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The particle size distribution of environmental DNA varies with species and degradation

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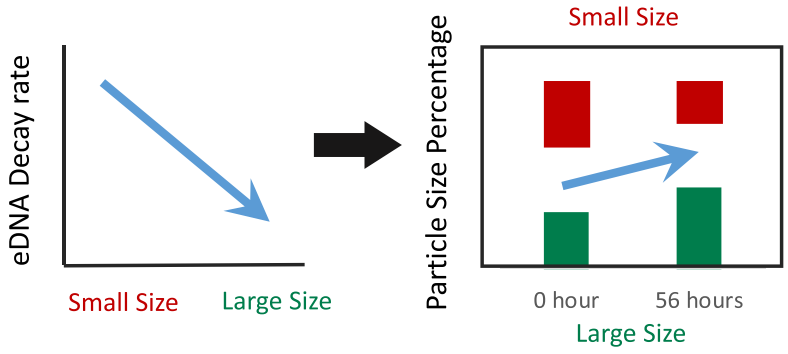
HIGHLIGHTS

- The persistence of eDNA may lead to a false-positive detection of organisms' presence.
- eDNA particle size distribution varies with species.
- Decay processes lead to a change in eDNA particle size distribution.
- eDNA particle size distribution has the potential to be a parameter to quantify eDNA decay.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental DNA (eDNA) analysis is frequently used as a non-invasive method to investigate species and biodiversity in ecosystems. However, such eDNA may represent both organisms currently present as well as species that released their DNA some point in the past, thereby representing a mix of current and historic biodiversity. This may lead to a false-positive detection of organisms' presence. As the eDNA particle size distribution (PSD) changes along with the decay process, it may facilitate solving the above problem. Here, we set up tank experiments with snails, zebrafish and daphnids, respectively, to monitor the change in eDNA PSD and eDNA degradation through time after removing organisms. We found that zebrafish eDNA decays more slowly for larger particle sizes. Across all species tested, the percentage of large size ranges tended to increase over time while the smaller sizes showed relatively fast decay rates. As a result, PSD changed consistently with eDNA decay, although initial PSD varied between species. In combination, we propose that eDNA PSD can be used to assess the current prevalence of organisms at an eDNA sampling location while avoiding false-positives on the presence of species. Our findings expand the applicability of eDNA for monitoring target species in freshwater ecosystems.

1. Introduction

Over the last decade, environmental DNA (eDNA) analysis has been successfully used to investigate the presence of aquatic macro-organisms (Alzaylaee et al., 2020; Brys et al., 2021; Holman et al., 2019; Mauvisseau et al., 2019). As aquatic macro-organisms continuously shed their genetic materials into the environment, the presence of species in that ecosystem can be inferred through detecting the DNA in water samples. This has proven to be especially successful for detecting rare and invasive species (Alzaylaee et al., 2020; Brys et al., 2021; Holman et al., 2019; Mauvisseau et al., 2019), and for analyzing community composition (Deiner et al., 2016; Djurhuus et al., 2018; Lacoursière-Roussel et al., 2018; Valentini et al., 2016). In various applications, eDNA analysis allows for more efficient and non-invasive surveillance compared with traditional methods, such as demersal

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trawl surveys (Salter et al., 2019), counting observations (Akre et al., 2019; Katano et al., 2017), or electrofishing (Pont et al., 2021). eDNA also has helped providing a better coverage of biodiversity patterns across temporal and spatial scales (Yamamoto et al., 2016).

However, the distribution of genetic material in combination with water flow in aquatic ecosystems can also obscure the exact position of organisms in the environment (Shogren et al., 2017). For example, after shedding, eDNA can persist for several days in a variety of water types (Barnes et al., 2014; Collins et al., 2018; Eichmiller et al., 2016; Strickler et al., 2015) and can move over hundreds of meters in aquatic systems, possibly leading to detection of eDNA while that organism is not or has never been physically present at that DNA sampling site (Bedwell and Goldberg, 2020; Deutschmann et al., 2019; Fremier et al., 2019). Therefore, both temporal and spatial factors can cause a mislinkage between the eDNA measured and the actual presence of the organism at a sampling site. This may lead to false-positive detection of species using eDNA techniques, i.e. when eDNA is detected but the species is absent (Buxton et al., 2021; Ficetola et al., 2015), which in turn can result in incorrect inferences on aquatic biomonitoring and corresponding water management measures.

While detailed spatial sampling in combination with modeling of water currents was demonstrated to be able to solve the spatial discrepancy between eDNA and organism presence (Thalinger et al., 2019; Yamamoto et al., 2016), resolving the temporal divergence between organism and eDNA presence in the ecosystem has remained difficult. To solve this temporal eDNA mismatch, one needs a method to evaluate the time interval between the disappearance of an organisms and the moment of eDNA sampling at a certain site. The exponential decay model $N(t) = N_0 e^{-\lambda t}$ has been commonly used to estimate eDNA decay rate (Eichmiller et al., 2016; Wood et al., 2020). After transforming this model to $t = -[\ln(N(t) / N_0)] / \lambda$, this model allows estimating the time period after shedding. This approach is complicated by two factors. First, this demands an understanding on the relative rate of eDNA decay, λ. Previous studies have shown that many environmental factors have an effect on eDNA decay rate, such as temperature, UV, and the density of organisms (Barnes et al., 2014; Eichmiller et al., 2016; Strickler et al., 2015). Estimating the decay rate and thus the retention time of eDNA, thus demands a thorough understanding on its environmental drivers. Second, in addition to the sampled eDNA amount (or concentration) $N(t)$, the initial amount (or concentration) of eDNA (N_0 in the equation above) is also needed, which is impossible to obtain with current technologies for most field studies. Up to date, there has been no study that has shown how to solve this problem, or allowed to assess the degree of eDNA degradation of field samples.

An alternative method to eDNA decay rate and concentration for evaluating the time interval since shedding eDNA is to use the characteristics of the eDNA particles themselves. eDNA includes various kinds of genetic materials from cell clusters to nuclear fragments with different sizes, as described by the eDNA particle size distribution (PSD). This term was first proposed by Turner et al. to study how organisms release their DNA (Turner et al., 2014). Since then, eDNA PSD has been used to study PSD differences between mitochondrial DNA and nuclear DNA, and to optimize filter sizes for eDNA capture (Jo et al., 2019b; Sassoubre et al., 2016; Turner et al., 2014; Wilcox et al., 2015). Importantly, the eDNA PSD also changes over the time period of degradation as the tissue, cells, and particles break down (Sassoubre et al., 2016). While previous studies (Jo et al., 2019b; Sassoubre et al., 2016; Turner et al., 2014; Wilcox et al., 2015) have shown that degradation occurs, they did not show whether changes in the eDNA PSD can be used to predict the degree of eDNA degradation. For that purpose, we need to comprehensively analyze the eDNA PSD over the entire time period from the removal of organisms up to the total disappearance of that particular eDNA from the environment. So far, such analysis has not been performed.

Previous research that used eDNA PSD have mostly used fish as a target species (Jo et al., 2019b, 2019a; Turner et al., 2014; Wilcox et al., 2015). Only one PSD study used an alternative species, *Daphnia magna* (Moushomi et al., 2019). The lack of different target species in PSD studies poses a problem as different species might release eDNA in different states (intracellular, intramembranous, extracellular, particulate) due to their general physiological differences, which might in turn affect the decay rate differently. This might result in situations where different species have shed their DNA at the same time but show different degrees of degradation at the same subsequent sampling point, purely because the states of eDNA for these species were different. For example, apart from faeces, fish and snails are expected to continuously shed off individual cells through their mucous layers as their primary source of DNA. Potentially, this could result in fish and snail eDNA to decay relatively fast. Besides, fish need to ventilate their gill chamber, while snails breathe air through their skin, lungs or gills. Additionally, snails physically crawl over surfaces with their foot, while fish mostly move through the water column. These differences potentially lead to differences in released eDNA states and decay rates. In contrast, the eDNA of exoskeleton-shedding invertebrates (such as *D. magna*) will first have to detach from the cellular structure of the exoskeleton before it is readily accessible to DNA degrading organisms. Their eDNA may therefore decay much slower than fish eDNA. Consequently, it is important to understand how different model taxa and the corresponding modes of shedding might affect the temporal change of the eDNA PSD before it can be used to determine when an organism was present in the ecosystem based on an eDNA sample.

Here we aim to use eDNA PSD for evaluating the changes in the degree of eDNA degradation over time, to ultimately move towards determining how long ago an aquatic organism was present in the ecosystem based on eDNA measurements. Specifically we were interested in studying i) the differences in PSD of released eDNA among model taxa, and ii) how eDNA PSD changes with the degree of degradation through time. For this purpose, three model species were evaluated separately; zebrafish (*Danio rerio*) covered by scales with collagen, daphnia (*D. magna*) with an exoskeleton and a snail species (*Lymnaea stagnalis*) with a shell and mucosal epidermis. Droplet digital PCR (ddPCR) was used to quantify the eDNA concentration of five particle size ranges (>5, 1.2-5, 0.8-1.2, 0.45-0.8 and 0.2-0.45 μm) of the different model organisms.

2. Materials and methods

2.1. Experimental setup and sampling

Three long-term and easily cultured model species (zebrafish, daphnia and pond snail) in Leiden University were chosen to represent the model taxa separately, a fish, a zooplanktic crustacean, and a snail, as their physiological differences probably lead to different states of released eDNA. The culturing of the snail and daphnia was done in a climate room at Leiden University, the Netherlands, from April to October 2019. The temperature set point of this climate room was 22 °C, with light between 7 am and 11 pm every day. They were cultivated for five days prior to experimentation, with densities of roughly 200 and 7 individuals/L, respectively. Zebrafish were maintained at Leiden University according to standard protocols (http://ZFIN.org), with densities of roughly 20 individuals/L at 26 °C. The culturing water of the zebrafish was transferred to the same climate room as above within 20 min prior to experimentation. We assumed that, the effect of the short time that it takes for 5 L water to decrease 4° on eDNA decay, compared to the 56 h of the whole process is limited. Additional information of the culturing procedure is provided in Text S1. The present study targets the eDNA PSD after removing the organisms. Therefore the different densities and culturing conditions between species do not influence the results.

For each of these three species, six tank experiments were run in the climate room as replicates and simultaneously for a given species (Fig. 1). For each experiment, 5 L water was sampled to a sterilized

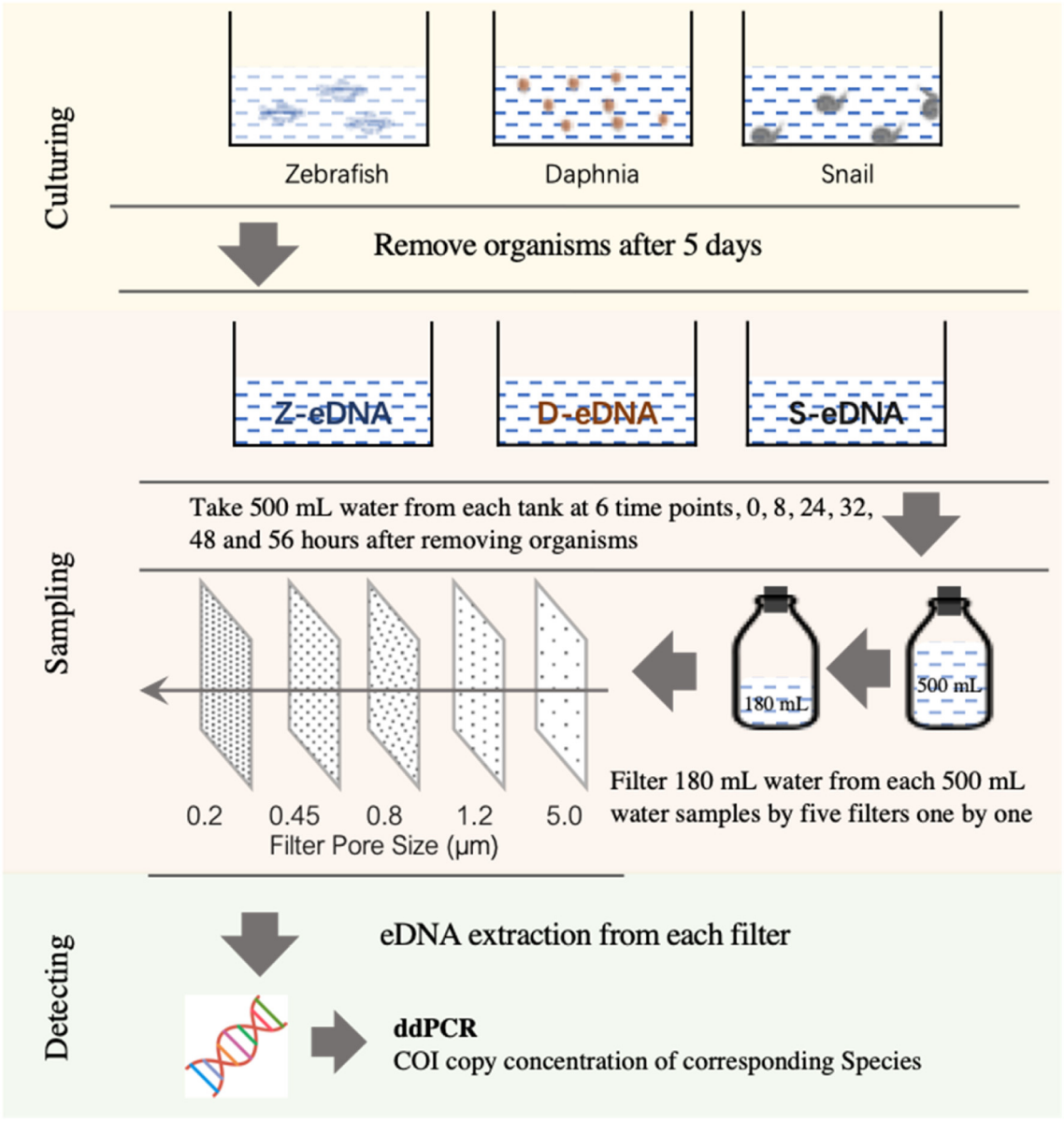

Fig. 1. Flow chart of the presented experiments. The whole experiment included three steps; culturing, sampling and detecting. First, zebrafish (*D. rerio*), daphnia (*D. magna*) and snail (*L. stagnalis*) were pre-cultured under particular physical situations (yellow background). Second, 500 mL water samples were taken from each tank after gently mixing the water at six time points, i.e. at 0, 8, 24, 32, 48 and 56 h after removing the organisms (*Z*-eDNA, zebrafish culturing water; D-eDNA, daphnia culturing water; S-eDNA, snail culturing water). 180 mL water of each 500 mL water samples was filtered using five polyethersulfone (PES) membrane filters with different pore sizes (5, 1.2, 0.8, 0.45 and 0.2 μm) sequentially (pink background). Last, DNA was extracted from every filter separately to evaluate the copy concentration of Cytochrome c Oxidase subunit 1 (COI) gene region through ddPCR species-special detection, using corresponding primers and probe (green background).

tank. The sterilized tank was covered with plastic wrap to protect the water from evaporation, and punctured using a sterilized tweezers for aeration. At 0, 8, 24, 32, 48 and 56 h after setting up a tank, 500 mL water samples were taken after gentle stirring. For each 500 mL water sample, after gentle stirring, 180 mL water was filtered by a plastic syringe (BD Plastipak™) using serial polyethersulfone (PES) membrane filters of pore sizes equal to 5, 1.2, 0.8, 0.45 and 0.2 μm, respectively (Fig. 1), to capture cells, mitochondria, incomplete mitochondria, and two size ranges of DNA combined with other organic materials separately. eDNA in the <0.2 μm size range was not included in the present study. The 5, 1.2 and 0.8 μm PES membranes were from Sterlitech whereas 0.45 and 0.2 μm filters were from Sartorius™. After filtering, each filter was immediately put into a 2 μL tube together with 700 μL CTAB Lysis buffer (AppliChem GmbH, DE) and stored at 4 °C. Prior to this process, all injectors, membrane containers, and glass wear had been soaked in 10% bleach over 10 min and washed by deionized water, then air dried on clean paper towels.

2.2. eDNA extraction and quantification

One day after filtering the water sample, eDNA extraction was performed following a standard CTAB protocol (Barnes et al., 2014; Turner et al., 2014) and eluted in 100 μL Tris-EDTA buffer solution (Sigma-Aldrich, US). In addition, tissue DNA of each species was extracted using the Qiagen blood and tissue kit following the manufacturer's protocol, as positive controls for further eDNA quantification. Zebrafish DNA was extracted from 5-days larva to meet the requirement of the local animal welfare committee of Leiden University (License number: 10612). All extracted DNA was tested by NanoDrop 2000 (Thermo Scientific) and stored at -20 °C till further quantification.

Quantification of eDNA concentration of target species was performed through measuring the copy number of cytochrome *c* oxidase I (COI) gene region using the QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad). The primers and probe assay for each of the three target species were designed using Geneious Prime software (V. 2019.2.1) according to the requirements of the ddPCR protocol (Droplet Digital™ PCR Applications Guide-Bio-Rad, https://www.bio-rad.com/). The COI gene sequence of *D. rerio* (GenBank: MK572160.1), *D. magna* (GenBank: MG317471.1) and L. *stagnalis* (GenBank: MG421376.1) were used as reference sequence for zebrafish, daphnia and snail separately. After that, the specificity of each primers and probe assay was assessed *in-silico* using the nucleotide blast function of NCBI (National Centre for Biotechnology Information; https://www.ncbi.nlm.nih.gov/). The results showed that each assay only amplifies the target species and locus. Primer sets and probes were ordered from Sigma-Aldrich (https://www.sigmaaldrich.com/).

Each 20 μL ddPCR reaction mix contained 8 μL DNA template, 900 nM of each forward and reverse primers, 250 nM of TaqMan probe, 10 μL ddPCR™ Supermix for Probes (No dUTP) and nuclease-free water, and transferred to the middle line of DG8™ Cartridges to generate droplets. 70 μL of Droplet Generation Oil for Probes was added to every oil hole on the cartridge. They were covered by DG8™ Gaskets, and smoothly put into the QX200 Droplet Generator. After about 3 min, 40 μL generated droplets of each sample was transferred to ddPCR™ 96-Well Plates. Each plate was only used for one species. Two negative controls using Tris-EDTA buffer solution and one positive control using the corresponding tissue DNA of the different species as template were set for each ddPCR plate. Thermal reactions were carried out as follows: 10 min at 95 °C, 40 cycles of 30 s at 94 °C, and 1 min at the annealing temperature of corresponding primers (Table 1), then 10 min at 98 °C before 4 °C conservation. Every sample was tested twice. Given the high accuracy among technical replicates and the nature of the ddPCR analysis (Doi et al., 2015; Nathan et al., 2014), two technical replicates was sufficient, and complies to procedures of other ddPCR studies (Jerde et al., 2016; Mauvisseau et al., 2019). After the PCR, each plate was placed into a QX200 Droplet Reader. Concentrations in copies/μL of each reaction were calculated by QuantaSoft (V.1.7.4, Bio-Rad) through merging the two replicate measurements, using the ratio between positive and negative droplets assuming a Poisson distribution (Miotke et al., 2014). Fluorescent thresholds for positive signals of droplets were determined based on the positive and negative controls following the Quantasoft manual. The ddPCR results were converted into eDNA concentrations of the culturing water (copies/L) based on the various dilutions involved in the analysis process.

2.3. Statistical analysis

R version 3.6.1 software was used to process all statistical analysis and generate figures in the present study. Two tanks of the snail culture water were excluded due to missing samples at two time points due to operation mistakes, allowing only four tank experiments of snails. In seven DNA samples from the second replicate experiment for snails, an extremely low concentration of the total extracted DNA (<0.1 ng/μL, NanoDrop results) indicated the failing of CTAB DNA extraction, and these were also removed from the statistical analysis. One tank experiment of daphnia was also removed, as its eDNA concentrations were much higher on the third day than on the second day, indicating contamination. One possible reason is that (too small to capture) larvae were left during the organism removal step at the beginning of the experiment, and captured on the filter after growing in the tank for three days. This situation did not occur in other tanks.

First, we investigated the differences in eDNA concentration over time and filter pore size, using a two-way ANOVA run for each species separately. To better understand the significant interactions (see results), a follow-up analysis was run using a one-way ANOVA, evaluating changes in the eDNA concentration of each size range and species combination. The *P* value threshold was Bonferroni-corrected within each species. After that, Tukey HSD post-hoc test on each one-way ANOVA were run. All eDNA concentrations, including zeros, were $\log_{10}(X + 1)$-transformed prior to analysis to meet the assumption of normality.

Second, to compare the eDNA PSD over species and time, the percentage of eDNA at each size range was calculated for each time point and every species. By using the ggpubr package (V.0.4.0, https://github.com/kassambara/ggpubr) in R, a Wilcoxon signed-rank test was performed to determine whether the initial percentage of eDNA was significantly different between species, for each eDNA particle size range separately. Next, an PERMANOVA test was carried out using the vegan package (V.2.5-7, https://cran.r-project.org/web/packages/vegan/) to evaluate the effect of time and species on eDNA PSD. Water samples with no eDNA at any particle size range were not included in the PSD analysis.

Third, eDNA decay rate constants were obtained using the easynls package (https://rdrr.io/cran/easynls/), through fitting eDNA concentration data to the exponential decay model $N(t) = N_0e^{-\lambda t}$, with N(t) and N_0 indicating the eDNA concentration at time T and time 0, respectively, and λ the decay rate constant. The eDNA retention time (hours) was estimated based on λ, and defined as the time to obtain a eDNA concentration equal to only 5% of the initial concentration (N_0) (Strickler et al., 2015), i.e. eDNA retention time (hours) $= -\ln(0.05) / \lambda$. A Kruskal-Wallis rank sum test was used to determine whether there were statistically significant differences in the decay rate constants and the estimated retention time between different eDNA particle size ranges. In the tanks with eDNA of daphnia and snails, the decay was much faster than in the tanks with zebrafish eDNA. This caused few time points with non-zero eDNA concentrations (>0.1 copies/μL), not allowing enough data to run the decay model for these two species. Therefore, only zebrafish data was used in this calculation. Two in thirty datasets (six tanks × five size ranges) of zebrafish for which the model could not fit the data (i.e. having a $R^2 < 0.3$ and an insignificant *P*-value for the fit) were excluded from further analysis.

3. Results

3.1. Temporal changes of eDNA concentration

For all three species, the eDNA concentration showed rapid declines at all size ranges over time (Figs. 2, S1, Table S1-3). Two-way ANOVA

Table 1
Species-specific primers and probes used for amplification of Cytochrome c Oxidase subunit 1 (COI) gene region in ddPCR detection in this study.

Species	Sequences (5′-3′)	Gene	Length (bp)	Amplicon length (bp) (bp)	ddPCR annealing temperature (°C)
Danio rerio	F: GGTGCTTGAGCCGGAATAGT	COI	20	73	55
	R: GTGCTCCTGGTTGGCTAAGT		20		
	FAM- ACCGCATTAAGCCTCTTAATCCGA -BHQ1		24		
Daphnia magna	F: CTAAGTTTGCCCGTTCTTGC	COI	20	81	55
	R: AGCCGGATCAAAGAACGAAG		20		
	FAM- AGCAATTACCATACTCTTAACTGACCGT -BHQ1		28		
Lymnaea stagnalis	F: CTAGCATAGTTGAAGGTGGG	COI	20	95	50
	R: GGCCAAATCCACAGATGAAC		20		
	FAM- CCCCTAAGAGGTCCTATTGCTCATGG -BHQ1		26		

tests indicated that both time and size range have significant effects on eDNA concentration for all species ($p < 0.01$ for all main effects). Additionally, time and size range showed an interaction for zebrafish ($p < 0.05$).

To further understand the different effect of time on the eDNA concentration among size ranges, subsequent one-way ANOVAs were run (Table 2). For daphnia, time did not show a significant impact on the eDNA concentration for the 0.2-0.45 and 0.45-0.8 μm size ranges. For snails, the effect of time was not significant for <1.2 μm, while very strong for all >1.2 size ranges. For zebrafish, on the other hand, time seemed to have similarly strong impacts at the different size ranges except for the >5 μm size range. Results for the Tukey HSD post-hoc illustrate the difference between time points depending on both size range and species (Fig. 2).

3.2. eDNA PSD differences with species and time

The initial eDNA PSD across the different filter sizes of different species normalized to percentages is shown in Fig. 3. For all three species, most eDNA was from particles larger than 1.2 μm, with very few eDNA occurring at the smaller particle size ranges. The Wilcox signed-rank test showed significant differences in the percentage of eDNA particles between species at both >5 μm and 1.2-5 μm ranges, especially between zebrafish and daphnia. At the smaller eDNA sizes (0.2-1.2 μm), no significant differences occurred except for the difference between snail to the other two species at 0.8-1.2 μm range.

Table 2
Results of the ANOVA test for the effect of time on eDNA concentrations at each size range.

Size range	Species	P value	
0.2-0.45 μm	Zebrafish	3.017e-05	**
	Daphnia	0.2218	
	Snail	0.04893	
0.45-0.8 μm	Zebrafish	0.004462	*
	Daphnia	0.02103	
	Snail	0.03421	
0.8-1.2 μm	Zebrafish	3.468e-05	**
	Daphnia	0.001675	**
	Snail	0.3197	
1.2-5 μm	Zebrafish	1.517e-09	**
	Daphnia	0.0001229	**
	Snail	0.001028	**
>5 μm	Zebrafish	0.03394	
	Daphnia	7.34e-06	**
	Snail	1.111e-05	**

Asterisks show the corresponding factors that are statistically significant after applying a Bonferroni correction (*, $P < 0.01$ (corresponding to an original $P < 0.05$); **, $P < 0.002$ (corresponding to an original $P < 0.01$)).

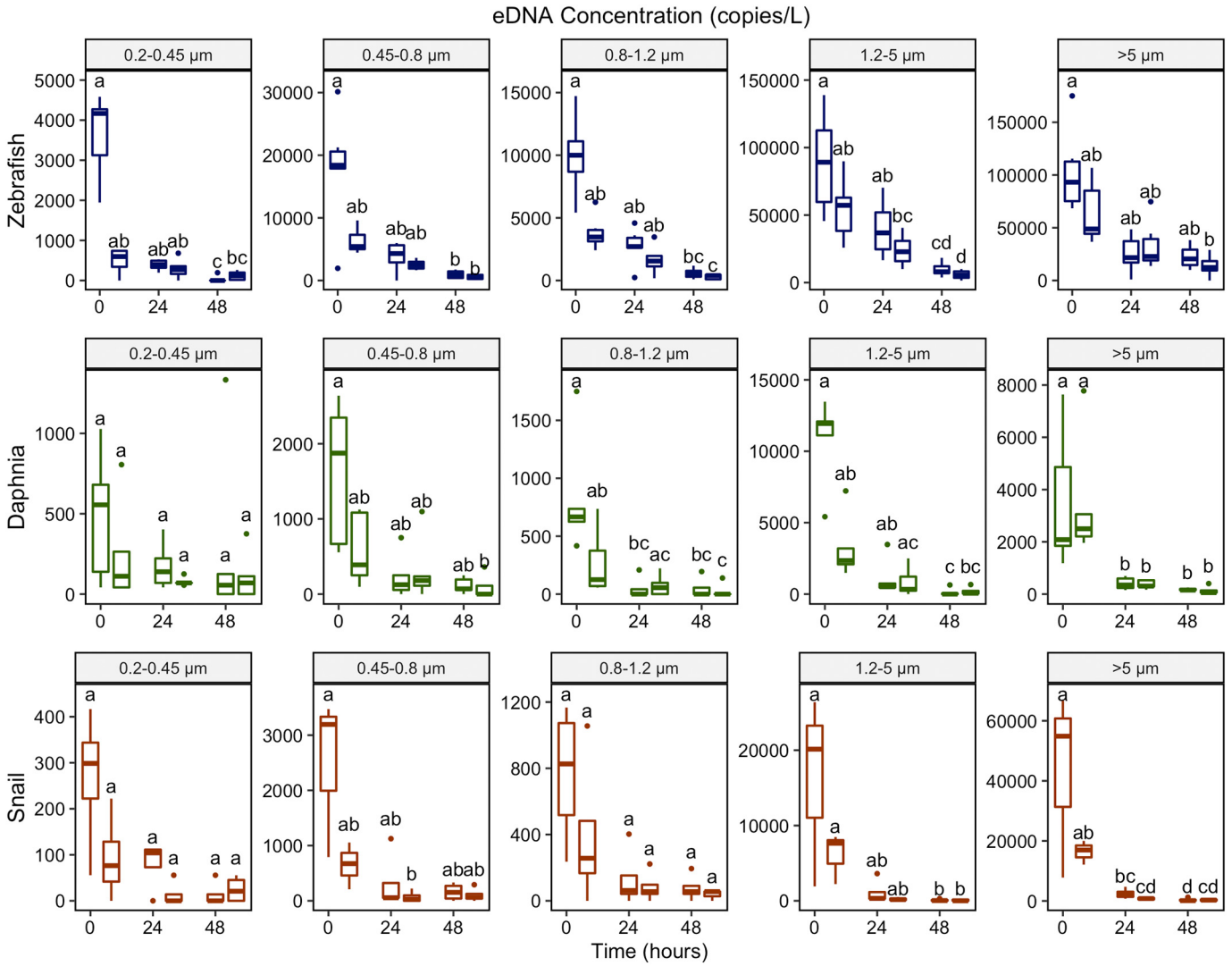

Fig. 2. The eDNA concentration (copies/L) and its temporal dynamics in culturing water at five particle size ranges of three species from 0 to 56 h after removing the organisms (blue, zebrafish; green, daphnia; red, snail). Note that the ordinate ranges are different. Different letters identify significant differences according to the Tukey HSD post-hoc tests on each one-way ANOVA, using $\log_{10}(X + 1)$-transformed eDNA concentrations.

The eDNA PSD of the three species from 0 to 56 h after removing the organisms is shown in Fig. 4. The PERMANOVA test supported the eDNA PSD differences between species when using data from all time points. Additionally, the test showed that eDNA PSD conspicuously changed with time ($P < 0.01$), while that change also varied between species (as indicated by a significant time * species interaction). The PERMANOVA test of each species individually indicated that the eDNA PSD changed significantly with time for all species ($P < 0.01$ for zebrafish and daphnia, and $P < 0.05$ for snail).

3.3. eDNA PSD decay rate

The zebrafish eDNA concentration of all size ranges exhibited an exponential decay. The decay rate constant showed a strong decrease from small to large size ranges ($p < 0.01$, Kruskal-Wallis Test), with a mean of 0.21 h^{-1} at the 0.2-0.45 μm range to 0.05 h^{-1} at the >5 μm range (Fig. 5, Table S4). Correspondingly, the retention time of eDNA showed an increase from small to large sizes ranges ($p < 0.01$, Kruskal-Wallis Test), with a mean of 16 h at the 0.2-0.45 μm range to 71 h at >5 μm (Fig. 5).

4. Discussion

In this study, we determined the eDNA concentration at five particle size ranges of three species separately at six time points after removing the organisms (Fig. 2), thereby assessing how the eDNA PSD changed with degree of eDNA degradation. Such understanding is critically important because the persistence of eDNA - which ranges from several days to months (Eichmiller et al., 2016; Jo et al., 2019c; Tsuji et al., 2017) - may result in false-positive inferences in field investigations. This strongly constrains the applicability of eDNA as a low-cost and effective strategy for investigating species and biodiversity in aquatic systems (Bakker et al., 2019; Klymus et al., 2017; Muha et al., 2019; Pochardt et al., 2020). Up to now, there had been no method that can determine how long ago the eDNA has been released and whether the aquatic organism is still present in the ecosystem, based on eDNA measurements. The eDNA PSD – and the changes therein- has the potential to be a parameter to quantify decay degree and thus to deal with these issues.

The eDNA concentration showed a significant decrease with time at most size ranges for all species, but not between each pair of time points. In most cases, these decreases were rapid and occurred in a matter of days, inducing fast changes in the degree of degradation of eDNA in these experimental conditions. We also observed that the change in eDNA concentrations varied between different sizes corresponding to one previous study (Jo et al., 2019a). Increases of eDNA concentration between some time points might have been caused by the heterogeneous distribution of the eDNA in some of the tanks. For the large size ranges, if DNA in the tanks exists as a combination of tissue particles and cells, capturing some of these tissue particles on the filter at some stage could increase the eDNA concentration considerably compared to earlier samples. For the smaller size ranges, the degradation of large size eDNA particles could generate an increase in small eDNA particles, leading to the increase of eDNA concentration at some time points. By comparing the zebrafish eDNA decay rate between size ranges, the

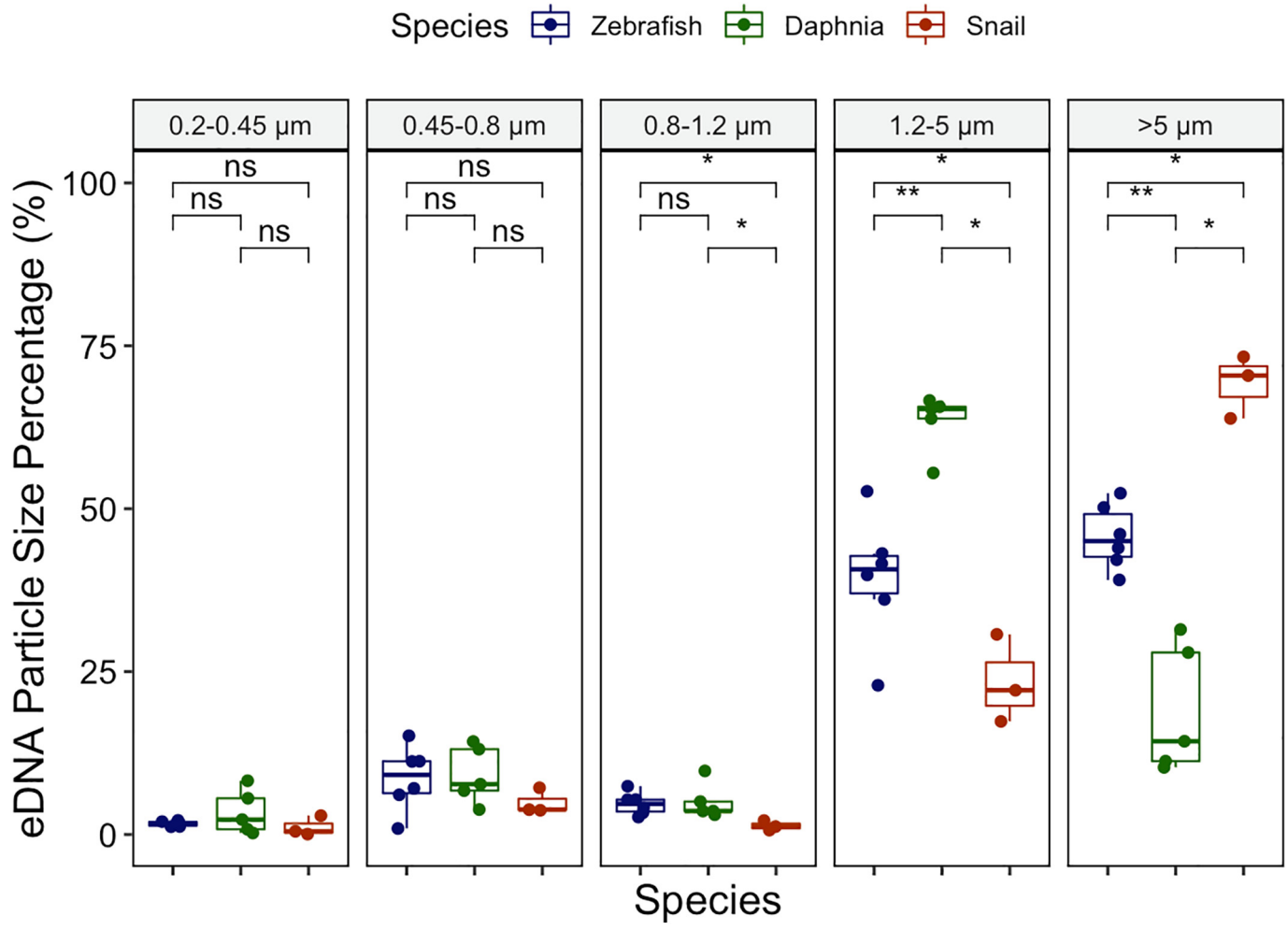

Fig. 3. The percentages of eDNA concentrations (copies/μL) at five particle size ranges for zebrafish(blue), daphnia (green) and snail (red) at time 0, with corresponding Wilcox signed-rank test *P*-values (**, 0.001-0.01; *, 0.01-0.05; ns, 0.05-1).

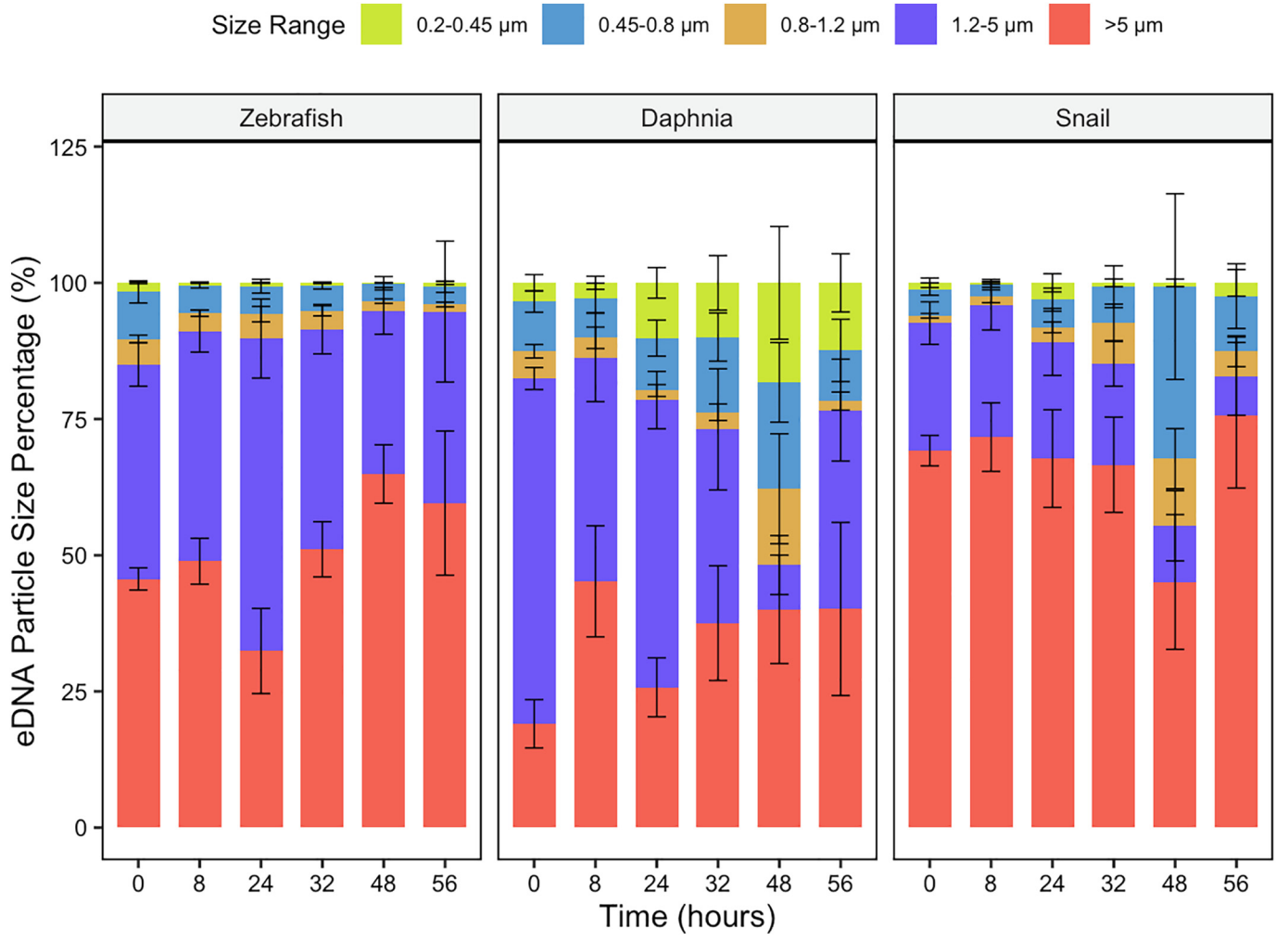

Fig. 4. The eDNA concentration percentage of different size ranges from 0 to 56 h after removing organisms of each species. Each color shows one size range. Error bars show the standard errors (SE).

present study allows us to understand how degradation varies with particle size. The decays were faster at the smaller size ranges with corresponding shorter retention times (Fig. 5), explaining the significant interaction too (Table 2).

In contrast to our expectations, the smallest size ranges thus disappeared more swiftly in our tank experiments. We had expected that, along with decay, larger particles would change into smaller particles, buffering the decrease of eDNA at the smaller size ranges over time as implied in a previous study (Jo et al., 2019a). However, this effect does not seem to occur (Fig. 4). In our study, any accumulation seems to be overruled by a faster inherent decay of smaller size eDNA particles. This faster decay of small size eDNA particles may be due to the different temperature used between these studies as Jo et al. also showed that temperature significantly affects the decay of smaller but not of larger size eDNA particles. This further indicates that without the protection of cell and organelle membranes, eDNA particles tend to be much more sensitive to drivers of decay in the environment. This mechanism possibly explains why eDNA from different sizes decays at different rates, which in turn explains the eDNA PSD changes with the degree of degradation, consistently across the species that we evaluated.

Based on the above findings, we propose that eDNA PSD has the potential to develop into a parameter to estimate the degree of degradation of obtained eDNA, allowing to assess the time period between the presence of organisms and sampling in field investigations. This provides a major potential to dealing with the issue of false-positives, in which eDNA is detected but the organism is not actually there: any deviation in the PSD towards the large size ranges compared to the PSD of the original eDNA when the species was still in the water points towards old DNA and suggests that the organism has likely left the location. We suggest implementing an evaluation of eDNA PSD in field sampling campaigns of eDNA, particularly for situations when false detections of species may have major management implications as may be the case for e.g. rare or indicator species.

We also suggest that the eDNA PSD can be used to trace the movement of populations. Through comparing the eDNA PSD of samples along the possible migration or dispersion pathway, the direction of migration or dispersion can be estimated. Based on our results, we expect the percentage of large eDNA size ranges to be related to the time since migrating from the corresponding sampling site. Thus, the migration direction is from the site with the higher to the lower proportion of the large eDNA size range (old to fresh eDNA), assuming that impacts of water movement are carefully accounted for.

Before eDNA PSDs can be used in field applications, a number of important obstacles demand further investigation. Foremost, we observed that different model taxa release their eDNA in different states. Our study is the first to evaluate the eDNA PSD of more than one target species at the same time. We found that the initial eDNA PSD showed differences between taxa. This suggests that aquatic macro-species with different interactions with environment and physiological characteristics indeed release their DNA differently and generate different eDNA PSDs. In turn, this indicates that when using eDNA PSD as a parameter to assess the degree of degradation and to detect potential false-

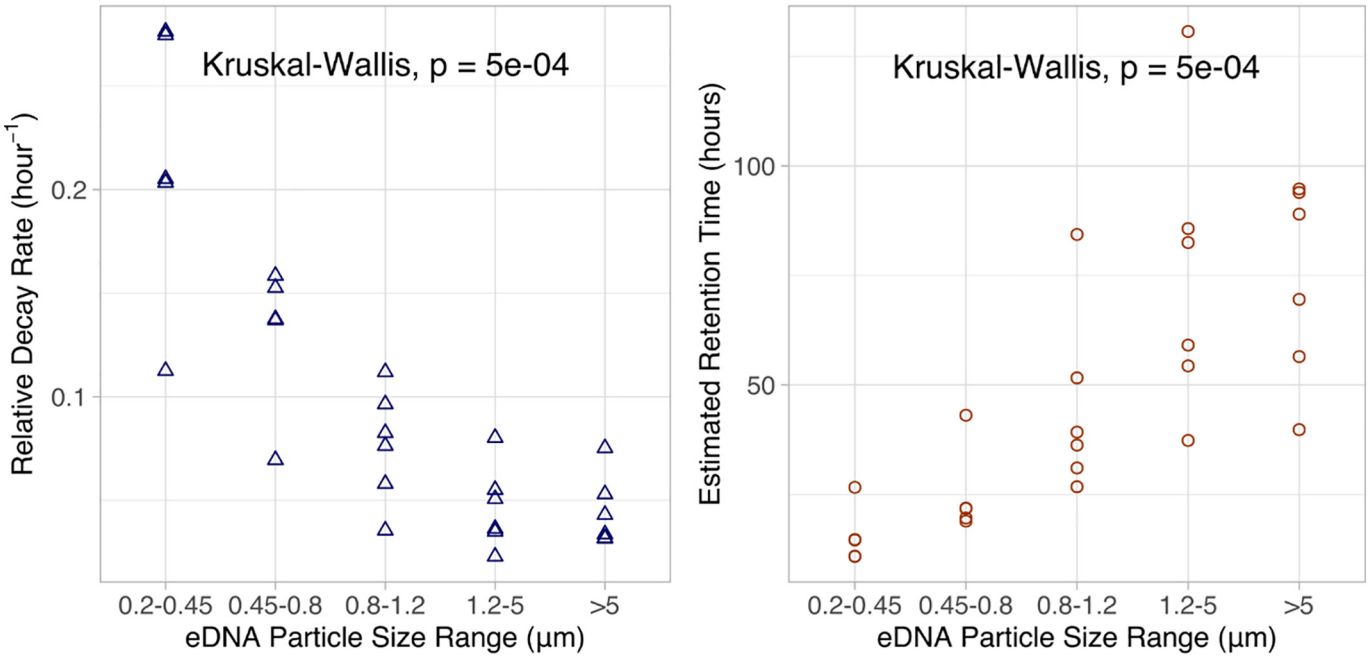

Fig. 5. The relative decay rate constant (blue triangles) and estimated retention time (red circle) of zebrafish eDNA at different size ranges. Each point represents one replicate.

positives, the evaluation criteria should be different for different target species. Besides, the eDNA PSD of target species in equilibrium with its environment will have to be evaluated prior to field applications and monitoring efforts. These differences in initial or equilibrium eDNA PSD may also explain why the changes in eDNA PSD over time varied between species, as shown by the significant interaction term between time and species (and consistent with (Moushomi et al., 2019)).

Next to differences between species, environmental conditions will also have to be taken into account as they might affect eDNA PSD differently. This could result in different eDNA PSD patterns in different environmental situations. For example, in tank experiments, fish including sardine, mackerel, and Japanese jack mackerel were found to release mostly eDNA in the >10 μm size range, which coincides with intracellular particles (Jo et al., 2019c, 2019a; Sassoubre et al., 2016). Also in the present study, snails and zebrafish released eDNA in these larger size ranges. However, in wild environments such as lakes and ponds, carp eDNA was found to be most abundant in a 1-10 μm range (Turner et al., 2014). Also for daphnia eDNA, the PSD may vary with conditions as it was found to be most prevalent in the 0.2-1 μm size range at 20 °C (Moushomi et al., 2019), while we found daphnia eDNA particles to be most abundant in the 1.2-5 μm size range at 22 °C. Further studies are certainly needed to evaluate how eDNA PSD varies between species, and how environmental factors impact the eDNA PSD of the same species.

Finally, we also found that the decay rates themselves were different for eDNA from different species (while in otherwise similar light and temperature conditions). While those differences may be due to differences in the eDNA state among species, they are equally likely to be due to differences in chemical and microbial conditions (induced by the presence of living individuals of different species). Enzyme concentrations and microorganisms are generally seen as drivers of eDNA decay (Collins et al., 2018; Salter, 2018; Shogren et al., 2018) and are a likely reason for the differences found. Further research is needed to better distinguish between the drivers of the possible differences in decay rate between eDNA of different species. With a better understanding of the decay rates in specific environmental conditions, estimates of the retention times of eDNA can be improved, which is useful for evaluating the actual presence of a species in addition to the eDNA PSD.

5. Conclusions

We have demonstrated that zebrafish eDNA decays more slowly for larger particle sizes, and that the eDNA PSD changes along the decay process after organisms have left the location for all three species. The eDNA PSD changed consistently towards larger size ranges across species. This provides a potential metric to assess the current prevalence of organisms at an eDNA sampling location and to avoid false-positives of the presence of species. This will largely expand the applicability of eDNA for monitoring species in the field, especially for aquatic systems and would eliminate one of the most important challenges in eDNA research. We also found that different species release their eDNA in different states. Therefore, further studies are demanded to understand how the eDNA PSD varies among species and different aquatic situations. Before such generic understanding is available, we recommend evaluating the eDNA PSD of target species in equilibrium with living individuals before application in monitoring.

CRediT authorship contribution statement

Peter van Bodegom, Krijn Trimbos and Beilun Zhao designed the experiments, analyzed the data, and wrote the draft of the manuscript. Beilun Zhao performed the tank experiments. The authors declare no competing financial interest.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149175.

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