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# Environmental DNA Particle Size Distribution and Quantity Differ Across Taxa and Organelles

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## ABSTRACT

The use of environmental DNA to detect species is now widespread in freshwater ecology. However, the detectability of species depends on many factors, such as the quantity of eDNA particles available in the environment and their state (e.g., free DNA fragments, organellar, or aggregated DNA particles). To date, the most advanced knowledge of the production and state of DNA particles concerns teleosts. Most often, these studies target mitochondrial genes, since they are present in multiple copies in a cell. However, it is likely that the characteristics of eDNA molecules vary greatly among taxa and genetic compartments, with direct consequences for species detection. Using an indoor mesocosm experiment, we compared the rate of mitochondrial and nuclear eDNA production and particle size distribution (PSD) of four distinct and common aquatic taxa (zebrafish, tadpole, isopod and mollusk). The tank water was filtered through a series of filters with decreasing porosity and mitochondrial and nuclear eDNA at each size fraction were quantified by qPCR. We found that the production and the size of eDNA particles varied greatly among taxa and genetic compartments. For most taxa, the number of nuclear eDNA particles released in water was higher than that of mitochondrial origin. The PSD of mt-eDNA showed a pattern common to all taxa: the relative number of particles increased from the smallest size fractions (0.2 μm and less) to the largest (over 1.2 μm), while the distribution of nu-eDNA was very different from one taxon to another. We also observed a high temporal variability in the quantity of eDNA particles and in PSD, although the latter was more complex to model. These results call for caution in how to sample and analyze eDNA in aquatic environments, particularly for organisms that emit small particles in small quantities such as isopods.

## 1 | Introduction

Organisms release DNA molecules into their environment through various processes such as excretion, reproduction, and epidermal sloughing. This genetic material transfers to the ecosystem and becomes environmental DNA (eDNA) which can be isolated from environmental samples such as water or sediments (Bohmann et al. 2014; Taberlet et al. 2018). About a decade ago, eDNA sampling and analysis emerged as a noninvasive and sensitive approach to infer the presence of aquatic species (Ficetola et al. 2008; Jerde et al. 2011). Rapid methodological development in the field including sampling and extraction protocols, and analytical technologies (i.e., high-throughput sequencing, quantitative PCR and droplet digital PCR) have democratized the use of eDNA to study biodiversity. In freshwater ecosystems, eDNA has been frequently sampled from water to detect rare, threatened, or invasive species (Biggs et al. 2015; Blackman et al. 2022; Takahara, Minamoto, and Doi 2013; Thomsen et al. 2012) and to describe whole communities of plants (Ji et al. 2021; Shackleton et al. 2019) and animals (Blabolil et al. 2021; Brantschen

et al. 2021). As the list of successful applications continues to grow, important international efforts have recently been made to standardize eDNA-based methods (Bruce et al. 2021) and integrate them into monitoring frameworks (Leese et al. 2018; Loeza-Quintana et al. 2020; Pilliod et al. 2019). Simultaneously, inquiries arise regarding the factors affecting the detectability of eDNA signals and the ecological inferences that can be drawn from them.

Among these factors, the production, state, transport, and persistence of eDNA particles in an ecosystem are of fundamental importance (Barnes and Turner 2016). Commonly referred as « eDNA ecology », these processes govern the distribution of eDNA in a specific location and consequently the detection, or non-detection, of a species. Since the publication of Barnes and Turner (2016), a significant advance in research has led to a better understanding of the dynamics of eDNA particles in aquatic systems, particularly regarding their transport and persistence. Overall, the literature shows that these processes are variable among ecosystems (Jo and Yamanaka 2022; Harrison, Sunday, and Rogers 2019). For example, in lotic ecosystems (i.e., streams, rivers), eDNA particles can be dispersed by the flow of water and detected at a distance ranging from few meters to several kilometers from their source (Deiner and Altermatt 2014; Fremier et al. 2019; Jane et al. 2015; Pont et al. 2018), whereas in lentic ecosystems, eDNA tends to stay within a perimeter close to the source (Dunker et al. 2016; Eichmiller, Bajer, and Sorensen 2014; Li et al. 2019; Moyer et al. 2014). In addition to hydrological factors, complex mechanisms of degradation, sedimentation and adsorption of eDNA particles are involved and, consequently, predicting eDNA fate in hydrosystems is very challenging. More recently, there has been a renewed interest in the nature of eDNA particles themselves, particularly their quantity and state (i.e., the physical form of eDNA molecules after being released by organisms), in order to better understand the temporal and spatial dynamics of eDNA in the environment (Kirtane, Kleyer, and Deiner 2023; Snyder et al. 2023; Mauvisseau et al. 2022; Stewart 2019).

Conceptually, eDNA is a pool of DNA molecules of varying states ranging from intracellular DNA (e.g., contained in tissues or cells) to extracellular DNA (e.g., organellar DNA, single- or double-stranded DNA fragments; Mauvisseau et al. 2022). Depending on their state, eDNA particles may have different structure, size, and weight, and thus specific spatio-temporal dynamics (Harrison, Sunday, and Rogers 2019; Jo, Arimoto, et al. 2019; Nagler et al. 2018). For example, heavier intracellular eDNA molecules ($>10\,\mu m$) could rapidly settle to the bottom of the water column and become undetectable (Snyder et al. 2023; Turner et al. 2014). Conversely, smaller particles such as mitochondria or free DNA fragments could be further subject to dispersion and dilution in the environment. Finally, the persistence of eDNA molecules in the environment also depends on their states once produced. For example, membrane-protected eDNA particles (e.g., mitochondria or cells) remain intact longer than eDNA in free forms such as extracellular fragments (Harrison, Sunday, and Rogers 2019).

The characteristics of eDNA particles sampled at a given time and location depend on their degradation and dispersal rate but also fundamentally on their production. Organisms can directly emit eDNA in different quantities, states, and forms. So far, analyses of eDNA production and particle states have been mostly done on teleosteans (Barnes et al. 2021; Jo, Arimoto, et al. 2019; Turner et al. 2014; Wilcox et al. 2015). Results showed a similar pattern between teleostean species: the quantity of eDNA produced is often correlated with organism biomass or abundance (Doi et al. 2017; Karlsson et al. 2022; Takahara et al. 2012) with a predominance of eDNA particles between 1 and $10\,\mu m$ (Barnes et al. 2021; Jo, Arimoto, et al. 2019; Jo, Murakami, et al. 2019; Wilcox et al. 2015). This suggests that teleostean eDNA is mainly in intracellular form in freshwaters (i.e., tissue or whole cells) originating from urine and fecal matter or from shed epithelial cells (Harrison, Sunday, and Rogers 2019). However, due to many biological differences, such as biomass, metabolism, respiration, and reproduction, it is likely that other aquatic taxa release eDNA at rather different quantities and states. More recent studies have shown that there can be significant intra- and inter-species variations in the eDNA production rate (Thalinger, Kirschner, et al. 2021; Thalinger, Rieder, et al. 2021).

Concerning the particle state, Moushomi et al. (2019) were the first to describe the eDNA particle size distribution (PSD) for a non-teleostean organism: the cladocera Daphnia (*Daphnia magna*). eDNA PSD is obtained using sequential filtration of water samples followed by a quantification of the number of eDNA particles found in each size fraction. PSDs do not directly describe the nature of each eDNA particle, but its shape is a useful approximation of the size of the eDNA particle emitted by an organism (Turner et al. 2014). Daphnia eDNA particles were smaller than those of teleostean, suggesting that the eDNA PSD may vary extensively among taxa. To test this hypothesis, Zhao, van Bodegom, and Trimbos (2021) analyzed the eDNA PSD and their degradation for three aquatic taxa simultaneously (a fish, a snail, and daphnia) and concluded that the three taxa release eDNA in different PSD. Altogether, these first results show that the PSD is highly taxa-dependent and reinforces the need to conduct similar experiments with other organisms with contrasting anatomies and physiologies. If taxa emit eDNA particles with very different characteristics, the sampling methods we use to sample eDNA might directly affect our capacity to monitor these taxa. For example, $0.45\,\mu m$ filters are routinely used to detect aquatic species from water samples. This is based on the assumption that most eDNA particles are collected in this size range. While this affirmation is likely to be true for most teleosteans, it is possible that other organisms release eDNA particles in other size ranges, which would therefore reduce their detectability.

Another important factor affecting eDNA detectability is the choice of a genomic marker. Most eDNA PSD analyses have only considered mitochondrial DNA (mt-DNA). Historically, mt-DNA has been widely used in DNA-based approaches due to (i) its presence in multiple copies per cell, increasing the probability of mt-DNA detection, and (ii) the large amount of mt-DNA sequences in databases especially for metazoans (e.g., MitoFish; Iwasaki et al. 2013), increasing the chance of taxonomic affiliation in metabarcoding studies. However, several authors have shown that the use of nuclear markers, particularly those present in many copies in the genome (e.g., ITS), allows sensitive and accurate detection of several fish species (Bylemans et al. 2017; Jo et al. 2017; Minamoto et al. 2017). Although nu-eDNA showed

promising results, its production and states are probably very different from mt-eDNA and further investigation is needed to evaluate its potential (Foran 2006; Minamoto et al. 2017). Altogether, the joint analysis of nu- and mt-eDNA production and PSD for a diverse set of aquatic taxa is warranted to propose robust and sensitive eDNA biomonitoring tools.

Here, we investigated the eDNA quantity and the particle size distribution of eDNA released by taxonomically distant taxa over time using mesocosm experiment. We selected four aquatic model taxa with contrasting life histories and physiologies: the zebrafish (*Danio rerio*), the tadpole of the marsh frog (*Pelophylax ridibundus*), the bladder snail (*Physa acuta*) and the water louse isopod (*A. aquaticus*). In addition, we examined how the genetic compartment and the timing of sampling influenced the eDNA quantity and PSD. We hypothesized that taxa, genetic compartment (mt-eDNA or nu-eDNA), and sampling timing would influence eDNA particle quantity and size distribution.

## 2 | Materials and Methods

### 2.1 | Model Taxa

To explore the influence of taxa, genetic compartment and sampling timing on eDNA production and PSD, we conducted an indoor tank experiment at the University of Lyon 1 (France), from November to December 2021. We chose four freshwater species easy to maintain in laboratory conditions with contrasted life histories and physiologies, leading probably to different eDNA production and PSD: adult zebrafish (*D. rerio*), the marsh frog tadpoles (*P. ridibundus*), the bladder snail (*P. acuta*) and the water louse isopods (*A. aquaticus*). Zebrafish (male and female) and tadpoles were purchased on specialized farms, the PRECI fish facility of the IFR128 Biosciences Gerland-Lyon Sud (Lyon, France) and François production (Pierrelate, France), respectively. Snails and isopods were sampled in October 2021, from two freshwater ponds located in an urban area (45°81′28″ N, 4°96′48″ E, 700 $m^2$ and 45°78′01″ N, 4°86′79″ E, 440 $m^2$, France, respectively). For each taxa, we chose individuals with equivalent sizes to limit variation in eDNA release due to biomass variation. Animals were acclimated to laboratory conditions during 7 days in tanks identical to those used during the experiment (see Section 2.2). Detailed information on transport and breeding conditions is available in the Appendix S1.

### 2.2 | Experimental Setup

For each taxa, three mesocosm experiments were simultaneously run as replicates, that is, a total of 12 tanks (Figure 1). The fish tanks and tadpole tanks were placed in the same climate controlled room, in the experimental platform ACSED dedicated to the rearing of vertebrates (http://www.umr5023.univ-lyon1.fr/plates-formes-de-recherche/fr-bioenvis/acsed), whereas snails and isopods were kept in two other separate climate-controlled rooms (University of Lyon, 69, France). Each mesocosm consisted in a glass tank (25×20×40 cm) containing 20 L of osmotic water, oxygenated with aquarium bubblers and with a constant temperature of 28°C ± 2°C for zebrafish, 22°C ± 2°C for tadpoles, and 21°C ± 2°C (mean ± SD) for snails and isopods and a 12-h daylight cycle. As the biomass per individual varies between the selected taxa, we estimated a density necessary to obtain an equivalent biomass between tanks. We calculated a theoretical biomass, based on measurements of the mass of the selected taxa available in our laboratory or in the literature (see Appendix S1). The density of individuals were 15 individuals per fish tank, 20 individuals per tadpoles tank, 100 individuals per snails tank and 150 individuals in isopods tank. This experiment was approved by the Animal Testing Committee from the University of Claude Bernard Lyon I (CEEA-55, agreement no. DR2020-34v2). Before the experiment, tanks, sampling, and filtration material (e.g., glass bottles, filtration devices) were decontaminated with 5% bleach (±2%), rinsed with osmosis water followed by a 15-min ultraviolet light (UV-C) treatment. The species were placed in separate rooms except the tadpoles and the fishes which were reared in the same room to follow the Animal Testing Committee requirements. The tanks were spaced 50 cm apart and covered with aluminum paper to protect

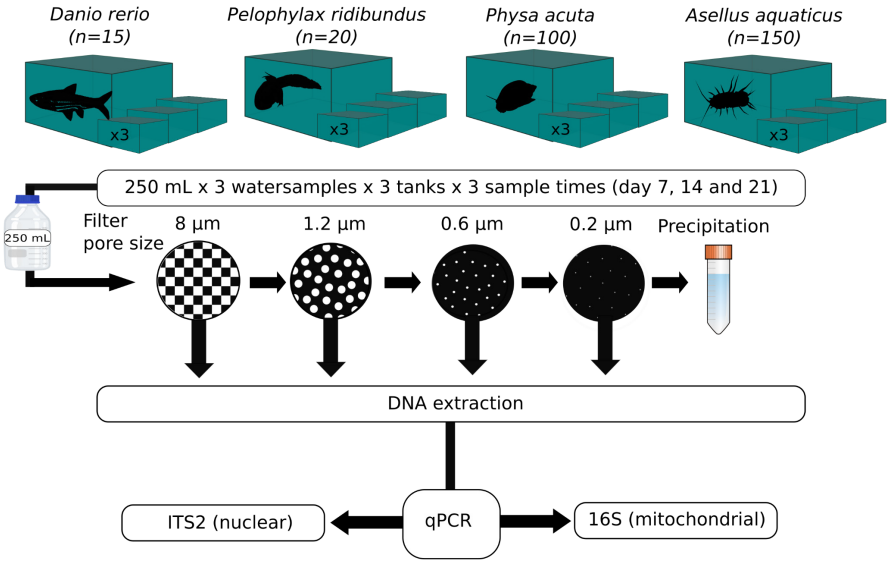


**FIGURE 1** | Experimental design. In total, the experiment included 3240 qPCR reactions (12 tanks × 9 water samples × 5 extractions × 2 genetic markers × 3 qPCR replicates).

the water from evaporation, and punctured using sterilized tweezers for aeration. Individuals were fed each day with non-animal food (i.e., autoclaved boiled salad for tadpoles, isopods, and snails and seaweed-based food for fish) to control for potential eDNA input resulting from feeding. Excess food was removed 1 h after feeding with nets decontaminated using 5% bleach.

## 2.3 | eDNA Sampling

We collected eDNA from the tanks at three sampling times: 7, 14, and 21 days after the start of the experiment. As the eDNA concentration is known to peak during the first few days of animal acclimation (Maruyama et al. 2014; Sansom and Sassoubre 2017), the first sampling was performed on day 7 in order to reach a theoretical stable eDNA concentration as described by Sansom and Sassoubre (2017). Then, since eDNA is a molecule sensitive to degradation, performing three sampling times allowed us to estimate the repeatability of eDNA quantification and PSD analysis. At each time point, we collected three 250 mL water samples per tank from the top to the bottom of the water column with a Pipet-Aid controller (Poly Labo, Paul Block & Cie, France) and single-use serological pipettes. Water volumes within tanks were kept constant by adding 750 mL of osmotic tap water after each sample collection. Water samples were transferred in 500 mL glass sterilized bottles. Immediately after sampling, water samples were filtered within a laminar flow hood (Noroit, H-BOX, France) at the ACSED platform (University of Lyon, France) using a glass vacuum filtration device (Merck Millipore, Germany). Water samples were sequentially filtered on filters with four decreasing porosities (8, 1.2, 0.6 and 0.2 μm pore size, MCE Membrane; Merck Millipore, Germany). Filters were placed into 5 mL LoBind eppendorf tubes and stored at −80°C until extraction. A volume of 14 mL of the remaining water fraction filtered at 0.2 μm was directly precipitated by adding 35 mL of 96% ethanol, 1.4 mL of 3 M sodium acetate, and 10 μL of glycogen in 50 mL falcon tubes (adapted from Ficetola et al. 2008). In total, we obtained five fractions of eDNA particle sizes: fraction F1 corresponding to particles < 0.2 μm, fraction F2 corresponding to particles from 0.2 to 0.6 μm, fraction F3 corresponding to particles from 0.6 to 1.2 μm, fraction F4 corresponding to particles from 1.2 to 8 μm and fraction F5 with particles 8 μm and over. The tubes containing the filters were then incubated for 3 days at −80°C.

## 2.4 | eDNA Extraction

The extraction procedure from filters and precipitates was carried out in a flow-hood reserved to very low DNA concentration and decontaminated with 5% bleach and followed by a 15-min UV-C treatment. DNA from filters was extracted using a modified protocol of the DNeasy Blood and Tissue kit (Qiagen DNA Blood and Tissue kit). Briefly, 500 μL of ATL buffer and 40 μL of proteinase K were added to the 5 mL tubes containing the filters, vortexed 15 s, and incubated at 56°C in a shaking water bath (Memmert GmbH + Co.KG, WB 14, Schwabach, Germany) at 150 rpm for 48 h. After incubation, 400 μL of AL buffer and 400 μL of ethanol 96% were added into the tubes and vortexed 15 s. Next steps were done following manufacturer's recommendations. The precipitated samples, after incubation at −80°C, were centrifuged at 1200 rpm for 35 min at 4°C. The pellets obtained were resuspended in 100 μL of 1× TE buffer and extracted with a commercial kit (Qiagen DNA Blood and Tissue kit). To trace potential contamination, we set up three types of contamination controls: (i) experimental controls corresponding to four glass tanks placed in each climate-controlled room containing only osmotic water and animal feed, (ii) filtration and precipitation controls corresponding to sterile water that was filtered and precipitated with the same equipment and protocols than eDNA samples, (iii) quantification controls corresponding to wells without DNA in qPCR plates. Extracted DNA samples were then placed at −20°C before quantification.

## 2.5 | Primer Design and qPCR Quantification

Quantitative PCR was used to quantify the eDNA released from each taxa, genetic compartment, and time replicates. Species-specific primers were designed to amplify a multiple copy nuclear gene (ITS2) and the mitochondrial (16S) gene of *D. rerio, P. ridibundus, P. acuta, and A. aquaticus* (Table 1). Target genetic regions were selected based on (i) the high genetic variability between species to avoid cross-amplification, (ii) the possible presence of multiple copies within a cell, and (iii) the availability of genomic data on the National Center for Biotechnology Information (NCBI). The primers were designed manually based on sequence alignments made with SeaView (Galtier, Gouy, and Gautier 1996, alignment available on zenodo 10.5281/zenodo.11208087). The amplification specificity of the primers was tested in silico using primer-BLAST (NCBI; Ye et al. 2012) as recommended by Klymus et al. (2020). The primer pairs were tested in vitro for amplification efficiency and specificity using genomic DNA extracted from tissue samples (1 ng/μL) from the target taxa, the three other taxa not targeted in the experiment and human DNA (detailed protocol in Appendix S1). The final primer sequences and concentrations, DNA targets, and primer annealing temperatures are shown in Table 1. The qPCR reaction volume was 10 μL consisting of 1× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA), 0.5 μM of primers, and 2 μL of DNA. All samples including DNA, positive and negative controls were run in triplicate in 96-well plates on a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). qPCR cycle started with an incubation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 s and an annealing/extension step at 64°C or 55°C for 20 s before a final melt curve from 65°C to 95°C (0.5°C increments). Each qPCR plate included a sevenfold dilution series of the purified DNA amplicon as standard, at a concentration between 0 and 2.6 ± 0.5 ng/μL quantified by a QuBit 3.0 assay. In total, we obtained 810 qPCR reactions per taxa, 297 reactions for all the qPCR controls, 163 reactions for the extraction controls, and 710 reactions for the experimental controls.

## 2.6 | Estimation of the Number of Mitochondrial Genome and Nuclear rDNA Loci

To explain the potential variation in eDNA copy number between the genetic compartment, we estimated the relative number of mitochondrial and rDNA loci in a cell for the four model species. The number of loci per cell and the relative copy number of the

mitochondrial and rDNA loci were estimated using whole genome shotgun sequencing (WGS) depth. Indeed, a gene in *N* copies is expected to harbor a sequencing depth that is *N* times the sequencing depth of a single copy gene. Except for *D. rerio*, complete genomes were not available for the taxa of this study, so we estimated the expected single-copy gene sequencing depth by dividing the sequencing effort (number of sequenced bases) by the estimated genome size. WGS was downloaded using fastq-dump (NCBI SRA toolkit, v3.0.9), and mitochondrial and rDNA loci with eftech (NCBI E-utilities, v20.9). The R1 reads were mapped on the mitochondrial genome and rDNA loci using bwa (v0.7.17-r1188; Li 2013) with the MEM algorithm. Average sequencing depth was estimated with samtools (v1.18; Danecek et al. 2021). We found WGS sequencing data for all the species used in this study except for *P. ridibundus* (Table 2). Instead, we used the available data for the closely related species *Pelophylax lessonae*. When mitochondrial genomes or rDNA loci were not available, they were reconstructed directly from the WGS reads using MitoFinder (v1.4.1; Allio et al. 2020) and aTRAM (v2.4.4; Allen et al. 2018) for the mitochondrial and rDNA loci, respectively. A single mitochondrial contig containing all the mitochondrial genes was reconstructed for *A. aquaticus*. Five rDNA contigs were assembled for *Pelophylax lessonae* covering the whole rDNA loci. Genome sizes were collected from genome sequencing projects (*D. rerio* and *P. acuta*) or from flow cytometry estimates (*A. aquaticus* and *Pelophylax lessonae*, Lefébure et al. 2017; Vinogradov 1998).

## 2.7 | Data Conversion and Analysis

### 2.7.1 | Data Processing and Conversion

We converted the quantity of eDNA per qPCR reaction (in ng/2 μL of reaction) into a number of eDNA copies per liter of tank water using Equation (1) below. In this equation, $X$ corresponds to the quantity of DNA amplicon (in ng), $6.022\times10^{23}$ is Avogadro's constant, which is used to calculate the number of DNA molecules in a mole, $N$ corresponds to the length of the double-stranded DNA amplicon and 650 g/mol is the average mass of 1 bp of double-stranded DNA. Finally, $1\times10^{9}$ is used as a conversion factor to convert grams into nanograms. This standardized unit is widely used for eDNA analysis and allows comparing of values across studies. Negative detections (NA) in qPCR triplicates were assigned with a concentration of zero (Ellison et al. 2006). The DNA concentrations were then averaged across the triplicates. Finally, we corrected for the number of copies of eDNA obtained by the

**TABLE 1** | Species-specific primer sets for amplification of 16S and ITS2 gene in qPCR.

| Species | Gene | Sequence (5′–3′) | Amplicon length (bp) | Annealing T (°C) |
|---|---|---|---|---|
| *Pelophylax ridibundus* | 16S | F-AAGTTTAACGGCCGCGGTACCC<br>R-ATCAGTTTCACTGATTGGAGAAAGG | 125 | 64 |
| *Pelophylax ridibundus* | ITS2 | F-GGTACCCCGGTCGACCCTC<br>R-GCAGCCCGTACGGCTAGGCC | 89 | 64 |
| *Danio rerio* | 16S | F-GAAGACCCTTTGGAGCTTAAGG<br>R-GAGGTTGTTTTTTACTCCGTGG | 125 | 64 |
| *Danio rerio* | ITS2 | F-CACCGTCTCTCGACCCGTGG<br>R-GGTCAGACCCACCGGCAGCC | 111 | 64 |
| *Physa acuta* | 16S | F-CCTTTCTATTGACACTAAAAGTGG<br>R-CTAGTCCAACATCGAGGTCAC | 118 | 55 |
| *Physa acuta* | ITS2 | F-ACTAAATCAATCGAGCTCGTC<br>R-GCTTGGACTTGAAACCACGG | 104 | 64 |
| *Asellus aquaticus* | 16S | F-GGTTTAAATGGCTGCAGTATCC<br>R-CTTGTGTAATAAAAAGCCTACCTC | 110 | 64 |
| *Asellus aquaticus* | ITS2 | F-AGCTAGTAAACGGTTAAGGC<br>R-CTACGACCCAAACGGCTTACG | 108 | 64 |

**TABLE 2** | Accession numbers of the reads and loci used to estimate copy number. Coordinates are given when only a subset of the sequence was used.

| | WGS reads | Mitochondrial genome | rDNA loci | Genome size (Gb) |
|---|---|---|---|---|
| *A. aquaticus* | SRR14415276 | Assembled with MitoFinder | AJ287055 (18S only) | 2.50 |
| *D. rerio* | SRR11676828 | NC_002333 | LR812082: 55888111–55,894,727 | 1.37 |
| *P. acuta* | SRR6240322 | NC_023253 | NW_026732181:458374–467,963 | 0.70 |
| *P. lessonae* | SRR11537215 | JN627426 | Assembled with aTRAM | 6.29 |

total biomass within a tank (number of individuals multiplied by their estimated individual biomass, see Section 2.2; Appendix S1).

$$\text{Number of DNA copies} = \frac{X \text{ ng} \times 6.022 \times 10^{23}}{(N \times 650 \text{ g/mol}) \times 1 \times 10^{9}} \quad (1)$$

#### 2.7.2 | Data Analysis

We hypothesized that taxa, genetic compartment and sampling time influence (i) the eDNA particle quantity (i.e., total number of eDNA copies/tank) and (ii) particle size distribution (PSD). To investigate the first hypothesis, we summed the number of eDNA copies collected at the five size fractions per water sample to obtain the total number of eDNA copies per liter of water in a given tank. To test the second hypothesis, the number of eDNA copies at each fraction size was kept; however, for certain combinations of factors (taxa, genetic compartment, and time), no eDNA copies were detected in certain size fractions, which led to statistical modeling problems due to partial separation. For this second set of statistical analysis, we therefore grouped the eDNA copies into three size fractions: $<0.2$ to $0.2\,\mu$m (F1 + F2), 0.2–$1.2\,\mu$m (F3), and $1.2\,\mu$m and over (F4 + F5).

We used a generalized linear mixed effects model (GLMM) to investigate the effect of taxa, genetic marker, and sampling time on the (i) total eDNA particle counts and (ii) eDNA PSD. We fitted two models: one GLMM with total eDNA particle count as the response variable and taxa, genetic compartment, sampling time, and their interactions as fixed effects. We then used a rate model to relate the rate of the number of eDNA copies per fraction to taxa, genetic compartment, time, and their interactions as fixed effects. To do so, we adapted a GLM approach based on the "Poisson Trick" parametrization, which enables the fit of a baseline multinomial model using a Poisson regression by integrating the categories (the dependent variable of the multinomial regression) as independent variables into the GLM associated with their interactions with the explanatory variables (e.g., taxa; see Lee, Green, and Ryan 2017 for more details). The logarithm of the total number of copies per replicate was used as offset (Faraway 2016; Hilbe and Turlach 2011). Both GLMMs were implemented in R (R version 4.2.3, R Core Team 2023) with the *glmmTMB* package (version 1.1.7, Brooks et al. 2017) using a negative binomial distribution. To address autocorrelation within the tank and taxa, we modeled within-tank samples nested within taxa as random effects. The dispersion parameter of the negative binomial distribution was allowed to vary between taxa. For each model (total eDNA particle counts or rate of copies per fraction size), the relative importance of taxa, genetic compartment, and time was assessed by comparing the corrected Akaike's information criterion (AICc) of possible subset models encompassing from one to three explanatory variables, with or without their interactions (see Table S1, Appendix S1). The models with the highest weight (derived from AICc values, computed with the "Weights" function of MuMIn package, version 1.47.5, Barton 2009) were conserved (Burnham, Anderson, and Anderson 2010). For each conserved model, we checked the model for overdispersion and examined residuals with the function "simulateResiduals" from the "DHARMa" package version 0.4.6. (Hartig 2022) and we tested the significance of each factor using ANOVA using the package "car." All plots were made with the ggplot2 package (Wickham, Chang, and Wickham 2016).

## 3 | Results

### 3.1 | Outliers and qPCR Assay Performance

Most individuals survived for the duration of the experiment, but 60% of snail individuals died in a single tank at the beginning of the experiment. In this tank, we quantified an abnormally high eDNA copy number (about 10 times higher) compared to the average DNA copy number in other tank replicates, corresponding to 90 eDNA samples (less than 3% of the samples). We therefore excluded these data from all the following analysis. In all qPCR analysis, the $R^2$ values, slope, *Y*-intercept, and PCR efficiency (%) of the calibration curves were $0.98 \pm 0.01$, $-3.38 \pm 0.07$, $0.6 \pm 0.6$, $97.84 \pm 2.99$ (mean ± SD) for mt-eDNA and $0.99 \pm 0.006$, $-3.24 \pm 0.3$, $2.007 \pm 1.4$, $104.1 \pm 11$ for nu-eDNA (see Appendix S1, Table S3). Despite the high qPCR efficiencies and previous successful specificity tests, we detected a specificity problem of the primers amplifying the ITS2 gene of *D. rerio* on eDNA samples. Indeed, we observed multiple peaks (i.e., misaligned from the main target DNA peak) on the melting curve at $C_t$ values below 30. This indicates a phenomenon of cross-amplification of non-target eDNA that we were unable to resolve despite our efforts to design more specific primer pairs without success (i.e., over 15 primer pairs tested). As this specificity problem can lead to a significant bias in the eDNA quantity estimates, we ran models with and without the ITS2 data from *D. rerio*. As the model findings were identical between the two models, we kept the *D. rerio* data allowing us to run a balanced model with all the parameters (species, compartment, and time), and discussed these results with caution.

### 3.2 | Contaminations Control

In the qPCR controls (i.e., wells without DNA), 12 positive detections (i.e., above zero eDNA copy) of target taxa occurred out of 297 qPCR reactions, with *Cq* values always above 30 (max = 114 copies/L/g, mean = 2 copies/L/g, SD = 10 copies/L/g). In the extraction controls (i.e., corresponding to filtered or precipitated sterile water), 64 positive detections occurred out of 163 qPCR reactions (max = 207 copies/L/g, mean = 24 copies/L/g, SD = 45 copies/L/g). These contaminations represent less than 0.15% of the average number of copies quantified in the tanks containing organisms (i.e., $1.5 \times 10^4$ eDNA copies/L/g of biomass). In the experimental control tanks (i.e., containing osmotic water without organisms and with food) placed next to the tanks containing organisms, positive detections occurred sporadically with varying eDNA quantity, depending on the taxa and the marker used. For mt-DNA, we measured between 21 and 41 positive detections of the targeted taxa per tank with an average of 12 copies/L/g in the control zebrafish tank, 157 copies/L/g in the control snail tank, 137 copies/L/g in the control tadpole tank, and 159 copies/L/g in the control isopod tank. This contamination corresponds to less than 1% of the amount of eDNA measured in tanks containing organisms. For nu-eDNA, particle quantities were on average two times higher than with mt-eDNA for most taxa (isopods, snails, and tadpoles), reaching

15,363 copies of eDNA/L/g in the zebrafish control tank, which represents more than 50% of the average quantity of nu-eDNA particles in tanks containing organisms. In the latter case, contamination increased with the duration of the experiment and was mainly associated with filter pore sizes of 0.6 and 1.2 μm, as well as with nuclear gene quantification. This outcome demonstrates the issue with the primer pair specificity for the ITS2 of *D. rerio* already reported in the previous section, which probably amplifies non-targeted eDNA that increased in quantity during the course of the experiment.

**TABLE 3** | Results of the ANOVA carried out on the outputs of the glmmTMB model testing the effect of the species, the genetic compartment, the sampling time and their interactions, on the total eDNA counts.

| Parameters | $X^2$ | Df | $p\ (>X^2)$ |
|---|---|---|---|
| Taxa | 256.86 | 2 | <2.2e-16*** |
| Genetic compartment | 228.71 | 2 | <2.2e-16*** |
| Time | 22.05 | 3 | 1.631e-05*** |
| Genetic compartment:Time | 0.45 | 2 | 0.798 |
| Genetic compartment:Taxa | 33.97 | 3 | 2.007e-07*** |
| Time:Taxa | 32.58 | 6 | 1.263e-05*** |
| Genetic compartment:Time:Taxa | 18.06 | 6 | 0.006** |

*Note:* $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical significance levels.

## 3.3 | Total eDNA Particle Counts

The complete model with all variables and their interactions had the highest AICc weight (0.830 on a [0–1] scale). This model reported that the type of taxa, the genetic compartment, and sampling time significantly influenced the total eDNA particle counts as revealed by their significant interaction (GLMM, $X^2 = 18.06$, $p < 0.001$; Table 3). We observed a strong variation of the number of eDNA copy between taxa where isopods released the lowest amount of eDNA (10 to $10^3$ copies/L/g), followed by snails ($10^3$ to $10^5$ copies/L/g), fish ($10^3$ to $10^4$ copies/L/g), and amphibians ($10^4$ to $10^5$ copies/L/g, Figure 2). On average per sampling time, the number of nu-eDNA particle was 1–40 times higher (Table 4) than that of mt-eDNA except for amphibians where there was little variation in DNA copy number between the two genetic compartments (mt-eDNA $= 1.9 \times 10^4$, nu-eDNA $= 1.7 \times 10^4$ averaged over the three sampling times and tank replicates). Over time, the quantity of mt-eDNA particles increased for isopods (from 65 to 4530 copies/L/g), decreased slightly for mollusks and teleosts, or remained relatively stable as for amphibians (Figure 2). Conversely, for nu-eDNA, the temporal variations followed rather different patterns, with notably an increased eDNA particle quantity for snails.

## 3.4 | Relative Number of rDNA and Mitochondrial Loci

Variation in the amount of eDNA copies found in the environment can be modulated by their level of duplication per cell. Indeed, under a simple dilution model, a single copy gene is expected to be less frequently found than a loci present in thousands of copies per cell. We estimated the number of copies per cell and the ratio

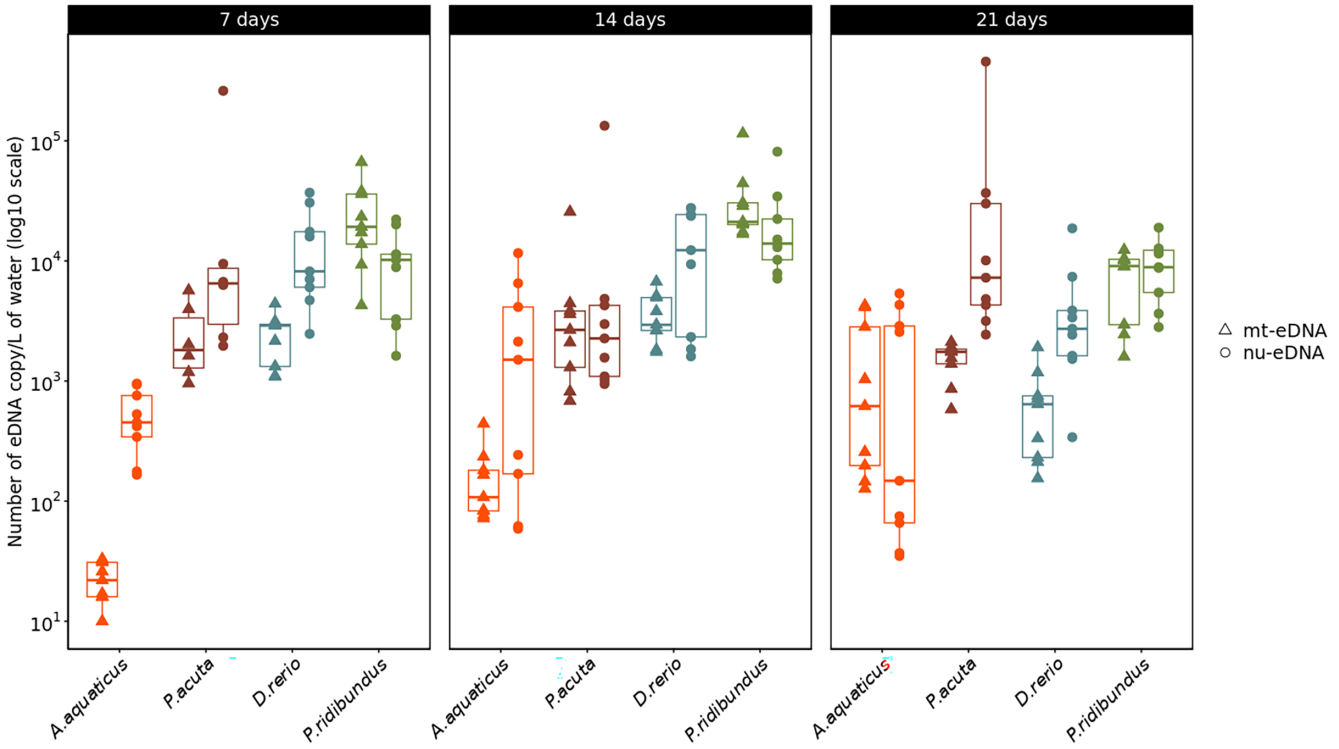


**FIGURE 2** | Number of mt-eDNA and nu-eDNA copies (eDNA copy/L of water in log scale) per species and sampling times (after 7, 14, and 21 days). Each point represents one water sample of one tank. The colors represent a species (orange = *Asellus aquaticus*, purple = *Physa acuta*, blue = *Danio rerio* and green = *Pelophylax ridibundus*).

of rDNA and mitochondrial copy number using whole genome shot-gun sequencing experiments publically available for the taxa used in this study, or a closely related one when not available. All taxa, except *Pelophylax*, displayed between six and nine times more rDNA loci than mitochondrial ones (Table 4). *Pelophylax* showed the opposite trend with about twice more mitochondrial than rDNA loci. These genomic ratios were within the observed eDNA copy number ratios observed in the mesocosms (Table 4).

**TABLE 4** | Estimation of the number of loci per cell using genome sequencing depth. Ratio r/m: Relative copy number between the rDNA and mitochondrial loci.

| Taxa | Expected depth | Mitochondrial depth | rDNA depth | Mitochondrial genome/cell | rDNA/cell | Ratio r/m | Observed eDNA ratio per sampling time (7, 14 and 21 days) | | |
|---|---|---|---|---|---|---|---|---|---|
| *A. aquaticus* | 6.7× | 387× | 2707× | 58 | 404 | 6.97 | 22.87 | 18.25 | 1.14 |
| *D. rerio* | 2.5× | 51× | 327× | 20 | 129 | 6.45 | 5.88 | 4.03 | 6.84 |
| *P. acuta* | 8.0× | 517× | 4553× | 65 | 569 | 8.75 | 18.64 | 3.38 | 40.31 |
| *P. lessonae* | 1.2× | 1345× | 541× | 1149 | 462 | 0.40 | 0.40 | 0.65 | 1.24 |

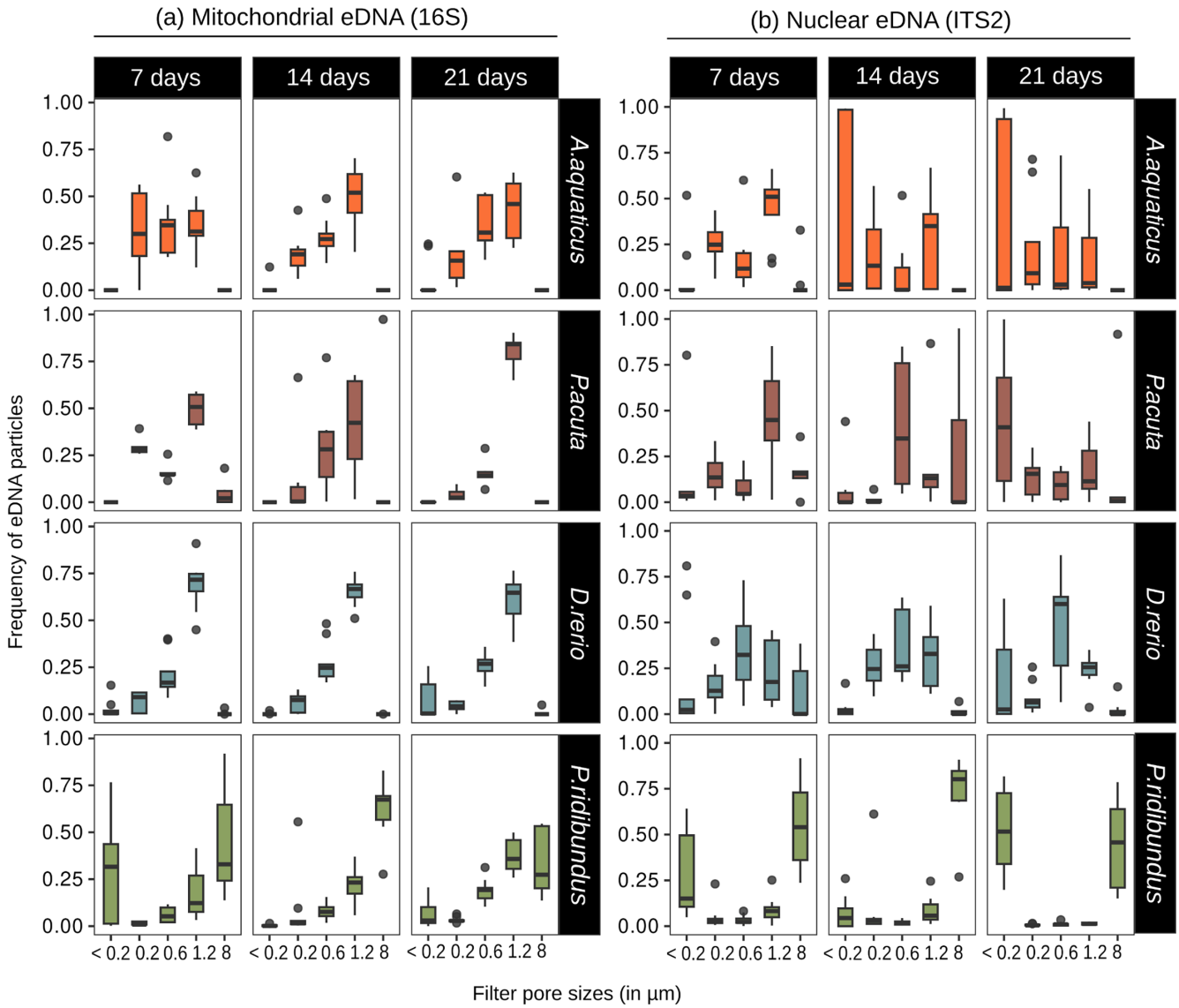


**FIGURE 3** | Frequency of (a) mitochondrial-eDNA and (b) nuclear-eDNA copies per particle size class (< 0.2–8 µm), per taxa and per sampling timing (after 7, 14 and 21 days since the start of the experiment).
*Note:* eDNA particle counts have been transformed into frequency to allow a better graphical representation.

### 3.5 | eDNA Particle Size Distribution

For mt-eDNA, the distribution of the raw counts into five particle size groups (Figures 3 and 4) showed a common pattern across taxa: the relative copy number increased from the smallest size fractions (0.2 μm and below) to the largest (above 1.2 μm), with an amplitude that depended on the taxa. For the majority of taxa (isopods, snails, and fish), the most abundant eDNA size fraction ranged from 1.2 to 8 μm, while for amphibians, it was 8 μm and above. For nu-eDNA, the PSD was very different from one taxon to another. The smallest fraction was the most dominant for isopods (< 0.2 μm) while the largest fraction was dominant in snails and tadpoles (Figures 3 and 4). For fish, the fractions were relatively well balanced, with the intermediate fraction (0.6–1.2 μm) being slightly more represented than the other two. Lastly, for tadpoles, the PSD of mt-eDNA and nu-eDNA was relatively comparable. The effect of time on the eDNA PSD was less clear: the nuclear smallest particles increased in frequency over time in the isopod, snail, and fish tanks, but apart from that, no common pattern was evident. Because some tanks had zero count for some size fraction, the statistical analysis of this dataset required the pooling of particles into three size fractions (see Section 2). By far the best model supported by this pooled dataset (AICc weight of 1) was congruent with the previous observations: While time was not significant, taxa strongly influenced the eDNA PSD as well as the genetic compartment through a significant interaction with the taxa (Table 5, Figure 5). All the mitochondrial PSD followed a distribution biased toward large particles, while the nuclear PSD was much more variable across taxa (Figure 5).

**TABLE 5** | Results of the ANOVA carried out on the outputs of the glmmTMB model testing the effect of the taxa and the genetic compartment and their interactions (represented by ":" between the variables) on eDNA copies per size fraction.

| Parameters | $X^2$ | Df | $p\ (>X^2)$ |
|---|---|---|---|
| Size fraction | 272.4085 | 3 | < 2.2e-16*** |
| Taxa | 25.7990 | 3 | 1.051e-05*** |
| Genetic compartment | 0.1146 | 1 | 0.73499 |
| Size fraction:Taxa | 186.3768 | 6 | < 2.2e-16*** |
| Size fraction:Genetic compartment | 82.4924 | 2 | < 2.2e-16*** |
| Taxa:Genetic compartment | 15.1280 | 3 | 0.002** |

*Note:* **$p < 0.01$ and ***$p < 0.001$ indicate statistical significance levels.

## 4 | Discussion

### 4.1 | eDNA Quantity Varies Across Taxa and Genetic Compartment

According to our hypothesis, we found that the eDNA particle quantity in water samples varied strongly across taxa. Despite being at similar biomass, fish and tadpoles were the two taxa

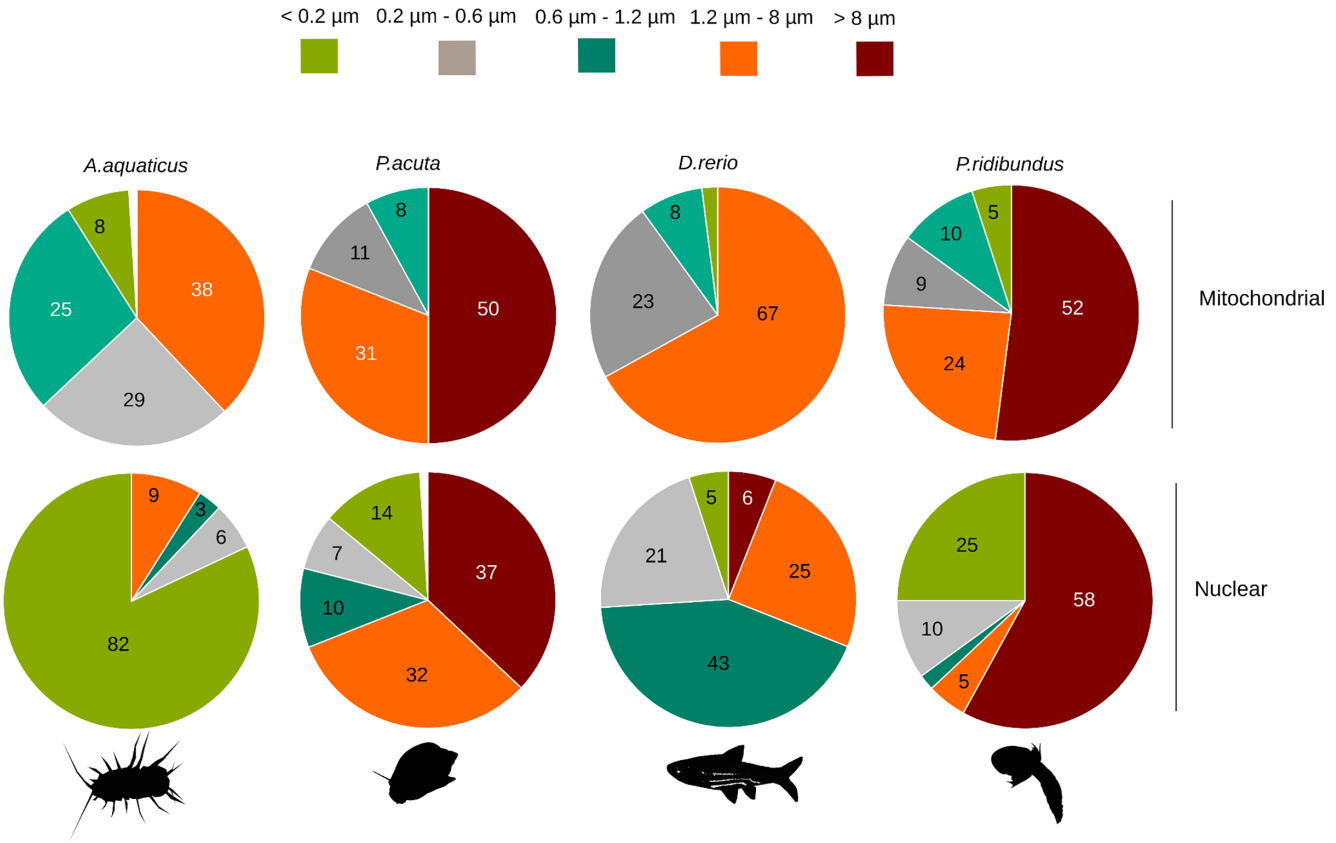


**FIGURE 4** | Pie chart showing the percentage of eDNA copies per particle size class, per taxa, and per genetic compartment averaged over the three sampling times.

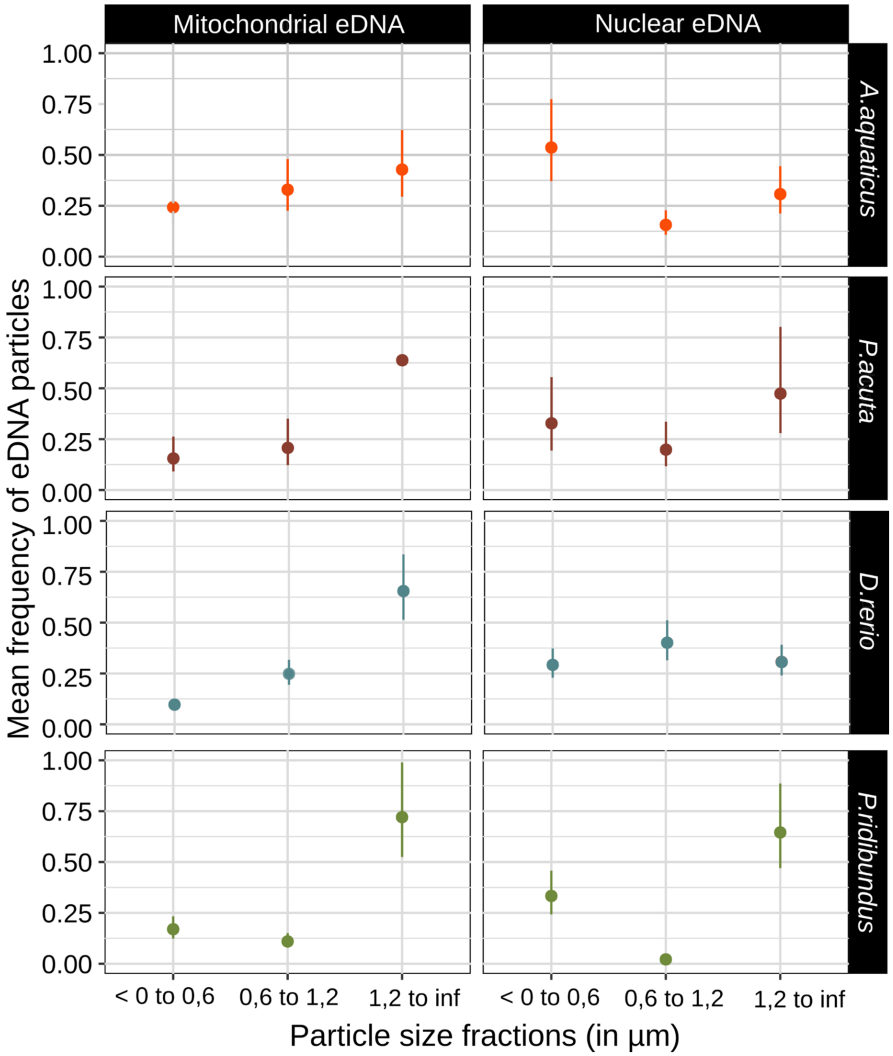


**FIGURE 5** | Effects of taxa, genetic compartment, and their interactions on the frequency of eDNA particles in a size fraction estimated by the GLMM.

releasing the highest amount of eDNA, regardless of the targeted genetic compartment and with values close to those reported in the literature ($10^4$ and $10^5$ copies/$L^{-1}$; Klymus et al. 2015; Maruyama et al. 2014; Sassoubre et al. 2016). While a positive relationship between eDNA quantity and organism biomass and/or abundance has been described mainly in teleosts (Harrison, Sunday, and Rogers 2019; Jo et al. 2020), our results showed that, at this very broad taxonomic scale, eDNA concentration is likely to be a very poor predictor of biomass variations. These findings are consistent with several studies focusing on non-teleost organisms, such as the hellbender salamander (Spear et al. 2015), northern crested newt (Biggs et al. 2015), crayfish (Dougherty et al. 2016; Larson et al. 2017), and green crab (Danziger, Olson, and Frederich 2022) which found no relationship between biomass and eDNA concentration. As we compare more and more distantly related taxa, it is likely that the biomass factor becomes less relevant compared to other biotic (such as stress, metabolism, life stage, reproduction, activity, diet, and development) as well as abiotic factors (including water temperature, UV radiation, and salinity) (reviewed in Stewart 2019; Rourke et al. 2022).

In our experiment, tadpoles released the largest amount of eDNA, between one and two magnitudes more than other taxa. We can propose several hypotheses to explain this high eDNA production rate. First, tadpoles eDNA could originate from the mucus secreted in large quantities by amphibians, especially under stressful conditions such as modifications in ionic conditions and temperature changes (Bernabò et al. 2013). It is therefore possible that the experimental conditions favored high excretion of mucus and eDNA. Second, the juvenile stage could promote eDNA excretion through cell turnover, as has been shown in several species such as bluegill sunfish (Maruyama et al. 2014) and the crayfish *Procambarus clarkii* (Tréguier et al. 2014). In the latter case, the authors suggest an increase in molting during the early stages of growth, leading to the release of eDNA.

Isopods showed the lowest rate of eDNA production in our experiment, between 3 and 4 magnitude lower than other taxa and despite the high density of organisms (150 individuals/tank). These results corroborate those of Andruszkiewicz Allan et al. (2021) who found that the rate of eDNA release from marine grass shrimp (*Palaemon* spp.) in microcosms was also very low compared to a

marine teleostean (*Fundulus heteroclitus*) and jellyfishes (*Aurelia aurita* and *Chrysaora* spp.). As all arthropods, isopods have a chitinous exoskeleton which can potentially limit the release of eDNA compared to mollusks, fish, and tadpoles. Although on average the rate of eDNA production in isopods is low, we observed peaks in eDNA quantities (up to $10^4$ copies/L/g at 14 and 21 days, Figure 2). We also observed exuviae in certain tanks, which are recognized as a source of DNA (Watts et al. 2005). Interestingly, research on crayfish indicates that these organisms release a relatively small amount of eDNA, with considerable variation over time. This suggests that crustaceans in general may excrete eDNA in pulses during molting and reproductive cycles (Dunn et al. 2017; Harper et al. 2018; Tréguier et al. 2014), in contrast to the possibly more constant release of eDNA by other organisms. Consequently, detecting these organisms with eDNA might pose greater challenges, potentially necessitating multiple sampling sessions for reliable detection. Further research is needed to analyze the temporal dynamics of eDNA production by arthropods and the underlying factors.

The concentrations of nu-eDNA were significantly higher than those of mt-eDNA for most taxa (isopods, mollusks, and teleosteans). This result corroborates those found in the literature (Jo, Murakami, et al. 2019; Moushomi et al. 2019) and can be explained by the level of duplication of mitochondrial and rDNA loci in a cell. Indeed, nu- and mt-eDNA relative copy numbers are a by-product of their cellular copy numbers. As suggested by Minamoto et al. (2017) and Dysthe et al. (2018), the nuclear gene targeted in our study (the ITS2 gene which is part of the ribosomal DNA loci) might be present at a higher genomic copy number per cell than the mitochondrial gene (16S gene). For the majority of the taxa in our study (isopods, snails, and teleosteans), this hypothesis was confirmed since we estimated that the number of ribosomal loci per cell was six to nine times higher than the number of mitochondrial ones (see Section 3.4). For tadpoles, the opposite pattern was found, and corroborates with the variations in the quantity of nu and mt-eDNA observed (Table 4). These findings support the idea that environmental DNA copy number variation across loci and organelles might be controlled by their cellular duplication level. Nevertheless, these cellular replication estimates remain approximate and would be more accurate using whole genome data and adding other factors to the analysis such as the loci GC content which can lead to sequencing biases (Benjamini and Speed 2012). In addition, the number of mitochondria per cell varies across cell types (Robin and Wong 1988) and even the number of rDNA cellular copies can vary during the development (Tao et al. 2020). The relative number of rDNA and mitochondrial loci per cell is therefore not fixed for a given species. Although further studies should be carried out to determine how the duplication rate may vary between species and impact species eDNA composition, the use of nuclear rDNA genes could be valuable for the detection of aquatic organisms (Minamoto et al. 2017; Dysthe et al. 2018), particularly for rare taxa or those releasing low amounts of eDNA such as isopods. Unfortunately, the genomic databases contain very few nuclear rDNA genes (including ITS sequences), which is currently challenging to design specific primers or perform taxonomic assignment potential leading to non-target amplification.

### 4.2 | eDNA Particle Size Distribution Varies According to Taxa and Genetic Compartment

Taxa and genetic compartment are major controlling factors of eDNA particle size distribution. For all taxa when targeting their mt-eDNA, we observed a general converging pattern: very few small eDNA mt-eDNA particles ($<0.2\,\mu m$) with an increasing frequency of larger particles ($1.2\,\mu m$ and above). This pattern is consistent with those found by the rare studies on the mt-eDNA PSD of teleostean species (i.e., Common Carp, Brook Trout, Turner et al. 2014; Wilcox et al. 2015) and on a snail (*Lymnaea stagnalis*, Zhao, van Bodegom, and Trimbos 2021). It confirms the hypothesis that eDNA particles are released in multiple states, but that most mitochondrial DNA particles in water samples likely correspond to subcellular (e.g., mitochondrial) or cellular particles.

The PSD of nu-eDNA showed a radically different pattern of that of mt-eDNA. We observed a taxa-specific particle size distribution, with an overall higher frequency of small eDNA particles ($<0.2\,\mu m$), especially in the isopod tanks. In contrast to mt-eDNA, there was a low frequency of large nu-eDNA particles, with the exception of amphibians, which surprisingly release eDNA in two main size ranges, that is, $<0.2\,\mu m$ or $>8\,\mu m$. These results differ from those of Jo, Arimoto, et al. (2019), Jo, Murakami, et al. (2019) who quantified the ITS1 of Japanese Jack Mackerel individuals (*Trachurus japonicus*) and found that most nu-eDNA particles were larger than $10\,\mu m$. This may be explained by the differences in experimental design between our study and that of Jo and colleagues, in which the water samples were taken 6, 12, and 18 h after the animals had been removed at the end of a week's stabling. Since we sampled the water after 7 days without removing the organisms, the eDNA particles are continuously released into the system while other particles have already begun a degradation process. Nevertheless, nu-eDNA seems much more unstable than mt-eDNA and further research is needed to understand the origin of these differences between the PSD of nu-eDNA and mt-eDNA. Developing microscopy-based methods could be relevant for this and would certainly make it possible to determine precisely the state and origin of eDNA particles.

### 4.3 | Effect of Sampling Time and Temperature on eDNA Counts and Particle Size Distribution

Because the mechanisms of degradation are complex, choosing the sampling time to study eDNA in microcosms is challenging. Our initial objective was to estimate the production and states of eDNA at an equilibrium state between eDNA production and degradation, to approximate what would be found in a natural environment. Since eDNA is very sensitive to degradation, we chose three sampling times spaced in time to estimate the replicability of our experiment (after 7, 14, and 21 days). Modeling the effect of time on eDNA particle size distribution was difficult due to the absence of eDNA copies in some size fractions. Consequently, we had to group the eDNA particles into three size fractions to avoid a problem of partial separation (see Section 2). This strategy may have masked a subtle effect of sampling time on PSD. Indeed, when we examined the raw data (i.e., ungrouped size

fractions), we observed a slight decrease in the frequency of the largest eDNA particles over time and, conversely, an increase of the frequency of the smallest eDNA particles (< 0.2 μm), mainly of nuclear origin. This suggests that once released, eDNA particles degradation rate and steady state depend on their genomic origin (i.e., nuclear or mitochondrial). In the study conducted by Jo et al. (2020), the results showed faster degradation of eDNA of nuclear origin (ITS1 gene) compared to mitochondrial eDNA (COI gene) after the removal of individuals. They hypothesized that the linear form of nu-eDNA could make it more sensitive to exonucleases. Foran (2006) reported also that mt-DNA degradation was slower than nu-DNA degradation in tissue. Altogether, it is likely that mt-eDNA degrades less quickly than nu-eDNA in aquatic environments. However, the environmental parameters and molecular characteristics that could explain this difference in degradation rate speed are still unknown.

In addition to these parameters, it is possible that the water temperature had an impact on the number of copies of eDNA in the tanks. Indeed, because zebrafish have an optimal temperature higher than the three other taxa, and because we did not want to induce a thermal stress, their mesocosms were set to a higher temperature. Higher temperatures can (i) increase the rate of eDNA release by boosting metabolism (Jo, Arimoto, et al. 2019; Jo, Murakami, et al. 2019; Takahara et al. 2012), which in turn may increase the excretion of mucus and shedding of epithelial cells, and (ii) accelerate the degradation of eDNA particles through enhanced enzymatic and microbial activity (Kasai et al. 2020; Tsuji et al. 2017). Among the four taxa studied, only the fish were kept at a higher temperature (28°C compared to 22°C ± 1°C for the other three taxa). Despite this temperature difference, amphibians (maintained at 22°C) released the highest quantity of eDNA particles, suggesting that the temperature effect, if it exists, is completely masked by the taxa effect. To date, mesocosm studies examining effects of temperature on eDNA production rates showed conflicting results: no effect in two studies (common carp, *Cyprinus carpio*, Takahara et al. 2012; bighead carp, *Hypophthalmichthys* spp., Klymus et al. 2015), and a significant increase in production rates in Mozambique tilapia (*Oreochromis mossambicus*; Robson et al. 2016). Nevertheless, temperature may have an impact on PSD in the fish tank by accelerating eDNA degradation, as we observed a significant decrease in the amount of total eDNA over time and an accumulation of small eDNA particles (Figure 2).

The common paradigm in eDNA mesocosm studies is that large particles of eDNA are emitted and then degrade into smaller and smaller particles with some parameter like temperature controlling the speed of this degradation process. Nonetheless, other environmental factors could potentially alter this dynamic, such as water composition or the presence of suspended solids. In the study by Brandão-Dias et al. (2023), it was found that some size classes of eDNA particles aggregate with some suspended materials (in particular fine particulate organic matter and clay) which might reduce, stop, or even reverse the large to small eDNA particle process. We recommend that future mesocosm studies introduce these parameters (e.g., suspended solids, turbidity, microbial activity) to better understand changes in eDNA PSD in natural environments.

## 5 | Conclusion

This study provides important information on the characteristics of eDNA, and in particular protostomian eDNA, which have often been overlooked in eDNA method development. We showed that the quantity and size of eDNA particles in a water sample are highly dependent on the taxa emitting it and on its genetic compartment origin. While eDNA can be present in multiple states in a water sample, we have shown that small nuclear particles tend to dominate the eDNA pool. This supports the use of filters with porosities below 0.45 μm and also encourages further monitoring tests using nuclear genes, in particular for taxa with an exoskeleton and releasing small quantities of eDNA particles.

### Author Contributions

H.V. and T.L. designed the experiment, developed the qPCR assays and methods for eDNA sampling and extraction, collected, processed, performed data analysis and wrote the paper. T.D. co-directed the research project and took part in writing the manuscript. M.L. played a major role in data modelling and writing. L.K.-D. and M.G. greatly helped develop the qPCR assay, sample, filtrate and precipitate water samples and extract eDNA.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The DNA sequences and primers used for each gene–species pair are available on zenodo (10.5281/zenodo.11208087), as are the data used to model the effect of taxa, genetic compartment and time on total eDNA and PSD, and the data from the negative controls.

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## Supporting Information

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