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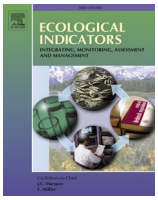

The true picture of environmental DNA, a case study in harvested fishponds

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ABSTRACT

The application of environmental DNA (eDNA) metabarcoding has revolutionised large scale biodiversity monitoring of aquatic ecosystems. Validation studies have been performed mainly in laboratories and mesocosm experiments, however large-scale field experiments are necessary to verify the robustness of eDNA based monitoring for more specific applications and different environmental conditions. Here, eDNA samples were collected from three fishponds with high fish density and broad species diversity during summer and autumn. This sampling design included a large number of spatial replicates evenly spaced across the pond surface and samples from the inflow, while pooled samples were used to test the effect of filtration volumes on detectability. Most common species were detected using eDNA, but rare species were often missed out under these high stocking densities. Average read counts and site occupancy positively correlated strongly with species abundance and biomass, with the exception of samples affected by PCR inhibition. Higher diversity detections were observed in autumn compared to summer samplings and in running compared to standing water. Fish communities detected in pooled samples reflect the overall community structure, and the species detectability increases with higher filtration volumes. This work highlights how eDNA based surveys can be optimised based on sampling conditions to achieve the highest overall detection, which has important implications for applying this method to aid management and policy initiatives.

1. Introduction

The knowledge of fish community composition is essential for fisheries management, but the traditional fishing methods are often invasive and species and/or size selective (Link, 2002, Kubečka et al., 2009). Environmental DNA (eDNA) metabarcoding of water samples has emerged as an alternative, non-invasive and highly sensitive method for describing biological community composition (Taberlet et al., 2018; Yamanaka & Minamoto, 2016). This approach is based on analysing DNA which is shed by organism into their environment (e.g., from mucus, remnants of skin, faces, blood and other body fluids) and has been applied successfully to monitor aquatic biota, especially fish (Wang et al. 2021, Keck et al., 2022). Collecting water sample is logistically and practically straightforward in most aquatic environments compared to traditional method. Due to their sensitivity eDNA methods have high detection probabilities for rare species which is a particular advantage for the early detection of invasive species or the monitoring of endangered species (Schmelzle & Kinziger, 2016). On the other hand, it is not possible to determine the size and age composition of a population, species hybrids, the sex ratio, or the fish condition by such metabarcoding methods (Olds et al., 2016).

Previous studies aimed at evaluating the effectiveness of this approach to accurately estimate relative abundance of a species in a fish community focused mainly on indirect comparison with traditional sampling methods (Hänfling et al., 2016; Keck et al., 2022; Perez et al., 2017) or direct comparison in controlled conditions (Davison et al., 2016; Evans et al., 2016). Comparisons between absolute fish abundance and eDNA metabarcoding estimates are rare and restricted to very small lentic water bodies (<1 ha; Li et al. 2019; Di Muri et al. 2020). Furthermore, the sensitivity of this approach needs to be evaluated in the context of different environmental conditions (Blabolil et al., 2021). For example, during the warm summer, increased DNA degradation

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occurs and eDNA's "lifespan" is short (hours), whereas, in cooler conditions with low activity of microorganisms and limited UV radiation, eDNA is detectable for a number of days, while in the frozen state for years and longer (Cristescuw and Hebert, 2018). The detection of rare species might depend on the overall fish density and species diversity in the community. The activity of fish, characteristic of species (e.g., territorial pike (*Esox lucius*) vs active asp (*Leuciscus aspius*)) and the sampling period (the highest amount of eDNA can be expected during fish spawning) is also crucial (Stoeckle et al., 2017).

Here, we compare the absolute fish community data from large aquaculture ponds with eDNA metabarcoding outputs. The studied ponds represent a very common type of waterbody in Central Europe; productive systems with a high fish density (Adámek et al., 2019; Roy et al., 2020). We aim to highlight important considerations for optimal sampling strategies. When water managers and stakeholders aim to apply such methods to novel habitats, it is important that sampling strategies are informed in an evidence-based manner. Specifically, we tested the relationship between semiquantitative eDNA metabarcoding proxies (relative read counts and site occupancy) and harvested fish abundance and biomass. We assessed the impact of sampling time (season) on eDNA metabarcoding performance in comparison to absolute fish community quantification. Finally, we consider sampling and filtration resolution, and the influence of increased sampling/filtration effort on species detectability.

2. Material and methods

2.1. Study sites

Three ponds in the Czech Republic were selected (Fig. 1), Pravikov (pond A), Kalich (pond B), and Kladiny Dolni (pond C) were of dimensions 2.60, 9.53, and 29.22 ha, maximum depth 2.0, 4.1 and 3.3 m and mean depth 0.91, 1.87, and 1.65 m, respectively. Fish were stocked between November 2018 and March 2019 (Supplementary Table 1) and harvested in autumn 2019 (24th September, 17th October and 13th November ponds B, A and C, respectively). All ponds were inspected twice per week to ensure a healthy fish community without excess mortality or poaching pressures. These ponds were drained to harvest the entire fish stock, which took four (pond A), ten (pond B) and twenty (pond C) days, respectively. Metal sieves (50 mm splicing) were installed in the discharge device during the whole season, supplemented by a net (10 mm knot-to-knot) during the fish harvest to prevent fish escape. Each pond was fed from the water of a small stream and for security reasons, inflows were kept open during the season. The water flow was consistent without any extreme flood event causing uncontrolled fish escape in or out the ponds. Additional fish stocking by local people cannot be excluded, but we trust the structure of the fish communities was stable from summer (after spring fish stocking, flushed from the upper catchment and natural reproduction) until the pond harvest in autumn. All harvested fish were determined to species level and sorted in individual fishing tubs. For each species a subset of 100–500 individuals was individually weighted, the rest weighted for each species jointly and the total species abundance estimated by proportion of total species biomass to average weight of the species.

2.2. eDNA sampling and laboratory processing

Water was sampled in summer (24th, 26th June, and 1st July) and autumn (13th, 15th, and 19th September) 2019 in ponds A, B, and C, respectively. Samples were taken at 39 equidistant points (mean ± SD distances 27.6 ± 4.9, 52.9 ± 8.8 and 97.0 ± 17.1 m in ponds A, B and C, respectively) across the ponds surface (Fig. 1) and from the inflowing brook to each pond (Supplementary Table 2). Water temperature (°C), concentration of dissolved oxygen (in mg/L and %), pH and conductivity (μS/cm) were measured by multiparametric handheld YSI PRO (https://www.ysi.com, USA) and transparency by Secchi disk at each

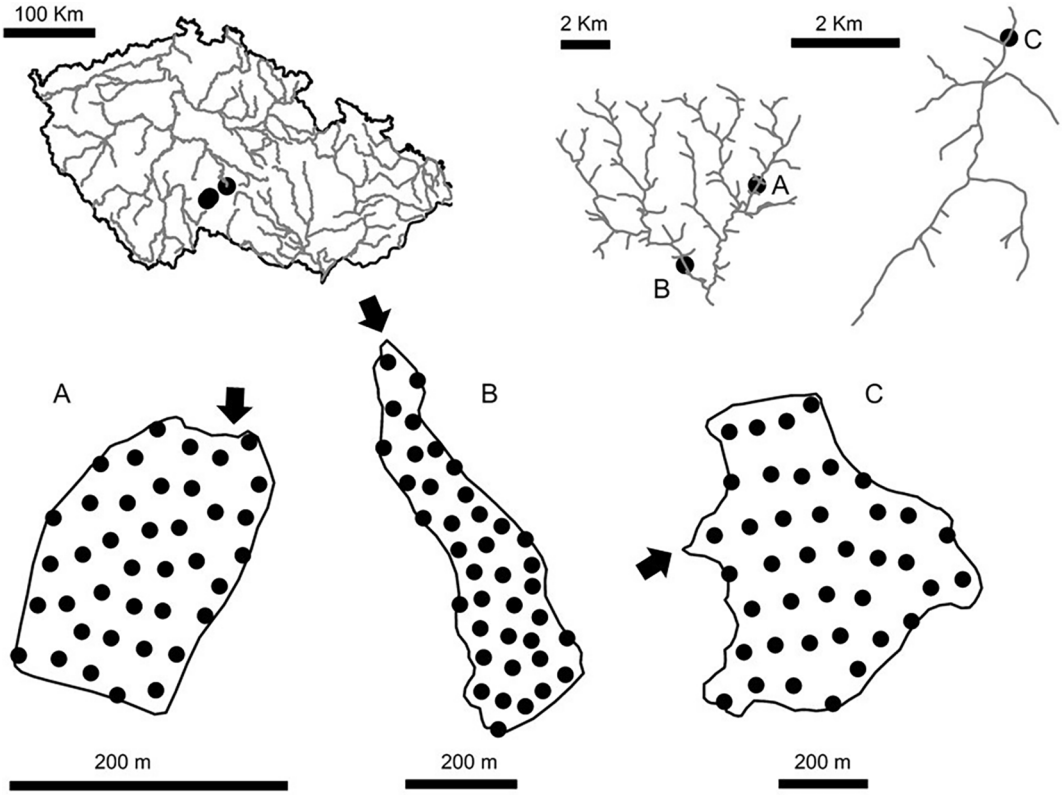

Fig. 1. Location of the studied ponds in the Czech Republic (upper left), location within catchments (upper middle and upper right) and position of sampling points in each pond (A Pravikov, B Kalich, C Kladiny Dolni). The arrows indicate inflows. All maps are North oriented.

sampling point.

At each pond five 400 mL subsamples were taken within 5, 10 and 15 m of the point in ponds A, B and C, respectively, and pooled in a two-litre sterile container. The samples were placed in a cool box filled with ice packs for storage at approximately 4 °C.

In the laboratory, 500 mL (2 × 250 mL) from each sample (39 from the pond and one from the inflow) was filtered through 47 mm diameter and 0.45 μm porosity sterile mixed cellulose acetate and cellulose nitrate open filters (Whatman, UK) within 24 h of sample collection. In addition, one litre of each sample (ponds and inflows) was pooled to make a mixed sample, from which three replicates each were filtered at volumes 500, 250, 125, 62.5, and 31.25 mL through the same grade filters. During each sampling event, three field/filtration blanks were included (n = 18) and processed together with water samples. The filters were stored in a sterile freezer at –23 °C until further processing. DNA was extracted following the Mu-DNA water protocol (Sellers et al., 2018).

The sequencing library was generated from uniquely indexed PCR amplicons. Primer pair 12S-V5-F (5́-ACTGGGATTAGATACCCC-3́) and 12S-V5-R (5́-TAGAACAGGCTCCTCTAG-3́), which amplifies a 73–110 (~106) bp fragment of the mitochondrial 12S ribosomal RNA (rRNA) gene in vertebrates (Riaz et al., 2011) were modified to nested metabarcoding approach by adding unique indexes, heterogeneity spacers, sequencing primers and pre-adapters. Each index was composed of a unique 8-nucleotide sequence allowing them to be bioinformatically linked back to the individual sample (Supplementary Table 3). The indexes were added to both the forward and reverse primers, to give 24 unique combinations of sample tags that can be arranged on a single plate (for details see Kitson et al., 2019). PCR negative (molecular grade water, n = 18) and positive (Zebra mbuna (*Maylandia zebra*) DNA, 0.05 ng/μl, n = 18) controls were included to detect possible contamination and inhibition within each sub-library. The first PCR for each sample, field/filtration blank, PCR negative control and PCR positive control was performed in 25 μL reaction volumes, including 12.5 μL Q5® High-Fidelity 2X Master Mix (New England Biolabs® Inc., MA, USA), 1.5 μL of each primer (10 μM, Integrated DNA Technologies), 0.5 μL Thermo Scientific™ Bovine Serum Albumin (Fisher Scientific, UK), 5 μL DNA, and 4 μL molecular grade water. PCR conditions were initial denaturation for 5 min at 98 °C, 35 cycles of 10 s at 98 °C, 20 s at 58 °C and 30 s at 72 °C, and final extension for 7 min at 72 °C. Three technical replicates were performed for each sample and then pooled to minimize PCR stochasticity. Pooled PCR products were visualized on a 2 % agarose gel stained with GelRed (Cambridge Bioscience, UK). PCR products were normalized into 18 sub-libraries (three per sampling campaign) based on the band strength observed for each sample (very bright = 5 μL, bright = 10 μL, faint = 15 μL, and very faint/no band = 20 μL) together with 10 μL of the field/filtration blanks and PCR negative controls, then 1 μL of the PCR positive controls (Alberdi et al., 2019). The normalized sub-libraries were purified using a double size selection protocol with Mag-Bind® TotalPure NGS magnetic beads (Omega Bio-tek, USA) to remove primer dimers and large secondary products by ratios of 0.9 × and 0.15 × magnetic beads to 100 μL of each sub-library.

The 18 purified sub-libraries were used as template DNA for a second PCR attaching Illumina indexes (Supplementary Table 3). The second PCR was performed in 50 μL reaction volumes, consisting of 25 μL Q5® High-Fidelity 2X Master Mix, 3 μL of each primer (10 μM), 4 μL purified product, and 15 μL molecular grade water. PCR conditions were initial denaturation for 3 min at 95 °C, 10 cycles of 20 s at 98 °C and 1 min at 72 °C, and final extension for 5 min at 72 °C. PCR was performed for sub-libraries in duplicate and replicates were pooled before visualization on a 2 % agarose gel stained with GelRed. The sub-libraries were purified using a double size selection protocol with magnetic beads to remove secondary products by ratios of 0.7 × and 0.15 × magnetic beads to 50 μL of each sub-library. The sub-libraries were normalized and pooled according to the number of samples each contained (without blanks and PCR negative controls) and their DNA concentration. The pooled library was purified again using the same volumes and ratios as the previous purification, and quantified by real-time quantitative PCR using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs® Inc., MA, USA) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, UK) following manufacturer's guidelines. Removal of the secondary product was verified using an Agilent 2200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA). Libraries were run at 13 pM with 10 % PhiX Control v3 on an Illumina MiSeq® using a MiSeq Reagent Kit v3 (600-cycle) (Illumina, Inc., CA, USA).

2.3. Bioinformatics analysis

Raw sequence reads were demultiplexed and analysed using metaBEAT v0.97.11 (https://github.com/HullUnibioinformatics/metaBEAT), a bioinformatics pipeline that incorporates open-source software. The program Trimmomatic 0.32 (Bolger et al., 2014) was used for quality trimming and removal of locus primers from the raw sequence reads. Average read quality was assessed in 5-bp sliding windows starting from the 3′ of the read, and reads were clipped until the average quality per window was above phred 30. All reads shorter than a defined minimum 90 bp read length were discarded. Sequence pairs were subsequently merged into single high-quality reads using the program FLASH 1.2.11 (Magoč & Salzberg, 2011). Reads surviving quality filtering and trimming were screened for chimeric sequences against a custom, curated reference database using the uchime_ref function implemented in vsearch 1.1 (https://github.com/torognes/vsearch). Sequences were compared against custom reference databases developed at the University of Hull for fish (https://github.com/HullUni-bioinformatics/Curated_reference_databases/tree/master/12S_Fish) together with the sequence for the positive control. Reads were then clustered using vsearch 1.1. Clusters represented by less than three sequences were considered sequencing errors and were omitted from further analysis. Nonredundant sets of query sequences were then compared to the reference database using BLAST (Zhang et al., 2000). BLAST output was interpreted using a custom python function, which implements a lowest common ancestor approach for taxonomic assignment, similar to the strategy used by MEGAN 5.10.6 (Huson et al., 2007). BLAST hits were only considered if they possessed 100 % identity query coverage. The sequences were assigned to species level with the exception European perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*) which can not be differentiated with this marker (Hänfling et al., 2016), further referred as *Perca* + *Sander* and used as "a single species". The field/filtration blanks and PCR negative controls were always without fish detections, Zebra mbuna was detected in PCR positive control together with three-spined stickleback (*Gasterosteus aculeatus*), that was not detected in regular samples and therefore treated as laboratory contamination (Supplementary Table 4).

2.4. Data analyses

To reduce possible false positives, records occurring with a sequence read frequency below 0.1 % were discarded. This threshold was applied to the proportional read counts for each taxon, which were calculated as read counts for a given taxon over the total read counts for each sample (Hänfling et al., 2016). The numbers of detected species by eDNA metabarcoding were compared by the generalized linear model (GLM) with season nested in pond identity, Poisson distribution log-link function, and Log-likelihood test. The relationship between the number of samples and the number of species was modelled by a rarefaction curve by iNEXT package (Chao et al., 2014). Species site occupancy was calculated as a percentage of samples with species presence compared to all samples in studied ponds. Because of eDNA and harvested fish abundance and biomass were not normal data distribution, log2 transformation was applied for the following statistical analyses. The relationships between eDNA metabarcoding data (average read counts and site occupancy) and fish in harvest (fish abundance and biomass)

per different species were compared by Spearman's rank correlation coefficient. The environmental variables and correlation coefficients were compared by paired *t*-test for two variables and analysis of variance (ANOVA) followed by Tukey HSD post-hoc test for more variables. Relationships between numbers of detected species and filtered volume were modelled by GLM with log-transformed water volume, season nested in pond identity, Poisson distribution log-link function, and Log-likelihood test. Finally, all records were plotted by a heatmap by package ggplot2 (Wickham, 2016). The analyses were carried out in R v.4.0.3 (R Developement Core Team, 2020).

3. Results

3.1. Environmental variables

The studied ponds were characterised by variable temperature and high primary production resulting in oxygen supersaturation and high pH in summer (Fig. 2). The measured environmental characteristics were significantly different between ponds, seasons (with exception of concentration of dissolved oxygen and conductivity) and the pond and inflow habitats (Table 1).

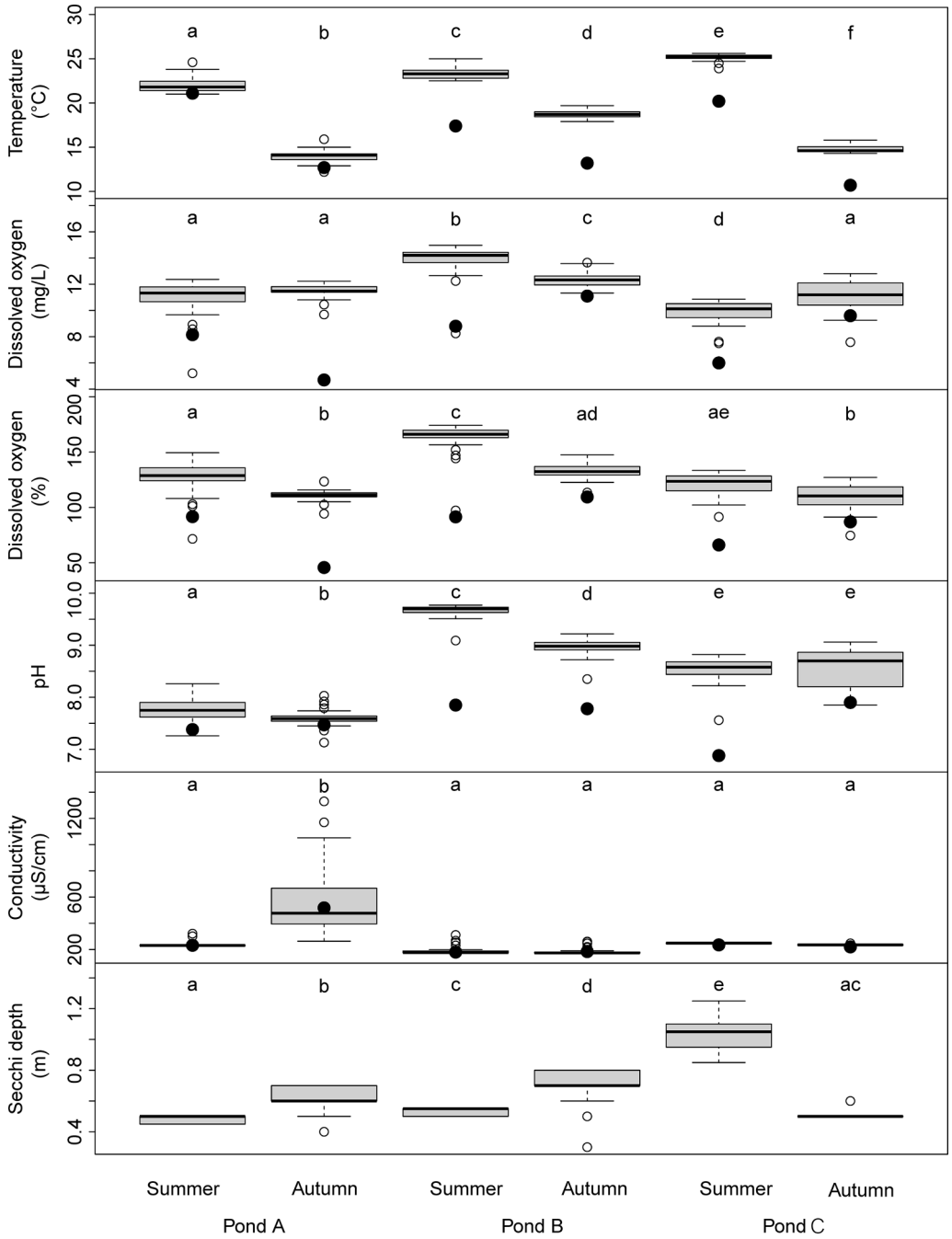

Fig. 2. Boxplots of the measured environmental variables. Letters above boxplots indicates statistically significant differences between experimental groups. Median values (thick lines), upper and lower quartiles (boxes), maximum and minimum values (whiskers), and outliers (empty dots) are shown. The black dots represent the values measured in inflows. The transparency in inflows was always up to the bottom and therefor is not shown.

Table 1
The results of statistical comparisons (analysis of variance) of environmental variables between ponds (A, B and C), seasons (summer and autumn), habitats (pond and inflow) and the interaction between the pond and season terms. The transparency in inflows was always up to the bottom.

	Pond			Season			Habitat			Pond:Season		
Characteristic	Df	F value	p value	Df	F value	p value	Df	F value	p value	Df	F value	p value
Temperature (°C)	2	415.5	<0.001	1	8285.9	<0.001	1	195.7	<0.001	2	394.2	<0.001
Dissolved oxygen (mg/L)	2	145.5	<0.001	1	0.3	>0.05	1	74.6	<0.001	2	38.1	<0.001
Dissolved oxygen (%)	2	220.3	<0.001	1	189.0	<0.001	1	106.6	<0.001	2	15.4	<0.001
pH	2	734.0	<0.001	1	60.6	<0.001	1	56.1	<0.001	2	41.8	<0.001
Conductivity (μS/cm)	2	86.0	<0.001	1	58.7	>0.05	1	0.3	<0.001	2	67.5	<0.001
Secchi depth (m)	2	220.2	<0.001	1	22.1	<0.001				2	462.1	<0.001

3.2. True fish community

In total 319,833 fish of 27,053.8 kg were harvested in the ponds. Eighteen fish species were captured in studied ponds, twelve in pond A, fourteen in B, and fifteen in C (Supplementary Table 5). The highest fish density in terms of abundance (1,854,393 ind./1000 m^3) and biomass (89,990 kg/1000 m^3) was in pond A, compared to broadly similar densities in pond B and C (429,780 and 413,872 ind./1000 m^3, 30,812 and 40,311 kg/1000 m^3, respectively). Eleven fish species (grass carp (*Ctenopharyngodon Idella*), common carp (*Cyprinus carpio*), pike, gudgeon (*Gobio gobio*), ruffe (*Gymnocephalus cernua*), *Perca* + *Sander*, topmouth gudgeon (*Pseudorasbora parva*), roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalmus*), wels catfish (*Silurus glanis*), and tench (*Tinca tinca*)) were detected in all three ponds. Freshwater bream (*Abramis brama*) was harvested in ponds A and C and six species were harvested only in one pond (B: bleak (*Alburnus alburnus*), stone loach (*Barbatula barbatula*), brown trout (*Salmo trutta*); C: white bream (*Blicca bjoerkna*), gibel carp (*Carassius gibelio*), burbot (*Lota lota*)). The most abundant fish species was ruffe (on average 34.8 %) followed by common carp and topmouth gudgeon (on average 24.4 and 20.5 %, respectively, Fig. 3). Common carp was the dominant fish species in biomass (on average 90.7 %), the second species grass carp reached on average 3.8 % biomass, and only pike and rudd exceed 1 % (Fig. 3).

3.3. eDNA metabarcoding

Most samples showed strong amplifications, apart from the summer samples from pond B where 75 % samples showed no or weak bands on an agarose gel after the 1st PCR. The total number of forward and reverse sequences across 384 samples (240 regular, 90 pooled eDNA samples, and 54 controls) was 39,727,310. Of these, 27,941,129 paired-end sequences passed the trimming quality filter and 25,755,257 were subsequently merged. 25,640,396 sequences remained after chimera detection and clustering. 15,572,644 sequences (61 % of the total) were assigned to fish with an average read count per sample of 47,190 (excluding control samples).

All samples detected 1 to 6 species, except for summer pond B where 64 % were negative. More species in total were detected in autumn compared to summer season in pond A (12/10) and pond B (9/2), with the same in pond C (11/11). The numbers of detected species in individual samples were different between ponds (Df = 2, loglikelihood deviance = 139.87, $p < 0.001$) and season (Df = 3, loglikehood deviance = 114.39, $p < 0.001$). (Fig. 4).

The only species detected in all campaigns was common carp with the highest average reads count of 28,961. Grass carp, roach, and rudd were detected in all campaigns except the summer pond B samples. Despite common occurrence, the numbers of reads of the three species

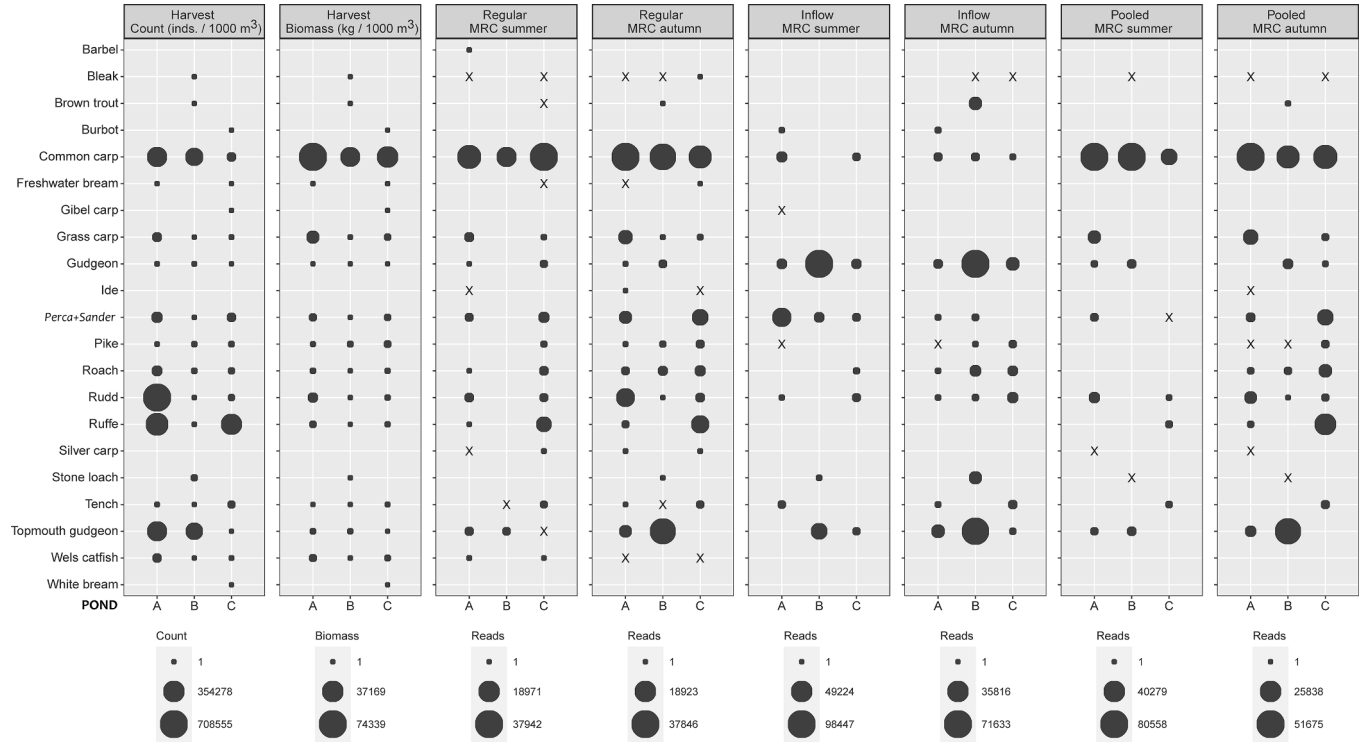

Fig. 3. Bubble plots depicting relative biomass, count and mean numbers of reads (MRS) using environmental DNA metabarcoding (circle size) of each species detected at studied ponds A, B and C. X symbol shows species detection below the threshold < 0.1 % of the proportional read counts for each taxon.

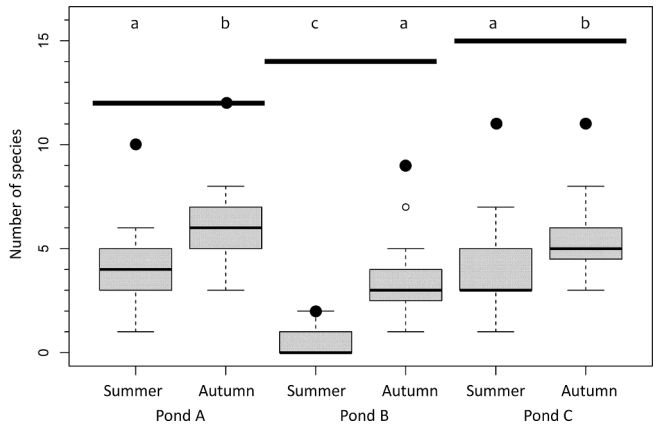

Fig. 4. Boxplots of the number of taxa detected in different pond environmental DNA (eDNA) samples in different seasons. Letters above boxplots indicates statistically significant differences between experimental groups. Median values (thick lines), upper and lower quartiles (boxes), maximum and minimum values (whiskers), and outliers (empty dots) are shown. The black dots represent the total number of taxa detected by eDNA in different seasons and solid lines the numbers of harvested taxa.

were an order lower compared to common carp (on average 1,265; 1,297; and 3,106; respectively). The site occupancy values were variable between ponds and seasons with exception of ubiquitous common carp. Between seasons, high consistency with site occupancy >70 % was observed for grass carp and rudd in pond A, and ruffe, *Perca* + *Sander* in pond C (Supplementary Fig. 1).

The rarest fish species detected only in one sample passing filtration threshold were stone loach (300 reads) and ide (*Leuciscus idus*) (361 reads) in pond A, stone loach (800 reads) in pond B, and freshwater bream (267 reads) and bleak (343 reads) in pond C.

3.4. Comparison between fish data from harvest and eDNA samples

The percentage of shared species detected at pond harvests and in both eDNA metabarcoding campaigns was 53.3, 14.3, and 47.1 in ponds A, B, and C, respectively. Adding the species detected only in autumn increases the proportion above 50 % in all ponds (Fig. 5). During the pond harvest 1 (freshwater bream), 5 (bleak, *Perca* + *Sander*, ruffe, tench, wels catfish) and 4 (burbot, gibel carp, topmouth gudgeon, white bream) species were captured but not detected in ponds eDNA compared to 3 (barbel, ide, silver carp (*Hypophthalmichthys molitrix*)), 0, and 2 (bleak, silver carp) species detected only in ponds eDNA in ponds A, B and C, respectively.

Significant positive relationships were observed between fish read counts and fish abundance/biomass (Fig. 6). The correlation coefficients were not significantly different between abundance and biomass relationships ($t = 2.11$, $df = 5$, $p = 0.088$), but were different between ponds (ANOVA, $F_{2,9} = 5.60$, $p < 0.05$) with significant differences between pond A and B (TukeyHSD, $p < 0.05$), and similar between seasons ($t = -0.611$, $df = 5$, $p = 0.568$). The strongest relationships were in pond A (Fig. 6 A, B) and the most consistent between summer and autumn seasons were in pond C (Fig. 6 E, F). The most different relationships were in pond B (Fig. 6 C, D).

Significant positive relationships were observed between fish site occupancy and fish abundance/biomass with exception of data from pond B in autumn (Fig. 7). The correlation coefficients were similar for abundance and biomass relationships ($t = 2.247$, $df = 5$, $p = 0.075$), but different between ponds (ANOVA, $F_{2,9} = 8.03$, $p < 0.01$) with significant differences between pond A and B, and B and C (TukeyHSD, $p < 0.05$), and similar between seasons ($t = -2.054$, $df = 5$, $p = 0.095$). The correlation coefficients were similar for average read count and site occupancy-based correlations with real fish abundance and biomass ($t = -0.984$, $df = 11$, $p = 0.346$).

3.5. Sampling effort in ponds

The numbers of samples required to gain 95 % of species were higher for summer samples compare to autumn ($t = -5.5$, $df = 2$, $p < 0.05$, Fig. 8) and the numbers were identical between ponds C and A (10/7 in summer and autumn, respectively) compared to pond B (14/9).

3.6. eDNA metabarcoding fish diversity in inflows

The numbers of detected species in inflows were higher in autumn compared to summer in pond A (7/5) and B (9/4) and identical in pond C (6/6). Gudgeon was the only detected species in all campaigns. Common carp, *Perca* + *Sander*, topmouth gudgeon, and rudd were detected in five of the six campaigns. The detected species in inflows were mostly identical to species detected in ponds (Fig. 3). Burbot was detected in summer and autumn only in inflow to pond A. In pond B *Perca* + *Sander* and in pond C topmouth gudgeon were detected in eDNA inflow in both seasons and in harvest, but not in eDNA in ponds.

3.7. eDNA metabarcoding fish diversity in pooled samples

The species composition in pooled samples reflects the species communities in studied ponds (Fig. 3). The only species detected in pooled samples and in pond harvest, but not in pond nor inflow eDNA

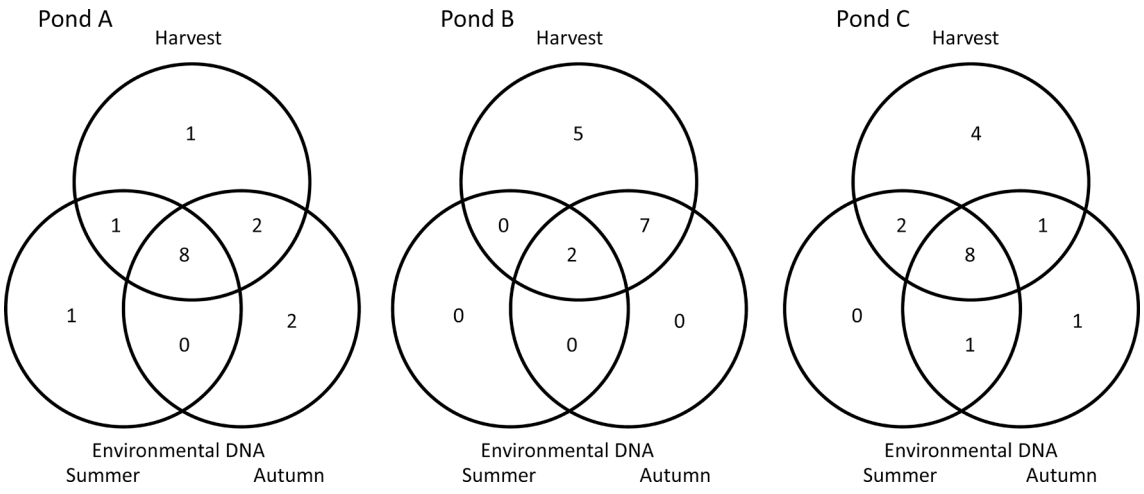

Fig. 5. Venn diagrams with numbers of detected taxa during ponds harvests, summer and autumn environmental DNA.

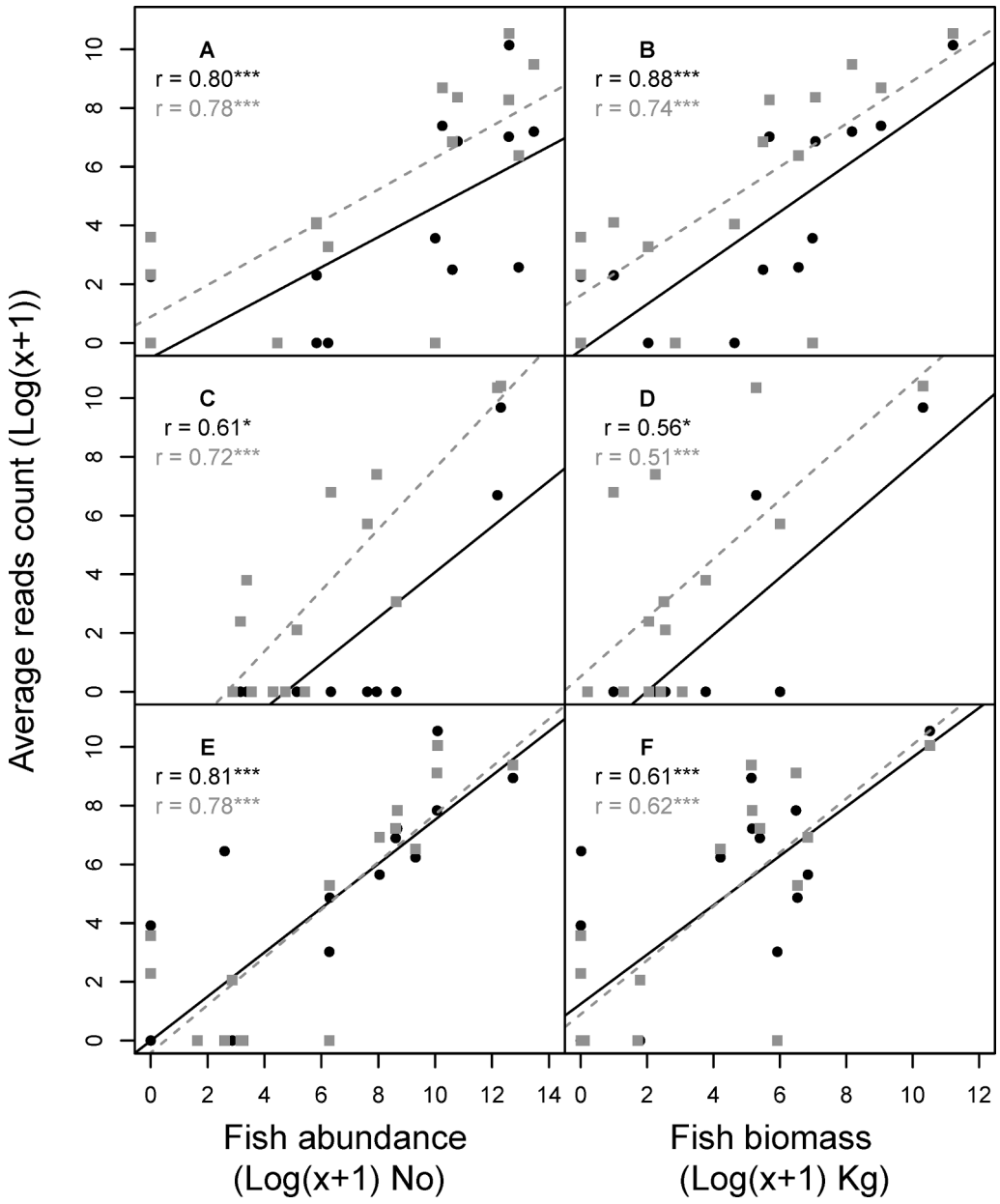

Fig. 6. Relationships between average read counts and fish abundance (A, C, E)/biomass (B, D, F) in ponds A (A, B), B (C, D) and C (E, F) in summer (black dots, solid line) and autumn (grey squares, dashed line). Spearman's correlations coefficients are added to each relationship (summer in black, autumn in grey) with significance $p < 0.001$***, <0.05*.

was gibel carp in autumn pond C. Common carp was detected in all samples except summer B of 250 mL filtered water without detection of any fish species. Negative samples were observed in summer, 66.7 % in pond B and 13.3 % in pond C. The average proportion of common carp reads was 64 % (13.3–100 %, without summer B 250 mL). The total numbers of detected species increase with sampled volumes ($Df = 1$, loglikehood deviance = 20.91, $p < 0.001$) and were different between ponds ($Df = 2$, loglikelihood deviance = 20.44, $p < 0.001$) and seasons ($Df = 3$, loglikehood deviance = 100.82, $p < 0.001$) (Fig. 9). The average numbers of detected species in pooled samples (500 mL filtered volume) were higher, with exception of summer samples in pond C, by 1 species compare to the average numbers of detected species in ponds, but not significantly ($t = 1.193$, $df = 5$, $p = 0.286$).

4. Discussion

In this study we were able directly compare absolute fish abundance and biomass from three large densely stocked ponds (2.6–29.2 ha) with results from eDNA metabarcoding. During the harvests >10 species were detected in each pond and >300,000 individuals and 27 tons of fish were individually determined to species level and from those >15,000 individually weighted. To our knowledge, this is the largest survey used for direct comparison with eDNA metabarcoding data. Our data showed that eDNA metabarcoding provides reliable species detection and accurate semi-quantitative estimates in these systems apart from samples which are affected by PCR inhibition.

This study can be considered as a case study for pond ecosystems, which are common worldwide (Downing et al. 2006). The fish communities in our study are typical for extensively managed large aquaculture ponds in Central Europe with one dominant commercial species (here common carp in biomass), a few supplementary commercially important species (e.g., grass carp, pike, wels catfish), supplementary prey species for predators (e.g., roach, rudd, ruffe) and additional minor species as accidental introduction with stocked fish (e.g., topmouth

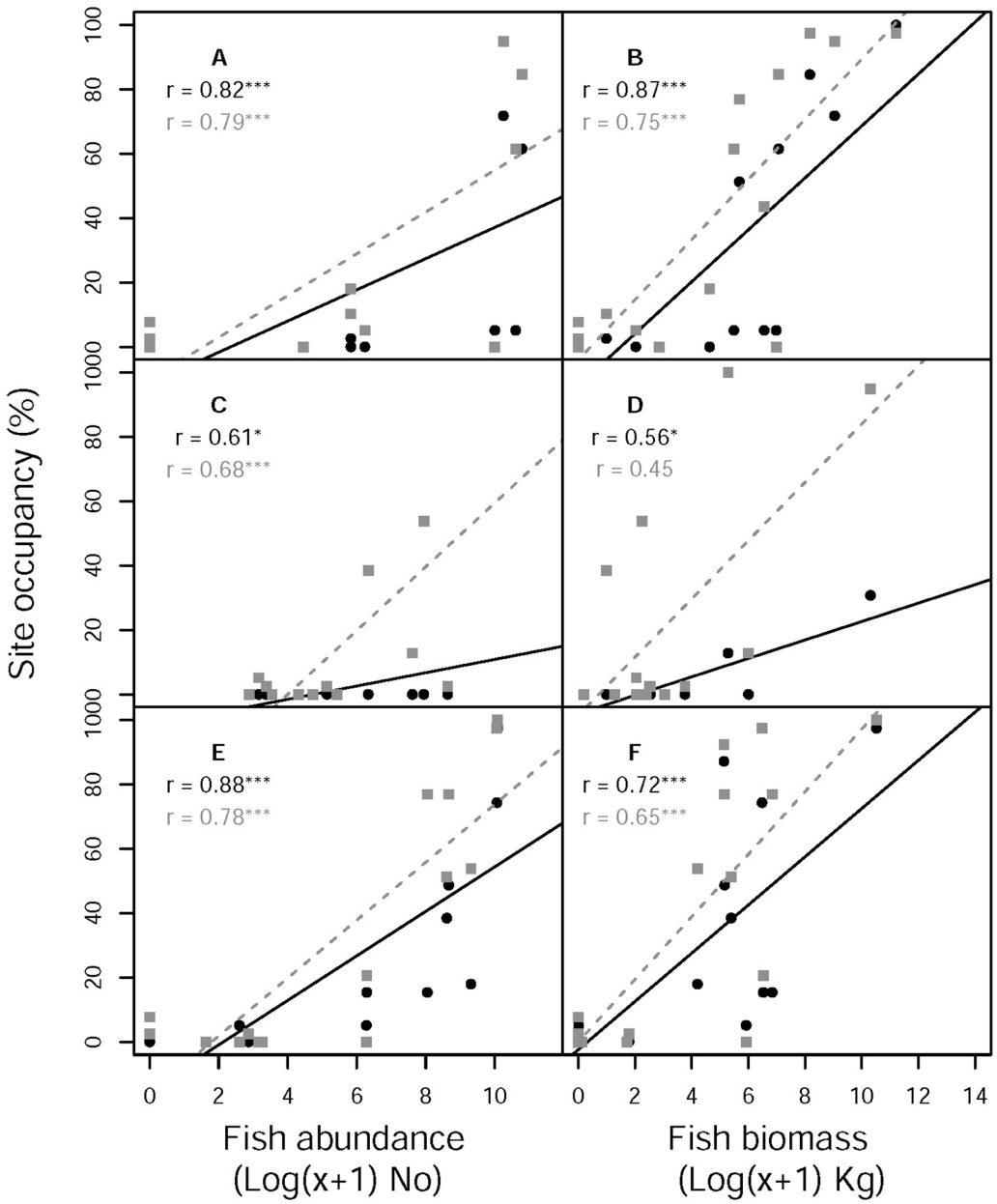

Fig. 7. Relationships between site occupancy and fish abundance (A, C, E)/biomass (B, D, F) in ponds A (A, B), B (C, D) and C (E, F) in summer (black dots, solid line) and autumn (grey squares, dashed line). Spearman's correlations are added to each relationship (summer in black, autumn in grey) with significance $p < 0.001$***, <0.05*.

gudgeon) or naturally occurred in the catchment (e.g., brown trout, stone loach). The commercially important fish were stocked as one to three years old specimens with high survival rate and narrow age distribution (Pechar, 2000), therefore, reducing the effect of different eDNA shedding rates from distinct life stages and age classes. Moreover, the ponds communities were similar in terms of overall fish densities and composition that positively influenced the reproducible and reliable quantitative characterisation.

4.1. Species detection

Despite a relatively low number of species detected in individual samples (1–6), the dominant species were detected in both sampling periods in the majority of individual samples. Furthermore, the total number of species detected across each pond and season was comparable to the numbers found in the fish harvest. This confirms previous studies regarding the effectiveness of eDNA metabarcoding to detect freshwater fish in lentic water bodies (Hänfling et al. 2016; Li et al. 2019; Zhang et al. 2020). Nevertheless, in our study some rare species which were present in the harvest were not detected in eDNA samples, especially during summer. It is possible that the high total fish densities and associated high overall fish DNA concentrations in the study ponds led to a masking of rarer species similar to rare elusive gobies (*Lythrypnus* sp.) in coastal marine protected areas (Gold et al. 2021). In a more natural environment with lower overall population density, the numbers of species detected may be higher as demonstrated in our previous study in protected drinking-water reservoirs (Blabolil et al., 2021). However, fish densities and community composition were comparable in Di Muri et al. (2020) and Li et al. (2019), where all species were detected from the albeit very small (<1ha) ponds.

In some cases, rare species were only detected by eDNA metabarcoding, but were not found in the pond harvest, i.e., barbel (*Barbus*

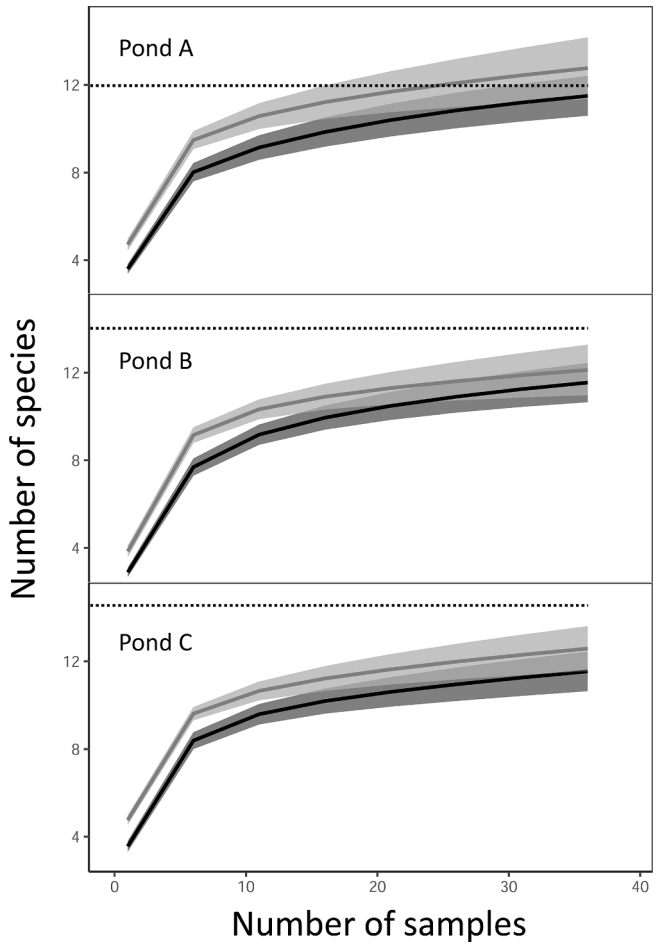

Fig. 8. Relationships between numbers of samples and cumulative numbers of detected species in summer (black line) and autumn (line in grey) in studied ponds. The horizontal dotted lines indicate the number of species harvested in each pond.

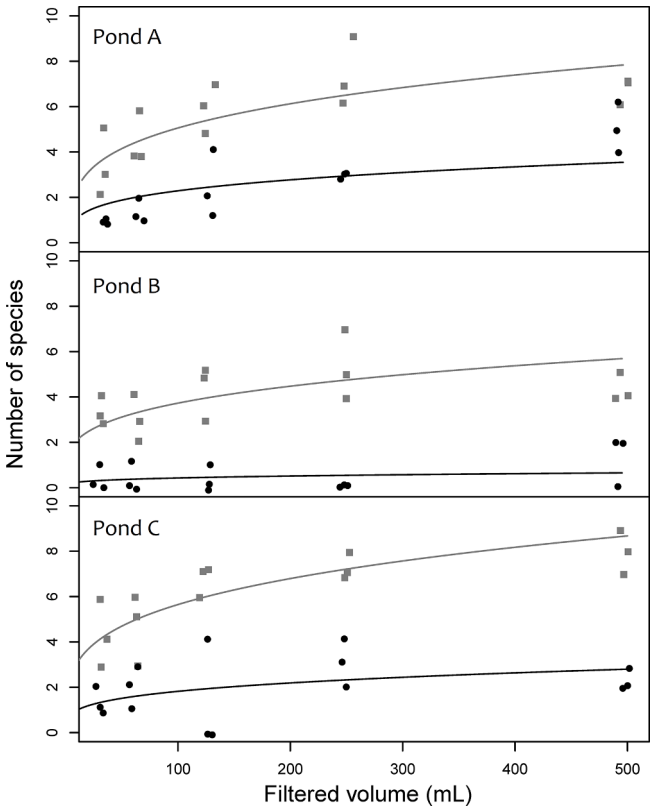

Fig. 9. Relationships between filtered volumes and number of detected species in summer (black dots) and autumn (grey squares) in studied ponds.

barbus) and ide, bleak and silver carp. Laboratory contamination is not likely as the detections were only in experimental samples, the negative controls were without fish detections and the only contamination was in one PCR positive control. It is possible that the rare and low abundance species were overlooked during the harvest of other fish, small specimens could be predated upon by larger stocked fish and thus not recorded in the harvest. The detections of rare species were more common in autumn compared to summer, when stronger water mixing due to more increased wind and more homogenous water columns occurred. Therefore, the eDNA could originate from resuspended sediment, as all the species were previously kept in the fishponds (Jaromír Křikava, Biofish s.r.o., personal communication). Another possibility is that the rare species detected with eDNA during the summer were no longer present when the ponds where drained in the autumn. Environmental DNA is more stable in sediment compared to water column and can be detected at time fish were dead or absent from the system (Sakata et al., 2020). The other explanation can be food waste for example silver carp is a popular food in the country or bleak used as bait fish by poachers.

Almost 40 % of cleaned sequences were assigned to non-fish taxa, (mainly human *Homo sapiens*), bacteria and degraded eDNA. Using different or additional primers that target bony fish, such as MiFish (Miya et al., 2015) and Teleo (Valentini et al., 2016) and have less non-fish bye-catch would therefore improve sequencing depth for fish and result potentially in higher detection probability of rare species. The reference database comprised all species living in the Czech Republic, and the BLAST similarity threshold of 98 % which was used is likely to account for any potential phylogeographic variation in barcode sequences (Gillet et al., 2018). Another potential option to increase the detection probability of rare species is the use of a PCR blocking primer designed to block the amplification of the most abundant species such as common carp eDNA (Rojahn et al., 2021) or to increase the number of PCR replicates per sample in order to further reduce stochastic variation during PCR (Harper et al., 2019). The detection probability was especially low for pond B during the summer. Most samples from this group showed and showed, no or very weak bands on an agarose gel, which is an indication of PCR inhibition, which can be the cause for false negatives (Bruce et al., 2021). Given that PCR inhibition was not prevalent in the autumn samples in this pond seasonal ecological variation is likely to account for this pattern. Previous studies have shown that complex humic substance are often responsible for PCR inhibition (Jane et al., 2015), but this is unlikely the case in these eutrophic ponds. From the ecological parameters measured here the summer samples of pond B stood out especially through a high pH $>$ 9 and elevated dissolved oxygen levels, but our dataset does not provide the opportunity to test the hypothesis that these factors caused PCR inhibition in our samples.

4.2. *Fish community structure*

In the artificial and heavily modified conditions of our study ponds, plastic, omnivorous species without particular habitat association prevailed (Blabolil et al., 2016). The dominant species, common carp, belongs to this category as is spread in many other countries in Europe (Souza et al., 2022). Dominance in biomass (i.e., common carp) seems to be more important than abundance (i.e., ruffe). Species traits should be taken into account as large-bodied common carp is highly mobile through water column compared to small-bodied ruffe associated with benthic habitat. The natural waterbodies in the Central Europe were small and middle size rivers with side oxbows (Hohensinner et al.,

2004). The fish community is not well adapted to lentic water conditions and majority of the species can be considered as ecologically plastic (e.g., freshwater bream, pike, roach). In terms of habitat associations, more species prefer benthic habitat (e.g., barbel, burbot, gudgeon, stone loach) compared to pelagic habitat (bleak, silver carp) (Kottelat & Freyhof, 2007). The detection of different ecological traits supports the applied sampling design in shallow waterbodies.

Two invasive species, topmouth gudgeon and gibel carp were detected (Lusk et al., 2010). These species were most probably accidental introduction of the commercial fish stock or were flushed from the upper catchment. Topmouth gudgeon is spawning multiple times during a vegetation season and therefore the population can increase almost exponentially (Kajgrová et al., 2022). The danger of gibel carp is in sexual parasitism (gynogenesis) during utilisation of same spawning area with other cyprinid species (Fuad et al., 2021). Populations of both invasive species suppress other (mostly native) species via food and space competitions. Early detection of such invasive species is therefore essential to maintain ecological stability.

The only detected endangered species was burbot, a typical cold-water species requiring high quality water and oxygen concentration (Blabolil et al., 2018). The burbot eDNA was consistently detected in inflow of pond A and 12 specimens were harvested in pond C. The inflows in both ponds have clear waters for the whole season and they might act as refugia for the species. Burbot is more active at low temperatures and the migration in pond C can be explained due to later pond harvest (13th November) compared to pond A (17th October).

Species-specific primers for invasive and endangered species have been developed and laboratory methods optimised. Previous studies confirmed the specific workflows as more sensitive compared to eDNA metabarcoding (Blackman et al., 2020; Harper et al., 2018). Therefore, the specific assays are recommended for early detection of invasions and/or measure of eradication effort success for topmouth gudgeon (Davison et al., 2019; Robinson et al., 2019) and gibel carp (Kamoroff & Goldberg, 2018). Environmental DNA metabarcoding was proven to detect invasive and endangered species and the complex information reflecting contribution of the species in a community is important for fisheries managers (Keskin, 2014).

4.3. Species eDNA correlates with the real fish community

The eDNA average number of reads and species site occupancy from ponds significantly positively correlated with fish abundance and biomass, with the exception of samples affected by PCR inhibition. Both eDNA proxies reflected the relative contribution of eDNA in the total dataset as confirmed previously in lakes in China (Zhang et al., 2020). During the PCR process, the template DNA is doubled in each step and thus the differences between common and rare DNA is increasing multiple times. Therefore, the reads counts have to be used in log-scale (with natural or 2 base) for correct interpretation. Our findings are in line with other studies corroborating the ability of eDNA metabarcoding to describe fish diversity in harvested ponds (Di Muri et al., 2020) and fish abundance estimates based on long-term data sets from trapping, gillnetting and recreational anglers' catches in lake Windermere in UK (Hänfling et al., 2016) and gillnetting in lake Aiguebelette in France (Civade et al., 2016). It is acknowledged that such correlation modelling is a simplification of the reality, as species can behave differently depending on age, physiology, life history and metabolic rate as well as on environmental conditions (Ruppert et al., 2019).

4.4. Importance of seasonal sampling

Physical, chemical and biological forces such as dilution, sedimentation and re-suspension, hydrolysis, oxidation and microbial activity, can influence eDNA persistence and dynamics within aquatic habitats (Goldberg et al., 2016). The studied ponds were close to each other, and sampling was conducted in a narrow time scale to ensure comparability and reliability of our results. More or equal species numbers were detected in autumn compared to summer, which may be due to prolonged eDNA persistence at colder temperatures, together with reduced microbial activities and lower UV-light degrading eDNA (Eichmiller et al., 2016). Summer sampling is more likely to reflect contemporary species distribution as fish are more active including higher metabolic rate and eDNA degrades faster (Eichmiller et al., 2016). At colder conditions, eDNA preserves for longer time and facilitates transport within the study system (e.g., water column mixing and possible sediment resuspension) as well as from connected running water (Blabolil et al., 2021). In our case, the temperature in autumn was mild ~ 15 °C and fish were pre-wintering, fish were active and the primary production was already behind summer peak (Sommer et al., 2012).

One-third of summer pond B samples were negative due to PCR inhibition suggesting a substance connected with the vegetation season caused this trouble. The oxygen supersaturation > 160 % and high pH > 9.6 were the highest in pond B indicating intensive primary production by phytoplankton. Vegetation matter (polysaccharides, polyphenols, pectin or xylan) is known to contain compounds that inhibit PCR (Schrader et al., 2012). Dead biomass and soil may contain humic and fulminic acids, which inhibit PCR even at low concentrations (Jane et al., 2015). The high pH could be another source of negative detection as Barnes et al. (2021) proved decreasing DNA detection with increasing pH in range 8.1 to 9.8. In lower ranges, the effect of pH can be negligible as Lacoursière-Roussel et al. (2016) demonstrated for pH from 5.6 to 7.9. We included BSA in PCR reactions to reduce inhibition (Strand et al., 2011) and attempted eDNA dilutions (McKee et al., 2015), however bands did not improve in post-PCR gel visualisation. For particularly problematic inhibition in environmental samples, the TaqMan Environmental Master Mix (Applied Biosystems) or using a variety of other DNA polymerases have been found to be effective for reducing inhibition in other studies (Albers et al., 2013).

4.5. Sampling strategy

Dominant species were easy to detect in our study, but more sample replicates were needed to capture rare and low abundance species in these high density ponds. The number of samples to detect 95 % of species by eDNA was slightly higher in summer compared to autumn, but in both cases close to 10 samples. In small 0.2 ha artificial ponds Di Muri et al. (2020) determined only six samples to detect all nine present fish species. The number of sampling replicates is therefore likely determined by the size of studied waterbodies and structure of the fish community.

Our pooled samples (pooled water aliquots of individual samples) consistently detected the common and in one case even a rare species (gibel carp). In the same water volume, the number of detected species were equal or higher compared to regular samples which is in contrast to previous studies (Di Muri et al., 2020; Zhang et al., 2020) failing to detect individuals of low-abundance taxa after water samples pooling. With the exception of a few samples, the pooled samples do not bring new information, however, in case of limited budget, the pooled samples can be cost-efficient. Sato et al. (2017) found similar fish communities in between regular and pooled samples and concluded the pooling might be useful potentially for among-site comparison of representative fish communities.

Higher volume of filtered water increases the eDNA capture yield and may increase the detection probability (Muha et al., 2019). In this study, pooled samples were tested, and less species were detected in volumes below 250 mL. The number of detected species in volume 250 and 500 mL was comparable. The filtered volume is dependent on filter porosity and numbers of particles in the water. The studied ponds were eutrophic with high phytoplankton production in summer, and the filtration of 500 mL through a filter of 47 mm diameter and 0.45 μm porosity took on average 35 min and 250 mL 15 min. Therefore, our study suggests that the lower volume could be more efficient overall. Li

et al. (2018) found the 0.45 μm filters performed the best in terms of total DNA yield, probability of species detection, repeatability within pond and consistency between ponds when studied filters of different pore-sizes. However, 0.8 μm filters provided a reasonable balance between filtration time and quantification efficacy and may be optimal in turbid, eutrophic, high fish density water bodies (Li et al., 2018).

5. Conclusion

These results provide further evidence that spatial and temporal replication in eDNA surveys is required for reliable estimates of species richness. We demonstrated that eDNA detection rate is enhanced with spatial and technical replication as well as with the increased filtered water volume.

This study provides evidence of the factors which influence the efficiency of eDNA metabarcoding campaigns in ponds environments when compared directly to known fish communities. Based on the knowledge of these factors, recommendations can be made to water managers and stakeholders regarding the optimal sampling seasons, sampling resolution requirements, filtration effort, and the overall inferences which can be reliably taken from outputs. By carrying out this direct comparison, this study further supports increased confidence in eDNA based decision-making, while also highlighting the importance of tailoring survey designs to project aims to maximise confidence.

CRediT authorship contribution statement

Petr Blabolil: Conceptualization, Methodology, Formal analysis, Resources, Visualization, Project administration, Writing – original draft. **Nathan P. Griffiths:** Investigation, Methodology, Writing – review & editing. **Bernd Hänfling:** Resources, Supervision, Methodology, Writing – review & editing **Tomáš Jůza:** Investigation, Writing – review & editing. **Vladislav Draštík:** Investigation, Writing – review & editing. **Jelena Knežević-Jarić:** Investigation, Writing – review & editing. **Romulo dos Santos:** Validation, Formal analysis, Writing – review & editing. **Tomáš Mrkvička:** Formal analysis, Methodology, Writing – review & editing. **Jiří Peterka:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2022.109241.

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