1 **Identifying distribution boundaries at the upper extent of fish in streams using**
2 **environmental DNA**

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4

5 **Introduction**
6 Delimiting geographic distribution boundaries of species is fundamental for conservation and
7 management decision-making. Forest management in the Pacific Northwest occurs across the
8 landscape, including at or near the upstream extent of fish distributions in headwater streams.
9 Stream reaches with fish have more regulatory protections and wider riparian buffers than
10 fishless reaches (Blinn and Kilgore 2001; Lee, Smyth, and Boutin 2004). Consequently, this nexus
11 has become the focus of questions for contemporary forest practices and fisheries.
12   Although there are multiple approaches that are accepted under Forest Practices Rules for
13 Washington to delimit the last-fish, electrofishing is currently the most widely used method
14 because it allows for detection in real time (WA DNR 2002). However, electrofishing can be
15 time-consuming, labor-intensive (Evans et al. 2017), can harm fishes, and is a poor tool for
16 detecting fish in low abundances (Peterson et al. 2004; Rosenberger and Dunham 2005).
17 Alternatively, environmental DNA (eDNA) is a rapidly evolving state-of-the-art method that
18 measures target DNA that is left behind in water and consequently does not harm fishes
19 (Goldberg et al. 2015; Wilcox et al. 2016). eDNA has been shown to be comparable to, or more
20 sensitive at, detecting fish than electrofishing in streams (Wilcox et al. 2016; Baldigo et al. 2017;
21 Evans et al. 2017; Ostberg et al. 2019), particularly when species are low in abundance (Dejean
22 et al. 2012; Pilliod et al. 2013; Sigsgaard et al. 2015; Itakura et al. 2019). Despite the expansion
23 of eDNA approaches into monitoring and inventory programs around the globe, issues remain
24 with detections of false positives and false negatives (Roussel et al. 2015, Guillera-Arroita et al.
25 2017). These management-relevant approaches have yet to be evaluated to understand their
26 abilities to detect the upper extent of fish in streams.
27   Here, we evaluate the relative reliability of eDNA of Coastal Cutthroat Trout
28 (*Oncorhynchus clarkii clarkii)* as a management tool to detect the upper extent of fish. To do so,
29 we compare the upper fish distribution from eDNA to standard electrofishing for a small number
30 (n=12) of forested streams in Washington. Coastal Cutthroat Trout are the fish generally found
31 the highest up in their stream network across their range (Budy et al. 2019). All sampling
32 coincided with the recognized defined sampling window for evaluating the upper extent of fish
33 under Forest Practices Rules in Washington (March 1st to July 15th). We (1) identify whether
34 eDNA can be used as a management tool to identify the upper boundary of fish by evaluating
35 whether it detects fish at the same sites as electrofishing within each stream and/or above the
36 boundary identified by electrofishing; and (2) identify operational limitations to using eDNA for
37 determining the last-fish. In addition, because the sampling of these 12 streams occurred within a
38 broader study across Washington and Oregon, we also (3) provide estimates of fish detection
39 probabilities of eDNA across a broader suite of sites (Penaluna et al. *in press)*. We predict that
40 eDNA will detect fish further upstream than electrofishing across streams because of its
41 acknowledged strength for identifying species in low abundance, as is often the case for fishes
42 near their upper distribution boundary. Ultimately, our results provide a comparison of a

43 standard field method and a rapidly advancing technique for examining presence of fish in small
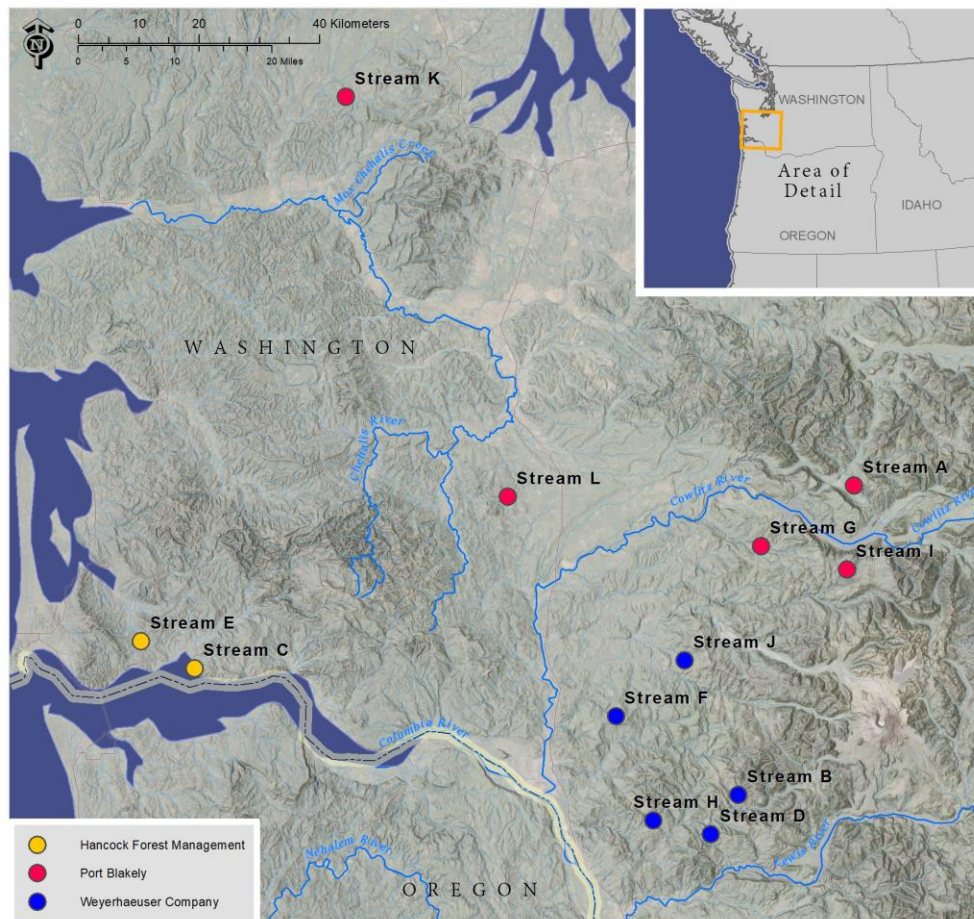44 streams.
45
46
47 **Methods**
48 *Study streams and sample design*
49   We sampled 12 streams in collaboration with Hancock Forest Management, Port Blakely,
50 and Weyerhaeuser Company on their land (Fig. 1). We worked with landowners to select streams
51 by prioritizing streams with previous information related to the upper extent of Coastal Cutthroat
52 Trout (*O. clarkii clarkii*). Our sampling framework relied on prior documentation of the
53 upstream extent of fish presence identified through a previous fish distribution survey, and,
54 consequently, we initiated sampling at least 175m downstream of these previous boundaries.
55   Environmental DNA samples were collected on the same day as electrofishing, but
56 immediately in advance of electrofishing to decrease contamination risk for eDNA and compare
57 approaches. We collected eDNA from eight discrete sampling sites located every 50 m moving
58 upstream (Fig. 2). Generally three sites were downstream of the last–fish, which was determined
59 at the time of electrofishing, and the remaining sites were upstream, except for streams C, D, E,
60 F, and I. To ensure eDNA sampling locations met the targeted sampling design (i.e., located
61 above and below of the last fish location as identified by electrofishing), additional eDNA
62 samples (>8) were often collected with subsequent processing limited to the 8 locations that met
63 the study design criteria. Sample spacing of 50m was selected to offer additional point
64 information on the detection probabilities of fish above and below where fish were noted during
65 continuous electrofishing. Consequently, the last-fish observed by electrofishing often occurred
66 between sites 3 and 4 with about 100 m downstream of that point and 250 m above.
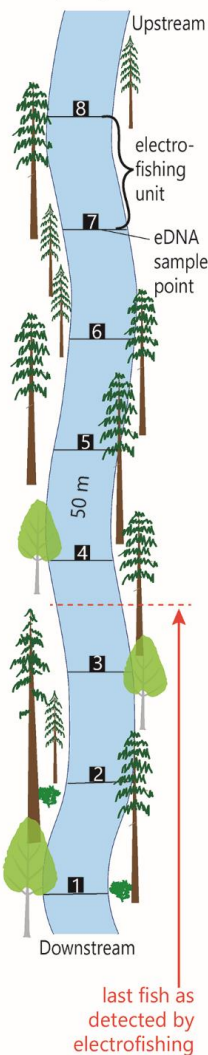
Figure 1. Map of twelve study streams in Southwest Washington. At each stream, the upper extent of fish was evaluated with electrofishing and eDNA.

eDNA sampling

At each stream, we collected 1L water samples in triplicate from the thalweg at the downstream end of each 50 m electrofishing sampling unit for each of the 8 sites. Samples were collected in triplicate to account for imperfect detection of eDNA (Hunter et al. 2015). We pumped sample water through 0.45 micron single–use cellulose nitrate filters (Sterlitech, Kent, WA) using a vacuum pump. Water was collected with either a 1L Nalgene bottle or a 1L disposable sterile Whirlpak bag and held in the stream to remain cool for 1–3 hours while other samples were collected from each site. Samples were picked up and sorted based on last–fish observed by electrofishing. Filters were loosely rolled and stored frozen in 5mL vials on wet ice during transport, and were frozen at –20°C within 6 hours of collection. Filters were stored at –20°C until DNA extraction. Bottles and tweezers were sterilized with a 50% bleach solution followed by a triple deionized water rinse before use.

sampling scheme

Upstream

electro-
fishing
unit

eDNA
sample
point

50 m

Downstream

last fish as
detected by
electrofishing

Figure 2. Schematic of eDNA sampling collection, including 8 eDNA sampling points at the
downstream end of each electrofishing unit. Generally, the last-fish detected by electrofishing
fell between sites 3 and 4, except for streams C, D, E, F, and I.

DNA was extracted from each filter using a modified protocol of the Qiagen DNeasy
Blood and Tissue kit (Levi et al. 2018). Specifically, we added 1.0 mm zirconia–silica beads to
the initial lysis buffer followed by a 15-minute vortex step to loosen the DNA from the filters.
Incubation in lysis buffer was increased to 48 hours. After incubation, 300ul of the lysed product
was transferred to a new 1.7ml microcentrifuge tube. Thereafter, we followed the manufacturer's
protocol for isolation of tissue. DNA was eluted in a total volume of 100ul. All DNA extractions
and PCR setup are done inside of separate hepa–filtered and UV–irradiated PCR cabinet (Air
Science LLC, Fort Meyers, FL) in a separate lab where no PCR products or other sources of high
concentration DNA are allowed.

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 9 possible replicates (3 field replicates x 3 qPCR or

101 technical replicates), but also recognize that a single positive sample provides weak evidence of
102 species presence relative to consistent positive samples across replicates over time (Jerde et al.
103 2011).
104
105 *eDNA quantitative PCR*
106   We used a species–specific assay for Coastal Cutthroat Trout that targets the cytochrome
107 oxidase I of the mitochondrial genome for trout in the study area. Each sample was run in
108 triplicate PCR reactions. PCR was performed using quantitative PCR (qPCR; Biorad). Each 20ul
109 qPCR reaction contained 6ul of DNA template, 10ul Environmental Master Mix 2.0
110 (ThermoFisher Scientific, Waltham, MA), 0.2 uM of both forward and reverse primers, 0.2um of
111 the TaqMan MGB probe, and sterile water. Additionally, each plate contained a four-point
112 standard curve using DNA obtained from Coastal Cutthroat Trout tissue. Extracted tissue was
113 quantified using a Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA) and diluted from
114 10^{-1} ng/ul to 10^{-4} ng/ul. PCR cycling conditions involved an initial denaturation step of 10 min at
115 95C to activate the HotStart Taq DNA polymerase, followed by 50 cycles of 95C for 15 s and
116 60C for 60 s. All reaction plates contained a negative control of water and extraction blanks.
117
118 *Electrofishing sampling and physical habitat surveys*
119   After eDNA water samples were collected at the downstream sampling point in each
120 stream, we sampled the entire 50 m electrofishing unit using standard backpack electrofishing to
121 determine the end-of-fish. We used a spatially continuous, single–pass backpack electrofishing
122 approach similar to that described by Torgerson et al. (2004) and validated by Bateman et al.
123 (2005). but sampling all accessible habitats We electrofished to compare relative abundance data
124 between both approaches, and consequently, our protocol differs from typical electrofishing to
125 identify the upper extent of fish where fish are not typically netted (WA DNR 2002).
126 Electrofishing settings were set to the appropriate settings for each stream. We measured total
127 length (mm) and weight (g; to tenths) of each fish captured. We processed fish, at least, at each
128 50-m reach break for a total of 350m of electrofishing per stream.
129   Physical stream habitat surveys were conducted for each 50-m reach, including channel
130 unit type (pool, riffle, cascade), channel unit length (m), depth (m), wetted-width (m), and
131 bankfull-width (m) at the lateral and longitudinal mid-point, gradient (recorded to nearest whole
132 number (%), and dominant substrate classification (boulder, cobble, bedrock).

133   **Table 1.** Stream characteristics for 12 study streams.

Ownership	Location	Stream	Latitude last-fish[1]	Longitude last-fish[1]	Electrofishing presence	eDNA presence	Last fish higher in stream with which method?
Port Blakely	Coast	Stream L	NA	NA	No fish detected	1,3	eDNA
Port Blakely	Coast	Stream K	NA	NA	No fish detected	1,2,3,4,5,6,7,8	eDNA
Weyerhaeuser	Cascades	Stream H	46.055817	-122.681817	1,2,3	1,2,3,6,7,8	eDNA
Weyerhaeuser	Cascades	Stream F	46.211533	-122.761283	1,2,3,4	1,2,3,4,6,8	eDNA
Port Blakely	Cascades	Stream I	46.428613	-122.260414	1	1,2,6	eDNA
Port Blakely	Cascades	Stream G	46.464469	-122.446467	1,2,3	1,2,3,4,8	eDNA
Weyerhaeuser	Cascades	Stream D	46.034383	-122.558367	1,2,3,5	1,2,3,4	electrofishing
Hancock	Coast	Stream E	46.321234	-123.785673	1,2,5	1,2	electrofishing
Weyerhaeuser	Cascades	Stream B	46.093533	-122.4993	1,2,3	1,2,3	same last-fish boundary
Weyerhaeuser	Cascades	Stream J	46.294667	-122.612517	1,2,3	1,2,3,4	same last-fish boundary
Port Blakely	Cascades	Stream A	46.553818	-122.243428	1,2,3	1,2,3	same last-fish boundary
Hancock	Coast	Stream C	46.281334	-123.669986	1,2	1,2	same last-fish boundary

134   [1]determined by electrofishing
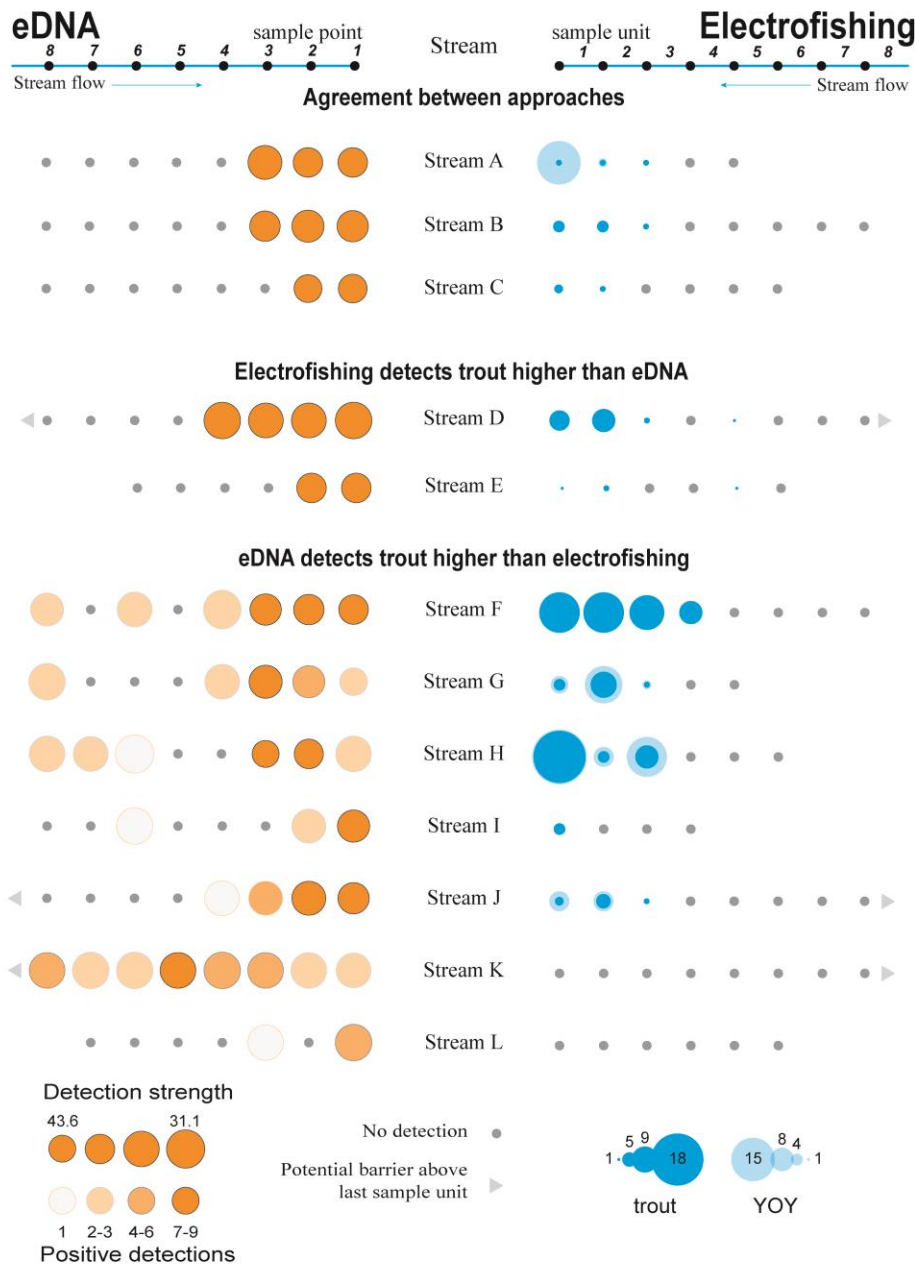
135
136 **Figure 3.** Patterns of agreement and disagreement in Coastal Cutthroat Trout detection between
137 methods using eDNA relative abundances and electrofishing over 8 sites across twelve sample
138 streams on Weyerhaeuser Company, Port Blakely, and Hancock Forest Management land in
139 Washington. In the upper section, we illustrate streams where both methods, eDNA (orange) and
140 electrofishing (blue), showed full agreement as to the upper extent of fish. Gray circles represent
141 no detection and gray x's represent not sampled for both methods. In the middle section, we
142 illustrate streams where electrofishing detected trout upstream of eDNA. Size of the symbols
143 represents eDNA detection strength (threshold cycle value: Cq) and fish abundance from
144 electrofishing (#/per 50 m sample unit). The lower section shows streams where eDNA detected
145 trout above electrofishing. Each row represents a single stream with arrows indicating stream

146   flow direction (eDNA is from left to center mirroring electrofishing, which is from right to
147   center). Dark orange shows higher detection amongst eDNA replicates, whereas light orange is
148   the opposite. Dark blue shows captures of adult Coastal Cutthroat Trout (trout) and light blue
149   shows captures of young-of-year (YOY), which could either be *O. mykiss* or *O. clarkii clarkii*.

150   *Data Analysis*
151   We compared the proportion of agreement between the detection of trout by eDNA and
152   electrofishing across streams and sites. We displayed information for all results across streams
153   and sites, including all field and qPCR replicates, to reveal the variability in eDNA results,
154   especially because we are near the lower detection limits of the focal species at the upper extent
155   of their distribution.
156
157   *Occupancy Modeling Approach using broader suite of sites in Washington and Oregon*
158   Because eDNA is heterogeneously distributed in water, there is imperfect detection and to
159   account for this imperfect detection, we used occupancy models to estimate detection
160   probabilities (Hunter et al. 2015). For the following analyses, we used the results from the 12
161   CMER streams in addition to 19 streams in both Oregon and Washington that followed the same
162   protocol for a more robust analyses that improves the confidence of the modeling.
163   We used a three-level occupancy model EDNAOCCUPANCY in R that uses Bayesian
164   methods of analysis of Markov Chain Monte Carlo (MCMC) methods of maximum-likelihood to
165   estimate model parameters [i.e., $\Psi(.)\theta(.)p(.)$] and include covariates (Dorazio and Erickson
166   2017). Accordingly, we can estimate fish detection probabilities while also estimating the
167   conditional probability of detecting trout DNA that may be present in a field sample or qPCR
168   replicate. The three levels of sampling included aspects of the nested sampling design innate in
169   eDNA sampling of location (stream x site), field sample, and qPCR replicate. In the model, Ψ is
170   the probability that the eDNA is present at a location, θ is the conditional probability that eDNA
171   occurred in a replicate sample given that it occurred at the location level, and p is the conditional
172   probability of detecting eDNA in a replicate qPCR reaction given that it occurred at the field
173   sample level.
174   We predicted that Ψ might vary across stream locations due to physical characteristics of
175   the stream sections. However, after initial data analyses, wetted width and depth were the only
176   characteristics that influenced the results and remained in the model. Accordingly, we evaluated
177   how Ψ might vary across the size of streams locations, including stream width and depth. In
178   addition, θ and p might be influenced by the abundances of trout or all fishes detected by
179   electrofishing due to eDNA inhibition or molecular competition in qPCR reactions. We
180   evaluated several models that included a different combination of covariates at different scales
181   (Ψ, θ, and/or p). Covariates were measured at the location-level including single-pass standard
182   electrofishing surveys that evaluated density of all fishes (#/50 linear m), and density of Coastal
183   Cutthroat Trout (#/50 linear m). Covariates encompassing habitat size included stream width (m)
184   and stream depth (m). We fitted and evaluated eight candidate models using available functions
185   for model-selection criteria from the EDNAOCCUPANCY package. Model-selection criteria
186   included the posterior-predictive loss criterion (PPLC) and widely applicable information
187   criterion (WAIC). We fitted each candidate model by running the MCMC algorithm for 11,000
188   iterations and retaining the last 10,000 for estimating posterior summaries. After selecting the
189   model with the greatest amount of support (lowest WAIC value and higher PPLC), we explored
190   the estimated relationships among covariates (i.e., stream width, stream depth, trout density, and
191   density of all captured fishes) and estimated model parameters Ψ and θ.
192   Lastly, we used results from the model [$\Psi(.)\theta(.)p(.)$] that included covariates and adopted
193   the approach described in Hunter et al. (2015) to compute the cumulative probability of detecting
194   Coastal Cutthroat Trout eDNA in K qPCR replicated sample (p^*), given that the sample
195   contained eDNA the model as $p^* = 1-(1-p)K$. This procedure allowed us to asses if we used an

196   adequate number of qPCR replicates to detect trout eDNA. We performed a similar analysis to
197   estimate the cumulative probability of occurrence of Coastal Cutthroat Trout eDNA in n water
198   samples (θ^*) collected from a location that contained eDNA using $\theta^* = 1-(1-\theta)n$.

199 **Table 2.** Parameter estimates (posterior mean ± SE) and model-selection criteria (PPLC and WAIC) for each candidate model of Coastal Cutthroat

200 Trout eDNA detections for 31 streams using same protocol. Streams included 12 CMER-funded streams and 19 non-CMER funded streams.

201 Values represent either probabilities or estimates of the coefficients of the relationship between the covariate(s) and detection probability of the

202 form $logit(\Psi) = \alpha_0 + \alpha_1*$ covariate $+ \alpha_2*$ covariate$_2$, or $logit(\theta) = \beta_0 + \beta_1*$ covariate $+ \beta_2*$ covariate$_2$, or $logit(P) = \delta_0 + \delta_1*$ covariate.

	Occupancy in location (Ψ)	Occupancy in sample (θ)	Detection in replicate (P)	PPLC	WAIC
$\Psi(.), \theta(.), P(.)$	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)	$\alpha_0 = 0.469\ (\pm0.013)$ $\alpha_1 = -0.153\ (\pm0.006)$ $\alpha_2 = 0.593\ (\pm0.012)$	$\beta_0 = 0.930\ (\pm0.004)$ $\beta_1 = 2.331\ (\pm0.025)$	$\delta_0 = 1.102\ (\pm0.001)$ $\delta_1 = 0.191\ (\pm0.001)$	222.008	0.4153
Ψ(depth), θ(trout+all fishes), P(all fishes)	$\alpha_0 = 0.649\ (\pm0.016)$ $\alpha_1 = 0.092\ (\pm0.004)$	$\beta_0 = 1.031\ (\pm0.006)$ $\beta_1 = 1.986\ (\pm0.028)$ $\beta_2 = 1.863\ (\pm0.017)$	$\delta_0 = 1.101\ (\pm0.001)$ $\delta_1 = 0.191\ (\pm0.001)$	222.756	0.4158
Ψ(width), θ(trout+all fishes), P(all fishes)	$\alpha_0 = 0.635\ (\pm0.011)$ $\alpha_1 = 0.504\ (\pm0.004)$	$\beta_0 = 1.046\ (\pm0.006)$ $\beta_1 = 1.892\ (\pm0.021)$ $\beta_2 = 1.918\ (\pm0.018)$	$\delta_0 = 1.102\ (\pm0.001)$ $\delta_1 = 0.191\ (\pm0.001)$	222.320	0.4158
Ψ(width), θ(trout), P(all fishes)	$\alpha_0 = 0.359\ (\pm0.007)$ $\alpha_1 = 0.410\ (\pm0.004)$	$\beta_0 = 0.928\ (\pm0.004)$ $\beta_1 = 2.169\ (\pm0.022)$	$\delta_0 = 1.104\ (\pm0.001)$ $\delta_1 = 0.192\ (\pm0.001)$	221.471	0.4167
$\Psi(.), \theta$(trout), P(all fishes)	0.63 (0.51, 0.79)	$\beta_0 = 0.938\ (\pm0.004)$ $\beta_1 = 2.231\ (\pm0.029)$	$\delta_0 = 1.104\ (\pm0.001)$ $\delta_1 = 0.194\ (\pm0.001)$	221.540	0.4172
Ψ(width+depth), θ(trout+all fishes), P(all fishes)	$\alpha_0 = 0.801\ (\pm0.011)$ $\alpha_1 = 0.851\ (\pm0.010)$ $\alpha_2 = -0.267\ (\pm0.006)$	$\beta_0 = 1.034\ (\pm0.006)$ $\beta_1 = 1.936\ (\pm0.017)$ $\beta_2 = 1.971\ (\pm0.019)$	$\delta_0 = 1.102\ (\pm0.001)$ $\delta_1 = 0.189\ (\pm0.001)$	222.940	0.4174
Ψ(depth), θ(trout), P(all fishes)	$\alpha_0 = 0.329\ (\pm0.008)$ $\alpha_1 = 0.122\ (\pm0.002)$	$\beta_0 = 0.936\ (\pm0.004)$ $\beta_1 = 2.184\ (\pm0.026)$	$\delta_0 = 1.104\ (\pm0.001)$ $\delta_1 = 0.193\ (\pm0.001)$	221.562	0.4181

203

204

11

Results

Three streams (25%) agreed to the upper extent of fish for both approaches (Table 1; Fig. 3). Trout eDNA was detected above the last observed fish with electrofishing in seven streams (58%) by 50–250 m. Two of these seven streams did not have any trout observed with electrofishing (Streams K and L). The most upstream trout detections with eDNA had fewer replicate eDNA detections than downstream sites in the same stream, often 1 to 3 of 9 total replicates. Two other streams (17%) resulted in fish observed 50–150 m higher with electrofishing than eDNA. All study streams had trout detections with eDNA.

Model results using broader suite of sites in Washington and Oregon

The model with covariates that had the greatest support revealed that (i) stream widths positively correlate with eDNA trout occupancy at location; (ii) electrofishing trout densities positively affect eDNA field samples; (iii) and electrofishing densities of all fish positively influence the quantity of positive qPCR replicates. Modeled results revealed that the predicted occurrence of trout eDNA was higher in wider stream locations (Table 2; Fig. 4). The occurrence of trout eDNA is increased in field samples with greater electrofishing trout density. eDNA detected trout at very low electrofishing densities of <5 trout per 50 lineal m. The occurrence of trout eDNA is greater in qPCR replicates with greater electrofishing fish density.

Estimates of detection probabilities of trout eDNA (P) suggested that qPCR was effective in detecting eDNA presence in a field sample (Model [($\Psi(.)$, $\theta(.)$, $P(.)$] in Table 2). The mean estimated detection probability collected by location was 0.89 (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. The cumulative probability of detecting trout eDNA (θ^*) resulted very high ranging from 0.980 to 0.995. This also suggests that the three eDNA samples collected was sufficient to include trout eDNA when the eDNA was present at that location.
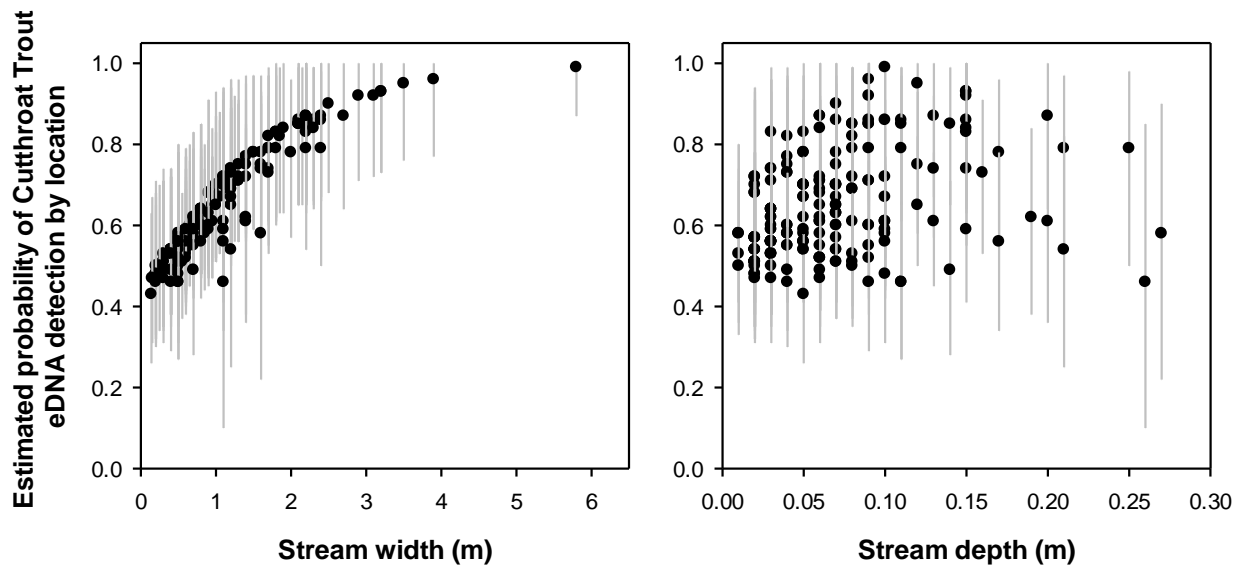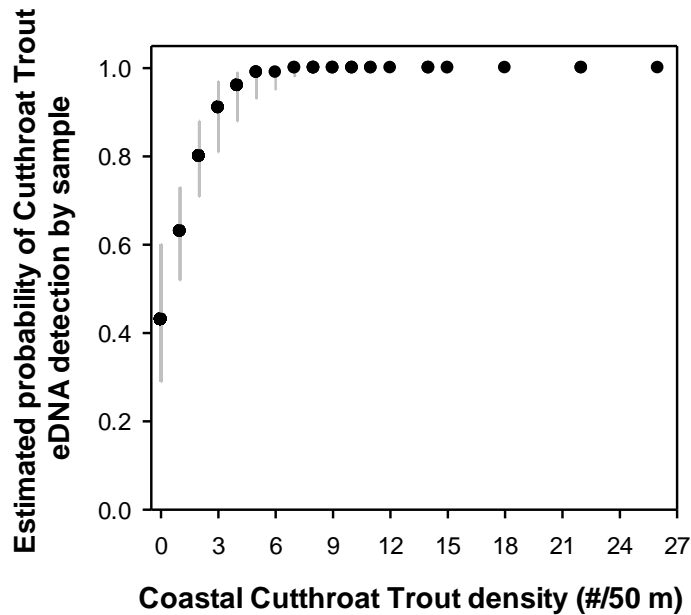

Figure 4. Estimated probabilities of occurrence of trout eDNA by location (Ψ) increases with stream width. Location is stream x site. Symbols are estimates of posterior means with 95% credible intervals for the model [Ψ(depth+width), θ(trout), P(all fishes)] described in Table 2.

237

Coastal Cutthroat Trout density (#/50 m)

Figure 5. Estimated probabilities of occurrence of trout eDNA in field samples (θ) increases with increased trout density from electrofishing. 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. Field samples are 1L biological replicates that were taken in triplicate.

Discussion

Can eDNA be used to identify the upper boundary of fish?

We provide evidence that eDNA constitutes an effective addition to approaches that should be considered to identify the upper extent of fish. While the last–fish boundary matches between approaches in a quarter of the streams, in over half of the streams trout DNA is detected further upstream with eDNA than trout have been detected with electrofishing. For streams with positive DNA detections of trout, the uppermost sites generally revealed a reduced detection signal relative to downstream sites from the same stream probably from a low concentration of target DNA upstream from fewer fishes being found at the uppermost edge of fish, or from false positives. We find that eDNA detects trout DNA when they occur in extremely low quantities, but its detection ability is imperfect and so it also misses detecting trout in low quantities in some circumstances (Streams D and E). For example, it is not always clear how to translate positive eDNA detections into actual living trout (or eggs) in the stream versus detection failure or true absence (e.g., Darling and Mahon 2011, Jerde et al. 2011, Wilson et al. 2014).

Can eDNA be used in addition to electrofishing to determine the end–of–fish?

The upper boundary of fish has the same boundary between both approaches for 25% of sampled streams, and eDNA detects the boundary higher in more than half of all sampled streams suggesting that it is more sensitive than electrofishing. Both streams where electrofishing detects trout above eDNA have one trout at their upper-most fish site potentially because that one fish was below the detection limits for eDNA and/or was disturbed or moved upstream by the eDNA

266 crew walked upstream first. Although eDNA is equal to or more sensitive than electrofishing, it
267 seems that using eDNA to define the upper extent of fish is near its detection limits. In most
268 cases, it is able to detect trout in low densities, but sometimes it also misses them. Electrofishing
269 has been the primary approach to identify the last-fish in streams for decades, but it appears that
270 its ability to detect fish at the upper extent of fish is generally equal to or less effective than
271 eDNA in these study streams. The lack of block net use while electrofishing may have pushed
272 some fish into upstream habitats as they fled, such as streams D and E. Electrofishing protocols
273 to determine last-fish do not typically use block nets, which ensure fish do not flee to adjacent
274 habitats (Peterson et al. 2005). Block nets are used to ensure fish do not flee to adjacent habitats,
275 however they are not typically used in electrofishing protocols to determine last-fish. At its
276 optimal, standard backpack electrofishing is most efficient for larger fish in shallower water with
277 ideal stream habitat conditions for conductivity, water temperature (<18°C), water transparency
278 (good visibility), and habitat characteristics (Price and Peterson 2010) . Trout have higher
279 capture probabilities than other fishes, such as those with coarse scales (cyprinids) or without
280 swim bladders (sculpins). Electrofishing offers data of catch in real-time and consequently
281 identifies the exact time and place that a fish was captured (Table 3). A main advantage of
282 electrofishing by an experienced crew is that they have the ability to detect many fish species
283 (although not equally across species or sizes), whereas eDNA detects only targeted fishes.

284
285 Table 3. Comparison of eDNA versus electrofishing approaches to delimit upper extent of fish.
286 **Bold face** denotes positive characteristics of method.

Metric	eDNA	Electrofishing
Assesses potential presence and absence of fish	**Yes**	**Yes**
Estimates relative abundance of fish	**Yes**	**Yes**
Archives fish as museum voucher	No	**Yes**
Obtains data on length, weight, or fish characteristics	No	**Yes**
Obtains genetic data*	**Yes**	**Yes**
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
Offers data instantaneously	No	**Yes**
Identifies exact time and place of fish	No	**Yes**
Potential for false positives**	Yes	**No**
Potential for false negatives	Yes	Yes

287 *genetic data can be obtained from eDNA samples if they are sequenced in addition to standard
288 qPCR analysis; **Electrofishing could have false positives if there are issues with field
289 identification of target species

290 *What are the operational limitations to the use of eDNA to determine the end–of–fish?*
291 eDNA warrants inclusion amongst the sampling approaches considered to identify the upper
292 extent of fish. We need to continue to push the boundaries of eDNA detections to identify where

293 the low eDNA detections for trout marks a distribution extension for the upper extent of Coastal
294 Cutthroat Trout (actual presence) versus where it does not reflect an actual fish in the stream
295 (detection failure). The effectiveness of eDNA depends on investigators being informed of the
296 potential location of last-fish to know where to start sampling with its utility potentially being
297 maximized when used as a complementary approach to standard methods. eDNA in streams
298 detects DNA of the target species from flowing water that are generally located upstream of the
299 sampling location, but the upstream distance DNA has travelled remains unknown, but is likely
300 variable by stream and flow conditions, whereas electrofishing can identify fishes in a specific
301 habitat type, such as a pool or riffle (Table 2). It is important to target all potential fishes with
302 eDNA to ensure that last-fish is detected with eDNA, which may mean using multiple
303 primer/probe sets. Although the precise time when DNA was shed into the environment by a
304 focal organism is not known with eDNA, it has been able to show abundances of run timing of
305 salmon (Levi et al. 2018), suggesting that sample timing needs to correspond to when fish are
306 present. After sampling, eDNA samples still have to be extracted and analyzed leading to a time
307 lag for results.
308   As managers start to incorporate eDNA surveys to detect last-fish, they may want to use
309 more than one criterion to define a positive eDNA detection as part of a decision-making
310 framework. For example, a threshold of a positive eDNA detection could be set for a given
311 number of replicates to separate a consistent series of strong detections from a few weak
312 detections, as well as incorporating information about potential barriers to fish movement and
313 other habitat characteristics (e.g., wetlands, habitat complexity). We suggest that as the
314 discussion of eDNA as a management tool continues it is important to distinguish between the
315 science of eDNA (e.g., methodological sensitivities, limitations) and the implications that are
316 derived from its information (e.g., fish presence). Although issues remain in the field of eDNA
317 with detections of both false-positive and false-negative errors (Roussel et al. 2015, Guillera-
318 Arroita et al. 2017), understanding such errors associated with using eDNA to delimit the last-
319 fish will help to define more robust monitoring and management outcomes.
320

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