.562	0.4181

Note: Values represent either probabilities or estimates of the coefficients of the relationship between the covariate(s) and detection probability of the form $\text{logit}(\Psi) = \alpha_0 + \alpha_1 \times \text{covariate} + \alpha_2 \times \text{covariate}_2$, or $\text{logit}(\theta) = \beta_0 + \beta_1 \times \text{covariate} + \beta_2 \times \text{covariate}_2$, or $\text{logit}(p) = \delta_0 + \delta_1 \times \text{covariate}$. Bold indicates favored model.

level, and p is the conditional probability of detecting eDNA in a replicate qPCR reaction given that it occurred in the field sample.

We assumed that Ψ might vary across stream locations owing to habitat size of stream width and stream depth. In addition, we hypothesized that θ and p might be influenced by the abundances of trout or all fishes detected by electrofishing because of eDNA inhibition or molecular competition. We evaluated several models that included a different combination of covariates affecting Ψ, θ, and/or p. Covariates were measured at the location level including single-pass standard electrofishing surveys that evaluated density of all fishes (no./50 linear m), and density of Coastal Cutthroat Trout (no./50 linear m). Covariates measuring habitat size included stream width (m) and stream depth (m). We fitted and evaluated eight candidate models using available functions for model-selection criteria from the eDNAoccupancy package. Model-selection criteria included the posterior-predictive loss criterion (PPLC) and Watanabe-Akaike information criterion (WAIC). We fitted each candidate model by running the MCMC algorithm for 11,000 iterations and retaining the last 10,000 for estimating posterior summaries. After selecting the best model, we explored the estimated relationships between covariates (i.e., stream width, stream depth, trout density, and density of all captured fishes) and estimated model parameters Ψ and θ.

Lastly, we used results from the simplest model (Ψ(.)θ(.)p(.)) that did not include covariates and adopted the approach described in Hunter et al. (2015) to compute the cumulative probability of detecting Coastal Cutthroat Trout eDNA in K qPCR replicated sample (p^*), given that the sample contained eDNA the model as $p^* = 1 - (1 - p)^K$. This procedure allowed us to assess if we used an adequate number of qPCR replicates to detect trout eDNA. We performed a similar analysis to estimate the cumulative probability of occurrence of Coastal Cutthroat Trout eDNA in n water samples (θ^*) collected from a location that contained eDNA using $\theta^* = 1 - (1 - \theta)^n$.

Results

The upper extent of fish for electrofishing and eDNA agreed for 17 streams (28%; Figs. 2, 3).

Trout DNA was detected above the electrofishing last-fish boundary in 31 streams (52%) by at least 50–250 m (Figs. 4, 5). Trout eDNA detections from the uppermost stream sites had fewer replicate detections (1–3 positive detections) relative to downstream sites in the same stream (Figs. 2–5). For 12 streams (20%), fish were detected higher in the stream with electrofishing than with eDNA, but by only a stream length of 50 m additional distance upstream. The single exception was for stream 10 where a perched population of trout (a population discontinuous from the downstream population) was noticed after sampling was completed. Upon this discovery, we reinitiated electrofishing sampling at sites 8 through 10 to confirm extent of fish, but because sites above 8 are beyond the sampling area for the study, we could not re-collect eDNA samples because electrofishing might have contaminated eDNA resampling.

We did not detect trout with either approach at three streams; however, one of these three streams contained another fish taxon, sculpins, which were detected by electrofishing (stream 9). There were multiple streams where we detected trout with only one of the two approaches. For example, trout DNA was not detected at three streams using eDNA (R.F. Salt, Brice, and Canyon Creeks), but trout were detected by electrofishing. Trout were not detected at an additional four streams using electrofishing (streams 28, 29, 30, and 31), but trout DNA was detected with eDNA. Coho Salmon juveniles were detected with electrofishing in stream 29 (Data S1).

Although multiple occupancy models had similar fit, the favored model with covariates revealed that (1) stream widths influenced eDNA trout occupancy at a location; (2) electrofishing trout densities affected eDNA field samples; (3) and electrofishing densities of all fish influenced qPCR replicates. Modeled results revealed that the occurrence of trout eDNA was higher in wider-stream locations (Fig. 6, Table 2). The occurrence of trout eDNA was higher in field samples with higher electrofishing trout density, but eDNA detected trout at very low electrofishing densities of <5 trout per 50 linear m (Fig. 7). The occurrence of trout eDNA was higher in qPCR replicates with higher fish density as detected by electrofishing (Table 2).

Estimates of detection probabilities of trout eDNA (p) showed that qPCR was effective in detecting eDNA presence in a field sample (Model ((Ψ(.), θ(.), p(.)) in Table 2). The mean estimated detection probability was 0.89 (range: 0.86–0.91), and consequently, the cumulative probability of detecting trout eDNA (p^*) was very high, ranging from 0.997 to 0.999. This suggests that three qPCR replicates per eDNA sample were sufficient to detect trout eDNA when it was present in a field sample. Similarly, the mean estimated detection probability of eDNA samples collected by location was 0.89 (range: 0.86–0.91) and the cumulative probability of detecting trout eDNA (θ^*) ranged from 0.980 to 0.995. This provides evidence that the three eDNA samples collected were sufficient to detect trout eDNA when the eDNA was present at that location.

Discussion

By comparing methods of electrofishing and eDNA to determine the upper extent of fish in streams, we evaluated their relative reliabilities when actual fish densities are naturally low. For streams with positive eDNA detections of trout, the uppermost sites generally revealed a reduced detection signal relative to downstream sites from the same stream, likely owing to a low concentration of target DNA from fewer fish. This, along with our other results, are evidence that eDNA is an appropriate tool to capture the transition from fish to no fish in a stream network taking its place alongside traditional high-effort catch-and-release or observational (e.g., snorkeling) tools. However, it also highlights the importance of evaluating the strength of evidence from both the detection strength (threshold cycle value: C_q) and number of positive detections (eDNA field replicates × qPCR replicates) of the eDNA results given the wide variability across the responses that may be counted as a positive eDNA detection. Here, if we are cautious and remove the data points where there is one positive detection (out of the nine possible detections at a site) from the study, we would not count fish as present at specific sites, especially upstream sites, but we would continue to see the same general pattern of eDNA detecting fish above electrofishing. As managers start to incorporate eDNA surveys to detect last-fish, they may want

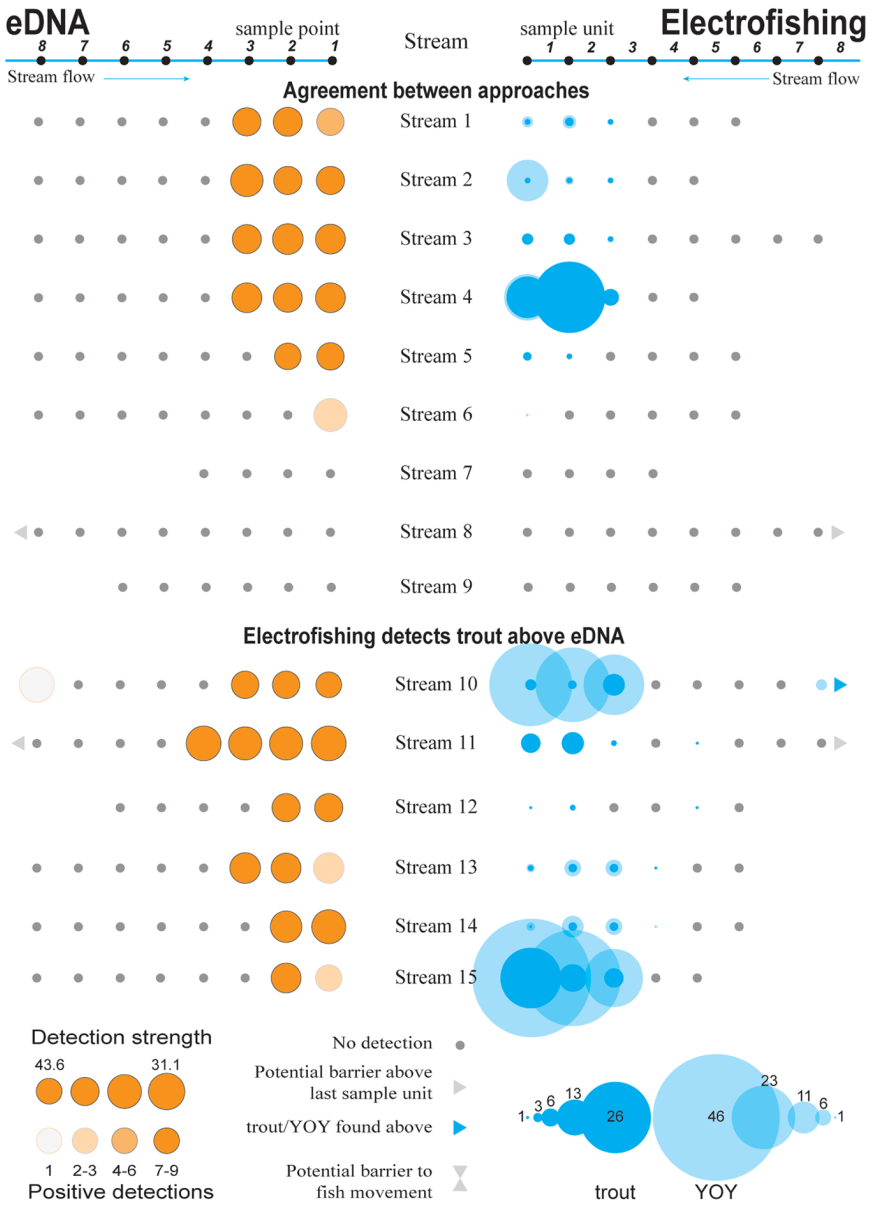

Fig. 2. Patterns of agreement and disagreement between environmental DNA (eDNA) and electrofishing sampling methods using relative abundances of Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* detected over eight sites across 15 streams in Washington and Oregon, USA. In the upper panel, we illustrate streams where both methods, eDNA (orange) and electrofishing (blue), showed full agreement as to the upper extent of fish. Gray circles represent no detection. In the lower panel, we illustrate streams where electrofishing detected trout upstream of eDNA. Size of the symbols represents eDNA detection strength (threshold cycle value: C_q) and fish abundance from electrofishing (no./per 50 m sample unit). Each row represents a single stream with arrows indicating stream flow direction (eDNA is from left to center mirroring electrofishing, which is from right to center). Dark orange shows higher detection among eDNA replicates, whereas light orange is the opposite. Dark blue shows captures of adult Coastal Cutthroat Trout (trout), and light blue shows captures of young-of-year (YOY), which could either be *Oncorhynchus mykiss* or *O. clarkii clarkii*.

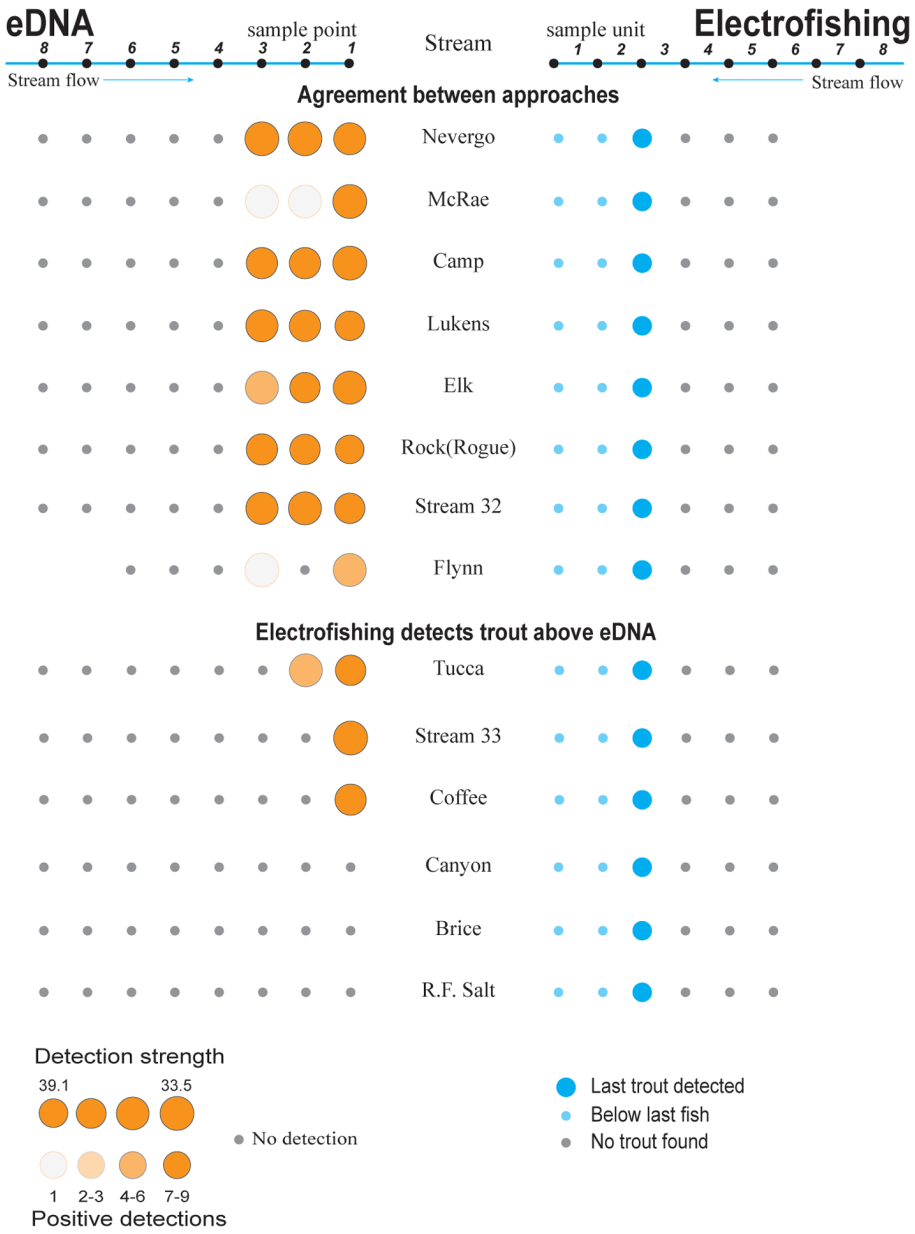

Fig. 3. Patterns of agreement and disagreement in Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* detection between methods using environmental DNA (eDNA) relative abundances and electrofishing (detection/no detection) over eight sites across 14 streams in Washington and Oregon, USA. In the upper panel, we illustrate streams where both methods, eDNA (orange) and electrofishing (blue), showed full agreement as to the upper extent of fish. Gray circles represent no detection. In the lower panel, we illustrate streams where electrofishing detected trout upstream of eDNA. Size of the orange symbols represents eDNA detection strength (threshold cycle value: C_q), whereas blue symbols represent fish detection from electrofishing (per 50-m sample unit). Each row represents a single stream with arrows indicating stream flow direction (eDNA is from left to center mirroring electrofishing, which is from right to center). Dark orange shows higher detection among eDNA replicates, whereas light orange is the opposite.

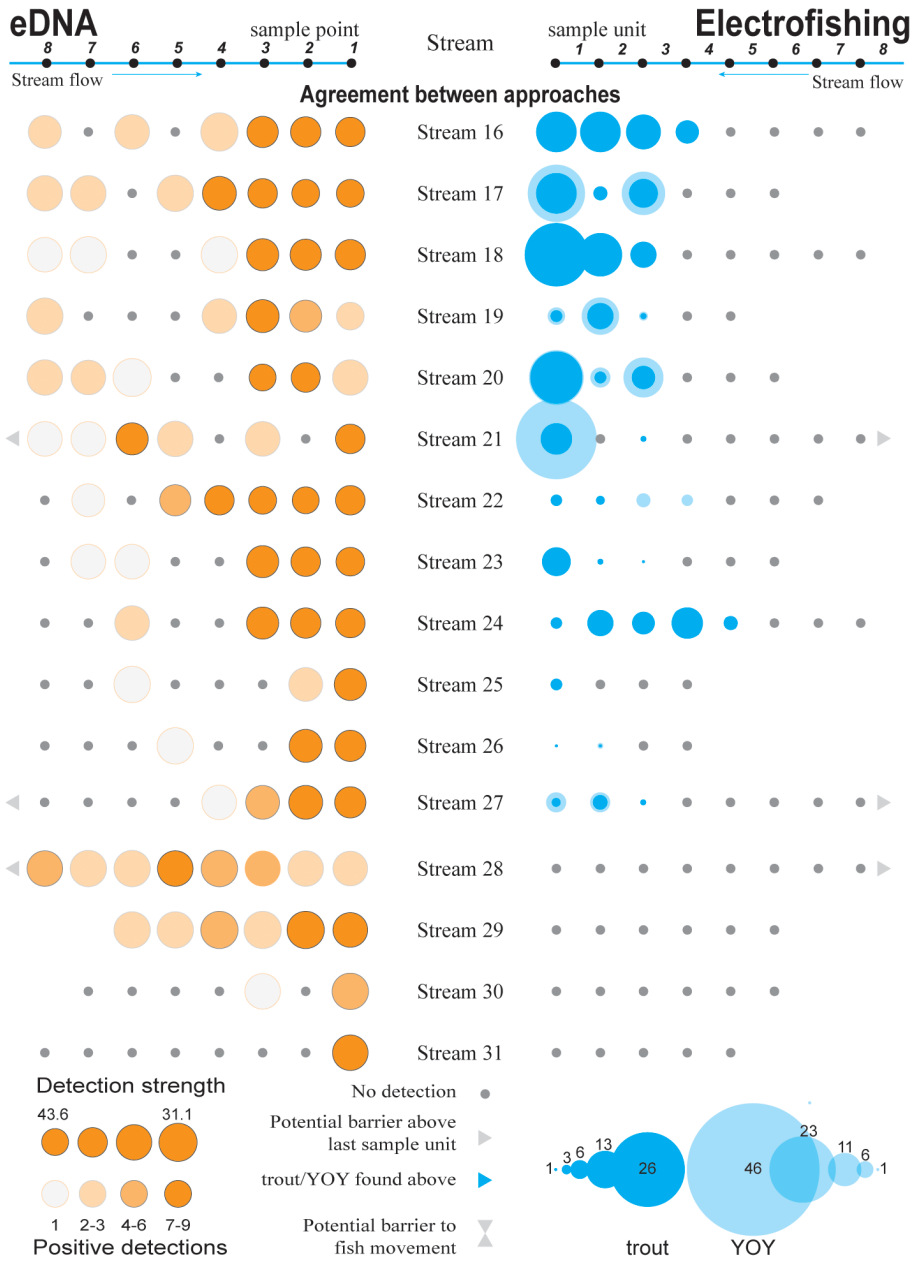

Fig. 4. Streams where environmental DNA (eDNA; orange) detected Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* upstream of electrofishing (blue) using eDNA relative abundances and electrofishing (detection/no detection) over eight sites across 16 streams in Washington and Oregon, USA. Gray circles represent no detection. Size of the symbols represents eDNA detection strength (threshold cycle value: C_q) and fish abundance from electrofishing (no./per 50 m sample unit). Each row represents a single stream with arrows indicating stream flow direction (eDNA is from left to center mirroring electrofishing, which is from right to center). Dark orange shows higher detection among eDNA replicates, whereas light orange is the opposite. Dark blue shows captures of adult Coastal Cutthroat Trout (trout), and light blue shows captures of young-of-year (YOY), which could either be *Oncorhynchus mykiss* or *O. clarkii clarkii*.

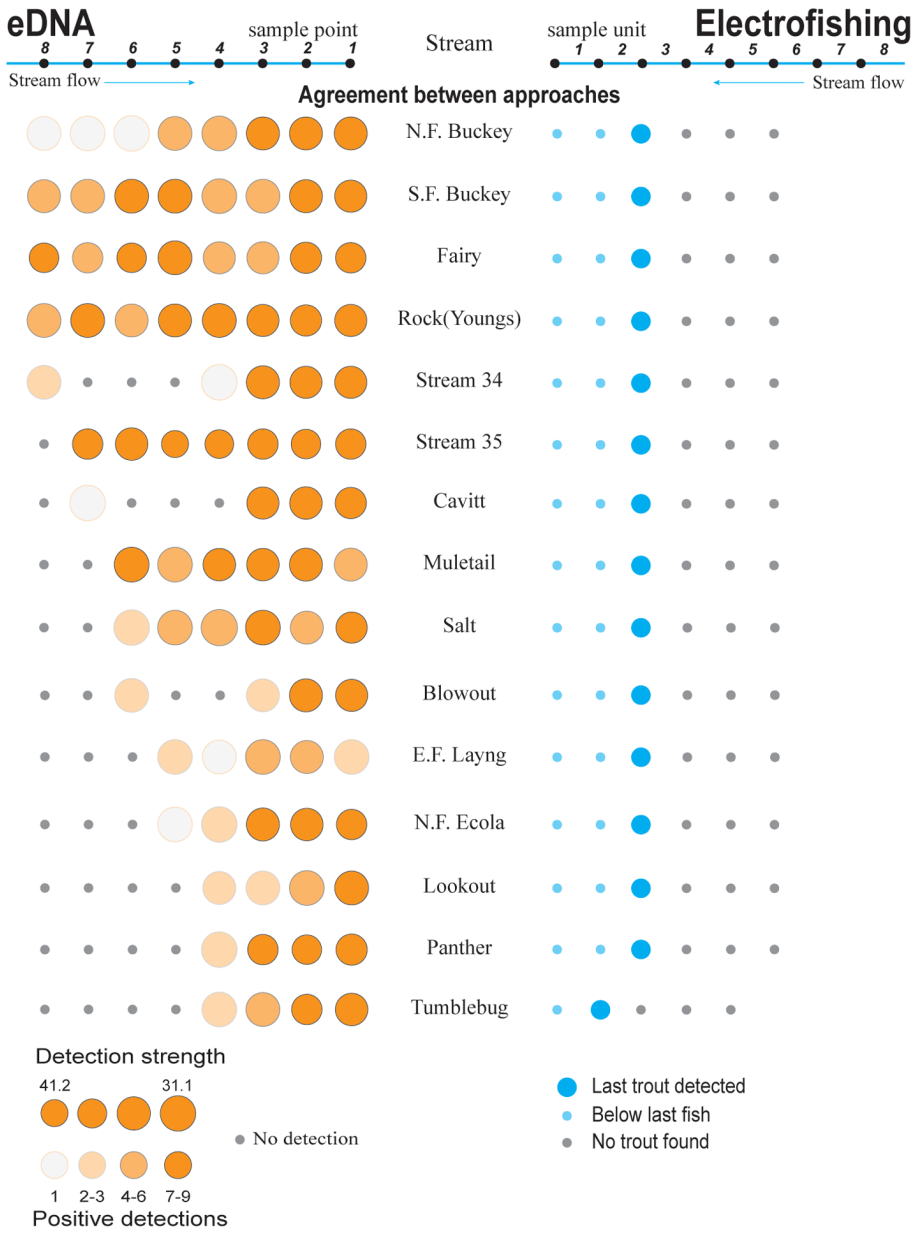

Fig. 5. Streams where environmental DNA (eDNA; orange) detected Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* upstream of electrofishing (blue) using relative abundances over eight sites across 16 streams in Washington and Oregon, USA. Gray circles represent no detection. Size of the symbols represents eDNA detection strength (threshold cycle value: C_q) and fish abundance from electrofishing (no./per 50 m sample unit). Each row represents a single stream with arrows indicating stream flow direction (eDNA is from left to center mirroring electrofishing, which is from right to center). Dark orange shows higher detection among eDNA replicates, whereas light orange is the opposite. Dark blue shows captures of adult Coastal Cutthroat Trout (trout), and light blue shows captures of young-of-year (YOY), which could either be *Oncorhynchus mykiss* or *O. clarkii clarkii*.

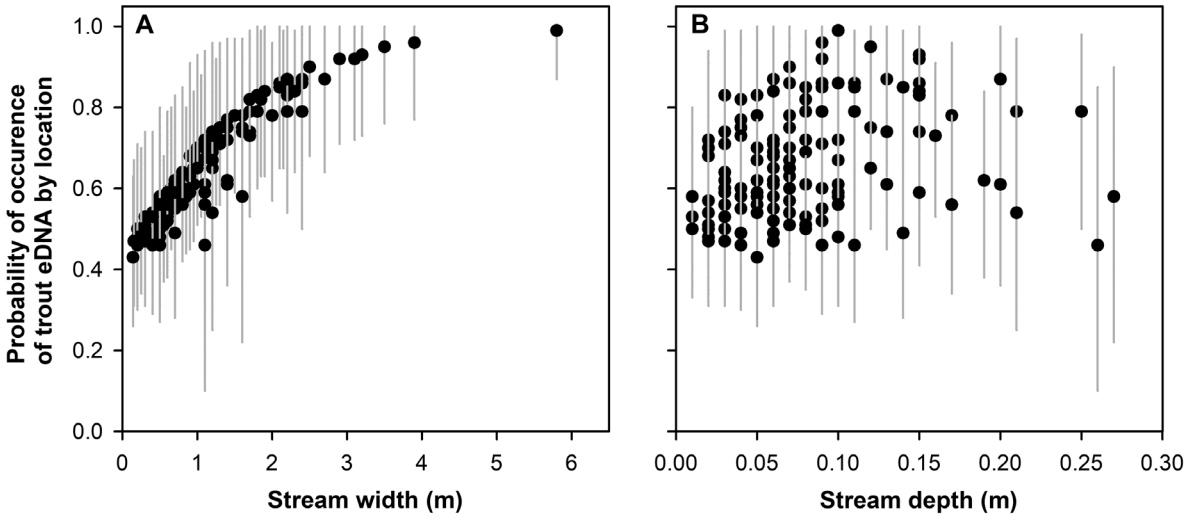

Fig. 6. Probabilities of occurrence of Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* environmental DNA by (A) stream width and (B) stream depth across locations (stream × site; Ψ) in Washington and Oregon, USA. Symbols are estimates of posterior means with 95% credible intervals for the model (Ψ(depth + width), θ(trout), *p*(all fishes)) described in Table 2.

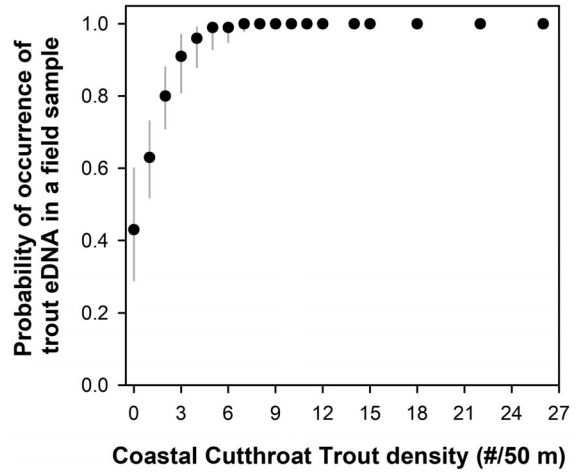

Fig. 7. Probabilities of occurrence of Coastal Cutthroat Trout *Oncorhynchus clarkii clarkii* environmental DNA (eDNA) by trout density from electrofishing in a field sample (θ) in Washington and Oregon, USA. Symbols are estimates of posterior means with 95% credible intervals of the model (Ψ(depth + width), θ (trout), *p*(all fishes)) described in Table 2.

to use more than one criterion to define a positive eDNA detection as part of a decision-making framework. For example, a threshold of a positive eDNA detection could be set for a given number of replicates to separate a consistent series of strong detections from a few weak detections, as well as incorporating information about potential barriers to fish movement (see Appendix S1: Figs. S1, S2 for barriers) and other habitat characteristics (e.g., wetlands, habitat complexity). We suggest that as the discussion of eDNA as a management tool continues it is important to distinguish between the science of eDNA (e.g., methodological sensitivities, limitations) and the implications that are derived from its information (e.g., fish presence). Below, we describe these aspects further as we discuss operational considerations for managers when using eDNA, including how many replicate samples are needed, how to define what is considered a detection, how to incorporate knowledge of site or landscape features that may limit fish distributions, which potential fish species will count as last-fish, where to start sampling, and more.

Our results demonstrate that eDNA is equally as effective as electrofishing and generally a more sensitive approach to detect last-fish. Trout DNA was detected further upstream with eDNA than by electrofishing in the majority of the streams we examined, effectively extending the upper extent of fish beyond the known boundary by up to 250 m, and potentially higher, although

some detections could be from false positives. Similarly, there were more detections of Bull Trout with eDNA than with electrofishing suggesting that Bull Trout are also more broadly distributed than previously thought (McKelvey et al. 2016). Our findings from the multiscale occupancy model revealed that when no trout were detected with electrofishing, the probability of occurrence of trout with eDNA was still relatively high (~0.4). We showed a consistent positive relationship between probability of eDNA detection of trout and trout density from electrofishing with a perfect detection probability at ≥5 trout per 50 m. These findings are consistent with Wilcox et al. (2016), which showed almost full detection at electrofishing densities of ≥3 fish per 100 m for Brook Trout *Salvelinus fontinalis* in Montana streams. We showed that the occurrence of trout eDNA by location (stream × site) increased with stream width, evidence that Coastal Cutthroat Trout were more abundant at downstream sites, or in larger streams. Studies evaluating the upper extent of Coastal Cutthroat Trout also found higher densities of trout in larger streams (Latterell et al. 2003, Cole et al. 2006). Only in some cases does electrofishing seem to outperform eDNA (see last paragraph below), highlighting some of the challenges of detecting fish at low densities and detection limits for both approaches.

Beyond sensitivity, we offer three additional examples from our study that detail advantages of eDNA, including the potential detection of multiple life stages, detection of animals in adjacent habitats, and distinguishing species-specific young-of-year. First, in some of our study streams, we suspect that eDNA may actually detect eggs in the ground (redds) and not juveniles or adults, owing to consistent eDNA signal across sites early in the sampling season and lack of detection with electrofishing (stream 28). Environmental DNA has been shown to detect redds as belonging to either Coho or Chinook salmon (Strobel et al. 2017). Another eDNA study found that eDNA concentration followed general patterns of salmon abundance, but variation in eDNA concentration is influenced by locally relevant habitat features of water temperature or behaviors such as spawning behavior or fish state (live, killed, or naturally dead; Tillotson et al. 2018). Second, because of hydrological connectivity between habitats, eDNA may be able to detect target DNA from habitats where it is difficult to effectively sample with other fishing approaches, such as in streams obstructed by large woody debris or in adjacent wetlands too shallow or vegetated for electrofishing. In our study, it is possible that the eDNA detections in stream 29 could have originated from trout in upstream wetlands. Third, young-of-year Rainbow Trout/steelhead and Coastal Cutthroat Trout are generally indistinguishable from each other in the field until they reach >75 mm (Total Length). Environmental DNA can resolve this misidentification shortcoming because individuals are detected based on genotype rather than phenotype or ontogenic stage as in some of our studied streams. Other advantages of eDNA include the lack permitting requirements to catch or handle fish, ease of fieldwork, potential for relative abundance estimates, and elimination of potential injuries to fish resulting from shocking and/or handling fish, which is especially important for at-risk wild populations or for individuals from populations with elevated conservation concerns.

The main drawbacks of using eDNA to identify last-fish boundaries are the occurrence of false positives or false negatives, processing time, information lost by not having a fish in hand (size, condition, disease symptoms, parasite load), and the need to know where to start sampling. Detecting the DNA of fish in a water sample implies that fish are present somewhere upstream of that collection point. False-positive errors, sensu stricto, occur when a positive signal is obtained even though the site does not contain any actual fish (true absence), which can occur as a result of contamination of the water sample in the field or laboratory, non-target binding owing to poor marker specificity, or PCR errors. We minimized these shortcomings of eDNA by: following a strict field-collection protocol; using clean laboratory space resulting in clean laboratory blanks; including HEPA-filtered and UV-irradiated PCR cabinets for extraction and PCR setup; having high marker specificity (Appendix S1: Table S2); and having three eDNA field replicates and three qPCR replicates. Future studies may also want to additionally consider field blanks, which are handled the same way as regular samples, to disregard the potential for

contamination in the field. However, there are situations when DNA is amplified in a sample, but not because fish or eggs are actually present at the site, rather, because DNA has been brought there from a distant source (allochtonous eDNA), or resides in sediments from long-past presence of target species (Turner et al. 2015). In these cases, positives may occur when DNA was dropped in by a passing predator, dragged through streams on the feet of animals as they pass (e.g., elk or cattle moving upstream), or the signal remains in the sediments, providing a long-lasting signal of species occupancy. For example, we suspect that the positive detections at site 6 in stream 25 occurred because multiple game trails cross the stream, facilitating the movement of trout signal by other animals.

False negatives, number of replicates, and persistence of eDNA signal in the water are important factors to consider when using eDNA surveys. False negatives occur when target DNA is not detected in the sample or because of lack of sensitivity during laboratory tests, but the organism is present in the system (Guillera-Arroita et al. 2017). In practice, target DNA may not be captured in each sample because uneven natural densities may vary with local conditions and from PCR inhibition. Consequently, eDNA may not be captured in every sample or detected in every laboratory replicate. Our results showed that three eDNA field replicates are sufficient to detect trout in streams, highlighting the need for increased sampling effort when the target species is in low abundance, such as at the upper extent of fish. More replicates may also be important in large water bodies when dilution may be a factor, such as for detecting the Northern Pike *Esox lucius* in the Columbia River (Carim et al. 2019). Environmental DNA degrades as a result of UV exposure, temperature, pH, and other factors, with fine-scale changes in detection occurring daily (Tillotson et al. 2018), and signal can persist in streams for one day to two weeks (Dejean et al. 2011, Thomsen et al. 2012) suggesting that a detected signal reveals current presence in stream water. Although eDNA is more sensitive than electrofishing at detecting the last-fish at most of our sampling sites relative to electrofishing, its broader use and effectiveness depends on investigators being informed of the potential location of the last-fish to advise on where to start sampling. An additional drawback is the non-immediacy of eDNA results, as samples must be extracted and analyzed by qPCR to assess last-fish distributions.

Dynamic flow regimes and spatial nesting in stream systems make eDNA point-sampling difficult to interpret. Our results suggest that fish probably had patchy rather than continuous distributions in a stream because upstream eDNA did not appear to accumulate downstream at relatively low flows. We found contrasting patterns of trout eDNA among sites within the same stream (sites 50 m apart), even though they are connected by the same flow of water. Environmental DNA is transported downstream in streams by flowing water, and consequently, a single eDNA sample theoretically contains genetic material from a considerable distance upstream (Jane et al. 2015, Wilcox et al. 2016, Tillotson et al. 2018). However, the majority of our study streams had sites without detections in-between sites with detections, evidence that eDNA can degrade quickly over space. Other studies from further downstream have reported that eDNA varies along a stream, is conserved over short distances of tens of meters (Tillotson et al. 2018), and degrades over longer distances (kilometers; Laramie et al. 2015, Tillotson et al. 2018, Ostberg et al. 2019), proposing that eDNA reflects a here-and-now presence of local abundances.

By potentially extending the upper extent of fish in most streams, our results suggest that we may need to redefine our understanding of headwater fish habitats, particularly barriers to fish movement. Trout in headwater streams have ecological importance because they contribute life-history variability to the overall population complex thereby safeguarding the population against temporally variable conditions (portfolio effect, Penaluna et al. 2018). In other words, stream-living trout found at the upper extent of fish support other life histories for *O. clarkii clarkii*, including sea-run, lake, and river forms. Across the Pacific Northwest, the upper limit of fish is determined mainly by physical habitat constraints, particularly stream size and gradient (Latterell et al. 2003, Cole et al. 2006, Fransen et al. 2006). Here, with eDNA methods, we illustrated that perceived physical barriers were inaccurate or were not actually a permanent barrier

in many study streams. For example, although waterfalls and steep cascades can be permanent barriers, the formation and destruction of debris jams are transient and likely short-term (seasonal to annual) barriers to fish movement and probably account for many of the discrepancies, but some may be from eDNA false positives. Changes in flows and flow paths at higher flows can facilitate passage by fish around transient barriers (Cole et al. 2006). Future work needs to better distinguish between permanent and transient barriers across streams.

In some cases, electrofishing does show an advantage over eDNA. Electrofishing detects fish higher in the stream network (<100 m upstream) than eDNA in a relatively small proportion of the streams, and generally when fish are in very low abundances, potentially owing to a lack of block net use while electrofishing. Block nets are used to ensure fish do not flee to adjacent habitats (Peterson et al. 2004), however they are not typically used in electrofishing protocols to determine last-fish. Because electrofishing provides real-time catch data and provides the exact time and place that a fish is captured, along with the ability to collect age-class counts and physical measurements, electrofishing has long been the main approach used to detect the upper extent of fish. Electrofishing can also detect many fish species (although not equally across species or sizes), whereas eDNA detects DNA of only targeted fishes. For example, electrofishing detects Coastal Cutthroat Trout as last-fish in 97% of streams, but sculpin (stream 9) or juvenile Coho Salmon (stream 29) are the last-fish in the remaining streams. Electrofishing minimizes false positives, unless there is species misidentification. Lastly, with a fish in hand, field observations by experienced ecologists or taxon specialists can also offer information that goes beyond quantitative and qualitative records, including observing external parasites, discoloration, and deformations.

Conclusions

The success of eDNA relative to electrofishing in determining the geographic boundary of fish represents a significant contribution to fisheries science with direct implications for species conservation and natural resource management, especially in forested systems such those studied herein. We found that our eDNA approach was robust in detecting fish even at very low natural abundances and generally detected fish higher in the stream network than electrofishing. Accordingly, we extended the upper extent of fish beyond the known boundary by up to 250 m in some streams owing to the study design; however, the boundary could potentially be even higher and should be further evaluated. We posit that eDNA merits inclusion among the sampling approaches considered to identify the upper extent of fish, but not as a replacement of traditional methods. Rather, multiple approaches could be used for improved reliability and to account for methodological shortcomings (Table 1). In particular, eDNA may be useful in streams where habitat is difficult to effectively sample using electrofishing because of either complex structures or upstream wetlands or when eggs are in the ground. Also, eDNA techniques will need to permit that managers target all possible fish species in a stream (e.g., parallel analyses of eDNA samples through high-throughput PCR [Wilcox et al. 2020] or use next-generation sequencing) so that streams are not mistakenly classified as fishless. Future studies can focus on understanding fish movement at their upper extent across seasons, redefine barriers to fish movement, and determine the contribution of these headwater fishes to the overall fish population to further elucidate their importance to overall population dynamics. Continued research is needed to push the boundaries of eDNA technology to better contend with false-positive and false-negative errors, better connect eDNA detections to actual fish abundances, and to make this approach more cost-effective and approachable for managers.

Acknowledgments

We thank Hancock Forest Management (agreement #18-MU-11261953-081), Port Blakely (agreement #18-MU-11261954-075), and Weyerhaeuser Company (agreement #19-RD-11261953-014) for partnering on this work. In particular, we thank Claudine Reynolds, Jenniffer Bakke, Lindsey Webb, Maryanne Reiter, and Jessica Homyack. Thanks to Dana Warren, Ashley Coble, Stan Gregory, Dede Olson, Pete Bisson, Gordie Reeves, Becky Flitcroft and two anonymous reviewers for constructive comments on the manuscript. We are

grateful to Dave Leer for his leadership in the field and to Tim Glidden, the Weyerhaeuser fish crew, and the field crew contracted by Hancock and Port Blakely for fieldwork. Thanks to Jenny Green, Loretta Ellenburg, Emily Hernandez, Eszter Munes, Howard Haemmerle, and J.Nick Tarvin for supporting the work. Kelly Christiansen developed bubble plots and map. Kathryn Ronnenberg helped to depict the sampling schematic and Table 1. Fish collections were authorized by Oregon Department of Fish and Wildlife scientific take permit 21223 for fish and #90 for amphibians in 2017 and by USFS Institutional Animal Care and Use Committee Permit #2018-010. Funding cooperators are Oregon State University College of Forestry Fish and Wildlife Habitat in Managed Forests Research and Washington State Department of Natural Resources Forest Practices Adaptive Management Program. Two streams where we conducted this research are on the HJ Andrews Experimental Forest, which is funded by the U.S. Forest Service, Pacific Northwest Research Station.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3332/full