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Monitoring of spatiotemporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA

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Abstract

To effectively monitor, manage and protect aquatic species and understand their interactions, knowledge of their spatiotemporal distribution is needed. In this study, we used a fine-scale spatiotemporal water sampling design, followed by environmental DNA (eDNA) 12S metabarcoding, to investigate occupancy patterns of a natural community of fish and amphibian species in a lentic system. In the same system, we experimentally estimated the spatial and temporal dispersion of eDNA by placing a community of different fish and amphibian species in cages at one side of the pond, creating a controlled point of eDNA emission. Analyses of this cage community revealed a sharp spatial decline in detection rates and relative eDNA quantities at a distance of 5–10 m from the source, depending on the species and its abundance. In addition, none of the caged species could be detected 1 week after removal from the system. This indicates high eDNA decay rates and limited spatial eDNA dispersal, facilitating high local resolution for monitoring spatial occupancy patterns of aquatic species. Remarkably, for seven of the nine cage species, the presence of a single individual could be detected by pooling water of subsamples taken across the whole water body, illustrating the high sensitivity of the eDNA sampling and detection method applied. Finally, our work demonstrated that a fine-scale sampling design in combination with eDNA metabarcoding can cover total biodiversity very precisely and allows the construction of consistent spatiotemporal patterns of relative abundance and local distribution of free-living fish and amphibian species in a lentic ecosystem.

KEYWORDS

eDNA, high-throughput DNA sequencing, metabarcoding, monitoring, relative abundance, water sampling

1 | INTRODUCTION

Detection of rare and elusive species and estimation of the community in which they occur are critical components for aquatic ecosystem conservation, especially for systems exposed to risks of species extinction and/or biological invasion. To effectively monitor, manage and protect populations and to fully understand interactions of aquatic species, information about their ecological distribution and habitat use in space and time is needed (Begon et al., 2005). For example, a single survey of species composition within an aquatic

system or habitat may offer insights into between-species interactions (e.g., co-occurrence patterns; Bhat & Magurran, 2007), whereas a time-series of surveys can reveal temporal patterns of habitat use within and among species (Brönmark et al., 2008). For fish and many aquatic amphibian species, conventional survey methods are based on capturing organisms using nets, pods, traps or electrofishing techniques (Radinger et al., 2019). In addition to being invasive and harmful, these methods are laborious and expensive (Lintermans, 2016). Due to the mobility and low detection probabilities of the organisms, such conventional methods are often ineffective, and practically limited because many aquatic habitats are inaccessible (Britton et al., 2011; Mackenzie & Royle, 2005; Maxwell & Jennings, 2005; Porreca et al., 2013).

In recent years, advances in molecular techniques based on aquatic environmental DNA (eDNA) have overcome several of these issues and have relieved logistical constraints (Jerde et al., 2011; Lodge et al., 2012). This methodology is generally based on the collection of shed cellular material that is suspended in the water column, and extraction of eDNA for taxonomic profiling. It is clear that these new methods are currently revolutionizing our ability to detect species and assess biodiversity in aquatic systems. However, without actual observation or trapping (Jerde et al., 2011; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), additional knowledge is needed to reach the full potential of eDNA surveys in offering insights into the spatial and temporal habitat occupancy of fish and amphibian species at a local scale (Bylemans et al., 2018; Li et al., 2019; Yamamoto et al., 2016; Zinger et al., 2019).

Overall, the possibility of detecting the presence of species in habitats or ecosystems via aquatic eDNA is strongly affected by the concentration of eDNA in the water sample, which, in turn, is determined by the sources (e.g., organismal emission) and sinks (e.g., decay), and advection and dispersion of eDNA through the system. For instance, the amount of eDNA that an organism releases into the water may depend on several factors, such as the type of organism (Goldberg et al., 2011; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), organism size and/or biomass (Dejean et al., 2011; Lacoursière-Roussel et al., 2016; Takahara et al., 2012), and life stage and breeding status (Goldberg et al., 2011; Maruyama et al., 2014). Decay rates of eDNA, on the other hand, can also be influenced by several factors such as: whether eDNA is extracellular or intracellular (Sassoubre et al., 2016; Turner et al., 2014); abiotic factors (sunlight, temperature, pH, turbidity, salinity, etc.; Barnes et al., 2014; Strickler et al., 2015; Turner et al., 2014); and biotic interactions with extracellular enzymes and/or microorganisms (Barnes et al., 2014; Jane et al., 2015). Once released, eDNA can disperse both horizontally and vertically in the water system, thereby affecting the probability of detection at a certain distance from the source (Bylemans et al., 2018; Deiner & Altermatt, 2014). A high dispersion rate and distance can also be expected to blur patterns of local eDNA emission and consequently diminish the potential to obtain insights into organismal habitat use or patch occupancy patterns. In contrast to lotic systems (such as streams and rivers), in which eDNA dispersal is found to range from several metres up to kilometres downstream from the source (Deiner & Altermatt, 2014; Jane et al., 2015; Pilliod et al., 2014; Pont et al., 2018), lentic systems (such as ponds and lakes) are expected to show much lower rates and shorter distances of eDNA dispersion (Dunker et al., 2016; Eichmiller et al., 2014; Li et al., 2019; Takahara et al., 2012; Yamamoto et al., 2016).

In this study, the major aim was to assess the spatiotemporal distribution of a natural fish and amphibian community ($n = 6$ species) in a seminatural pond based on eDNA metabarcoding. Therefore, once a week, we sampled water from the study system during a 4-week period, using an integrative spatial sampling design to examine species-specific occupancy patterns both in space and in time. In the same system, we experimentally estimated the spatial and temporal resolution of eDNA dispersal, by placing a community of different fish and amphibian species in cages on one side of the pond, here called the "cage" community ($n = 9$ species), thereby creating a controlled point of eDNA emission. The latter allowed us to estimate the eDNA dispersal rate and distance per species in combination with the impact of organismal abundance, all under otherwise natural conditions. We used eDNA metabarcoding as a powerful and efficient approach to determine detection rates and relative quantities of each of the fish and amphibian species within the study system (i.e., the natural and cage community) simultaneously, and finally, we tested how long after removal the cage community species can still be detected.

2 | MATERIALS AND METHODS

2.1 | Study system and experimental set-up

The study was carried out in a seminatural pond of the Research Institute for Nature and Forest (INBO) in Linkebeek, Belgium (50°45′58.8″N, 4°20′07.7″E). This pond is used for fish breeding and (re)introduction programmes of rare and vulnerable species in Belgium. The size of the pond is 30 × 40 m, with an average depth of 1.5–2 m. The experiment was conducted from 14 June to 12 July 2016. During this time interval no extreme rain or wind events occurred, and water conditions were relatively constant, with mean pH = 7.81 ± 0.13, mean conductivity = 558.1 ± 67.4 mS cm^{-1}, mean Na = 25.3 ± 0.9 mg L^{-1} (see Appendix S1 for more details). The free-living fish and amphibian community in the pond (further denoted as "natural" community) consisted primarily of five fish species: *Lota lota*, *Cyprinus carpio*, *Carassius carassius*, *Leuciscus leuciscus* and *Leuciscus idus*, together with some naturally occurring amphibians, such as *Lissotriton vulgaris* and species belonging to the *Pelophylax* cf. *bedriagae* complex. On the other hand, the community placed in the cages at one side of the pond consisted of seven fish species: *Barbatula barbatula*, *Ctenopharyngodon idella*, *Lepomis gibbosus*, *Misgurnus bipartitus*, *Phoxinus phoxinus*, *Rhodeus amarus* and *Leuciscus idus*, and two amphibian species: *Triturus cristatus* and *Lithobates catesbeianus* at varying numbers of individuals (see Figure 1). Individuals from the two amphibian species used in the cage community were all in their larval stage. Four

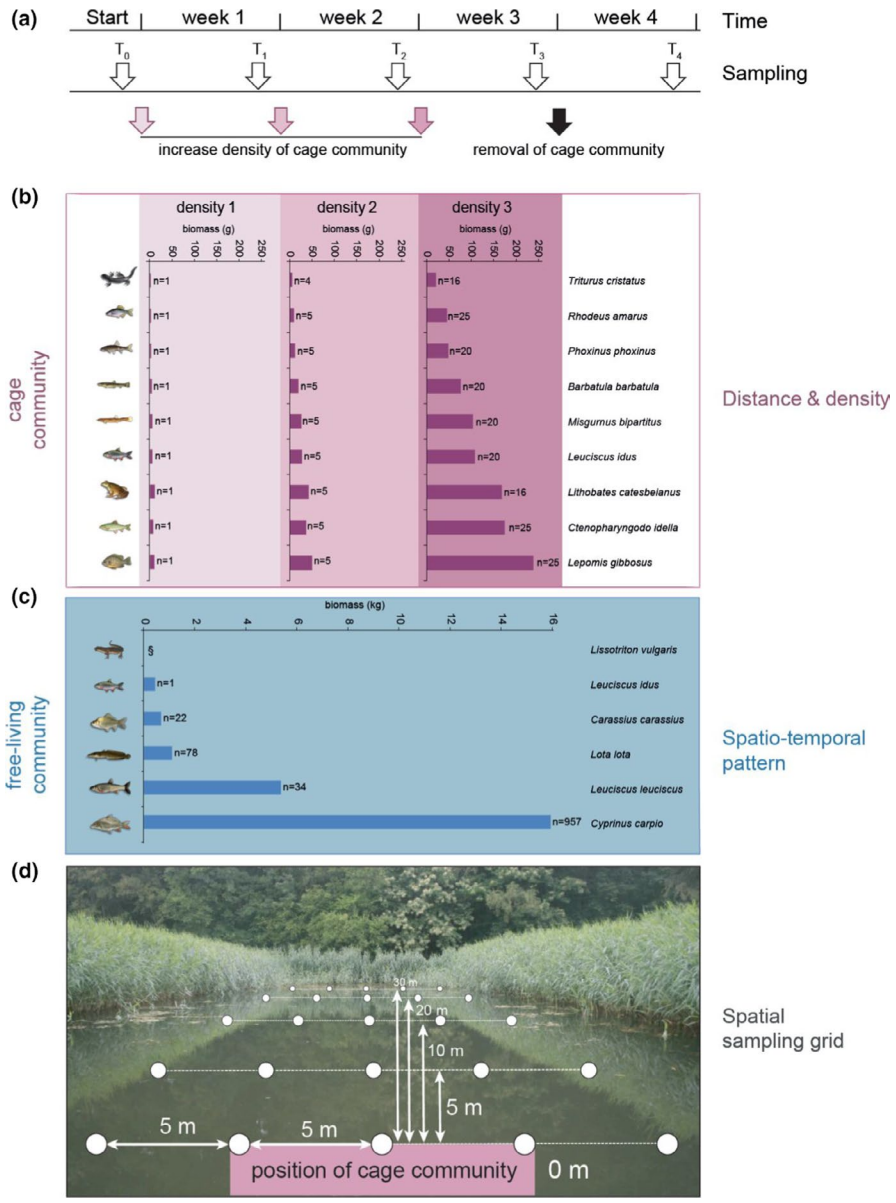

FIGURE 1 Sampling scheme of the natural and cage community in a natural pond. The cage species, consisting of seven fish and two amphibian species, were placed in cages on one side of the pond at the lowest density at the start of week 1. The density (number of individuals) and biomass (represented by the bar plots) of each of these cage species was gradually increased at the start of week 2 and week 3. The natural community consisted of five free-living fish species and one amphibian, the density and biomass of which were determined after complete drainage of the pond at the end of the experiment (except for *Lissotriton vulgaris*). Water sampling was performed at five time points (T_0–T_4) according to a grid: at each distance (0, 5, 10, 20 and 30 m) along a transect parallel to the cages, five subsamples were taken (each at 5 m from each other) perpendicular to the length of the pond. The cage community was used to investigate the dispersion rate and detection distance as a function of cage density, whereas the spatiotemporal distribution of the free-living natural community was monitored to investigate occupancy patterns

cages were used to keep each of these species at a fixed point in the pond (Figure 1), and the cages remained located at that point during the entire experiment. Before the onset of the experiment (T_0), three control water samples were taken from the pond (each consisting of 25 pooled subsamples of 650 ml spread across the entire surface of the pond) to estimate the eDNA composition of the natural community in the absence of the species belonging to the cage community (Figure 1). At the beginning of the cage experiment (T_1), we placed one individual of each cage species into one of the four fixed cages (Figure 1). Over a period of 3 weeks, the number of individuals of these cage species (further denoted as "density") was weekly increased, from one individual per cage at the lowest density (density 1), to four or five individuals per cage at the second (density 2), and up to 16 or even 25 individuals per cage at the highest density (density 3) (see Figure 1 for more details). Each individual was weighed to the nearest 0.1 g before being placed in the cage. Because our findings revealed that eDNA emitted by the caged species completely disappeared over a period of 1 week, we can assume that the different density treatments used are independent. One week after the start of the caging experiment with the lowest density, and following each increase in the density of the caged species, water samples were taken at five different distances from the cages (0, 5, 10, 20 and 30 m) (Figure 1). At each distance, five subsamples of 650 ml were taken along a transect parallel to the positioning of the cages. These subsamples were pooled per transect to obtain one homogenized pooled sample per distance (3.25-L sample) (Figure 1). This was repeated three times at each distance per density applied, to obtain three independent field replicates per time point and distance. At the same time, we also took three pooled samples using the same sampling grid, each consisting of all 25 subsamples at the different distances (further denoted as "pooled samples"). In each of these weekly sampling rounds (T_1 to T_4), we started sampling from the largest distance of the cages (i.e. 30 m and thus probably

the lowest eDNA concentration of the cage community) towards the cages to avoid potential cross-contamination. All water samples were taken just below the water surface (±10 cm), using a long sterile sampling pole with a sterile Whirl-Pak stand-up bag at the top (B01365, Nasco). During each sampling round two field/material blanks were included, one at the beginning and one at the end of sampling, using mineral water to test for potential cross-contamination during field sampling. All reusable field material was decontaminated with 2% Virkon S (Antec - DuPont) as a biosafety precaution and to avoid potential DNA cross-contamination (U.S. Fish and Wildlife Service). When the water sampling was finished at the highest density of the cage community (after T_3), all the caged species and cages were removed from the pond. One week later (T_4; Figure 1), we sampled the pond on each of the different distances once more to test whether eDNA of the cage species was still detectable in the water column. Over the course of the entire experiment we took 75 water samples (consisting of 675 subsamples of 650 ml). None of the individuals of the cage community died or escaped from the cages during the entire experiment.

Finally, to obtain estimates of the number and biomass of each of the free-living species, the pond was completely drained at the end of the experiment using a net placed at the outlet to avoid any fish or amphibian escaping from the system. Once caught, all fish were identified to the species level and sorted, after which total biomass (to the nearest gram) and number of individuals were determined per species. Numbers and biomasses of the observed free-living amphibian species that frequented the pond during the experiment could not adequately be determined because they were able to emerge from the water during the experiment.

2.2 | Water filtering and eDNA extraction

From each water sample, including the blanks, 200 ml was filtered through an enclosed 0.45-μm pore size PVDF Sterivex-HV filter capsule (SVHVL10RC, Merck Millipore). This was repeated for each of the three independent water samples (i.e., field replicates) per distance for each density or time point. For each water sample a new sterile 60-ml Luer-Lock syringe and new gloves were used. At the end of each filtration, the remaining water inside the capsule was expelled by pushing air through the capsule until it dried completely and capped it at both ends. After filtration, all filters from each time series were immediately stored at −20°C until DNA extraction.

In a next step, the DNA on each of the frozen filters was extracted following the $SX_{CAPSULE}$ method (suitable for filters without preservation buffer as in Spens et al., 2017) by using the DNeasy Blood & Tissue Kit (Qiagen) (Appendix S2, step 1). The extraction process was performed in a dedicated PCR-free room for low-copy-number template extractions, with controlled DNA-free, high-efficiency particulate air-filtered compartments with positive air pressure, to avoid any contamination of eDNA samples. Besides field blanks, we also included three extraction blanks to detect potential contamination at the extraction stage. The eDNA extract was purified with MagNA beads and quantified with a Quantus fluorimeter according to the manufacturer's instructions (Appendix S2, steps 2–3), prior to amplification.

2.3 | PCR amplification, library preparation and sequencing

DNA amplification was performed with two short amplicons of the mitochondrial 12S gene. The first primer assay consisted of the degenerated fish Teleo-primers (teleo_F, 5′-ACACCGCCCGTCACTCT-3′; teleo_R, 5′-CTTCCGGTACACTTACCRTG-3′), which amplify an ~102-bp amplicon (for further details see Valentini et al., 2016). The second primer assay used the vertebrate-specific Riaz primers (12S_F1, 5′-ACTGGGATTAGATACCCC-3′;12S_R1, 5′-TAGAACAGGCTCCTCTAG-3′), which amplify an ~142-bp fragment (Riaz et al., 2011). Both primers have previously been shown to be highly useful to detect European freshwater fish species (Hänfling et al., 2016; Kelly et al., 2014; Port et al., 2016), and the Riaz primers additionally amplify amphibian species appropriately (Riaz et al., 2011).

Inline index sequences were attached at each primer, finally resulting in a unique dual-matched indexing for each PCR (polymerase chain reaction) which reduces sample misassignment during read demultiplexing (see Appendix S2, step 4 for further details). Variable length indices were used to increase the chance that neighbouring clusters on the Illumina Flowcell sequence the internal PCR-amplified target fragment out-of-phase. PCR was carried out in a 25-μl reaction volume containing 3 μl of the eDNA template (1–30 ng μl^{-1}), 2.5 μl 10 × PCR Buffer II (100 mm TrisHCl; 500 mm KCl), 0.5 μl dNTPs (20 mm), 2 μl primer mix (2.5 μm each of indexed forward and reverse primer), 0.5 μl bovine serum albumin (10 mg ml^{-1}), 2.5 μl $MgCl_2$ (25 mm), 0.2 μl AmpliTaq Gold DNA Polymerase (5 U μl^{-1}) and 13.8 μl water (see Appendix S2, step 4 for details). The primer assay was performed with 45 (Riaz) or 50 (Teleo) PCR cycles, and for each DNA sample, at least three technical PCR replicates were performed. At this stage, we also included three negative PCR controls in each library to test for potential contamination in the laboratory. A selection of PCR products was checked by agarose gel-electrophoresis to confirm the correct size of the amplified fragment per primer assay, and used for crude quantification with a Promega Quantus fluorimeter to confirm sufficient PCR amplification (see Appendix S2, steps 5–6). A standard volume of PCR product per individual sample was pooled prior to the ligation, independent of the actual concentration per sample. Samples were split into three pools (one pool per set of PCR replicates) as the same set of inline indices were used per PCR replicate. Per PCR replicate set, the Riaz and Teleo amplicon pools were crudely quantified and combined, at roughly equimolar amounts, to further reduce the number of pools for ligation. For each Riaz + Teleo pool, the DNA concentration was quantified, cleaned up with MagNA beads and again quantified using a Quantus Fluorometer (Appendix S2, steps 7–10). The length distribution of the DNA fragments was

analysed per pool using the Qiaxcel DNA high-resolution kit according to manufacturer's instructions (Appendix S2, step 11). The KAPA PCR-free Hyper Prep Kit was used for Y-shaped indexed Illumina TruSeq adapter ligation to each pool of amplicons (Appendix S2, steps 12–14). Finally, the ligation products were cleaned using SPRI beads, fragment length distributions were analysed again by using a Qiaxcel DNA High Resolution Kit, followed by a quantitative (q) PCR analysis to quantify the library (Appendix S2, steps 15–17). The resulting product was then sequenced on a HiSeq3000 instrument using 2 × 150-bp paired-end sequencing at the sequencing facility of OMRF (Appendix S2, step 18).

2.4 | Sequence read processing and taxonomic assignment

2.4.1 | Bioinformatic analyses, quality check and data cleaning

The sequenced libraries have some specific characteristics: (i) unique dual index combinations with indices of variable length and (ii) PCR fragments with inline indices that are ligated in both directions with respect to the Illumina i5 and i7 Flowcell adapters (non-directional ligation). To properly demultiplex the samples, we have to take these properties into account: we need to check for the combined presence of the two variable-length indices in both the forward and the reverse direction. To do this, we developed a custom demultiplexing script. This shell script uses the tool SABRE version 1.000 (https://github.com/najoshi/sabre), which allows fast demultiplexing using variable-length sample indices. However, SABRE does not provide support for dual indices (unique pairs of variable-length indices on both ends of the sequenced fragment) nor for nondirectionally cloned libraries. Therefore, the custom demultiplexing script calls SABRE multiple times to assign the read-pairs to samples: subsequently for forward and reverse reads, as well as in both directions. A read pair is only assigned to a certain sample if both its forward and its reverse index match without errors. After the demultiplexing, the forward and reverse reads were merged using PEAR version 0.9.11 (Zhang et al., 2014) with a minimum overlap of 20 nucleotides, and a final fragment length of a minimum 60 and maximum 195 nucleotides for the Riaz fragment and a minimum 20 and maximum 140 nucleotides for the Teleo fragment. After merging, the primers were removed both from the 5′ and the 3′ end using CUTADAPT version 1.15 (Martin, 2011), allowing for a maximum of 15% errors in the primer sequence. Only fragments where both primers were present were retained. Next, a quality filtering step was done using VSEARCH version 2.7.1 (-fastq_filter) (Rognes et al., 2016) with a maximum expected error of 0.5. After these preprocessing steps, the reads were renamed (to include the sample name in their fasta header) and concatenated to a single fastq file for further processing using the OBITOOLS software version 1.2.13 (Boyer et al., 2016). Dereplicated sequences ("obiuniq") with a read count of a minimum 80 over all samples were kept for further analysis ("obigrep"). PCR and/or sequencing errors were removed by applying the "obiclean" program with a maximum number of differences between two variant sequences of 1 (option -d) and a threshold ratio between rare and abundant counts of 0.05 (option -r). Only sequences with the head status in at least one sample were kept (option -H). Next, a taxonomy was assigned to the cleaned sequences using "ecotag" from OBITOOLS with default parameters. The reference database contains full or partial 12S fragments of fish, amphibians and possible contaminants expected in Flemish waters (a combination of in-house determined Sanger sequences and public data). Finally, the sequences were sorted by abundance ("obisort") and written to output in table format ("obitab").

To filter out low-frequency read noise that passed the previous filtering steps, and may originate from PCR artefacts or contamination during the library construction or sequencing process, data were additionally filtered by including a species-specific threshold. This was set on a relative abundance of 0.005.

Preliminary screening of the data obtained from both primer pairs revealed that for seven out of the 11 fish species, the relative abundance estimated with the Teleo primer assay was nearly equal to the relative abundance estimated with the Riaz primers for the same sample, and across all observations a strongly significant and positive correlation was observed between the two primer assays ($r = .989$; $p < .0001$; Appendix S4a). However, for some of the study species, the Teleo primer assay could not reliably amplify the target amplicon, and only the Riaz primer assay resulted in successful and efficient amplification. This was the case for two fish species, *Carassius carassius* and *Lota lota* (Appendix S4b), and the three amphibian species in the natural and cage community (Appendix S4c). Finally, the Teleo amplicons of *Leuciscus idus* and *Leuciscus leuciscus* provide identical sequences, precluding estimation of species-specific relative abundance for these two species. As we aim to reconstruct the spatiotemporal distribution of the fish and amphibian community as a whole, the general analyses were based only on the Riaz amplicon data. However, we cross-validated relative abundance values of the fish species using the Teleo amplicon data.

Raw sequence data (Riaz and Teleo amplicons) was uploaded to the Sequence Read Archive (SRA) of NCBI under BioProject no. PRJNA616325. The demultiplexing script as well as a shell script used for all analyses described above are available at Zenodo (https://doi.org/10.5281/zenodo.3731310). The custom reference databases used for taxonomic assignments are available at Zenodo (https://doi.org/10.5281/zenodo.3730934).

2.5 | Data analysis

2.5.1 | Detection rates and abundance indexing

For each water sample we first calculated detection rates of eDNA per species as the proportion of positive PCR replicates per sample. This was only done for the caged species, to test the impact of distance from the source on detection success. For the free-living species, this estimate was not meaningful as detection success was positive in almost

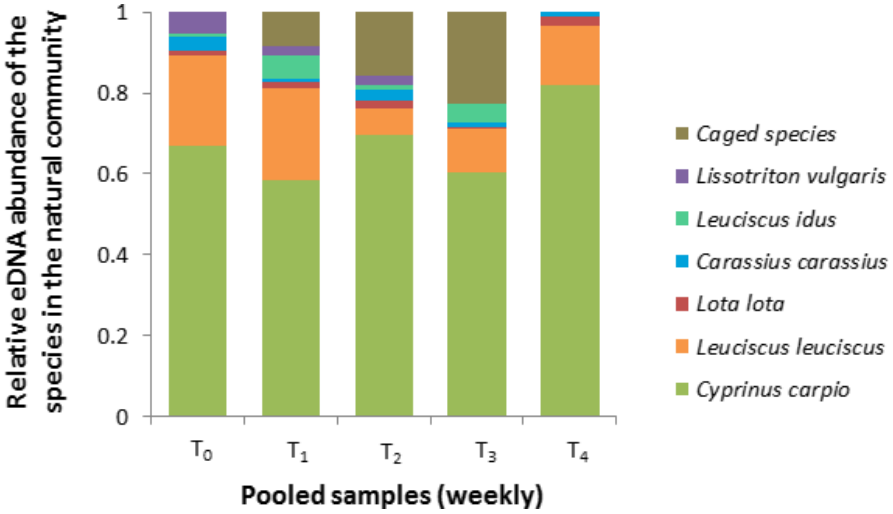

FIGURE 2 Relative abundance of the fish and amphibian species in the natural community with the total contribution of the caged species, estimated by pooled water samples taken across the pond at five subsequent weekly sampling time points (T_0–T_4). Relative abundance is calculated as the ratio of reads per species to the total number of reads per pooled water sample and expressed on a scale between 0 and 1

all PCR replicates. Next, to obtain a standardized estimate of eDNA abundance for each species (both the caged and free-living species) across all samples, the eDNA index was used (Djurhuus et al., 2020; Kelly et al., 2019). This species-specific eDNA index varies between zero and one, and is a double-transformation in which first read counts are converted into proportions within a sample (i.e., number of reads per species relative to the total number of reads in that sample), and second the resulting proportion of each species is scaled to the largest observed proportion of that species across all samples in the study:

$$\mathrm{eDNA}_{ij} = \frac{\frac{Y_{ij}}{\sum_i Y_i}}{\max_j\left(\frac{Y_{ij}}{\sum_i Y_i}\right)}$$

This normalization reflects the intuition that absolute raw read counts in metabarcoding data sets, standing alone, do not provide reliable information about the abundance of a species present, because they are affected by many analytical steps, such as water volume used, variation in eDNA extraction efficiency, differences in PCR amplification efficiency and unequal pooling of libraries before sequencing. Each of these aspects can influence the number of reads assigned to a particular species in a sample and potentially bias patterns of interest. Reads derived from species that were not of interest to this study were removed before calculation of the relative abundance, such as reads assigned to human, mammals and some amphibian species, such as *Lissotriton helveticus* and *Rana temporaria* that only sporadically showed up in a few samples at very low abundances.

2.5.2 | Statistical analyses

First, generalized linear mixed models (GLMMs, GLIMMIX procedure) were used to test whether the detection rate and eDNA index of the relative abundance of each of the caged species was significantly affected by distance from the source (i.e., the cages), density at which they were present in the cages and identity of the species. Both response variables were included by using the binomial distribution and a logit link function. Initially, each model incorporated all main factors and their interactions. The AIC was then used as a goodness-of-fit criterion for model evaluation (Bolker et al., 2009), following a hierarchical simplification procedure starting with removal of highest-level interactions first until the best model was obtained.

To test whether the eDNA index of the relative abundance of each of the species in the free-living natural community showed species-specific differences along the spatial sampling gradient (i.e., position in the pond), and to assess whether this was significantly affected by the timing of sampling and species identity, a GLMM was again used with the eDNA index included as response variable. Again all two- and three-way interactions were initially included in the model, followed by backwards hierarchical simplification of the model based on the AIC values. All analyses were done in SAS 8.02 (Littell et al., 1996).

3 | RESULTS

3.1 | Overall eDNA composition

Metabarcoding and sequencing yielded a total of 14.9 million reads. Of these, 11.0 million reads (73.7% of the total number of reads) were retained after filtering, removal of chimeric sequences and species that were not of interest for this study (see Materials and Methods). Out of 10 field blanks, two showed limited but positive amplification of fewer than 100 read sequences (belonging to the species *Cyprinus carpio* and *Leuciscus leuciscus*), representing about 0.001% of the mean total number of sequences that were detected in an eDNA sample, which is negligible. All extraction negative blanks and PCR negative controls showed no positive amplification, indicating the absence of contamination during sample extraction and amplification.

Screening of the natural species composition in the pond, based on the pooled samples taken prior to addition of the cage community

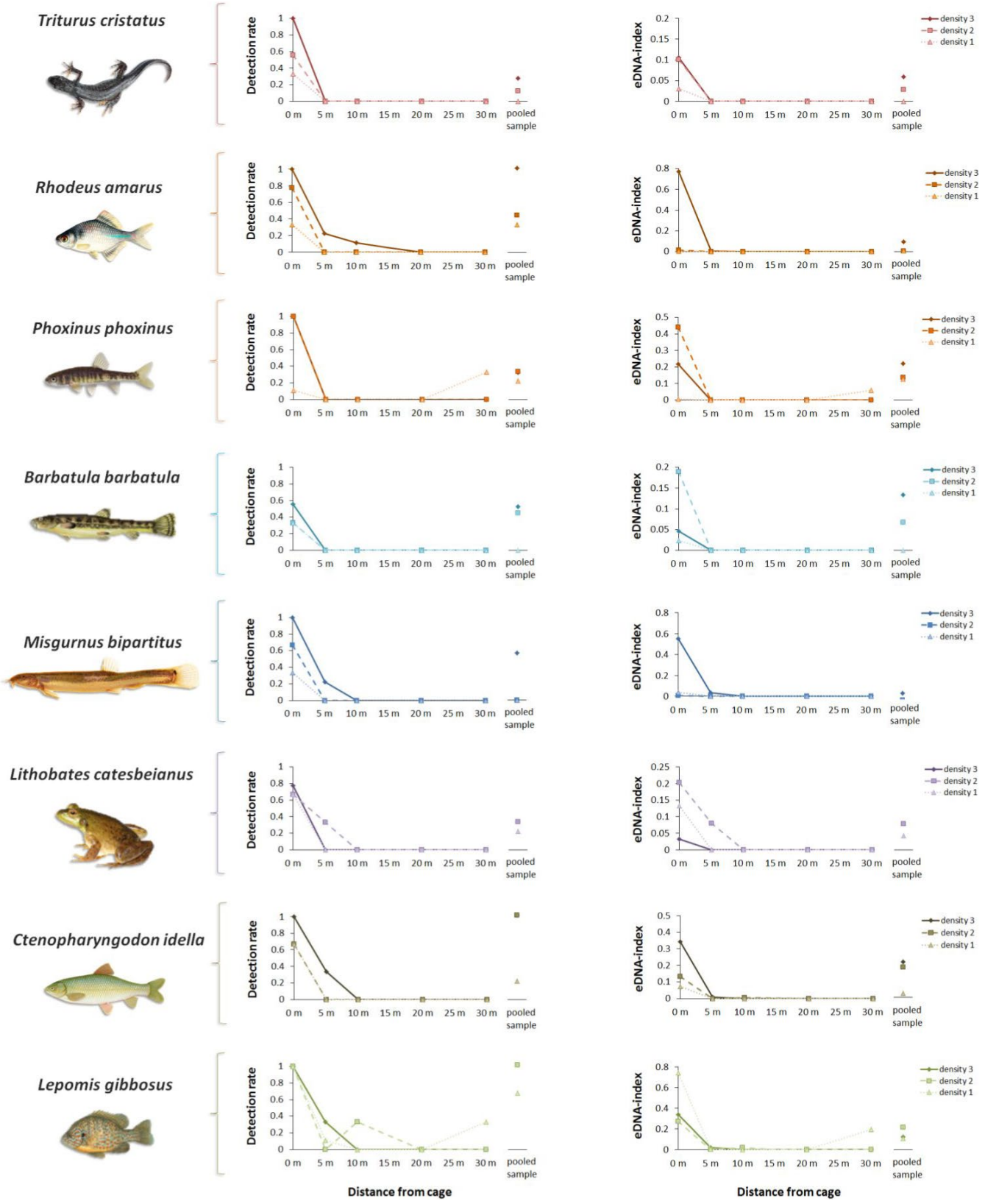

FIGURE 3 Detection rates and standardized eDNA indices of relative abundance of the fish and amphibian species included in the cage community estimated by water samples taken at increasing distance from the cages and with increasing density of the cage species. See Figure 1 for the sampling grid and densities

(i.e., samples taken at T_0; Figure 1), confirmed the presence of five fish species, that were also caught after drainage of the pond, and one amphibian species (see Figure 2). Each of these species was free to move in the pond and all but one (*Leuciscus idus*) were not included in the cage community. For *Leuciscus idus*, one free-living individual was caught at the end of the experiment (which was not expected at the beginning of the experiment), but this species wasalso included in the temporal cage community. Conversely, eight species included in the cage community were not present in the natural community of the pond. The overall eDNA composition of the natural community was relatively constant over the entire study period (see Figure 2), except for *Lissotriton vulgaris*, which gradually decreased during the sampling period (from 5.3% at T_0 towards undetectable levels at T_4). *Cyprinus carpio* was by far the most dominant species in the pond (average relative abundance of 71.1 ± 8.9%), whereas the other species were far less abundant (<15%) (Figure 2). From T_1 onwards, the cage community collectively contributed to the total eDNA of the pond, and displayed a gradual increase from on average 8% at the lowest density (T_1) to up to 20% at the highest density (T_3). Remarkably, 1 week after the cage community was removed from the pond (T_4), these caged species could no longer be detected, either in the pooled samples (Figure 2) or in any of the samples taken at the different distances in the pond, not even at the location (0 m) where the cages had been positioned.

3.2 | Empirical estimation of eDNA dispersion rate and detection distances

Analyses of the eDNA patterns of the species unique to the cage community revealed a strong spatial clustering around the cages, as expected (Figure 3). Overall, both the eDNA detection rates and the standardized eDNA indices of relative eDNA abundance that the caged species locally emitted significantly decreased with increasing distance from that point source (Table 1). These patterns were significantly affected by the density at which the species were present in the cages, determining the eDNA dispersion rate and final distance at which it could be detected (spatial decrease with distance from the source), as indicated by the significant interaction effect between distance and density (Table 1). Similar patterns were also found for the eDNA index values, although in some cases, such as for *Lithobates catesbeianus* and *Barbatula barbatula*, lower densities seemed to show a stronger relative contribution to the total eDNA compared to higher densities (Figure 3). Finally, eDNA of *Ctenopharyngodon idella*, *Lepomis gibbosus*, *Misgurnus bipartitus* and *Lithobates catesbeianus* disperse farther (up to 10 m from the source) compared to other species, such as *Barbatula barbatula* and *Triturus cristatus*, which could only be detected at a maximum distance of around 5 m from the cages (Figure 3).

Interestingly, the presence of each of the species in the cages could also be detected in a pooled water sample, made by pooling all subsamples taken at each of the 25 different sampling points across the pond (Figure 3). The only situations where the caged species was not detected in pooled samples were in the case of single individuals of *Misgurnus bipartitus* and *Triturus cristatus*.

TABLE 1 Results of the generalized linear mixed model (GLMM) displaying distance (i.e., distance at which a sample was taken from the cages), density (i.e., number of individuals per species in the cages) and species effects on (i) the observed detection rate and (ii) the standardized eDNA index of relative abundance estimated for each of the nine species (seven fish species: *Barbatula barbatula*, *Ctenopharyngodon idella*, *Lepomis gibbosus*, *Misgurnus bipartitus*, *Phoxinus phoxinus*, *Leuciscus idus*[a] and *Rhodeus amarus*, and two amphibian species: *Triturus cristatus* and *Lithobates catesbeianus*) that were used in the cage community in a cage at a fixed location in the pond

	df[b]	*F*-value	*p*
Detection rate			
Distance	1, 349	0.27	.6043
Density	1, 349	20.85	**<.0001**
Species	7, 349	1.38	.2146
Distance × Density	1, 349	14.74	**<.0001**
Distance × Species	7, 342	0.80	.5886
Density × Species	7, 335	0.51	.8264
Distance × Density × Species	7, 328	0.62	.7373
eDNA-index			
Distance	1, 349	3.70	**.0452**
Density	1, 349	7.16	**.0078**
Species	7, 349	0.93	.4837
Distance × Density	1, 349	5.18	**.0234**
Distance × Species	7, 335	0.29	.9558
Density × Species	7, 342	0.49	.8435
Distance × Density × Species	7, 328	0.12	.9971

Note: Significant values ($p < .05$) are indicated in bold.

[a]Note that *Leuciscus idus* was omitted from the data prior to analyses, as this species also occurred in the free-living community.

[b]Note that the denominator degrees of freedom (*df*) depend on the number of factors that were included in the model, as a backward hierarchical elimination procedure was applied based on the AIC values and nonsignificant factors.

3.3 | Spatiotemporal distribution of species within the natural community

During the 4 weeks of spatial sampling, individual species of the natural community showed significant differences in their relative abundance, expressed in terms of the standardized eDNA index of relative abundances, but their composition did not change significantly over time, as indicated by the lack of a significant interaction between species and time point (Table 2). Species of the free-living natural community also showed significant spatial differences in their eDNA patterns (Figure 4), indicated by the significant interaction between species and space. However, per species, spatial patterns were relatively consistent over time (Table 2; Figure 4). For

TABLE 2 Results of the generalized linear mixed model (GLMM) displaying distance (i.e., longitudinal distance across the pond), time (i.e., weekly sampling during one month) and species effects on the standardized eDNA index of relative abundance for the natural fish and amphibian community (consisting of four fish species[a]: *Lota lota*, *Cyprinus carpio*, *Carassius carassius* and *Leuciscus leuciscus*, and one salamander, *Lissotriton vulgaris*)

	df[b]	*F*-value	*p*
eDNA-index			
Distance	1, 290	4.02	.0541
Time	1, 289	0.40	.5268
Species	4, 290	131.52	**<.0001**
Distance × Time	1, 284	0.00	.9499
Distance × Species	4, 290	5.98	**<.0001**
Time × Species	4, 285	0.89	.5083
Distance × Time × Species	4, 280	0.20	.9383

Note: Significant values ($p < .05$) are indicated in bold.

[a]Note that *Leuciscus idus* was omitted from the data prior to analyses, as this species also occurred in the cage community.

[b]Note that the denominator degrees of freedom (*df*) depend on the number of factors that were included in the model, as a backward hierarchical elimination procedure was applied based on the AIC values and nonsignificant factors.

instance, *Leuciscus leuciscus* displayed low eDNA index values close to the 0-m sampling point which gradually increases towards the 30-m sampling point. *Lota lota* and *Lissotriton vulgaris*, on the other hand, showed higher eDNA index values towards the 0-m sampling point. Interestingly, *Leuciscus idus*, present both in the natural and in the cage community, displayed a composite eDNA profile (Figure 5). Moreover, in the absence of the caged individuals at T_0 and T_4, the free-living *Leuciscus idus* displayed an elevated eDNA index value towards the side of the pond away from the location of the cages, similar to the pattern with *Leuciscus leuciscus*. Strikingly, a locally increased density of *Leuciscus idus* in the cages at T_{1-3} is reflected in a local increase of the relative abundance in the spatial eDNA distribution near the 0-m sampling point. This local peak disappeared again after removal of the cage community at T_4 (Figure 5), showing again the quick and local response of eDNA to temporal changes in the spatial distribution of the species.

4 | DISCUSSION

In this study we used a fine scale spatiotemporal sampling design in a lentic system to evaluate the effect of sampling on detection success and the capacity to reveal spatial distribution patterns of fish and amphibian species using eDNA metabarcoding. An observational study of the natural community, combined with a cage experiment to quantify dispersion rate and distance, offered very detailed information on preservation of macrobial eDNA (denoted by Pawlowski et al., 2020) in water samples and revealed consistent species-specific spatial occupancy patterns of the free-living fish and amphibians over time, showing the potential to obtain deeper insights into fish and amphibian ecology via eDNA approaches.

4.1 | Limited eDNA dispersal in space and time

In aquatic environments, it is generally assumed that eDNA does not disperse over large distances, especially in lentic systems. However, empirical evidence under natural conditions is rather scarce (but see Li et al., 2019) and mostly confined to lotic systems (e.g. Bylemans et al., 2018; Deiner & Altermatt, 2014; Jane et al., 2015; Pilliod et al., 2014; Wilcox et al., 2016). Our observations of a localized caged community showed that the detection rates and standardized patterns of eDNA (as quantified using the eDNA index) emitted by seven fish and two amphibian species decreased sharply with increasing distance from the source, and decreased strongly within a radius of 5 m from the cages. Additionally, we also found remarkable species-specific differences in eDNA dispersal distances, with some species, such as *Triturus cristatus* and *Barbatula barbatula*, showing a very limited range at which they can be detected (<5 m), whereas others, such as *Ctenopharyngodon idella*, *Lepomis gibbosus* and *Lithobates catesbeianus*, were characterized by a higher distance of eDNA dispersal, reaching up to 10 m from the source. A probable explanation for this observation might be found in the substantial differences in total biomass among species included in the cages (see Figure 1), although other factors, such as species-specific differences in eDNA emission rates (e.g., Goldberg et al., 2011; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), primer efficiency (e.g., Piñol et al., 2019) or decay rates (e.g., Sassoubre et al., 2016), can be expected to affect eDNA dispersal rates too.

The distance at which a locally occurring species or population could be detected increased significantly with the number of individuals included in the cages. These findings corroborate observations of Dunker et al. (2016), who reported a significant distance effect on eDNA concentrations detected around stocking locations of *Esox lucius* in some Canadian lakes. Although macrobial eDNA is expected to show much larger dispersion distances in streams and rivers as there the water column itself moves and carries the eDNA with it, significant downstream decays have also been documented (Jane et al., 2015; Pilliod et al., 2014) and can result in aggregated eDNA "clouds" in such lotic systems too (Deiner & Altermatt, 2014; Wilcox et al., 2016). In marine environments, recent work by Jeunen et al. (2019) and West et al. (2020) has indicated limited eDNA transport despite extensive tidal and oceanic movements, allowing habitat partitioning studies based on eDNA metabarcoding surveys.

The very limited macrobial eDNA dispersal distances observed around our cage community can, at least partly, be explained by the limited horizontal water current in our study system. In addition, fast eDNA decay rates are expected to maintain these patterns, especially under natural conditions. Our findings indeed confirm that 1 week after removal of the cage community at its highest density, none of the species could any longer be detected in the study system, even at the location where the cages were positioned. These

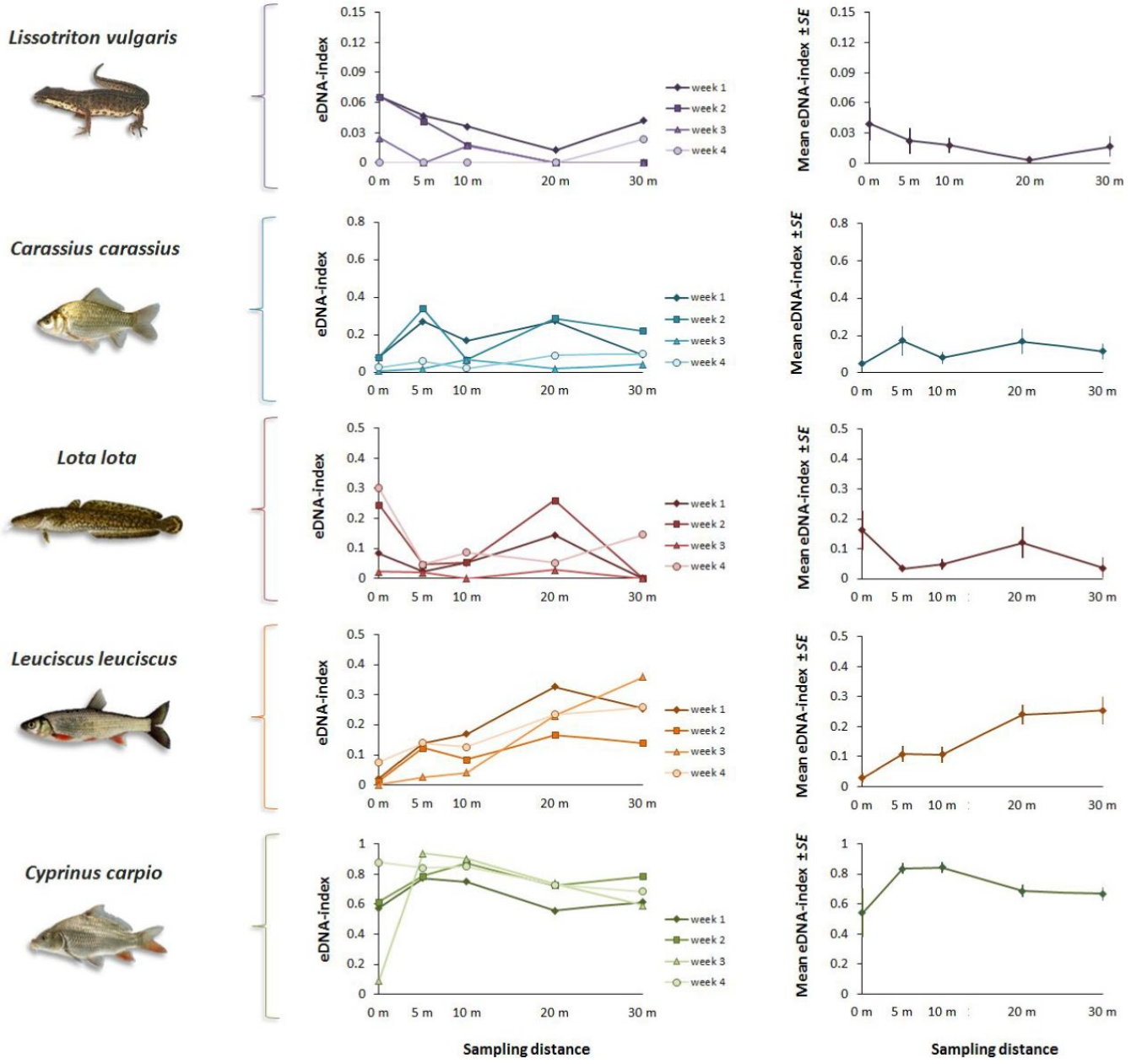

FIGURE 4 Standardized eDNA indices of relative abundance of the fish and amphibian species occurring in the free-living community, estimated by water samples taken at increasing distance from the cages at subsequent weekly sampling time points (weeks 1–4). See Figure 1 for the sampling grid and biomass

observations are in accordance with previous studies, especially for fish, which demonstrate that eDNA degraded below detection thresholds within a period of 1 week (Barnes et al., 2014; Sassoubre et al., 2016; Thomsen, Kielgast, Iversen, Møller, et al., 2012). Some studies for amphibian species, however, reported longer decay periods of several weeks (Dejean et al., 2011; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), and eDNA of a mud snail (*Potamopyrgus antipodarum*) was still detected in the water after 1 month (Goldberg et al., 2013). The fast decay rates observed in our study can probably be attributed to the fact that the experiment was carried out during summer, with high water temperatures, UV radiation and microbial activity, aspects which are documented to accelerate eDNA degradation (e.g., Barnes et al., 2014; Strickler et al., 2015; Tsuji et al., 2017).

4.2 | Effective detection of biodiversity

The possibility of detecting the presence of species in an aquatic environment depends on the concentration of its eDNA in the water sample, but even more importantly on whether the eDNA "cloud" (i.e., spatial aggregation of macrobial eDNA in the water column) of such target species is effectively sampled. In this context, the densities used in our cage community accurately mimicked the

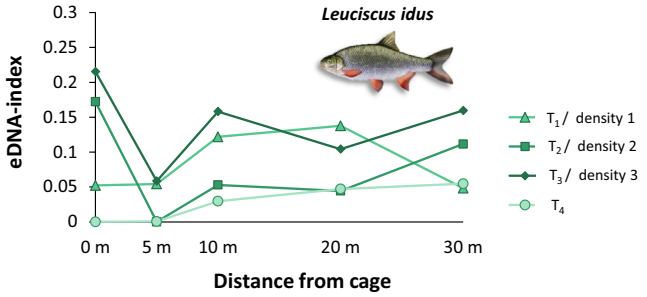

FIGURE 5 Standardized eDNA index of relative abundance of *Leuciscus idus* estimated by water samples taken at increasing distance from the cages at subsequent weekly sampling time points (weeks 1–4). See Figure 1 for the sampling grid and densities

occurrence of rare species with a very local habitat use. Moreover, the total biomass of the cage community only marginally contributed to the total fish biomass in the study system (from 0.2% at the lowest density to 4.0% at the highest density). Based on the sharp decline of eDNA concentrations with increasing distance from a locally positioned source population, we suggest that the collection of multiple samples, with a fine spatial sampling resolution, is needed to appropriately survey total fish and amphibian diversity, especially if small and immobile populations occupy such lentic systems. Several eDNA studies in lotic systems similarly suggested that this, although on another scale, is also needed in lotic systems (e.g., Bylemans et al., 2018; Deiner & Altermatt, 2014). Under such a dense sampling design (intersubsample distances around 5–10 m in our study system), we observed a high detection sensitivity, with almost complete species coverage in the pooled samples. Moreover, for six out of the nine caged species, we were able to detect the presence of only one individual in the study system using such pooled samples. These findings thus suggest that the potential negative impact of dilution effects on detection success is rather small. However, Sato et al. (2017) and Zhang et al. (2020) recently documented that pooling of subsamples taken at a much larger scale can have a significant impact on the detection resolution of rare species, which they attributed to dilution effects. The development of an appropriate sampling design for a certain lentic system thus needs to balance the trade-off between subsampling (grid) density and the sensitivity (i.e., detection thresholds) of the method used, in our case metabarcoding. The eDNA dispersion rate thus determines the maximal inter-subsample distances in the sampling grid in the study system: at large dispersion rates, fewer sampling points are needed and vice versa. In other words, not including a sample point within the eDNA dispersion radius of rare and locally distributed species may result in the exclusion of that species from the pooled water sample, and will therefore certainly result in false negative detection. However, the smaller these distances, the higher the total number of pooled subsamples and thus the higher the dilution factor. If the eDNA of a low-abundance species is included in the eDNA extract, the detection threshold (sensitivity) of the eDNA approach used downstream in the workflow determines the maximal dilution factor allowed to avoid false negative detection. However, such dilution effects can, to some extent, be counterbalanced by increasing the number of PCR replicates per sample. An effective sampling design thus ideally needs to combine a sampling grid that is narrow enough to cover both the action radius and expected eDNA dispersal rates of each of the species in the study system, with the pooling of a maximal number of subsamples that still allows subsequent detection. If the size of the study system exceeds these thresholds, one needs to split such water bodies into multiple pooled samples for testing. Future eDNA-based studies thus certainly need to focus on the effectiveness and limitations of spatial sampling strategies in lentic systems, including the size of study system, its species composition, the size of the populations, and the species mobility, physiology and ecology.

4.3 | Species-specific occupancy patterns

The obtained patterns of strongly limited eDNA dispersal in space and time indicate the potential to assess habitat preferences and between-species interactions of natural fish and amphibian communities based on eDNA distribution patterns. Our findings of the natural, free-living community indeed provide consistent spatial and species-specific eDNA patterns during the 4-week study period. Whereas some species, such as *Carassius carassius*, showed a rather homogeneous distribution across the pond, others, such as *Leuciscus leuciscus*, displayed a preferential spatial distribution in the system. The eDNA pattern of *Leuciscus idus* additionally demonstrates this point. While we locally changed the abundance of this species via our cage community, one large individual of this species also appeared to be present in the free-living community, where it showed a similar spatial distribution as its much more dominant sister species, *Leuciscus leuciscus*. In other words, the spatial eDNA pattern of *Leuciscus idus* thus represents a composite profile of that free-living individual, showing a peak in abundance at one side of the pond, and patterns resulting from the individuals included in the cages at the different densities, at the other side of the pond (Figure 5). In contrast to our observations, previous studies in ponds and small lakes have reported a lack of such fine-scale eDNA distribution patterns of aquatic organisms, as patterns tended to be much more homogeneous (Evans et al., 2017; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). On the other hand, some studies also documented spatial heterogeneity in eDNA distribution patterns among species, but mostly at a much larger spatial scale (e.g., Hänfling et al., 2016). For instance, Dunker et al. (2016) and Eichmiller et al. (2014) reported spatial eDNA distribution and local aggregation of free-roaming *Esox lucius* and *Cyprinus carpio* in large lakes. Spatial patterns in relative eDNA abundance also appeared to concur with species-specific habitat use in both marine (e.g., Jeunen et al., 2019; Port et al., 2016; Salter et al., 2019; West et al., 2020) and lotic ecosystems (e.g., Bylemans et al., 2018; Thalinger et al., 2019).

Taken together, our findings clearly show consistent spatial occupancy patterns over a 4-week period and at fine spatial resolution in a lentic system. Nevertheless, because each lentic system may have its own unique surface water flow patterns, circulation dynamics,

habitat heterogeneity, and species assemblage and distribution, the spatial representation of eDNA-based biodiversity and spatial occupancy patterns may be highly variable and will require case-by-case evaluation in different ecosystems.

4.4 | Conclusions

Although the advent of eDNA-based techniques is revolutionizing the fields of effective, high-throughput and noninvasive biomonitoring and biodiversity assessments, careful evaluation of how macrobial eDNA "behaves" in different habitats and what the limitations are remains essential for accurate interpretation and application of biodiversity monitoring and spatial detection. For instance, it is generally known that the presence of eDNA in a body of water is governed by its production rate, transport and persistence, processes which all depend on the targeted organisms, their biomass and the ecosystem considered. Nonetheless, one of the most critical, yet manageable steps in eDNA analyses is the collection of environmental samples in space and time. With this work we have shown that the spatial sampling design, especially in a lentic system, can profoundly affect the detection of the fish and amphibian community via eDNA. This is critical for the development of effective sampling protocols allowing accurate species detection and abundance estimates in lentic ecosystems. It is also crucial for the establishment of protocols to reveal spatial heterogeneity in eDNA patterns and potential habitat preferences or species interactions. In this context, we have demonstrated that eDNA metabarcoding may provide a powerful tool for the spatial monitoring of aquatic species distributions at a very local scale.

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AUTHOR CONTRIBUTIONS

R.B. conceived the study; R.B., D.H., S.N. and J.A. performed the fieldwork, S.N. and A.S. conducted all laboratory work, whereas sequence data analyses and statistical analyses were performed by R.B., A.H., T.R. and D.H. The manuscript was written by R.B., T.R. and A.H., with input from all authors.

DATA AVAILABILITY STATEMENT

Raw sequence data have been uploaded to the Sequence Read Archive (SRA) of NCBI under BioProject no. PRJNA616325. The demultiplexing script as well as a shell script used for all analyses described above are available at Zenodo (https://doi.org/10.5281/zenodo.3731310). The custom reference databases used for taxonomic assignments are available at Zenodo (https://doi.org/10.5281/zenodo.3730934).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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