Lateral and longitudinal fish eDNA distribution in dynamic riverine habitats

Running title: spatio-temporal eDNA distribution

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Abstract

Assessing the status and distribution of fish populations in rivers is essential for management and conservation efforts in these dynamic habitats and currently, environmental DNA (eDNA) is established as an alternative and/or complementary approach to the traditional monitoring of fish species. In lotic systems, a sound understanding of hydrological conditions and their influence on the local target DNA detection probability and quantity is key for the interpretation of eDNA-based results. However, the effect of seasonal and diurnal changes in discharge and the comparability of semi-quantitative results between species remain hardly addressed. We conducted a cage experiment with four fish species in a glacier-fed, fish-free river in Tyrol (Austria) during summer, fall, and winter discharge (i.e. 25-fold increase from winter to summer). Per season, water samples were obtained on three consecutive days at 13 locations downstream of the cages including lateral sampling at 1-2 m distance across the wetted width. Fish eDNA was quantified by species-specific endpoint PCR followed by capillary electrophoresis. Close to the cages, lateral eDNA distribution was heterogenous, mirrored cage placement within the stream, and showed the diluting effect of increased discharge. Additionally, the eDNA signals were significantly lower for fish species of larger individual size at comparable per-species biomass. For further downstream distances with laterally homogenous eDNA distribution, the signals decreased significantly with increasing distance independent of longitudinal discharge changes. This study exemplifies the importance of prevailing hydrological conditions for the interpretation of eDNA-based data across seasons. To control for heterogenous eDNA distribution and enable comparisons over time, sampling schemes in lotic habitats need to incorporate hydrological conditions and species biology.

Keywords: lotic, environmental DNA, hydrology, sampling scheme**,** *Salmo trutta*, *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Phoxinus phoxinus***,**

Introduction

In times of rapid environmental changes there is a growing need for biomonitoring in both terrestrial and aquatic systems (Cardinale et al., 2012; Tickner et al., 2020). Reliable and cost-effective approaches for species detection are thus key for tracking species in time and space and informing conservation and management efforts (Jetz et al., 2019). Molecular methods detecting environmental DNA (eDNA) released by organisms into their environment have the capability to accommodate this demand as they are non-invasive, sensitive, and enable the processing of large sample numbers (Barnes & Turner, 2016; Deiner et al., 2017; Thomsen & Willerslev, 2015). In aquatic habitats eDNA can be used for monitoring of taxa in both lotic and lentic systems with a focus on endangered or invasive species and flexible year-round application (Beng & Corlett, 2020; e.g. Harper et al., 2019; Thomsen & Willerslev, 2015).

Rivers and streams contain, absorb, and transport eDNA of aquatic and terrestrial species and are thus ideal for cross-habitat species detection (Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Sales et al., 2020), albeit the dynamic nature of these ecosystems leads to constantly changing conditions during sampling (Shogren et al., 2017; Willett, McCoy, Taylor Perron, Goren, & Chen, 2014). Nevertheless, aspects such as a species' upstream distribution limits (Carim et al., 2019; Robinson, de Leaniz, & Consuegra, 2019) or local abundance and its change over time (Doi et al., 2017; Levi et al., 2019; Thalinger, Wolf, Traugott, & Wanzenböck, 2019) have been successfully examined in lotic systems via eDNA. Often, these efforts are combined with traditional monitoring techniques to confirm molecular results and facilitate their interpretation (Evans, Shirey, Wieringa, Mahon, & Lamberti, 2017; Wilcox et al., 2016) and there is generally a good consensus between molecular and non-molecular data.

However, changes in hydrological conditions have profound influence on the distribution and persistence of eDNA in the water column (reviewed by Harrison, Sunday, & Rogers, 2019), which makes the interpretation of local eDNA signals or comparisons between sampling campaigns challenging. The longitudinal eDNA detection probability in

rivers directly depends on dilution, transport, deposition, resuspension, and degradation (reviewed by Harrison et al., 2019). These effects were examined for small streams (Fremier, Strickler, Parzych, Powers, & Goldberg, 2019; Shogren et al., 2017; Wilcox et al., 2016) and large river systems (Deiner & Altermatt, 2014; Pont et al., 2018), and eDNA was mostly found to behave similar to fine particulate organic matter. Recently, studies also focused on lateral eDNA distribution, describing a “plume” downstream of the source and gradual lateral homogenization with increasing downstream distance (Laporte et al., 2020; Wood, Erdman, York, Trial, & Kinnison, 2020). The discharge-dependent changes of these patterns or species-specific effects were, however, neglected.

Alpine rivers exemplify the benefits of eDNA-based monitoring as well as and the challenges associated with sampling campaigns in such dynamic ecosystems. The prevailing low water temperatures permit only humble population densities and a limited species inventory at risk of biodiversity loss due to increased climate change effects (Settele et al., 2015). Anthropogenic influences in the form of straightened riverbeds, hydropower plants, and dams add to this strained situation and intensify the need for monitoring the remaining natural fish populations (Faulks, Gilligan, & Beheregaray, 2011; Fette, Weber, Peter, & Wehrli, 2007). The discharge in these rivers varies with changing seasons from winter drought to high water levels in spring and summer (snow melt and glacial melt) to intermediate conditions in fall. Additionally, the melting processes induce substantial diurnal discharge changes in spring and summer (Bard et al., 2015). Therefore, traditional monitoring via electrofishing is only possible outside of protected periods, spawning seasons, and at low discharge and turbidity in fall and early winter. eDNA-based approaches can potentially overcome these limitations, but only after accounting for the spatio-temporal dynamics of Alpine rivers and by optimizing sampling schemes and the interpretation of the derived results.

In this study, we compared changes in lateral and longitudinal eDNA distribution between seasons in a glacier-fed Alpine river. The experiments were conducted with caged fish and we used species-specific endpoint PCR combined with capillary electrophoresis

(celPCR) to investigate how longitudinal and lateral eDNA detection probability and signal strength vary on a small scale (<20 m) between summer, fall, and winter, (i.e. high, medium, and low water levels) with up to 25-fold variations in discharge. Additionally, the longitudinal change in eDNA signal strength was examined at an intermediate scale (~1 km) for fish species of different size, but constant total biomass.

Materials and Methods

Study site

The three cage experiments took place in the Melach, a fish-free, glacier-fed, Alpine river in Lüsens, Tyrol (Latitude 47°7'0,32", Longitude 11°8'4,92", WGS1984) in August (22^{nd}-26^{th}) 2016, November (21^{st}-25^{th}) 2016, and September (26^{th}-29^{th}) 2017. The Melach shows typical seasonal and daily fluctuations in discharge and sediment load associated with glacially influenced rivers in the Alps (Sertić Perić, Jolidon, Uehlinger, & Robinson, 2015). Following the European river zonation, the sampling site is located in the epirhithral of the river at an elevation of 1700 m a.s.l. Permanent fish populations cannot be established in this part of the river due to extreme discharge situations. Additionally, a transverse structure at the downstream end of the examined range prohibited potential upstream migration.

Experimental design

All handling of study animals conformed to Directive 2010/63/EU, and permission for field work was granted by the fishing area manager H. Raffl. Prior to the cage experiment, the absence of fish was confirmed via electrofishing, starting 1.3 km downstream at the transverse structure. For each of the three trials, two steel cages 1 × 1 × 0.6 m with “mesh” size of 10 mm and 5 mm, respectively, were installed at the same location in full current of the river for the duration of the experiment (four days), secured against drift, and equipped with a few stones to provide some natural structure. Four fish species were used in the experiment: A cyprinid species (Eurasian minnow – *Phoxinus phoxinus* L. 1758) was placed

in the cage with the narrow mesh size and three salmonid species (brown trout – *Salmo trutta* L. 1758, brook trout – *Salvelinus fontinalis* M. 1814, and rainbow trout – *Oncorhynchus mykiss* W. 1792) were placed in the other cage. On days two, three, and four of the experiment, 200 g fish per species (never less for salmonids) were placed in the cages, equalling two to five individuals per salmonid species and 40 to 90 *P. phoxinus* individuals. At the end of each sampling day, fish were removed from the cages and exchanged for new individuals. To prevent multiple use of individuals, fish were kept in separate tanks thereafter; in November the availability of *P. phoxinus* was limited and the same individuals were used throughout the experiment.

Two-liter water samples were used for the detection of fish eDNA. On the first day of each trial (prior to fish placement in cages), control samples were taken from at least five locations between the cage positions and the transverse structure. On days two, three, and four, water samples were taken daily at 13 locations downstream of the cages (1.3 km, 555 m, 423 m, 323 m, 223 m, 130 m, 65 m, 33 m, 16.8 m, 8 m, 4 m, 2.5 m, and 0.5 m) at approximately the same daytime. Due to changes in the structure of the riverbed, the distance between the cages and the first seven transects sometimes varied one to two meters between experiments, which was accounted for during data analyses. At 0.5 m to 130 m distance, two to four water samples were taken in transects, depending on the width of the river. Further downstream, only one sample was taken per location. In November, only two to three transect samples were taken due to limited discharge. Each day, sampling was carried out from the most downstream location moving upstream towards the cages. In September, two such sampling runs were carried out per day.

Water samples were collected right below the stream surface using 2 L wide-neck bottles, which were treated with chlorine bleach (3.6 g sodium hypochlorite per 100 g liquid) overnight and thoroughly washed using fish-DNA-free tap water. Filtration was carried out on 500 ml filter towers with a peristaltic pump (Solinst; Model 410) and glass fibre filters with 47 mm diameter (1.2 µm mesh width, Whatman GF/C) followed by storage at –80 °C until further processing. In case of filter clogging (high turbidity; only in August), up to three filters

158 were utilized per sample. During all water processing steps, DNA-free gloves were worn and
159 frequently changed. All multi-use equipment was soaked in chlorine bleach for at least ten
160 minutes between samples and thoroughly rinsed using MilliQ water. Forceps for filter
161 handling were singed three times prior to each use. Every ten samples, 2 L of MilliQ water
162 were filtered as negative controls to check for cross-contamination during fieldwork, whereas
163 a sample from the fish transport container served as daily positive control.

164 Total discharge and its lateral differences were measured with a FlowTracker
165 (Sontek, USA) during the August trial, and with salt and a TQ Tracer (Sommer Messtechnik,
166 Austria) during the November and September trials. In August, discharge increased during
167 the sampling time (10 AM to 5 PM) but remained longitudinally constant due to the strong
168 glacial influence. Therefore, diurnal discharge was measured once and supported by point-
169 measurements at 3 PM. In September and November, the inflow of side streams caused an
170 increase along the 1.3 km sampling range, with hardly any changes in the course of a day.
171 Hence, measurements were carried out daily directly at the cages, at 223 m, 555 m, and
172 1,300 m downstream, and supported by a diurnal measurement per trial. Discharge at each
173 water sampling point (in space and time) was extrapolated; no rainfall occurred during any of
174 the sampling days. For all three trials, turbidity was measured with a turbidity meter using an
175 infrared light source (AL250T-IR, Aqualytic, Germany). Water temperature, pH, conductivity,
176 and oxygen saturation were obtained with a multi-parameter probe (WTW, Germany).

177

178 *Laboratory processing*

179 All molecular work was carried out in a clean-room laboratory at the University of Innsbruck
180 (Austria) compliant with ancient-DNA processing standards. First, filters were lysed using
181 190 µL TES (0.1 M TRIS, 10mM EDTA, 2% sodium dodecyl sulphate; pH 8) and 10 µL
182 Proteinase K (VWR, 20 mg/mL) each, followed by incubation over-night on a rocking platform
183 at 56°C. Then, filters were transferred to plastic inserts with a perforated bottom and
184 centrifuged for 10 min at 14,000 rpm. The entire resulting lysate was extracted on the
185 Biosprint 96 platform (QIAGEN) using a custom DNA-uptake protocol which combined the

DNA contained in up to 900 µL lysate prior to extraction with the "BS 96 tissue protocol" according to the manufacturer's instructions; except for elution in 100 µL TE. Lysates of filters stemming from the same water sample were also combined during the uptake process. Each 96-well plate contained four extraction negative controls and all resulting eluates were stored at -32°C until further processing.

Target DNA was amplified using species-specific primers in endpoint PCRs coupled with capillary electrophoresis (celPCR). PCRs were run on Nexus Mastercyclers (Eppendorf), with each plate including at least one positive control (DNA extract from target species) and one negative control (molecular grade water). Previously published primers (Thalinger et al., 2016) and a newly developed primer pair for *S. trutta* (Table 1) were utilized. During PCR optimization, primers were tested for specificity against other fish species and aquatic invertebrates occurring in Central European freshwaters (Thalinger et al., 2016). Additionally, we specified assay sensitivity following Sint et al. (2012): reliable positive amplifications were possible for all primer pairs from 10 DNA ds. Each 10 µL PCR master mix contained 1 × Multiplex reaction mix (QIAGEN), 0.5 µM of each primer, 30 mM TMAC, 5 µg BSA (Bovine Serum Albumin) and 3.2 µL DNA extract. Optimized thermocycling conditions were: denaturation at 95°C for 15 min, 35 cycles of 94°C for 30 s, 64°C (66°C for *P. phoxinus*) for 3 min and 72°C for 60 s followed by final elongation at 72°C for 10 min. Target DNA signal strength was determined via capillary electrophoresis on the QIAxcel (Qiagen) with the associated software QIAxcel Screengel version 1.6.0.10 using the method AM320-30s. The fluorescent signal measured in relative fluorescence units (RFUs) was used as a semi-quantitative measure of target DNA (Thalinger et al., 2019) and signals ≥0.08 RFU were deemed positive. To verify the applicability of this approach, 40 samples positive for *S. trutta* were re-tested with droplet digital PCR (SI 1). All extraction and PCR negative controls resulted negative, however, in the August and the November trial six of 16 negative controls showed low levels of contamination (≤0.2 RFU), hence, fluorescence values of potentially affected field samples were down-corrected by the respective values.

Statistical analysis

All data were analyzed in R (R Core Team, 2019) and visualized with “ggplot2” (Wickham, 2016), “ggpubr” (Kassambara, 2019), and “viridis” (Garnier, 2018). We generated eDNA heatmaps per fish cage, i.e. for salmonids and *P. phoxinus,* for each of the three trials up to 20 m and 65 m downstream distance. The lateral distance from the orographically left shore (x), the longitudinal distance from the respective cage (y) and mean RFUs (z) were used for the linear interpolation of irregular gridded data with the “akima” package (Akima & Gebhardt, 2016). eDNA signals were not extrapolated towards the edge of the water body and RFUs were interpolated on a 5×5 cm grid. To identify the distance at which lateral eDNA distribution was homogenous, salmonid RFUs were tested for normal distribution with a Shapiro-Wilk-test, followed by Kruskal-Wallis-tests per transect and trial. Hence, only data with downstream distances ≥ 130 m were used for the subsequent analyses. Differences in detection rates and eDNA signal strengths amongst salmonid species were examined per trial with Z-tests and Kruskal-Wallis tests, respectively; p-values were Holm-Sidak-corrected. No differences in detection rates and eDNA signal strength were detected, thus salmonid samples remained pooled for subsequent analyses. The same tests were applied comparing detection rates and signal strength between *P. phoxinus* and the salmonids per trial. For all tests of eDNA signal strength, only samples testing positive were taken into account, as non-detections in this case were random and can be attributed to the sampling process (Hagenaars & McCutcheon, 2002).

The relationship between RFUs and downstream distance from the cages was examined by fitting Linear Mixed-Effects Models (function “lmer”; package “nlme” (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2020)). To account for a) the non-linear relationship between RFUs and absolute target copy numbers, b) the influence of discharge and c) a non-normal data distribution, RFUs were exponentiated, divided by the respective discharge, and subsequently ln-transformed. Downstream distances were also ln-transformed and after initial inspection, data obtained in August were not included (see Results). The environmental variables turbidity (0 – 1.055 NTU), oxygen saturation (10.11 – 11.25 mg/L),

242 water temperature (4.2 – 7.3°C), conductivity (49.3 – 69.2 µS/cm) and pH (7.45 – 7.6) were
243 also not included as their fluctuations were considered negligible. Linear-Mixed-Effects
244 models were built starting with an intercept-only model and gradually including fixed effect
245 (distance) and random effects (month and fish family; Table 2). Model fit was compared by
246 AIC and BIC and changes in Log Likelihood Ratios (see SI 2 for the complete R code).
247
248
249 **Results**
250 Altogether, 306 water samples were analyzed: 84, 168, and 54 from the August, September,
251 and November trial, respectively and average discharge during sampling 0-20 m downstream
252 of the cages was 835 L/s in August, 174 L/s in September, and 61 L/s in November. Fifty
253 percent of the 1,224 PCRs resulted positive for one of the four fish species with the majority
254 of detections occurring in September and November. Heatmaps of eDNA signals up to 20 m
255 downstream of the cages revealed eDNA plumes below the respective fish cage and much
256 weaker or no signals at small lateral distances of ~1 m (Fig. 2). In August, cages were
257 placed in the center of the stream and positive samples within 20 m distance were almost
258 exclusively obtained from sampling points with no lateral offset (Fig. 1 and 2). In September,
259 the main water flow out of the salmonid cage was observed towards the orographically right
260 edge of the river; the main water flow from the *P. phoxinus* cage was on the opposite side.
261 This situation was mirrored by high target eDNA signals downstream of the respective cage,
262 and low eDNA signals at the opposite sides. In November, eDNA signals were highest for
263 both salmonids and *P. phoxinus* and albeit the eDNA distribution pattern was similar to
264 September, lateral differences decreased at smaller downstream distance (Fig. 2).
265 At 130 m distance from the cages, salmonid signals were laterally homogenous
266 during all three trials ($p_{corr} > 0.05$). The eDNA signals from 130 m to 1.3 km downstream
267 distance displayed distinct patterns: in August, only few samples resulted positive with low
268 values for both *P. phoxinus* (0.25 RFU ± 0.16 RFU SD) and the salmonids (0.23 RFU ± 0.3
269 RFU SD); discharge and eDNA signal strength did not show distinct longitudinal changes

(Fig. 3). In September, signals remained at a similar level for *P. phoxinus* (0.34 RFU ± 0.23 RFU SD) and salmonids (0.27 RFU ± 0.15 RFU SD). In November, mean *P. phoxinus* eDNA signals were highest (0.34 RFU ± 0.22 RFU SD), and salmonid signals remained similar to September (0.23 RFU ± 0.21 RFU SD). The longitudinal increase in discharge was similar in September and November with a 2.1-fold increase from 201 L/s to 335 L/s and a 2.7-fold increase from 71 L/s to 167 L/s, respectively. Discharge-corrected eDNA signals of *P. phoxinus* and the salmonids showed a stronger decline along the sampling range in November (Fig. 3). Of the samples for which a homogenous lateral eDNA distribution was expected, 57% of the PCRs were positive for *P. phoxinus* and 25% were positive for salmonid species in August. In September and November, all *P. phoxinus* PCRs resulted positive in comparison to ~2/3 of the salmonid reactions (Fig. 4). These differences were significant in August ($Chi^2 = 5.79$, $p_{corr} < 0.05$) and September ($Chi^2 = 13.62$, $p_{corr} < 0.001$). Additionally, eDNA signal strength in November was significantly higher for *P. phoxinus* compared to the salmonids ($W = 635.5$, $p_{corr} < 0.01$; Fig. 4).

Of the six Linear Mixed-Effects models fitted to September and November eDNA signals, model “e” containing ln-transformed distance as fixed effect and the random effects month (random slope and intercept per trial) and fish family (random intercept only) best described the data (Table 3 and 4). Model “f”, additionally containing random slopes per fish family and month (Figure 4; bottom panel), resulted in a higher BIC and the change in log-likelihood was not significant between the two models. Model “e” confirmed a significant ($p < 0.001$) negative relationship between eDNA signals and downstream distance after correcting for changes in discharge (Table 4). Permitting different eDNA signal strengths at the upper end of the examined range between months and between fish families within each month proved vital for model fit; the same was true for incorporating the variation in eDNA signal decline between months.

Discussion

298 Our results demonstrate profound spatio-temporal changes in eDNA distribution induced by
299 seasonal discharge conditions. Lateral and longitudinal extent of the eDNA plumes
300 downstream of the caged fish mirrored their position inside the river and at higher discharge,
301 lateral mixing occurred further downstream. Additionally, the small-sized *P. phoxinus*
302 individuals emitted a significantly stronger eDNA signal than the larger salmonid individuals
303 at low discharge in November. Along the 1.3 km flow path, discharge-corrected eDNA
304 signals declined significantly and this effect was most pronounced in November.

305 The eDNA signals recorded at a small lateral and longitudinal scale (<20 m) coincide
306 with previous results describing a plume-shaped distribution of eDNA downstream of the
307 source (Laporte et al., 2020; Wilcox, McKelvey, Young, Lowe, & Schwartz, 2015; Wood et
308 al., 2020). Additionally, the position of the salmonid and *P. phoxinus* plume mirrored the
309 lateral position of the respective cage when these were not aligned in flow direction in
310 September and November. This finding complicates the interpretation of field-derived eDNA
311 results when local conditions differ across the wetted width because individual species prefer
312 specific micro habitats and this choice is furthermore affected by hydrological conditions (e.g.
313 seeking shelter from high flow velocities) (Aarts & Nienhuis, 2003). Based on our
314 experimental setup with constant fish biomasses, it was not possible to confirm the results of
315 Wood et al. (2020) who found that the release of higher eDNA quantities increases detection
316 probability at higher lateral distance. However, our data show a comparable effect induced
317 by lower discharge and slower flow velocities (Wondzell, Gooseff, & McGlynn, 2007) at
318 constant fish biomass. Future studies examining lateral and longitudinal eDNA distribution
319 should therefore take biomass-induced variations and hydrological effects into account.

320 The salmonids (average individual mass 101 g) had lower detection probabilities and
321 lower eDNA signals than *P. phoxinus* (average individual mass 3.5 g) at similar total
322 biomass. eDNA and biomass in most cases exhibit a positive relationship in controlled and
323 natural settings (e.g. Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Takahara,
324 Minamoto, Yamanaka, Doi, & Kawabata, 2012; Thalinger et al., 2019), but the influence of
325 fish size is not well studied. Maruyama et al. (2014) found elevated levels of eDNA release

per gram in juvenile fish, but attributed this effect to higher metabolic activity (Vinberg, 1960). In the present study, several scenarios can explain the difference in detection probability and signal strength: the *P. phoxinus* individuals, albeit mostly not juvenile, could indeed have had higher metabolic rates, which are common for smaller fish species (Clarke & Johnston, 1999). The increased surface to volume ratio of the smaller fish is another potential explanation as more *P. phoxinus* surface was exposed to the water flow. The eDNA release rates could generally differ between cyprinids and salmonids, which also exhibit structural and physiological differences (Freyhof & Kottelat, 2007). The salmonids were definitely better adapted to the high flow conditions within the cages in August when some *P. phoxinus* did not survive the exposure to 1,500 L/s discharge. However, the difference in eDNA signal strength was most pronounced in November at ~60 L/s. It is unlikely that the effect was induced by the species-specific primers, as detection patterns and signal strength did not differ within the salmonids and the PCR assays were extensively tested for specificity and equal sensitivity (Sint et al., 2012; Thalinger et al., 2016). Most likely, the effect is a combination of the aforementioned physiological differences and could be even stronger when fish are compared to other aquatic groups such as amphibians, mussels, or crayfish (Bedwell & Goldberg, 2020; Robinson, Uren Webster, Cable, James, & Consuegra, 2018; Wacker et al., 2019).

As expected for glacier-fed Alpine rivers and streams, discharge changed substantially (25-fold) between trials (Bard et al., 2015). The associated diluting effect was clearly visible from eDNA signal strengths directly downstream of the cages: average *P. phoxinus* signals at this location ranged from 0.15 RFU in August to 1.27 RFU in November. Few of the samples taken at high discharge >1,000 L/s tested positive, but in this situation the 200 g fish mass per species were less than 0.2 ‰ of the passing water mass. The summer situation was most challenging to examine because discharge and turbidity increased quickly from morning (~270 L/s and ~22 NTU) to afternoon (~1,100 L/s and ~130 NTU). In contrast to classic fish monitoring via electrofishing, where sampling in such harsh conditions is not feasible, eDNA-based methods are not restricted to low flow conditions outside of protected

periods and spawning seasons. Based on our results, spring and summer sampling in glacier-fed Alpine rivers should, however, not take place at high discharge in the afternoon and evening and is also not advisable during floods and after strong rainfalls. In September and November discharge remained almost constant over time, but side streams led to a more than 2-fold increase within the examined 1.3 km range. So far, most eDNA studies only discuss this factor (Harrison et al., 2019; Laramie, Pilliod, & Goldberg, 2015; Wood et al., 2020) even though the incorporation of these dilution effects was vital for our analysis.

After controlling for the changing discharge, the eDNA signals of both *P. phoxinus* and the salmonids declined with increasing distance as expected from previous work on eDNA deposition and degradation (Harrison et al., 2019). At constant longitudinal and temporal discharge, average transport distance (S_P) and depositional velocity (v_{dep}) are used to describe eDNA deposition (Pont et al., 2018; Shogren et al., 2017; Wilcox et al., 2016). The calculation of v_{dep} relies on flow velocity and depth data, which can fluctuate considerably in natural and semi-natural rivers and are directly influenced by discharge. Therefore, we refrained from calculating this factor. S_P is commonly described as the slope parameter of a first order exponential decline $S_P = 1/-k$ (but also see Wood et al., 2020). In our case this would result in an average transport distance of ~1,800 m and ~930 m for *P. phoxinus* eDNA in September and November, respectively (based on data from locations with homogenous lateral eDNA distribution) and confirm the positive correlation between river size in general and transport distance (Deiner & Altermatt, 2014; Jane et al., 2015; Pont et al., 2018; Shogren et al., 2017). However, S_P is non-generalizable between streams (Harrison et al., 2019) and thus of limited use outside a systematic framework incorporating the complex flow regime of Alpine rivers.

Our work aids to the understanding of how eDNA signals obtained from field-collected samples can be interpreted for species monitoring and conservation. Sampling campaigns carried out in dynamic habitats such as Alpine rivers and streams need to account for the heterogenous lateral eDNA distribution, adapt the sampling scheme to habitat preferences of the target species, and address the prevailing discharge situation. In a best-case scenario,

382 the target species has distinct habitat preferences, and discharge is low and constant during
383 the entire sampling period. Then, eDNA quantities measured directly downstream of suitable
384 habitats are likely to be directly correlated with local target species biomass (Hinlo,
385 Lintermans, Gleeson, Broadhurst, & Furlan, 2018). Otherwise, only discharge measurements
386 at each sampling location can prevent flawed inferences (Thalinger et al., 2019). The
387 detection of rare species, however, is best accomplished by determining a suitable distance
388 between sampling points and preliminary investigations of the respective eDNA shedding
389 rates (cp. Wood et al., 2020). The population densities and the position of individuals within
390 lotic systems should not be inferred from eDNA signals without any *a priori* knowledge on
391 local hydrology. Comparisons between species are also not advisable without previous tests
392 under controlled conditions. Therefore, we advocate for the reporting of the sampling
393 position, local discharge, time, and species biology during field sampling. In the future, these
394 data can be incorporated in hydrological models specifically designed for eDNA-based
395 species monitoring.
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405
406
407 **Conflict of interest**

MT is the co-founder of Sinsoma GmbH, a for profit company dedicated to DNA analyses in environmental studies; DK is employed by Sinsoma. CM and RS are co-founders of the ARGE Limnologie GesmbH, a for profit consultancy specialized in aquatic ecology.

Author contributions

MT, JW, CM, and RS conceived the study; the experiment was designed by BT, MT, RS, and CM. Data were acquired and analyzed by BT, DK, and YP. BT wrote the first draft of the manuscript which was revised by DK, YP, JW, and MT.

Data Archiving Statement

All data on fish, discharge, eDNA signals, environmental conditions, sampling and comparison between celPCR and ddPCR have been uploaded to Figshare and are available at https://doi.org/10.6084/m9.figshare.12380642.v1

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597

598 **Table 1:** Primer pairs used for the molecular analysis of the eDNA samples.

Species	Primer name	5' - 3'	Target gene	Fragment length (bp)	Reference
Phoxinus phoxinus	Pho-Pho-S639 Pho-Pho-A648	CGTGCAGAAGCGGATATAAATAC CCAACCGAAGGTAAAGTCTTATTG	16s	128	Thalinger et al. 2016
***Salvelinus* sp.**	Sal-vel-S651 Sal-vel-A651	ATAGTCGGCACCGCCCTT TAACGAAGGCATGGGCTGTT	COI	112	Thalinger et al. 2016
Oncorhynchus mykiss	Onc-myk-S655 Onc-myk-A655	TCTCCCTTCATTTAGCTGGAATC GCTGGAGGTTTTATGTTAATAATGGTC	COI	82	Thalinger et al. 2016
Salmo trutta	Sal-tru-S1002 Sal-tru-A1002	TCTCTTGATTCGGGCAGAACTC CGAAGGCATGGGCTGTAACA	COI	89	

599

600 The respective target taxon, primer sequence, target gene, fragment length in base pairs and the source for previously published primers are
601 displayed. Please note that the *Salvelinus* sp. primer pair was designed to amplify both *S. fontinalis* and *Salvelinus umbla*.

602

603 **Table 2:** The set of Linear Mixed-Effects models used to investigate the relationship between eDNA signals (RFU) and distance from the eDNA
604 source.

#	model description
a	ln(exp(RFU)/discharge ~ 1
b	ln(exp(RFU)/discharge ~ 1 , random: ~1|month
c	ln(exp(RFU)/discharge ~ 1 , random: ~1|month/fish family
d	ln(exp(RFU)/discharge ~ ln(distance) , random = ~1|month/fish family
e	ln(exp(RFU)/discharge ~ ln(distance) , random = list(month = ln(distance), fish family = ~1)
f	ln(exp(RFU)/discharge ~ ln(distance) , random = list(month = ln(distance), fish family = ln(distance))

605

606 Model structure is displayed as coded for in R. a) is the intercept only model, b) contains random slope for month of trial, c) random slopes for trial
607 and fish families with each trial, d) introduces the fixed effect “ln-transformed discharge”, e) adds random slope per month, f) adds random slope
608 per month and fish family within each month.

609

610 **Table 3:** Comparison of Linear Mixed-Effects model performance during the stepwise building process.

#	AIC	BIC	log-likelihood	sign. change in log-likelihood
a	311.13	317.66	-153.57	
b	117.72	127.51	-55.86	<.0001
c	104.85	121.16	-47.4	<.0001
d	-44.15	-24.58	28.0	<.0001
e	-80.23	-54.13	48.12	<.0001
f	-78.21	-45.59	49.1	0.37

611

612 The Akaike information criterion (AIC), Bayesian information criterion (BIC), log-Likelihood and it's change are used for comparison between

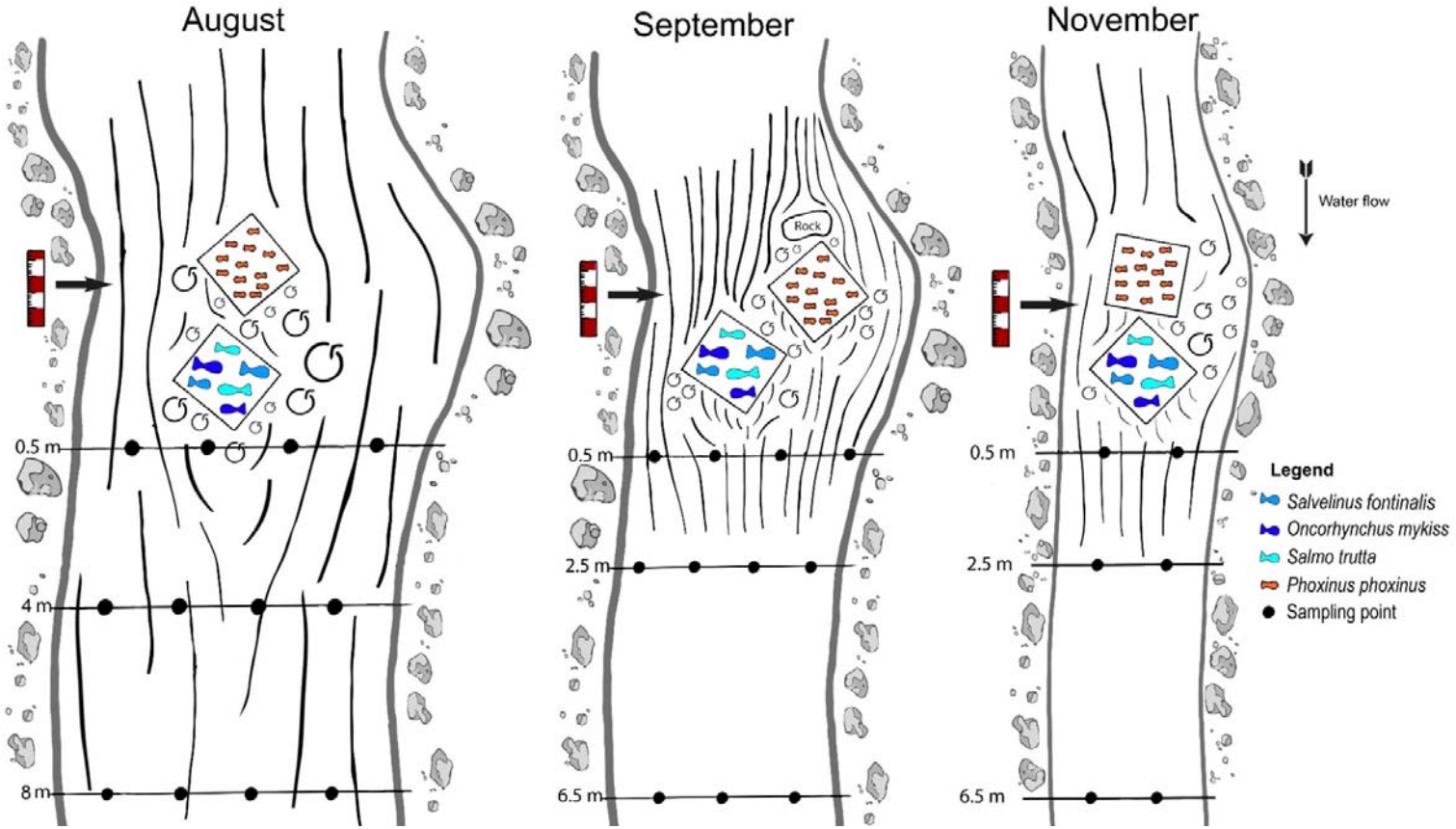
613 consecutive models (level of significance: $p < 0.05$).

Table 4: Linear Mixed-Effects model describing the relationship between eDNA signals and downstream distance.

Random effects	parameter estimate	lower 95% CI	upper 95% CI	**Fixed effects**	parameter estimate	lower 95% CI	upper 95% CI	se	t-value	p-value	**Estimated deviation**	random effect	intercept	ln(distance)
month												September	-1.21	0.13
sd(*intercept*)	1.22	0.44	3.42	*intercept*	-2.50	-4.22	-0.78	0.88	-2.86	< 0.01		November	1.21	-0.13
sd(ln(distance))	0.13	0.05	0.38	ln(distance)	-0.38	-0.57	-0.19	0.1	-3.92	< 0.001		Sept. / *P. phoxinus*	0.03	
cor	-1.00	-1.00	0.24	cor	-1							Sept. / salmonids	-0.04	
												Nov. / *P. phoxinus*	0.11	
fish family												Nov. / salmonids	-0.09	
sd(*intercept*)	0.09	0.04	0.22											

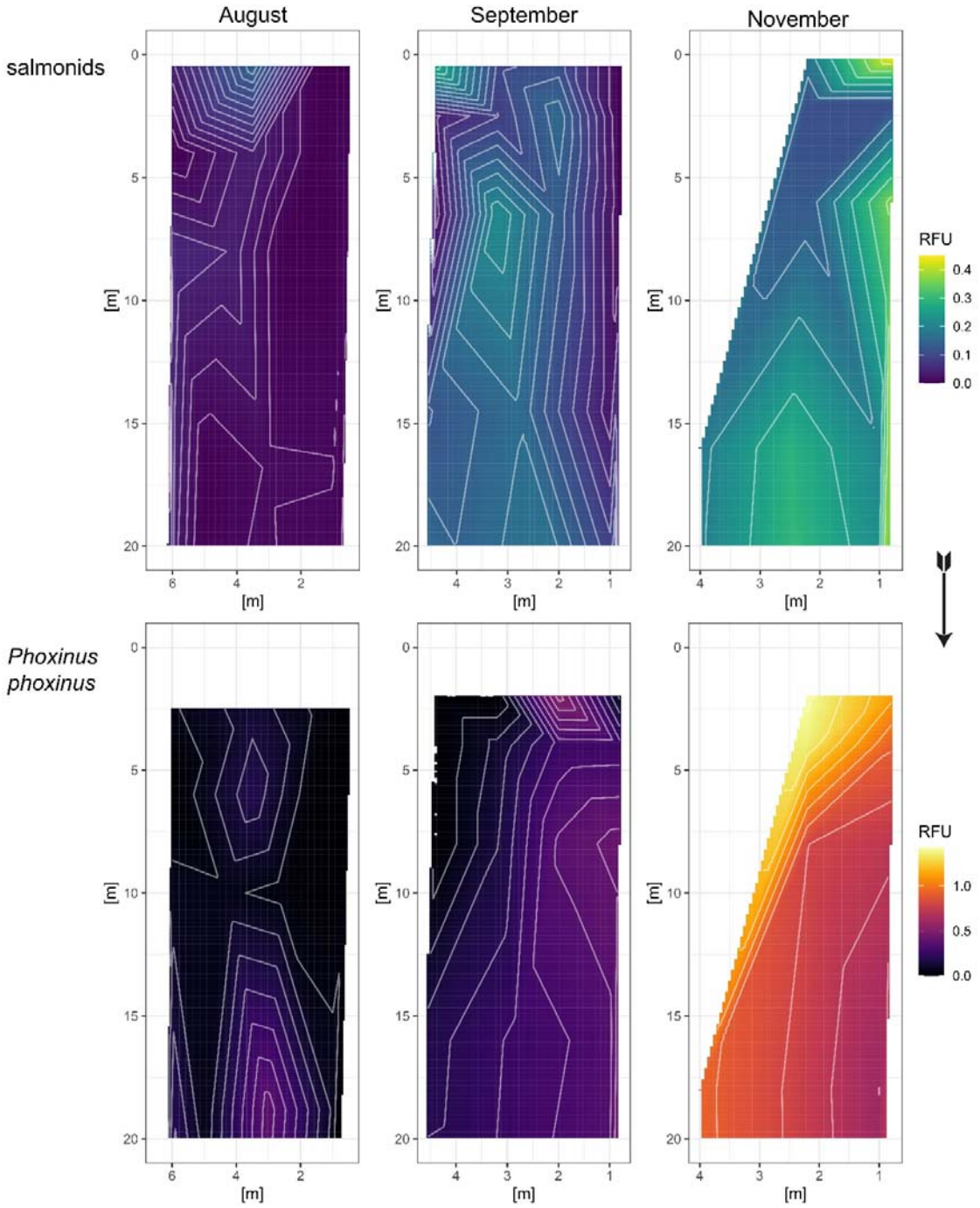
Parameter estimates and confidence limits are given for fixed effects, in addition to the correlation (cor) between intercept and ln(distance). For random effects, these values are provided for the standard deviations (sd). Additionally, the estimated deviations of intercept and ln(distance) from the fixed effects parameter estimate are provided per random effect.

620 **Figure 1:** Schematic view of the cage placement in the stream and the hydrological conditions prevailing during the August, September, and
621 November trial. Larger flowlines and eddies code for stronger currents. Due to reduced discharge, relative location and number of eDNA samples
622 was not kept uniform between trials. Note that additional sampling distances up to 1.3 km downstream of the cages are not displayed.



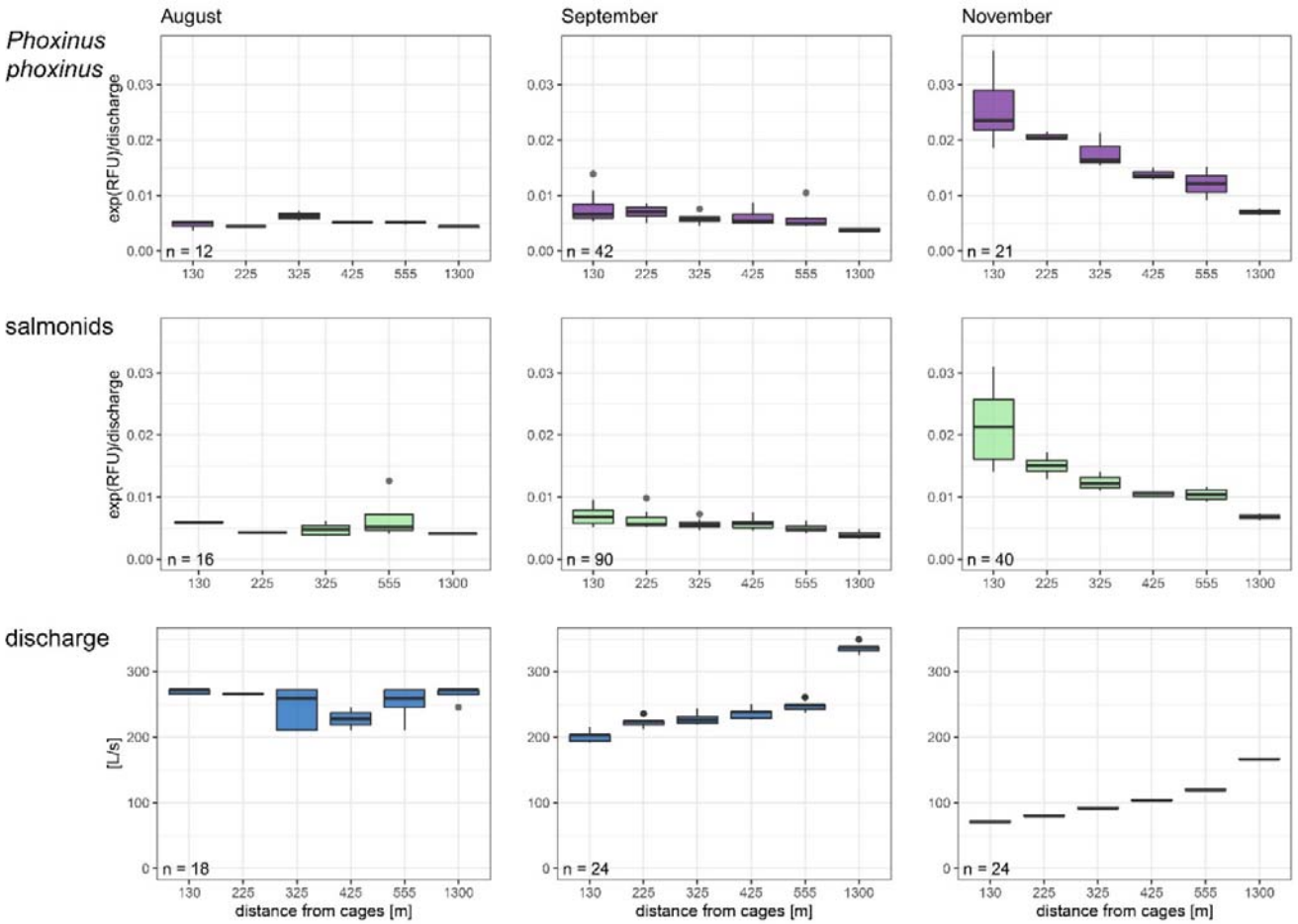
623

624 **Figure 2**: Heatmaps showing the small-scale eDNA distribution until 20 m downstream of the
625 cages during the three examined discharge situations. As eDNA levels differed between
626 *Phoxinus phoxinus* and the salmonids, different colour scales where used. However, coloring
627 per taxon remains constant for the three trials. Isotherms display interpolated differences of
628 0.05 RFU; the irregular shape in November reflects an increase in wetted width downstream
629 of the cages.

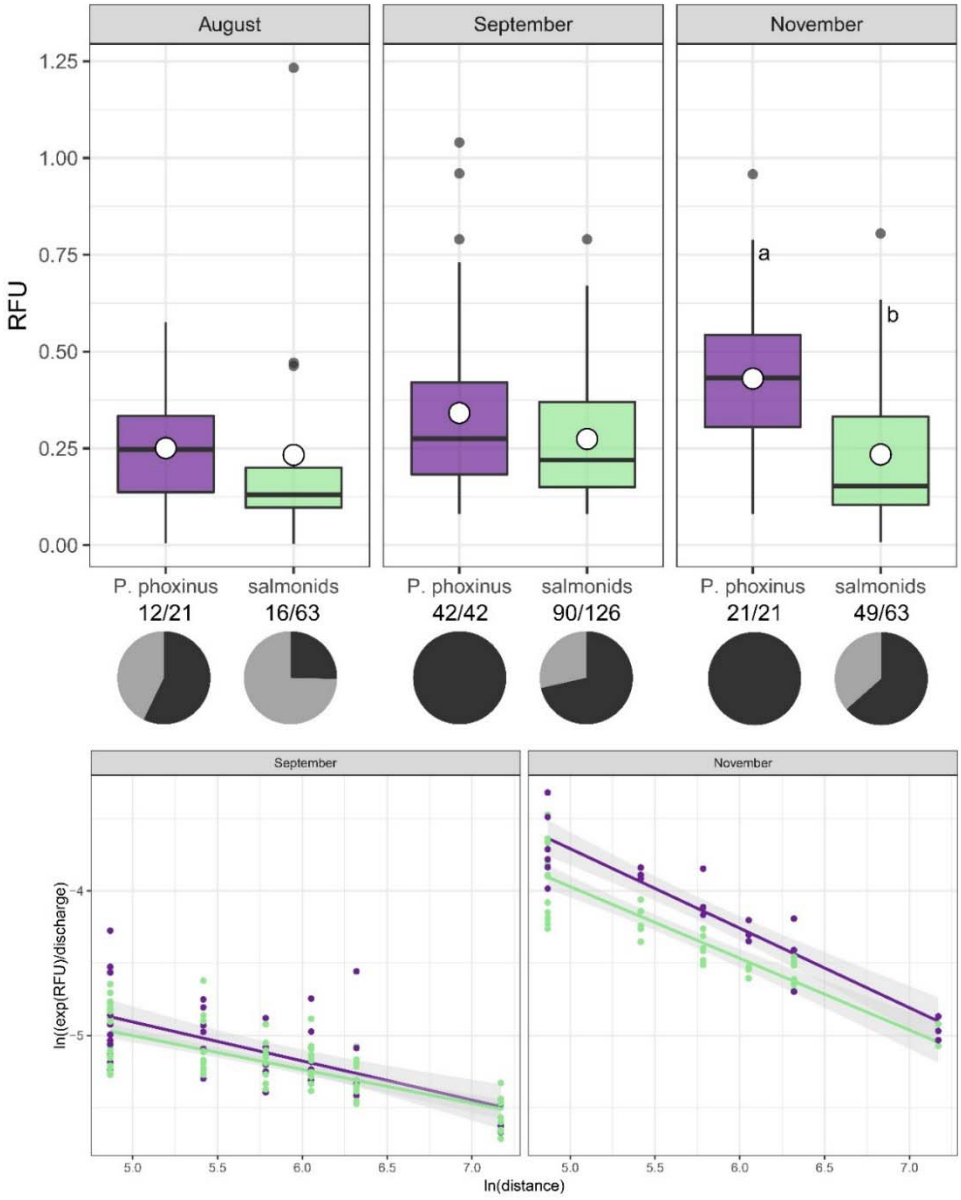


630

631 **Figure 3:** The discharge-corrected eDNA signal strengths of *P. phoxinus* and the salmonid species are displayed for downstream distances with
632 homogeneous lateral eDNA distribution for August, September, and November. Additionally, the discharge at each distance (three values each) is
633 displayed.



634 **Figure 4**: The pie charts display the proportion of PCR positives for *P. phoxinus* and the
635 salmonid species per trial; dark shading codes for successful detection; the number of positives
636 and total reactions are shown between pie charts and boxplots. The RFUs of positive samples
637 are displayed as boxplots; white circles code for mean values. RFUs of *P. phoxinus* are
638 significantly higher than salmonid RFUs ($p < 0.05$) for the November trial. The bottom panel
639 shows species-specific eDNA signals obtained during September and November from 130 m to
640 1.3 km downstream of the cages (distance and RFUs are transformed); regression lines are
641 displayed separately per species and month (cp. model "f").



642