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Ingestion and Toxicity of Polystyrene Microplastics in Freshwater Bivalves

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Abstract: The ubiquity of microplastics in aquatic ecosystems has raised concerns over their interaction with biota. However, microplastics research on freshwater species, especially mollusks, is still scarce. We, therefore, investigated the factors affecting microplastics ingestion in the freshwater mussel *Dreissena polymorpha*. Using polystyrene spheres (5, 10, 45, 90 µm), we determined the body burden of microplastics in the mussels in relation to 1) exposure and depuration time, 2) body size, 3) food abundance, and 4) microplastic concentrations. *D. polymorpha* rapidly ingested microplastics and excreted most particles within 12 h. A few microplastics were retained for up to 1 wk. Smaller individuals had a higher relative body burden of microplastics than larger individuals. The uptake of microplastics was concentration-dependent, whereas an additional food supply (algae) reduced it. We also compared the ingestion of microplastics by *D. polymorpha* with 2 other freshwater species (*Anodonta anatina*, *Sinanodonta woodiana*), highlighting that absolute and relative uptake depends on the species and the size of the mussels. In addition, we determined toxicity of polystyrene fragments (≤63 µm, 6.4–100 000 p mL^{-1}) and diatomite (natural particle, 100 000 p mL^{-1}) in *D. polymorpha* after 1, 3, 7, and 42 d of exposure, investigating clearance rate, energy reserves, and oxidative stress. Despite ingesting large quantities, exposure to polystyrene fragments only affected the clearance rate of *D. polymorpha*. Further, results of the microplastic and diatomite exposure did not differ significantly. Therefore, *D. polymorpha* is unaffected by or can compensate for polystyrene fragment toxicity even at concentrations above current environmental levels. *Environ Toxicol Chem* 2021;40:2247–2260. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Microplastics; Toxic effects; Mollusk toxicity

INTRODUCTION

Plastics are part of almost every aspect of modern human life. However, the rising global plastic production (PlasticsEurope 2018) coincides with plastic pollution in nature. In particular, the fragmentation of plastic debris results in a global distribution of micrometer-sized plastic particles, so-called microplastics (1–1000 µm; Hartmann et al. 2019), in the aquatic environment. Accordingly, there is emerging concern over the potential environmental impacts of microplastics.

In recent years, research on microplastic exposure and toxicity has especially focused on marine bivalves. Their high filtration activity results in a higher ingestion of microplastics compared to other taxa (Setälä et al. 2016), rendering bivalves especially susceptible to microplastic exposure. Uptake of microplastics has repeatedly been demonstrated in wild and cultured marine bivalves (Li J et al. 2019). The current microplastic body burden varies intensively from ≤1 (Railo et al. 2018) up to several hundred particles per individual (Mathalon and Hill 2014). Numerous experimental studies further confirm that bivalves ingest microplastics of different size, shape, and polymer type (see Brillant and MacDonald 2000; Bråte et al. 2018; Li L et al. 2019).

While there is sufficient evidence demonstrating that bivalves ingest microplastics, less is known regarding the ingestion kinetics. In theory, microplastic ingestion will depend on multiple factors, including the physicochemical properties of microplastics (e.g., size, shape, polymer type), the exposure and depuration time of the individuals, biological traits of

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species (e.g., feeding type), microplastic bioavailability, and the presence of other particulate matter. Previous publications investigated some of these relevant factors in mussels (Brillant and MacDonald 2000; Capolupo et al. 2018; Woods et al. 2018; Fernández and Albentosa 2019; Gonçalves et al. 2019; Rist et al. 2019a, 2019b). However, the use of different experimental designs (e.g., different types of microplastics and exposure periods) limits the comparability of the existing data and prevents a general assessment of microplastic uptake kinetics. Furthermore, understanding better the mussel microplastic ingestion kinetics will improve our understanding of a potential microplastic uptake by humans through mussels as a food source (Mercogliano et al. 2020). Yet, understanding the factors driving microplastic ingestion by bivalves is important to identify species that are particularly susceptible to microplastic exposure and to design adequate toxicity experiments.

To address this knowledge gap, we studied the ingestion and egestion of microplastics in the freshwater species *Dreissena polymorpha* in 4 experiments with similar design. For this, we exposed *D. polymorpha* to a mixture of 5-, 10-, 45-, and 90-μm polystyrene (PS) spheres to analyze how the factors exposure and depuration time (experiment 1), body size (experiment 2), food abundance (experiment 3), and microplastic concentration (experiment 4) affect the body burden of microplastics in *D. polymorpha*.

Dreissenids are a well-established test organism in freshwater ecotoxicology (Péden et al. 2019), but because of their small size and invasive character, they may not be representative of larger species (e.g., Unionidae in European freshwater systems). We, therefore, repeated ingestion experiments 1 and 2 with the 2 larger freshwater species *Anodonta anatina* and *Sinanodonta woodiana* to evaluate species- and size-specific variations.

In addition to microplastic ingestion, further data are needed regarding the toxicity of microplastics in freshwater bivalves. Previous research on *Corbicula fluminea* (Rochman et al. 2017; Guilhermino et al. 2018; Oliveira et al. 2018; Baudrimont et al. 2020) and *D. polymorpha* (Magni et al. 2018, 2019, 2020; Binelli et al. 2020) provided contradicting results. Although some of these studies identified significant microplastic-induced neurotoxicity, oxidative stress, and a change in feeding behavior, others did not observe such effects. Thus, we exposed *D. polymorpha* over 1, 3, 7, and 42 d to 6.4 to 100 000 particles (p) mL^{-1} using PS fragments (≤63 μm) and analyzed effects on the clearance rate as well as energy reserves (protein, glycogen, lipid content) and oxidative stress levels in the midgut gland (synonyms "digestive gland," "hepatopancreas") of *D. polymorpha*. In addition, we exposed the mussels to diatomite (100 000 p mL^{-1}) to compare the toxicity of microplastics and naturally occurring particles.

MATERIALS AND METHODS

Mussel culture

Dreissena polymorpha were collected from Oberwald Lake in Mörfelden-Walldorf, Germany (49°59′0.242″N, 8°35′48.666″E). *Sinanodonta woodiana* and *A. anatina* were purchased from local fish shops and cultures. In the laboratory, all bivalve species were cultured in aerated Organisation for Economic Co-operation and Development (OECD) medium (Organisation for Economic Co-operation and Development 2016) at 14 °C water temperature and a 16:8-h light:dark cycle. *Sinanodonta woodiana* and *A. anatina* were kept in 150-L tanks with up to 30 individuals $tank^{-1}$. *Dreissena polymorpha* was cultured in a 50-L tank with approximately 200 individuals $tank^{-1}$. Mussels used in the ingestion experiments were allowed to acclimatize for at least 1 wk, whereas those used in the toxicity study (*D. polymorpha*) were cultured for at least 4 wk prior to the experiments. Twice a week, at least half of the medium was renewed. Mussels were fed with algae (*D. subspicatus*) ad libitum at least thrice a week.

Particle characterization

For the ingestion experiments, we used 5-, 10-, 45-, and 90-μm plain, fluorescent PS spheres. We used spheres with a homogenous size because this allows for investigation of the size dependency of microplastic ingestion and depuration. The 10-, 45-, and 90-μm PS spheres were purchased from PolyScience (Fluoresbrite YG microspheres; excitation 441 nm, emission 486 nm). The 5-μm spheres were obtained from MagSphere (excitation 538 nm, emission 584 nm). We suspended the PS spheres in ultrapure water (microplastic stock suspensions) and determined the particle concentration and size distribution in the stock suspensions with a Coulter counter (Multisizer 3; Beckman Coulter; details in Supplemental Data, S1).

For the toxicity study, we used PS fragments (≤63 μm) because of the higher environmental relevance of fragments compared to spheres (De Sá et al. 2018) as well as diatomite (Sigma-Aldrich). The PS fragments were prepared from orange fluorescent drinking cups (excitation 360–370 nm) by cryomilling, followed by sieving with a 63-μm sieve. The diatomite particles were sieved in the same way to obtain the size fraction ≤63 μm. In our previous work, we confirmed that the cups were made of PS and had low but detectable concentrations of chemicals which could not be matched to substances commonly used in plastics (see Weber et al. [2020] for details). Further, scanning electron microscopic images of PS fragments as well as diatomite particles are published in Weber et al. (2021). These images indicate that both microplastic and diatomite powder included nano-sized particles. As stated in Weber et al. (2021), nanoparticle abundance was not quantified because micro-sized particles would have blocked the nanoparticle tracking analysis instrument.

We determined the particle number per powder mass (number mg^{-1}) and the particle size distribution with a Coulter counter (size range 2–60 μm). The microplastic and the diatomite powder contained 287 526 and 4 632 990 p mg^{-1}, respectively. Particle size distribution of the PS and the diatomite suspensions increased exponentially with decreasing particle size; 90% of the microplastic and the diatomite particles were smaller than 12.4 and 11.8 μm, respectively (for detailed methods and results see Weber et al. [2020]).

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Relevant factors affecting microplastic ingestion and depuration by D. polymorpha

Basic exposure scenario. All ingestion experiments with *D. polymorpha* were performed using the same basic exposure scenario (see Supplemental Data, S2.1). Ten-liter glass tanks (19 × 29.3 × 19 cm) were filled with 7.5 L aerated OECD medium and quartz sand (5 cm layer, previously annealed at 200 °C for 24 h). Each tank was equipped with a pump (Tetra IN400plus; Tetra) set to the lowest rate to create a constant circular water flow and to keep the particles suspended in the water column. The extent to which the particles remained in the water phase was characterized in an extra experiment. Despite a constant water flow, PS spheres settled, with smaller microplastics remaining longer in the water column. From 6 h onward, only 5- and 10-μm PS spheres were present in the water phase; and after 12 h, 50.2% of the 5-μm spheres and 77.3% of the 10-μm spheres had cleared from the water phase (for details on methods and results, see Supplemental Data, S2.2).

Twelve hours prior to the start of the experiments, 6 mussels were transferred to each tank. Algae (*D. subspicatus*) were added 1 h before the start of the experiment to stimulate filtration behavior. Each experiment started when microplastics were added to the tanks by pipetting the microplastic stock suspensions directly below the water surface into the flow of the pump. Because of the short exposure times (1–48 h), the water was not exchanged during the ingestion experiments. Throughout each experiment, we visually monitored each mussel hourly and recorded whether its valves were opened or closed. Only mussels which were open at least at 50% of the monitored time points (e.g., ≥6 time points throughout a 12-h exposure period) were classified as "active mussels" and further analyzed. In the following sections, we used this basic exposure scenario to examine the impact of the factors exposure and depuration time, body size, food abundance, and exposure concentration on microplastic ingestion by *D. polymorpha*.

Exposure and depuration time (experiment 1). The impact of the exposure time on microplastic ingestion was analyzed by exposing *D. polymorpha* (1.8–2.2 cm maximal shell length) for 1, 3, 6, 12, 24, and 48 h to a mixture of 5-, 10-, and 45-μm PS spheres at 3 p mL^{-1} each. Further, we added 90-μm PS spheres (0.1 p mL^{-1}) to examine whether *D. polymorpha* is able to ingest also larger microplastic particles. We applied these at lower concentrations because of high material costs, and the respective data were, thus, analyzed separately. We used one separate tank with 6 individuals for each time point. Algae were added to each tank at a concentration of 1 mg L^{-1} total organic carbon (TOC). Four "active" individuals from each tank were analyzed for their microplastic body burden.

For analysis of the impact of depuration time on the body burden of microplastics in *D. polymorpha*, we exposed 30 mussels (5 tanks with 6 individuals each) in the presence of algae (1 mg L^{-1}) to PS spheres (sizes and concentrations as stated in the previous paragraph) for 12 h. After the exposure, 28 active mussels were randomly selected and transferred into tanks filled with microplastic-free OECD medium. Individual *D. polymorpha* were held there for 1, 3, 6, 12, 24, 72, and 168 h (one tank per depuration time point with 4 individuals each). Directly after the transfer and subsequently every 24 h afterward, mussels were fed 1 mg L^{-1} TOC algae. Because mussels are able to reingest excreted microplastic, we transferred the mussels into new tanks with fresh medium after 24, 72, and 120 h to minimize microplastic reuptake.

Body size (experiment 2). In the second experiment, we evaluated the relationship between body size and microplastics in *D. polymorpha*. For this, *D. polymorpha* individuals from 3 different size classes (1.0–1.5, 1.8–2.2, and 2.5–3.0 cm) were exposed in the presence of algae (1 mg L^{-1} TOC) to PS spheres (sizes and concentrations as above, see section *Exposure and depuration time [experiment 1]*) for 12 h. For each size class, 2 tanks were set up with 6 individuals each. After the exposure, microplastic body burden was analyzed in 8 active out of the 12 exposed individuals per size class.

Food abundance (experiment 3). We evaluated how algae abundance affects the microplastic body burden by exposing *D. polymorpha* (1.8–2.2 cm) for 12 h to PS spheres (sizes and concentrations as above, see section *Exposure and depuration time [experiment 1]*) in the presence of 3 algae concentrations (0.2, 1, or 5 mg L^{-1} TOC algae). The microplastic to algae ratios (based on particle numbers) in the 3 exposures were 1:5589, 1:27 798, and 1:135 477, respectively, based on the sum concentration of 5-, 10-, 45-, and 90-μm spheres and of algae cells in each tank. Algae were added 1 h before the start of the experiment to stimulate filtration activity. For each algae concentration, 2 tanks with 6 mussels each were established and 8 active individuals per treatment analyzed.

Microplastic concentration (experiment 4). We investigated the relationship of microplastic concentration and microplastic body burden in *D. polymorpha* (2.5–3.0 cm) by exposing the mussels in the presence of algae (1 mg L^{-1} TOC) for 12 h to 5-, 10-, and 45-μm PS spheres at either 0.3 or 3 p mL^{-1} each. Again, we also added 90-μm PS spheres (0.01 or 0.1 p mL^{-1}, respectively). The 10-fold lower concentration of 0.3 p mL^{-1} was chosen to resemble environmental concentrations already reported for freshwater systems (Leslie et al. 2017; Lahens et al. 2018). For both concentrations, 2 exposure tanks were prepared (6 mussels per tank) and 8 active mussels were analyzed per concentration.

Analysis of microplastic body burden in D. polymorpha. After each experiment, *D. polymorpha* individuals were thoroughly rinsed with tap water and frozen at –80 °C. After defrosting, the shells were removed. All tissues were lyophilized to determine the total dry weight of each mussel. Afterward, tissues were lysed in 20 to 40 mL 10% potassium hydroxide solution at 55 °C for 24 to 48 h. The lysate was filtered on glass fiber filters (pore size 1.25 μm; VWR). Each filter was analyzed visually with a fluorescence microscope (BX50, ×40 magnification;

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Olympus), and the number of fluorescent spheres on the whole filter was determined for each microplastic type (for details see Supplemental Data, S2.2). The body burden in the mussels was characterized both separately for each microplastic type and as the total number of ingested microplastics (total body burden). The latter corresponds to the sum of 5-, 10-, and 45-µm PS spheres. Because of the divergent exposure concentrations (see section *Exposure and depuration time [experiment 1]*), the results for the 90-µm spheres are not included in the total microplastic body burden and are presented separately.

Quality assurance. The background contamination with fluorescent particles in *D. polymorpha* tissues was determined by analyzing mussels (1.0–1.5, 1.8–2.2, 2.5–3.0 cm) from the culture which had not been exposed to microplastics. We lysed 3 *D. polymorpha* individuals from each size class, as described in *Analysis of microplastics body burden in D. polymorpha*, and corrected all data from the ingestion experiments for the microplastic body burden in those control mussels (see Supplemental Data, S3.2) by subtracting the average number of microplastic-resembling particles per control mussel from the microplastic body burden in exposed individuals (separate data correction for each particle type).

Comparison of microplastic ingestion between freshwater mussel species

We further compared microplastic ingestion by *D. polymorpha* with other freshwater mussel species. Originally, we intended to repeat the experiments just described with the native species *A. anatina*. However, the number of available *A. anatina* specimens was too low. Therefore, we used a second species (*S. woodiana*) with similar morphology and ecology and limited the comparative studies to experiments 1 and 2.

We repeated experiment 1 with *S. woodiana* (9.5–12.0 cm). For the depuration experiment, we exposed 24 mussels (4 tanks with 6 individuals each). After the exposure, 16 "active" *S. woodiana* (for definition, see section *Basic exposure scenario*) were randomly selected and transferred into tanks filled with microplastic-free OECD medium. We limited the number of depuration time points to 4 (12, 24, 72, and 168 h) because of the limited number of mussels available.

We repeated experiment 2 with *S. woodiana* and *A. anatina* (2 size classes each: 6.0–8.0, 9.5–12.0 cm). For each species and size class, 2 tanks were set up with 6 individuals each. After the exposure, microplastic body burden was analyzed in 8 active out of the 12 exposed individuals from each treatment. Because of the mussels' inactivity in some treatments, the experiment was repeated with a third tank to obtain 8 active individuals per treatment.

For analysis of the microplastic body burden in *S. woodiana* and *A. anatina*, we did not analyze the whole body as we did for *D. polymorpha* because of the large body size and, thus, insufficient tissue lysis. Instead, we removed the mantle, gills, and foot. In a prior experiment, we demonstrated that microplastic levels in the mantle, gills, and foot were low compared to the other tissues; and we, thus, consider the number of microplastics in the removed tissues negligible (for details see Supplemental Data, S3.1). After dissection, we lyophilized both the mantle, gills, and foot and the remaining tissues to determine the total dry weight of each mussel. The mantle, gills, and foot tissue was discarded afterward, whereas the remaining tissue was analyzed for the microplastic body burden as described in *Analysis of microplastics body burden in D. polymorpha*. Again, we determined background contamination by lysing control mussels and correcting the results from the ingestion experiments accordingly (for detailed results, see Supplemental Data, S3.2).

Microplastic toxicity in D. polymorpha

Exposure scenario. *Dreissena polymorpha* were exposed to either 6.4, 160, 4000, or 100 000 p mL^{-1} PS fragments (≤63 µm) or 100 000 p mL^{-1} diatomite for 1, 3, 7 (acute exposure), and 42 d (chronic exposure). We also included a negative control without microplastic/diatomite. Each particle concentration was tested in a separate glass tank (14 × 20 × 20 cm) with 3 L of OECD medium and 40 mussels (2.0–2.3 cm). To keep the experiments manageable, we set up one set of 6 glass tanks (one tank per treatment) for the acute exposures (1, 3, and 7 d; for all 3 time points mussels were sampled from the same tank), whereas for the chronic exposure we set up a separate set of 6 glass tanks (42 d; scheme of the experimental design in Supplemental Data, Figure S2).

The required masses of microplastic and diatomite were weighed for each treatment and added directly to the medium in the aquaria. For the 6.4 p mL^{-1} treatment, we used a 100-fold diluted stock suspension in OECD medium, which was applied to the respective tank. Each tank was constantly aerated through 2 glass pipettes to enhance particle dispersion in the water phase. The mussels were fed with algae (*D. subspicatus*, 0.25 mg TOC $individual^{-1}$) daily. In the chronic exposure experiment, the medium was completely renewed every 7 d by transferring the mussels to new tanks prepared as described.

Mortality was recorded daily, and dead individuals were removed. After 1, 3, 7, and 42 d, the clearance rate of 10 individuals per treatment was determined. In case of acute exposures, individuals were reintroduced into their corresponding tanks afterward. Further, at each time point, 10 individuals were frozen in liquid nitrogen and stored at –80 °C for energy reserve and stress metabolite analysis.

Clearance rate, energy reserves, and stress metabolites. We quantified the clearance rate of *D. polymorpha* by placing 10 mussels per treatment and time point individually in an algae suspension and determining algae concentrations in the medium as chlorophyll fluorescence (in relative fluorescence units [RFUs]) prior to and after 45 min (Tecan; GENios; excitation 440 nm, emission 680 nm). The starting concentration was 4000 ± 360 RFU (±standard deviation), representing $1.21 \times 10^7 \pm 1.08 \times 10^6$ algae cells mL^{-1}. The clearance rate is the difference in RFUs before and after 45 min. We used *Raphidocelis subcapitata* (formerly *Pseudokirchneriella*

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subcapitata) instead of *D. subspicatus* because it is unicellular, allowing for more accurate fluorescence analyses.

As biochemical endpoints, we analyzed the energy content as well as oxidative stress markers in the midgut gland of *D. polymorpha*. The midgut glands from 10 individuals per treatment were dissected, wet-weighed, homogenized, and frozen at –80 °C. Energy reserves in the midgut gland homogenates were measured as the protein content according to Bradford (1976) and as the glycogen content (anthrone assay) and the total lipid content (sulfo-phospho-vanillin assay) according to Benedict (2014). Oxidative stress was quantified as the malondialdehyde (MDA) content (an important biomarker for lipid peroxidation) as well as the remaining antioxidant capacity. Concentrations of MDA were measured using the thiobarbituric acid reactive substances (TBARS) assay (Hodges et al. 1999; Furuhagen et al. 2014). The remaining antioxidant capacity in the midgut gland was measured with the oxygen radical absorbance capacity (ORAC) assay (Ou et al. 2001; Furuhagen et al. 2014). Further methodological details are provided in Weber et al. (2020). Because of highly divergent results for midgut gland with a wet weight <5 mg compared to midgut gland >5 mg, we excluded results on energy reserves and oxidative stress from individuals with an midgut gland <5 mg ($n = 7$–10).

Statistics

All statistical analyses were performed with IBM SPSS Statistics (Ver 25) using one-way or 2-way analysis of variances (ANOVAs). Prior to each analysis, we tested for normality (Shapiro-Wilks test), variance homogeneity (Levene test), and heteroscedasticity (*F* test). Very few treatments violated the normality criteria. In this case, we reperformed the statistical analysis after outlier exclusion but did not observe changes in the results. All data were visualized using GraphPad Prism 8.4.3 (GraphPad Software).

Statistics for the ingestion experiments with D. polymorpha. We analyzed the effects of the factors exposure time and depuration time (experiment 1), individual size (experiment 2), food abundance (experiment 3), and microplastic concentration (experiment 4) on the microplastic body burden by applying separate one-way ANOVAs for each factor (dependent variable, absolute or relative microplastic body burden; fixed variables, factors listed). In addition, we performed Tukey's posttests to analyze differences between the treatments of each experiment (full-factorial comparison of all treatments). Data for total microplastic body burden were log-transformed (depuration time, individual size, microplastic concentration), square root–transformed (food abundance), or not transformed (exposure time) for the statistical analysis.

Statistics for the comparison of microplastic ingestion between species. We analyzed the effects of the variable species in combination with the variable exposure time, depuration time (experiment 1), or individual size (experiment 2) as well as their interaction on the total microplastic body burden (absolute or relative) in the analyzed freshwater mussels with 2-way ANOVAs. Both variables were integrated as fixed variables, whereas the total microplastic body burden was used as a dependent variable. Further, for the results of experiment 2 we performed Tukey's posttest to determine homogenous subgroups in regard to the different species (*D. polymorpha*, *S. woodiana*, *A. anatina*) as well as size classes (1.0–1.5, 1.8–2.2, 2.5–3.0, 6.0–8.0, 9.5–12.0 cm). Data for total microplastic body burden were log-transformed (depuration time, individual size) or square root–transformed (exposure time) for the statistical analysis.

Statistics for the microplastic toxicity study with D. polymorpha. In the toxicity study, the effects of microplastic concentration and exposure time (both fixed variables) as well as their interaction (microplastic concentration × exposure time) were determined with 2-way ANOVAs for each endpoint (clearance rate, protein, glycogen, total lipids, MDA, antioxidative capacity) as a dependent variable. Data for the dependent variable were integrated as either log- (glycogen, lipids, MDA [TBARS], Trolox equivalents [ORAC]), square root–(protein), or third root–transformed (RFU [clearance rate]).

Statistical comparison of the microplastic and the diatomite exposure (both 100 000 p mL^{-1}) with 2-way ANOVAs was performed as described for the toxicity study but with the variable particle type instead of microplastic concentration. Data for the dependent variable were transformed as described in the previous paragraph.

RESULTS

Factors affecting microplastic ingestion and depuration by D. polymorpha

Exposure and depuration time (experiment 1). Exposure time significantly affects total microplastic number in *D. polymorpha* ($p < 0.01$). The total microplastic body burden (5-, 10-, 45-µm PS spheres) in *D. polymorpha* was highest after 1 h and decreased afterward, with another peak after 12 h (Figure 1A). These 2 peaks were significantly higher compared to 48 h of exposure ($p < 0.05$). The 10-µm PS spheres were found in highest quantities except after 12-h exposure, when the quantity of 5-µm PS spheres exceeded that for 10-µm spheres.

Similarly, the depuration time had a significant effect on microplastic number ($p < 0.001$). The total microplastic body burden significantly decreased from 1 h of depuration onward compared to the 12-h exposure without any depuration phase (12(+0) h). Further, the total body burden after 1 h of depuration was still significantly higher compared to 3, 24, 72, and 168 h of depuration ($p < 0.05$). The decrease was most distinct for 5- and 10-µm PS spheres (Figure 1B). After 7 d, microplastic numbers had decreased to 0.3% (0.5 p $individuals^{-1}$ [median]) of the original body burden after 12(+0) h (151.0 p $individuals^{-1}$). We did not detect 90-µm spheres in *D. polymorpha* tissues in experiment 1 or in any of the following experiments.

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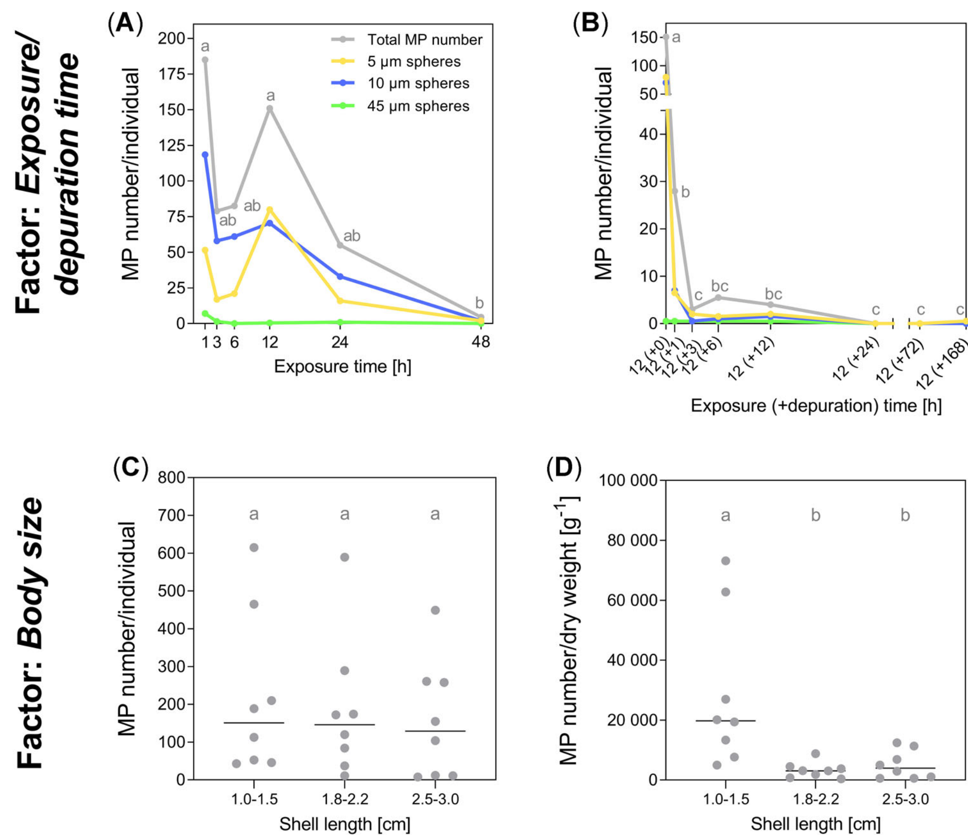

FIGURE 1: Impact of exposure and depuration time (**A**, **B**) as well as body size (**C**, **D**) on microplastic (MP) ingestion by *Dreissena polymorpha*. Mussels were (**A**) exposed to microplastic (5-, 10-, and 45-µm polystyrene [PS] spheres, 3 p mL^{-1} each; 90-µm PS spheres, 0.1 p mL^{-1}) for 1 to 48 h or (**B**) exposed for 12 h (12 (+0)) and then transferred to microplastic-free medium for up to 168 h. $n = 4$ for each time point. Data points indicate median values. (**C**, **D**) Mussels from 3 size classes were exposed to microplastic for 12 h, and the (**C**) absolute total body burden (sum of 5-, 10-, and 45-µm spheres) as well as the (**D**) relative total body burden (per dry wt) were determined ($n = 8$). Lines indicate the median. Statistics: one-way analysis of variance with Tukey's posttest; different letters indicate significant differences between the treatments. No 90-µm spheres were detected in *D. polymorpha* in any of the exposure experiments.

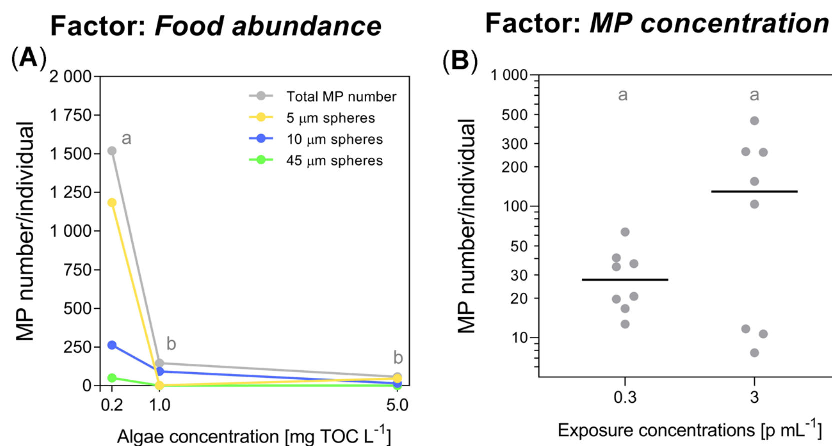

FIGURE 2: Impact of food abundance (**A**) and microplastic (MP) concentration (**B**) on microplastic ingestion by *Dreissena polymorpha*. (**A**) Mussels were exposed to microplastic (5-, 10-, and 45-µm polystyrene [PS] spheres, 3 p mL^{-1} each; 90-µm PS spheres, 0.1 p mL^{-1}) for 12 h in the presence of 3 algae concentrations ($n = 8$, data points indicate median). (**B**) Mussels were exposed to microplastic (5-, 10-, 45-, and 90-µm PS spheres) for 12 h at concentrations of 0.3 and 3 p mL^{-1} each (90-µm spheres, 0.01 and 0.1 p mL^{-1}) for 12 h. Data points represent the total body burden (sum of 5-, 10-, and 45-µm PS spheres) per individual. The line indicates the median. Statistics: one-way analysis of variance with Tukey's posttest; different letters indicate significant differences between the treatments. No 90-µm spheres were detected in *D. polymorpha* in any of the exposure experiments. TOC = total organic carbon.

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Body size (experiment 2). Ingestion of microplastics by *D. polymorpha* varied considerably within the 3 tested size classes. The absolute microplastic body burden in individuals of the 3 size classes did not differ significantly (Figure 1C; $p > 0.05$). When considering the relative body burden (microplastic number per dry wt), the smallest individuals had significantly higher microplastic numbers compared to larger individuals (Figure 1D; $p < 0.01$).

Food abundance (experiment 3). *Dreissena polymorpha* ingested a lower amount of microplastic when more food was available. The decrease of the total body burden was, however, not linear but most pronounced between 0.2 and 1 mg TOC L^{-1} algae (equivalent to 5.08×10^4 and 2.53×10^5 algae cells L^{-1}; Figure 2A). The mussels did not take up 45-μm spheres when fed 5 mg TOC L^{-1} algae.

Microplastic concentration (experiment 4). In *D. polymorpha*, the total body burden increased when exposing the mussels to a 10-fold higher microplastic concentration, the increase was, however, not significant ($p > 0.05$; Figure 2B). Further, the increase was not proportional: a 10-fold increase in microplastic concentrations resulted only in a 5.1-fold (30.7 vs 156.9 microplastic individual^{-1}) higher microplastic burden.

Comparison of microplastic ingestion between freshwater mussel species

We reperformed experiment 1 (factors exposure and depuration time) with *S. woodiana* as a second species and compared these data with the results for *D. polymorpha* to determine species-specific differences (Figure 3A and B). Considering the microplastic ingestion after various exposure periods, total body burden (sum of 5-, 10-, 45-μm PS spheres) differed significantly between *S. woodiana* and *D. polymorpha* ($p < 0.001$), with *S. woodiana* ingesting much more microplastic than *D. polymorpha* (Figure 3A). In *S. woodiana*, the body burden peaked after 3 and 6 h, whereas in *D. polymorpha* microplastic levels peaked after 1 and 12 h. Consequently, no significant effect of exposure time was observed ($p > 0.05$). The same applies to the interaction of both factors ($p > 0.05$).

In the depuration experiments, both the variables species ($p < 0.001$) and depuration time ($p < 0.001$) as well as their interaction ($p < 0.01$) significantly affected microplastic body burden in the mussels. In both species, microplastic numbers reduced with increasing depuration time (Figure 3B). However, microplastic depuration was faster in *D. polymorpha* with almost complete microplastic clearance within 12 h, whereas the lowest body burden in *S. woodiana* was reached after 72 h. After 7 d of depuration, microplastic clearance in both species was similar (*D. polymorpha*, 99.7%; *S. woodiana*, 95.8%). However, whereas in *D. polymorpha* only few microplastic particles remained in the mussel tissues, we detected >100 microplastics in *S. woodiana*.

When comparing absolute body burdens in *D. polymorpha*, *A. anatina*, and *S. woodiana* of various sizes (experiment 2; Figure 3C), neither body size nor species nor their interaction had a significant effect. The Tukey's posttest, however, showed that *D. polymorpha* as well as the 3 smaller size classes (1.0–1.5, 1.8–2.2, and 2.5–3.0 cm) form homogenous subgroups which differ from the remaining subgroups consisting of *A. anatina* and *S. woodiana* as well as the 2 larger size classes (6.0–8.0 and 9.5–12.0 cm). Absolute microplastic ingestion by *D. polymorpha* was, therefore, lower compared to the 2 larger freshwater species.

With regard to relative body burden in the freshwater mussels (Figure 3D), the variables individual size ($p < 0.001$) and species ($p < 0.05$) had a significant effect, although no interaction was observed ($p > 0.05$). For the variable species, all 3 species formed separate subgroups (Tukey's posttest), with *D. polymorpha* having the highest and *A. anatina* the lowest microplastic number in its tissues. With regard to individual size, 3 subgroups were identified (1.0–1.5 cm; 1.8–2.2, 2.5–3.0, and 6.0–8.0 cm; and 6.0–8.0 and 9.5–12.0 cm), which indicates that the relative body burden increases with decreasing individual size.

All 3 species ingested high proportions of 10-μm spheres (Figure 3E). Also, *D. polymorpha* (especially 1.0–1.5 cm) ingested large quantities of 5-μm spheres, whereas in the larger species, *S. woodiana* and *A. anatina*, 45-μm spheres were more abundant. Thus, smaller mussel species seem to ingest smaller microplastics. The same trend applied to differently sized individuals within the 3 species (except for *D. polymorpha*, 1.8–2.2 cm). The burden of smaller microplastics (5 and 10 μm) increased with smaller body size. In contrast to *D. polymorpha*, the 2 larger species also ingested 90-μm PS spheres. After a 12-h exposure, we detected up to 37 (6.0–8.0 cm) and 49 (9.5–12.0 cm) 90-μm PS spheres in *A. anatina* as well as up to 47 (6.0–8.0 cm) and 56 (9.5–12.0 cm) 90-μm spheres in *S. woodiana*.

Microplastic toxicity in *D. polymorpha*

No mortality occurred in any treatment during the acute exposure (1, 3, and 7 d) of *D. polymorpha* to PS fragments or diatomite. In the chronic exposure (42 d), mortality remained low, with 0% in the 100 000 p mL^{-1} microplastic treatment, with 10% in the control, 160 and 4000 p mL^{-1} microplastic treatment, as well as with 12.5% in the 6.4 p mL^{-1} microplastic and 100 000 p mL^{-1} diatomite treatment.

The exposure of *D. polymorpha* to microplastics (6.4–100 000 p mL^{-1}) for 1, 3, 7, and 42 d did not induce significant effects ($p > 0.05$) on the energy reserves (proteins, glycogen, lipids) or oxidative stress levels (MDA content, remaining antioxidant capacity) in the midgut gland of the analyzed individuals (Figure 4 and Table 1). However, we observed significant microplastic effects on the clearance rate of *D. polymorpha* ($p < 0.05$; Table 1), with a pronounced increase in the 100 000 p mL^{-1} treatment after 7 and 42 d of microplastic exposure (Figure 4F). In contrast, the exposure time significantly affected the energy reserves and oxidative stress level in *D. polymorpha* ($p < 0.01$; Table 1). However, there was no clear linear trend between the exposure time and the endpoints except for the remaining antioxidative capacity, which decreased with increasing exposure time (Figure 4E).

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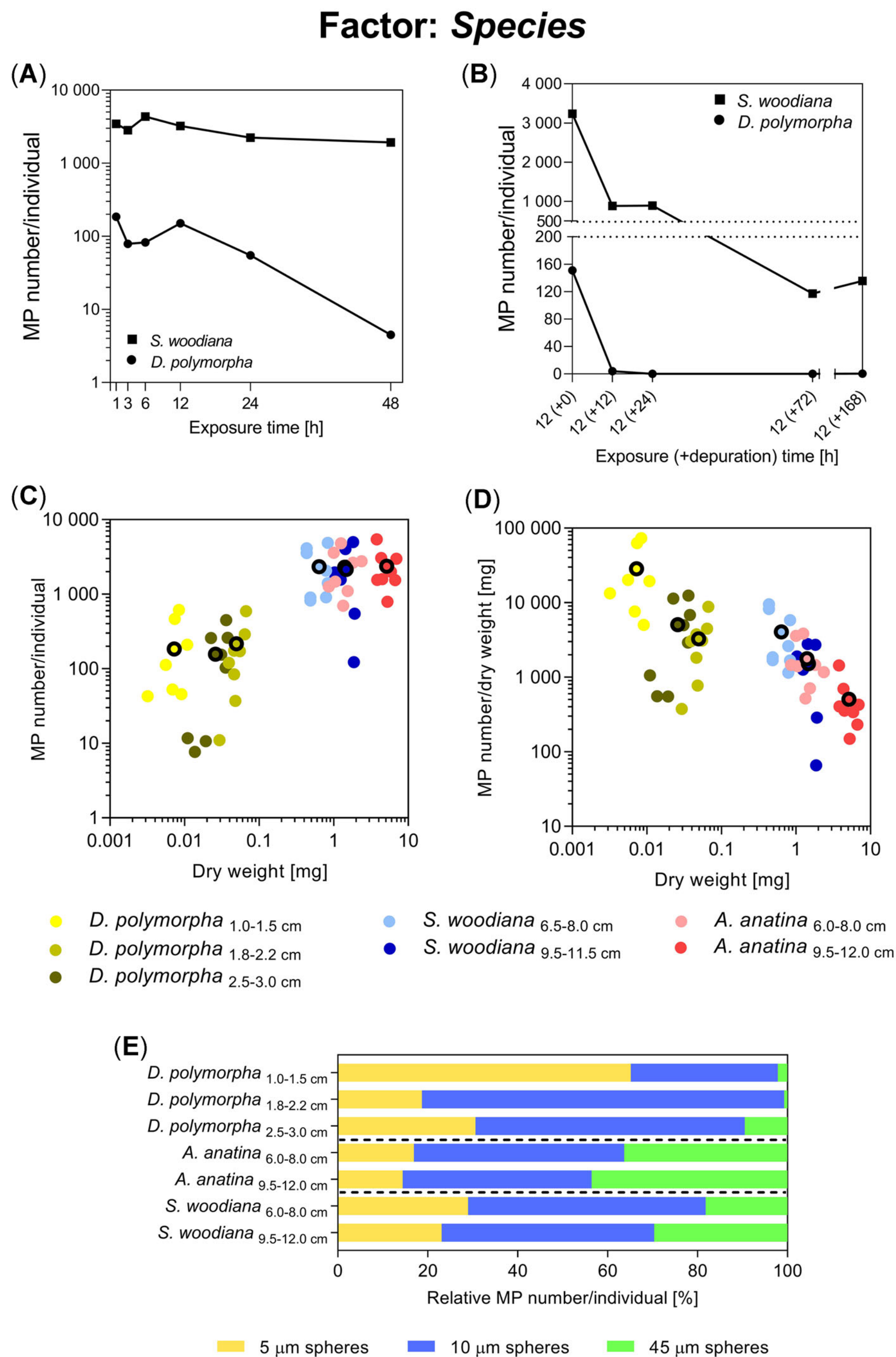

FIGURE 3: Comparison of the total body burden of microplastics (MP) in *Sinanodonta woodiana* and *Dreissena polymorpha* after different exposure (**A**) or depuration (**B**) time periods as well as comparison of the (**C**) absolute (per individual) and (**D**) relative body burden (total microplastic number per dry wt) in *D. polymorpha*, *S. woodiana*, and *Anodonta anatina* of various body sizes (12-h exposure). (**E**) Ratios of the microplastic types in mussels of different size classes (12-h exposure). (**A**, **B**) *Sinanodonta woodiana* and *D. polymorpha* were exposed as described in Figure 1A and B ($n = 4$, data points indicate median). (**C**–**E**) *Sinanodonta woodiana*, *A. anatina*, and *D. polymorpha* were exposed as described in Figure 1C and D ($n = 8$, black circles indicate geometric mean).

In addition, we statistically compared the 100 000 p mL^{-1} microplastic and diatomite exposures for each endpoint considering the variables particle type and exposure time as well as their interaction. Significant differences were only observed for the exposure time ($p < 0.05$; Supplemental Data, Table S4) regarding the protein content, MDA (TBARS), and the antioxidative capacity (ORAC). The particle type did not cause significant effects ($p > 0.05$). Particle effects due to an microplastic and diatomite exposure did not, therefore, differ in *D. polymorpha*.

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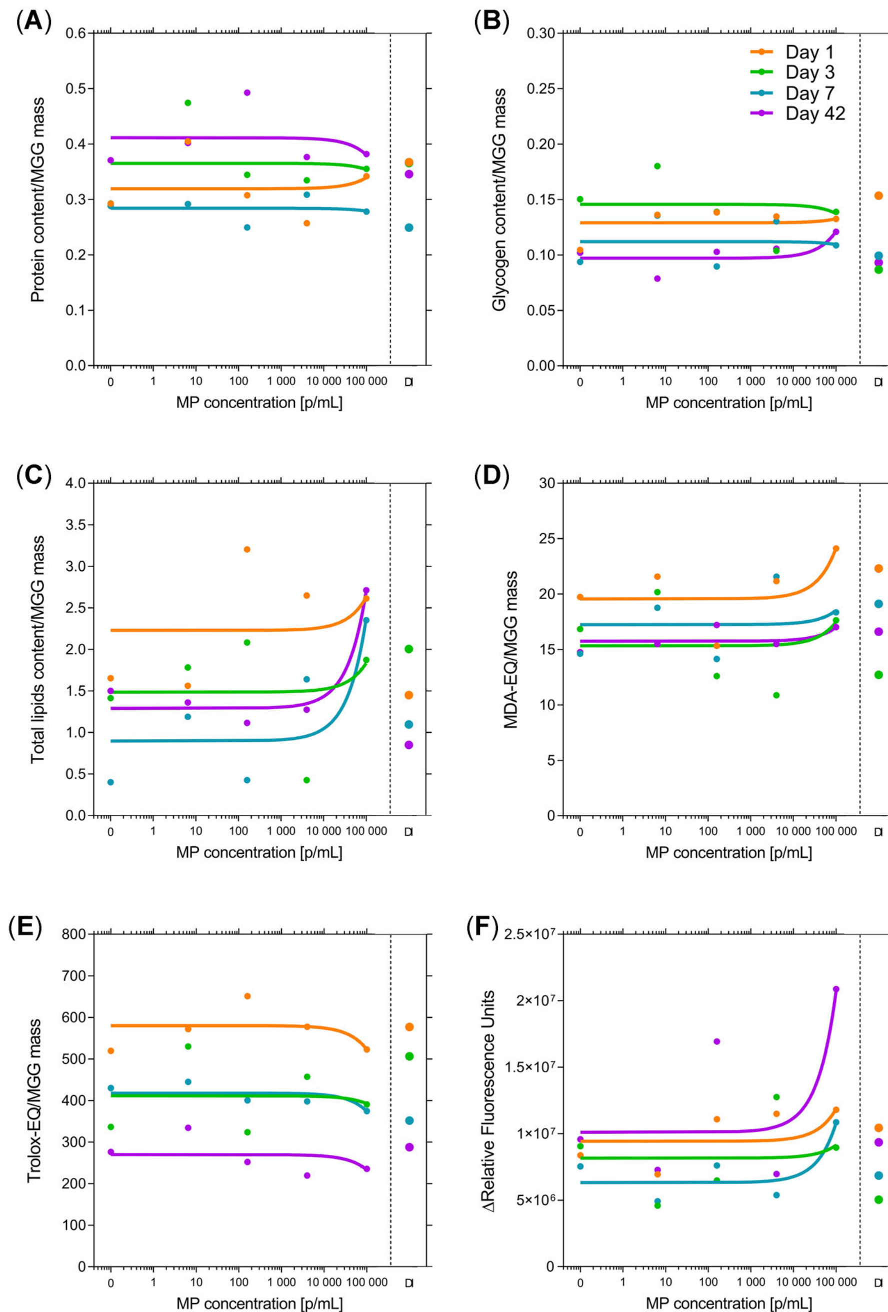

FIGURE 4: Chronic toxicity of polystyrene microplastic fragments (≤63 µm, 6.4–100 000 p mL^{-1}) and diatomite (≤63 µm, 100 000 p mL^{-1}) in *Dreissena polymorpha* exposed for 1, 3, 7, and 42 d. Endpoints were (**A**) protein, (**B**) glycogen, (**C**) total lipids, (**D**) malondialdehyde content, and (**E**) the remaining antioxidant capacity (Trolox equivalents) in the midgut gland, as well as (**F**) clearance rate. Dots indicate mean of each treatment; lines indicate linear regressions (separate regression for each exposure duration). (**A**–**E**) $n = 7–10$, (**F**) $n = 10$. MGG = midgut gland. MDA = malondialdehyde; EQ = equivalent.

DISCUSSION

Factors affecting microplastic ingestion by D. polymorpha

Bivalves selectively filter-feed on particles from the water column. The mussels' ciliated gills as well as ciliated labial palps separate digestible food particles from the remaining particle load by transporting the former to the digestive system and rejecting the latter as pseudofeces (Ward and Shumway 2004; Vaughn et al. 2008; Tuttle-Raycraft and Ackerman 2019). This selection mechanism is, however, inefficient for microplastics because we detected plastic particles in almost every individual after a respective exposure. Accordingly, more knowledge on the factors affecting the ingestion is required to better understand the mussels' exposure to microplastics.

Exposure and depuration time. *Dreissena polymorpha* rapidly ingested microplastic, with a maximum body burden reached after 1 and 12 h (experiment 1). The excretion of microplastic was similarly fast in *D. polymorpha*, with a significant

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TABLE 1: Two-way analysis of variance results for the effects of the variables "microplastic concentration" and "exposure time" and their interaction on the clearance rate, energy reserves (proteins, glycogen, total lipids), and oxidative stress (TBARS, ORAC) of *Dreissena polymorpha*

Variable		Clearance rate	Proteins	Glycogen	Total lipids	TBARS	ORAC
Microplastic concentration	*df*	4	4	4	4	4	4
	F	3.205	2.099	0.483	0.918	2.132	1.545
	p	**0.014**	0.083	0.748	0.455	0.079	0.192
Exposure time	*df*	3	3	3	3	3	3
	F	0.861	7.671	5.320	6.348	4.670	11.254
	p	0.463	**< 0.001**	**0.002**	**< 0.001**	**0.004**	**< 0.001**
Microplastic concentration × exposure time	*df*	12	12	12	12	12	12
	F	0.990	1.117	1.680	1.356	1.133	0.910
	p	0.460	0.350	0.075	0.192	0.337	0.538

Bold indicates significance.
TBARS = thiobarbituric acid reactive substances; ORAC = oxygen radical absorbance capacity; MP = microplastic; DI = diatomite.

reduction of microplastic body burden after 1 h of depuration and a reduction to fewer than 10 particles from 3 h onward. These results agree well with data on marine bivalves; for example, *Mytilus edulis* ingests polyethylene terephthalate fibers with a maximum body burden after 6 h (Woods et al. 2018), and *Geukensia demissa* excretes ingested polyethylene spheres within 12 h (Khan and Prezant 2018). This confirms that maximal microplastic burden in mussels is reached already after a few hours of exposure. Furthermore, decreasing microplastic burdens following peak levels highlight the fast egestion of microplastic particles by mussels but possibly also indicate decreasing exposure concentrations throughout prolonged exposure periods.

Despite fast excretion, few microplastics remained in *D. polymorpha* after 7 d of depuration. Therefore, mussel clearance mechanisms do not eliminate all internalized microplastics within 1 wk after exposure. The residual particles may have been either retained in the digestive tract or translocated into tissues or the circulatory system. In previous research, microplastics (≤25 µm) were detected not only in the stomach and intestine but also in the associated midgut gland ducts and diverticula (blind-ending tubules [Owen 1974]) and the circulatory system of bivalves (Von Moos et al. 2012; Guilhermino et al. 2018; Magni et al. 2018; Pittura et al. 2018; Gonçalves et al. 2019). These translocation processes may prevent the removal of the microplastic particles from the body and, thus, cause prolonged retention in mussels such as *D. polymorpha*.

When considering microplastic ingestion after extended time periods (>12 h), we observed a constant decrease of the microplastic body burden without reaching a steady state. This indicates that the microplastic exposure or bioavailability decreased during the experiment, possibly because the microplastic sedimented or the mussels had taken up the available microplastic and excreted it as (pseudo)feces. We did not examine feces production and deposition in our study and, therefore, cannot quantify its contribution to bioavailability reduction. Nonetheless, we investigated the settlement of particles in an additional experiment (Supplemental Data, S2.2) and observed that from 6 h onward, only 5- and 10-µm spheres remained in the water phase and that after 12 h only approximately half the 5-µm spheres and one-third of the 10-µm spheres remained in the water phase. This indicates that from 12 h onward, microplastic uptake by *D. polymorpha* was possible lower over time, whereas a constant depuration resulted in an overall decreasing microplastic body burden. From this, we conclude that stable microplastic burden in mussels from wild populations are only possible in case of continuous microplastic bioavailability and ingestion by the mussels.

Body size. The absolute microplastic ingestion (per individual) varied distinctively within each size class of *D. polymorpha*. Accordingly, no significant differences between the 3 size classes were observed. This indicates that the interindividual differences in absolute microplastic burden in mussels may be unrelated to body size. Considering the relative body burden (microplastic per dry wt), instead, microplastic ingestion was significantly higher in smaller individuals. Hence, smaller mussels (e.g., juveniles) in the environment may be exposed to relatively higher microplastic levels. This is possibly related to higher relative feeding activity. In *Mytilus* spp., the weight-specific pumping rate increases exponentially with decreasing body size. The same is true for the weight-specific gill area (Jones et al. 1992; Duinker et al. 2007). Higher relative pumping rates and larger relative gill areas allow smaller mussels to take up more microplastics per body mass.

Food abundance. Providing algae as food caused a significant, dose-dependent decrease in microplastic ingestion in *D. polymorpha* (experiment 3). Rist et al. (2019a) observed the same trend for *M. edulis* larvae, which were exposed to 2-µm PS spheres in the absence and presence of algae. In adult *M. edulis* instead, higher algae concentrations increased the ingestion of 30-nm PS spheres (Wegner et al. 2012). Wegner et al. (2012) discuss that nanospheres may adsorb on the algae as a potential reason for their findings. Because we used microplastics with a similar or larger size than *D. subspicatus* cells (8 × 5 µm; Hessen and Van Donk 1993), indirect microplastic ingestion through adsorption on algae was probably not a relevant pathway. Considering environmental conditions, these results indicate that high food abundance may reduce overall microplastic ingestion by bivalves. Especially in freshwater systems with high primary production or in seasons with elevated food availability (e.g., algae bloom in spring),

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mussels may take up fewer microplastics than in oligotrophic systems.

Microplastic concentration. *Dreissena polymorpha* ingested microplastics in a concentration-dependent manner (experiment 4). The increase in microplastic uptake was, however, not linear such that animals exposed to a 10-fold higher concentration had 10-fold higher body burdens. Therefore, microplastic body burdens in mussels may behave rather logarithmically, suggesting the existence of a maximal level. A logarithmic relation between microplastic water concentrations and body burdens has previously been reported for *M. galloprovincialis* larvae (Capolupo et al. 2018) and *M. edulis* (Woods et al. 2018). Limited microplastic uptake at very high concentrations in the water phase is mostly related to pseudofeces production. When the gill cilia which sort particles prior to ingestion (see section *Ingestion of microplastics in 3 mussel species*) are overloaded, excess particles are gathered, embedded into pseudofeces, and rejected (Ward et al. 1993). However, overloading of the bivalves' sorting mechanism with microplastics is possibly not very relevant in nature because the highest reported environmental concentrations of 0.2 to 0.5 p mL^{-1} (Leslie et al. 2017; Lahens et al. 2018) are much lower than the concentrations used in the laboratory studies mentioned above (Capolupo et al. 2018 [50–10 000 p mL^{-1}]; Woods et al. 2018 [3–30 p mL^{-1}]).

Ingestion of microplastics in 3 mussel species

The ingestion experiments with *D. polymorpha* pointed out that numerous factors affect microplastic ingestion in this species, including exposure and depuration time, body size, food abundance, and microplastic concentration. Species-specific differences (e.g., size, morphology) may, however, be another relevant factor influencing microplastic ingestion by freshwater mussels. In light of a broader assessment of microplastic ingestion in freshwater species, we repeated the experiments for the factors exposure and depuration time (experiment 1) as well as body size (experiment 2) with the 2 larger freshwater mussel species, *A. anatina* and *S. woodiana*.

Distinct differences between the 3 freshwater species were seen with regard to the absolute microplastic ingestion. The smaller *D. polymorpha* ingested fewer microplastics than the 2 larger species (experiment 1) when considering the absolute body burden. Between the 2 larger species instead, absolute microplastic ingestion did not differ distinctively. Higher microplastic ingestion in larger species may be caused by either higher absolute filtration rates of larger mussels as reported by Kryger and Riisgård (1988) or a higher bioavailability of microplastics to the larger, sand-dwelling unionids. The latter would be true if the sand-dwelling species are able to take up additional microplastics from the sediment. Based on an additional experiment (see Supplemental Data, S6), we did, however, show that microplastic uptake by unionids from the sediment phase is rather limited and that the filtration rate is, thus, the determining factor for absolute microplastic ingestion. Accordingly, body size seems to be a relevant indicator for microplastic body burden, with larger species ingesting higher microplastic quantities. Differences between species of similar size, instead, seem to be of limited relevance for absolute microplastic ingestion.

In contrast, the relative microplastic body burden (per dry wt) was higher in smaller specimens, with both species and individual size having a significant effect. Variations in relative microplastic ingestion are possibly caused by differences in relative filtration rate between mussels of different species and size. Kryger and Riisgård (1988) observed that smaller bivalves (e.g., *D. polymorpha*) often have a higher relative filtration rate (per mussel dry wt) compared to larger species (e.g., Unionidae) but that filtration rates may also vary between species of similar size (e.g., *Sphaerum corneum* vs *D. polymorpha*). This suggests that smaller species may be more susceptible to microplastic ingestion than larger ones but that species-specific variations may also affect the overall microplastic exposure in mussel individuals.

Further, we compared the ingestion and depuration behavior of *D. polymorpha* and *S. woodiana* over various exposure and depuration time periods. Ingestion behavior over a period of up to 12 h was rather similar because both species reached peak levels within 12 h. Depuration, instead, differed in both species, with *D. polymorpha* excreting microplastics faster than *S. woodiana*. *Dreissena polymorpha* removed ingested PS spheres almost completely within 12 h, whereas microplastic levels in *S. woodiana* decreased further until 72 h of depuration. Because we compared 2 species with different size, it remains unclear whether the difference is species- or rather size-specific. The latter has already been shown for *Mytilus chilensis*, in which smaller individuals had a higher weight-specific excretion rate compared to larger ones (Navarro and Winter 1982). Hence, smaller mussels have a higher relative microplastic body burden but also excrete microplastics faster than larger mussels. In smaller mussels, higher relative uptake may, therefore, be compensated for by a higher microplastic depuration.

Interestingly, the 3 freshwater species ingested microplastic size-dependently. Although 10-µm PS spheres were most abundant in all species, *D. polymorpha* also ingested large quantities of 5-µm spheres, whereas the larger species tended to ingest larger particles (45- and 90-µm spheres). Variations in feeding size selectivity are possibly due to differences in the particle selection mechanism and, more specifically, to morphological variations of the lamellated gills (Ward and Shumway 2004; Rosa et al. 2018), as well as the selection mechanism in the stomach. Lamellated gills are composed of numerous filaments with associated laterofrontal cirri (bundle of cilia) with which inhaled particles are selected from the inhaled water current (Ward and Shumway 2004; Silverman et al. 1999). Jørgensen et al. (1984) have shown that the retention potential of the laterofrontal cirri is species-dependent. Although *D. polymorpha* efficiently retains particles as small as 1.5 µm with the laterofrontal cirri, in *Anodonta cygnea* 4-µm particles pass through the cirri. The difference in laterofrontal cirri morphology may, thus, be a reason for higher ingestion of 5-µm spheres in *D. polymorpha*.

Following the lamellated gills, particle selection involves further sorting steps on the labial palps as well as within the

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digestive system. As a final step, particles are separated in specialized grooves in the stomach and either redirected into the digestive tubules of the midgut gland or into the intestine for direct excretion (Ward and Shumway 2004). Ten Winkel and Davids (1982) reported that the stomach of *D. polymorpha* preferentially selects for algae with a size between 10 and 50 µm, with a maximal preference for 20-µm algae cells. This size preference was also seen in our ingestion experiments because all tested freshwater species mostly ingested 10-µm spheres. Still, a high burden of 5-µm PS spheres, especially in *D. polymorpha*, indicates that stomach sorting is not exclusively limited to 10- to 50-µm particles.

Besides interspecies differences, we also observed that the size selectivity varied in each species. For all 3 tested species, smaller individuals ingested larger quantities of 5- and 10-µm spheres compared to larger individuals (experiment 2). Again, this is probably related to morphological changes once a bivalve grows: for *M. edulis*, the gill and labial palp area as well as the distance between gill filaments enlarge with increasing body length (Kiørboe and Møhlenberg 1981; Jones et al. 1992), and larger interfilamentary spaces will reduce the retention of small particles. This points toward a higher relevance of an ingestion of small microplastics for smaller individuals (e.g., juveniles).

Microplastic toxicity in D. polymorpha

The exposure of *D. polymorpha* to PS fragments (≤63 µm) for 1 to 42 d at concentrations up to 100 000 p mL^{-1} caused a significant increase in clearance rate, whereas mortality, metabolic endpoints (energy reserves), and oxidative stress were not altered. Hence, the increase in clearance rate, especially in the 100 000 p mL^{-1} microplastic treatment, may be an adaptation mechanism to compensate for potential microplastic-related effects (e.g., reduced food uptake). Considering the exposure time, we observed a significant general change in energy allocation as well as basic stress levels in *D. polymorpha* throughout the 42-d exposure. Because *D. polymorpha* individuals were accustomed to the medium as well as the food source as early as 4 wk prior to the toxicity study, we assume that changes in endpoints over the exposure time were not caused by the exposure design itself but may rather be related to stress reactions caused by the water changes and the transfer of the mussels into new exposure tanks. Such a stress reaction could have, thus, also caused the observed decrease of the antioxidative capacity with increasing exposure time. The definite reason, however, remains unclear.

Interestingly, we also did not observe significant differences between the effects of the microplastic and diatomite treatments. This suggests that *D. polymorpha* is rather insensitive to an exposure to high concentrations of microplastic and natural particles alike. These results contrast with those of earlier studies in which mussels reduced their clearance rate with increasing turbidity, resulting in a lower food uptake (Aldridge et al. 1987; Tuttle-Raycraft and Ackerman 2019). Mussels are able to compensate for such reduced feeding by increasing the particle selection efficiency via morphological adaptations (Payne et al. 1995; Tuttle-Raycraft and Ackerman 2019). Hence, compensation mechanisms to microplastic exposure (increase in clearance rate) in our study seem to differ from those for high turbidity. It will, however, require additional studies to elucidate whether these differences are stressor-dependent (microplastic vs natural particle turbidity) or a result of different adaptation periods (42-d microplastic exposure vs lifelong exposure in turbid environment). Notwithstanding, compensation mechanisms seemed to be efficient enough to protect *D. polymorpha* from microplastic effects even at concentrations far higher than currently reported for freshwater environments.

The absence of negative effects in our study only partially agrees with previous research. In a systematic literature search in May 2020 (PubMed, search "microplastics" OR "microplastic" AND "mussel" OR "bivalve," studies evaluating toxicity after depuration excluded), we identified 8 toxicity studies with freshwater mussels (*D. polymorpha* and *C. fluminea*; see Supplemental Data, Table S5). Two studies did not report any microplastic-induced effects, whereas one study observed effects on all and 5 studies on some of the analyzed endpoints. Although the majority of the endpoints remained unaffected (Supplemental Data, Table S5), these reports show that freshwater bivalves may be, at least to some extent, susceptible to microplastic exposure. However, applied microplastic concentrations in the toxicity studies with *D. polymorpha* and *C. fluminea* exceeded those currently reported for freshwater ecosystems (0.52 p mL^{-1} [Lahens et al. 2018]). It, thus, remains unknown whether the observed effects are relevant in the environmental context.

Interestingly, effects on feeding, histology, neurology, and oxidative stress metabolites varied intensively, with similar endpoints being affected in some but not in other studies. Effects may be species-specific; however, even when comparing the same species (*D. polymorpha*), marked differences were observed (e.g., effects on catalase and glutathione peroxidase in Magni et al. [2018, 2020]). We, therefore, believe that complex effect patterns are also a consequence of differences in exposure scenarios (especially exposure time and concentration), microplastic properties (e.g., polymer type, size, shape), and the sensitivity of endpoints. Accordingly, drawing general conclusions on microplastic risks for bivalves may be overly simplistic. An identification of the most sensitive species and their respective traits provides a way forward. Beyond that, a better understanding of the biological mechanisms protecting mussels from high loads of suspended particles as well as of the mechanism of microplastic toxicity in mussels is required. This knowledge is fundamental to determining whether microplastics can indeed bypass defense mechanisms in bivalves and, thus, represents a risk to wild mussel populations.

CONCLUSION

We investigated the kinetics of microplastic ingestion and egestion in *D. polymorpha*. Comparing multiple relevant factors, we demonstrate that exposure and depuration time, body size, food abundance, and microplastic concentration affect overall microplastic ingestion. *Dreissena polymorpha* rapidly ingested microplastics and excreted the majority of the particles within

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12 h, with only a few particles being retained for more than 1 wk. Smaller individuals ingested more microplastics compared to larger individuals relative to their body size. An additional supply of food reduced the uptake of microplastics.

Further, we compared microplastic ingestion in *D. polymorpha* with 2 larger unionid species (*A. anatina*, *S. woodiana*). Absolute microplastic ingestion was higher in the 2 unionid species but did not differ between the unionids. Relative microplastic ingestion, instead, differed significantly with regard to both the species and the body size of the individuals, with smaller mussels ingesting more microplastics. With regard to microplastic size, smaller species as well as smaller individuals within each species also ingested smaller microplastics.

We also analyzed the toxicity of PS fragments (≤63 µm, 6.4–100 000 p mL^{-1}) in *D. polymorpha*. Exposure for up to 42 d caused a significant increase in the clearance rate but did not affect energy reserves or oxidative stress. Further, no significant difference between the effects of microplastics and natural particles (diatomite) was observed. Enhanced filtration rate may be a compensatory mechanism rendering *D. polymorpha* rather insensitive to microplastic exposure even at very high concentrations. Taking into account previous research on microplastic toxicity in bivalves, this does not imply that mussels in general are not susceptible to microplastic exposures. Divergent toxicity data probably originate from species-specific differences or variations in experimental design. A better understanding of the traits and mechanisms rendering some species and endpoints more sensitive than others is needed to prioritize species potentially at risk.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5076.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (martin.wagner@ntnu.no).

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