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Deliverable 3.1 **Review of experimental practices and informative endpoints in MP studies**

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Work package 3: **Impacts of MP, associated contaminants, and biofilms on Baltic biota**

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Review of experimental practices and informative endpoints in MP studies (D3.1)

This deliverable is a contribution to Task 3.1: *Establishing environmentally realistic exposure settings and preparing MP for experimental exposure*. The focus of this task is on selecting model MPs, exposure scenarios, and endpoints when conducting experimental studies within WP3. The review is based on published and unpublished works addressing primarily testing with benthic and pelagic invertebrates because these are the main test organisms in current ecotoxicological studies with MP as well as in the experiments envisioned in WP3.

The full report is published as a peer-reviewed paper (Ogonowski *et al.*, 2018), with the data openly available in the Supplementary Information. It is also acknowledged that more thorough metadata analysis should be conducted using this dataset (and, perhaps, complementing it with a few newer studies given the rapid publication rate on the subject) to extract more valuable information regarding the important parameters of experimental design, endpoints, and responses. This analysis in planned and will be conducted in the nearest future.

RESEARCH QUESTIONS PERTINENT TO THE REVIEW

Today, microplastic (MP) pollution is perceived as an environmental threat. For a reliable risk assessment of MP, knowledge about interactions with biota is needed. Various biological and ecological impacts of MP have been suggested and experimental studies addressing mechanisms and severity of these impacts are being conducted worldwide. However, the assessment of MPs as environmental pollutants is a new field, with much unsettled methodology (Connors *et al.*, 2017), and extensive testing with standard organisms under reproducible laboratory conditions with well-characterized MP is not yet available. To evaluate the hazard of MP within a risk assessment context, we need to critically evaluate experimental designs that are currently being used and to identify ecologically sound endpoints in our experimental studies.

The feeding as one of the primary targets of MP has been of primary concern mainly because ingestion of larger plastic debris has been observed to cause clogging of appendages and gastrointestinal blockage in relatively large and easily observable animals, such as turtles, fish, and birds (Wright *et al.*, 2013). When discussing MP as environmental hazard, similar effects are generally anticipated in organisms at lower trophic levels (Wright *et al.*, 2013; Galloway *et al.*, 2017). Such parallelism should, however, be handled with caution (Ogonowski *et al.*, 2018).

Microplastics are not the only particles in the water that have a potential to affect biota. Non-edible particles in the microplastic size range, such as mineral (clay, sand, etc.) and organic particles (cellulose, lignin, chitin, amber, etc.), are ubiquitous in the aquatic environments, reaching seston-associated concentrations in the range of g/L, which – by far – exceeds ecologically plausible MP concentrations, at least in the water column (Lenz *et al.*, 2016; Phuong *et al.*, 2016). Similarly, food particles for zooplankton and fish are thousands to million times more abundant than MP (Figueiredo and Vianna, 2018). In the sediments and for common deposit-feeders, inorganic particles represent *the environment*. Moreover, the evolutionary histories of the deposit-, suspension- and filter-feeders, the animals that encounter microplastics at the base of the food webs, both in pelagic and benthic environments, imply the great adaptive capacity to handle mixtures of edible and non-edible particles (Gulati and DeMott, 1997; Ward *et al.*, 2000; Ward and Shumway, 2004). These animals are, therefore, well-equipped to handle exposure to a variety of refractory materials and to sustain high growth and reproductions rates as long as food resources

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are sufficient (Arruda *et al.*, 1983). Deposit feeders generally ingest large quantities of sediment to extract organic components, but also many suspension- and filter-feeders, and, particularly, the non-selective filter-feeders, frequently face turbid environments with high concentrations of refractory matter and non-edible particles, which are generated by terrestrial runoff, currents, and extreme weather-induced bottom sediment resuspension (Gulati and DeMott, 1997). These conditions are well acknowledged as stressful in aquatic ecology (Pelletier *et al.*, 2017) and regulated by water quality standards, although it is also acknowledged that limited data exist concerning biological responses of aquatic animals to suspended sediment at dosages commonly associated with suspended sediment plumes and dredging projects (Pelletier *et al.*, 2017). Similar to the experimental studies with MP, much of the available data come from bioassays that measured acute responses and required high concentrations of suspended sediments to induce the measured response, usually mortality. Therefore, a reliable and meaningful risk assessment requires the identification and understanding of the differences between MP and natural particles in their interactions with biota.

These aspects have been largely ignored in the microplastic research. Furthermore, the current lack of proper particle characterization (Potthoff *et al.*, 2017) and the inappropriate treatment of plastic as a single substance (Andrady, 2017; Horton *et al.*, 2017) have hampered the identification of relevant modes of action, understanding of interactions between MP and biota, and, consequently, the advancement towards a risk assessment. As a consequence, most experimental studies suffer from inadequate exposure conditions and poor understanding of the effect mechanisms.

We summarized recently published studies addressing effects of MP from an ecological perspective and relevance for risk assessment of MP in aquatic environments. In this summary, particular attention was paid to:

- Effects of MP and reference particles using endpoints across different levels of biological organization;
- Crucial aspects of the experimental design, i.e., including relevant control treatments and selection of the appropriate reference particle type(s); and
- Exposure conditions that facilitate ecological relevance of MP effects on feeding and growth; these are the main physiological responses addressed in our experimental studies in WP 3.

METHODS

Data origin. Published data on experiments designed to address concentration-dependent effects across different levels of the biological organization were compiled through online searches in Scopus and Google Scholar using keywords and phrases including *microplastic*, *microbead*, *microparticle*, *nanoparticle*, *clay*, *TSS* (total suspended solids), *storm water*, *polyethylene*, *polystyrene*, *aquatic*, *toxicity*, *LOEC,* and *LC50*. Additional targeted searches were conducted from reference lists of the relevant papers. Because searches often failed to return older literature (pre-1980s), additional hand searches of available literature in the authors' possession were conducted. The studies included in the data set were limited to those presenting adequate background information on the experimental conditions, including presence and type of food provided during the exposure, data on particle-free controls, and the range of the concentrations tested. Also, we included only those studies that reported statistically significant effects ($p < 0.05$) for any of the measured responses. A total of 28 articles published in over the last 50 years (1962-2017; Appendix

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I, Table A1) were evaluated, and the data were extracted as a metadata set suitable for further analysis. For simplicity, the studies used for our review focus exclusively on the physical effects, although the approach used here is fully applicable for analysis of chemical and combined effects.

Variables used for comparisons. LOEC values reported for plastic (synthetic polymers, usually purchased from a commercial supplier) and mineral microparticles, such as sand and clay, but in some cases also uncharacterized natural sediment. Log_{10}-transformed lowest observed effect concentration (LOEC, mg L^{-1}) in various species exposed to a suspension of microplastics or mineral particles; for tests with benthic animals, the particle suspension was simply added to the test system and allowed to settle. The responses were measured at different levels of biological organization (macromolecules, cell, organ, individuals, population, and community) at varying exposure conditions; these levels were defined following a conventional approach in ecotoxicology (Connon *et al.*, 2012). The reported values were plotted and used for statistical tests to compare responses across the levels. As the LOEC concentration, we used the lowest test concentration that resulted in a significantly different deviation of the response (positive or negative) compared to a particle-free control.

Statistics. We transformed the reported (or estimated here) LOEC values with the Box-Cox model and used the transformed values as a dependent variable in the generalized linear models (GLM) with error structure following a normal distribution and a log link as implemented in Statistica 8.0 (StatSoft Inc.). The distribution of the model residuals was evaluated using q-q plots. By GLMs, we evaluated the effects of the biological organization level (*Effect level*) as an ordinal variable (ordered from the lowest [macromolecular] level to the highest [community] level; and *Material* (microplastics vs. mineral particles) as a categorical variable on the LOEC values, including the interaction effect.

RESULTS AND DISCUSSION

Despite the growing number of research studies on the matter, and general acceptance of MP as hazardous waste (Rochman *et al.*, 2013; Jahnke *et al.*, 2017), the experimental reports do not provide convincing evidence on the effects that (1) occur at concentrations comparable to those in nature, (2) can be attributed to the MP exposure *per se* and not the exposure to non-edible particulate material, and (3) have consequences at the ecosystem level. One of the major issue in MP ecotoxicology is that the mechanisms of MP toxicity are currently unknown, and poorly addressed. Microplastics exposure potentially includes a particle effect (physical), a chemical effect, and the combined effect, i.e., particle + chemical effects. However, it is unclear and rarely addressed whether these effects are unique to the MP or related to the mere presence of the particulate material in the water or sediment. The latter has major implications for the regulations and risk assessment of MP in the environment. Therefore, the ecologically relevant impacts of MP pollution remain to be assessed.

Endpoints and the observed effects

Effect studies commonly indicate adverse effects of MP on feeding, reproduction, and metabolism (Eerkes-Medrano *et al.*, 2015; Galloway *et al.*, 2017). Logically, these effects may result from MP intake via the gastrointestinal tract and, accordingly, a decreased intake of the edible particles. The decreased caloric intake is likely the reason for the MP exposure effects observed across biological organization levels. The main question, therefore, is whether these responses are unique to MP or

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can also be observed when organisms are exposed to any chemically inert suspended matter or simply a low caloric intake? The comparison of the reported MP effects and suspended mineral particles in various test organisms and across the different levels of the biological organization suggests that effect concentrations are often comparable (Fig. 1 and Fig. A1, Appendix I). For example, silt and clay, two naturally occurring particles, exert adverse effects on benthic and planktonic filtrators by decreasing the filtering activity and fecundity, which reduces growth and lifespan (Kirk, 1991b, 1991a; Berry *et al.*, 2003, 2016, 2017).

High variability in LOEC values, with no apparent differences between the particle types for suborganismal responses (Table 1, Fig. 1), was observed. Also, the variability of LOEC values was significantly higher fore MP than mineral particles (Table 1; Fig. A2, Appendix I), which could be related to the broader range of the concentrations in the MP expose experiments. Additional uncertainty sources are poor characterization of the sediments used for the experiments as well as variations in size distribution, material and settling of the test particles during the exposure, which implies lower than nominal exposure concentrations for the animals feeding on suspended particles. Notably, the most observations but also the highest variability were found for the individual-level responses, i.e., feeding, somatic growth, and reproduction, i.e., responses related to the active uptake of the particles by the test animals.

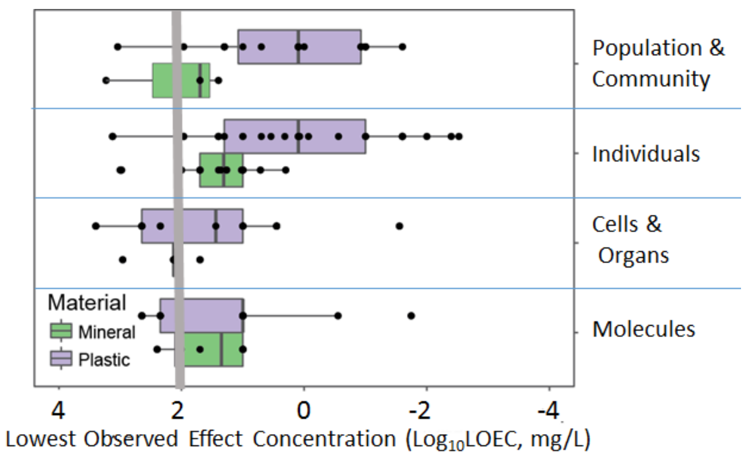

Fig. 1. Effects of particle suspensions (Plastic, including MP, all polymers, vs. Mineral particles, including mixed sediment,and specific minerals such as clay and sand) on various species and endpoints for different organization levels. The primary data are gathered from 28 studies conducted at controlled experiments and published during 1962-2017. The full presentation of the dataset and the analysis are published elsewhere (Ogonowski *et al.* 2018).

The solid vertical line denotes acceptable levels of TSS in stormwater (100 mg L^{-1}).

Table 1. Descriptive statistics for LOEC values (mg L^{-1}) reported for endpoints in studies using mineral and microplastic particles as test substances. Note that tests with low (<10 mg L^{-1}) concentrations of mineral particles would not be meaningful, because these concentrations are very common in surface layers of freshwater and estuarine water bodies; this explains the differences in the test concentration range and thus – at least partly – the difference in the LOEC values.

Material	Median	Min	Max	n
Mineral	50	2.0	1750	33
Plastic	10	0.00025	2500	80

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As appeared upon inspection of the data plot (Fig. 1), the output of the full GLM (Table 2A) indicated that the interaction effect was significant, which means that the differences between the exposure to microplastic and mineral particles for *Material* were not consistent across the *Effect level* values. A follow-up evaluation of the *Material* effect was conducted, by comparing the LOEC values for suborganismal responses (pooled *Macromolecules*- and *Cell*-level data) between the MP and mineral particles (Table 2B; Fig. 2). The same was done for the responses at higher levels (pooled *Individual*- and *Population*-level data). While no significant difference was observed for the suborganismal responses (Table 2C), LOEC values for *Individual* and *Population* responses were significantly higher for mineral particles compared to microplastics. However, to evaluate the validity of this difference in MP effects between the organization levels, both particles types should be compared under the same conditions.

The significantly lower LOEC values reported in the experiments with microplastics for the higher-level responses may be – at least partially - explained by the difference in specific gravity between the microplastics (close to 1 g cm^3) and mineral particles (2-3 g cm^3) leading to both slightly lower LOEC values and faster removal of the sediment particles from the water during the exposure, and hence overestimated LOEC values. Also, microplastics used in such experiments have smaller and more uniform particles compared to the size spectra of natural sediment (Wilber and Clarke, 2011; Lenz *et al.*, 2016). The latter implies that on a particle count basis, the experimental microplastic concentrations would be higher than those of sediment particles. As clearance rate in most non-selective feeders is a function of particle abundance (Best and Thorpe, 1983), the number of particles in a searchable volume is more important than mass-based concentrations. Consequently, controlled experiments are needed to conclude whether microplastics have indeed lower LOEC values and thus higher toxicity compared to the naturally occurring particles.

Table 2. GLM output for the effects of (A) *Material*, *Effect* level, and their interaction on the LOEC values (mg L^{-1}), and *Material* on the LOEC values at (B) lower levels of biological organization (*Macromolecules* and *Cell* levels pooled) and (C) higher levels of biological organization (*Individual* and *Population* levels pooled); the effects were reported for endpoints in studies using mineral and microplastic particles as test substances.

A

Explanatory variable	Estimate	S.E	Wald stat.	p-value
Material (mineral)	-0.17	0.12	2.12	0.15
Effect level	-0.10	0.04	6.61	**0.01**
Material × Effect level	0.10	0.04	6.31	**0.01**

B

Effect level	Estimate	S.E	Wald stat.	p-value
Material (mineral)	-0.03	0.07	0.16	0.70

C

Effect level	Estimate	S.E	Wald stat.	p-value
Material (mineral)	0.23	0.10	5.51	**0.02**

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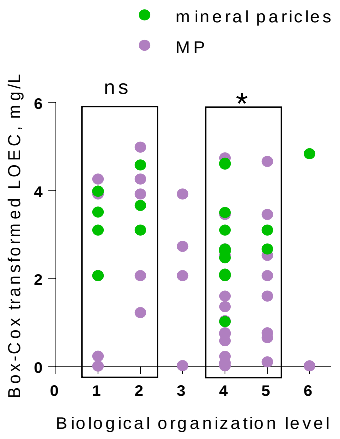

Fig. 2. Box-Cox transformed LOEC values across different levels of biological organization (*X* axis: 1 – Macromolecules, 2 – Cells, 3 – Organ, 4 – Individual, 5 – Population, and 6 – Community). There was no significant effect of material (mineral vs. plastic) at the lower levels of organization (1 and 2, pooled), whereas mineral particles had significantly higher LOEC values compared to plastics at the higher levels of organization (4 and 5, pooled). See Table a1 for the data sources and Table 2 for statistical details.

At the organismal level, the effects of MP exposure are often related to the ingestion of non-palatable material and thus decreased caloric intake (Galloway *et al.*, 2017). The most commonly used endpoint are feeding or clearance rate (>80% of all studies) and somatic or reproductive growth. The addition of MP to the feeding suspension or organic-rich sediment causes dilution of the food source for non-selective feeders (von Moos *et al.*, 2012; Au *et al.*, 2015; Ogonowski *et al.*, 2016) and/or prolonged gut residence time (Au *et al.*, 2015; Ogonowski *et al.*, 2016; Dawson *et al.*, 2018). Another mechanism that has been suggested is adherence of microplastics to feeding appendages in cladoceran and copepods hindering filtration efficiency (Cole *et al.*, 2013; Young *et al.*, 2018) and even causing direct mortality (Jemec *et al.*, 2016). However, similar clogging of filtering devices in filter-feeders can also be caused by increased turbidity or filamentous algal blooms (Palmer and Williams, 1980; Ward and Shumway, 2004; Darchambeau, 2005). Very few reports are available on the effects of textile fibers (Jemec *et al.*, 2016), but the shortcomings of the experimental design and lack of control particles preclude a meaningful evaluation of whether the observed effects of MP are different from, for example, filamentous algae.

At the suborganismal levels, changes in oxidative status accessed by biomarkers (antioxidant enzymes, total oxidative capacity, lipid peroxidation and carbonylated proteins) and gene expression induced by MP exposure were reported (von Moos *et al.*, 2012; Heindler *et al.*, 2017; Jeong *et al.*, 2017). However, it is noteworthy that there is no indication for higher sensitivity of the suborganismal endpoints to particle exposure compared to the higher-level responses (Fig. 1). Moreover, it cannot be ruled out that such biomarker responses reflect the decreased caloric intake rather than direct toxicity of the microplastics *per se*. In most cases, the selection of the biomarkers is poorly justified and the fact that biomarker and gene expression responses can also be related to the variations in the caloric intake (Furuhagen *et al.*, 2014) is often ignored, which makes the interpretation of these responses challenging.

Important shortcomings of the experimental design

Experimental studies on microplastic ecotoxicity have been very diverse. Over 80 different species have been tested ranging from worms, zooplankton, crustaceans, algae, and mussels to fish (Connors *et al.*, 2017); however, only a handful of studies were suitable for the dataset used for this evaluation, resulting in a selection of 23 species over 7 taxonomic categories (Fig. 3). Among the taxa used in the experimental evaluations of MP and mineral particle effects, crustaceans and

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mollusks clearly dominate. Also, most of the invertebrates used as model suspension-feeders, are benthic species.

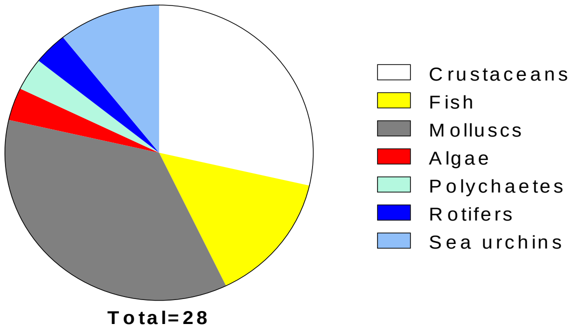

Fig. 3. Representation of taxa used as test organisms in studies included in our dataset (see also Table A1, Appendix I for the full list). Studies employing mixed communities are not shown; thus, only monospecies test systems are included in the diagram.

Recent studies indicate that most MP particles in the sea will sink and eventually end up in the sediments (Van Cauwenberghe *et al.*, 2013), and thus, benthic organisms are most relevant target of MP pollution. However the studies on the occurrence of MP in benthic sediments and organisms are few making realistic exposure concentrations difficult to estimate and there are still no documented links between MP occurrence and negative effects for wild biota (GESAMP, 2016). Moreover, field sampling only provide a “snapshot” of what is in the guts of organisms at a certain time point and therefore studies measuring turnover rates of different MPs in marine biota are needed when aiming to understand actual MP exposure over time and link this to possible hazardous impacts. These kind of studies are currently ongoing within WP3.

The challenges in evaluating particle effects have previously been discussed in the context of nanoparticle toxicity testing (Stone *et al.*, 2010; Handy *et al.*, 2012) and, recently, in the microplastics research (Connors *et al.*, 2017; Ogonowski *et al.*, 2018). Maintaining exposure is more difficult with particulate materials compared to conventional chemicals, particularly for the experiments addressing interactions between animals and MP in suspensions. The following aspects were identified as requiring specific attention when designing experimental studies aiming at quantification of MP effects in biota and reporting the results.

Lack of reference particles and proper controls. In experimental evaluation of biological response to suspended MP, hazard literature for suspended solids could be used to benchmark MP responses and put observations into a broader environmental context. Natural particles, of defined sizes and physicochemical properties, could serve as experimental controls for direct/physical adverse effects. For non-selective deposit feeding organisms that are exposed to MP mainly via ingestion of large quantities of sediment, the influence of additional inert particles from MP may be of less importance, particularly at relevant exposure concentrations (Redondo-Hasselerharm *et al.*, 2018). Thus, the inclusion of control particles may not be as critical, although this cannot be ruled out without testing.

The selection of the reference particles is not a trivial task. As MP overlap with the size categories of many naturally occurring particles including: clay (<2 µm), silt (2–50 µm), and sand (50 µm –2 mm) (Handy *et al.*, 2012), the selection of such reference particles could be based on size. Another option is for all investigators to use a small set of microplastics supplied by commercial vendors that have a narrow range of sizes and reliable certificates of analysis. Such reference materials would add credibility to any adverse effects found by unknown materials.

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We were able to identify only four studies that have used particle controls to estimate the relative toxicity of MP. In these studies, the following types of reference material were used: (1) kaolin clay when testing effects of polyethylene (PE) fragments and plastic spheres in the water flea *Daphnia magna* (Ogonowski *et al.*, 2016); (2) silica when testing effects of polyhydroxybutyrate (PHB), polymethylmethacrylate (PMMA) and polyethyleneimine polystyrene (PS-PEI) in a range of organisms spanning from bacteria to freshwater invertebrates (Casado *et al.*, 2013; Straub *et al.*, 2017), and (3) natural sediment when testing effects of carboxylated (COOH) and aminated (NH_2) polystyrene spheres on the branchial function of the shore crab *Carcinus maenas* (Watts *et al.*, 2014). These studies reported some effects of microplastics that were not observed in the reference treatments, although it must be acknowledged that very high exposure concentrations were applied. Without a clear understanding of the differences between the artifacts due to the abnormally high concentrations of total suspended matter in general and MP in particular, no clear conclusions on the MP hazard level can be drawn.

Environmental relevance of test concentrations. The ultimate goal is to predict the risks of microplastic pollution for natural populations within the context of all other environmental factors. Since the risk is a function of hazard and exposure, we need quantitative measures of both. Currently, much effort is devoted to the quantification of ambient concentrations of MP in the water and sediments. It was soon recognized that quantifying <100-µm plastic particles in environmental matrices is analytically challenging (Hidalgo-Ruz *et al.*, 2012; Huvet *et al.*, 2016; Maes *et al.*, 2017). Moreover, even for >100-µm microplastics, experimental particle concentrations reflecting those reported in the field surveys would in most cases be meaninglessly low. As a result, unrealistically high exposure concentrations are being used in laboratory experiments (Lenz *et al.*, 2016); see also Fig. A1. When the effects of exposure are expected to be weak, the use of exceedingly high concentrations may still be motivated to obtain measurable effects and identify relevant modes of action (Huvet *et al.*, 2016). However, high exposure levels can also lead to manifestations of effects that are transient under natural conditions, i.e., experimental artifacts, which may invalidate the extrapolation of the experimentally observed effects and mode of action to the field situations. For example, physical adherence of MP to appendages and carapaces of crustaceans was reported as one of the exposure effects because this may affect swimming properties and escape behavior (Cole *et al.*, 2013). In *Daphnia*, adverse effects were observed only at extremely low food:MP ratios due to the formation of aggregates that would not be likely to occur *in situ* (Ogonowski *et al.*, 2016). Thus, such effects are likely to result from the high experimental concentrations and low food:MP ratios, and thus may be irrelevant at environmental concentrations of MP and prey.

There is a general opinion that there is an urgent need for laboratory exposure conditions to mimic environmental concentrations (Phuong *et al.*, 2016). It is likely, however, that high concentrations will continue to be used, at least for establishing dose responses, which are the golden standard in ecotoxicology. When conducting such experiments with unrealistically high concentrations of MP, it is particularly important to benchmark measured responses to naturally occurring particles of similar size and shape (Ogonowski *et al.*, 2016). When testing MP effects in the experimental setting with animals that are provided with food during the exposure, it is important to use biologically relevant food:MP ratios (Ogonowski *et al.*, 2016, 2018). For most consumers, this is challenging, because *in situ* microplastic abundance is too diluted compared to the density of prey for particles to be found and ingested by such animals as copepods, fish larvae and chaetognaths (Figueiredo and Vianna, 2018).

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Insufficient information regarding MP concentrations. To compare effect data, reporting particle concentrations in comparable units is crucial. Because of incomplete reporting of property attributes, such as specific gravity and particle size, which should be provided by the MP suppliers, it is often impossible to perform the unit conversion for experimental effects data. It is particularly important for toxicity endpoints that depend on particle encounter rate (e.g., zooplankton feeding). When presenting exposure concentrations, both particle concentrations (abundance) and mass concentrations should be clearly stated (Connors *et al.*, 2017); moreover, providing information for these calculations facilitate quality assurance of the experimental publications.

Insufficient information regarding size, shape and physical properties of MP. It is generally appreciated that particular properties of particles (mobility, surface properties, and bioavailability), including particles made of synthetic polymers, may affect their biofilm burden, behavior, uptake, gut residence time, and, ultimately, effects on consumers and microorganisms in the environments (Stone *et al.*, 2010; Potthoff *et al.*, 2017; Rummel *et al.*, 2017). Similar to nanoparticles (Stone *et al.*, 2010), several properties have been suggested to affect MP toxicity including particle size, shape, surface topography, density, charge, functionality (–COOH, $-NH_2$, $-SO_3$), and polymer identity. It would be highly desirable to understand how these properties contribute to the observed effects to predict risks associated with specific materials and their aging. However, the MP used in most exposure experiments so far represent only a few types and do not include the most relevant MP found in the field that are weathered and colonized by diverse biofilms (Jahnke *et al.*, 2017).

In the reviewed studies, the majority of MP (>80%) were primary spherical microplastics, often from Cospheric or Polysciences, and often (>40%) with added fluorescence markers to facilitate their identification in the environments and biota. It is generally assumed that these markers do not affect the particle effects, but these assumptions are rarely tested (Booth *et al.*, 2016). Recent studies, however, have indicated that staining MP with Nile Red fluorescent dye is a promising method that enables experiments with a wide range of MP with sizes and shapes more relevant to those found in the field, instead of being restricted to commercially available fluorescence-labeled primary MP (Erni-Cassola *et al.*, 2017). Experiments using these methods are presently carried out within WP3.

The most commonly used materials were polystyrene and polyethylene (>90%). Consequently, the assessment of the plastic material effect on the LOEC of MP was not possible, due to the insufficient descriptive data in the reports. Also, very few studies used fragmented plastics (i.e., secondary MP) precluding a systematic evaluation of their effects in comparison with primary MP.

Need for new and biologically relevant endpoints. Experimental endpoints used for detecting particle effects have been equally divergent, including ingestion/egestion, energetics, cellular function, histopathology, behavior, respiration, mortality, growth, and reproduction. Many of these endpoints lack standardized methods, and the statistical power and repeatability of the bioassays are unknown. Therefore, it is challenging to apply these observations to a traditional risk assessment paradigm.

Much more experimental studies are needed to understand relationships between physicochemical properties of the particle surface, their uptake and appendage movements in consumers, and long-term effects of continuous exposures (Karami, 2017). The methodological challenge in the ecotoxicological assays with MP is a search for endpoints that would improve the mechanistic understanding of the effects on food intake and growth observed in the laboratory and the field. For example, the behavior is increasingly reported as a sensitive and early indicator of toxicant stress in aquatic organisms (Chevalier *et al.*, 2015), and various techniques are being developed to

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derive behavioral endpoints that not only sensitive but can also inform us about specific changes in the functioning of a system as a result of exposure. This approach can be used for comparing movements of a filter-feeder exposed to different particle types, and thus when testing whether the test animal perceives MP differently from other naturally occurring particles. Demonstrating that MP can affect locomotion and foraging behavior in other ways than, for example, mineral particles is crucial for predicting potential risks of plastic pollution. The behavioral endpoint are being used in WP3 experiments with both pelagic and benthic test species.

Another relevant endpoints with regard to the uptake of MP via gills in the aquatic animals are various parameters related to respiration and the associated processes, such as ionic composition of the body liquids and osmoregulation (Watts *et al.*, 2014, 2016). Much can be learn from the research on nanoplastics and similar uptake routes caused by these particles (Rist and Hartmann, 2018). At the cellular level, respiration is the conversion of organic molecules and oxygen to energy, driving processes such as basal metabolism, locomotion, and secondary production (e.g. growth and reproduction). Therefore, even low alterations in these processes might affect the energy balance.

Need for standardization of ecotoxicological assessment. Standardized toxicity tests for MP in test organisms and ecologically representative species and systems (including field studies) to understand the ecological impacts of MP are badly needed (Karami, 2017). Whether existing standard laboratory tests and endpoints can be applied to MP toxicity assessments is debatable, particularly for studies addressing bioavailability of MP and their additive chemicals (especially particle translocation and chemical bioaccumulation). Also, how dose-response relationships can be developed for MP to understand the full range of their potential impacts (Koelmans *et al.*, 2017); and how these could be tied into higher-level biological effects, such as using the Adverse Outcome Pathway (AOP) approach (Ankley *et al.*, 2010). A positive example of such approach has been recently published demonstrating how linear dilutions of MP in sediment can be used to derive such dose-response relationships and threshold values that can be used in hazard assessment of MP (Redondo-Hasselerharm *et al.*, 2018). The authors have also suggested that a combination of effect threshold data in species sensitivity distributions may represent a more refined approach as part of a higher tier in the assessment of physical effects of MP. Finally, a comprehensive metaanalysis can be conducted in order to identify both key ecological hazard research gaps and the appropriate methodologies for conducting hazard tests with microplastic materials to fill these research gaps.

CONCLUSIONS

- Microplastics are a minor fraction of the microparticles naturally present in the water and the sediment; both MP and natural mineral particles can induce similar effects in biota, but the comparative studies are too few for the well-grounded conclusions;
- Much experimental data on the effects of various particulate materials exist in the literature on the effects of total suspended solids on aquatic organisms, and these data can be synthesized to evaluate potential effects of MP with specific size distributions in biota.
- Flawed experimental designs preclude diagnostics of MP effects and their mode of action in various organisms. To understand environmental risks of microplastics and to address their specific effects, we need adequate controls in our experimental studies and environmentally

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realistic conditions. Relevant MP particles and reference particles must be used to identify MP-specific effects;

- When designing the exposure experiments, particular attention should be paid to ecologically relevant endpoints and parameters that would facilitate mechanistic understanding of the MP effects across different levels of biological organization, and peculiarities of these effects compared to naturally occurring particulate materials with similar size distributions;
- MP impacts should be assessed based on ecological soundness. Future research needs to focus on understanding the effect mechanisms of microplastic exposure in various biota and selection of sensitive species and relevant environmental settings so that we can identify populations and environments at risk.

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Appendix I.

Table A1. List of the test organisms and bibliographic references that were used to extract the data. The reference number is used as a marker for the data points in Figure A1. Whenever the mass concentration (mg L^{-1}) was missing in the original reference (+), concentration values have been estimated from the linear particle dimensions, numerical concentrations, and specific weight of the polymer. In all the experiments included in this synthesis, particles had been added freely to the test medium at the beginning of the experiment except for the study by Paul-Pont et al. [20], where microplastics were added to the feeding suspension and administered daily. In this particular case, the accumulated concentration at the end of the exposure was used (++).

Reference	Test organism	Material
[1]	*Hyallella azteca*	Plastic
[2]	*Plecoglossus altivelis*	Mineral
[3]	*Arenicola marina*	Plastic
[4]	*Scenedesmus obliquus*	Plastic
[5](+)	*Gammarus fossarum*	Plastic
[6]	*Crassostrea gigas*	Plastic
[7]	*Placopecten magellanicus*	Mineral
[8]	Mixed benthic community	Mineral
[9]	Mixed benthic community	Plastic
[10]	*Brachionus koreanus*	Plastic
[11]	*Tripneustes gratilla*	Plastic
[12]	*Tigriopus japonicus*	Plastic
[13]	*Penaeus japonicus*	Mineral
[14]	*Crassostrea virginica*	Mineral
[15](+)	*Paracentrotus lividus*	Plastic
[16]	*Oncorhynchus mykiss*	Mineral
[17]	*Mercenaria mercenaria*	Mineral
[18](+)	*Daphnia magna*	Mineral
[18](+)	*Daphnia magna*	Plastic
[19]	*Pomatoschistus microps*	Plastic
[20](++)	*Mytilus edulis*	Plastic
[21]	*Evechinus chloroticus*	Mineral
[22]	*Haliotis iris*	Mineral
[23]	*Daphnia hyalina*	Mineral
[23]	*Daphnia magna*	Mineral
[24]	*Crassostrea gigas*	Plastic
[25]	*Mercenaria mercenaria*	Mineral
[26]	*Mytilus edulis*	Plastic
[27](+)	*Carcinus maenas*	Plastic
[28]	*Mytilus edulis*	Plastic

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Figure A1. LOEC values reported for plastic and mineral microparticles. Log_{10}-transformed lowest observed effect concentration (LOEC, mg L^{-1}) in various species exposed to a suspension of microplastics or mineral particles; the values are summarized using 28 experimental studies. The responses were measured at different levels of biological organisation (macromolecules, cell, organ, individuals, population and community) at varying exposure conditions.

The reported values are plotted as a reference number of the study (Table A1). As the LOEC concentration, we used the lowest test concentration that resulted in a significantly different response (in any direction) compared to a particle-free control. Solid vertical lines show median values for each group. Observe that most values, irrespective of the particle type, are below the acceptable daily discharge limit for total suspended solids (TSS; 100 mg L^{-1}) in stormwater shown as the vertical, dashed line (U.S. EPA, 2015).

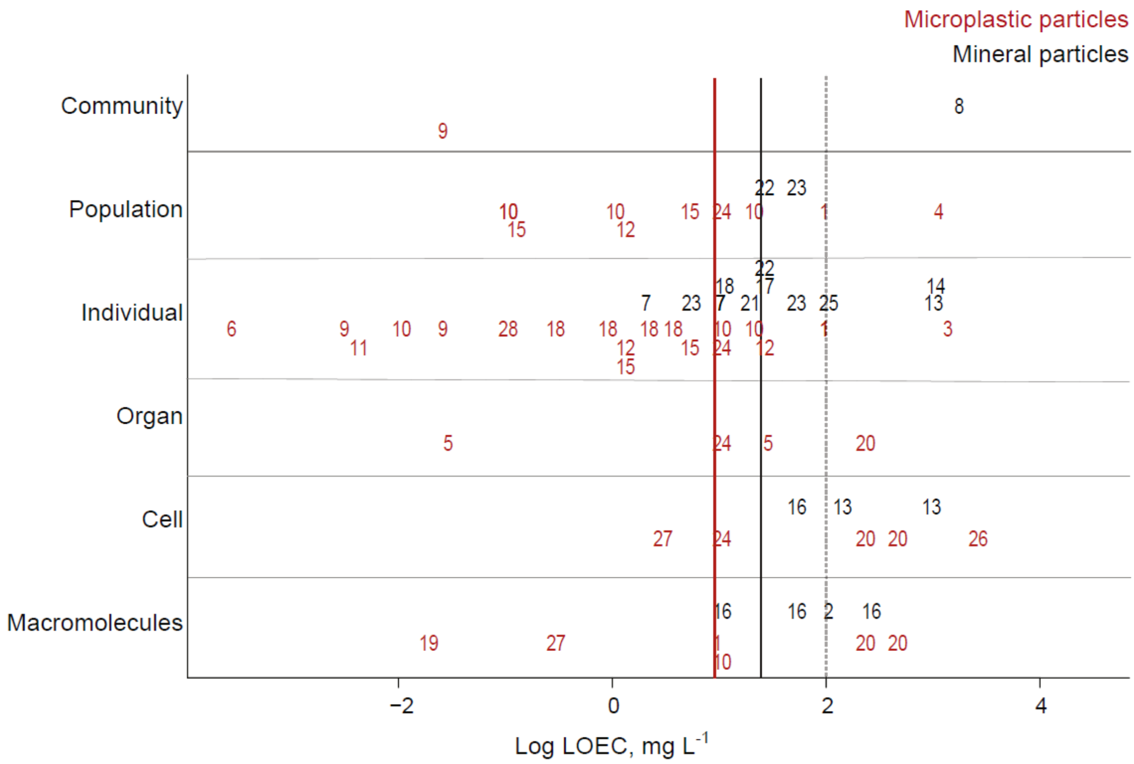

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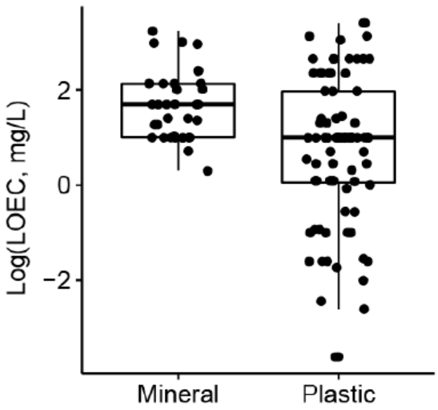
Figure A2. Log_{10}-transformed LOEC-values for mineral and plastic particles, respectively. Horizontal lines show median, boxes — interquartile range (IQR), whiskers — 1.5 × IQR and dots — data points shown in Fig. A1.



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