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ORIGINAL ARTICLE

Experimental assessment of downstream environmental DNA patterns under variable fish biomass and river discharge rates

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Abstract

The development of environmental DNA (eDNA) methods toward implementation as a cost-effective, nonlethal tool for fish biomonitoring in lotic environments requires insights on the temporal and spatial distribution of eDNA in river systems. Yet, little is known on how downstream eDNA dispersal is affected by the combination of river discharge and source biomass effects. In this study, we aimed at unraveling the effect of source- and system-specific processes on the stream reach of eDNA. We used a longitudinal cage study in two river sections characterized by a significantly different discharge rate, where two invasive and two native fish species were introduced under two contrasting biomass treatments. Using droplet digital PCR (ddPCR) analyses, we found that eDNA concentrations become strongly reduced 2 km downstream from the source, an effect that is strengthened with increasing river discharge and coinciding dilution effects. Higher discharge rates resulted in equal or even higher detection probabilities at increasing distance from the source. The introduction of high fish stock biomass resulted in an increase of eDNA concentrations, as well as detection probabilities and in parallel reduced the stochasticity of the measurements. A peak in eDNA concentrations at a downstream distance ranging between 300 m and 2 km confirms the complexity of plume-shaped downstream eDNA patterns. Our results showed interspecific variation in eDNA emission and suggest species-specific differences in eDNA persistence. This study underlines the impact of both river discharge rate and source biomass on downstream eDNA detection and dispersal patterns.

KEYWORDS
droplet digital PCR, eDNA quantification, environmental DNA, fish population biomass, interspecific eDNA emission, lotic ecosystems

1 | INTRODUCTION

The use of environmental DNA (eDNA) (allochtonous DNA released by macroorganisms) which can be isolated from environmental samples such as water, sediment or even air (Bedwell & Goldberg, 2019), to detect and quantify the occurrence of organisms in the environment, developed spectacularly in the past decade (Bruce et al., 2021; Everts et al., 2022; Mauvisseau et al., 2021; Wood et al., 2021). Especially in aquatic environments, eDNA-based methods are applauded as a promising tool to monitor vertebrate species (Curtis

et al., 2020; Fremier et al., 2019; Wang et al., 2021) to a point where they can outcompete conventional methods in terms of higher detection sensitivity, lowered sampling effort and associated survey costs (Bruce et al., 2021; Cordier et al., 2021; Wood et al., 2021). Molecular biomonitoring via eDNA is particularly useful for the detection of rare or cryptic species without causing harm neither to their populations nor to their environment (Rojahn et al., 2021; Strickland & Roberts, 2019; Wood et al., 2021). Although considerable progress has been made on several technical and methodological issues associated with eDNA-based monitoring, such as primer design (Farrington et al., 2015; Wilcox et al., 2013) and selection (Everts et al., 2021), DNA extraction (Deiner et al., 2015; Spens et al., 2017), inhibition abatement (McKee et al., 2015), and selection of the PCR method (Brys et al., 2021; Xia et al., 2018), empirical insights on the behavior of eDNA and associated constraints in a wide range of aquatic environments are still lacking (Wood et al., 2021). More particularly, additional knowledge on patterns and factors affecting eDNA displacement in space and time is still needed (Thalinger, Kirschner, et al., 2021; Wood et al., 2021). Such insights are especially needed for (i) the understanding of the methods' efficacy and interpretation of obtained eDNA data (Fremier et al., 2019; Pont et al., 2018) and (ii) the large-scale implementation of this methodology as a more efficient, robust and replicable monitoring tool for management decision making (Bruce et al., 2021). Challenges remain regarding the spatial range at which species can be appropriately detected and quantified and how eDNA concentrations change in response to the distance at which the target organisms are shedding their eDNA depending on variable discharge rates (Coulter et al., 2019).

Spatial dispersion and heterogeneity of eDNA particles in "closed" lentic systems are already well-understood (see for instance Brys et al., 2021). In lotic waters, which can be defined as relatively "open" systems, the translation of eDNA patterns to upstream localized populations is far less understood and is subjected to much larger constraints (Bylemans et al., 2018; Fremier et al., 2019; Rourke et al., 2021; Thalinger, Kirschner, et al., 2021). Similar to other aquatic environments, the final eDNA concentration measured at a downstream sampling point is expected to be a function of species-specific eDNA emission rates and a source community's abundance or total biomass (Coulter et al., 2019; Horiuchi et al., 2019; Murakami et al., 2021) (Figure 1). During its downstream transport, eDNA can be exposed to the process of precipitation into sediment layers or biofilms (short-term removal process), diminishing its initial concentration in the water column further away from the source (Fremier et al., 2019). Simultaneously, eDNA undergoes a continuous and multiphasic process of decay (long-term removal process), which can be influenced by e.g. water temperature, turbidity, acidity, UV, and microbial activity (Harrison et al., 2019).

Finally, eDNA concentrations in the water column are expected to be significantly subjected to river-specific characteristics, such as the stream velocity and river discharge rate (Pont et al., 2018; Wood et al., 2021). Moreover, a higher river discharge rate can be hypothesized to increase the spatial range of eDNA detection of a species (Curtis et al., 2020). However, uncertainties remain on whether the latter is due to the resulting increased dispersion of eDNA molecules, or whether such larger river systems are housing much larger populations that are emitting more eDNA in the water column. In addition, a larger discharge rate can also be expected to increase the effect of dilution on eDNA concentration (Figure 1), during which dispersion and advection may even halt the spatial extent of the eDNA detection. Indeed, the hypothesized monodispersal model for eDNA transport (Pont et al., 2018; Wilcox et al., 2016) has taken a more complex form of this longitudinal eDNA pattern. For instance, due to the presence of a plume dynamic, with eDNA being initially concentrated and transported only in the midstream, but accumulating in the stream margins more downstream, the eDNA detection probability and measured concentration are inherently affected (Wood et al., 2021). As a result, eDNA dispersal patterns are

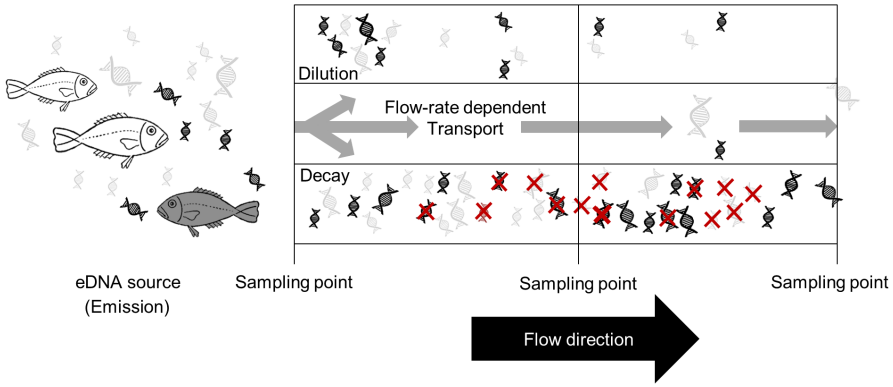

FIGURE 1 Schematic overview of the different parameters under study. In a lotic environment, the eDNA as emitted by an eDNA source (fish) is both degraded during the process called "eDNA decay" as well as diluted in the water column. The final eDNA concentration measured at a downstream sampling point is the result of these processes and their interaction with the flow rate-dependent transport defined as stream velocity and discharge. Understanding how source biomass, stream velocity, and discharge rate affect the downstream eDNA dispersal patterns will facilitate a more correct eDNA detection and quantification of fish present in a lotic water body

most likely shaped by a complex interplay between river-dependent characteristics and features of the eDNA source itself, which in turn determine the range at which a species can be detected and the concentration at which the eDNA can be measured at a certain distance downstream from the source. This is crucial information when attempting to generate reliable predictions of the fish species present within a certain transect of the stream or river (Bedwell & Goldberg, 2019; Thalinger, Kirschner, et al., 2021).

In this study, we investigated the effect of source biomass and discharge rate on downstream eDNA dispersal patterns and the eDNA detection and quantification rate (Figure 1). In order to do so, we conducted a longitudinal cage study in two different sections (upstream and downstream) of a species-poor stream. We introduced two invasive alien fish species, and two rare, valuable native fish species that initially did not occur in the study system, in two contrasting population sizes. This way, we aspired to gain knowledge on the spatial scale at which eDNA patterns can provide information on detection and quantification of upstream fish populations in small river systems, as well as on species-specific dispersal patterns of eDNA and with it, we aimed to increase our understanding of eDNA "ecology" in relation to lotic water transport properties.

2 | MATERIALS AND METHODS

2.1 | Study system and experimental setup

The study was carried out in a natural stream called the Barbierbeek, located between Sint-Niklaas and Kruibeke, Belgium (Figure 2a). Historical pollution has currently left the river very poor in number of fish species. The strong meandering nature of this stream makes it additionally usable and a representative example of an unaffected river system in terms of flow and discharge rates. Traditional fish monitoring and a preliminary eDNA screening with generalist vertebrate primers (Kelly et al., 2014), revealed that only five species were present in this system prior to the onset of the experiment (T0): *Leucaspius delineatus* (Heckel, 1843), *Pseudorasbora parva* (Temmink & Schlegel, 1846), *Gasterosteus aculeatus* (Linnaeus, 1758), *Pungitius pungitius* (Linnaeus, 1758), and *Carassius gibelio* (Bloch, 1782) (*unpublished results*). The cage study was conducted from April 15 to 25, 2018 in two different sections of this stream, further denoted as the upstream section and downstream section (Figure 2b, c). Before and during the onset of the experiment, no extreme rain or wind events occurred, and water conditions were relatively constant (Table 2). At each distance and sampling point, mean stream velocity (measured

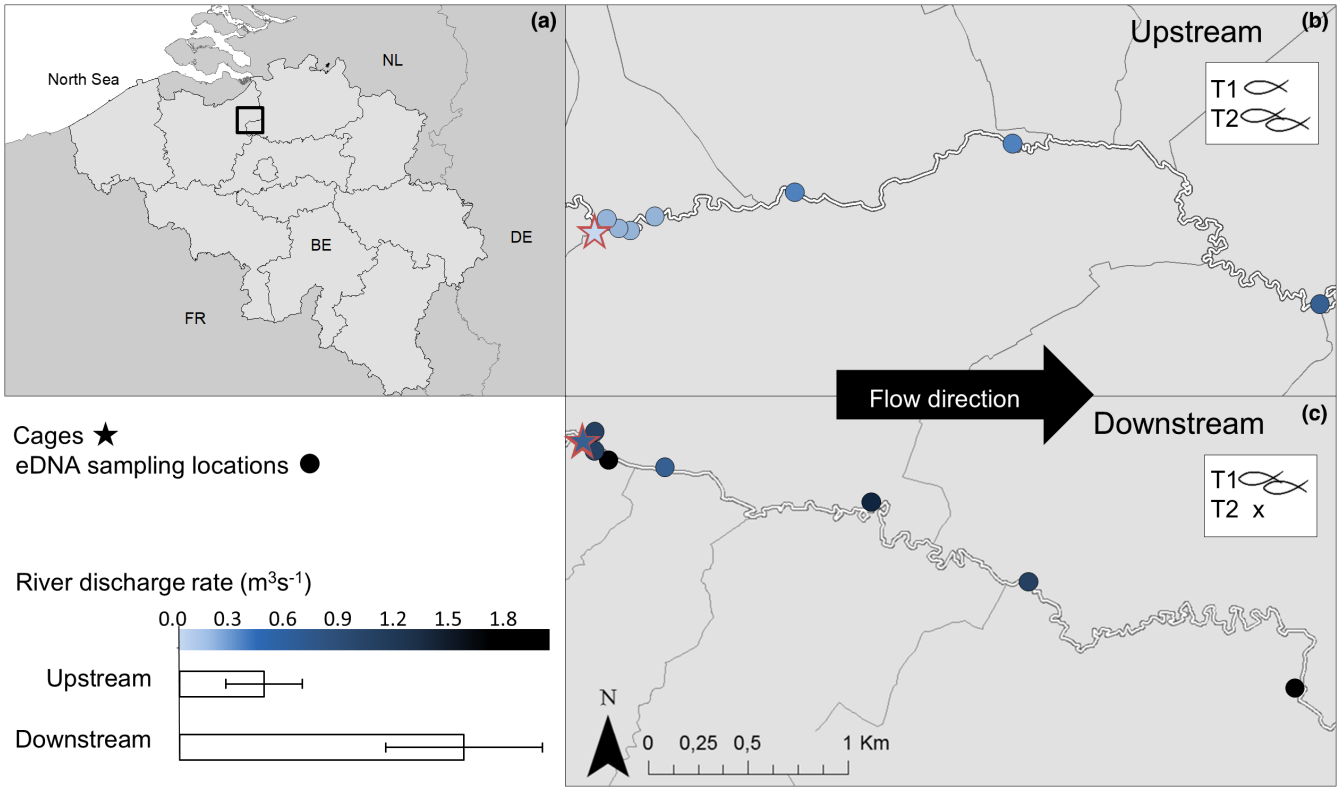

FIGURE 2 Study site depicted as two parts of the Barbierbeek, situated between Sint-Niklaas and Kruibeke, Belgium (a). Indicated are the positions of the cage communities (stars) as well as the different sampling locations (circles) in the upstream (b) and downstream (c) part of this river. Per sampling location, the average river discharge rate (m^3s^{-1}) is shown (color hue), while mean river discharge is given by the bar graph on the left. The cross-section surface area encompassed on average 1.26 $m^2 \pm 0.38$ upstream, versus 2.34 $m^2 \pm 0.59$ in the downstream section. The black arrow depicts the flow direction. At T1, a small fish community (low biomass) was introduced in the upstream section, while simultaneously a large community (high biomass per species) was introduced downstream. This high biomass community was moved and added to the upstream cages at T2

TABLE 1 Information related to the introduced fish populations in number of individuals and the corresponding total biomass (g) for the downstream section as well as the two quantities in the upstream section. Note that one individual of *P. semilunaris* died between transportation of the downstream to the upstream section. Per treatment, the mean length per individual (cm) ± the standard error is given

	Downstream			Upstream (1)			Upstream (2)		
Latin name	Nr	Biomass (g)	Length (cm)	Nr	Biomass (g)	Length (cm)	Nr	Biomass (g)	Length (cm)
Lota lota	9	122.91	18 ± 1.60	2	27.31	18 ± 1	11	150.22	18 ± 1.51
Rutilus rutilus	30	848.01	14.70 ± 1.74	5	111.50	13.7 ± 1.29	35	989.34	14.70 ± 1.64
Proterorhinus semilunaris	50	305.99	2.7 ± 0.60	3	21.33	5 ± 0.83	52	318.23	2.7 ± 0.59
Neogobius melanostomus	50	480.04	7.93 ± 1.71	2	18.42	9 ± 0.1	52	499.24	7.93 ± 1.69

as the mean travel duration of five floaters over a 25 m transect), mean depth (measured perpendicular to the stream flow direction and taken at both shores as well as at the deepest point of the stream section) and width of the intersection were measured, to calculate the river discharge rate at that point. The latter was then calculated by multiplying the cross-sectional area (width × mean depth) by the mean stream velocity (Figure 2). The mean river discharge rate of the upstream section was $0.42 \pm 0.19 m^3 s^{-1}$ and significantly lower ($F_1 = 27.59$, $p < 0.001$) than the mean discharge of the downstream section, $1.41 \pm 0.39 m^3 s^{-1}$ (Figure 2).

In the first phase of the experiment (T1), we placed a cage community in each of the two stream sections (Figure 2b, c), 5 km apart from each other, containing four species: the native *Lota lota* (Linnaeus, 1758) and *Rutilus rutilus* (Linnaeus, 1758), and the invasive non-indigenous Ponto-Caspian gobies, *Proterorhinus semilunaris* (Heckel, 1837) and *Neogobius melanostomus* (Pallas, 1814). *R. rutilus* individuals were obtained from the professional fish farm Gebroeders Vandeput & Zonen; *L. lota* individuals originated from the INBO breeding facilities at Linkebeek, and *P. semilunaris* and *N. melanostomus* were caught in the Belgian part of the Meuse, as this river harbors large populations of both invasive fish species. All fish were handled in a directed manner to reduce discomfort and stress during the transportation. The length of the fish was measured, and total biomass of the cage communities per species was determined prior to inclusion in the nets (Table 1). Initially, a cage community consisting of each of these four species in low biomass was introduced upstream, whereas downstream these species were introduced at the same time in much higher biomasses (see Table 1). The fish species were divided over multiple tube-shaped life nets, of 1 m in diameter and 5 m long (further denoted as "cage"), that were placed next to each other at a fixed point in the stream. A total of 5 days of acclimatization time was given to these caged "artificial" populations, after which water samples were taken at 8 different distances downstream from these cages (0, 25, 50, 100, 300, 1000, 2000, and 4000 m) (see Figure 2). At each distance, a 10 L water sample was taken cross-sectional to the stream, using a Vampire sampler pump (Buerkle, Bad Bellingen, Germany) with disposable silicone tubing that was moved through the entire water column during sampling. This sampling procedure was repeated three times at each distance, to obtain three independent field replicates per distance and river section.

TABLE 2 Environmental parameters characterizing the chemistry of the water in both the upstream and downstream section of the study system. Values represent the mean ± standard error of repeated observations throughout the experiment

	Upstream	Downstream
pH	7.97 ± 0.0	8.26 ± 0.3
Water temperature(°C)	16.1 ± 0.85	20.7 ± 0.50
Conductivity ($mS\,cm^{-1}$)	844.5 ± 16.26	822.67 ± 0.58
O2 ($mg\,L^{-1}$)	51.7 ± 17.68	86.67 ± 3.12
Turbidity (FNU)	16.9 ± 1.89	14.33 ± 1.53

To avoid potential cross-contamination during sampling, we started sampling from the largest distance and thus lowest potential eDNA concentration of the target fish species in the upstream section. We first started in the upstream river section, containing the low biomass fish stock in the life nets, and repeated this strategy in a next step also in the downstream section in which the high biomass fish stock was introduced. Sterile nitrile gloves were worn during sampling and all reusable material was decontaminated with 2% Virkon S (Antec – DuPunt) between each water sampling at the different distances as a biosafety precaution and to further avoid potential DNA cross-contamination (U.S. Fish and Wildlife Service). When the water sampling was finished, the high biomass fish populations from the downstream cages were moved and added to the cage community in the upstream stream section (T2) (Figure 2), upon which again 5 days of acclimatization were allowed. Then, the same sampling procedure was repeated for that upstream part, after which all the caged fish and life nets were removed from the stream. It was not possible to simultaneously move the low biomass fish populations toward the downstream section because of the potential risk of eDNA seepage from high biomass populations into the downstream river section. As a consequence, effects of variable biomass can only be tested in the upstream section, whereas potential effects of differences in discharge between the upstream and downstream section can only be tested for the high biomass treatment. None of the individuals of the cage community died nor escaped from the cages during the entire experiment, by exception of one individual of *P. semilunaris* (Table 1).

2.2 | Water filtering, eDNA extraction, and ddPCR

From each 10 L water sample, 300 ml was filtered immediately after sampling, through an enclosed 0.45-µm pore size PVDF Sterivex-HF filter capsule (SVHVL10RC, Merck milipore), after homogenizing the entire water sample. For each of the three independent water samples (i.e., field replicates), we used a new sterile 60-ml Luer-Lock syringe and gloves. At the end of each filtration, the remaining water inside the capsule was expelled by pushing air through the filter capsule until it dried completely, and filters were capped at both sides. All filters were then stored at −20°C in a BlueLine box (delta T, Fernwald, Germany) for transportation to the laboratory until DNA extraction for optimal fixation. Prior to and at the end of each sampling round (i.e., stream transect and biomass treatment), a field negative control was included using 2 L of deionized water to test for potential cross-contamination during sampling, resulting in 6 filters in total during the entire experiment.

DNA extraction from filters was conducted using the $SX_{CAPSULE}$ method (suitable for filters without preservation buffer as in Spens et al., 2017) with the DNeasy Blood & Tissue Kit (Qiagen), following the manufacture guidelines (for more details see also Brys et al. (2021)). Contamination of eDNA samples was avoided by the use of a dedicated PCR-free room for low copy number template extractions, with controlled DNA-free, highly efficient particulate air-filtered compartments with positive air pressure. Potential contamination at the extraction stage was further detected by the inclusion of three extraction blanks, that is one per extraction batch. All reusable laboratory materials were, after use, decontaminated by use of UV lights (10–15 min). The DNeasy PowerClean Cleanup Kit (Qiagen) was used to additionally purify the DNA extracts (manufacturer guidelines), upon which they were eluted in 100 µl of TE. All eDNA samples were analyzed in duplicate (i.e., laboratory replicates).

Species-specific primer/probe assays compatible for ddPCR as developed by Brys et al. (2021) and Mauvisseau et al. (2021) were used to quantify species-specific eDNA concentrations of three of the study species, namely *L. lota*, *R. rutilus*, and *N. melanostomus*. For the detection and quantification of *P. semilunaris*, an additional primer/probe assay was developed and validated on its specificity and sensitivity via silico and in vitro tests (Brys et al., *unpublished results*). The latter assay targets a 90-bp fragment situated on the mitochondrial cytochrome b (cytb) locus (Table 3). Droplet digital PCR (ddPCR) was performed using a QX200 ddPCR system (Bio-Rad, Temse, Belgium) in 20 µl. Each reaction contained 10 µl of Bio-Rad ddPCR supermix for probes (no deoxyuridine triphosphate), 750 nM of each primer, 375 nM of each probe (IDT, Leuven, Belgium) and 4 µl of template DNA and was adjusted to the final volume of 20 µl by adding diethylpyrocarbonate (DEPC) water (Sigma-Aldrich, Overijse, Belgium). After droplet generation, ddPCR was continued with 40 cycles of denaturation. Optimal primer annealing temperature per species can be found in Table 3. Each ddPCR plate included 6 negative ddPCR blanks and three positive reference samples of the targes species we are testing for. Following amplification, visualization of target-positive and target-negative droplets was performed by a QX200 droplet reader (Bio-Rad).

2.3 | Data processing

Following ddPCR, calculation of eDNA copy numbers (per µL reaction volume) was performed by QuantaSoft software (1.7.4

TABLE 3 Species-specific primer/probe assays for the four target species with, target gene, product size, melting temperature, and primer/probe length. For further details on primer/probe design, see Mauvisseau et al. (2021)

Species	Gene	Size	Temp	Length (bp)
Lota lota	COI – CytC	175 bp	56°C	
Forward	5′- CTGGGGCTTCTGTTGACCTT-3′			20
Reverse	5′-AGGAGTAGGACGGCTGTA-3′			19
Probe	5′- GGGTCTCATCAATTCTTGGAGC-3′			19
Rutilus rutilus	CytB	94 bp	56°C	
Forward	5′-AACCGCGTTTTCATCGGTGAC-3′			21
Reverse	5′-GAAGAAGAAGGATGCTCCATTAG-3′			23
Probe	5′-TCAACTACGGCTGACTTATCC-3′			21
Proterorhinus semilunaris	CytB	90 bp	54°C	
Forward	5′-CGTTATCTTGGCCGTCACC-3′			19
Reverse	5′-TTATCTGCATTAGAGTTAAGGCC-3′			23
Probe	5′-CCTACTCTTTCTACACCAAACAGGAT-3′			26
Neogobius melanostomus	COI	105 bp	54°C	
Forward	5′- ACTTCTGGCCTCCTCTGGT-3′			19
Reverse	5′-GTCAAGTCGACGGATGCTC-3′			19
Probe	5′- AGGAACCGGGTGGACAGTTTAC-3′			22

Bio-Rad) and was estimated using the ratio between positive and negative droplets within a sample, using Poisson statistics (Miotke et al., 2014). Positive droplets beyond the fluorescence threshold (amplitude > 2000) (QuantaSoft instructions) were counted as positive events and resulted in number of copies, which were corrected for the water volume filtered per sample. Corrected values were used in further analyses.

All statistical analyses were carried out using at least an alpha-value of 0.05 and with package *stats* in RStudio version 4.2.0 (RStudio Team, 2022). To assess which factors determined eDNA concentrations at different locations downstream of the cage, we first compared the absolute eDNA concentrations from each of the four targeted fish species over distance from the source. In a first step, multicollinearity of the variables of interest was tested via the variance inflation factor (VIF) to retain only independent explanatory variables in the models. Since stream velocity was highly collinear with discharge rate, only discharge rate was kept as the most biologically relevant variable in the model. A linear mixed-effects model (*lme*) was applied (additionally using the R package *nlme*), to test the impact of (a) river discharge rate, (b) fish species identity, (c) distance from the source, and (d) source biomass as well as each of the interaction terms as explanatory variables on the downstream eDNA concentrations (log[x + 1] transformed) (see Table A1). In these analyses, distance from the cages was included as a nested random factor per stream section, and a serial correlation over distance term was additionally included to correct for the dependence of a downstream concentration by the concentration measured upstream from that point.

To assess the robustness of eDNA quantification across field and laboratory replicates, we calculated the coefficient of variation (CV) for each of the four targeted species, by dividing the mean of the obtained eDNA concentrations at each location and each biomass treatment by their standard deviation. We then performed a two-way ANCOVA, to investigate the effect of (a) species and (b) eDNA concentration (log[x + 1] transformed) on the CV, followed by a Bonferroni post hoc test with the Benjamin-Hochberg FDR method for multiple hypotheses testing.

To compare the detection probability of each species at a given location downstream from the source, the eDNA concentrations were transformed into binary values. A generalized linear mixed-effects model was then fitted, using the additional R package *lme4*, including the *glmer* function with binomial (link = "logit") as parameter family.

To obtain an estimate of species-specific eDNA emission rate per body mass immediately behind the cage (0 m), the absolute eDNA concentrations of the four fish species present at high biomass cages were normalized by dividing the measured eDNA copies per µL by the total fish biomass of each species (g) (Table 1). This standardization allows to compare species-specific eDNA emission rates among the four target fish species in a natural stream measured under two different discharge rates (i.e., upstream versus downstream section). A two-way ANOVA was used to test the effect of (a) species and (b) stream section (with coinciding differences in river discharge rate) on the standardized eDNA concentrations (copies per µL, per L filtered water) measured at the cage locations (0 m).

3 | RESULTS

3.1 | Data quality

All extraction negative blanks and PCR negative controls showed no positive amplification for each of the target species, indicating the absence of contamination during field sampling, extraction, and/or amplification.

3.2 | Downstream patterns of fish eDNA

The linear mixed-effects model including a term for autocorrelation (L. Ratio) best explained the downstream patterns of fish eDNA (Table A1). Overall, the mean eDNA concentrations (copies per µL DNA extract, per L filtered water) were highest directly at the cage (0 m), and significantly decreased over distance ($F_{1\text{–}10}$ = 150.95, $p < 0.001$, Figure 3, Table 4). This downstream decline is interrupted by a flare-up of higher eDNA concentration resulting from the high biomass source roughly 300 m downstream in both the upstream and downstream sections of the stream. A similar second peak in eDNA concentration also occurred in the low biomass situation, but more downstream, at distance of 1000–2000 m from the source. Overall fish eDNA concentrations were significantly and positively affected by biomass ($F_{1\text{–}360}$ = 300.21, $p < 0.001$, Table 4), whereas discharge rate also had a significant yet negative impact on the eDNA concentration measured ($F_{1\text{–}10}$ = 54.11, $p < 0.001$, Table 4). In addition, significant interaction effects were found between sampling distance and both total biomass ($F_{1\text{–}360}$ = 40.83, $p < 0.001$, Table 4) and species ($F_{3\text{–}360}$ = 3.48, p = 0.016, Table 4), whereas species, in turn, also significantly interacted with biomass ($F_{1\text{–}360}$ = 65.27, $p < 0.001$, Table 4).

Each of the four fish species showed similar spatial patterns of their eDNA concentrations measured downstream from the source, although their eDNA concentrations significantly differed among each other ($F > 22.48$, $p < 0.001$, Figure 3, Table 4). Regardless of distance from the source, the eDNA concentration of *L. lota* was significantly lower than that of the other three species (Figure B1), with *N. melanostomus* consistently showing the highest eDNA concentrations in both the upstream and downstream transects and for both biomasses.

3.3 | Robustness of eDNA measurements

The variability among replicates expressed as the CV of the mean eDNA concentrations significantly declined with increasing eDNA concentration (log [x + 1]) (F_1 = 9.6210, p = 0.002) (Figure 4a). In addition, the CV significantly differed among the four species (F_3 = 2.8165, p = 0.044), while no interaction occurred between

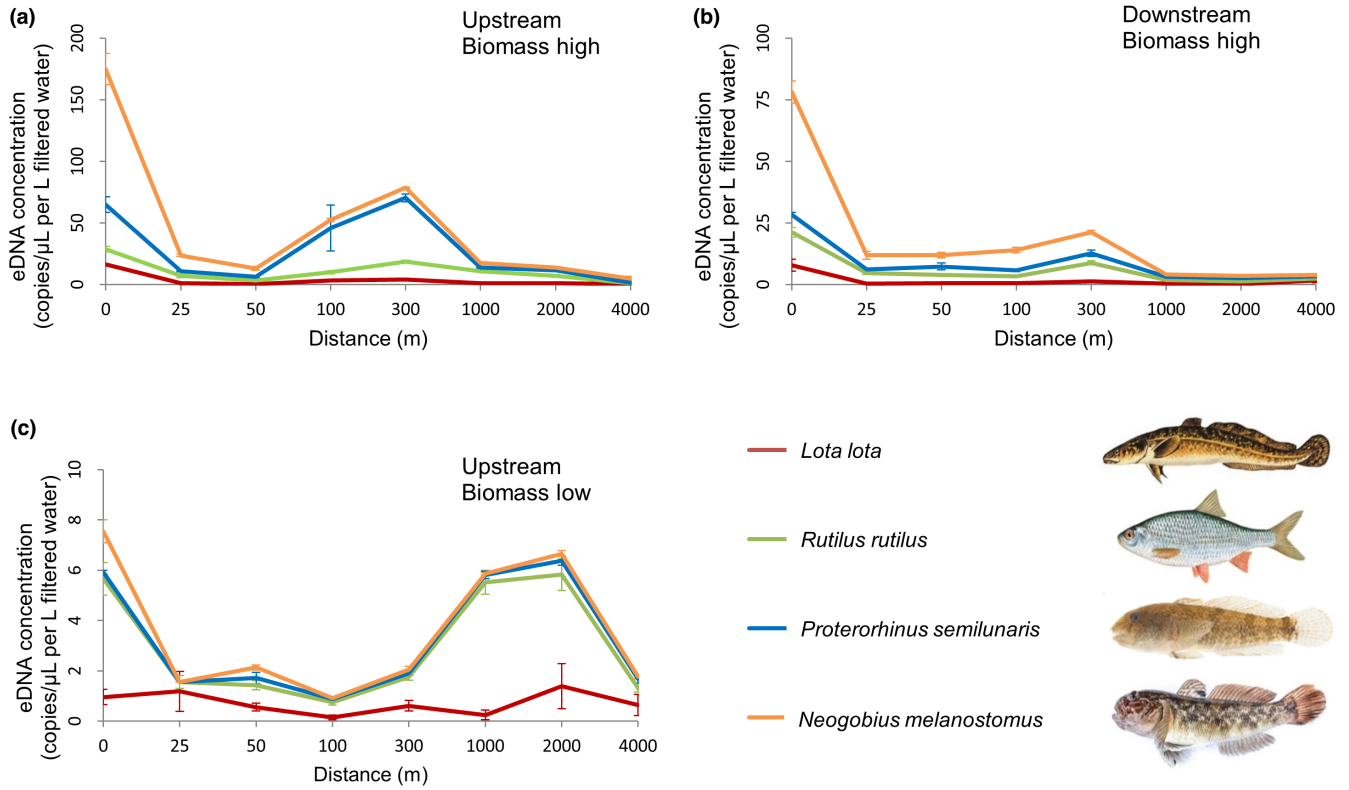

FIGURE 3 Mean eDNA concentration in absolute number of copies per µL DNA extract, standardized per L water filtered, at increasing distance downstream from the cages in two different stream sections, with a high (b) and low (c) initial biomass in the upstream section

TABLE 4 Results of the linear mixed-effects models displaying the effects of species, distance from the cages, discharge and biomass (total biomass (g) of the fish present in the cages as calculated per species and treatment) on the eDNA concentration subdivided into (i) discharge effects (i.e., using the data of both stream sections, but only of the high biomass communities) and (ii) biomass effects (i.e., using two biomass treatments in one stream section).

	numDF	denDf	F-values	*p*-value
Discharge effects				
Species	3	357	39.47	**<0.0001**
Distance	1	10	150.95	**<0.0001**
Discharge	1	10	54.11	**<0.0001**
Species:Distance	3	357	6.98	**0.0001**
Discharge:Stream	1	10	0.70	0.4225
Distance:Discharge	1	10	0.03	0.8728
Biomass effects				
Species	3	360	22.48	**<0.0001**
Distance	1	6	3.18	0.1248
Biomass	1	360	300.21	**<0.0001**
Species:Distance	3	360	3.48	**0.0162**
Distance:Biomass	1	360	40.83	**<0.0001**
Species:Biomass	3	360	65.27	**<0.0001**

Significant *p*-values are marked in bold.

eDNA concentration and species ($F_3 = 1.7823$, $p = 0.157$). Moreover, significant pairwise differences occurred between the adjusted CV means of *L. lota* and *R. rutilus* ($F_{89} = 2.49$, $p = 0.015$) and between *R. rutilus* and *N. melanostomus* ($F_{89} = -2.72$, $p = 0.008$) (Figure 4b).

The detection probability significantly differed among species ($\chi^2 = 18.03$, $p < 0.001$) and was significantly affected by the discharge rate ($\chi^2 = 5.50$, $p = 0.019$), as well as by the source's biomass ($\chi^2 = 15.39$, $p < 0.001$) (Figure 5, Table 5). More specifically, *R. rutilus* showed a consistent higher detection probability irrespective of the sampling distance whereas the other species displayed a larger variation in detection probability, especially beyond the 300 m sampling point (Figure 5b). The detection probability was significantly and negatively affected by the interaction of both biomass and distance from the eDNA source population ($\chi^2 = 11.77$, $p < 0.001$). At low biomass, the target fish species are less likely detected (<25%–50%) 25 m downstream of the cage (Figure 5a, b).

3.4 | Interspecific differences in eDNA emission rate

The eDNA concentrations per gram body mass directly measured behind the cages (0 m) were significantly different among the four species ($F_3 = 79.88$, $p < 0.001$) and were additionally significantly

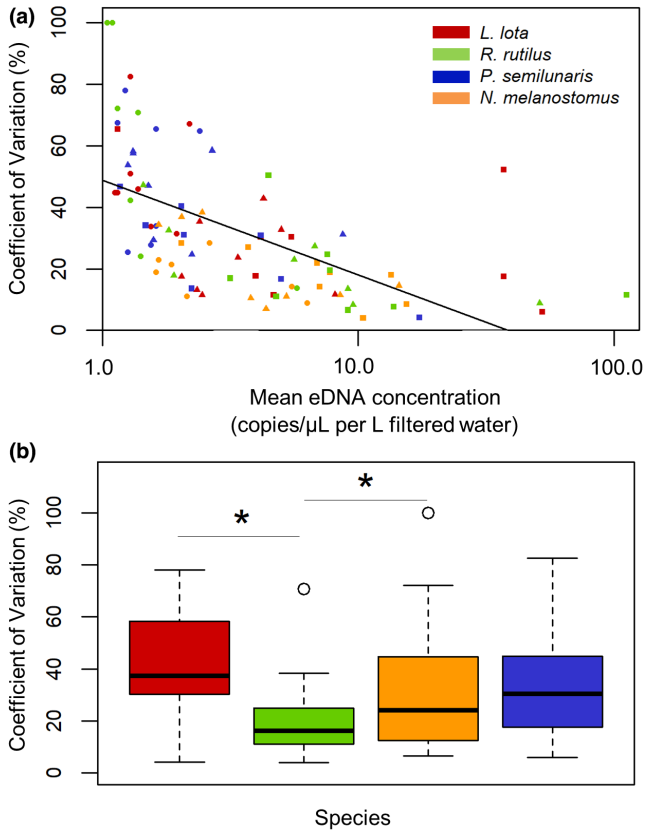

FIGURE 4 (a) Relationship between the coefficient of variation and mean eDNA concentrations per species. The x-axis represents a logarithmic scale. Symbols display the combination of stream section and level of abundance (• upstream, low biomass - ■ upstream, high biomass - ▲downstream, high biomass). Significant differences between species are indicated by an asterisk (*) (b)

lower in the downstream section compared with the upstream section ($F_1 = 28.28$, $p < 0.001$) (Figure 6). Specifically, eDNA concentrations were significantly lower for *R. rutilus* ($p < 0.001$) compared with the other species, and this difference was greater in the upstream section compared with the downstream section ($p = 0.009$).

4 | DISCUSSION

Despite the rapid developments in the quantitative monitoring of diverse fish communities via eDNA-based methods, uncertain fate determination of eDNA in lotic environments is constraining the use of these methods for directed conservation actions (Wang et al., 2021). The monitoring and assessing of target species and biodiversity in lotic systems via eDNA-based methods, requires a change in perspective: namely the need to distinguish between detecting the presence of the organism itself at a certain location (as done via conventional fish survey methods, for example, traditional electrofishing and netting) from detecting the species by proxy of eDNA in the water current (Fremier et al., 2019). This distinction is hindered by the lack of insights on how stream reach and hydrogeomorphic characteristics affect eDNA dispersal in lotic systems (Bylemans et al., 2018; Fremier et al., 2019; Thalinger, Kirschner, et al., 2021). In this context, one of the most relevant proxies for eDNA stream reach is river discharge rate (Thalinger et al., 2019; Thalinger, Kirschner, et al., 2021; Wilcox et al., 2016).

The handful of studies that used artificial populations and cage experiments in combination with quantitative species-specific eDNA methods to determine stream reach of fish eDNA in river systems (Figure 7) suggest that the maximum distance at which target eDNA can be successfully detected tends to increase with rising river discharge rate. The observed maximum distance of successful detection and measured discharge rates in our study system fit well in this general pattern. Moreover, in the two river sections under study, the detection probabilities at the maximum distance (4 km) of identical introduced fish populations of the four target species were highest in the downstream section characterized by a significantly higher discharge rate than the upstream section.

However, when taking overall eDNA measurements into consideration, it appeared that the concentrations of each of these fish species under study were significantly lower in the downstream section. This observation can be attributed to dilution effects resulting from higher discharge rates and associated water run-off downstream, especially in the strongly meandering river system we

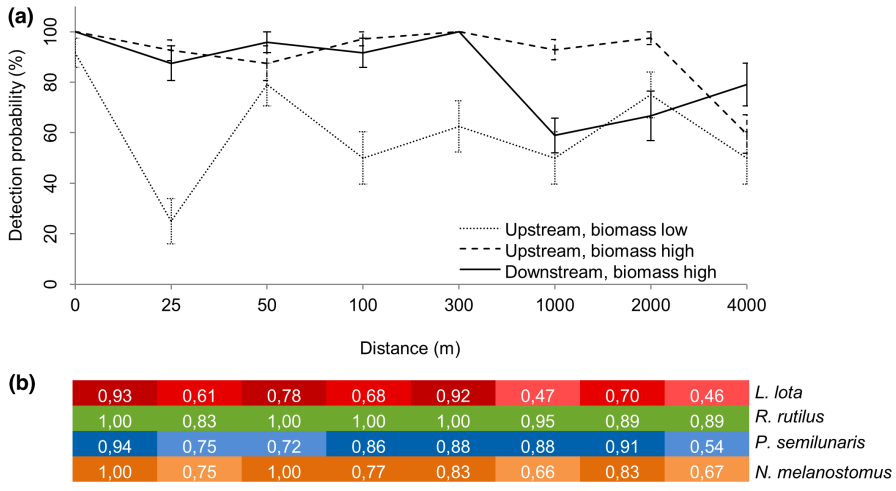

FIGURE 5 (a) Detection probability (binary values for presence/absence in field replicates) of eDNA over sampling distance from the cages for the three treatments (error bars represent SE) irrespectively of species. (b) This same eDNA detection probability over distance is now shown per species. Colors red, green, blue, and orange respectively represent the species *L. lota*, *R. rutilus*, *P. semilunaris*, and *N. melanostomus*

TABLE 5 Results of the generalized mixed-effects model displaying the effects of species, distance, discharge, and biomass (total biomass (g) of the fish present in the cages as calculated per species and treatment) on the eDNA detection probability subdivided into (i) discharge effects (i.e., using the data of both stream sections, but only of the high biomass communities) and (ii) biomass effects (i.e., using two biomass treatments in one stream section).

	DF	Chisq	Pr(>Chisq)
Discharge effects			
Species	3	18.0329	**0.000433**
Distance	1	0.0980	0.754199
Discharge	1	5.4974	**0.019044**
Species:Distance	3	5.9725	0.112955
Discharge:Stream	1	0.1869	0.665472
Distance:Discharge	1	0.0005	0.981503
Biomass effects			
Species	3	0.3009	0.9598543
Distance	1	0.1297	0.7187745
Biomass	1	15.3857	**8.765e-05**
Species:Distance	3	4.9494	0.1755430
Distance:Biomass	1	11.7731	**0.0006009**
Species:Biomass	3	1.2325	0.7452101

Significant *p*-values are marked in bold.

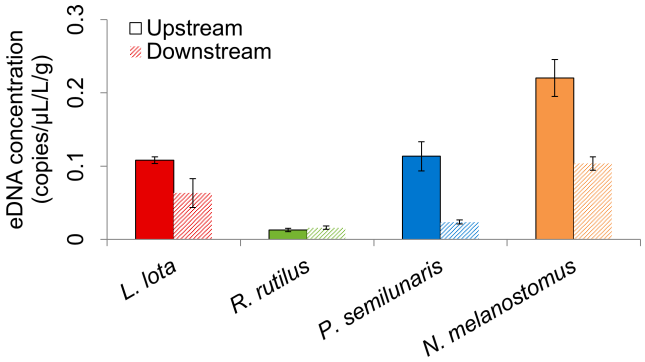

FIGURE 6 Mean eDNA concentration in number of copies per µL DNA extract, standardized per L water filtered, corrected for the sources' biomass. Copies per gram biomass-caged fish are displayed only for the 0 m sampling distance, thus displaying interspecific differences in eDNA emission in a natural river

studied (Mächler et al., 2019). At first sight, the lower eDNA concentrations per liter water under higher discharge rates (Figure 3a, b), in combination with relatively similar or even higher detection probabilities (Figure 5) seem to be contradictory. Nevertheless, this observation can likely be attributed to the fact that under higher discharge rates eDNA may disperse much faster, thereby being less disposed to eDNA decay over time. This observation is in agreement with what is yet documented in literature and summarized in Figure 7, showing that with high discharge rates, eDNA is detected over larger distances regardless of its concentration. Similar observations are also made using community-based eDNA methods like metabarcoding, as reported by Laporte et al. (2021), who found, for instance, that in a river system with a discharge rate of 16,800 m^3s^{-1} (in comparison to $1.41 \pm 0.39 m^3s^{-1}$ in our downstream section), brown trout (*Salmo trutta*) was still detected up to 5 km downstream of the shedding source, although its relative abundance decreased to marginal levels (<1.0%) already after 100 m. This suggests that even in medium to large-scale lotic systems (Cantera et al., 2022; Laporte et al., 2022) local assessment of fish communities might be possible, while the maintained detection at large distances allows biodiversity assessment at basin scale.

Our data additionally showed that the introduction of a higher biomass fish stock in the cages resulted in an overall and significant increase in eDNA concentrations measured across the different distances downstream from these sources. This observation corroborates the growing body of empirical evidence that eDNA concentrations can indeed provide useful proxies for abundance and/or biomass of fish populations in aquatic environments (Capo et al., 2021), even in lotic environments (Maruyama et al., 2018 and reviewed in Rourke et al., 2021). In natural systems, however, such patterns are often exposed to substantial variation resulting from interaction effects with several abiotic factors, such as discharge rate or water volume of the system under study (Rourke et al., 2021; Tillotson et al., 2018). The inclusion of those parameters in prediction models is therefore expected to generate more reliable quantitative eDNA data that better predict the spatial and temporal behavior of aquatic species within and among river systems in the future (Everts et al., 2022; Wilcox et al., 2016; Yates et al., 2021). Large river systems can, on the other hand, be expected to house bigger fish populations too, thereby, at least partly, overcoming dilution effects resulting from increased discharge rates, thereby affecting the quantitative eDNA-based population estimates and interpretation. Indeed, Laporte et al. (2021) found that 41 out of 49 studied fish species in the St. Charles River (Canada) remained to be detected in the most downstream sampling sites, namely the most abundant species.

Wood et al. (2021) recently reported that the emitted eDNA of a local fish population in streams and rivers can be exposed to more complex plume dynamics in contrast to the previously documented monodispersal decline in eDNA concentrations. The results presented here confirm these observations and showed that sampling close (<25 m) to our caged populations surprisingly resulted in relatively low eDNA concentrations compared to a peak that flared up further away from the source depending on the biomass of the populations in the cages. Moreover, in case of the high biomass populations, the peak was observed at a distance of 300 m downstream from the source, in both stream sections, whereas this peak popped up at a distance of 1–2 km with lower caged fish biomass. The occurrence of such plume-shaped patterns in eDNA concentrations can be explained by the fact that locally emitted eDNA may undergo a continuous "breakdown and homogenisation phase" wherein particle fragmentation and lateral mixing result in smaller, more evenly dispersed eDNA particles, translating in higher

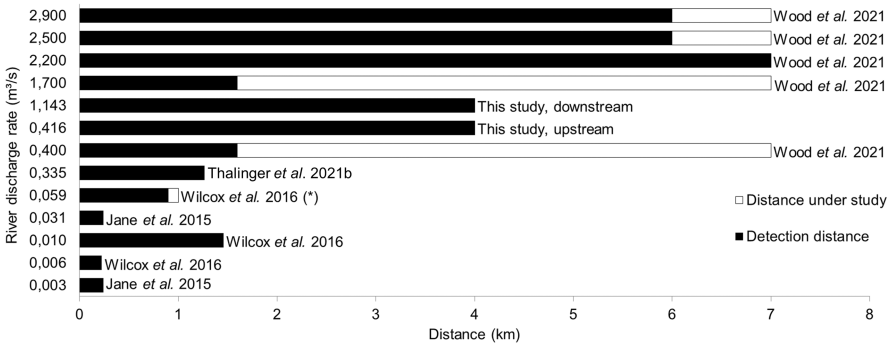

FIGURE 7 Summary of the existing (not exhaustive) literature on how eDNA detection distance increases with increasing river discharge rate. Total bar length displays the extent of the sampling distance, also the distance under study. The proportions of the bars that are filled in black, represent the distance up until which eDNA was actually detected. Only one reference performed an observational study (*), where all other references used caged fish experiments. The studies from this summary are all using qPCR, while this study was performed using ddPCR. Important notes on interpretation and extrapolation relate to methods for discharge calculation, as well as size of the river system under study

eDNA concentrations per liter water (Laporte et al., 2020; Wood et al., 2021). In addition, free dispersing eDNA in lotic systems can also be exposed to a bankside aggregation along the borders of the stream or river (Wood et al., 2021). Knowledge on local hydrology is therefore a prerequisite to infer population densities from eDNA signals in lotic systems (Carraro et al., 2021; Thalinger, Kirschner, et al., 2021). The impact of such effects can be minimized by applying a cross-sectional sampling strategy of the stream or river under study, as was done in this work.

Our findings also showed that when eDNA concentrations diminish, a significant increase of stochasticity effects occurs, which in turn can affect the accuracy of the measurements, especially when screening for low abundant species. Such variability in eDNA concentrations can partly be allocated to the clumped nature of eDNA (Mauvisseau et al., 2021; Wood et al., 2021) and can be expected to be influenced by the discharge rate of a system. Although our results showed that eDNA concentrations of the caged fish species dropped to marginal levels beyond the 1–2 km sampling points, the overall detection probabilities remained surprisingly high, even at a distance of 4 km from the source. This observation highlights the sensitivity of ddPCR methods for fish detection. It further emphasizes its practical use as a tool for early detection of invasive alien species or monitoring of endangered species occurring at low abundances, facilitating practical advice on eDNA detection scale and sampling interval in lotic systems.

Murakami et al. (2021) and Sassoubre et al. (2016) documented that intraspecific variation in eDNA emission is relatively low in stable, experimental conditions and regardless of the presence of other fishes. In natural populations, however, individual variation in size and biomass can be expected to be much larger and may additionally affect the overall population-level rates of eDNA emission (Yates et al., 2019; Yates et al., 2021). Indeed, eDNA emission generally increases with individual biomass but, on the other hand, a few large individuals emit less eDNA particles than more conspecifics of equivalent or smaller biomass (Yates et al., 2021). Although to a lesser extent than occurring in natural populations, such effects of population-level allometry might also have affected the eDNA patterns observed in this work, but is expected to have minimal influence as size variation was very limited within each of the four species used and between the treatments within the same species (Table 1). The eDNA concentrations measured in this work seem, however, greatly exposed to species-specific differences in eDNA emission rates. Again, part of such interspecific variation can also be affected by allometric differences among the studied species, and such size-structure data should be given further consideration in future eDNA research. In case of our studied species, it can be assumed that the larger roach (*R. rutilus*) individuals used released smaller amounts of eDNA per gram body mass, in comparison with the used round gobies (*N. melanostomus*) that were on average four times smaller (Table 1). The differences in biomass per individual among the other three species (excluding *R. rutilus*) are, on the other hand, much smaller (by maximum a factor two). Such substantial interspecific variation in eDNA production further tends to be correlated with differences in physiology and metabolism (Jo et al., 2019; Thalinger, Rieder, et al., 2021) as well as the ecology and behavior of fish species (Rourke et al., 2021; Thalinger, Rieder, et al., 2021). We found, for instance, that the small round goby (*N. melanostomus*) released much larger amounts of eDNA per gram body mass into the water column, in comparison with the roach (*R. rutilus*). The increase per gram body mass eDNA emission of the small round goby might also be attributed to its higher swimming activity which according to Thalinger, Rieder, et al. (2021) coincides with higher eDNA release into the environment. Additionally, distinct differences in external characteristics may influence the eDNA emission as it does for the brown bullhead (*Ameiurus nebulosus*), a scaleless species that releases epithelial cells remaining in suspension much longer than scales that rapidly sink toward the riverbed (Caza-Allard et al., 2022). The latter may also, partially explain the lowered eDNA concentrations of burbot (*L. lota*) in comparison to the small round goby in our study. Taking into account such differences is especially important when using eDNA approaches to monitor vulnerable or invasive species, without under- or overestimating

their population size in the water body under study. *R. rutilus*, for instance displayed much lower eDNA concentrations compared with the other study species, while this species' eDNA displays a surprisingly constant detection probability across the different measuring points downstream from the source. This suggests that this species' DNA bears a higher persistence (Barnes et al., 2014; Dejean et al., 2011; Díaz-Ferguson & Moyer,). On the contrary, *L. lota* (displaying very low eDNA concentrations) and *N. melanostomus* (highest eDNA concentrations) both display much higher variability among measurements and coinciding variation in detection probability at increasing distances downstream from the source. The overall higher detection probability over distance of *R. rutilus* points on these potential interspecific differences in eDNA persistence in the aquatic environment, directly linked with the lowered eDNA decay rate for this species. Such strong interspecific differences in eDNA emission and decay rate highlight the importance of source characteristics that inherently impact population size estimates based on eDNA patterns and the resulting directed management and conservation actions (Rourke et al., 2021).

We conclude that the eDNA patterns found in our natural and strongly meandering river system are significantly shaped by a complex interplay between both source dependent as well as water body-specific characteristics. Moreover, our findings clearly showed that river discharge rate, as well as source biomass both significantly affect the longitudinal eDNA observed in our study system. Furthermore, we recommend for future eDNA sampling strategies in lotic systems to build as much as possible on a priori knowledge on species-specific eDNA emission- and decay rates, the vulnerability and ecological status of the species under study, as well as detailed information on the study system including river discharge rate, in order to accurately detect and quantify fish populations in riverine systems.

AUTHOR CONTRIBUTIONS

Rein Brys designed the experiment. The field collection was performed by Rein Brys, Sabrina Neyrinck, and Charlotte Van Driessche. Sabrina Neyrinck conducted the molecular analyses, upon which Charlotte Van Driessche performed the statistical data analyses, prepared the figures, and wrote most of the manuscript with significant contributions from all co-authors. Teun Everts and Rein Brys assisted in the further interpretation of the data. All co-authors provided comments and helped revising the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available from the Dryad Digital Repository. https://doi.org/10.5061/dryad.rjdfn2zfc.

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APPENDIX A

MODEL SELECTION

We ran four different types of models to test the impact of (a) river discharge rate, (b) fish species identity, (c) distance from the source and (d) source biomass as well as each of the interaction terms as explanatory variables on the downstream eDNA patterns. First a basic eDNA concentration (log[x + 1] transformed) over distance model was used, in which a generalized least squares (*gls*) function is applied. Second, we ran a linear mixed-effects model (*lme*) incorporating the effect of variability in eDNA concentrations measured over distance due to the presence of an eDNA plume and coinciding bankside aggregation of the eDNA (see Wood et al., 2021). For this model, the additional R package *nlme* was used. In this conditional model, the different distances from the cages were nested per stream section and were incorporated as random effects. The latter allows to model a potentially different response in eDNA concentrations over different distances from the source, which cannot be tested in the previous GLS model with only its fixed effects. A serial correlation over distance was included in both of previously described models, by including an autocorrelation term to correct for the dependence of a downstream concentration by the concentration-measured upstream from that point. This resulted in the third and fourth model respectively. Model selection was carried out via the log-likelihood ratio test. Due to the unbalanced experimental setup, we separately tested discharge effects (using the data obtained from both stream sections, but only of the high biomass communities) and potential biomass effects (using only the data obtained from the two biomass treatments in the upper stream section).

TABLE A1 Additional information on the model selection where four models to explain the longitudinal eDNA over distance patterns were tested, starting from the most complex model (1) that included the random effects over distance and stream as well as autocorrelation over distance, (2) the same linear mixed-effects model but without autocorrelation, (3) a linear model using generalized least squares again including a term for autocorrelation over distance and last, (4) the same GLS linear model was used but excluding autocorrelation. The log-likelihood ratio test indicated that the most complex model most correctly represents the eDNA patterns as found in this experiment for both discharge and biomass effects. The log-likelihood ratio method was chosen over the Akaike information criterion for maximum likelihood, as the latter adds a penalty (negative bias) for models with higher parameter complexity

	Model	Df	AIC	BIC	logLiK	Test	L.Ratio	p-value
Discharge effects								
	1	16	204.43	266.91	−86.21			
	2	15	202.43	261.01	−86.21	1 vs 2	0.00	1
	3	14	135.74	190.42	−53.87	2 vs 3	64.68	<0.0001
	4	13	364.57	415.34	−169.28	3 vs 4	230.83	<0.0001
Biomass effects								
	1	17	325.71	392.05	−145.85			
	2	16	323.71	386.15	−145.85	1 vs 2	0.00	1
	3	15	170.05	228.59	−70.03	2 vs 3	151.66	<0.0001
	4	14	409.21	463.84	−190.60	3 vs 4	241.15	<0.0001

APPENDIX B

DETAILED RESPONSE OF *L. LOTA*

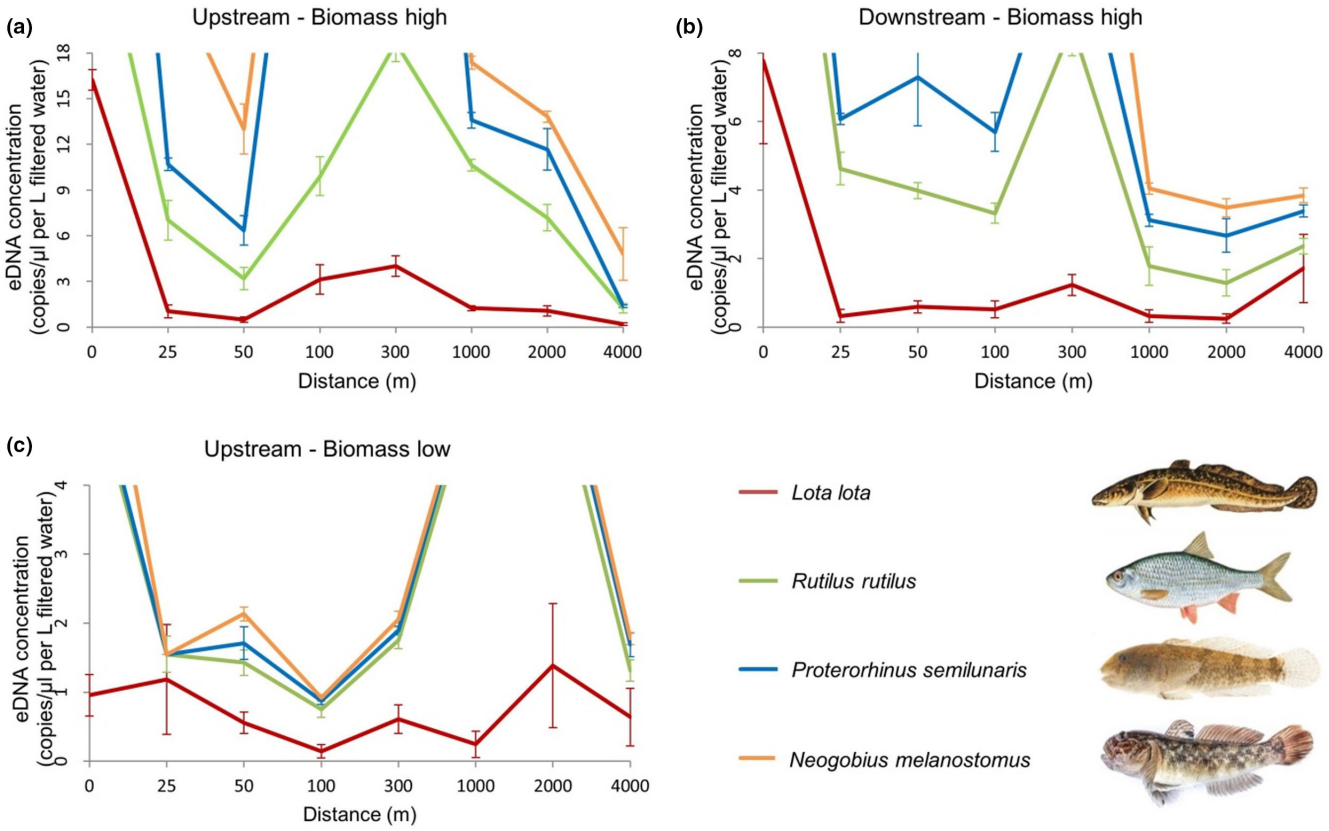

FIGURE B1 Mean eDNA concentration in absolute number of copies per µl DNA extract, standardized per L water filtered, estimated by water samples taken at increasing downstream distance from the cages in two different stream sections, with two different start biomasses in the upstream section, that is low biomass and high biomass. The scale of the y-axis has been adjusted to further investigate the downstream eDNA patterns for *Lota lota*. Indeed, though less pronounced as the other three species, due to overall lower eDNA concentrations, a characteristic increase of *L. lota* eDNA concentration at the 100–300 m sampling point was again found for the high biomass sources. The eDNA concentration downstream of the low biomass source is more subject to stochasticity by low copy numbers, rather than a clear plume dynamic as found with the other species