Effects of microplastics mixed with natural particles on *Daphnia magna* populations

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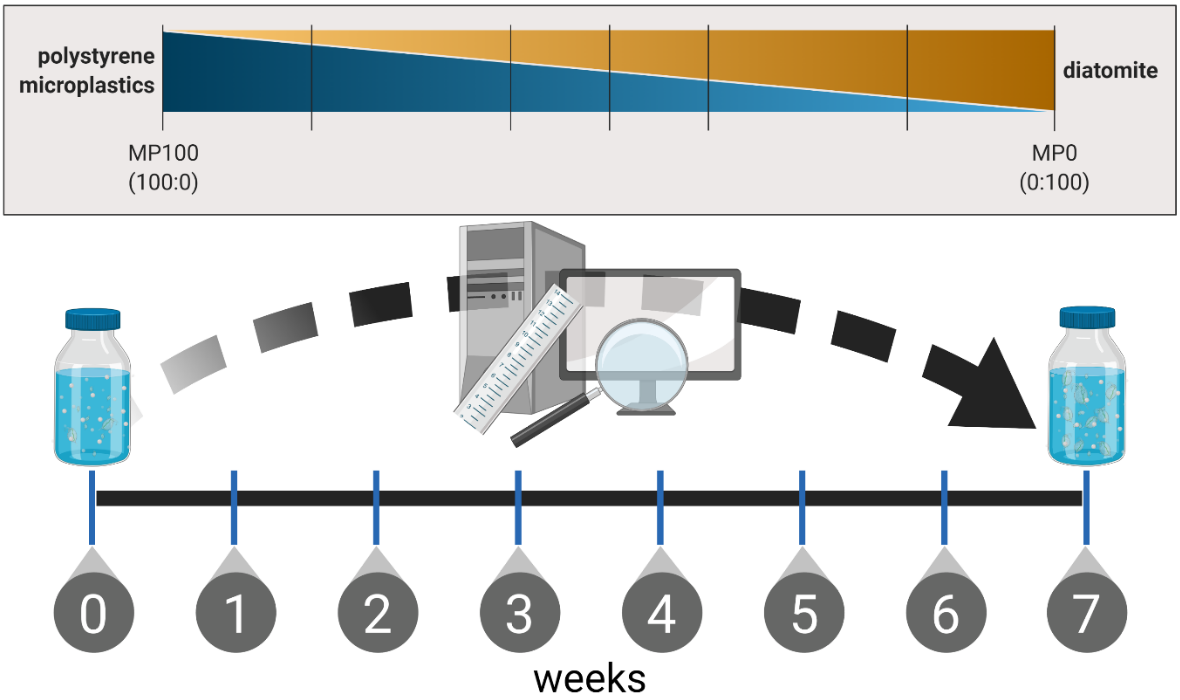
Abstract

The toxicity of microplastics on *Daphnia magna* as key model for freshwater zooplankton is well described. While several studies predict population-level effects based on short-term, individual-level responses, only very few have validated these predictions experimentally. Thus, we exposed *D. magna* populations to irregular polystyrene microplastics and diatomite as natural particle (both ≤63 µm) over 50 days. We used mixtures of both particle types at fixed particle concentrations (50,000 mL^{-1}) and recorded the overall population density, the size of the individual animals, and resting egg production. Particle exposure adversely affected the population density and structure and induced resting egg production. The terminal population size was 31–42% lower in exposed compared to control populations. Interestingly, mixtures containing diatomite induced stronger effects than microplastics alone highlighting that natural particles are not *per se* less toxic than microplastics. Our results demonstrate that an exposure to synthetic and natural particles has negative population-level effects on zooplankton. Understanding the mixture toxicity of microplastics and natural particles is important given that aquatic organisms will experience exposure to both. Just as for chemical pollutants, better knowledge of such joint effects is essential to fully understand the environmental risks of complex particle mixtures.

Environmental Implications

While microplastics are commonly considered hazardous based on individual-level effects, there is a dearth of information on how they affect populations. Since the latter is key for understanding the environmental impacts of microplastics, we investigated how particle exposures affect the population size and structure of *Daphnia magna*. In addition, we used mixtures of microplastics and natural particles because neither occurs alone in nature and joint effects can expected in an environmentally realistic scenario. We show that such mixtures adversely affect daphnid populations and highlight that population-level and mixture-toxicity designs are one important step towards more environmental realism in microplastics research.

Graphical Abstract



Highlights

- *Daphnia* populations exposed to mixtures of microplastics and diatomite
- Effects on population density, structure, and resting egg production
- Diatomite as natural particle was more toxic than microplastics
- Particle mixtures induce negative population-level effects
- Particle mixtures represent more realistic exposure scenario

Keywords

particulate matter, population dynamics, suspended matter

Introduction

Small plastic particles, microplastics, are a ubiquitous pollutant in the aquatic environment. They can interact with and affect a broad range of species across all levels of biological organization, including zooplankton such as the Cladoceran *Daphnia magna*. In the environment, microplastics are only one type of non-food particles organisms interact with and microplastics as well as naturally occurring particles have been shown to negatively affect daphnids, sometimes across generations (Kirk 1991; Robinson, Capper, and Klaine 2010; Ogonowski et al. 2016; Rist, Baun, and Hartmann 2017; Martins and Guilhermino 2018; Schür et al. 2020). Nonetheless, as non-selectively filter-feeding organisms, daphnids are well-adapted to non-food particles. This is achieved through a number of behavioral and physiological mechanisms, including a reduction in feeding rate, regurgitation of boluses, and the ability to remove adhering particles from the filtering setae via the post-abdominal claw (Burns 1968a; 1968b; Kirk 1991; Ogonowski et al. 2016). Since exposure in the environment is never to a singular kind of particle (synthetic or natural) and their effects in comparison to microplastics are often overlooked, authors have argued that exposing animals to particle mixtures is more environmentally relevant (Gerdes et al. 2018; 2019). Additionally, the currently available literature is strongly biased towards acute exposure scenarios, even though, due to their short generation time and the environmental persistence of microplastics, daphnids are exposed continuously over generations and not just intermittently (Rozman and Kalčikova 2021). Thus, a long-term, continuous exposure throughout an individual's lifetime, as well as following generations, is a more realistic scenario (Schür et al. 2020; 2021). Daphnids as r-strategists form large, often short-lived, populations. Population growth rates are high, but quickly reach a carrying capacity limited by space and/or food. Such stressors are then often met with the formation of resting eggs that can resurrect the population once conditions have returned to a more favorable state (Smirnov 2017). In accordance with these considerations, we designed an experiment in which *D. magna* populations with a defined age structure and size were continuously exposed to mixtures of microplastics and the natural particle diatomite at constant particle numbers and constant food levels. The aim of this study was to compare the effects of microplastics to natural particles and their mixtures on the population level in a more realistic scenario.

Materials and Methods

Daphnia culture

Ten *D. magna* individuals were cultured in 1 L of Elendt M4 medium (OECD 2012) at 20 °C with a 16:8 h light:dark cycle. The daphnids were fed with the green algae *Desmodesmus subspicatus* thrice a week at 0.2 mg carbon per individual per day (mgC daphnid^{-1} d^{-1}). The medium was fully renewed once a week.

Particle preparation

The irregularly shaped microplastics were produced from polystyrene coffee-to-go-cup lids as described in Schür et al. (2020). Diatomite was purchased from Sigma Aldrich (CAS: 91053-39-3). Particles were sieved to ≤ 63 µm to achieve particles in a size range that is available for daphnids for ingestion (Scherer et al. 2018). Additional characterization of the material and the two particles types (size distributions, surface charge, electron microscopy images etc.) can be found in Schür et al. (2021) and Scherer et al. (2019). Particle suspensions were prepared in Elendt M4 medium based on measured particle concentrations (Multisizer 3, Beckman Coulter) and used throughout the experiment. A new microplastic stock suspension of was prepared after day 37.

Experimental design

The initial daphnid populations consisted of 3 adults (2 weeks old), 5 juveniles (1 week old), and 8 neonates (< 72 h old) held in 1 L glass vessels containing 900 mL Elendt M4 medium (OECD 2012). Each population was kept for 50 d and fed a constant ration of 0.5 mgC d^{-1} of *D. subspicatus.* All treatment groups were exposed to a total of 50,000 particles mL^{-1} of varying ratios of microplastics and diatomite (n = 3, Table 1).

Populations were fed thrice per week, and the medium was exchanged on days 7, 14, 21, 28, 37, 42, and 50. During each feeding, vessels were covered with a lid and gently inverted to re-suspend the particles. With each medium exchange, populations were sieved, transferred to an hourglass, and photographed. ImageJ (Schneider, Rasband, and Eliceiri 2012) was used to then quantify living animals (Figure 1) and the number of resting eggs (Figure 2) as well as measure body lengths (Figure 3). Resting eggs are seen as indicators of population stress like insufficient food or high population density (Smirnov 2017). Individual body lengths were measured from the center of the eye to the base of the apical spinus (Ogonowski et al. 2016). Body lengths were categorized into three size/age classes in accordance with Agatz et al. (2015). The size classes are neonates (≤ 1400 µm), juveniles (1400–2600 µm), and adults (> 2600 µm).

Table 1: Ratios and absolute nominal particle concentrations of microplastics and diatomite in the treatment groups of the population experiment.

Treatment group	**Microplastics**		**Diatomite**	
	%	Particles mL^{-1}	%	Particles mL^{-1}
Control	0	0	0	0
MP100	100	50,000	0	0
MP80	80	40,000	20	10,000
MP60	60	30,000	40	20,000
MP50	50	25,000	50	25,000
MP40	40	20,000	60	30,000
MP20	20	10,000	80	40,000
MP0	0	0	100	50,000

Statistical analysis

The data was visualized using R (R Core Team 2021) with RStudio 2021.09.2+382 and the tidyverse package (Wickham et al. 2019). The impact of exposure time and treatment on population sizes and structure was analyzed using a Mixed-effects model with Geisser-Greenhouse correction and Dunnett's multiple comparison test against the corresponding control group in GraphPad Prism for Mac 9.3.1. The number of resting eggs on day 50 of the experiment was compared against the control group using a one-way ANOVA with Holm-Šídák's multiple comparisons test in GraphPad Prism for Mac 9.3.1. The body length of individuals in each population was compared using Kruskal-Wallis tests followed by Dunn's multiple comparison tests. Boxplots are created with the geom_boxplot() function of the ggplot2 package (Wickham 2016) in accordance with Mcgill et al. (1978). Significance levels are indicated by asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Overall, the experiment included three main endpoints: absolute population size (i.e., total number of individuals per population at each time point), body lengths of the individuals comprising each population, and the number of resting eggs (*ephippiae*) per population. All populations, both exposed to particles and of the control group, grew rapidly with regards to the number of individuals during the first two weeks, with little variability between the three replicates per treatment group (Figure 1). This is because the available food was sufficient for such small populations coupled with low population densities acting as triggers for rapid population growth. All population sizes peaked at day 14, declined from day 21 onwards, and reached their lowest recorded size on day 50.

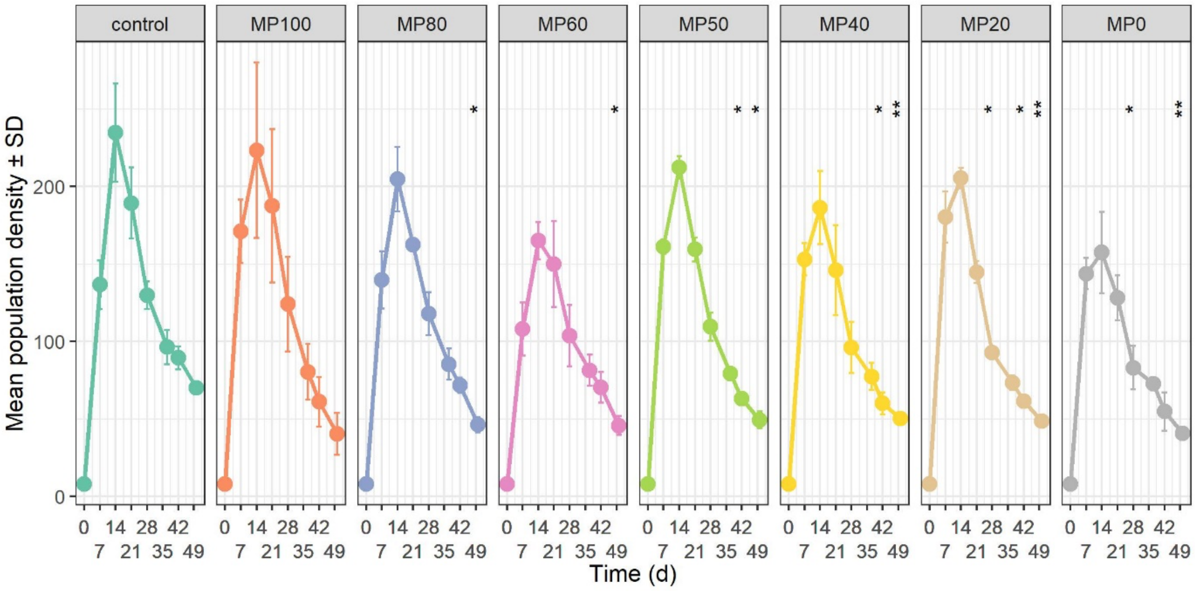


Figure 1: Mean population density of *Daphnia magna* exposed to polystyrene microplastics (MP100), diatomite (MP0), or their mixtures over 50 d. The error bars represent the standard deviation, significant differences compared to control populations are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$.

We observed a concentration-dependent effect in the populations exposed to particles in such that in the phase of rapid decline (days 21–42), daphnid populations exposed to particle mixtures that contained more diatomite had a lower population size (Figure S1). For instance, populations exposed to particle mixtures with 80 and 100% diatomite (MP20, MP0) were significantly smaller than the control populations on day 28 ($p < 0.05$, mixed-effects model). The same was true for populations exposed to particle mixtures with 50, 60, and 80% diatomite on day 42 ($p < 0.05$). Notably, this effect decreased over time and the terminal population density in all treatments was 31–42% lower compared to control. This difference was statistically significant for all treatments except the populations exposed to 100% microplastics (MP100).

Resting egg formation occurred in all populations, including controls, after day 14 (Figure S2) but to varying degrees. Since the production of resting eggs is a stress response (Smirnov 2017), this indicates a rapid onset of stress caused by increasing population densities and/or decreasing food levels. The particle exposure had a significant effect on the total number of resting eggs produced, with the populations in the MP60 ($p = 0.023$), MP40 ($p = 0.003$), MP20 ($p = 0.011$), and MP0 ($p = 0.008$) groups producing circa 100 ephippiae compared to 70 in the control populations. Similar to the population density, this points towards a stronger effect of diatomite compared to microplastics.

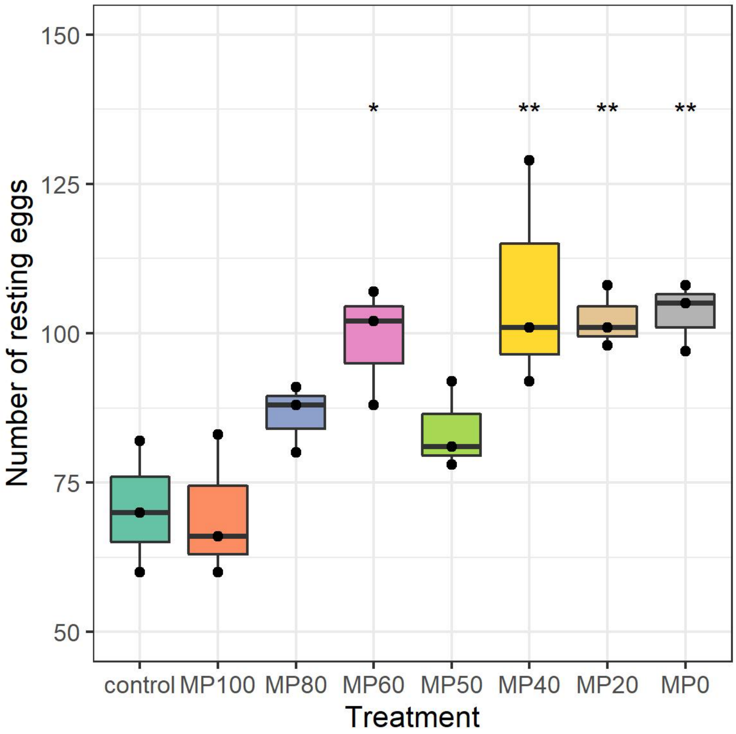
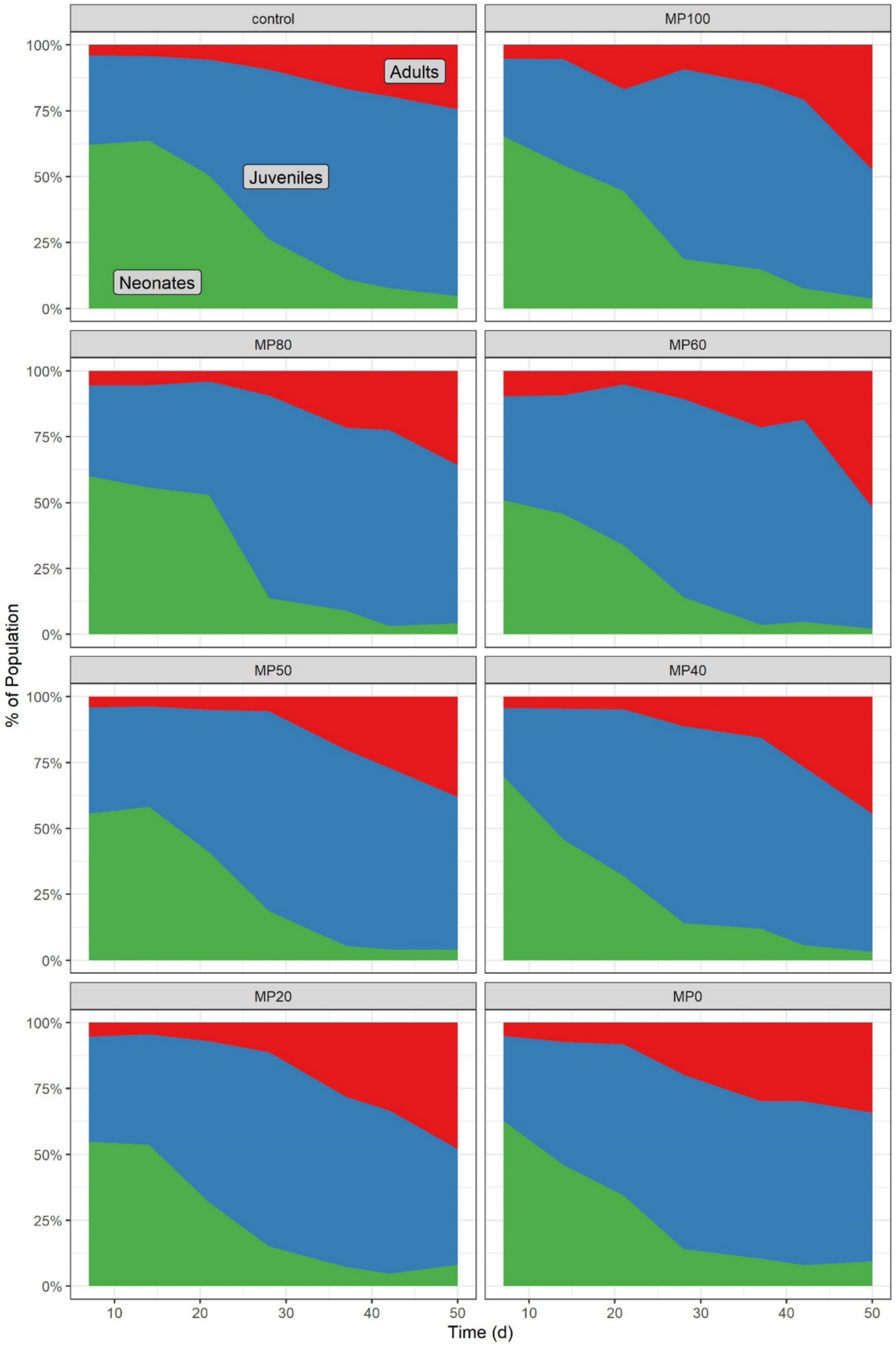


Figure 2: Total number of resting eggs produced by *Daphnia magna* populations exposed to polystyrene microplastics (MP100), diatomite (MP0), or their mixtures over 50 d (n = 3). Significant differences compared to control populations are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$.

We measured the body length of each individual in a population weekly and used this to describe the population structure by categorizing the daphnids into neonates, juveniles, and adults. The initial population growth is largely driven by the production of neonates (Figure 3).

As a result of the lower reproduction from day 14 onwards, the population structure shifts towards juveniles and adults. Overall, particle exposure had no strong effect on population structure, and we did not find significant effects except for populations exposed to the particle mixture containing 60% microplastics (MP60), which had significantly less juveniles and more adults compared to control populations at the end of the experiment ($p < 0.05$, mixed-effects models based on the relative ratios). However, individuals in particle-exposed populations were in many cases significantly larger than in control populations most likely because of the lower reproduction in these treatments (Kruskal-Wallis tests, Table S4).



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185 **Figure 3: Population structure of *Daphnia magna* exposed to polystyrene microplastics**
186 **(MP100), diatomite (MP0), or their mixtures over 50 d.** Data presented as mean relative
187 ratios of neonates (green), juveniles (blue), and adults (red) compared to the overall population
188 density (n = 3).

Discussion

We exposed *D. magna* populations to 50,000 particles mL^{-1} of either polystyrene microplastics, diatomite, or mixtures of both over the course of 50 d. Particle exposure affected the population density and resulted in populations consisting of 31–42% less individuals than control populations at the end of the experiment. This effect on population density is most likely due to particle exposures having a negative impact on reproduction (as had previously been shown by Ogonowski et al. (2016) and Schür et al. (2020)), especially during the phase of rapid population decline (days 14–28). The reproductive toxicity of particles is also reflected in the population structure with particle-exposed populations consisting of larger and, thus, older individuals than control populations. Taken together, this demonstrates that mixtures of synthetic and natural particles have negative effects at the population level in *D. magna*.

The fact that microplastics as well as their mixtures with natural particles affected the terminal population density and structure highlights that the well-documented individual-level toxicity of microplastics and other particles in daphnids translates into impacts at the population level. While multiple studies predict effects of microplastic exposures on population growth rates based on individual level responses (*e.g.*, Martins and Guilhermino (2018); Guilhermino et al. (2021)), to the best of our knowledge, only two other studies have investigated the population level effects of microplastics in daphnids. Bosker et al. (2019) reported that exposure to polystyrene beads caused a significant decline in population size and biomass but did not affect the size of individuals or *ephippiae* production. Besides using another type of microplastics, their general approach was different from ours as they grew populations to holding capacity before starting particle exposure at day 30. This probably reduced the overall stress level induced by continuous particle exposures. Al-Jaibachi et al. (2019) observed the initial decline but subsequent recovery of daphnid populations in MP-treated mesocosms, while no effect on other species was observed. Here, high variability and unknown influencing factors from the mesocosm setup impede the comparison between the two studies. Nonetheless, all three studies demonstrate that microplastic effects also manifest on the population level, which is considered highly relevant for assessing the environmental risks of these particles.

We used multiple mixtures of microplastics and diatomite at a fixed numerical concentration to explore a more realistic exposure scenario (i.e., microplastics as part of a more diverse set of suspended solids) and investigate whether the mixtures' toxicity is driven by plastic or natural particles. Indeed, our results show that diatomite is more toxic to daphnid populations than microplastics. With regards to terminal population density, resting egg production, and population structure, exposure to pure diatomite induced stronger effects than to pure microplastics (Figures 1-3). In the treatments with particle mixtures, we often observed a concentration-dependent response with mixtures containing more diatomite being more toxic. This is particularly obvious for the population density at days 14–28 and the resting egg production. Accordingly, mixtures consisting of more diatomite are more toxic.

The reason for the higher toxicity of diatomite compared to microplastics may be its porous and spiky structure. Diatomite has biocidal properties (European Food Safety Authority (EFSA) (2020)) and its absorptive and abrasive capacities will damage insect cuticles (Korunic 1998) and may injure the digestive system (Scherer et al. 2019). Diatomite has been used as natural reference material in previous microplastics studies. In the freshwater mollusks *Dreissena polymorpha* and *Lymnea stagnalis*, diatomite was in general not more toxic than polystyrene microplastics (Weber, Jeckel, et al. 2021; Weber, von Randow, et al. 2021) but induced a stronger effect on the antioxidant capacity in the former species (Weber, Jeckel, and Wagner 2020) at identical numerical concentrations. In *Chironomus riparius* larvae, diatomite was toxic but less so than polyvinyl chloride microplastics at identical mass-based concentrations (Scherer et al. 2019). Since one of the main mechanisms of its toxicity appears to be the desorption of waxes from the cuticle, arthropods, such as chironomids and daphnids, may be particularly sensitive to diatomite exposures.

Our study shows that some natural particles can be more toxic than a mixture of natural particles and microplastics or microplastics by themselves. Earlier work compared the effects of the natural particle kaolin with polystyrene microplastics similar to those used in this study in a multigenerational study with daphnids (Schür et al. 2020). There, we found that kaolin had no effect, while microplastics affected all recorded endpoints in a concentration-dependent manner with effects increasing over generations. This shows that transferring findings on one particle type to another is not straightforward and microplastics may be more toxic than some but not all natural particles. Particle shape may play an important role in case of diatomite but might be less relevant for other natural particles. Just as for microplastics, the toxicity of natural particles will depend on their individual set of physicochemical properties and cannot be easily generalized without a better mechanistic understanding (see Scherer et al. (2019) for an in-depth discussion).

Finally, our study was not designed to mimic environmental concentrations of microplastic or natural particles. Instead, our aim was to investigate the toxicity of mixtures of both, because this exposure scenario is more realistic compared to the use of only microplastics in toxicity studies. Given that, in nature, aquatic organisms will most likely be exposed to natural and synthetic particulate matter concurrently, a better understanding of the joint toxicity is needed to develop realistic predictions of environmental risks.

Conclusions

Our study demonstrates that an exposure to microplastics and diatomite alone as well as in mixture has negative population level effects in *D. magna*. This corroborates previous predictions based on individual-level responses. Our findings are relevant because adverse impacts on populations of a keystone zooplankton species will have ecological consequences. However, the fact that we used one very high particle concentration calls for follow-up studies to generate concentration-response relationships. We used mixtures of plastic and the natural particle diatomite because we deem this exposure scenario more realistic and found that diatomite is more toxic than microplastics. This contradicts the common assumption that natural particles are benign and highlights that – just as with microplastics – the toxicity of a particle type depends on its individual set of physicochemical properties. This calls into questions whether general comparisons, such as microplastics are more or less toxic than something else, are meaningful. It also highlights the challenge of finding an adequate reference particle when attempting to perform such comparisons. Finally, we believe that investigating the mixture toxicity of synthetic and natural particles is valuable given that aquatic organisms will experience exposure to both. Similar to chemical pollutants, better knowledge of such joint effects is essential to fully understand the environmental risks complex particle mixtures pose to aquatic species.

Author contributions

Christoph Schür: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Project administration, Writing - original draft, Writing - review & editing

Joana Beck: Data curation, Investigation, Writing - review & editing

Scott Lambert: Conceptualization, Methodology, Writing - review & editing

Christian Scherer: Conceptualization, Methodology, Investigation, Writing - review & editing

Jörg Oehlmann: Funding acquisition, Project administration, Resources, Writing - review & editing

Martin Wagner: Conceptualization, Formal analysis, Funding acquisition, Resources, Project administration, Visualization, Resources, Writing - review & editing

Declaration of interest

Martin Wagner is an unremunerated member of the Scientific Advisory Board of the Food Packaging Forum (FPF). He has received travel funding from FPF to attend its annual board meetings and from Hold Norge Rent (Keep Norway Beautiful) to speak at one of their conferences. The other authors declare no conflict of interest.

Acknowledgments

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Supplementary Material

The supplemental data are available ###.

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