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Alterations in swimming behavior of *Daphnia* exposed to polymer and mineral particles: towards understanding effects of microplastics on planktonic filtrators

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Abstract

Concerns have been raised that microplastics (MP) can impact aquatic organisms by compromising their nutrition. However, little is understood about the mechanisms of the adverse effects in suspension-feeders that routinely ingest particles of various nutritional value, including various mineral particles. We compared effects of non-edible particles (MP and kaolin) mixed with microalgae on the swimming behavior of a planktonic filtrator *Daphnia magna*; incubations with only algae served as controls. The following questions were addressed: (1) Are there differences in swimming movements between the daphnids exposed to MP and those exposed to kaolin, and (2) Whether occurrence of biofilm on the particle surface affects daphnid filtering and jumping movements, and (3) How these effects differ between kaolin and MP. We found that both particle types altered filtration-related movements albeit in opposite way: kaoline decreased the time spent on filtration, whereas MP increased it. Exposure to both kaolin and MP resulted in higher jumping activity. The differences between the particle-specific responses were amplified in the coated particles, indicating that daphnids exposed to MP might spend more energy, and even more when the MP are carrying biofilm. The increased swimming activity may translate into inefficient feeding, changes in energy balance and growth.

Keywords: Microplastics, Swimming behavior, Planktonic filtrators, Daphnia, Time budget

1. Introduction

Microplastics (MPs) have recently been recognized as emerging environmental contaminants. However, the potential impacts of MPs for aquatic animals are not well understood, although they are commonly referred as causing various feeding disturbances. A major methodological issue related to testing MP effects is the inadequacy of standard ecotoxicological methods that have low sensitivity and little relevance for detecting ecologically relevant effects (Ogonowski *et al*. 2016).

In nature, filter- and suspension-feeders are the most likely consumers capable of ingesting MPs. Thus, these animals are an important “entrance” for MP to aquatic food webs. Experimental studies have shown that filter- and suspension-feeders frequently ingest different types of MPs, with concomitant effects on food intake, growth, and reproduction, albeit only at very high MP concentrations. These effects, however, result from the food dilution with nutritiously inert material and do not as such represent a toxic response. Moreover, in such experiments, commercially available plastic beads, so-called virgin MPs, are commonly used. Such particles have no coating with dissolved organic material or microorganisms (biofilms), which may affect interactions between the particle and filtering apparatus. When MPs or any other particle occur in natural plankton assemblages or sediment communities, their surfaces become coated with various organic substances, such as carbohydrates or peptides, and colonized by various microorganisms, such as bacteria, algae, fungi and protists, interacting within complex biofilms. Therefore, it is relevant to understand how filtrators interact with biofilm-covered MPs and whether these interactions are different from those with virgin MPs commonly used in the experiments. Motion analysis is a set of techniques for quantifying movement patterns and behavioral responses to external stimuli. The application of motion analysis in aquatic ecotoxicology addresses effects of abiotic stressors and incorporates data quantifying movements at a range of frequencies, thus providing a holistic analysis of animal motion. This type of analytical approach is applicable to a wide range of species, at various developmental stages. Some types of movements can be particularly informative for a given organism, but it may also be an advantage to incorporate a range of movements into a single analysis (Tills *et al*. 2013). The latter approach is particularly useful for analyzing movements of a filter-feeder exposed to edible and non-edible particles when evaluating whether MPs can be perceived differently from other naturally occurring particles, such as clay, lignin, and cellulose. Demonstrating that MP can affect filtration behavior in other ways than, for example, mineral particles is crucial for predicting potential risks of plastic pollution. We studied swimming behavior of the cladoceran *Daphnia magna*, a common non-selective filter-feeder and a model species in ecology and ecotoxicology. The daphnids were exposed to the natural clay (kaolin) and MPs of similar particle size. Moreover, both particle types were coated with protein to produce an artificial biofilm that was expected to change surface properties and increase resemblance of our experimental particles to weathered particles *in situ*. To characterize surface charge of the test particles, we measured zeta potential. In planktonic filtrators, the particles are only captured by a feeding apparatus when the Reynolds number is low due to the laminar flow and slow fluid movement (Gerritsen and Porter 1982). This implies that particles having different surface charge will have different retention capacity and thus the probability of being ingested (Matz and Jurgens 2001). Therefore, the surface charge should be considered when studying interactions between MPs and filtering processes.

The following questions were addressed:

(1) Are there differences in swimming movements between the daphnids exposed to MPs and those exposed to kaolin;

(2) Is coating with BSA affects zeta potential and daphnid swimming;

(3) Are there differences in coating induced effects between the kaolin and MPs?

2. The experiment

2.1. Test species and its feeding movements

Daphnia magna is a planktonic cladoceran, ecologically important, common, and easily cultured in the laboratory. It is also a standard species in ecological and ecotoxicological testing, including feeding inhibition assays (McWilliam and Baird 2002). Daphnids have two doubly-branched antennae (frequently half the length of the body or more) that are used for swimming and flattened leaf-like limbs inside the carapace (thoracic legs) that produce a current of water which carries food and oxygen to the mouth and gills. Small particles (generally <50 µm) are filtered out by fine setae on the thoracic legs and channeled along a groove at the base of the legs to the mouth. Daphnids feed on suspended particles: phytoplankton, bacteria, and fungi, but also decaying organic material. Although there is some evidence of preferential feeding on certain types of algae, it is generally believed that all particles of suitable size are ingested without any selective mechanism (Dodson *et al*. 1997). Therefore, non-edible particles commonly present in seston are also ingested. When particularly rough material or tangled masses are introduced between the mandibles, they are removed by spines on the first legs and then kicked out of the carapace by the post-abdomen. Alterations in daphnid swimming behavior in response to various stressors has been studied, and different motion patterns have been characterized using electromagnetic signals at different frequencies (Gerhardt *et al*. 2005). Here, we used the same approach to analyze motion response in *D. magna* exposed to food in the presence of non-edible material (MPs and kaolin) and to compare this response between the particle types.

2.2. Culture conditions

We used *Daphnia magna*, test strain *Klon 5* Federal Environment Agency, Berlin, Germany, cultured in M7 medium (artificial lake water; OECD, 2004; OECD, 2008). The animals were kept in groups of ~25 individuals in 2 L containers and fed a mixture of the green algae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* three times a week. For the experiments, adult females with body length 2-3 mm were used.

2.3. Test particles

Kaolin (Sigma-Aldrich; size 4 ± 1 µm, density 2.6 g/cc), a clay mineral, with soft consistency and earthy texture, was used as a source of natural particles that daphnids would encounter even in the most pristine systems. As MP, we used fluorescent green microspheres (Cospheric, Santa Monica CA, USA; 1-5 µm, 1.3 g/cc). Bovine Serum Albumin (BSA) was used for coating of MP and kaolin as described by Taghon (1982). The particles incubated with BSA (2 mg/ml) were stored in the fridge for 48 h to ensure a complete coating. Using Zetasizer Nano Z (Malvern Instruments), zeta potential was measured in the non-coated and coated particles suspended in M7 at room temperature in a folded capillary cell (DTS1060) at a modulator frequency of 1,000 Hz. The cell was rinsed with M7 medium between the samples to minimize contamination.

2.4. Exposure system and treatments

Multispecies Freshwater Biomonitor® (MFB; LimCo International, Germany) was used for movement recording. The MFB measures the activity of test animals by sending high frequency signals from one pair of electrodes to another in a small cage. The cages were closed at both ends with nylon gauze (500 µm) fastened by screw-on rings (Figure 1A). Each cage contained a single *Daphnia* and was immersed in a separate 600-ml aquarium filled with M7 medium (Figure 1B). The exposures were conducted at room temperature and ambient light, from 10-11 am to 10-11 pm. The experimental design was 2 × 2 with particle type (Kaolin vs. MP) and coating (coated vs. non-coated) as treatment factors. The particle concentrations were 1.34×10^5 and 1.45×10^5 for non-coated and coated MP, respectively, and 1.12×10^5 and 1.01×10^5 for non-coated and coated kaolin, respectively. The concentrations of particles and algae were determined by a Spectrex laser optical counter (SPECTREX Inc. USA). Test particles and algae (5×10^5 cells/ml) were added to the aquaria at the start of the incubation. The eight experimental units were run simultaneously; 4 of them served as controls (only algae and daphnids) and 4 as particle tests (MP or kaolin, algae and daphnids). Algae and test particle settling at the bottom of the aquaria were resuspended on a few occasions during the exposure using a pipette. Upon termination of the exposure, the daphnids were retrieved and their body length was measured. No mortalities were recorded.

2.5. MFB readings and endpoints

Daphnia movements caused changes in the amplitude of the electrical signals generated at certain frequencies. The signal is depending on the type of activity and the size of the animal. In our pilot experiments, the MFB recordings were positive only in the range of 0.5-3.5 Hz; therefore, only this range was considered. A quantitative recording of the movements was made automatically for 12 h, starting every 10 min, with a trace of 10 sec. The raw data files containing the MFB records were analyzed according to the signal amplitudes and frequencies by discrete fast Fourier analysis (Gerhardt *et al*. 1998). Based on the set of pilot experiments with various chemical substances known to affect specific motion types (caffeine, diclofenac, CO_2-saturated water) and published studies (Gerhardt *et al*. 2005), we defined the filtering activity as corresponding to the routine low-amplitude movement of the thoracic legs and recorded at 0.5-1.0 Hz. Movements recorded at 2.0-3.5 Hz represented more chaotic and agitated movements related to high-amplitude strokes by antenna as well as discarding of non-edible material by post-abdominal claws and cleaning of the feeding appendages.

2.6. Data analysis and statistics

First, the recorded signals were averaged for 0.5-1.0 Hz (filtering movements) and 2.0-3.5 Hz (jumping movements). Then, the average proportions of time spent on filtering (Filtering activity) and jumping (Jumping activity) over the observation period (12 h) were calculated. The values for Filtering and Jumping activities were Box-Cox transformed to approach normal distribution and divided by the individual body length to remove size-related differences. Then, the resulting values were normalized to the respective values in the controls (*Daphnia* fed only algae and incubated during the same run) and these control-normalized values were used as swimming proxies. For statistical comparisons, we used two-way repeated measures ANOVA (RM-ANOVA) with either *Particle type* and *Coating* (when testing effects on zeta potential) or *Particle type* and S*wimming proxy* (when testing effects on swimming behavior) as fixed factors. Assumption of normality and homogeneity of data were met (Levene test). Individual-based subject matching applied in the ANOVA analysis was significant in all cases, because Filtering and Jumping activities are not independent within a time budget of an individual. Tukey's test was used for pair-wise comparisons. Data are shown as means and standard deviations, α was set at 0.05.

3. Results and discussion

3.1. Effect of coating on zeta potential

Zeta potentials revealed that both test particles had negative net charges; moreover, these values were within the range of values found for algal prey (Rosa *et al*. 2017). In both particle types, coating lead to more neutral zeta potential. This change, however, was significant for MP only as indicated by the significant interaction effect ($F_{1,8}$ = 10.16; p = 0.013; Figure 2A). There were significant differences in zeta potential between the non-coated (q_8 = 8.167; $p < 0.001$) but not between the coated particles (q_8 = 1.792; $p > 0.05$). The coating was expected to result in a less negative charge and increased adhesive and aggregative capacities of both particle types (Taghon 1982, Matz and Jurgens 2001). This was indeed observed in MP, but not in kaoline that was more neutral than MP, which might have been the reason behind the lack of its response to BSA coating. However, given that both particle types with or without coating had weakly negative charge, they were all likely to be readily ingested by the animals. Nevertheless, the significantly more negative charge in virgin MPs implies that studies employing such particles may underestimate particle intake rate considering numerous reports on the relationships between particle retention rate and its surface charge in various filter- and suspension-feeders (Taghon 1982). In *Daphnia magna*, particle capture was also found to be a function of wettability, with hydrophilic particles being retained at a higher proportion than hydrophobic ones (Gerritsen and Porter 1982). Therefore, another parameter that would be of relevance for MP characterization is their wettability (Rosa *et al*. 2017).

3.2. Effect of test particles on swimming behavior

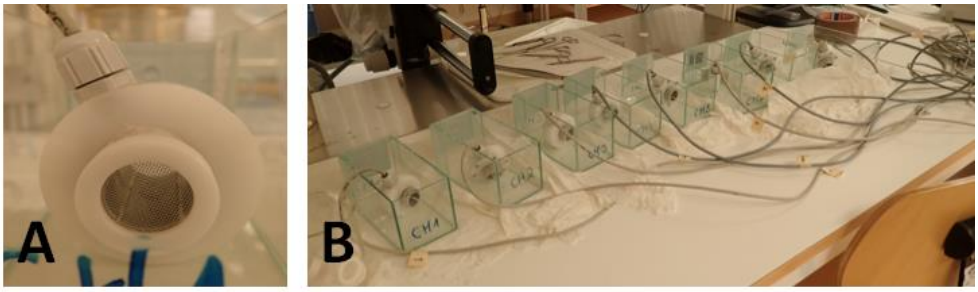

Figure 1. MFB test chamber for a single animal exposure (A), and the set of the experimental aquaria used for simultaneous measurements with 8 channels (B). The measurement principle is based on the animal functioning as resistant in an alternating current between electrodes at opposite walls of the test chamber. Movements of the animal change the conductivity and the electrical field between a second, non-current carrying pair of electrodes and generate specific electrical signals for different kinds of behavior (swimming, locomotion, etc.).

The daphnids exposed to non-coated MP spent 25-35% more time on both Filtering and Jumping activities compared to those exposed to kaolin (Figure 2B, left panels). Although in the non-coated particle treatments none of the swimming proxies were significantly different from the controls, the overall difference between MP and kaolin was significant ($F_{1,6} = 15.50$, $p = 0.008$). In the coated particle treatments, Filtering and Jumping activities responded differently in the two particle types as indicated by a significant interaction effect ($F_{1,6} = 84.11$, $p < 0.0001$; Figure 2B, right panel). In the coated MPs, the Jumping activity increased significantly compared to both coated kaolin ($q_6 = 13.31$, $p < 0.0001$) and the controls (one-sided t-test, $p < 0.05$), whereas the Filtering activity was similar to that in kaolin and controls. Although the Filtering activity in the coated particles was not significantly different from the controls, the daphnids exposed to any test particle coated with BSA spent on average 35% less time filtering compared to those fed algae in the absence of the non-edible particles or with non-coated particles (Figure 2B). These findings suggest that both kaolin and MP may alter swimming behavior yet in opposite way, with a slight decrease of filtration-related movements in kaolin and their increase in MPs. Moreover, the presence of coating resembling biofilm was found to amplify this difference between the particles. This implies that daphnids would spend more time performing the non-feeding movements when swimming in suspension with MPs, and even more when the MPs are carrying biofilm. The increased jumping activity of filtrators exposed to microparticles may translate into changes in energy balance and growth. *In situ*, all particles would have much more developed biofilms compared to the BSA coating applied here. Therefore, it is possible that a stronger biofilm effect on swimming behavior would be expected in weathered MP with heavy biofilm burden. It is necessary to note that very high concentrations of the test particles were used here, such concentrations would correspond to highly turbid waters with a low ratio between the densities of algae and inert particles. At such densities, both natural mineral particles and synthetic polymers are likely to exert negative effects on various filter-feeder species by decreasing feeding efficiency (Boenigk and Novarino 2004). Nevertheless, the differences between responses to kaolin and MPs indicate more severe swimming alterations and potentially higher foraging costs due to >2-fold increase in jumping and corresponding decrease in filtering activity brought about by presently unknown MP-animal interactions. The nature of these effects and the importance of particle physicochemical properties is still unclear and requires further investigation. In previous studies on *Daphnia* movement analysis, swimming speed has been the favorite variable to use, but some other measures of swimming behavior have also been proposed (Dodson *et al*. 1997). Here, we report two useful proxies for daphnid swimming behavior related to food intake and time budget for feeding. Together they provide an efficient way for rapid screening of alterations in feeding behavior that are detectable long before the responses in food intake and growth can be detected. In testing MP effects, separation of the animal's foraging habits and behavior from its environmental setting is artificial, and the results can be misleading. While plastic litter is indeed a source of environmental pollution that should be combated, the immediate danger of microplastics for aquatic animals is often overstated. The effects reported so far are largely related to the effects that would be caused by any inert particles, such as those ubiquitously present in natural waters, and the downstream effects on food intake, growth, and reproduction. Intelligent testing of MP effects should

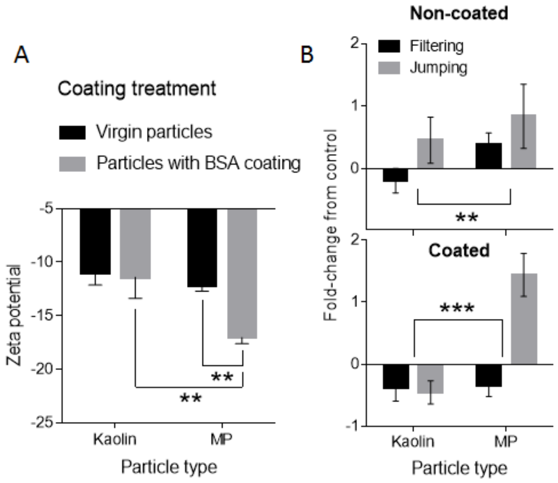

Figure 2. Experimental results for coating effects testing. (A) Effect of BSA coating on the surface charge measured as zeta potential (mean ± SD, n = 5) in kaolin and microplastics (MP). Tukey's test was used for the pair-wise comparisons, the significant effects are indicated with asterisks. (B) Swimming behavior assayed as percent time spent on Filtering and Jumping activities (mean ± SD, n = 4) in *Daphnia magna* exposed to the test particles with and without coating. The activity values show fold-change RM-ANOVA relative to controls (i.e., daphnids exposed to algal food only). Two way RM-ANOVA comparison for *Particle type* (both swimming proxies) is shown with asterisks; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

therefore include methods and techniques that help to understand the mechanisms behind these effects and evaluate whether they actually differ between the MP and naturally occurring suspended solids.

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