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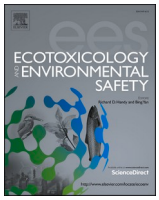

Dietary exposure to four sizes of spherical polystyrene, polylactide and silica nanoparticles does not affect mortality, behaviour, feeding and energy assimilation of *Gammarus roeseli*

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ABSTRACT

The abundance and persistence of plastic nanoparticles in aquatic habitats are considered a threat to marine and freshwater biota. However, the risk assessment of plastic particles is complicated due to various factors that need to be considered, including composition, size and environmental abundance. This study investigated the behavioural response of a key river species, *Gammarus roeseli*, to dietary exposure of plain biodegradable and non-biodegradable plastic as well as to natural small micro- and nanoparticles. Mortality, feeding, swimming velocity and energy assimilation endpoints were examined by considering four particles sizes ranging from 30 to 1000 nm in two concentrations. Contrary to our expectations, neither decreasing size nor increasing abundance of each tested particle impacted any of the examined endpoints. Likewise, dietary exposition with biodegradable plain polylactide did not induce other or stronger effects than non-biodegradable plain polystyrene or natural silica micro- and nanoparticles, as all three particle types did not lead to adverse effects on *G. roeseli*. These findings also suggest that the functional role of *Gammarus roeseli* as a shredder is not impaired due to particle occurrence within the exposure range of this study.

1. Introduction

Plastic particles in the environment are considered an emerging threat due to their various chemical compositions, increased use, and inappropriate disposal (Lambert et al., 2014; Shen et al., 2019). Particles can have a three-fold effect on organism survival: the direct effect of the plastic particle, the leaching of additives from the plastic particle, and the potential vector effect for ab- /adsorbed chemicals and pathogens (Shen et al., 2019; Triebskorn et al., 2019). Regarding the first aspect, particle size and abundance are two key factors that govern uptake, exposure and potential ecotoxicological effects of plastic particles in the environment (Kögel et al., 2020; Zimmermann et al., 2020). There is evidence that particle sizes can range from macro- to nanoscale due to degradation processes (Lambert and Wagner, 2016). With further ongoing degradation of plastic in the environment, the abundance of very small particles in the micro- and nanoscale is expected to increase in the future (Kögel et al., 2020; Lambert and Wagner, 2016). Plastic nanoparticles are believed to be more hazardous than macro- and microparticles. Especially, the increasing abundance of plastic nanoparticles has become the focus of research. Finer particles can be ingested by smaller and thus more diverse groups of organisms (Besseling et al., 2019; Kögel et al., 2020; Shen et al., 2019), in which a greater diversity of exposure pathways such as pulmonary inhalation and dermal uptake can occur (Kögel et al., 2020; Stapleton, 2019). Once in the organism, it is assumed that nanoparticles < 1 μm can be transferred into gut cells and induce cytotoxicity (Firdessa et al., 2014; Pitt et al., 2018; Shen et al., 2019; Triebskorn et al., 2019). Likewise, when transferred into cells, particle excretion is delayed for an indefinite period. The higher possibility of ingestion and intracellular uptake contribute to the greater potential of nanoparticles to accumulate in the food web (Pitt et al., 2018).

To prevent overestimation or misinterpretation of plastic particle toxicity, their comparison with natural particles of the same size and shape is necessary (Götz et al., 2021; Ogonowski et al., 2018). Natural

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particles are typically present in the environment at much higher concentrations (Triebskorn et al., 2019) and are thus adaptable by most organisms. Some species even depend on certain concentrations to provide required levels of turbidity for feeding and predator avoidance (Evan Ward and Shumway, 2004; Lummer et al., 2016; Hasenbein et al., 2013). Direct comparisons between exposure effects of plastic nanoparticles with naturally occurring particles can distinguish effects of the material from those that are simply a result of physical (e.g. shape-related) properties.

Further, most studies have focused on high particle concentrations viable for hazard assessment, but not environmentally relevant for risk assessment. The observed effects appeared at the cellular and organism levels. For example, reduced survival rate and locomotion behaviour were observed, and gene expression and cell-stress-response were upregulated (Prüst et al., 2020). Observed effects also include reduced cell viability in algae, and impacts on the physiology of three aquatic invertebrates (González-Pleiter et al., 2019; Hazeem et al., 2020). Zimmermann et al. (2020) investigated the toxicity of three plastic particle types, including the biodegradable polylactide, differentiating the toxicity of plastic with additives, leached additives and plain particles. They found that plastic toxicity is not always chemically driven, because they observed that the plain polylactide particle - but not the leached chemicals - reduced survival in *D. magna*.

In addition, studies reporting no effects of plastic micro- and nanoparticles are rare, possibly reflecting a rejection of publishing such results (De Sá et al., 2018). The effect assessment of plastic particles is also impeded by the number and complexity of factors that are compared (Kögel et al., 2020; Paul et al., 2020). This makes the observed effects inconsistent, leading to a controversy about the hazard potential and real ecotoxicological risk of plastic particles. The classification is even less strict regarding plastic nanoparticles because the database is poorly filled (Paul et al., 2020).

This study's primary objective was to assess the ecotoxicological effects of micro- and nanoparticles on a key species of stream ecosystems, *Gammarus roeseli*. In this context, four hypotheses were examined regarding size-, concentration- and composition-related plastic particle toxicity. In addition, general natural- and not plastic-specific particle toxicity was determined. In particular, the following four hypotheses were tested: (I) smaller nanoparticles < 100 nm cause more harm than bigger ones due to cytotoxicity and enhanced accumulation; (II) higher concentrations of micro- and nanoparticles lead to earlier and stronger toxicity responses in aquatic organisms like *Gammarus roeseli*; (III) biodegradable plastics induce other toxicity than non-biodegradable plastics; and, (IV) plastics are more toxic than natural particles of the same size, shape and concentration.

Therefore, we investigated the dietary exposure of particles for two weeks to the shredder *Gammarus roeseli*. It is a key species in riverine systems (Boeker and Geist, 2015) and has been recently recognised as a standard invertebrate species for freshwater river-dwelling organisms for laboratory risk assessment (Kunz et al., 2010) and stressor assessment in the wild occurring in high numbers in streams of the study region (Pander et al., 2022).

2. Material and methods

The four hypotheses were examined by considering lethal and sublethal endpoints based on a comparison of two plastic types and one natural particle of the sizes 1000, 500, 100 and 30 nm, at two concentrations. The examined particle types were (1) plain polystyrene as a non-biodegradable and one of the most investigated plastic particle types for easier comparisons with other studies; (2) plain polylactide because of its biodegradable properties and supposed different toxicity mechanism than polystyrene and as a rare examined plastic particle type; (3) silica as a natural reference particle to exclude false-positive general particle effect observations.

2.1. Organism and acclimatisation

Gammarus roeseli were sampled in April 2021 using a dip net in the River Moosach near the Aquatic Systems Biology Unit of TUM in Freising (Germany) and transported in river water to the laboratory. Afterwards, they were size-selected by sieve passage with a diameter of 1.5 and 3 mm. A homogenous size class (8.3 ± 1.8 mm, based on a subsample of $n = 40$) was chosen for the experiment according to Beggel et al. (2016). After size selection, gammarids were acclimatized for one week in an aquarium filled with aerated artificial water (EN ISO, 1998) and glass stones to the steady conditions of the climate chamber with 13 °C ± 0.5 °C and a 16:8 h light:dark cycle. The water parameters were measured weekly. The artificial water was prepared a day before use and aerated for one day in the climate chamber. Parameters before use were 9.8 ± 0.1 mg O_2/L, 13.5 °C ± 0.3 °C, pH $= 7.9 \pm 0.1$ and 669 ± 5 µS/cm. Phyll-tabs (Götz et al., 2021) and Phyll-flakes (Tetra GmbH, Germany) were fed ad libitum during acclimatization. Based on pre-experiments which revealed no sex-related differences, animals were then randomly distributed into the test units.

2.2. Particle concentration

Three spherical particle types in four sizes were tested in this experiment: plain polystyrene (PS,[4] 1000/500/100/30 nm), plain polylactide (PLA,[5] 2000/500 nm) and plain silica (Silica, 1000/500/100/30 nm). All particles were provided in suspension in water without further detergents of 50, 25 or 10 mg/mL by the company micromod (Germany, Rostock), except for the 30 nm PS particles which were provided in suspension by BS Partikel (Germany, Mainz). According to the manufacturer's protocol, plain silica particles have a hydrophilic surface with terminal Si-OH-groups without additional functional groups. The plain PS particles have no additional surface coating or functional groups, but can have rare negative loaded sulphate groups on the surface. In the absence of reliable environmental nanoparticle concentrations, the concentration used was based on Triebskorn et al. (2019) to match reported concentrations of 100–900 plastic microparticles/L in the River Elbe of which 1.8% were PS, mostly (90%) with a mean diameter of 20 µm. The mean environmental concentration of 10 PS microparticles/L with 20 µm size and an approximate spherical shape was assumed to mirror a realistic concentration.

Therefore, ten 20 µm PS-particles/L were set as environmental relevant concentration. This corresponds in theory to a weight of 4.31 ng based on the density 1.03×10^{-9} mg/µm^3 of PS specified by micromod. Subsequently, the same mass was used for the environmentally relevant concentration for each particle size. This decision was based on the simplified assumption that the microparticles degrade to nanoparticles without mass loss. Thus, two concentrations were chosen: one environmentally relevant concentration (ERC[6]) of 4.31 ng PS (low concentration = L) and one concentration of 431 ng, 100-fold higher than the environmentally relevant concentration (high concentration = H). The particles were homogeneously embedded in the food matrix for dietary exposure, as described by Götz et al. (2021). The tabs consist of agar, cellulose and Phyll (Tetra GmbH) and can be customized with supplements of interest. This method provides a stable distribution of the particle during the experiment without leaching and the possibility to connect the mass of consumed food to the amount of ingested particles.

G. roeseli were exposed to 4.31 ng/mg tab dry weight (tdw[7]) of each size of the PS nanoparticles. The theoretically equivalent particle concentrations are listed in Table SI1. The environmental concentration of silica as natural reference particles is supposed to be much higher than

[4] Polysytrene
[5] Polylactide
[6] Environmental relevant concentration
[7] Tab dry weight

that of PS, while PLA is assumed to occur at substantially lower concentrations. To use comparable concentrations, the ERC for these two particle types was based on the particle concentrations of PS and their specific density provided by micromod (Table SI1). To ensure similar particle concentration, 4.19 ng PLA/mg tdw and 8.38 ng silica/mg tdw were used for exposure. Subsequently, the 100-fold higher concentration was 431 ng PS/mg tdw, 419 ng PLA/mg tdw and 838 ng silica/mg tdw. The particle suspensions were pipetted into the DECOTAB[8] mixture during production (Götz et al., 2021) or, for the ERC, previously diluted in distilled water.

2.3. Bioassay

To test the four hypotheses, the experiment comprised a total of 22 treatments (Table 1). To minimize stress originating from predation or competition for food, the setup consisted of smaller numbers of three acclimatized gammarids per beaker, but a higher number of six replicates per treatment including the control, according to Götz et al. (2021). Three glass stones were placed into each beaker as a refuge for the gammarids, to prevent cannibalism and to reduce stress. The 1-L beakers were filled with 500-mL artificial water and placed randomly into the climate chamber. Eighteen gammarids were allocated to starvation treatment for test duration and separated into a 250-mL beaker with 150-mL artificial water and two glass stones to avoid cannibalism. These gammarids were not fed and remained unexposed to particles. Exposure time was two weeks. Water was completely changed once a week. After the organisms were allocated to a beaker, exposure was initiated by adding the particle-loaded phyll-tabs according to the respective treatments. The tabs were exchanged weekly. For the measurement of the status of the acclimatized gammarids right before the start of the experiment, 40 specimens from the gammarids, pre-acclimatized for one week, were instantly prepared for energy assimilation measurement and stored at −20°C until analysis.

Table 1
Summary of the 22 treatments with shortcuts and combinations. The first indicator stands for the treatment, C = Control, H = Hunger/Starvation, Silica = silica, PS = polystyrene, PLA = polylactide. The second indicator stands for the particle size in nm. The last indicator gives the concentration. "L" in the shortcut or "Low" is the environmentally relevant concentration of 4.31 ng/L PS, 4.19 ng/L PLA and 8.38 ng/L silica, and "H" in the shortcut or "high" is the 100-fold higher concentration.

Treatment	Particle type	Particle Size [nm]	Conc.
C.0.0 (Control)	-	–	–
H.0.0 (Starvation)	-	–	–
Silica.100	Silica	1000	High
Silica.100	Silica	1000	Low
Silica.50	Silica	500	High
Silica.50	Silica	500	Low
Silica.10	Silica	100	High
Silica.10	Silica	100	Low
Silica.3	Silica	30	High
Silica.3	Silica	30	Low
PS.100	Polystyrene	1000	High
PS.100	Polystyrene	1000	Low
PS.50	Polystyrene	500	High
PS.50	Polystyrene	500	Low
PS.10	Polystyrene	100	High
PS.10	Polystyrene	100	Low
PS.3	Polystyrene	30	High
PS.3	Polystyrene	30	Low
PLA.200	Polylactide	2000	High
PLA.200	Polylactide	2000	Low
PLA.50	Polylactide	500	High
PLA.50	Polylactide	500	Low

[8] DEcomposition and COnsumption TABlet

2.4. Measurements

2.4.1. Mortality

Mortality was monitored daily, and dead gammarids were removed from the test. Gammarids were counted as dead when no movement of antennae or pleopoda was observed.

2.4.2. Feeding

To determine the mass of food consumed, the loaded phyll-tabs were dried for three days at 45 °C in a drying cabinet (Memmert GmbH), and initial dry weight (dw_I[9]) was determined with a fine scale (Sartorius, 0.01 ± 0.02 mg). Afterwards, tabs were pre-wetted in distilled water for a day for easier feeding and transferred into the allocated treatment. After one week of exposure, the tabs were removed, exchanged with new tabs (pre-weighed and pre-wetted) and dried for two days at 45 °C in the drying cabinet. The final dry weight (dw_F[10]) was also determined. Simultaneously, tabs of each treatment were handled the same but without gammarids in the beaker to determine weight loss during the week independent of gammarids feeding on the tabs (dw_L[11]). The mass eaten per week of all living gammarids in the beaker was calculated by subtracting the percentual proportion of the calculated dw_L from the dw_I and then subtracting the dw_F. Subsequently, the mass eaten per gammarid and day was calculated by dividing the mass eaten per week by the sum of the number of feeding gammarids on each day.

2.4.3. Swimming behaviour

The swimming behaviour of the individual gammarids was monitored at the beginning of the experiment and subsequently every seventh day (Bartonitz et al., 2020). First, individual gammarids were carefully removed with a spoon from the beaker and transferred into a Petri dish with a diameter of 5.5 cm filled with 10 mL of artificial water. The dishes were placed on light boards and at a 30 cm distance under a camera. The gammarids were tracked with Ethovision XT 11 (Noldus, Netherlands) for 10 min and with a sample rate of 25 frames per second. Thus, for each gammarid, the velocity in cm/s was measured and summarised in an Excel sheet. Afterwards, gammarids were transferred using a spoon into a new beaker according to the treatment.

2.4.4. Dry weight determination

The dry weight of the gammarid tissue (except head and gut) was measured before the energy assimilation determination to allow connection with the other endpoints. Tissues were dried for one day in a drying cabinet (U 40, Memmert, Germany) at 45 °C and weighed after being cooled down for 30 min in a desiccator.

2.4.5. Energy assimilation

The determinations of lipid, glucose and glycogen were based on the assays from Charron et al. (2014) with some modifications. The determination of protein content was based on the Bradford assay (Bradford, 1976) as described by Walker (2002). The dried gammarid tissue (without head and gut) were homogenised in a 1.5-mL tube with a mortar fixated in a dremel (Micromod 50/e, Proxxon). Then, 200 µL methanol was added, and the remains were homogenised the second time. Another 700 µL methanol was rinsed over the mortar into the tube to wash any sample residues. After homogenisation using a vortexer, the sample was divided into three aliquots: 300 µL was transferred into a 1.5-mL tube for lipid measurement, 300 µL was transferred into the second 1.5-mL tube for glucose and glycogen measurement. Next, 2 × 50 µL was transferred to two glass test tubes for protein measurement.

[9] Dry weight initial
[10] Dry weight final
[11] Dry weight loss

Lipid and glucose/glycogen measurement assays were further conducted as described in Götz et al. (2021) with some adjustments. The lipid aliquot was divided into two samples of 400 μL after cooling for 20 min for measurements in duplicate. The used amount of reagents in each assay was 2.5 mL instead of 5 mL. For protein measurement, 50 μL distilled water was added to 50 μL of the sample in each glass test tube. Also, 50 μL of distilled water and 50 μL of methanol were pipetted into an extra glass test tube as blank. Finally, 1 mL Bradford reagent was added to each glass test tube. After 2 min, the colour change from brown to blue allows the photometric measurement of the absorption at 595 nm against the blank. The amount of proteins was calculated using a calibration curve.

2.5. Statistical analysis

R (v.4.1.0), Rstudio (RStudio, 2015) and Jamovi (The jamovi project, 2021) were used for statistical analyses. Each treatment per measurement was tested for normal distribution with the Shapiro–Wilk test and, due to some treatments without normal distribution and the small sample number, tested with the robust Fligner–Kileen test for homogeneity in variance. Based on the results, The Friedman test for repeated measurements and, in case of significance, the Durbin–Conover post hoc test was used to test for changes within each treatment over time. To test for differences between treatments, the parameter-free Kruskal–Wallis test was conducted. When differences were observed ($p < 0.05$), the Wilcoxon for pairwise comparisons for unpaired measurements with Bonferroni–Holm continuity correction was used as a post hoc test to find the specific treatments, which were different from each other.

3. Results

3.1. Mortality

There was a significant increase in mortality for each single treatment over time ($p < 0.001$). Despite this result, there was no difference between the treatments after one week ($p = 0.921$) and two weeks ($p = 0.781$). Furthermore, 61.1% ± 32.8% of the gammarids in control and 44.4% ± 50.2% of the starved gammarids died within the two weeks. In the particle-exposure treatments, mortality was between 39.9% ± 25.1% (Silica.30.L) and 83.3% ± 27.9% (PS.1000.L) after two weeks. The other treatments also varied within this range.

3.2. Feeding

Overall, the gammarids in each treatment constantly fed during the two weeks (within treatment comparisons between week one and two, every $p > 0.05$) and significantly more than the starved gammarids ($p = 0.031$). In the first week, gammarids fed with Phyll-tabs loaded with a high concentration of 500 nm silica particles slightly consumed less than those from the treatments with PLA.2000.H, PS.100.H, PS.500.H, Silica.100.H and Silica.1000.H ($p = 0.048$). There were no further differences in the amount of mass eaten by the treatments in the first and second week, including the control. Each control gammarid fed with unloaded Phyll-tabs consumed on average 184.6 ± 68.7 μg/d*gammarid in the first and 237.6 ± 78.6 μg/d*gammarid in the second week. In the particle treatments, mean mass consumption ranged from 141.5 ± 39.3 (Silica.500.H) to 335.2 ± 140.9 (PS.100.L) μg/d*gammarid in the first week. There was more variation in feeding within the treatments in the second week including some outliers (Fig. 1); the minimum consumed food was 231.8 ± 85.1 μg/d*gammarid (PS.1000.H) and the maximum was 1164.1 ± 1638.7 μg/d*gammarid (PS.500.H). A table with the mean mass eaten and the corresponding ingested amount of particles per gammarid and day for each treatment is provided in the supplementary information (Table SI2). The calculation of the amount of ingested particles was based on the findings of Götz et al. (2021), where the uptake of the particles embedded into the food has been systematically examined. In this context, it is known that leaching of particles from the tab can be neglected. As an example for the calculation of the ingested amount of particles: In the treatment PLA.2000.H, the consumption per gammarid in one of the beakers was in theory

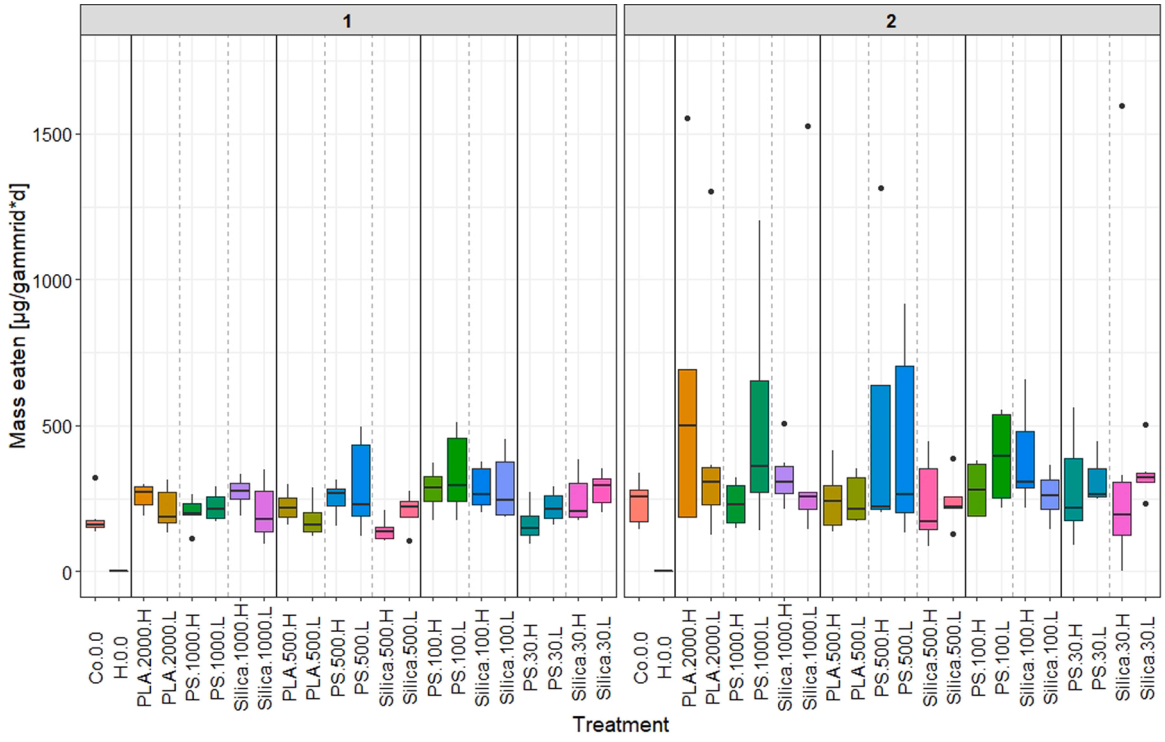

Fig. 1. Mass eaten per day and gammarid in week one (1) and two (2). Gammarids were exposed to four sizes (30, 100, 500 and 1000 nm) and two concentrations, High = H, Low = L, of spherical micro- and nanoparticles of plain polystyrene, polylactide and silica. The number of measured gammarid samples per treatment ranged from n = 3 (PS.1000.L) to n = 11 (Silica.30.L).

291.3 μg/d of the Phyll-tab in the first week. The tab was loaded with 419 ng PLA-particles/mg, which leads to a calculated mass of 122.1 ng PLA-particles ingested per day. A gammarid from the treatment PLA.2000.L with nearly the same amount of food consumed (297.48 μg/d*gammarid) ingested around 1.3 ng PLA-particles per day. Overall, the gammarids treated with the low dose ingested particles in the one-digit nanogram level, while those fed with the high dose ingested around 100-fold higher amounts of particles in the lower three-digit nanogram range.

3.3. Swimming behaviour

None of the gammarids in any treatment swam significantly different to those in other treatments at test start ($p = 0.339$), after one week ($p = 0.267$) or after two weeks ($p = 0.071$), including the comparison of the treatments to the control. Comparing the velocity within each treatment from start to end of the test, the starved gammarids swam slower after two weeks ($p = 0.008$), while the velocity of the gammarids from the control did not change ($p = 0.368$). The gammarids from the particle treatments did not change their swimming speed over the two weeks of exposure, except the gammarids treated with PLA.500.L ($p = 0.014$) and Silica.1000.L ($p = 0.007$). In both treatments, the gammarids swam significantly faster after two weeks. Control gammarids swam between 0.71 ± 0.32 and 0.43 ± 0.38 cm/s. The other treatments varied in the same range from minimum (0.50 ± 0.24 cm/s; H.0.0) to maximum (0.82 ± 0.77 cm/s; PLA.500.L) at test start. After two weeks, the range of the velocity of the gammarids in the treatments varied from 0.24 ± 0.14 cm/s (H.0.0) to 0.91 ± 0.30 cm/s (Silica.1000.L).

3.4. Dry weight determination

Particle exposure did not influence dry weight of the dissected gammarids ($p = 0.852$, n = 3 (PS.1000. L) to n = 11 (Silica.30.L) and 39 for acclimatisation). The dried body remains mean weight was 2.30 ± 0.39 mg. Gammarids from the Silica.1000.H treatment weighed the most with 2.95 ± 0.93 mg, and gammarids from the Silica.500.H treatment weighed only 1.54 ± 1.19 mg.

3.5. Energy assimilation

The four storage substances, protein, lipid, glucose and glycogen, were measured in the body of the gammarids without head and gut. None of the four substances changed due to the particle treatments ($p = 0.130$–1.000) after the two weeks exposure and, hence, were the same as in gammarids from the control, starvation and acclimatisation (Fig. 2).

4. Discussion

To our knowledge, this is the first study which examines nanoparticle effects on *Gammarus roeseli* based on comparing dietary exposures to mineral particles, non-biodegradable and biodegradable plastic particles. Surprisingly, exposure to small nanoparticles of 30 nm did not result in increased signs of toxicity on the organism level compared with larger particles up to 1000 nm. In contrast to our hypotheses, none of the four tested particle sizes induced any adverse effect in the examined endpoints independent of whether the environmentally relevant ng concentration or the 100-fold higher concentration was used. Our further hypotheses were likewise not confirmed. Primarily, there was no observable difference in the reaction of *G. roeseli* to the plastic and the natural particles. Also, the biodegradable PLA did not induce higher or other toxicity responses in the investigated endpoints than the non-biodegradable PS. The dietary exposure did not result in alterations in survival, feeding, swimming behaviour or energy assimilation of *G. roeseli* within the experimental duration of two weeks. Therefore, no concentration, particle type or size-dependent effects were evident. This suggests that *G. roeseli* will not be negatively affected in their performance and behaviour if it ingests micro- and nanoscale particles in the environment. Thus, even if the nanoparticles translocate into tissues (Firdessa et al., 2014; Triebskorn et al., 2019), there is no effect showing up to the organism level.

The gammarids fed with Phyll-tabs without particles consumed on average half as much food as observed by Götz et al. (2021). However, the consumption is still in the acceptable range, like for the other to micro- and nanoparticles exposed gammarids. This suggests that the functional role of *Gammarus* as a shredder is not impaired due to particle occurrence. For the interpretation of the energy reserves, it should be noted that the lack of significance of the differences between treatments might be due to the relatively small sample size of some treatments. Nevertheless, the amount of measured lipids in the dissected gammarids from each treatment is similar to the amount of lipids of the natural state and Phyll-tab-fed gammarids of another study (Götz et al., 2021). Only glucose and glycogen were consistently less stored in the present gammarids independent of the treatment, including acclimatization, than in previously tested gammarids (Götz et al., 2021). The small amount of glucose stored in the gammarids is the same or lesser as in gammarids starved for 16 (Götz et al., 2021) and 23 d (Charron et al., 2015). This discrepancy is likely due to an additional environmental stressor affecting the gammarids' survival. Mortality induced by the setup can be excluded as the controls in preceding experiments of gammarids from the same population in well-fed states resulted in low mortality (Bartonitz et al., 2020; Götz et al., 2021). In contrast, it indicates a preceding malnutrition of the gammarids of our study (Charron et al., 2015; Götz et al., 2021), which seems to continue over the experiment as the time of two weeks may not be enough to fill up the reserves. The gammarids were caught in April in contrast to those from Götz et al. (2021), which were caught in November. In this spring, the habitat contained remarkably less vegetation and detritus, which most likely led to the malnutrition of the gammarids. This additional stressor due to preceding starvation affects the survival, but not the behaviour relating to the investigated endpoints. In contrast, the gammarids consumed the offered tabs and subsequently ingested the micro- and nanoparticles as intended. Therefore, particle uptake und subsequent exposition of the gammarids can be considered as given since particle uptake via the DECOTAB was confirmed in a previous study by Götz et al. (2021). Additionally, organisms treated with more than one stressor normally react more sensitively than they would with a particular one (Marcogliese and Pietrock, 2011; Vellinger et al., 2012). Consequently, the pre-starved population of the caught and tested gammarids would likely not additionally be affected regarding swimming, feeding and energy assimilation by the occurrence of those particles as tested here. In addition, multiple stressors affecting gammarids in the wild can also result in sub-optimal conditions and high mortalities in natural exposures (Pander et al., 2022). From an ecotoxicological point of view, comparing treatments of defined stressor exposure in non-optimal states can exacerbate the effect strength. The absence of such effects in our study may be indicative that no larger effects would occur under optimal conditions.

Generally, the risk of negative environmental consequences caused by nanoparticles is rising since the degradation and disintegration of plastic debris will most likely increase the abundance of smaller-sized particle fractions. This probability for increased risk is based on the assumption that nanoscale particles can pass epithelial barriers and translocate into tissues, which induces cytotoxicity (Shen et al., 2019). Hence, we wanted to evaluate whether environmentally relevant concentrations of nanoparticles induce toxicity affecting the population of *G. roeseli* correlating with the decreasing size from 1000 to 30 nm. Due to the dietary exposure, the uptake and subsequent exposure of the particles can be predicted when feeding occurred (Götz et al., 2021). However, the ingested nanoparticles were not documented by detection methods in our study. The lack of effects do not correspond with those

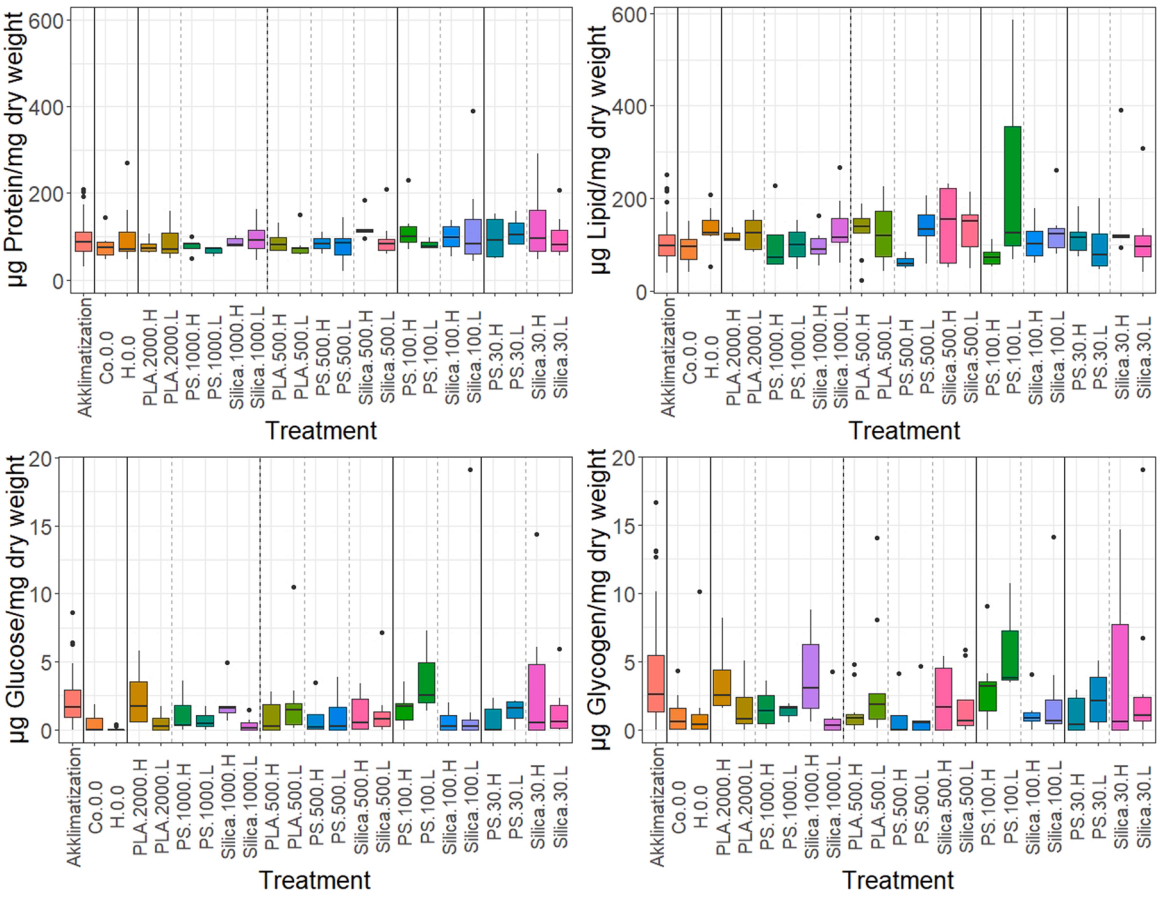

Fig. 2. The amount of protein, lipid, glucose and glycogen in µg per mg dry weight of the gammarid bodies exposed to different sizes and concentrations of three micro- and nanoparticles after acclimatization and two weeks. The particle types were spherical polystyrene, polylactide and silica in 30, 100, 500 and 1000 nm. The number of measured gammarid samples per treatment ranged from n = 3 (PS.1000.L) to n = 11 (Silica.30.L) and 39 for acclimatization.

from other studies, as 70 nm PS nanoparticles induced mortality and delayed development for *D. pulex* (Liu et al., 2019), and 20–500 nm PS nanoparticles reduced cell viability, chlorophyll-*a* concentration and increased levels of reactive oxygen species (ROS[12]) in *Chlorella vulgaris* (Hazeem et al., 2020). Nevertheless, most studies greatly exceed the environmental concentrations we examined in our study in the nanogram range and used milligrams of the particles. Our observations suggest that PS and PLA nanoparticles do not impact the individuals' behaviour and, very likely, the performance of a population of *G. roeseli* in the mass concentrations that are currently assumable for PS nanoparticles in the environment. These observations do not correspond with other studies, where biodegradable plastic particles were found to be more toxic than non-biodegradable plastic or mineral particles. For example, secondary nanoparticles (75–200 nm) of the biodegradable plastic polyhydroxybutyrate (PHB) reduced cellular growth and affected physiological parameters of *Anabaena* sp. and *Chlamydomonas reinhardtii* within three days of exposure and *Daphnia magna* after two days (González-Pleiter et al., 2019). Furthermore, fragmented PLA microparticles (< 59 µm) at a high mg concentration reduced survival and reproduction of *Daphnia magna*, being 35-times more toxic than the natural kaolin microparticles, which also reduced the reproduction rate but only in a much higher concentration (Zimmermann et al., 2020). The EC_{50} was calculated as 23.6 mg PLA/L, and the smallest investigated concentration of PLA microparticles in their study did not reduce reproduction. The latter finding corresponds with ours, as the examined concentrations in the ng range did not affect the investigated endpoints.

Still, the missing significance in the effects may also be due to a variability in the response originating from the undifferentiated sexes of the gammarids, which is known to be a possible factor (Sornom et al., 2010; Charron et al., 2014). Nevertheless, the conclusion seems likely that the environmentally relevant nanoparticle mass concentrations in the lower nanogram range calculated from the microparticle mass concentrations from Triebskorn et al. (2019) used in our study will not impact *Gammarus roeseli* when exposed to them in their habitat.

Finally, we assessed whether the plastic particle toxicity is similar to the toxicity of naturally occurring particles. The direct comparison showed that neither plastic particles nor mineral particles impacted the examined organisms. Although this could be interpreted as evidence that the in other studies interpreted plastic particle toxicity could likewise be a general particle toxicity, this assumption needs to be verified with effect-inducing concentrations. Nevertheless, most of those studies did not compare their observed responses with responses to natural nanoparticles of the same size and shape (González-Pleiter et al., 2019; Hazeem et al., 2020; Liu et al., 2019). Therefore, not a specific plastic nanoparticle effect but rather a general nanoparticle effect was proven. Studies investigating plastic particle effects compared with natural particles are occasional but important (Triebskorn et al., 2019). The study of Zimmermann et al. (2020) is the first but rare evidence that the toxicity of biodegradable plastic particles is different to natural particles.

Regarding the assessed endpoints, most of the studies examine mechanistic and metabolic relevant endpoints. The focus of our study was on ecologically relevant endpoints for risk assessment reflecting the performance of particle-exposed organisms in the environment. The future, integration of additional physiological endpoints and

[12] Reactive oxygen species

microindicators reflecting metabolic pathways and gene or protein expression patterns with the same setup may make a useful addition and contribute to a more mechanistic understanding of the underlying factors. In the context of the reviewed literature, our study is a rare example that micro- and nanoparticles of polystyrene and biodegradable polylactide do not induce adverse effects on aquatic keystone organisms. With this study we provide another basic dataset for further plastic nanoparticle risk assessment by examining a realistic ERC.

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Disclaimer

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CRediT authorship contribution statement

A. Götz: conceptualisation, data curation, formal analysis, investigation, methodology, visualisation, writing - original draft, review and editing; **S. Beggel**: conceptualisation, supervision, formal analysis, methodology, writing - review and editing; **J. Geist**: conceptualisation, supervision, resources, writing - review and editing.

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.

Data availability statement

Rawdata are open access in Dryad Database. DOI:10.5061/dryad.547d7wmbj. Calculation tools are available from the corresponding author (geist@tum.de).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113581.

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