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Ingestion and effects of micro- and nanoplastics in blue mussel (*Mytilus edulis*) larvae

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Abstract

It is well known that mussels are exposed to microplastics but ingestion and potential effects on mussel larvae are not well understood. We quantified ingestion and egestion of 100 nm and 2 μm polystyrene beads in blue mussel larvae after 4h exposure and 16h depuration using different plastic-to-microalgae ratios. Effects on growth and development of mussel larvae were investigated at 0.45, 28.7 and 287 $\mu\text{g L}^{-1}$ within 15 days of exposure. We found that, on a mass basis, larvae ingested a higher amount of 2 μm than 100 nm beads, while egestion was independent of particle size and the plastics-to-algae ratio. Although particle egestion occurred readily, microplastics remained inside the larvae. Larval growth was not affected but abnormally developed larvae increased after exposure to polystyrene beads. Malformations were more pronounced for 100 nm beads and after longer exposure time.

Keywords: microplastics, quantification, egestion, growth rate, development, malformation

Introduction

The ubiquity of microplastics in the marine environment has resulted in a large number of organisms being directly exposed to these particles. A variety of taxa of different trophic levels and feeding strategies interact with marine microplastics (Avio et al., 2017). Especially filter-feeding animals are prone to ingest microplastics passively together with their normal food, which has been demonstrated for zooplankton (Cole et al., 2013), bivalves (Santana et al., 2016), barnacles (Goldstein and Goodwin, 2013) and even filter-feeding whales (Besseling et al., 2015; Fossi et al., 2016). Blue mussels (*Mytilus edulis*) are among the most widely studied organisms in this respect due to their ecological importance as a keystone species in benthic ecosystems (Bonanno and Orlando-Bonaca, 2018; Ragnarsson and Raffaelli, 1999). Ingestion of microplastics has been observed in the field (Qu et al., 2018; Van Cauwenberghe et al., 2015) as well as in laboratory studies (Browne et al., 2008; Wegner et al., 2012). It has been shown that a variety of factors can influence the amount of ingested particles, such as particle size, concentration and shape. Accordingly, a broad range of ingested particle numbers have been reported (Li et al. 2019). As mussels are adapted to an ecosystem with a high load of inorganic particles microplastics may simply be egested with the faeces (Van Cauwenberghe et al., 2015; Van Cauwenberghe and Janssen, 2014). However, after 3 days of depuration in the lab, mussels that had been collected from a farm still had two thirds of the initial microplastics burden in their bodies (Van Cauwenberghe and Janssen, 2014). Egestion was thereby less efficient for smaller particles. Furthermore, Browne et al. (2008) found polystyrene beads of 3 and 9.6 µm to translocate into the circulatory system of blue mussels where they persisted for more than 48 days. Also, adverse effects of microplastic exposure on a cellular as well as a physiological level in mussels have been reported, including oxidative stress and inflammatory responses (Paul-Pont et al., 2016; von Moos et al., 2012), reduced feeding and respiration, and increased mortality (Rist et al., 2016; Wegner et al., 2012).

So far research on interactions between blue mussels and microplastics has focused on adult stages. No study has investigated microplastic ingestion and effects on blue mussel larvae. However, knowledge on the impact of plastic pollution on planktonic larvae is particularly relevant since early life stages are especially sensitive to environmental stressors (His et al., 1999; Widdows, 1991) and their survival determines dispersion and recruitments of populations. Ingestion of plastic particles, mostly in the nanometer size range, has been observed in marine planktonic larvae of other species, such as larvae of the Pacific oyster (*Crassostrea gigas*) (Cole and Galloway, 2015), the Mediterranean mussel *Mytilus galloprovincialis* (Beiras et al., 2018; Capolupo et al., 2018), the sea urchin *Paracentrotus lividus* (Beiras et al., 2018; Della Torre et al., 2014), the brine shrimp *Artemia franciscana* (Bergami et al., 2016), the

copepods *Acartia clausi* and *Tigriopus fulvus* (Beiras et al., 2018) and the rotifer *Brachionus plicatilis* (Beiras et al., 2018; Manfra et al., 2017).

Ingestion of microplastics has been shown to cause different adverse effects on planktonic invertebrate larvae. Cole and Galloway (2015) found reduced feeding of *C. gigas* larvae during exposure to 1 μm polystyrene beads at a concentration of 1000 mL^{-1} . Abnormal embryo-larval development increased in *C. gigas*, *M. galloprovincialis* and *P. lividus* as an effect of 50 nm PS-NH₂ beads with EC50 (median effect concentration) values of 0.15 (36h exposure), 0.142 (48h exposure) and 2.61 mg L^{-1} (48h exposure), respectively (Balbi et al., 2017; Della Torre et al., 2014; Tallec et al., 2018). In *P. lividus* this was accompanied by changes in the expression of genes responsible for cell death (Della Torre et al., 2014). An increased mortality rate was observed when *B. plicatilis* was exposed to the same particles at a concentration of 2.5 mg L^{-1} and higher (Manfra et al., 2017).

The blue mussel *M. edulis* is among the most studied species with respect to microplastics. Still, no information is available on microplastic ingestion and effects on its, possibly, most sensitive development stage: the larvae. This is needed to evaluate in what way this keystone species interacts with, and is affected by, microplastics throughout its life cycle. The general aim of the present study was therefore to investigate the ingestion and effects of microplastics on larval stages of the blue mussel. Our specific objectives were to determine: (i) whether *M. edulis* larvae ingest plastic particles in the nano- and micrometer size range, (ii) quantify the ingestion and egestion rates of plastics particles and (iii) examine the influence of food concentration on the ingestion and egestion of microplastics. Additionally, we investigated how prolonged exposure to micro- and nanoplastics affects larval growth and development.

Materials and methods

Mussel cultivation and larvae rearing

The blue mussels (*M. edulis*) used for this study came from two different populations. For the first experiment (Ingestion & egestion test) mussels were collected less than a week in advance on the island of Skaftö (Sweden), and kept in a flow through system with natural seawater at a temperature of 7°C, salinity of ~33‰ and ambient pH. For the second (Prolonged egestion test) and third (Larval growth test) experiment blue mussels were collected from long lines on a mussel farm in Limfjorden near Sallingsund (Denmark). These were kept in an aquarium with filtered sea water (FSW) (15°C, salinity ~33‰) of which half of the water volume was renewed daily. To feed the mussels *Rhodomonas salina* was added daily to reach a concentration of 10,000 cells mL^{-1} . *R. salina* was cultured in 1 L bottles at 20°C under constant

illumination, aeration and stirring. The culturing medium was modified from DS/ISO 14669 (DS/ISO 1999) and prepared with FSW. The algal culture was left to grow until a cell density of approximately $1 \cdot 10^6$ cells mL^{-1} was reached. It was then stored at 4°C in the dark until use. Cell densities were determined with a particle counter (Multisizer 3, Beckmann Coulter).

Spawning of the mussels was induced by a sudden increase of 10°C in water temperature. For this, mussels were individually placed in beakers with 200 mL of FSW. The eggs of different females were mixed and the water was filtered with a $100\ \mu\text{m}$ mesh to remove bigger particulate matter. Sperm of 4 different males was added and fertilization took place at ambient temperature. After 20 min eggs were collected with a $10\ \mu\text{m}$ mesh and cultures with densities of 20 eggs mL^{-1} were prepared in glass flasks. Fertilization success was confirmed microscopically by checking for cell division (Olympus CX31, 40x). The cultures were kept at 15°C with gentle aeration and the larvae were fed with *R. salina* from day 5 onwards. Every second day the concentration of *R. salina* in the cultures was adjusted to $150\ \mu\text{g C L}^{-1}$ as determined by the size and density of the algae and their equivalent carbon content (Mullin et al., 1966). After one week the water in the cultures was renewed with fresh FSW.

Microplastic particles

The model micro- and nanoplastic particles used in the present study were fluorescent (Ex: 440 nm, Em: 486 nm) polystyrene (PS) beads with a diameter of 100 nm and $2\ \mu\text{m}$. They were neutrally charged and had no surface functionalization. The particles were purchased from Phosphorex and supplied in a suspension of 1% solids in deionized water with 0.1% Tween20 and 20mM sodium azide. The maximum concentrations of Tween20 and sodium azide in any of our experimental exposure suspensions were 0.00001% and $0.18\ \mu\text{g L}^{-1}$, respectively. These lie far below concentrations at which an effect on the organism can be expected (Cherr et al., 1990; Ostroumov, 2003). The stock suspensions were stored at 4°C in the dark and vortexed immediately before preparing exposure suspensions for the experiments. Particle properties (size distribution and charge) in FSW were determined in the exposure setup used for Experiment 3 (Larval growth test). The results showed a high stability of the $2\ \mu\text{m}$ PS beads in the exposure suspension, while the 100 nm beads agglomerated with time (details in supplementary information).

Experiment 1: Ingestion & egestion test

The aim of this experiment was to quantify the ingestion and egestion of micro- and nanoplastics in blue mussel larvae. The body burden, which was defined as the mass of PS beads per individual at a certain time point, was measured after 4h of exposure to different ratios of food (*R. salina*) and plastic particles, and after a subsequent 16h depuration period in clean FSW. The ratios between food and microplastics were

based on an algal density of 10,000 cells mL⁻¹ of which different percentages (100%, 75% and 50%, volume-based) were replaced by PS beads. Respectively, the tested ratios were 0:1 (only plastic), 1:3, 1:1 and 1:0 (only algae control). This corresponds to concentrations of plastic particles in these treatments of 1.40, 1.05, 0.70 and 0 mg L⁻¹, respectively (Table 1). These ratios were chosen as a mechanistic approach to understand the interaction of blue mussel larvae with plastic particles in dependence on the ratio of plastics and food algae.

The experiment was conducted in Falcon tubes with 50 mL of exposure suspension and 20 larvae mL⁻¹ at 15°C in the dark (n=3). At the beginning of the test, the larvae were 12 days old. After the exposure duration of 4h, half of the treatments were sampled and the other half was transferred to clean FSW for depuration. For the transfer to depuration, larvae were collected on a 90 µm mesh and carefully rinsed to remove any particles from the surface. They were then transferred to Falcon tubes with 50 mL of clean FSW and 10,000 cells mL⁻¹ of *R. salina*. After 16h of depuration the larvae were sampled. The sampling of larvae after exposure and depuration, respectively, was initiated by adding a few drops of 16% paraformaldehyde (PFA) and letting the dead larvae settle. The supernatant was then removed and clean FSW was added as a washing step. Again, the larvae were left to settle and the supernatant was removed. The larvae were then transferred to glass vials and stored at 4°C in the dark until quantification of PS beads.

The body burden of PS beads in the larvae (all larvae of one replicate pooled, i.e. 1000) was quantified by measuring the particle fluorescence. A procedure that has previously been developed for *Daphnia magna* (Rist et al., 2017) was applied. In brief, larval tissues were digested with Proteinase K and the fluorescence intensity of the resulting solution was measured with a fluorescence spectrophotometer (F-7000, Hitachi) (for details see Rist et al., 2017). The particle mass in the larvae was calculated from the fluorescence intensity by using a standard curve.

Table 1: Ratios of algae to plastic used in Experiment 1, with the respective concentrations of algal cells and polystyrene (PS) beads of 2 µm and 100 nm

Algae to plastic ratio	Algal cells (number mL ⁻¹)	PS mass (mg L ⁻¹)	2 µm PS (number mL ⁻¹)	100 nm PS (number mL ⁻¹)
0:1	0	1.40	2.0·10 ⁸	4.4·10 ¹¹
1:3	2500	1.05	1.5·10 ⁸	3.3·10 ¹¹
1:1	5000	0.70	1.0·10 ⁸	2.2·10 ¹¹
1:0	10000	0	0	0

Experiment 2: Prolonged egestion test

A follow-up study of Experiment 1 was performed to investigate how a longer depuration time can influence the body burden of PS beads in blue mussel larvae. The same setup and procedure was used as in Experiment 1 but exposure was only conducted at an algae-to-plastic ratio of 1:1 and the depuration period was prolonged to 48h. During the depuration period the larvae were fed twice with 10,000 cells mL⁻¹ of *R. salina* (once per day). Larvae had the same age as in Experiment 1 and sampling and particle quantification were done as before.

Experiment 3: Larval growth test

We exposed blue mussel larvae to 3 different concentrations of 100 nm or 2 µm PS beads for 2 weeks to examine the potential effect of plastic particles on larval growth and development. The concentrations of PS beads were chosen to simulate high current and possible future concentrations of micro- and nanoplastics in the coastal marine environment. In a Swedish harbor, close to the habitat of the first mussel population used in the present study, an average of 1000 to 2000 and a maximum of 102,000 plastic particles (>80 µm) m⁻³ were found (Noren, 2007). We estimated what particle number this would correspond to if they fragmented to 2 µm and 100 nm particles under the assumption that all reported particles were spherical with a size of 80 µm (for detailed calculation, see supplementary information). On this basis we calculated the respective number of 2 µm and 100 nm spheres that fill the same total volume. The average observed concentration of 1500 particles m⁻³ (= 0.0015 mL⁻¹) by Noren (2007) thus resulted in 96 particles mL⁻¹ of the 2 µm beads and 0.77·10⁶ mL⁻¹ of the 100 nm beads, or a mass of 0.42 µg L⁻¹. This was chosen as the lowest concentration in the test. The intermediate concentration was based on the reported maximum number (~100,000 m⁻³) and corresponded to 6398 mL⁻¹ of the 2 µm beads and 0.51·10⁸ mL⁻¹ of the 100 nm beads, or a mass of 28.2 µg L⁻¹. As the highest concentration we chose 10 times the amount of the intermediate concentration to constitute a future worst case scenario.

The exposure experiment was conducted in 1 L glass bottles filled with 250 mL of exposure suspension (FSW without beads (control) or containing either 2 µm or 100 nm PS beads) and at a larval density of 6 mL⁻¹ (n=4). At the beginning of the experiment mussel larvae were 7 days old. Bottles were kept at 15°C in the dark with gentle aeration and larvae were fed with 10,000 cells mL⁻¹ of *R. salina* every second day. After one week the exposure suspensions were renewed with fresh FSW and new particles. To examine the larval growth and development within the 2 weeks of exposure, samples of larvae were taken on days 4, 6, 8, 11, 13 and 15. Samples of 9 mL each were fixed with 4% PFA and stored at 4°C in the dark until analysis. Additionally, some extra larvae of the same start culture were fixed at the beginning of the experiment (day 0). Ten larvae of each sample were imaged with a microscope (Zeiss Axioskop) with a connected camera

(Leica DFC 320) and the software Image-Pro Plus. The larval size was determined by measuring the shell length parallel to the hinge from which growth rates were calculated. The absence or presence of malformations was recorded based on His et al. (1997).

As an additional experiment we performed a functional response test to investigate if the presence of the 2 μm beads influenced larval feeding at a plastic concentration of 2.8 $\mu\text{g L}^{-1}$ (or 640 beads mL^{-1}). We had 4 different treatment groups: only algae or algae and mussel larvae, both with and without microplastics. Five concentrations of *R. salina* were implemented in triplicates: 1000, 2000, 5000, 10000, and 20000 cells mL^{-1} . Based on larval clearance rates reported in the literature (Sprung, 1984a) the number of larvae per bottle was chosen to reach a reduction of algal cells by 35% at each algal concentration. The respective numbers of larvae per bottle were 66, 81, 101, 153 and 286. *R. salina* suspensions were amended with B1 growth medium (1 mL L^{-1}) to avoid a differential algal growth between treatments with and without larvae due to nutrient excretion from the mussel larvae. The test was performed in 35 mL glass bottles in FSW, which were closed air-free and kept on a rotating plankton wheel (0.4 rpm) for 24h at 15°C and a light/dark cycle of 12:12h. Of the “only algae” group, additional bottles were prepared for every algal concentration at the beginning of the test to measure and confirm the initial number of cells mL^{-1} . After 24h the mussel larvae were filtered out with a 50 μm mesh and fixed with Lugol solution for microscopy. To have the same handling for all groups, the filtration was also done in the treatment groups without larvae. In the filtered suspension the algal concentration was measured with a particle counter (Multisizer 3, Beckmann Coulter) and the growth rate of the algae was calculated according to Frost (1972).

Data analysis

All figures and statistical analyses were made with the free statistical computing software R (version 3.2.5 (2016-04-14)). In Experiment 1, differences between the exposure groups were analyzed with a simple ANOVA (analysis of variance) for each particle size. The normality of residuals was tested with the Shapiro-Wilks-W-Test and homogeneity of variances with the Fligner-Killeen Test. In case of a significant difference ($p < 0.05$) a post hoc test (Tukey's HSD) was conducted. To analyze whether the depuration sample differed significantly from the respective exposure sample, planned contrasts were calculated. This was preferred over an ANOVA with a subsequent post hoc test since it was not meaningful to compare all exposure with all depuration samples. In Experiment 2 the difference between exposure and depuration sample was tested with a Welch's t-test for every particle size. In Experiment 3, linear regression was used to determine larval growth rates and differences between these (i.e. differences between the slopes) were analyzed using ANCOVA (analysis of co-variance). Tukey's HSD was again used as a post hoc test in case of a significant outcome. For the data on malformations it was first tested whether there was a time-

dependence of malformation in the controls using ANOVA. As this was not the case the control values of all sampling days were pooled. The differences between the treatment groups and the pooled control were analyzed with ANOVA and subsequently Tukey's HSD in case of a significant finding ($p < 0.05$).

Results and Discussion

Ingestion of polystyrene beads

The method of quantifying the body burden of PS beads by measuring the fluorescence intensity was successfully applied to blue mussel larvae. We found that the larvae readily ingested plastic particles of both sizes (micro- and nanoplastics) under all studied conditions. The robustness of the quantification method is illustrated by the fact that the particle masses per animal were similar for the same treatments in both experiments (4h ingestion at a ratio of 1:1 in Experiments 1 and 2) (Fig. 1). This was the case even though the experiments were conducted with mussels of different populations. The finding implies that the ingestion behavior of blue mussel larvae of the same age was comparable between populations. However, we also observed a high degree of variation within single treatment groups, especially ingestion at a ratio of 1:1 in Experiment 1, making it more difficult to compare the groups with each other. After a 4h ingestion period in Experiment 1 we found on average 0.8, 0.3, and 0.5 ng per larvae of the 100 nm beads and 4.9, 3.4, and 3.1 ng per larvae of the 2 μm beads at an algae-to-plastic ratio of 0:1, 1:3 and 1:1, respectively. Despite an identical particle mass in the water, the ingested mass of 2 μm beads was at least 6 times higher than of the 100 nm beads. The size of 2 μm lies within the normal prey size spectrum of blue mussel larvae (1 to 9 μm) and it is slightly smaller than the size of maximum retention efficiency (2.5 to 3.5 μm) (Riisgård et al., 1980). It can therefore be expected that the 2 μm particles were actively filtered by the larvae whereas the 100 nm beads were too small for that and they most likely entered the digestive tract passively with the water. In agreement with our results, other studies have observed the presence of nanometer-sized particles in larvae of other marine invertebrate species, such as oysters (Cole and Galloway, 2015) and sea urchins (Della Torre et al., 2014). However, in oyster larvae of similar age (10 days post fertilization) the opposite ingestion behavior was observed: a higher amount of nano-sized (160 nm) than micro-sized (1.84 μm) PS beads was found per larva (Cole and Galloway, 2015).

We found that the particle body burden of the larvae tended to decrease with decreasing plastic particle mass in the water. Interestingly though, the decrease in the larval body burden was not directly proportional to the decrease in PS concentrations. For instance, body burdens substantially decreased from a ratio of 0:1 (only plastic) to 1:3 (25% less plastic) by 62% for the 100 nm and 31% for the 2 μm beads.

However, when comparing the ratio of 0:1 to 1:1 (50% less plastic), body burdens only decreased by 35% for the 100 nm and 36% for the 2 μ m beads (Fig. 1). Due to a relatively high variability within the treatment groups the differences between the ratios of 0:1 and 1:3 or 1:1 were not statistically significant ($p > 0.05$, except for the 100 nm 1:3 group). Blue mussel larvae have the ability to actively select food particles and reject others by the cilia of the velum or by pseudofaeces (Sprung, 1984a). As no substantial decrease of particle ingestion was observed though at a ratio of 1:1, our results suggest that no selection for algae and rejection of PS beads took place when algae were added. It has also been described that inert particles can be sorted in the stomach and passed directly to the intestinal tract (Widdows, 1991), resulting in a shorter gut passage time. However, if that had been the case, a decrease in larval body burdens of at least 50% would have been expected at a ratio of 1:1 in comparison to 0:1 (plastic only). Since the decrease was only 35% and 36% for the 100 nm and 2 μ m beads, respectively, this speaks for a buildup of plastic particles in the organism where particle ingestion is faster than particle egestion. To investigate if a high particle mass in the organism builds up already at low plastic concentrations, it would be interesting to look at higher algae-to-plastic ratios. The fact that the particle mass inside the mussel larvae was not directly related to the plastic mass in the water phase indicates that, with our current knowledge, it is not possible to make extrapolations of particle contamination inside the organisms from analyses of microplastics in the water phase and vice versa. In a study with larvae of the Mediterranean mussel, Capolupo et al. (2018) observed a clear increase of the number of ingested 3 μ m PS beads with increasing particle concentration. Similar to our results though, the ingested amount was not proportional to the particle concentration in the water and for 9 days old larvae it even decreased for the highest concentration. This may be related to the fact that the authors observed preferential ingestion of algal cells over the PS beads (Capolupo et al., 2018).

Fig. 1: The particle mass (ng) of 100 nm (A) and 2 μm (B) polystyrene beads was determined in blue mussel larvae after exposure (grey bars) to different ratios of algal cells and plastic particles (based on volume) and subsequent depuration in clean water (white bars). The side left of the dashed line shows the results of Experiment 1 with 4h exposure and 16h depuration (mussel population from Sweden). The right side shows the results of the second experiment with 4h exposure and 48h depuration (mussel population from Denmark). Data is presented as means ± SD (n=3). The letters show the differences between the exposure groups. The asterisks () indicate a significant difference of the depuration group compared to the respective exposure group ($p < 0.05$).*

Egestion of polystyrene beads

Blue mussel larvae showed egestion of both particle sizes under all tested conditions. The particle mass per larvae after the depuration phase was significantly lower in comparison to the exposure phase (Fig. 1). The only exception was the group exposed to 100 nm beads at a ratio of 1:3, which also showed a comparably low particle body burden after egestion, but the difference to the body burden at the end of the ingestion period was not statistically significant ($p > 0.05$). In this treatment, the ingestion was found to be surprisingly low and the variation in body burdens of animals at the end of the egestion period rather high. We found on average 0.18, 0.1, and 0.12 ng of particles per larvae of the 100 nm beads and 0.53, 0.37, and 1.44 ng particles per larvae of the 2 μm beads at algae to plastic ratios of 1:0, 1:3 and 1:1, respectively (Fig. 1). In comparison to the respective particle mass after ingestion this corresponded to a decrease of 77, 67 and

76% for the 100 nm and 89, 89 and 54% for the 2 μm particles, respectively. The degree of egestion was quite similar for all treatments, which means that this process was independent of particle size and the ratio of algae and plastics. For all treatments it was evident that even though the larvae were able to egest the particles for 16h, they exhibited a residual mass of particles at the end of the egestion period. This shows that the particles have the potential to stay in the organisms for a period of time that exceeds the normal gut passage time. Bivalve larvae usually have a short gut passage time, for instance 6h in oyster larvae (Chaparro et al., 2001; Reinfelder and Fisher, 1994).

To investigate if a complete egestion of particles could be achieved by a prolonged egestion period, Experiment 2 was set up with using the same ingestion period (4h) but with a prolonged (48h) egestion period. For practical reasons, the adult mussels used in this experiment had a different origin than those from Experiment 1. Surprisingly, we found higher particle body burdens after 48h than after 16h in clean water for both particle sizes even though the ingestion was at the same level (Fig. 1). One possible explanation could be that the particles were re-ingested during the prolonged egestion period. However, it remains unclear whether mussel larvae re-ingest excreted particles and the amount found seems higher than what would be expected after a re-ingestion. While the similarities in ingestion behavior could indicate that the origin of the organisms is of minor importance for this type of studies, the egestion data may point in another direction. Possibly the egestion behavior differed between the two populations. A long egestion time has also been reported for larvae of the Mediterranean mussel, for which it took 8 days to reach a complete clearance of 3 μm PS beads (Capolupo et al., 2018). If the observation of a long prevalence of plastic particles in the organisms is consistent and can be confirmed in future studies, this may have serious implications as it increases the likelihood for adverse effects on the organism. Furthermore, bivalve larvae are prey of many marine animals (Cerruti, 1941) and a high residence time of microplastics inside the larvae could increase the transfer of microplastics to higher trophic levels in marine food webs.

Larval growth and development

The growth of the mussel larvae was linear in the control and all treatment groups as has also been described in the literature (Sprung, 1984b). At the start of the experiment, at which the larvae were 7 days old, they had a mean size of 114 μm , which increased to 145-166 μm on average in the different groups after 15 days. We found larval growth rates between 2.0 and 3.3 $\mu\text{m day}^{-1}$ (Fig. 2). The measured sizes fall within the range that is expected for the developmental stage of veliconcha larvae that begins roughly 3 days postfertilization and lasts for 20-22 days during which larval size increases from 92 to 240 μm (Bayne,

1964). The growth of *M. edulis* larvae is strongly dependent on temperature and food concentration in the water (Pechenik et al., 1990; Sprung, 1984b). Pechenik et al. (1990) reported a maximum growth rate of $8.1 \mu\text{m day}^{-1}$ (16°C) at a density of 300,000 cells of *I. galbana* mL^{-1} . While we used *R. salina* for feeding, we obtained similar growth rates to those observed by Pechenik et al. (1990) at algal densities of 10,000 to 30,000 cells mL^{-1} , namely 1.3 to $3.1 \mu\text{m day}^{-1}$. Ventura et al. (2016) used *R. salina* for feeding and found lower growth rates of 1.1 to $2.2 \mu\text{m day}^{-1}$ which can, however, be explained by the lower temperature of 9°C in their study in comparison to 15°C in the present study. We found no significant differences between growth rates in the different treatment groups, with the only exception of the group exposed to a low concentration ($0.42 \mu\text{g L}^{-1}$) of $2 \mu\text{m}$ beads, which exhibited a significantly lower growth rate of $2.0 \mu\text{m day}^{-1}$ in comparison to the other $2 \mu\text{m}$ treatment groups (2.9 – $3.3 \mu\text{m day}^{-1}$). An effect of plastic exposure on larval physiology is expected to be concentration-dependent, where stronger effects are expected at increasing concentrations. Here, the opposite is the case as an effect was only observed at the lowest particle concentration. There was also no gradient in the response from a low to high concentration. Therefore, this cannot be interpreted as a clear effect of the plastic exposure. Based on these observations, it can be summarized that larval growth was not affected by the exposure to 100 nm and $2 \mu\text{m}$ PS beads at either of the three exposure levels ($0.42 \mu\text{g L}^{-1}$, $28.2 \mu\text{g L}^{-1}$, $282 \mu\text{g L}^{-1}$) during 15 days in the chosen exposure system. However, a chronic exposure for an extended duration or in a different system, for instance a flow-through system, might lead to different results.

Fig. 2: Blue mussel larval growth during 15 days of exposure to two sizes of polystyrene beads (100 nm (upper panel) and $2 \mu\text{m}$ (lower panel)) at a low ($0.42 \mu\text{g L}^{-1}$), intermediate ($28.2 \mu\text{g L}^{-1}$) and high ($282 \mu\text{g L}^{-1}$) concentration. Larval size was measured in four replicates six times during the incubation period. Growth rates were determined by linear regression. The initial size at day 0 was determined in the larvae culture used for all treatments and was included for the regression analysis of all groups.

Although growth rates were not affected by the presence of micro- and nanoplastics, we observed different malformations in the blue mussel larvae during the 2 week experiment (Fig. 3). Abnormally developed larvae were observed in all treatments, including the controls, but a higher fraction of larvae with malformations was observed with increasing concentrations of PS beads and exposure time (Fig. 4). In larvae exposed to 100 nm PS beads 40 to 60% were found to be abnormally developed at all particle concentrations at day 6 and from day 11 to 15. In the group exposed to a low concentration the number of animals with malformations on day 15 was not different from that of the control group. The fraction of abnormally developed larvae was significantly higher than in the control on days 6, 11 and 15 (also close to being significant on day 13, $p=0.06$) at a high particle concentration. In the treatment groups exposed to 2 μm PS beads 40 to 60% of all larvae showed signs of abnormal development from day 11 on irrespective of the particle concentration (with the exception of the group exposed to a low particle concentration on day 15). Significant differences to the control were found at a high particle concentration on days 11 and 15 as well as a low concentration on day 11. In general, the variability within treatment groups was rather high, which makes it more difficult to find a significant difference to the control. However, when looking at the individual replicates per group it can be seen that differences were also substantial in groups for which no statistical significant difference was found (Fig. S1 in supplementary information). It has been suggested that the fraction of abnormally developed larvae should not exceed 25% in the control (Balbi et al., 2017), a criterion that is fulfilled for the 100 nm beads control (on average 20% [16,24]_{95%}) but violated for the 2 μm beads control group (on average 27% [21,32]_{95%}). This could be an effect of non-ideal culturing conditions and may overshadow a treatment effect. But still, significant differences to the control were found for several treatment groups, which clearly indicates that the exposure to plastic particles can cause shell malformations in blue mussel larvae.

Fig. 3: Commonly observed larval phenotypes during the 2 weeks exposure to 100 nm and 2 µm polystyrene beads. Normally developed veliconcha larva (A), convex hinge (B), cupped (C) and protruding mantle (D). Photos were taken on day 13 of the experiment.

Fig. 4: Larval malformations during 15 days of exposure to two sizes of polystyrene beads (100 nm (A) and 2 µm (B)) at a low ($0.42 \mu\text{g L}^{-1}$, white bars), intermediate ($28.2 \mu\text{g L}^{-1}$, light grey bars) and high ($282 \mu\text{g L}^{-1}$, dark grey bars) concentration. The number of larvae showing signs of malformation was counted in a subsample of 10 larvae from every replicate ($n=4$). The control groups were pooled and the mean is illustrated as a horizontal line with the dashed lines showing the SD ($n=24$). The asterisks (*) indicate a statistically significant difference between a treatment group and the control ($p<0.05$).

Overall, an effect of particle exposure on normal larval development was more pronounced and observed after shorter exposure time for the 100 nm PS beads in comparison to the 2 µm PS beads. A possible

explanation for this could be the difference in particle numbers between the two bead sizes, which were approximately 8000 times higher for the 100 nm beads at the same mass concentration. Both, mass and number based exposures have frequently been applied in studies with micro- and nanoplastics (e.g. Beiras et al., 2018; Cole and Galloway, 2015). However, for biological effects particle numbers are assumed to be decisive as it is the individual particles that are the entities with which the organisms interact (Connors et al., 2017). Smaller particles may be more hazardous as they are more likely to cross biological barriers and translocate from the point of initial uptake into other parts of the body (da Costa et al., 2016). An increasing number of abnormally developed larvae was also observed in *M. galloprovincialis* after a 48h exposure to amino-modified 50 nm PS beads at concentrations between 10 and 1000 $\mu\text{g L}^{-1}$ with an EC50 value of 142 $\mu\text{g L}^{-1}$ (Balbi et al., 2017). The concentrations are comparable to our intermediate (28.2 $\mu\text{g L}^{-1}$) and high (282 $\mu\text{g L}^{-1}$) concentrations, at which we saw similar effects. At concentrations between 2,500 and 10,000 $\mu\text{g L}^{-1}$ Balbi et al. (2017) observed a delay in development. In our study we did not observe such a delay but the concentrations used by Balbi et al. (2017) also far exceeded our highest exposure concentration. Despite the immediate similarity between results it should be stressed that there were considerable differences between the studies, such as the used particles (size and surface modification), the species, larval age and exposure duration, making the results not directly comparable. In a study with oyster larvae an increase in malformations caused by plain 50 nm PS beads without surface modifications was only observed at very high concentrations of 10 and 25 mg L^{-1} , whereas an EC50 value of 150 $\mu\text{g L}^{-1}$ (36h) was found for the same particles as used by Balbi et al. (2017) (Tallec et al., 2018). Plain 2 μm PS beads had no effect on the development of the oyster larvae. The authors concluded that toxicity is size- and functionalization-dependent, with much stronger effects of positively charged particles. The fact that we observed effects on larval development caused by neutrally charged PS beads at low concentrations might be related to the big difference in exposure duration in our experiment (2 weeks) in comparison to most other exposure studies with micro-/nanoplastics and marine invertebrate larvae that used exposure times of 24 to 48h (e.g. Balbi et al., 2017; Della Torre et al., 2014; Manfra et al., 2017; Tallec et al., 2018). In a study with sea urchin larvae a 5 day exposure to 300 PE microspheres mL^{-1} resulted in decreased survival and a small effect on larval growth (Kaposi et al., 2014). Cole and Galloway (2015) exposed oyster larvae to 1 and 10 μm PS beads for 8 days and found no effects on feeding or growth at concentrations below 100 particles mL^{-1} . This is comparable to our treatment group with 2 μm PS beads at a low concentration (0.42 $\mu\text{g L}^{-1}$, 96 particles mL^{-1}) for which we did not observe an effect until day 8 either. However, on day 11 this group exhibited a significantly higher fraction of abnormally developed larvae, which illustrates again the importance of choosing longer exposure periods.

In the functional feeding response test the presence of larvae surprisingly did not result in a reduction but in an increase of algal growth rates across all concentrations (Fig. S2 in supplementary information). It was confirmed microscopically that the larvae ingested the algae as the red color of *R. salina* was visible in the gut. However, either larval feeding rates were much lower or algal growth rates much higher than expected. A likely mechanism is that nutrients excreted by the larvae caused the enhanced algal growth. Although this remains to be investigated for larval stages, it is known that bivalves can stimulate the growth of algae by releasing nitrogen and phosphorus in a dissolved form or with faeces and pseudofaeces (Cognie and Barillé, 1999; Kautsky and Wallentinus, 1980). Furthermore, zooplankton communities have been shown to stimulate phytoplankton growth (Coello-Camba et al., 2017). A stimulation of algal growth by the larvae is also supported by the fact that the degree to which growth was enhanced depended on the ratio of algae to larvae. While the ratio increased from 530 algal cells per larva at 1000 cells mL⁻¹ to 2448 at 20000 cells mL⁻¹, the growth stimulation decreased. Hence, the addition of nutrients at the beginning of the test was not sufficient and should be increased in future studies to outweigh the nutrient excretion by the larvae. However, more importantly there was no statistically significant difference ($p>0.05$) between treatments with and without 2 µm PS beads at a concentration of 2.8 µg L⁻¹ (Fig. S2 in supplementary information). It can thus be concluded that the presence of microplastics did neither affect the growth of *R. salina* nor the feeding rates of the mussel larvae. This is also in accordance with the observation that larval growth was not affected by the presence of microplastics since it could be expected that a change in feeding behavior ultimately affects growth.

Due to the technical limitations of detecting and identifying plastic particles below 10 µm in the environment, it remains unknown in what quantities the smallest fraction of micro- and nanoplastics is present in the natural habitat of blue mussel larvae. The presence of nanoplastics has however been reported in the North Atlantic Subtropical Gyre (Ter Halle et al., 2017) and the formation of nano-sized particles during plastic fragmentation has been demonstrated under laboratory conditions (Lambert and Wagner, 2016). We chose our concentrations based on high reported values of marine microplastics that we assumed to fragment into a small micrometer and nanometer size range. It remains unclear to what degree our concentrations reflect current conditions in the marine environment. The development of methods to detect and quantify nanoplastics and small microplastics in field samples is therefore urgently needed to evaluate the environmental realism of exposure studies. Most likely, the chosen concentrations still exceed real levels in the environment but they are lower than what has been used in many other studies with mussels (e.g. Avio et al., 2015; Canesi et al., 2015; von Moos et al., 2012; Wegner et al., 2012). Our findings support the conclusion that mussel larvae could be a sensitive organism and life stage for future effect studies with micro- and nanoplastics.

Conclusion

While it is well established that adult blue mussels ingest different types of microplastics, the present study is the first to investigate ingestion and effects of micro- and nanoplastics on *M. edulis* larvae. It was observed that the larvae readily ingested PS beads with sizes of 100 nm and 2 μm within an exposure period of 4h. To quantify the body burden of PS beads the measurement of particle fluorescence provided a robust and sensitive method. Micron-sized beads were ingested to a much higher degree, on a mass basis, than beads in the nanometer size range, which may be attributed to the active filtering of the former. For both sizes, the resulting body burdens followed the trends of the remaining particle masses in the water phase but were not directly comparable. This means that deducting the particle mass in the larvae from a measured concentration in the water and vice versa is most likely not possible. The larvae effectively egested the PS beads, independent of particle size and the ratio of algae to plastics in the water. However, a residual mass of particles was present in the organisms after 48h in clean water. In future studies it would be interesting to prolong the depuration period even more and use a setup that excludes the possibility of re-ingestion of particles. A two weeks exposure to 0.42, 28.2 and 282 $\mu\text{g L}^{-1}$ did not affect larval growth rates but led to an increase of abnormally developed larvae. Based on the assumption that bigger particles detected in the environment fragment to small micro- and nanometer sizes, 0.42 and 28.2 $\mu\text{g L}^{-1}$ can be considered environmentally realistic, while 282 $\mu\text{g L}^{-1}$ presents a possible future scenario. The occurrence of these malformations was more pronounced for the smaller beads, at higher concentrations and after longer exposure times. It remains to be investigated how these developmental malformations may affect the larval dispersion, settling and further development of the mussels.

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