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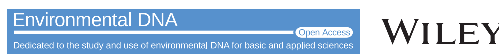


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# Stirring up the relationship between quantified environmental DNA concentrations and exoskeleton-shedding invertebrate densities

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## Abstract

The application of eDNA techniques for the detection, monitoring, and conservation of biodiversity holds great promise. While many studies apply eDNA techniques in aquatic systems to determine the presence or absence of a given species, using eDNA for the purpose of species density or biomass predictions remains a challenge, especially for freshwater invertebrates that shed exoskeletons. Here, we aimed to determine whether and how eDNA concentrations relate to exoskeleton-shedding invertebrate densities. We used microcosms holding different densities of a common invertebrate freshwater species, *Daphnia magna*. During 2 weeks, we monitored temporal dynamics of eDNA and the eDNA/density relationship by taking water samples and quantifying eDNA concentrations with the droplet digital PCR. The setup included one treatment without and one with homogenization before sampling, to test the effects of admixture on the relation between eDNA concentration and density. *Daphnia magna* individuals were removed after 1.5 weeks to track DNA degradation rates. In the stagnant water setup, hardly any DNA was detected before *D. magna* removal. Within days after removal, eDNA concentrations became undetectable. No significant correlation between *D. magna* density and eDNA concentrations was observed. In the homogenization treatment, a significant positive correlation between eDNA concentration and densities was demonstrated for the days around *D. magna* removal, albeit with some within-treatment variability. Our results show that, given adequate time for eDNA production and degradation to stabilize, positive correlations between eDNA and organism densities in water with sufficient homogenization are detectable for exoskeleton-shedding invertebrates. Therefore, our study indicates that—although difficult—using eDNA to quantify freshwater exoskeleton-shedding invertebrate densities may be possible under field conditions if circumstances result in frequent homogenization of the water column.

KEYWORDS

crustacean, *Daphnia magna*, digital droplet PCR, DNA concentrations, eDNA/density relationship, exoskeleton-shedding invertebrates, homogenization, Quantification

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# 1 | INTRODUCTION

In a time of severe global biodiversity decline, ecological studies are ever more needed to achieve science-based conservation and management of biodiversity (Cardinale et al., 2012; Dirzo et al., 2014; Pereira et al., 2010). This might be even more pressing in freshwater ecosystems, where species richness is declining at a faster rate than in terrestrial and marine ecosystems (Dudgeon et al., 2006; Macadam & Stockan, 2015). To comprehend the decline in species diversity, both community composition and the densities of individual species and their fluctuations within the communities must be understood (Baird & Hajibabaei, 2012; Berkes, 2007; Magurran et al., 2010; McCarthy et al., 2014). The density of species within communities is often described by relative abundance (Hubbell, 2001; Legendre & Gallagher, 2001; Magurran, 2004), generally based on morphological techniques. Unfortunately, these sampling regimes are usually invasive, time-consuming, expensive, and require specialist taxonomic expertise. Particularly, the latter is often not available (Baird & Hajibabaei, 2012; Beja-Pereira et al., 2009; De Bie et al., 2012).

Monitoring methods that use environmental DNA (eDNA), that is, fragments of DNA prevailing in air, water, and soil identified through PCR and next-generation sequencing techniques, hold the promise to overcome the shortcomings of traditional sampling methods (Deiner et al., 2017; Deiner et al., 2016; Makiola et al., 2020; Porter & Hajibabaei, 2018; Thomsen & Willerslev, 2014; Valentini et al., 2016). Theoretically, this technique allows for species monitoring through direct isolation of DNA from the environment (Bohmann et al., 2014; Cristescu, 2014; Rees et al., 2014; Thomsen & Willerslev, 2014; Valentini et al., 2016). So far, most eDNA studies have been directed toward the detection (presence/absence) of invasive or rare eukaryotic species, where eDNA techniques have proven less biased and labor-intensive than traditional methods (Bálint et al., 2018; Goldberg et al., 2016; Harper et al., 2019) Although interesting from a rare species perspective, such assessments generally do not yield a better understanding of ecosystem functioning. Therefore, the eDNA method needs to be further developed to assess abundance of species important for ecosystem functioning.

Studies that have related eDNA concentrations to species density (biomass/abundance) in freshwater ecosystems have focused almost exclusively on fish or amphibian species (Evans et al., 2016; Hänfling et al., 2016; Lacoursière-Roussel et al., 2016; Olds et al., 2016), even though invertebrate species generally contribute much more to aquatic biodiversity than vertebrates (Baxter et al., 2005; Moore, 2006; Pereira et al., 2012). Moreover, invertebrates are used as important indicators of water quality, particularly in stagnant and isolated water bodies (Bonada et al., 2006; Joao et al., 2012; Ojija & Laizer, 2016; Rizo-Patrón V. et al., 2013). There are two main reasons for the emphasis on vertebrates in aquatic eDNA studies to date. Firstly, the mode of eDNA shedding by fish and amphibians, a continuous shedding of skin cells, is highly predictable (Klymus et al., 2014). Secondly, these species have relatively high mobility in aquatic environments, which homogenizes eDNA concentrations (De Bie et al., 2012). In combination, this allows for a density estimate based on the eDNA concentration in a given water sample (Barnes & Turner, 2016).

Much less work has been done on species that exhibit different modes of DNA shedding (Barnes & Turner, 2016) that might complicate detection with eDNA methodologies. Many invertebrates, such as crustaceans and insects, mostly release eDNA into their environment by molting their exoskeletons (Chequer et al., 2019; Deiner & Altermatt, 2014). This mode of eDNA shedding might obscure eDNA concentration–density relationships since connected cell structures, containing DNA, might not homogenize as easily, will settle down faster, and will therefore be harder to detect as separate DNA-bearing cells or mitochondria (Barnes & Turner, 2016; Carim et al., 2016). As a potential consequence, most eDNA studies on these exoskeleton-shedding invertebrates have shown low to minimal detection rates (Carim et al., 2016; Tréguier et al., 2014). Improved detection is hampered by the lack of knowledge on eDNA production and degradation, processes that together determine the eDNA concentration at any time point (Thomsen et al., 2012). The contradiction between the high abundance of exoskeleton-shedding invertebrates yet low detection rates using eDNA calls for studies that research quantitative relationships and temporal dynamics between density and eDNA concentration for exoskeleton-shedding invertebrates.

To move toward a better understanding of the relationship between species densities and resulting eDNA concentrations for exoskeleton-shedding invertebrates, we carried out a microcosm experiment with two treatments and increasing densities of *D. magna*. Specifically, we were interested in determining (a) the temporal dynamics of eDNA in relation to production and degradation and (b) the effects of homogenization on the relationship between eDNA concentration and *D. magna* density. Droplet digital PCR (ddPCR) has been shown to be more accurate and sensitive in absolute quantification of target DNA than the commonly used quantitative PCR (qPCR), especially when concentrations are low (Doi et al., 2015; Hindson et al., 2013; Nathan et al., 2014). Furthermore, ddPCR analysis does not need calibration curves nor many replicates, yet still has a higher reproducibility than qPCR and is less sensitive to PCR inhibitors, making it more cost-effective (Doi et al., 2015; Hindson et al., 2013; Nathan et al., 2014; Yang et al., 2014). Therefore, to deal with detection limitations due to low eDNA concentrations, we quantified eDNA concentrations using droplet digital PCR (ddPCR).

# 2 | MATERIALS AND METHODS

## 2.1 | Experimental setup and sampling

*Daphnia magna* was used as a model for exoskeleton-shedding invertebrate since it is often a dominant invertebrate taxon in freshwater bodies, especially in stagnant water (Ebert, 2005). Additionally, it is relevant as a model organism because, like all arthropods, the

majority of eDNA shedding happens during the molting of its exoskeleton (Chequer et al., 2019; Deiner & Altermatt, 2014). This behavior compares nicely to other exoskeleton-shedding invertebrates, such as crayfish, that have been successfully detected using eDNA techniques in previous studies (Carim et al., 2016; Dunn et al., 2017; Larson et al., 2017; Tréguier et al., 2014). Some obvious differences between crayfish and *Daphnia* might lead to differences in eDNA detection: (a) crayfish have higher biomass per individual and (b) often occur on or close to the sediment, while daphnids are found throughout the entire water column, have a higher metabolic rate, and are often present in greater numbers, much like the majority of arthropod species (including most insects) living in fresh water, thus making them a potentially appropriate species for eDNA studies (Deiner & Altermatt, 2014; Delong et al., 2014; Tréguier et al., 2014). Furthermore, using *D. magna* is ecologically relevant as it is an often-used model organism for toxicity tests (Barmentlo et al., 2018; Traudt et al., 2017). Lastly, it is also easy and inexpensive to breed and keep, matures early, and does not show predator–prey like behavior (Harris et al., 2012), even at relatively high densities.

We conducted two treatments to investigate the distribution of *D. magna* eDNA and its relationship with density, using the same general setup as described below (Figure 1). The experiment was conducted in a climate chamber where temperature (22°C), humidity (80%), and light (setpoint 35%, on 7 a.m., off 11 p.m.) were kept constant throughout the experiment. The treatments are representative of two common situations in stagnant water bodies, one without admixture and one with admixture. More importantly, both situations may affect where the eDNA is present in the water column and thereby impact the eDNA detection and the relationship between eDNA concentration and density. In the first treatment (further referred to as the No-homogenization treatment), we avoided disturbing the medium in the microcosm. In the second treatment, we stirred the medium within the microcosm vigorously before water was extracted, thereby increasing homogenization of any eDNA present (hereafter referred to as Homogenization treatment).

We performed both treatments with five different densities of *D. magna*: 0, 10, 15, 25, and 50 individuals within a volume 320 ml *D. magna* medium OECD Elendt M4 in each microcosm (OECD, 2012). The densities ranged from 33 ind/l to 167 ind/l. This represents a good proportion of the range of densities observable in typical field situations (Barmentlo et al., 2018).

We used neonate individuals selected within 24 hr after hatching from a *D. magna* culture. Since this species reproduces clonally, which results in neonate individuals at the same life stage having equal biomass (Deiner & Altermatt, 2014), we will further only refer to densities. A total of 2 mg of inactivated spirulina powder (raw organic food) was dissolved in 2 ml of OECD medium. Subsequently, 5 droplets of this solution were fed to the *D. magna* individuals using a Pasteur pipette, every other day. The effect of *D. magna* density on eDNA concentrations was assessed for 9 days, after which the *D. magna* were removed to assess eDNA degradation in the microcosms. Density zero (0) was used to monitor and correct eDNA concentrations for potential eDNA contamination between microcosms. Except for density zero, we performed the setup in both treatments in triplicate to correct for sample variance.

For eDNA extraction, one 15 ml water sample was taken daily or twice-daily in every microcosm using a 25-ml volume pipette (Greiner Bio-one). Water samples were taken on days 2–14 at 8:30 a.m. From days 6 to 10, we also took samples at 8:15 p.m. (days 6.5, 7.5, 8.5, 9.5, 10.5) to better quantify eDNA production and degradation dynamics (Figure 1). In total, 234 water samples were taken. Morning samples were extracted during the afternoon, and evening samples were extracted during the next morning. To ensure volume continuity throughout the treatments, 15 ml of new medium was added after each sampling time. Volume remained constant during the removal of *D. magna* (between sampling time day 9 and day 9.5; see Figure 1) by catching all individuals with a Pasteur pipette, placing them in a Falcon tube, and returning any medium in the Falcon tube to the respective microcosm over a strainer. Assuming constant daily evaporation, the obtained eDNA concentrations were

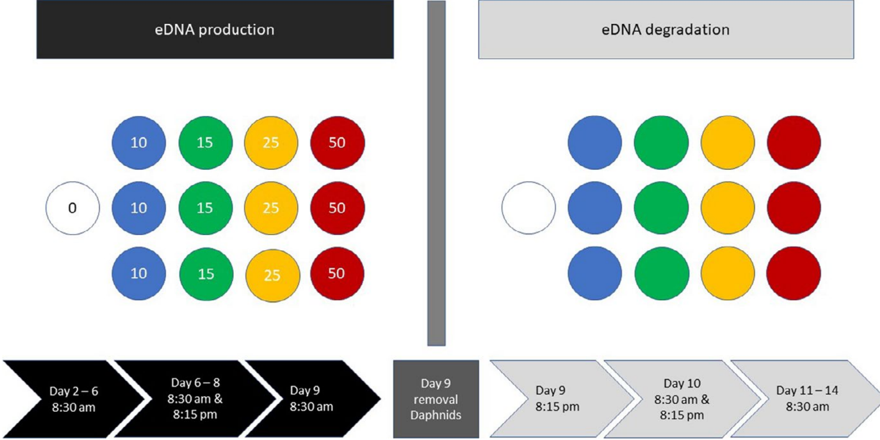


**FIGURE 1** Schematic visualization of the experimental setup including number of replicate microcosms used per density (one circle represents one microcosm), the timeline with the sampling times per day (gray arrows), and the removal of*Daphnia*individuals (gray square). Additionally, the different densities in the microcosms are indicated by colors (white 0, blue 10, green 15, orange 25, and red 50) and by numbers when the*Daphnia*were present (eDNA production phase of the experiment)

corrected for volume loss in individual microcosms by comparing the beginning and end volume of the different microcosms.

A recent modeling study demonstrated that the proportion of the water body sampled (rather than the volume of water) is of significant importance to the detection probability of eDNA (Willoughby et al., 2016). The amount of water extracted here was 5% of the total water body from our microcosm at every sampling time which should be more than adequate. A strainer was placed at the surface of the microcosm to ensure that *D. magna* would not be sampled. Before each sample, a new pipette was taken, and the strainer was cleaned. Since introducing *D. magna* to a new environment might induce a stress response, resulting in higher metabolic activity and therefore higher eDNA production, we started taking water samples at the second day following introduction to the microcosms (Boersma et al., 1999; Garreta-Lara et al., 2018).

As exoskeletons are particulate, the DNA present in these structures might not easily homogenize in the water and could thereby potentially disrupt the relationship between eDNA concentration and density. For example, some exoskeletons remain intact, whereas others might break into pieces, making it easier for the DNA-bearing cells and mitochondria connected to these parts to distribute through the water column. Potentially, detection success and thereby the detection of an eDNA concentration/density relationship might be dependent on the amount of disintegration of these exoskeletons. Additionally, especially in stagnant water bodies, eDNA measurements will not sample these exoskeletons directly, as the exoskeletons will likely settle to the bottom. Instead, measurements will be reliant on the amount of DNA that has detached from the exoskeletons and diffused into the water column, while the exoskeletons were breaking down. Hence, to keep measurements comparable to field situations and minimize the obscuring effect of exoskeletons on eDNA concentrations, exoskeletons were removed from microcosms during the first sample moment of every day. However, by doing this, "the DNA source" might have been excluded from the DNA buildup process. Therefore, exoskeletons were only removed once daily, so that "DNA-containing" cells and mitochondria would have the time to detach from the exoskeletons and provide a DNA signal in the water column. Additionally, we evaluated whether the removed number of exoskeletons was more strongly related to eDNA concentrations than the density of the organisms. As we used neonate individuals, no reproduction took place in our setup, and therefore, no increase in densities was observed during the treatments. We did have to remove some dead individuals. A total of three dead individuals were found (and removed) in the No-homogenization treatment from two of the three microcosms holding the highest density (50 individuals) in the 2 days prior to the removal of the *D. magna*. Hence, we assume that this event had limited effect on the eDNA production during the short remainder (only 1 or 2 days) of the treatment and have not corrected for this variation. Individuals were not replaced as their growth phase and therefore their shedding patterns would not have been compatible to the remaining individuals in the microcosms, which might in turn have influenced the DNA concentrations and obscured its relationship with densities.

## 2.2 | eDNA capturing and extraction

To correctly represent eDNA concentration–density relationships, it is crucial to capture both intracellular eDNA and extracellular eDNA, which can be done with a precipitation protocol (Deiner & Altermatt, 2014; Dejean et al., 2011; Doi et al., 2015; Turner et al., 2014). We followed the precipitation protocol by Ficetola et al. (2008) with a minor modification. Instead of centrifuging at a speed of 5500 *g* for 35 min, we centrifuged at 7,100 *g* for 30 min to ensure pellet fastening on the wall of the tube. After removal of the supernatant, eDNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as described by Ficetola et al. (2008) with some minor modifications to raise yield and concentration of the resulting DNA extract: Instead of adding 100 μl at once, we twice added 50 μl AE buffer to the spin column followed by an incubation step of 5 min. Subsequently, the extracts were stored at −20°C until PCR analysis.

## 2.3 | ddPCR analysis

We quantified eDNA using droplet digital (ddPCR) analysis. We performed ddPCR for mitochondrial cytochrome oxidase I gene since mitochondrial DNA has substantially greater copy numbers than nuclear DNA, which increases the detection rate (Mills et al., 2000). Based on mitochondrial cytochrome oxidase I gene, *D. magna*-specific primers (forward: 5′TGT ATG AGC GGT TGG AAT CA 3′ and reverse: 5′GCA AGA ACG GGC AAA CTT AG 3′ amplifying a total sequence length of 57 base pairs) were designed by making use of primer-3 (Rozen & Skaletsky, 1996). For parameter settings and considerations in primer-3 and further PCR protocol optimization, we used the steps as described in the Bio-Rad Droplet Digital PCR Applications Guide. Each ddPCR mixture contained 2 μl DNA extract, 0.2 μl 100 nM forward and reverse primers, 10 μl Bio-Rad EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), and 9.6 μl Milli-Q adding up to a final volume of 22 μl. Of this 22 μl PCR mixture, 20 μl was transferred onto a DG8 Bio-Rad cartridge containing 8 wells and covered with a DG8 rubber gasket. Each DNA sample was run once. Additionally, blank samples were run in every plate containing 3 μl Milli-Q instead of 3 μl DNA solution to check and correct for contamination. The ddPCR mixture was emulsified with Bio-Rad generator oil and partitioned in 10,000–20,000 droplets using a Bio-Rad QX-200 droplet generator. Of the resulting emulsion mixture, 40 μl of the produced droplet mixture was pipetted into a semi-skirted TwinTec 96-well plate. The plates were sealed with pierceable sealing foil, using the PX1 PCR Plate Sealer (Bio-Rad). PCR was performed in a Bio-Rad C1000 touch thermal cycler using the following program: 5 min at 95°C, and 40 cycles of 30 s at 95°C and 60 s at 55°C with ramp rate of 2.0°C/s, followed by signal cancelation 5 min at 4°C and 5 min at 90°C and a hold at 12°C. After PCR amplification, the PCR plate was transferred to the Bio-Rad QX-200 droplet reader. To quantify the number of target copies, we used Bio-Rad's QuantaSoft software version 1.7.4. Droplets were

assigned as positive or negative by thresholding against the height of their respective fluorescence amplitude. The number of positive and negative droplets was used, through the Poisson modeling, to calculate the concentration of the target and reference DNA sequences and their corresponding 95% confidence intervals. The threshold for a positive signal was set based on a positive control sample using the QuantaSoft manual instructions. By using the separation value between the threshold and the center of the negative droplet band from the positive control sample, we subsequently determined threshold values in the test samples. Droplets above the threshold were counted as positive events. The blank samples containing only Milli-Q water were used as negative controls for the test samples. Count estimates for each sample were compared with the maximum confidence interval (95%) of the negative controls to determine whether DNA concentrations were statistically different from zero. The resulting concentration measurements in molecules/20 µl were used for further statistical analyses. If more than one negative sample contained more than 2 positive droplets after thresholding, the plate was rerun.

### 2.4 | Statistical analysis

To assess the effect of *D. magna* densities on eDNA concentrations on different days of the setup, we conducted robust multiple-model estimation (RMME) as described in Evans et al. (2016). Through multiple iterations, such models assign greater weight to central data points (data closely fitting the model at each iteration), while further data points are weighted less and data points with weight numbers of zero are identified as outliers. This is especially useful in eDNA studies as DNA concentrations are often too low to be counted as positive concentrations and therefore are noted as zeros (Evans et al., 2016). Therefore, distribution of eDNA data is often skewed toward zero. The reweighting process retains the maximum fraction of possible outliers without corrupting the estimate, through a bisquare redescending score function (Evans et al., 2016). This analysis was completed using the *lmrob()* function in the R-package *robustbase* (Finger 2010). Measured eDNA data of all days before and after the removal of *D. magna* were also combined to test whether there was eDNA buildup and degradation over time, respectively, and whether this temporal dynamic was affected by density (density and day). To account for eDNA contamination between samples, we created a contamination probability distribution. This Poisson distribution was described by the contamination found in density zero microcosms (mean contamination of 0.39 and 2.2 molecules/µl for the No-homogenization and Homogenization treatments, respectively). Estimated contamination (randomly drawn samples of the contamination distribution) was subtracted from measured eDNA concentrations. Robust models for each day including the contamination subtraction were run 1,000 times, and mean intercepts and slopes were determined. For days with significant slopes within an treatment, data were amalgamated to test whether the slopes differed significantly between days. Contamination was accounted for as above, and 1,000 sets of two linear mixed-effects models were run (including microcosm as random effect) to test whether the eDNA concentrations were best described using density only, or an interaction between density and day. Similarly, within each treatment eDNA concentrations of all days before and after the removal of *D. magna* were also combined to test whether there was eDNA buildup and degradation over time, respectively. To assess whether any temporal dynamic was affected by density, we again used 1,000 sets of contamination-corrected data to compare models with a day only fixed effect and a day*density interaction. We also evaluated whether the number of exoskeletons removed was more strongly related to measured eDNA concentrations than the density of the organisms, by comparing linear mixed-effects models with the number of exoskeletons and density as fixed effects, respectively, and microcosm as a random effect. Mixed-effects models were fitted using the *lme4* R-package (Bates et al., 2015). To determine whether interaction models significantly better described the data than the simpler models, we used the *ANOVA()* function of the associated *lmerTest* package and describe the distribution of *p*-values of 1,000 sets of model comparisons (Kuznetsova et al., 2017). All data visualizations and statistical analyses were conducted in R 3.4.2 and R Studio 1.1.414 (R Core Team 2012).

## 3 | RESULTS

In the No-homogenization treatment, eDNA concentrations remained undetectable until day 7 (Figure 2). DNA concentration peaked on sampling point day 9.5 shortly after individuals were removed. After the individuals had been removed, eDNA concentrations gradually decreased toward 0 for all densities tested (Figure 2). Daily RMME models did not demonstrate significant correlations between eDNA concentration and *D. magna* densities at any sampling time (table 1, Figure 3). However, combining all the data prior to removal of *D. magna* (day 9) showed a significant buildup of eDNA over time, which was significantly affected by *D. magna* density ($p < .02$). Upon removal, there was a significant degradation of eDNA ($p < .001$), which was not affected by *D. magna* density ($p > .25$).

In the Homogenization treatment, where the medium was homogenized prior to eDNA sampling, DNA was detectable from the first sampling time at day 2 until day 12 (Figure 2). Environmental DNA concentrations were variable across days and densities, resulting in erratic patterns between day 2 and day 12. Assessing the buildup of eDNA over time prior to removal of *D. magna* in this treatment (day 9) showed a significant trend of eDNA over time, which was significantly affected by *D. magna* density ($p < .035$). After individuals had been removed between day 9 and day 9.5, eDNA concentrations showed a decrease toward zero. This significant degradation of eDNA ($p < .002$) was not affected by *D. magna* density ($p > .08$). While low, density 0 demonstrated detectable eDNA concentrations on days 2, 4, 5, 10, and 10.5 (Figure A1, Appendix 1) and this was used to estimate contamination (see Methods). RMME models demonstrated significant positive correlations between eDNA concentration and *D.*

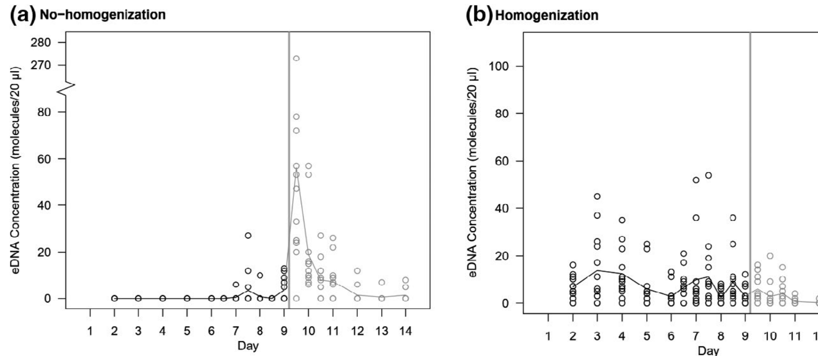


**FIGURE 2** Temporal dynamics of *Daphnia magna* eDNA concentration in (a) the No-homogenization treatment and (b) the Homogenization treatment, when the medium was stirred prior to eDNA sampling. Points demonstrate *D. magna* eDNA concentration in the different microcosms plotted per day. The vertical gray line indicates the time of *Daphnia* removal, while the sampling period before and after removal is indicated in black and gray, respectively. For visualization purposes, the black and gray lines show daily averaged eDNA concentration before and after *Daphnia* removal, respectively. Temporal dynamics per *D. magna* density is shown in Figure A1 (Appendix 1)

**TABLE 1** Results of the robust multiple-model estimation for the relationship between eDNA concentration and *Daphnia magna* density in the No-homogenization treatment

| Sampling time | *n* models converged | Intercept (*SE*) | Intercept *t*-value | Intercept *p*-value | Slope (*SE*) | Slope *t*-value | Slope *p*-value |
|---|---|---|---|---|---|---|---|
| Day 2 | 142 | −0.61 (0.37) | −1.66 | 0.94 | 0.00 (0.01) | 0.00 | 0.50 |
| Day 3 | 127 | −0.61 (0.37) | −1.67 | 0.94 | 0.00 (0.01) | 0.01 | 0.50 |
| Day 4 | 123 | −0.55 (0.37) | −1.50 | 0.92 | 0.00 (0.01) | −0.18 | 0.57 |
| Day 5 | 115 | −0.63 (0.39) | −1.63 | 0.93 | 0.00 (0.01) | −0.04 | 0.52 |
| Day 6 | 140 | −0.6 (0.36) | −1.68 | 0.94 | 0.00 (0.01) | 0.02 | 0.49 |
| Day 6.5 | 111 | −0.65 (0.37) | −1.74 | 0.95 | 0.00 (0.01) | 0.10 | 0.46 |
| Day 7 | 259 | −0.55 (1.19) | −0.46 | 0.67 | 0.00 (0.04) | −0.04 | 0.51 |
| Day 7.5 | 587 | −0.59 (5.34) | −0.11 | 0.54 | 0.01 (0.18) | 0.03 | 0.49 |
| Day 8 | 255 | −0.54 (1.87) | −0.29 | 0.61 | 0.00 (0.06) | −0.03 | 0.51 |
| Day 8.5 | 123 | −0.61 (0.37) | −1.64 | 0.94 | 0.00 (0.01) | −0.02 | 0.51 |
| Day 9 | 925 | **7.27 (2.59)** | **2.81** | **0.01** | −0.16 (0.09) | −1.83 | 0.95 |
| Removal of *Daphnia magna* individuals | | | | | | | |
| Day 9.5 | 1,000 | 45.8 (41.44) | 1.11 | 0.15 | −0.19 (1.41) | −0.13 | 0.55 |
| Day 10 | 1,000 | 14.18 (10.42) | 1.36 | 0.10 | −0.06 (0.35) | −0.17 | 0.57 |
| Day 10.5 | 1,000 | **13.38 (4.1)** | **3.26** | **0.004** | −0.21 (0.14) | −1.50 | 0.92 |
| Day 11 | 1,000 | 6.15 (4.94) | 1.24 | 0.12 | 0.03 (0.17) | 0.16 | 0.44 |
| Day 12 | 433 | −0.53 (2.47) | −0.22 | 0.58 | 0.00 (0.08) | 0.00 | 0.50 |
| Day 13 | 264 | −0.6 (1.36) | −0.44 | 0.66 | 0.00 (0.05) | 0.02 | 0.49 |
| Day 14 | 563 | −1.6 (1.74) | −0.92 | 0.81 | 0.08 (0.06) | 1.31 | 0.11 |

*Note:* For each sampling time, the table shows the intercept and slope and their standard error (*SE*), and associated *t*- and *p*-values, estimated from up to 1,000 contamination correction models (see Methods section for details, only converged models used). Significant intercepts ($p < .05$) are indicated in bold; none of the slopes were significant.

*magna* density on days 8 ($t = 3.90$, $p < .01$), 8.5 ($t = 2.95$, $p = .01$), 9 ($t = 3.20$, $p < .01$) before, and 9.5 ($t = 2.56$, $p = .01$) after *D. magna* removal (Figure 4). On day 4, RRME models showed a marginally significant positive correlation between eDNA concentration and *D. magna* density ($t = 1.62$, $p = .07$, table 2, Figure 4). Separate models testing whether the effect of density on eDNA concentrations differed between days (when there was a significant relationship) showed that the inclusion of a density*day interaction did not significantly perform better than models with density only ($p > .1$).

In both treatments, the measured eDNA concentration was significantly better predicted by a model including *D. magna* density than a model including the number of exoskeletons removed (No-homogenization: Chisq = 0.8274, $p < .001$; Homogenization: Chisq = 1.484, $p < .001$).

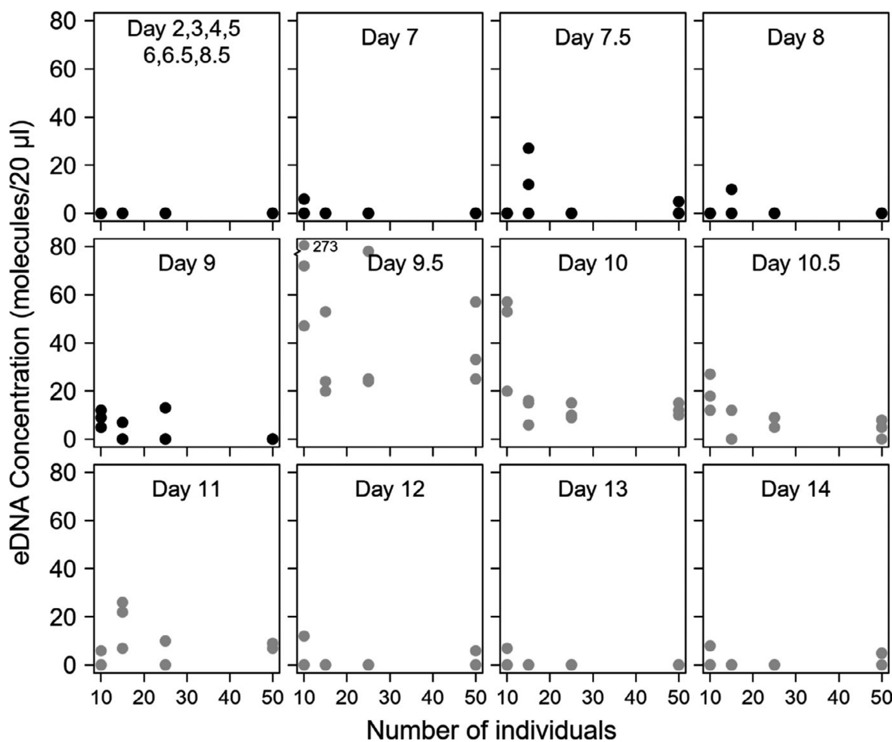


**FIGURE 3** Relationship between eDNA concentrations and *D. magna* density in the No-homogenization treatment over time. Robust multiple-model estimation, RMME, did not show any significant correlation between eDNA concentration and density (see Table 1). Measurements before and after removal of *D. magna* are indicated in black and gray, respectively

## 4 | DISCUSSION

Here, we report one of the few studies that has quantified the temporal dynamics of eDNA using a microcosm approach with different densities of a freshwater exoskeleton-shedding invertebrate species. Our study demonstrates that the extent of mixing in the medium is crucial for eDNA detection and abundance estimates of an exoskeleton-shedding species. Furthermore, once accumulation is sufficient, that is, once an organism stays in the same surroundings for a sufficient period of time (which seems likely in a natural setting) and if enough homogenization occurs (flowing systems), detection is possible and robust.

Our results suggest a major impact of homogenization on detection success. In the No-homogenization treatment, DNA signals were only found at the day of and days after the removal of *D. magna* individuals irrespective of their density. This indicates that the DNA detected after the *D. magna* removal was already present in the microcosms, although not in the higher parts of the water column. The DNA-bearing material had likely settled to the bottom of the microcosms since only minimal handling, and therefore minimal homogenization, took place during this stage of the setup. In this treatment, microcosms were disturbed for *D. magna* removal at day 9, which could have instantaneously stirred up DNA material and redistributed it throughout the medium. Potentially, heavy particles dislodged from exoskeletons that were previously removed may have also been stirred up and subsequently sampled, thereby causing disproportionately high values at this particular time point (see Figure 2a at day 9. 5). The disturbance and the resulting DNA peak on sampling day 9 probably also explain the positive and significant eDNA buildup found for this treatment. This would imply that disturbance of the DNA material in the microcosms right before and after *D. magna* removal caused the eDNA to become instantly detectable.

This is likely caused by the fact that, similar to many freshwater invertebrates, eDNA release of crustaceans is related to the release of exoskeletons which settle at the bottom. Several studies already indicated that at least some DNA-bearing material of aquatic organisms will settle in stagnant waters, probably depending on the state of the DNA, i.e. particulate, intramembranous, or extracellular (Barnes & Turner, 2016; Klymus et al., 2014). This might be especially the case for organisms that shed particulate DNA such as skins or exoskeletons and do this, like many invertebrates, in a discontinuous manner (Barnes & Turner, 2016; Dunn et al., 2017; Tréguier et al., 2014). Our results indicate that even when exoskeletons are removed, this settling of larger particulate DNA appears to occur. When sampling takes place close to the water surface (which is common in eDNA studies), this could potentially hamper the detection of these exoskeleton-shedding invertebrates through eDNA techniques in stagnant waters especially for invertebrate species that spend their entire life cycle in or close to the sediment. Depending on the sediment type, weather conditions, or species-specific seasonality patterns, this could result in a varying rate of DNA degradation and stirring up of the DNA material, respectively, and thereby a varying eDNA detection potential (Buxton et al., 2017; Strickler et al., 2014; Takahara et al., 2019).

In line with this hypothesis, the Homogenization setup resulted in a strongly increased probability of detection, and our results showed successful and continuous eDNA detection after day 2 of the treatment until *Daphnia* removal. Additionally, significant buildup was detected in the Homogenization treatment before

**TABLE 2** Results of the robust multiple-model estimation for the correlation between eDNA concentration and *Daphnia magna* density in the Homogenization treatment

| Sampling time | *n* models converged | Intercept (*SE*) | Intercept *t*-value | Intercept *p*-value | Slope (*SE*) | Slope *t*-value | Slope *p*-value |
|---|---|---|---|---|---|---|---|
| Day 2 | 1,000 | 3.44 (2.73) | 1.26 | 0.12 | 0.02 (0.09) | 0.19 | 0.43 |
| Day 3 | 1,000 | **25.67 (7.26)** | **3.54** | **0.00** | −0.53 (0.25) | −2.16 | 0.97 |
| Day 4 | 999 | 3.11 (5.7) | 0.55 | 0.30 | 0.31 (0.19) | 1.62 | 0.07 |
| Day 5 | 1,000 | 6.74 (4.98) | 1.35 | 0.10 | −0.17 (0.17) | −1.02 | 0.83 |
| Day 6 | 999 | 0.24 (3.08) | 0.08 | 0.47 | −0.01 (0.11) | −0.07 | 0.53 |
| Day 6.5 | 1,000 | −0.25 (4.18) | −0.06 | 0.52 | 0.16 (0.14) | 1.10 | 0.15 |
| Day 7 | 1,000 | 1.49 (10.76) | 0.14 | 0.45 | 0.01 (0.37) | 0.02 | 0.49 |
| Day 7.5 | 1,000 | 13.17 (10.61) | 1.24 | 0.12 | −0.32 (0.36) | −0.89 | 0.80 |
| Day 8 | 981 | −4.3 (1.39) | −3.1 | 0.99 | **0.18 (0.05)** | **3.90** | **0.001** |
| Day 8.5 | 1,000 | −4.18 (4.72) | −0.89 | 0.80 | **0.47 (0.16)** | **2.95** | **0.007** |
| Day 9 | 1,000 | −3.88 (1.8) | −2.16 | 0.97 | **0.2 (0.06)** | **3.20** | **0.004** |
| Removal of *Daphnia magna* individuals | | | | | | | |
| Day 9.5 | 1,000 | −2.44 (2.93) | −0.83 | 0.79 | **0.26 (0.1)** | **2.56** | **0.01** |
| Day 10 | 997 | −2.4 (4.31) | −0.56 | 0.71 | 0.05 (0.15) | 0.32 | 0.38 |
| Day 10.5 | 999 | 4.34 (2.89) | 1.5 | 0.08 | −0.13 (0.1) | −1.35 | 0.90 |
| Day 11 | 987 | −1.4 (1.54) | −0.91 | 0.81 | −0.01 (0.05) | −0.16 | 0.56 |
| Day 12 | 980 | −1.88 (1.36) | −1.38 | 0.90 | −0.01 (0.05) | −0.13 | 0.55 |
| Day 13 | 961 | −2.18 (1.31) | −1.66 | 0.94 | 0 (0.04) | 0.00 | 0.50 |
| Day 14 | 964 | −2.15 (1.3) | −1.65 | 0.94 | 0 (0.04) | −0.01 | 0.51 |

*Note:* eDNA concentrations were corrected for contamination as described in the method section. The table shows intercept and slope and their standard error (*SE*), and associated *t*- and *p*-values estimated from up to 1,000 contamination correction models (see Methods section for details, only converged models used). Significant slopes and intercepts ($p < .05$) are indicated in bold. Significant slopes have been drawn in Figure 4 for visualization.

*Daphnia* removal. Moreover, a significant and robust correlation between eDNA concentration and *D. magna* density was only present after sufficient time had allowed eDNA to accumulate (i.e., around the time of *Daphnia* removal). As has been demonstrated in other research, background bacterial degradation needs time to initiate at the beginning of the treatment before *Daphnia* introduction as the DNA in the microcosms was too limited for the bacteria to proliferate (Nevers et al., 2018; Sassoubre et al., 2016; Takahara et al., 2012; Thomsen et al., 2012). Only when enough time had passed for the buildup progress and background degradation to stabilize across densities, did this result in detectable correlations between densities and eDNA concentrations. Another explanation for the relatively late detectability of a relationship between eDNA concentrations and density could be differential eDNA production patterns between *Daphnia* densities. Different eDNA production patterns have previously been reported for different fish species and have been linked to among others differences in metabolism (Kelly et al., 2014). Indeed, we demonstrated higher eDNA concentrations during the sampling moments before *Daphnia* removal in the lower density microcosms compared with the highest density used, which could have potentially obscured the relationship between eDNA concentrations and densities. A previous study demonstrated that high *Daphnia* densities are negatively correlated with metabolic rates (Delong et al., 2014). Possibly, metabolic rates were inhibited more strongly at high densities than at lower densities (Boersma et al., 1999; Garreta-Lara et al., 2018). Moreover, crowding can induce similar responses as food shortages (Garreta-Lara et al., 2018), which reduce growth and molting rates (and hence eDNA shedding (Chang & Mykles, 2011; Hartnoll, 2001)). It remains unclear which of these processes is the best explanation for our results. Probably, both have played a role here simultaneously.

Together, this suggests that a certain period of eDNA production and degradation stabilization is needed before correlations between eDNA concentrations and densities can be demonstrated and will remain relatively stable over time. It is plausible that in field situations, where aquatic systems and therefore eDNA production and degradation are assumed to be in equilibrium, this is less of a problem.

In this study, the complete degradation of eDNA (which was either released from exoskeletons or nonexoskeleton bound) occurred within 5 days after *Daphnia* removal (Figure 2), indicating that eDNA presence is closely connected to species presence. Similar decreases in eDNA concentration have been shown in previous studies that investigated eDNA persistence in aquatic environments (Dejean et al., 2011; Thomsen et al., 2012). However, as our analysis demonstrated that eDNA concentrations were mostly influenced

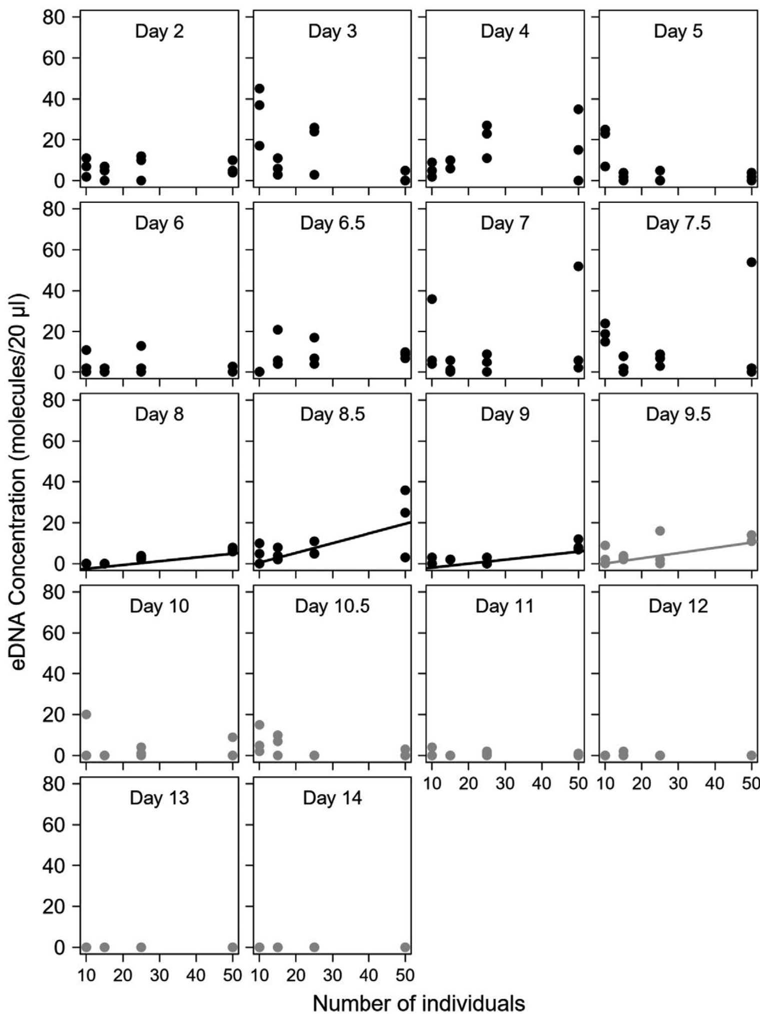


**FIGURE 4** Results of the Homogenization treatment showing the correlation between eDNA concentration and density of *Daphnia magna* at different sampling times. Lines indicate those days where robust multiple-model estimation (RRME) showed a significant relationship between concentration and density (see Table 2 for more detailed results). Measurements before and after removal of *D. magna* are indicated in black and gray, respectively

by densities and not by removal of the exoskeletons, these cellular structures will probably still be a considerable DNA source in the field. Hence, as a proportion of the eDNA in the field will originate from disintegrating exoskeletons and thereby the sloughing of individual cells, DNA may persist longer in field situations compared with the persistence found in this study. This will likely enhance the probability of detecting eDNA in streams or other aquatic ecosystems with sufficient currents for homogenization or admixture. Indeed, a previous field study showed that in moving water, eDNA of *D. longispina* could be successfully detected throughout most of the sample sites (Deiner & Altermatt, 2014). In an aquarium study, mimicking stagnant water, a relationship between crustacean biomass and eDNA concentration could be demonstrated only when individuals were egg bearing, resulting in high eDNA concentrations (Dunn et al., 2017). This further supports the notion that eDNA concentrations and therefore a robust relationship with densities or biomass are potentially more problematic in stagnant water than in other water types.

Overall, our study indicates that using eDNA for detection and monitoring of density or biomass of freshwater exoskeleton-shedding invertebrate communities is only feasible in particular conditions. Given sufficient time for the eDNA production and degradation to stabilize and only in water bodies with enough movement for admixture, positive correlations between eDNA and organism densities are likely to be found. This contrasts with several studies on fish and amphibians for which a wider range of conditions seems to be suitable for the detection of correlations between eDNA concentrations and organism densities (Evans et al., 2016; Hänfling et al., 2016; Lacoursière-Roussel et al., 2016; Pilliod et al., 2013; Saitoh et al., 2016; Valentini et al., 2016). Since a large proportion of invertebrates shed exoskeletons and invertebrates, in turn, represent a large proportion of freshwater fauna, the results from this study likely reflect the situation for a significant part of the freshwater biodiversity (Albrecht et al., 2007; Baxter et al., 2005; Covich et al., 1999; Dettner, 2019; Moore, 2006; Pereira et al., 2012; Wallace & Webster, 1996). This would indicate that densities of this very diverse group of organisms might be quantifiable

by eDNA techniques after all, in conditions described above. The ability to use eDNA to measure freshwater invertebrate densities, in addition to community composition, will greatly increase comprehension of the decline of this important indicator group for ecosystem quality and improve its conservation prospects.

To further improve density estimates for exoskeleton-shedding species in stagnant waters, site occupancy modeling could be used, as these models can infer densities from eDNA presence/absence data alone (Schmelzle & Kinziger, 2016). Also, other sampling regimes could be explored. A previous study that focused on eDNA detection of crustaceans showed that adjusting sampling strategies, by sampling low to the bottom and thus closer to the eDNA, accommodates for detection difficulties due to settling DNA and resulted in successful detection of freshwater shrimps (Carim et al., 2016). Furthermore, it could be useful to reiterate an experiment as performed here but with the exoskeletons left behind, to provide a comprehensive source of eDNA and which is simultaneously relevant for aquatic systems. Moreover, this could allow for a more empirically representative relationship between eDNA concentrations and species abundance, as exoskeletons are also left behind in field situations. Finally, different strategies for estimating biomass of freshwater invertebrate DNA might be implemented. For example, calibration studies could be performed where individuals of different freshwater species might be sampled and counted through traditional techniques, combined with physically grinding to homogenize DNA and subsequently analyzed through metagenomic analysis (Elbrecht et al., 2017). These calibration measurements and a similar methodology, except for the counting of individuals, might be applied in subsequent field studies. Although such a method would not get rid of the invasiveness of traditional surveys, it may be much faster and cheaper than morphological identification. In turn, the number of samples could be increased which would allow for an increased temporal or spatial coverage of the study area. Furthermore, as entire individuals are sampled, ground, and analyzed for DNA, any obscuring effects of different eDNA shedding rates between populations on eDNA concentrations would pose less of a problem. We suggest that future studies further investigate the potential of these techniques for the improvement of freshwater invertebrate detection and abundance estimation, which might eventually lead to a robust application of DNA techniques for a total freshwater census.

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

KBT designed the research, performed most of the research, analyzed part of the data, and wrote most of the paper. EC did most of the statistical analysis and co-wrote the paper, and AS performed part of the research. CJMM co-wrote the paper. MS co-wrote the paper. LB did some of the preliminary work that resulted in the protocol used in the paper. PvB contributed to the research design and co-wrote the paper.

## DATA AVAILABILITY STATEMENT

We will extract the ddPCR results as csv files add evaporation and exoskeleton data and store them in the publicly accessible repository Dryad.

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## APPENDIX 1

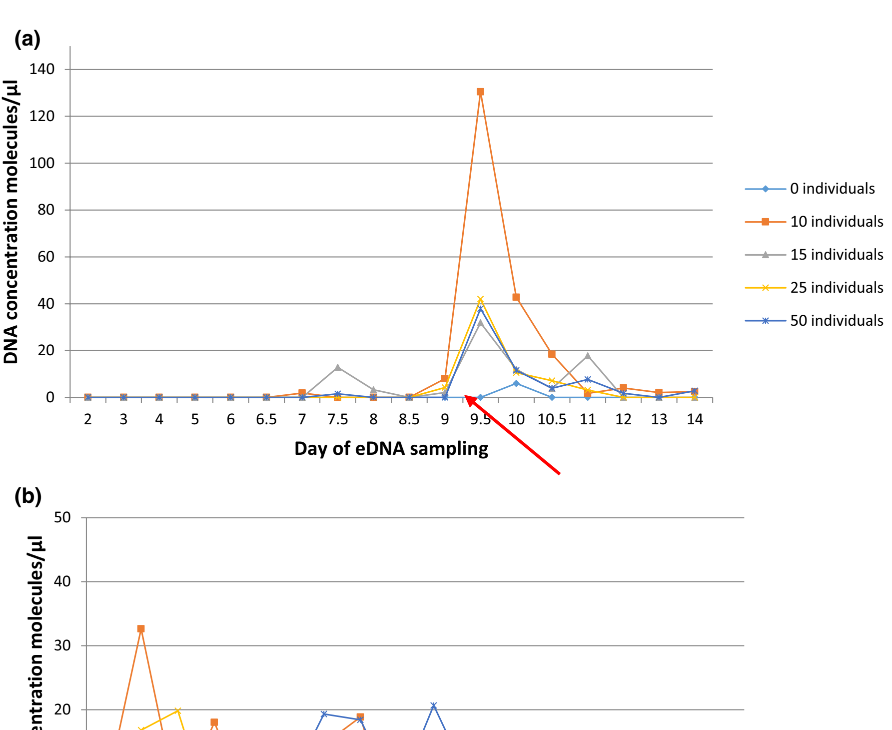


**FIGURE A1** The *Daphnia magna* eDNA concentration, in molecules/µl, of the 5 different densities used in the No-homogenization experiment (a) and the Homogenization experiment (b), averaged for 3 replicates over a time period of 14 days. Daphnia removal took place between day 9 and 9.5 and is indicated with an arrow. Note the different *y*-axis scales for figure a and b