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Ingestion and toxicity of microplastics in the freshwater gastropod *Lymnaea stagnalis*: No microplastic-induced effects alone or in combination with copper

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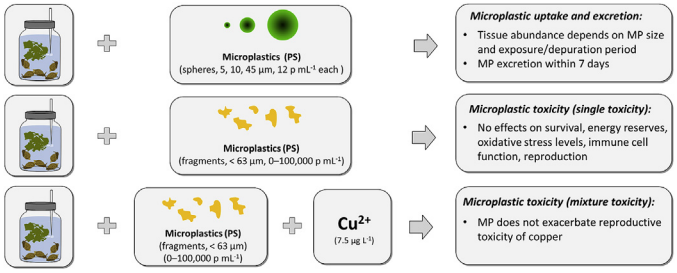
HIGHLIGHTS

- *Lymnaea stagnalis* readily ingests and egests PS microplastics (5–90 μm).
- Ingestion and excretion of spherical PS (5–45 μm) is size- and time-dependent.
- Almost complete depuration of ingested spherical PS within 7 days.
- No significant chronic toxicity of irregular PS (<63 μm).
- In mixture, irregular MP does not exacerbate the reproductive toxicity of copper.

GRAPHICAL ABSTRACT



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ABSTRACT

The interaction of microplastics with freshwater biota and their interaction with other stressors is still not very well understood. Therefore, we investigated the ingestion, excretion and toxicity of microplastics in the freshwater gastropod *Lymnaea stagnalis*.

MP ingestion was analyzed as tissues levels in *L. stagnalis* after 6–96 h of exposure to 5–90 μm spherical polystyrene (PS) microplastics. To understand the excretion, tissue levels were determined after 24 h of exposure followed by a 12 h–7 d depuration period. To assess the toxicity, snails were exposed for 28 d to irregular PS microplastics (<63 μm, 6.4–100,000 particles mL^{-1}), both alone and in combination with copper as additional stressor. To compare the toxicity of natural and synthetic particles, we also included diatomite particles. Microplastics ingestion and excretion significantly depended on the particle size and the exposure/depuration duration. An exposure to irregular PS had no effect on survival, reproduction, energy reserves and oxidative stress. However, we observed slight effects on immune cell phagocytosis. Exposure to microplastics did not exacerbate the reproductive toxicity of copper. In addition, there was no pronounced difference between the effects of microplastics and diatomite. The tolerance towards microplastics may originate from an adaptation of *L. stagnalis* to particle-rich environments or a general stress resilience. In conclusion, despite high uptake rates, PS fragments do

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not appear to be a relevant stressor for stress tolerant freshwater gastropods considering current environmental levels of microplastics.

1. Introduction

The realization that plastic pollution is ubiquitous in aquatic ecosystems worldwide has raised concern on the possible consequences for aquatic life (SAPEA, 2019). Previous research has focused on microplastics (MP, 1–1000 μm, Hartmann et al., 2019) due to their global distribution (Rezania et al., 2018). While the ingestion of MP has been demonstrated in field studies for numerous taxa (Eerkes-Medrano et al., 2015; Lusher, 2015), the implications of MP ingestion are still unclear. In previous toxicity studies, MP concentrations inducing adverse effects varied by orders of magnitude (reviewed by Anbumani and Kakkar, 2018; Strungaru et al., 2019; Triebskorn et al., 2019) leading to sometimes divergent interpretations whether MP pose a current and future risk for the aquatic environment (Adam et al., 2018; Burns and Boxall, 2018; Guzzetti et al., 2018).

Previous toxicity studies have mostly focused on the MP effects on fish, bivalves and crustaceans (de Sá et al., 2018). In contrast, effects on gastropods are rarely studied and, therefore, underrepresented in current risk assessments. MP ingestion by gastropods has already been demonstrated both in the field and laboratory (Courtene-Jones et al., 2017; Gutow et al., 2016, 2019; Karlsson et al., 2017; Watermann et al., 2017). Gastropods ingest spherical and irregular particles of various polymer types.

Knowledge on MP toxicity in aquatic gastropods, however, is currently limited to five peer-reviewed studies. In the marine slipper snail *Crepidula onyx*, 2–5 μm polystyrene (PS) spheres reduced settling and growth of juveniles at high (60,000 and 140,000 particles mL^{-1} (p mL^{-1})) but not at low (10 p mL^{-1}) concentrations (Lo and Chan, 2018). Qu et al. (2020) reported significantly enhanced filtration rates in the freshwater gastropod *Cipangopaludina cathayensis* after exposure to 0.7 μm PS MP (20 mg L^{-1}). In the freshwater mudsnail *Potamopyrgus antipodarum*, high MP concentrations in food (250 mg MP and 100 mg food in agar) did not cause any toxicity on growth, development, reproduction and survival (Imhof and Laforsch, 2016). In the pond snail *Lymnaea stagnalis*, nylon particles (1% w/w in sediments) caused no changes in wet weight or microbiome (Horton et al., 2020). Finally, Doyle et al. (2020) did not observe a correlation between environmental MP levels and the individual emergence behaviour in *Littorina littorea* populations.

Given the scarcity of data on gastropods, the aim of this study was to investigate the ingestion and excretion of MP (5–90 μm PS spheres) as well as its effects (irregular PS, < 63 μm, 6.4–100,000 p mL^{-1}) on adult freshwater pond snail *L. stagnalis*. Effects were determined after 28 d of exposure with energy reserves, oxidative stress, immune cell phagocytosis activity and reproduction as endpoints.

Further, we compared the toxicity of MP to that of natural particles. High particle loads in aquatic ecosystems reduce the food supply and quality of benthic invertebrates (Camargo and Alonso, 2017). As a consequence, snails can adapt their feeding strategy (Calow, 1975) but may also adapt evolutionary to high loads of suspended particulate matter. Therefore, the toxicity of MP needs to be benchmarked against that of natural materials (Scherer et al., 2018). Here, we used diatomite (DI, 100,000 p mL^{-1}) to compare the effect of synthetic and natural particles.

In the real world, MP will be just one amongst many stressors (Backhaus and Wagner, 2019) and mixture toxicity studies can be an important second step towards more environmentally realistic study scenarios (Syberg et al., 2015). Inorganic copper (Cu) is a relevant stressor for freshwater organism with *L. stagnalis* being one of the most sensitive species (Brix et al., 2001; ECI, 2008). Cu concentrations as low as 2–10 μg L^{-1} affect survival, growth, metabolism and reproduction in *L. stagnalis* (Atli and Grosell, 2016; Brix et al., 2011; Das and Khangarot, 2011; Ng et al., 2011). Accordingly, we studied the joint effects of Cu^{2+} and MP on *L. stagnalis*. We exposed the snails to 7.5 μg L^{-1} Cu^{2+} and either MP (6.4–100,000 particles mL^{-1}) or DI (100,000 p mL^{-1}) using the energy reserves, oxidative stress and reproduction as endpoints.

2. Materials and methods

2.1. Lymnaea stagnalis culture

L. stagnalis individuals were obtained from an in-house culture at Goethe University and raised in aerated 50 L tank with 100–200 individuals per tank (maximal density according to OECD (2016): 5 individuals L^{-1}) at 20 °C water temperature (optimal water temperature for *L. stagnalis* growth and development: 16–20 °C, Van der Schalie and Berry, 1973). Tanks were filled with ISO medium prepared in accordance with OECD guideline no. 243 (OECD, 2016, *Lymnaea stagnalis* Reproduction Test). The ISO medium was composed of 294 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 123 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 64.7 g L^{-1} $NaHCO_3$ and 5.75 mg L^{-1} KCl. Once to twice a week, 50% of the water volume in each tank was renewed. *L. stagnalis* was fed three times a week with both butterhead lettuce (*Lactuca sativa* var. *capitata*, organic quality according to EU-Eco-regulation or stricter) and Tetramin (Tetra GmbH, Melle, Germany) *ad libitum*.

2.2. Particle preparation

For the MP ingestion and excretion study, non-functionalized 10, 45 and 90 μm PS spheres (density: 1.05 g cm^{-3}) were purchased from Polysciences Europe (Fluoresbrite YG microspheres, Hirschberg an der Bergstraße, Germany, excitation: 441 nm, emission: 486 nm). Non-functionalized 5 μm PS spheres were obtained from Magsphere (Pasadena, CA, USA, excitation: 538 nm, emission: 584 nm).

For the toxicity studies, we produced irregular MP with a broad particle size range from yellow-orange fluorescent PS drinking cups (excitation: 360–370 nm) by cryomilling. DI particles were purchased from Sigma-Aldrich (Taufkirchen, Germany). From both the MP and DI, we isolated the particle fraction <63 μm by sieving. The polymer type and chemical content of the drinking cups was determined by Attenuated Total Reflection-Fourier Transform Infrared-spectroscopy (ATR-FTIR-spectroscopy) and pyrolysis-GC-MS. A low level of chemicals was detected in the PS cups but the compounds could not be matched to common substances from polymer industry (further details on particle preparation and polymer analysis in Weber et al. (2020)).

The purchased PS sphere stock suspensions for the ingestion and excretion study were diluted in ultrapure water and their numerical particle concentrations were determined with a Coulter

Counter (Multisizer 3; Beckman Coulter, USA). For the toxicity studies, we determined particle concentrations (number per mg powder) and size distribution in the <63 μm MP and DI powder using the Coulter Counter (size range: 2–60 μm). For both the MP and DI powder, particle concentrations increased exponentially with decreasing particle size. Within the 2–60 μm size range, 95% of the MP and the DI particles were smaller than 9.7 and 13.6 μm, respectively (details in S1.1 and S2.1).

For qualitative assessment of particles <2 μm, MP and DI powder were imaged with a scanning electron microscope (SEM, details in S1.2). SEM imaging confirms that nanoparticles are present in the MP and DI powder (Fig. S5) but a quantitative analysis (e.g., by nanoparticle tracking analysis) was not possible due to the abundance of large particles that would have blocked the instrument.

2.3. Ingestion and excretion study

We analyzed the MP ingestion by exposing *L. stagnalis* simultaneously to a mixture of 5, 10 and 45 μm fluorescent PS spheres (12 p mL^{-1} each) over an exposure period of 6, 12, 24, 48 or 96 h. In addition, we added 90 μm PS spheres (2 p mL^{-1}) to test whether the snails are also able to ingest larger MP. We used PS spheres with homogenous sizes instead of polydisperse fragments to investigate the size-dependency of MP ingestion and excretion.

For each exposure period, we filled one glass jar (diameter: 10 cm, height: 18 cm) with 1 L ISO medium and added five snails (2.5–3.0 cm, apex to basal lip) and 10 g lettuce as food source (see 2.1). The jars were filled with ISO medium first before the prediluted PS sphere stock suspensions with known particle concentrations (see 2.2) were added to the ISO medium. Stock suspensions were stirred thoroughly prior to pipetting to allow homogenous particle distribution. Throughout the experiment, the jars were gently aerated using glass pipettes and water loss (due to evaporation, up to approximately 20 mL d^{-1}) was adjusted daily by adding ultrapure water if necessary.

In previous experiments, we observed that (despite a constant water movement through aeration) the 5, 10, 45 and 90 μm PS spheres as well as the PS fragments used in the toxicity experiments sedimented within few hours to days, with smaller spheres staying longer in the water phase compared to larger spheres (unpublished data). Since *L. stagnalis* not only grazes on surfaces but also floats freely in the water column and on the water surface (OECD, 2016), both suspended and settled particles were bioavailable throughout the experiments.

For the excretion study, snails were exposed for 24 h as described above (five jars with five individuals each), then transferred into glass jars with aerated, MP-free ISO medium and kept in those for another 12, 24, 48, 96 h or 7 d (one jar with five individuals per depuration period). The water in the jars was exchanged daily to remove excreted particles and minimize reuptake. Directly after the transfer of *L. stagnalis* into the MP-free medium as well as after each water exchange, snails were fed with 0.5 g lettuce $individual^{-1}$ d^{-1}.

After the exposure, the shell and soft tissue of all individuals were thoroughly rinsed with tap water to remove attached MP and directly frozen at −80 °C. After defrosting, the shell of each individual was removed, and the soft tissue of each gastropod was lysed individually in 20–40 mL 10% potassium hydroxide solution at 55 °C for 24–48 h. The resulting lysate was filtered on glass fiber filters (VWR, Darmstadt, Germany, pore size: 1.25 μm). The total number of 5, 10, 45 and 90 μm PS spheres on each filter was determined visually using a fluorescence microscope (BX50, 40× magnification, Olympus, Hamburg, Germany).

For quality assurance, another five individuals were exposed for 48 h as described above but without added MP to determine the background contamination throughout the exposure and lysis. We found one particle each in two out of five snails resembling 5 μm PS spheres but none with the color and shape of 10, 45 or 90 μm spheres. Thus, we corrected the ingestion and excretion rates of 5 μm PS spheres for this background contamination (−0.4 particles per individual).

2.4. Microplastics toxicity study

Toxic effects of MP were analyzed by exposing *L. stagnalis* over 28 d to irregular PS MP (<63 μm) at concentrations of 6.4, 160, 4000 and 100,000 p mL^{-1} ($MP_{6.4}$, MP_{160}, $MP_{4,000}$, $MP_{100,000}$; concentrations correspond to the size range 2–60 μm). In addition, we included a control without MP (control) and a treatment with DI (100,000 p mL^{-1}, $DI_{100,000}$, for test design see Fig. S1). The study was designed according to OECD guideline no. 243 (OECD, 2016). For each of the six treatments, six glass jars were filled with 1 L ISO medium (gentle aeration through glass pipettes), the corresponding particles and five snails (2.5–3.0 cm; 30 individuals per treatment). For the $MP_{4,000}$, $MP_{100,000}$ and $DI_{100,000}$ exposures, particles were weighed in and added to the 1 L ISO medium present in each jar (based on the particle number per plastic powder mass, see 2.2 and S2.1). For the two lower MP concentrations ($MP_{6.4}$, MP_{160}), we prepared 100-fold concentrated suspensions which were then diluted in the exposure vessels as MP mass was too low to weigh it in. Prior and throughout the dilution, the suspensions were stirred intensively to minimize particle agglomeration and maximize homogenization. As we did not use any surfactant, we cannot exclude that MP particles (especially PS fragments) aggregated throughout the exposures causing heterogenous MP distribution in the exposure vessels. Due to mobility of *L. stagnalis* (see 2.3), the effect of a heterogenous particle distribution on bioavailability, however, was probably small.

All snails were exposed at 20 ± 1 °C and a 16:8 h day-night-cycle. For water exchange, the experimental set-up was completely renewed every 3–4 d and gastropods were transferred into new jars (filled as described above). Egg clutches in the former jars were removed and analyzed to quantify reproductive output (see 2.7). Feces was collected from each vessel and analyzed qualitatively for MP presence with a fluorescent microscope (Olympus, BX50, Hamburg, Germany). In addition, we analyzed the water quality (water temperature, oxygen content, pH, conductivity, water hardness) in two out of six jars per treatment (one jar for water hardness). After the transfer of the snails into the new jars, they were fed with lettuce (0.5 g $individual^{-1}$ d^{-1}). Each jar was checked daily for mortality and dead individuals (motionlessness after touching, abnormal foot position and decomposed tissues) were removed. Based on this, mortality rates and time of survival were calculated (2.6). Throughout the toxicity study, the OECD validity criteria (mortality and reproduction in the control, water parameters) were fulfilled excepted for slightly deviations of the average exposure temperature (18.8 °C compared to 20 ± 1 °C) as well as water conductivity and hardness (detailed results in S2.4). The removal of individuals from the exposures may have affected particle uptake by the remaining individuals in the low (6.4 p mL^{-1}) but not in the higher MP treatments, as the total MP number (≥1.6×10^5 p jar^{-1} in the exposure vessels with ≥160 p mL^{-1}) by far exceeded the particle numbers measured in *L. stagnalis* (maximal ingestion of 2235 p $individual^{-1}$ at an exposure concentrations of 36 p mL^{-1}, see 3.1).

Three days prior to the 28 d exposure, individuals were acclimatized to the test conditions. The experiment was started after this period as in more than half of the jars an egg clutch had been laid (OECD guideline prerequisite). After 28 d of exposure, we sampled hemolymph from 6 to 7 individuals per treatment by

irritating the foot with a plastic pipette tip (careful rubbing of the foot with the tip), collected the released hemolymph and directly analyzed the phagocytic activity of the hemocytes (see 2.9). The remaining individuals were directly frozen in liquid nitrogen and stored at −80 °C for analyses of energy reserves and oxidative stress in the midgut gland (MGG, see 2.8).

2.5. Mixture toxicity study with copper and microplastics

In a second toxicity experiment, we analyzed whether MP modulates the toxicity of Cu as primary stressor. The experiment included six treatment groups with 7.5 μg L^{-1} Cu (actual concentration according to chemical analytics, spiked as Cu^{2+} from $CuCl_2$) and 0, 6.4, 160, 4000 and 100,000 p mL^{-1} irregular PS MP (Cu, Cu + $MP_{6.4}$/MP_{160}/$MP_{4,000}$/$MP_{100,000}$) or 100,000 p mL^{-1} DI (Cu + $DI_{100,000}$). In addition, we included a control treatment without Cu and MP particles to verify test validity (for test design see Fig. S2). For each of the seven treatments, seven glass jars with five snails (2.5–3.0 cm, 35 individuals per treatment) each were used. To avoid Cu leaching from the jars or the glass pipettes throughout the experiment, all glass ware was placed in 70 °C pre-subboiled (DST-4000, Savillex, Minnesota, USA), distilled HNO_3 solution (1.3% (v/v)) for at least 7 d and afterwards cleaned several times with ethanol and ultrapure water. The jars were filled with MP and medium first (as described in 2.4) before we spiked the Cu using a 100-fold concentrated stock solution. The snails were introduced 1 h later to allow for an equilibration of the MP and Cu distribution in the jars. The following experiment was performed as described in 2.4. Again, reproduction as well as the energy reserves and oxidative stress in the MGG were analyzed as endpoints. Hemocyte phagocytosis analysis could not be performed due to technical failure. OECD test validity criteria were fulfilled excepted for slight deviations of water hardness, pH and oxygen content in very few treatments (results in S2.4).

With each water exchange every 3–4 d (complete renewal of the set-up), water samples for Cu analysis were taken. One sample (49 mL) of the unspiked ISO medium as well as one sample (49 mL) of the ISO medium spiked with 7.5 μg L^{-1} Cu^{2+} (sampling 1 h after Cu spiking to allow for Cu distribution in the jar) were taken from freshly prepared new exposures (0 d). Further, after the 3–4 d exposure, we pooled the water of all jars from the same treatment and took one sample (49 mL) from each treatment (throughout each water exchange). Each water sample was aspired with a new syringe (B. Braun, Omnifix Luer Lock, latex-free, 20 mL), directly sterile-filtered (Spartan syringe filters, regenerated cellulose, pore size: 0.45 μm, Whatman, Sigma-Aldrich, Taufkirchen, Germany) into pre-leached DigiTubes (cleaned with HNO_3 as described above, DigiTubes: 010-500-264, SCP Science, Quebec, Canada), preserved with 1 mL subboiled HNO_3 (65%) and stored at 5 °C in the dark until being analyzed. Filtration of the water samples ensured the removal of all particles ≥0.45 μm (e.g., MP or food) from the water samples which could have affected the Cu concentrations in the water phase. However, a potential influence of particles <0.45 μm on the Cu analysis cannot be ruled out. Total Cu concentrations (all copper species) in the water samples were measured with ICP-QQQ-MS (details in S1.6).

After the 28 d exposure, four snails per treatment were transferred to pure ISO medium (with 0.5 g lettuce $individual^{-1}$ d^{-1}) for another 3 d to allow the excretion of Cu-contaminated remains in the digestive system. Afterwards, individuals were frozen, their shell was removed and wet and dry weight (after freeze-drying) was determined. Individuals from each treatment were pooled and tissue Cu concentrations (total concentration of all copper species) were measured using an MLS turboWAVE system and ICP-QQQ-MS (see S1.6). In addition, background Cu tissue concentrations were analyzed in a pool of four *L. stagnalis* individuals from the culture frozen prior to the experiment.

To understand the distribution of Cu in the exposure vessels, we further performed a separate Cu distribution study (details in S1.5). For this, both unspiked and Cu-spiked (7.5 μg L^{-1}, spiked as Cu^{2+} from $CuCl_2$) ISO medium was incubated (without snails and lettuce) for 3 d in presence and absence of PS MP (6.4, 160, 4000 or 100,000 p mL^{-1}) or DI (100,000 p mL^{-1}) to evaluate potential impacts on Cu water concentrations. Further, Cu-spiked ISO medium was also incubated with 1.5, 4.5 and 7.5 g lettuce to determine Cu adsorption on the food. Cu concentrations in the water (sample taken as described above) as well as on the lettuce were measured both before the start of the Cu distribution study as well as 3 d after. Cu concentrations on MP were not quantified because currently no appropriate reference material (MP with adsorbed metals) is available for ICP-QQQ-MS analysis. For snail tissues and lettuce, we used standard mussel tissue and standard white cabbage as reference materials (S1.6).

2.6. Mortality and time of survival

Total mortality in each treatment was determined as ratio of dead individuals compared to the original number of snails in each treatment. The time of survival reported as "total survival days" was determined for each jar as the sum of days which the five individuals per jar survived (maximum: 140 d). In case of mortality, survival days of the dead individual were calculated as $((t+1)+t)/2$ with t = last day on which the individual was alive (OECD, 2016).

2.7. Reproduction

The reproductive output of *L. stagnalis* was quantified by determining the total number of laid eggs and egg clutches in each jar over a period of 28 d. The number of eggs in each clutch was determined using a stereo microscope. As endpoints, we calculated the number of eggs per egg clutch and the number of eggs or egg clutches per survival days (2.6) in each of the six (MP toxicity experiment) or seven (mixture toxicity experiment) jars per treatment.

2.8. Energy content and oxidative stress in the midgut gland

MGGs from ten individuals per treatment (at least one individual from each jar per treatment) were dissected, weighed and homogenized (S1.7) to analyze the energy content and oxidative stress. Homogenates were stored at −80 °C until being analyzed. In brief, energy reserves in the MGG homogenates were measured as protein (Bradford, 1976), glycose (anthrone assay, Benedict, 2014) and total lipid content (sulfo-phospho-vanillin assay, Benedict, 2014). Oxidative stress was determined as malondialdehyde content (MDA, biomarker for lipid peroxidation) based on Furuhagen et al. (2014) and the remaining antioxidant capacity in the MGG was measured with the ORAC assay (Ou et al., 2001; Furuhagen et al., 2014). The antioxidant capacity is expressed as Trolox equivalents (reference antioxidant) per MGG mass [μmol mg^{-1}]. Further details on energy and oxidative stress assays are reported in Weber et al. (2020).

2.9. Hemocyte phagocytosis activity

The immune function of *L. stagnalis* was assessed through the phagocytic activity of the hemocytes in response to foreign stimuli. The methodology was applied with some modifications as described by Weber et al. (2020, methods and modifications are summarized in S1.8 and S2.6). In brief, hemocytes of seven snails

per treatment (at least one individual from each jar per treatment) were exposed individually to 1 μm PS microspheres (25 spheres cell^{-1}, Fluoresbrite YG, PolyScience, Hirschberg an der Bergstraße, Germany) for 3 h and the number of hemocytes with ≥3 phagocytized microspheres was compared to the total number of living cells with a BD FACSVerse (BD Biosciences, Heidelberg, Germany, see S2.6). For some of the samples, the minimal number of live cells (>5000) required for the FACS analysis was not reached resulting in 5–7 remaining replicates per treatment.

2.10. Data analyses

MP ingestion and excretion were statistically analyzed using a general linear model (GLM, IBM SPSS Statistics, version 25) with the two fixed factors "MP particle size" and "exposure period" (ingestion study) or "depuration period" (excretion study) as well as their interaction term (MP size×exposure period or MP size×depuration period). The number of MP in the *L. stagnalis* individuals was used as dependent variable and was square root transformed (ingestion study) or log (x+0.5)-transformed (excretion study) to maximize variance homogeneity. Prior to each GLM, normality (Shapiro-Wilks test) and variance homogeneity (White test) requirements were tested. Requirements were fulfilled except for the treatment with 45 μm spheres for 24 h, (ingestion study, $p = 0.04$) and with 5 μm spheres for 168 h depuration (excretion study, $p = 0.03$). As we exposed the snails to a mixture of MP with different sizes, the analysis is based on the assumption that these do not interact, that is, affected the ingestion of the other MP.

For the toxicity experiments and the Cu distribution study, statistical differences between the treatments were determined using either a one-way ANOVA with Sidak's post-test (in case of normal data distribution and variance homogeneity) or a Kruskal-Wallis test with Dunn's post-test (in case of non-normal data distribution or variance heterogeneity). Normality was checked using Shapiro-Wilk tests. To test for variance homogeneity Bartlett tests were used. In the two toxicity studies, for each endpoint a separate analysis was performed (details in Figs. 2 and 3, S11). Replicates numbers per treatment were n = 10 for energy reserves and oxidative stress (see 2.8), n = 5–7 for immune cell phagocytosis (see 2.9) and n = 6 (MP toxicity study) or n = 7 (Mixture toxicity study, see 2.7) for reproduction. In the Cu distribution study, every test was performed with 3 jars each (see 2.5, S1.5).

3. Results

3.1. Microplastics ingestion and excretion

MP abundance in the tissues of *L. stagnalis* after an exposure to a mixture of 5, 10 and 45 μm PS spheres over 6–96 h significantly depended on the "particle size" (GLM, df = 2, $p < 0.001$), the "exposure period" (df = 4, $p < 0.001$) and interaction of both factors (df = 8, $p = 0.01$). With regard to MP size, 5 μm spheres were more abundant (range: 59 ± 44 (±SD to 1572 ± 683 individual^{-1}) in *L. stagnalis* compared to 10 μm (range: 61 ± 66 to 328 ± 178 p individual^{-1}) and 45 μm spheres (range: 110 ± 107 to 353 ± 453, Fig. 1a). The snails ingested also 90 μm PS spheres (Fig. S6) but we did not compare the uptake quantitatively due to the lower exposure concentration. Considering the exposure period, MP levels markedly increased during the first hours of exposure (mostly due to enhanced ingestion of 5 μm PS spheres) with a peak at 24 h and a subsequent decrease afterwards.

Similarly, the MP excretion depended both on the factors "particle size" (df = 2, $p = 0.001$) and "depuration period" (df = 5, $p < 0.001$), while no significant interaction of both factors was observed (df = 10, $p > 0.05$). The total particle numbers in *L. stagnalis* decreased most distinctively within the first 12 h of depuration (Fig. 1b, Fig. S7). However, the excretion was not continuous over time as MP levels were lower after 12 h compared to 24 h depuration. After that, the tissue levels decreased exponentially.

Overall, snails excreted smaller MP faster than larger MP (Fig. S7). For instance, after 48 h of depuration, the tissue levels had decreased by 95.7% (5 μm PS spheres), 91.0% (10 μm) and 87.5% (45 μm) compared to tissue levels after 24 h MP exposure without subsequent depuration period (Fig. S7). After 7 d of depuration, the mean tissue levels of all MP types had decreased by more than 99%.

3.2. Microplastics toxicity study

Throughout the MP toxicity study, feces of *L. stagnalis* contained MP which confirms ingestion of the PS fragments by the snails (Fig. S8). Qualitative visual analysis suggested a higher MP ingestion with increasing particle concentrations. Despite the high ingestion of PS fragments, MP exposure did not significantly affect energy reserves (Fig. 2a–c), oxidative stress (Fig. 2d and e) and reproduction (Fig. 2g–i) in *L. stagnalis* (Kruskal-Wallis tests with

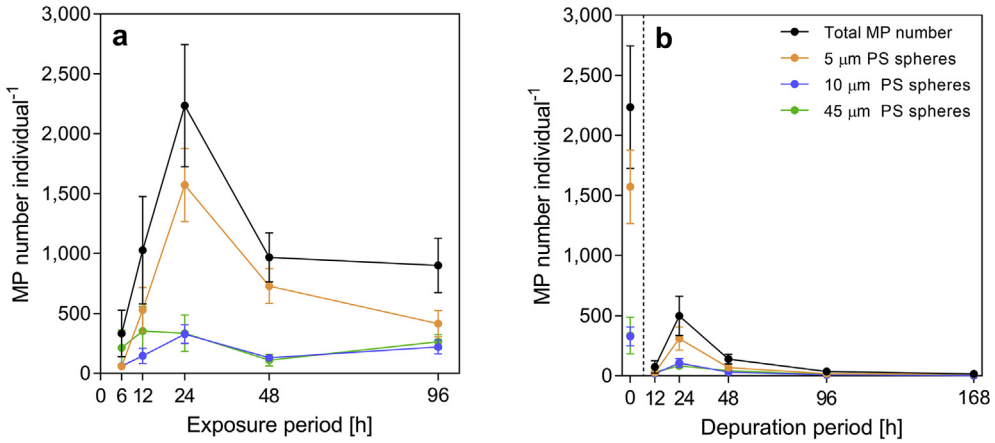

Fig. 1. Ingestion and excretion of microplastics (MP) by *L. stagnalis*. (a) Mean (±standard error) number of MP per individual after 6–96 h of exposure to a mixture of 5, 10, and 45 μm PS spheres (12 p mL^{-1} each, n = 5 per exposure period). (b) Mean (±standard error) number of MP per individual after 24 h of exposure to 5, 10 and 45 μm PS spheres (12 p mL^{-1} each, 0 h = 24 h in a) followed by a 12–168 h depuration period in MP-free medium (n = 5 per depuration period).

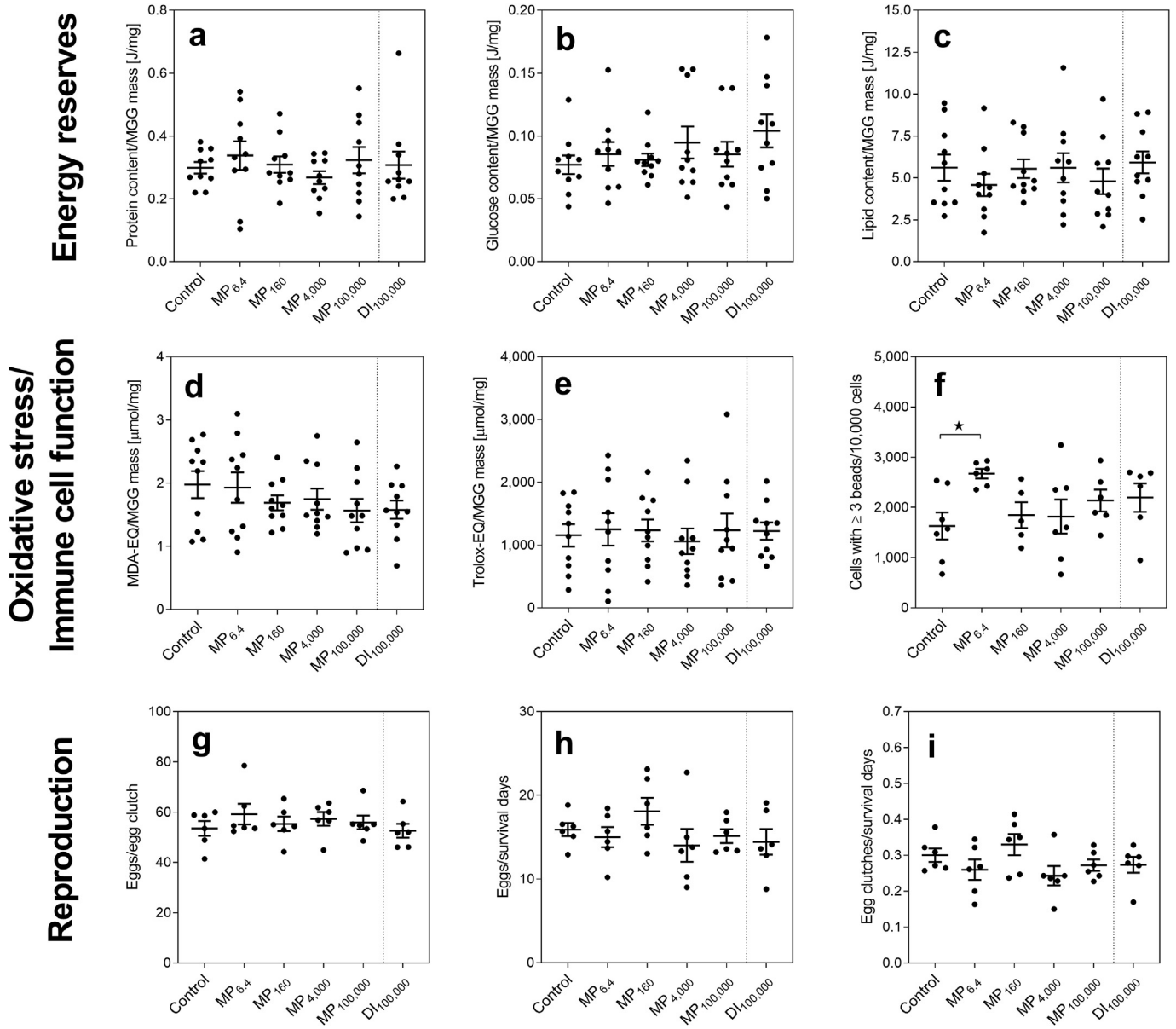

Fig. 2. Effects of irregular PS microplastics (MP, < 63 µm, 6.4–100,000 p mL^{-1}) and diatomite (DI, 100,000 p mL^{-1}) on the energy reserves ((a) protein, (b) glycose and (c) lipid content in the midgut gland (MGG)), oxidative stress ((d) MDA content and (e) remaining antioxidant capacity in the MGG), immune cell phagocytosis ((f) hemocyte phagocytosis activity) and on the reproduction ((g) average clutch size, (h) number of eggs, (i) number of egg clutches) of *L. stagnalis*. (a–e): n = 10, (f): n = 5–7, (g–i): n = 6. Statistics: Comparison: Control vs. all MP-treatments + $MP_{100,000}$ vs. $DI_{100,000}$; a, b, c, g: Kruskal-Wallis test with Dunn's post-test; d, e, f, h, i: one-way ANOVA with Sidak's post-test, ★ = $p < 0.05$.

Dunn's post-test/one-way ANOVA with Sidak's post test, $p > 0.05$). The phagocytic activity of hemocytes increased significantly in snails from the $MP_{6.4}$ treatment compared to control (Fig. 2f, one-way ANOVA with Sidak's post-test, df = 5, $p < 0.05$). No difference was observed in the other exposure groups compared to the control. Importantly, there was a high inter-individual variation for almost all endpoints. A statistical comparison of the treatment groups $MP_{100,000}$ and $DI_{100,000}$ did not reveal significant difference for any endpoint.

3.3. Mixture toxicity study with copper and microplastics

3.3.1. Copper analyses

Background contamination in the ISO medium was 7.4 ± 0.2 µg L^{-1} (±SD) and did not change significantly after 3 d of incubation with and without MP (Fig. S10a, Kruskal-Wallis test with Dunn's post-test, $p > 0.05$). However, Cu concentrations tended to be generally lower after 3 d compared to fresh ISO medium (Fig. S10a). Spiking of the ISO medium increased Cu concentrations by 7.9 µg L^{-1} (nominal: 7.5 µg L^{-1}) to 15.4 ± 0.8 µg L^{-1}. A 3 d exposure decreased mean Cu concentrations significantly by 23.8% compared to the freshly spiked ISO medium (Fig. S10c, one-way ANOVA with Sidak's post-test, df = 4, $p < 0.01$).

An additional incubation with MP or lettuce did not significantly affect the Cu concentrations compared to the spiked medium without MP after 3 d (Fig. S10b: Kruskal-Wallis test with Dunn's post-test, $p > 0.05$; Fig. S10c: one-way ANOVA with Sidak's post-test, df = 4, $p > 0.05$). Nevertheless, Cu concentrations on the lettuce significantly increased after 3 d in the Cu treatments (Fig. S10d, one-way ANOVA with Sidak's post-test, df = 3, $p < 0.05$). Here, Cu

concentrations per lettuce wet weight were highest in the treatments with 1.5 g lettuce and decreased with increasing lettuce mass (Fig. S10d). Accordingly, 1.5, 4.5 and 7.5 g lettuce adsorbed in total 4.3, 4.9 and 4.5 μg Cu, respectively, which was equivalent to 28–32% of the overall copper in the water phase. As we did not quantify Cu concentrations on the MP, it can, however, not be discriminated between Cu that directly adsorbed to the lettuce surface and Cu from Cu-contaminated MP which had aggregated on the lettuce surface.

In the mixture toxicity study, control treatments filled with ISO medium contained a mean total Cu concentration of 9.5 ± 1.4 μg L^{-1} in the freshly prepared exposures throughout each water exchange (0 d). Spiking with copper significantly increased Cu levels (at 0 d) to 17.2 ± 1.3 μg L^{-1} (spiked: 7.4 μg L^{-1}, nominal: 7.5 μg L^{-1}; Kruskal-Wallis test with Dunn's post-test, $p < 0.05$, Fig. S11a). Feeding with lettuce introduced an extra 0.49 μg Cu g^{-1} lettuce (wet weight) into each jar. Cu concentrations in the ISO medium decreased after 3–4 d both in the unspiked (−23%) and spiked medium (−41%). However, even after 3–4 d exposure (directly prior to the next water exchange), Cu concentration was still significantly higher in the Cu treatment compared to the control (Kruskal-Wallis test with Dunn's post-test, $p < 0.05$). A co-exposure with MP did not alter Cu water concentrations compared to the Cu exposure without MP (Fig. S11a).

Cu concentrations in *L. stagnalis* tissues increased in all test groups, including the control, throughout the 28 d exposure compared to pre-experimental tissue concentrations (Fig. S11b). In individuals from the Cu + $MP_{6.4}$ and Cu + MP_{160} groups, tissue concentrations were higher (15.1 and 11.3 μg Cu g^{-1} tissue) compared to individuals from the unspiked control (9.2 μg Cu g^{-1} tissue) and the Cu exposure (10.8 μg Cu g^{-1} tissue). In the Cu + $MP_{4,000}$, Cu + $MP_{100,000}$ and Cu + $DI_{100,000}$ groups, instead, average Cu tissue concentrations were lower than in the control and the Cu exposure (7.0–8.6 μg Cu g^{-1} tissue).

3.3.2. Copper toxicity

In the mixture toxicity experiment, exposure to Cu alone affected the reproduction of *L. stagnalis* significantly by reducing the number of eggs per clutch (Fig. 3f, one-way ANOVA with Sidak's post-test, $df = 6$, $p < 0.01$) but not the total number of eggs or egg clutches per survival days (Fig. 3g and h, one-way ANOVA with Sidak's post-test, $df = 6$, $p > 0.05$). Further, Cu exposure did not affect the energy reserves (Fig. 3a–c) or induce oxidative stress (Fig. 3d and e).

3.3.3. Effects of the additional MP exposure

The combined exposure to Cu and $MP_{4,000}$ significantly reduced the number of eggs per egg clutch compared to the control (Fig. 3f, one-way ANOVA with Sidak's post-test, $df = 6$, $p < 0.05$), while no significant differences compared to control were observed for any of the other combined treatments (Fig. 3f, $p > 0.05$). Further, none of the combined treatments affected reproduction (number of laid eggs or egg clutches per survival days, Fig. 3g and h), energy reserves (Fig. 3a–c) or oxidative stress (Fig. 3d and e) in the MGG. Furthermore, the effects of a co-exposure of Cu + $MP_{100,000}$ and Cu + $DI_{100,000}$ did not differ significantly for any endpoint.

4. Discussion

4.1. Microplastics ingestion and excretion

L. stagnalis is a continuous and primarily herbivorous grazer that sometimes also feeds omnivorously (Jager, 1971; Veldhuijzen, 1974). It has a specialized muscular stomach (gizzard) which grinds up large particles into a digestible size (Carriker, 1946) allowing the ingestion of food particles with a very broad size range. In case of PS spheres, *L. stagnalis* was able to ingest MP in the size range between 5 and 90 μm. The ingested quantities differed significantly both in regard to particle size and exposure period. Here, 5 μm spheres were more abundant in the snails compared to larger MP.

One reason for the size-dependent MP uptake may be a size-selectivity in *L. stagnalis* feeding. Usually, *L. stagnalis* is classified as unselective herbivore because its scraping apparatus, the radula, is not able to selectively scrape specific food items from a mixture (e.g., a biofilm, Groendahl and Fink, 2016). This implies that *L. stagnalis* cannot discriminate between different particle sizes. However, because our results demonstrate a significantly higher uptake of smaller MP, *L. stagnalis* individuals may have still either fed size-selective to a certain extend or smaller particles were more bioavailable to the individuals.

The importance of particle bioavailability on particle ingestion has recently been highlighted by Scherer et al. (2017). They observed a higher uptake of 10 μm and 90 μm compared to 1 μm PS spheres in the freshwater snail *Physa acuta* and discuss that larger MP had settled faster and, therefore, had become more bioavailable for the grazing snails. For *L. stagnalis*, particle sedimentation would have had less impact on overall bioavailability as it can both float freely in the water phase and graze on surfaces (see 2.3). Differences in MP availability due to a heterogenous distribution may still have originated from the formation of hetero-aggregates of small and large PS MP as well as adsorption to the lettuce. Here, 5 μm PS spheres may have stayed longer in the water phase (see 2.3) and, thus, could have better adsorbed to the lettuce, increasing their bioavailability compared to larger particles. Indeed, previous research has shown that 1–2 μm PVC particles frequently adsorb on phytoplankton and form hetero-aggregates (Long et al., 2017; Zhang et al., 2017). However, as we did not analyze the size-specific particle distribution in our exposure vessels, its contribution to the observed size-dependent MP ingestion remains unknown.

Regarding particle excretion, *L. stagnalis* can process and egest particles within 4 h after ingestion (Veldhuijzen, 1974). Egestion of MP was similarly rapid with particle abundance in the individuals decreasing mostly within 12 h. While the slightly higher MP concentrations after 24 h of depuration might be due to a re-uptake of MP-containing feces (coprophagy, Noland and Carriker, 1946; Scheerboom and Van Elk, 1978), particle abundance in *L. stagnalis* individuals dropped to below 5% of the initial level after 96 h for all particle types. This implies that long-term retention of MP particles in *L. stagnalis* over several days is rather limited.

The retention of MP by *L. stagnalis* is comparable to other aquatic invertebrates. The freshwater crustacean *Gammarus fossarum* excretes ingested polyhydroxybutyrate and polymethyl methacrylate particles (32–63 μm) almost completely within 64 h (Straub et al., 2017). In the bivalve *Mytilus galloprovincialis*, 90% of 1, 10 and 90 μm PS spheres pass the digestive tract within 12–84 h, with smaller spheres being excreted faster than larger ones (Kinjo et al., 2019). In goldfish, MP retention was modelled to range between 15 h and 6 d (Grigorakis et al., 2016).

The fast egestion of MP and the rather constant MP tissue levels of 10 and 45 μm spheres in the ingestion study suggest that *L. stagnalis* has fed on and excreted MP continuously. In contrast, we observed a marked increase in the abundance of 5 μm spheres in the snails after 24 h. This was not due to a prolonged retention in the digestive tract as *L. stagnalis* excreted smaller MP faster than larger ones. A potential explanation is that a change in feeding activities (Ter Maat et al., 2007) in combination with a heterogenous MP distribution (e.g., adsorption of MP to the lettuce) have caused this phenomenon, but the exact reason remains to be investigated.

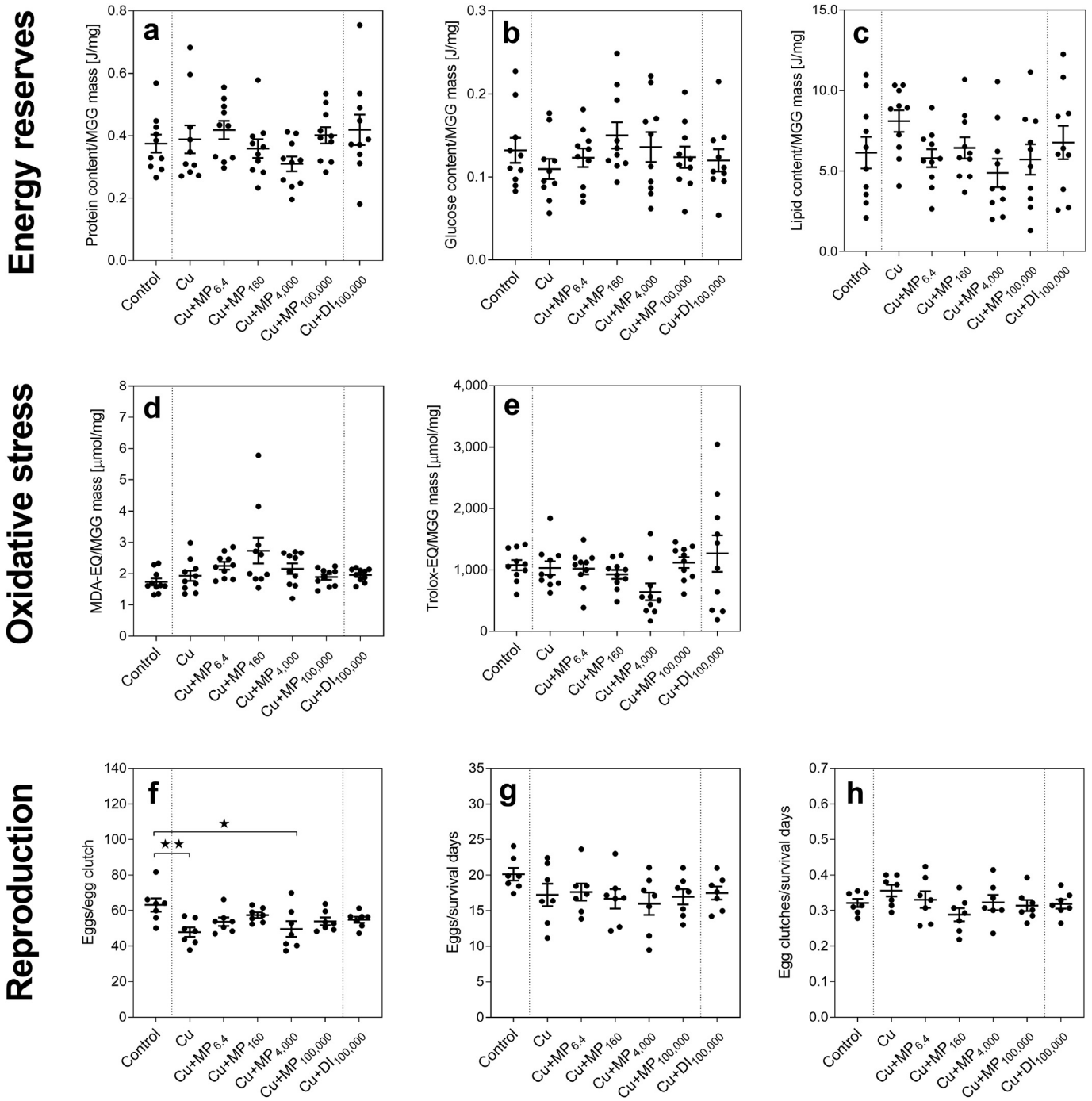

Fig. 3. Effects of copper (Cu, spiked as Cu^{2+}, 7.5 µg L^{-1}) alone or in combination with microplastics (irregular PS MP, < 63 µm, 6.4–100,000 p mL^{-1}) or diatomite (DI, 100,000 p mL^{-1}) on the energy reserves ((a) protein, (b) glycose and (c) lipid content in the midgut gland (MGG)), oxidative stress ((d) MDA content and (e) remaining antioxidant capacity in the MGG) and on the reproduction ((f) average clutch size, (g) number of eggs per survival days, (h) number of egg clutches per survival days) of *L. stagnalis*. Replicate numbers: a–e: n = 10, f–h: n = 7. Statistics: Comparison: Control and Cu vs. all Cu + MP treatments + Cu + $MP_{100,000}$ vs. Cu + $DI_{100,000}$; a, d, e: Kruskal-Wallis test with Dunn's post-test; b, c, f–h: one-way ANOVA with Sidak's post-test, ★ = $p < 0.05$, ★★ = $p < 0.01$.

4.2. Microplastics toxicity

An exposure to PS fragments (<63 µm) did not induce significant effects regarding survival, reproduction, energy reserves or oxidative stress in *L. stagnalis*, neither at environmentally relevant (6.4 p mL^{-1}) nor at very high MP concentrations (100,000 p mL^{-1}).

These results contradict data by Lo and Chan (2018) who reported strong MP effects on the veliger larvae of the slipper limpet *C. onyx* exposed to 2–5 µm PS spheres (at 60,000 and 140,000 p mL^{-1}). MP also affected *C. onyx* juveniles after larval settling. These MP effects may be due to the different feeding behavior of the *C. onyx* larvae compared to adult *L. stagnalis*. While the latter are grazers, *C. onyx* veliger larvae are planktotrophic filter feeders (Chiu et al., 2007; Li and Chiu, 2013) which rely on high algae abundance throughout the first days of growth. Starvation during early development reduces larval growth with long-term effects (Chiu

et al., 2008). In addition, varying effect levels may also be related to the different life stages of the snails (juvenile *C. onyx* vs. adult *L. stagnalis*). However, a higher sensitivity of juveniles compared to adults was not observed in *P. antipodarum*, a grazing freshwater snail like *L. stagnalis* (Imhof and Laforsch, 2016). This may indicate that, in some species, the feeding type might be more relevant for MP toxicity than the life stage. Yet, this assumption deserves further investigation in future MP toxicity studies with gastropods tested under comparable exposure conditions.

We observed some effect of MP on immune cell phagocytosis. Here, exposure to 6.4 p mL^{-1} MP significantly increased the phagocytic activity of snail hemocytes. This effect was, however, not observable at higher MP concentrations pointing towards a MP-unrelated cause.

In conclusion, MP exposure had no adverse effects on *L. stagnalis*. As nanoplastics were present in the PS powder we used, this is probably also true for the nanofraction that was part of the polydisperse MP suspension. The lack of effects is especially surprising as the qualitative analysis of MP in the feces suggests high ingestion rates. For example, feces of snails exposed to 100,000 p mL^{-1} consisted almost exclusively of MP. Because the ingestion of high quantities of non-digestible particles will reduce food and energy uptake (Gardon et al., 2018), *L. stagnalis* must have developed mechanisms to compensate changes in food quality and quantity. A previous starvation experiments with freshwater gastropods has shown that gastropods increase their ingestion rate with rising proportion of non-digestible material in the food source (Calow, 1975). Further, *L. stagnalis* can also increase its digestion efficiency by enlarging the gut space, enhancing MGG activity and increasing MGG digestive juice secretion (Calow, 1975). In addition, the presence of MP in the stomach may have facilitated the mechanical gridding of ingested food, enhancing assimilation efficiency and, thus, compensating effects due to reduced food uptake. Increasing food scavenging and/or a more efficient food digestion could have prevented adverse effects from reduced feeding success.

MP effects may have also been limited due to the highly adapted digestive system. As deposit feeder, *L. stagnalis* has a highly specialized selection system in the pylorus (stomach) which separates digestible and non-digestible particles. Digestible particles are transported to the MGG, while non-digestible particles are transferred to the intestine for direct excretion. Only particles <0.4 μm are transported into the digestive gland (Veldhuijzen, 1974). We, therefore, assume that MP >0.4 μm were directly transported from the pylorus into the intestine for egestion without reaching the digestive gland limiting MP-induced effects on metabolism. While smaller MP present in the suspension may have passed into the MGG, they did not induce any effects on the examined endpoints.

Adaptations of deposit feeders such as *L. stagnalis* towards the ingestion of high loads of non-digestible particles may, therefore, be a key mechanism limiting MP toxicity. This assumption is also supported by the fact that an exposure to high concentration of natural DI particles did not induce adverse effects. *L. stagnalis*, therefore, seems to react similarly to natural and plastic particles. Accordingly, grazing freshwater gastropods might have a limited sensitivity to MP exposure due to their evolutionary adaption to a particle-rich environment.

This conclusion must, however, be considered in the light of a potential stress tolerance of the *L. stagnalis* culture used in this study (see 4.3.1). Vinebrooke et al. (2004) hypothesized that a tolerance towards one stressor is positively correlated to a co-tolerance towards additional stressors. For instance, in the freshwater snails *Biomphalaria glabrata*, a parental exposure to a predator threat increased the tolerance to cadmium in its offspring (Plautz et al., 2013). As the snails in our laboratory culture were inadvertently exposed to low levels of copper in the ISO medium over several generations, they may have developed a certain stress tolerance towards copper as well as MP. While this could be investigated using snails cultured under especially pristine conditions, other stressors (including metals) are abundant in natural environments. Thus, a hypothetical pre-adaptation to stress might be common in nature.

In summary and considering MP concentrations in freshwater systems of up to 0.5 p mL^{-1} (Lahens et al., 2018), the current levels of MP do not seem to pose an overt risk to gastropods. MP toxicity may, however, increase in combination with other environmental stressors. In a second experiment, we therefore co-exposed *L. stagnalis* to a mixture of MP and Cu.

4.3. Mixture toxicity of copper and microplastics

4.3.1. Copper analysis

The unspiked ISO medium contained a background concentration of up to 9.8 μg L^{-1} Cu. Such high concentrations are usually toxic to *L. stagnalis*, especially for juveniles (Atli and Grosell, 2016; Brix et al., 2011; Das and Khangarot, 2011; Ng et al., 2011). The copper contamination in our experiments originated from the in-house distilled water supply. As this contamination probably had already existed for several months, multiple *L. stagnalis* generations in our culture had been raised in this water. We, thus, believe that the specimens used in our study have adapted to elevated Cu levels.

Both in the unspiked and Cu spiked medium, copper concentrations decreased in the water phase throughout the 3–4 d exposure suggesting some adsorption of copper on the vessels. A further addition of MP did not cause distinct changes in Cu concentrations in the water phase although we cannot exclude some adsorption of copper to MP (as shown by Holmes et al. (2012) on polyethylene pellets). Further, we observed Cu adsorption to the lettuce. Elevated Cu concentrations on the lettuce may originate from directly adsorbed Cu or (indirectly) from Cu-contaminated MP attached to the lettuce surface. Besides dermal uptake of dissolved Cu through passive diffusion at cell membranes (Grosell et al., 2002), dietary uptake is estimated to be the second relevant exposure pathway for freshwater gastropods (Hoang et al., 2008). Cu adsorption to the lettuce (both directly and indirectly) suggests that, besides dermal uptake, dietary exposure was a second relevant Cu exposure pathway for *L. stagnalis*.

4.3.2. Copper toxicity

The exposure to 7.5 μg L^{-1} Cu significantly reduced the number of eggs per clutch, while all other reproductive endpoints (eggs and clutches survival d^{-1}) remained unaffected. These effects are partially in accordance with a previous study in which exposure to 5.6 and 10 μg L^{-1} Cu significantly reduced the total egg number and the number of eggs per egg clutch, respectively (Das and Khangarot, 2011).

Cu exposure did not induce oxidative stress in *L. stagnalis*. We expected Cu to activate the antioxidant system of *L. stagnalis* as Cu toxicity is mostly caused by the generation of reactive oxygen species (Gaetke and Chow, 2003). High levels of reactive oxygen species usually activate the antioxidant system of mollusks to prevent tissue damage (Manduzio et al., 2005). For instance, exposure of *L. stagnalis* to 2 μg L^{-1} Cu activated the antioxidant system in the hepatopancreas (Atli and Grosell, 2016). However, we did not observe a significant oxidative stress induction, probably because of the previous Cu contamination and corresponding Cu tolerance in the *L. stagnalis* culture (see 4.3.1), such as, the expression of metallothioneins (metal-ion binding proteins) as well as the formation of metal-rich granulae (Ng et al., 2011). These mechanisms may have limited oxidative stress in *L. stagnalis* tissues. However, these detoxification mechanisms are energetically

costly (Moolman et al., 2007). Accordingly, *L. stagnalis* might have allocated less energy to reproduction while maintaining the energy storages (glycogen, lipids) in the MGG.

4.3.3. Effects of the additional MP exposure

In regard to reproduction, the number of eggs per clutch was significantly reduced in the Cu + $MP_{4,000}$ treatment but not the Cu + $MP_{100,000}$ treatment compared to the control. The energy reserves and oxidative stress were not significantly affected by a treatment with MP/DI and Cu compared to the control. Further, also no difference between the mixed exposure treatments and the Cu treatment were observed for any of the tested endpoints. Accordingly, an additional exposure to MP or natural particles did not exacerbate the limited toxicity Cu induced alone. MP was, therefore, neither a relevant single stressor nor a relevant stressor in combination with Cu for *L. stagnalis*. Again, this conclusion especially refers to our snail population that may be stress tolerant (see 4.2).

So far, knowledge on mixture toxicity of MP with additional chemical stressors is very limited for gastropods. For *L. stagnalis*, Horton et al. (2020) observed that in a 96 h mixed exposure to polybrominated diphenyl ethers (PBDE, 94–3000 ng g^{-1} sediment) and MP (1% nylon w/w in sediments), snails in PBDE-only treatments lost significantly more weight compared to treatments with PBDE and MP. In contrast, a mixed exposure of methamphetamine (0.1–50 mg L^{-1}) and MP (0.7 μm PS, 20 mg L^{-1}) enhanced mortality and filtration rates compared to a methamphetamine-only exposure of the freshwater gastropod *C. cathayensis* (Qu et al., 2020).

With regards to bivalves, previous studies exposed either the freshwater species *Corbicula fluminea* or the marine mussel *Mytilus edulis* to MP in combination with florfenicol, fluoranthene or mercury chloride (Guilhermino et al., 2018; Magara et al., 2018; Oliveira et al., 2018; Paul-Pont et al., 2016). Similar to the gastropods, these studies reported highly divergent results and suggested either no interaction, antagonistic or synergistic effects of MP and a chemical.

A key factor affecting the interaction is the sorption of the chemical to MP which mediates the uptake, but also elimination of the chemical by mollusks. The partition of a chemical between the solid (MP) and the liquid phase, however, depends on the physicochemical characteristics of both the polymer particles and the chemicals. Potential joint effects of MP and chemicals on mollusks in the environment will, therefore, depend on the abundance, type and interaction of MP and chemicals in the surrounding habitat complicating general predictions on mixture toxicity effects in the environment.

5. Conclusion

This study investigated the ingestion and toxicity of PS MP in the freshwater gastropod *L. stagnalis*. MP ingestion and excretion depended both on the particle size as well as the duration of the exposure and depuration period. Here, the snails ingested more 5 μm compared to 10 and 45 μm PS spheres and highest tissue levels were observed after 24 h of exposure. Further, *L. stagnalis* excreted MP within few days with <1% of the original particle abundance remaining after 7 d of depuration.

Despite the high MP ingestion, a 28 d exposure to PS fragments (<63 μm) at concentrations up to 100,000 p mL^{-1} did not affect survival, reproduction, energy reserves and oxidative stress of *L. stagnalis*. This suggests that current MP concentrations in freshwater ecosystems do not represent an overt risk for *L. stagnalis* populations when considering MP as sole stressor. The same was true when we exposed snails to a mixture of Cu and MP. Here, MP did not exacerbate the reproductive effects induced by Cu alone.

Limited MP effects may relate to potential stress tolerance in our *L. stagnalis* culture due to the presence of low levels of Cu may have limited MP and Cu effects. Such pre-adaptation to stress may be common in natural environments. Further, as continuous, unspecific grazer, *L. stagnalis* is well adapted to tolerate the ingestion of non-digestible material. These morphological and physiological adaptations may also have compensated the toxicity of MP. Accordingly, future research should focus more closely on the adaptation of aquatic biota to particle-rich environments and its relationship to MP toxicity.

CRediT author statement

Conceptualization and Methodology: AW and MW, Investigation: AW, MvR and AV (all experiments); BM, MvA and EF (copper analysis), Formal analysis: AW, MvR and AV, Visualization: AW, Writing - Original Draft: AW, MW, BM, MvA, Writing - Review & Editing: all authors, Supervision and Funding acquisition: MW.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128040.

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