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Microplastics versus natural mineral particles. How to create and test them while maintaining environmental relevance

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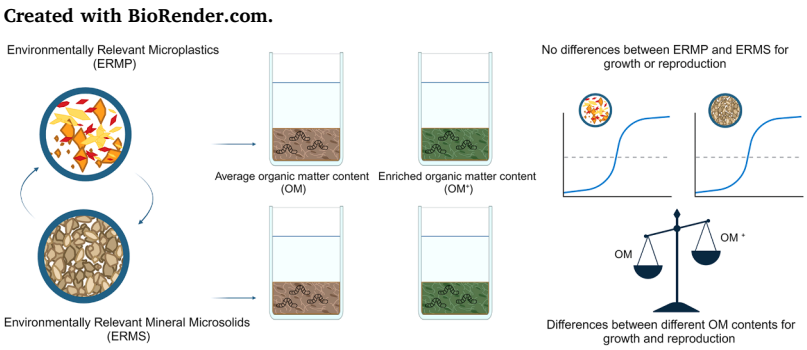
HIGHLIGHTS

- New method to create microplastic mixtures with realistic polydispersity.
- Effects compared to those of an equally diverse mix of mineral particles.
- *L. variegatus* egests more microplastic than mineral particles.
- No difference in growth or reproduction effects between plastic and mineral particles.
- The new method enables realistic testing of plastic vs. mineral particle effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Whether microplastics cause different effects than inert natural particles, and how to create relevant testing materials, are key questions in microplastics research. We prepared Environmentally Relevant Microplastic (ERMP) and Mineral Microparticle (ERMS) mixtures with similar levels of polydispersity and tested their 28-day chronic effects on the reproduction and growth of *L. variegatus* at two different organic matter (OM) contents (average and enriched). Additionally *L. variegatus* was exposed to ERMP and ERMS to study the particle egestion for 14 days. We observed no differences in growth or reproduction between ERMP and ERMS at particle concentrations of up to 10 % (v/v). In contrast, organisms exposed to enriched OM content increased their growth with 30 % and increased reproduction with 20 %. For ERMP with an enriched OM content, reproduction was reduced with an effect threshold EC_{50} of 13.68 ± 5.54 % (v/v). After 14 days of exposure to 5 % ERMP, the egestion of faecal pellets was higher compared to exposure to 5 % ERMS, suggesting that in order to acquire the same amount of nutrition, *L. variegatus* is spending more energy. With this study, we demonstrate that refinements in the manufacturing of environmentally diverse particle mixtures can contribute to a more realistic testing of particle effects.

1. Introduction

Microplastics (MP) are pervasive in aquatic environments and can be ingested by organisms, potentially leading to various negative effects, such as reduced growth rates and energy levels, physiological stress, cell death, developmental abnormalities, altered lipid metabolism, and

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intestinal damage [1-5]. Key factors determining microplastic toxicity include concentration, particle size, shape, exposure duration, and co-factors like species traits and food quality [6,7,4,8,9]. While much is still unknown about the underlying mechanisms, the strongest evidence in invertebrate species points to 'food dilution,' where inert material reduces food assimilation, and to oxidative stress [7,9]. There are however, several research gaps that need to be addressed to better assess the ecological risk of microplastics [6,7].

If food dilution is indeed a dominant effect mechanism, this could be validated by comparing the effects of different types of inert particles. After all, for food dilution, only the collective volume of the ingested particles is relevant. A concrete example of this approach is comparing the effects of microplastics with those of inert natural particles of similar size and shape, such as sand, silt, or clay particles. In microplastic effect testing, a treatment with inert non-polymer particles is often referred to as a 'positive control' treatment. The need to include positive particle controls in effect tests, as well as to investigate differences in effects between material types while keeping particles otherwise as similar as possible, is a frequently mentioned research priority [10-12,8]. However, to date, only a few studies have been conducted comparing the effects of MP with non-polymer test materials [10,13-15]. Furthermore, these studies used monodisperse or only slightly polydisperse particles, making them only of limited environmental relevance.

Therefore, a second research gap is the lack of toxicological effect thresholds measured for microplastics with an environmentally relevant degree of polydispersity (i.e., Environmentally Relevant Micro-Plastic; ERMP). There are mathematical alignment methods to eliminate differences in the degree of mono- or polydispersity between mixtures of microplastic particles [16], but these involve uncertainty due to the uncertainty in the parameters used. By measuring effect thresholds using realistic physical mixtures, these uncertainties are avoided [17]. Measuring effects thresholds in this way is novel and presents a significant challenge in itself, but is essential for drawing accurate ecological conclusions. In order to use ERMP in a test, one should first need to be able to produce it, and for that, it is necessary to know what the relevant properties of ERMP are [18]. As mentioned, for effects related to 'food dilution,' the relevant property is the collective volume of the ingested polydisperse ERMP mixture [16], which should therefore contain all sizes of an environmentally relevant microplastic particle mixture in the correct proportions. The same applies to translocation-based effect mechanisms, such as inflammation and oxidative stress, for which the collective reactive surface of the translocated particles is important [18,19]. The need to produce ERMP to ensure realism in effect tests, together with the aforementioned first research gap regarding positive controls, implies that these positive controls should also be produced with the same degree of polydispersity. After all, the intention is for a particle-positive control to differ only in material type, but not, or as little as possible, in terms of particle size, shape, and the distribution of those properties. We are not aware of studies specifically aimed at keeping toxicologically relevant characteristics such as size and volume approximately equal, and also equal to the polydispersity observed in nature.

Effects of microplastics on benthic invertebrates are preferably studied in as ecologically relevant conditions as possible, using natural sediment. However, it cannot be ruled out that potential effects of ERMP and polydisperse positive controls also depend on habitat factors such as the amount or type of sediment organic material. Recently, we detected a significant difference in the effects of ERMP on *L. variegatus* in sediments with varying organic matter (OM) content [17,20]. While Redondo-Hasselerharm et al. [20] found no effect on the growth or reproduction of *L. variegatus* in sediments with a very high OM content of 32 % [20], de Ruijter et al. [17] reported negative effects on the growth and reproduction of *L. variegatus* (growth: $EC_{50} = 0.77 \pm 0.29$ % d.w.; reproduction factor: $EC_{50} = 2.51 \pm 0.44$ % d.w) when the OM content in the sediment was 4.5 times lower [17]. This suggests that sediment OM content is an important factor influencing the effects of microplastics, which has not been investigated so far.

Based on these connected research gaps, the aim of our study is twofold. First, we aim to compare the effects of two different inert materials, i.e., ERMP and environmentally relevant mineral microsolids (ERMS), on the benthic invertebrate species *L. variegatus*, while ensuring their particle size distribution is as similar as possible at two different levels of sediment organic matter (OM). Second, we aim to provide a novel method and recipe for creating polydisperse mixtures of microplastics or mineral particles (i.e., as 'positive controls') needed for such effect tests, based on a pre-known environmentally realistic particle size distribution. We claim that this methodological innovation represents a significant advancement in the field, enabling more accurate and relevant ecological risk assessments.

To achieve this goal, we utilized the model species *L. variegatus* to compare the effects of ERMP on growth and reproduction with those of a mixture of ERMS. We chose *L. variegatus* because it has proven to be a sensitive species in previous microplastic effect tests [17], and because it is a sediment-dwelling worm that ingests and processes many particles. Following the rigorous QA/QC criteria outlined by de Ruijter et al. [7], we conducted standardized chronic (i.e., 28-day) dose-response tests. The ERMS consisted of a diverse mixture of various clay, silt, and sand fractions with the same polydispersity as the ERMP mixture, and the method designed to prepare both mixtures was considered a secondary objective of the study, as mentioned. This approach allows us to contextualize the effects of ERMP in relation to natural particles. Similarities in — or absence of differences in — the effects between ERMP and ERMS are examined in the context of known effect mechanisms, such as food dilution [16,21]. Given that the implications of an effect mechanism like food dilution are closely related to food availability, we also studied the egestion of faecal pellets over a 14-day exposure period [20,22].

2. Materials & methods

2.1. Preparation of environmentally realistic microplastic particles (ERMP)

Environmentally realistic microplastic particles were designed to closely mimic the properties of microplastics found in the environment including size, shape and polymer distributions [18], and were manufactured as follows. Plastic granulates and flakes consisting of PE, PP, PET, PS and PA were all cryogenically milled under the same conditions, specifically at −50 °C at the industrial grinding company Netzch Lohnmatecknik GmbH (Bobingen, Germany). Our objective was to grind the plastic granulates and flakes into the following size distribution: $D_{10} = 20$ μm, $D_{50} = 80$ μm, $D_{90} = 500$ μm (Table S1), however it is important to note that each polymer type has a unique size distribution (Fig. S1). This variation arises because, by using the same grinding energy for all polymers, relative differences in polymer strengths and resistances are preserved, which also affect the relative size distributions of individual polymers in the environment [18].

The polymer identity was confirmed using ATR-FTIR (see SI, pages S13–20). To verify size and shape distributions, we captured high resolution pictures ($n > 100$ particles) for each polymer type and size class using an Olympus SZX10 stereomicroscope (Figs. S5.1−S5.9). These images were then analyzed with ImageJ to determine for major- and minor axis [23]. In total nine classes of polydisperse particles were identified, with lengths ranging from 3 to 145 μm (PA), 5 to 185 μm (PS), 5 to 229 μm (PET), 9 to 348 μm (PP), 15 to 589 μm (PE_1), 7 to 590 μm (PE_2), 3477 to 5000 μm (PE_{big}), 2858 to 5000 μm (PET_{big}), and 4371 to 5000 μm (PP_{big}) (Table S1). To ensure that the particles remained within the standard maximum size for microplastics, they were sieved twice through a 5 mm sieve. However, it should be noted that a few particles longer than 5 mm were found, likely having passed the sieve perpendicular to their longitudinal axis. Nevertheless, based on particle number this represented a negligible amount within the microplastic

mixture. The density of each polymer type, as well as the final mixture was measured using a gas pycnometer (Ultrapyc1200, Quantachrome Instruments) for powders and porous materials. The polymers PA, PS, PE, PP and PET showed densities of 1.16 ± 0.001, 1.10 ± 0.001, 0.97 ± 0.002, 0.93 ± 0.001 and 1.30 ± 0.015 g cm^{-3} (n = 3) (Table S1), respectively. The proportions at which the different polydisperse polymer powders had to be mixed to obtain an ERMP mixture were based on an a priori *in silico* design, which is detailed in the next section. Finally the ERMP mixture had a density of 1.00 ± 0.006 g cm^{-3} (n = 3).

2.2. Designing environmentally realistic microplastic particles (ERMP) from polydisperse polymer particles with a limited size range

Because we know the particle size and polydispersity (the slope of the power law) for each particle class, we can simulate the properties of any mixture made from these 9 classes *in silico*. This allows us to optimize how the classes should be blended to closely match the power law exponent measured for ERMP in the environment. To create a size distribution similar to that found in the freshwater environment [18], we calculated the required proportions of the nine polydisperse polymer powders a priori using Eq. 1, taking into account the upper (UL; μm) and lower size limit (LL; μm) of each size class and a mean power law exponent parameter of 3.25 ± 0.19 (α) [18].

$$A = \int_{LL}^{UL} C \quad x^{-\alpha} dx = \frac{UL^{1-\alpha} - LL^{1-\alpha}}{1-\alpha} \tag{1}$$

To obtain an accurate particle size distribution based on ImageJ analysis of microscope pictures, multiple pictures were combined until a number of > 100 particles was obtained (Figs. S5.1–S5.9). For each polymer type, we determined the actual lower limit (LL) and upper limit (UL) through image analysis (Table 1, column 'LL', 'UL'). In some cases, a size class had varying sizes and multiple magnifications were required to capture all the particles within the sample. The higher magnifications used to observe the smaller particles accurately can be considered as subsamples and were therefore rescaled based on the difference in magnification factor. Subsequently, for each size class the amount of particles per dataset was calculated (Table 1, column 'Observed ImageJ Count'). Using the major axis diameter (length) (φ_l) and the minor axis diameter (width) (φ_s) and the polymer density (ρ), we calculated the mass per unique dataset (Table 1, column 'observed mass') using Eq. (2) [24].

$$M_{Simon} = \frac{4}{3} \left(\frac{\varphi_l}{2}\right)\left(\frac{\varphi_s}{2}\right)\left(\frac{0.67\varphi_s}{2}\right)\pi\rho \tag{2}$$

We targeted an average number of particles in each size fraction for the LL and UL values using Eq. 1, where α = 3.25 represents the power-law slope of the targeted particle size distribution. Based on these values, a relative targeted number concentration (%) was calculated (Table 1, column 'targeted particles (%)'). Since the observed and targeted relative fractions vary for each polymer, a multiplication factor (MF) was computed (MF = targeted % /observed %). For example, for PA, the targeted percentage was 58.4, while the observed percentage was 17 %, resulting in an MF of 58.4 / 17 = 3.51. By applying these multiplication factors to the observed weights per polymer fraction, we derived a corrected weight for each polymer fraction (column 'Required mass'). These weights are low and the weight of a mixture made from these polymer fractions is too low for experimental use. However, the calculated weight fractions for each polymer (Table 1, under the column 'Recipe mass %') can be applied to achieve any desired total weight for the final mixture. To verify the mean power law exponent parameter for the final mixture, the required proportions were adjusted *in silico* by multiplying the datasets accordingly. Subsequently, power law distributions for the separate polymers and the final mixtures were fitted using maximum likelihood estimation [25,26] and n = 100 bootstraps as previously designed by Kooi et al. [18]. All calculations were performed using the R package poweRlaw [27].

Finally, based on the design, we created in the laboratory the physical ERMP mix with varying polymer type, size, and shape, in mass proportions corresponding to those occurring in the freshwater sediment environment (PE > PET > PP > PS > PA), and a natural polydispersity characterized by an average exponent parameter of 3.57 ± 0.10 (Figs. S1 and S2) [28]. Our research questions concern the particle effects of microplastics, rather than particle-associated chemicals. Therefore, to eliminate any additives present in the plastic, the microplastics were washed with methanol three times and mixed on a shaker table for at least two hours per wash, with a final overnight wash [7,21].

2.3. Preparation of environmentally relevant mineral microsolids (ERMS)

Similar to the environmentally relevant polydisperse microplastic mixture, a mixture of inert non-polymer particles was designed and created from nine classes of mineral particles (clay, fine sand, coarse sand, very coarse sand and fine gravel) (Table S2). High resolution pictures (n > 100 particles) were taken per size class with an Olympus SZX10 stereomicroscope (Figs. S5.10–S5.18). The particle properties of these nine classes were determined using ImageJ and exhibited unique, yet slightly overlapping size distributions (Fig. S4). Kaolin particles ranging from 5 to 39 μm were purchased from Sigma Aldrich. Very fine sand to coarse sand with grain sizes ranging from 14 to 413 μm (Zilverzand), 16 to 533 μm (Ophoogzand), 16 to 353 μm (Speelzand), and 42 to 787 μm (Brekerzand), were collected from Karwei construction shop (Wageningen, the Netherlands). Very coarse sand particles ranging from 86 to 907 μm (Rayher Hobby) and 22 to 876 μm (MICA Decoration) were ordered from Shop partners b.v. Finally, very fine gravel with particles ranging from 12 to 4047 μm (Nurzur Dekoration) and 50 to 5071 μm (Eurosand) were ordered from hobby shop Boetiek Chloë and Dutch Quality Products respectively (Table S2).

The mineral-based ERMS mixture was made to exhibit a similar level of polydispersity as the ERMP and followed the same design procedure. A mean exponent parameter of 2.37 ± 0.03 was obtained (Figs. S3 and S4). Although no additives or sorbed chemicals were expected for these

Table 1
Design table: How to design a polydisperse mixture with environmentally relevant microplastics.

Polymer	LL (μm)	UL (μm)	Targeted alpha	Avg number	Targeted particles (%)	Observed mass (g)	Observed ImageJ count	Observed particle (%)	Required factor	Required mass (g)	Recipe mass (%)
PA	2.8	145	3.25	0.0428	58.4	8.34E−05	1638	17	3.51	2.93E−04	11.34
PS	5.2	185	3.25	0.0107	14.7	2.53E−04	1763	18	0.82	2.07E−04	8.02
PET	5.5	229	3.25	0.0097	13.2	9.55E−04	3374	34	0.39	3.68E−04	14.23
PE	6.8	590	3.25	0.0060	8.2	1.14E−03	899	9	0.90	1.02E−03	39.46
PP	9.2	348	3.25	0.0030	4.1	3.26E−04	976	10	0.41	1.34E−04	5.20
PE	14.7	589	3.25	0.0011	1.4	1.53E−03	818	8	0.17	2.65E−04	10.26
PET_big	1749	5000	3.25	0.0000	2.8E−05	1.48E+ 01	164	2	0.00	2.47E−04	9.56
PE_big	3843	5000	3.25	0.0000	2.3E−06	1.39E+ 01	112	1	0.00	2.86E−05	1.11
PP_big	3881	5000	3.25	0.0000	2.2E−06	1.03E+ 01	105	1	0.00	2.15E−05	0.83

mineral particles, they underwent the same methanol washing treatment as the ERMP mixture [7,21]. The density of the sand and clay as well as the final mixture was measured using a gas pycnometer (Ultrapyc1200, Quantachrome Instruments) for powders and porous materials. The particles from Eurosand, MICAdecoration, Rayherhobby, Nurzurdekoration, Brekerzand, Speelzand, Ophoogzand. Zilverzand and Kaolin had densities of 2.69 ± 0.004, 2.68 ± 0.004, 2.64 ± 0.003, 2.86 ± 0.003, 2.64 ± 0.006, 2.64 ± 0.003, 2.64 ± 0.015, 2.64 ± 0.012 and 2.71 ± 0.055 g cm^{-3} (n = 3), respectively (Table S2). Finally the ERMS mixture has a density of 2.68 ± 0.004 g cm^{-3} (n = 3).

2.4. Sediment

Clean freshwater sediments were collected from the experimental field station of Wageningen University (the Sinderhoeve, Renkum, The Netherlands). Subsequently, sediments were sieved through a 2 mm sieve and stored at −20 °C in order to preserve OM and kill any organisms present. The sediment had an OM content of 4.21 % ± 0.03 (n = 5) [17], which can be considered as a typical, average OM content for freshwater sediments. Background microplastic concentration was assessed (see SI Text) and calculated to be less than 0.0072 % (w:w), which was considered negligible.

2.5. Test organisms and test design

L. variegatus were cultured at the Aquatic Ecology and Water quality Management Department, Wageningen University and Research (Wageningen, The Netherlands), in Dutch Standard Water (DSW) at 20 ± 1 °C. They were fed twice a week with organic nettle powder. Additionally unbleached kitchen paper was added as a living substrate. DSW was renewed weekly.

The ERMP and the ERMS mixtures (see Section 2.3) were each mixed into the sediment in order to obtain six doses: 0 %, 0.3 %, 1.0 %, 2.5 %, 5.0 % and 10.0 % (v/v). These doses were selected based on previously measured threshold effect concentrations for growth and reproduction of *L. variegatus* (growth: EC_{50} = 0.77 ± 0.29 % d.w.; reproduction factor: EC_{50} = 2.51 ± 0.44 % d.w. de Ruijter et al., [17] and are also consistent with environmentally relevant concentrations, which have been measured at levels up to 3.6 % in natural sediments [29]. For a more detailed motivation the reader is referred to our earlier publication [17]. Exposure to ERMP and ERMS was standardized by applying doses based on particle volume. This equalizes the number of encounters between organisms and the particles, as well as the bioavailable fractions for particle ingestion and food dilution. However, equalizing by volume does result in a difference in dose based on the weight of the particles. In terms of weight, ERMS doses were 2.68 times higher than those for ERMP. Weight percentages were: 0 %, 0.26 %, 0.88 %, 2.21 %, 4.43 % and 8.91 % (wt). Particle number concentrations were: 0, 3.49 × 10^6, 1.18 × 10^7, 2.97 × 10^7, 5.94 × 10^7 and 1.20 × 10^8 microplastics kg^{-1}. Experimental units were prepared in triplicate. For the systems with enriched OM, 1.0 g of organic nettle powder was added to the sediment. Subsequently, the systems were manually homogenized using a stainless steel spoon (Fig. S8, S9). Afterwards, DSW was gently added at a 4:1 water-to-sediment ratio. The systems were randomized and left to acclimatize for two weeks before adding the organisms. In each experimental unit, 20 organisms were added. The exposure duration was 28 days, and the temperature was maintained at a constant 20 ± 1 °C. Dissolved oxygen, pH, temperature, conductivity, salinity and NH_3 concentrations were measured twice a week. DSW was periodically refreshed, and air supply was checked daily. During the exposure period, the test systems were covered with aluminum foil to avoid contamination from the laboratory environment. After 28 days, the organisms were sieved, counted and then dried for 96 h at 37 °C before being weighed per replica. For ERMP treatments, exposure was verified by analyzing whole body tissue and egestion samples for microplastic contents using micro-FTIR, which is reported elsewhere [17]. In a parallel experiment, with a similar approach as the 28 day test, the egestion rate of *L. variegatus* was measured following [20] and [22], at concentrations of 0 %, 5 % and 10 % (v/v) of either ERMP or ERMS in triplicate for 14 days. Faecal pellets were collected from the sediment surface using a pipette every two days and stored in aluminum foil cups. Subsequently, the pellets were dried at 37 ± 0.5 °C for 48 h and weighed with a Cubis Micro balance (Goettingen, Germany).

Test design, materials, handling of materials, control of background contamination and exposure conditions adhered to the QA/QC criteria as defined and previously described by de Ruijter et al. [7] and de Ruijter et al. [17]. A summary of how these criteria were met is provided as Supporting information (Table S3).

2.6. Data analysis

All statistical analyses and graphs were conducted in R [30], using the drc package for dose-response curves and effect concentrations (EC_x) R [31]. Continuous reproduction and growth data were fitted to 2 to 4 parameter log-logistic and Weibull models, with the best model selected using the Akaike Information Criterion (AIC) and visual inspection. Model assumptions were checked via Q-Q and residual plots. Dose-effect relationships were tested using the likelihood ratio test, and EC_{10} and EC_{50} values with 95 % confidence intervals were calculated using the ED () function.

The effects of material type (ERMP or ERMS), OM content, and exposure concentration on reproduction and growth were analyzed with a three-way ANCOVA. Additionally, a repeated three-way ANOVA tested the effect of material type, concentration (0 %, 5 %, 10 %), and day. Interactions were explored using ANOVA, AIC, and interaction plots, with model assumptions verified through diagnostic plots and tests (Shapiro-Wilk and Levene's). Residuals of reproduction and growth data were normally distributed, while egestion data required log transformation. Tukey's method was used for multiple comparisons between treatments and concentrations.

3. Results & discussion

3.1. Effects of microplastics and mineral particles on reproduction

We exposed worms for 28 days to increasing doses of particles, mixed in the sediment. Water quality measurements temperature, pH and dissolved oxygen were consistent through time and showed no apparent differences between treatments (Table S4). Conductivity 884 ± 68.41 µS cm^{-1} was higher than recommended, however no differences between treatments were detected (Table S4) [32]. The average number of living worms in the controls increased with a factor of 2.5 ± 0.10, 1.98 ± 0.32, 2.08 ± 0.31, 1.8 ± 0.48 compared to the start of the test for the treatments ERMP enriched OM content, ERMP average OM content, ERMS enriched OM content and ERMS average OM content, respectively. This indicates that all exposures adhered to OECD guidelines regarding control reproduction, and test conditions were adequate [32].

Our primary objective was to compare the effects of ERMP with an inert non-polymer material exhibiting a similar level of polydispersity. In a direct comparison of the effects of ERMP on reproduction with those obtained for ERMS, no statistically significant differences were observed (three-way ANCOVA, p = 0.174) (Table S6). This implies that neither material is significantly more toxic than the other, even at the highest tested dose of 10 %. While this lack of differences does not confirm the existence of specific effect mechanisms such as food dilution (since no effects were observed), it does reduce the likelihood of material-specific mechanisms. In contrast, a non-specific mechanism like food dilution relies solely on the volume ingested. Thus, it is possible that the ingested volumes for both material types were limited enough to prevent food dilution from occurring. In this context, the results are not inconsistent with a food dilution mechanism. Similarly, previous studies by Silva et al. [33] and Redondo-Hasselerharm et al. [20] also did not find any

reproductive effects on *L. variegatus* after a 28-day exposure.

Despite these findings, our dose-response model extrapolated a statistically significant effect threshold above the highest tested dose (EC_{50} = 13.68 ± 5.54 % (v/v); $1.64 \times 10^8 \pm 6.59 \times 10^7$ microplastics kg^{-1}) for ERMP with enriched OM treatment (Table S5; Fig. 1). However, because the EC_{50} value exceeds the highest tested concentration of 10 % (v/v), this raises concerns about the reliability of the threshold, which therefore must be interpreted with caution. Nevertheless, this EC_{50} is close to the highest 'hotspot' concentrations reported for Liangfeng River sediments in China with 2.21×10^8 number kg^{-1} of dw. For the other treatments, including ERMP with average OM content, ERMS with enriched OM content, and ERMS with average OM content, no significant dose-effect relationships could be established (Table S5; Fig. 1).

Significant differences in the reproduction rate were found between the treatments depending on the OM content in the sediment (three-way ANCOVA, $p = < 0.001$) (Table S6). Where the OM content was higher, the reproduction rate was also higher with a factor of 1.2 for the treatment ERMP (Tukey HSD, ERMP OM enriched vs ERMP OM average, $p = 0.022$) and ERMS (Tukey HSD, ERMP OM enriched vs ERMS OM average, $p = 0.003$) (Fig. 1; Table S6). This confirms the important role of food quality and abundance as factors of habitat quality for benthic invertebrates [34,35]. Furthermore, this underscores the significance of food conditions when designing microplastics effect studies.

3.2. Effects of microplastics and mineral particles on growth

Chronic exposure to ERMP or ERMS with concentrations of up to 10 % (v/v) caused no significant effect on the growth of *L. variegatus,* and no significant dose effect relationships were observed (Fig. 2; Table S7). This is in accordance with previous studies that exposed *L. variegatus* to PS and PE, respectively [20,33]. No differences on the growth were detected between the mixtures ERMP and ERMS (three--way ANCOVA, $p = 0.592$)(Table S8). Hence, also for this endpoint, neither material appeared significantly more toxic than the other, supporting our interpretation of the reproduction data. Interestingly, sediment OM content appeared to be a more important factor explaining the observed growth differences (three-way ANCOVA, $p = < 0.001$) (Table S8). With enriched OM content, the growth of *L. variegatus* increased on average by a factor of 1.3. These differences were apparent between the treatments ERMP OM enriched and EMS OM average (Tukey HSD, $p = 0.013$) and ERMS OM enriched and ERMS OM average (Tukey HSD, $p = 0.011$)(Table S8).

3.3. Effects of microplastics and mineral particles on egestion

Water quality measurements temperature, pH and dissolved oxygen were consistent over the 14 days of exposure and showed no apparent differences between treatments (Table S9). After 14 days, the controls had an average reproduction factor of 0.91 ± 0.11 and growth of 10.77 ± 3.24 mg dw.

The egestion of feces by *L. variegatus* was not significantly different between the two mixtures ERMP and ERMS (three-way repeated ANOVA, $p = 0.071$)(Fig. 3; Table S10). Only for one exposure concentration 5 % (v/v), ERMS. *L. variegatus* exposed to ERMP egested more than when exposed to ERMS (three-way repeated ANOVA, Mix x Concentration, $p = 0.004$) (Tukey HSD, $p = 0.010$)(Fig. 3; Table S10). We can only speculate about the explanation. As egestion is a proxy for ingestion this indicates that for the same amount of nutrition ingested more energy is spent. Another explanation could be due to the density differences in the particles. As ERMS had a 2.68 times higher density than ERMP, it might take more energy to process the sand and clay particles compared to the microplastics, resulting in less egestion of these particles. However, it remains unclear, as this would also be expected at other doses, which is not observed.

When the dose of ERMP is increased up to 10 % (v/v), egestion decreases significantly (Tukey HSD, $p = 0.029$) and shows no difference compared to the control (Tukey HSD, $p = 1.000$) (Fig. 3; Table S10). We have no conclusive explanation for this observation; we can only speculate how material-specific differences such as particle density or aggregation behavior might affect the egestion of particles. A possible explanation is that at higher concentrations microplastics were more aggregated or encapsulated in the sediment, rendering them less bioavailable for *L. variegatus*. For the treatment with ERMS the egestion rate is not concentration dependent; no differences are found between

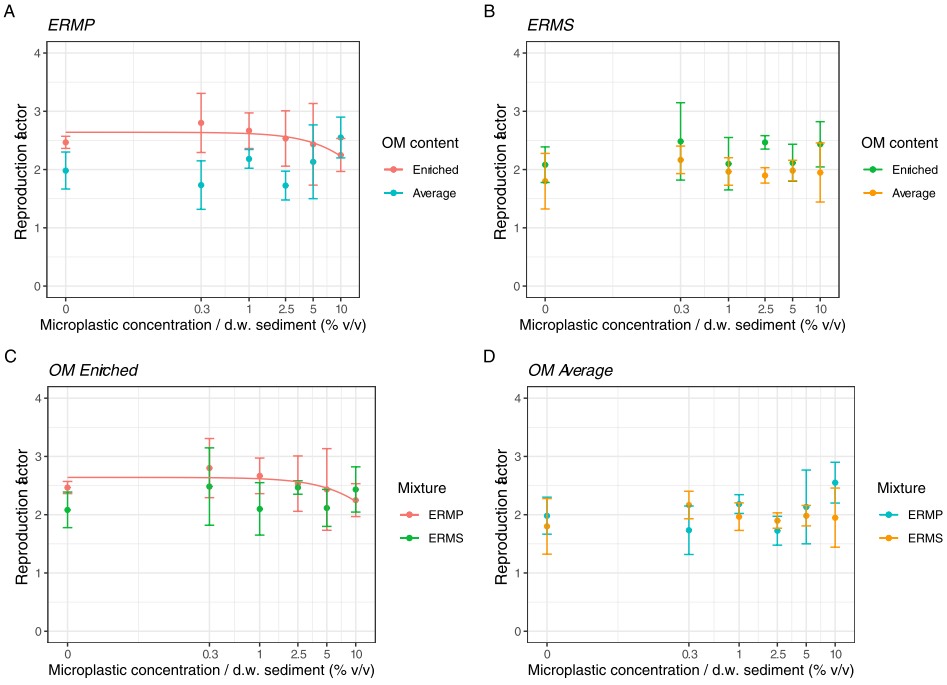

Fig. 1. Mean reproduction factor (± s.d.) of *L. variegatus* after chronic exposure to ERMP or ERMS in sediment with enriched versus average OM content. Note that concentrations are on a log scale. Additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

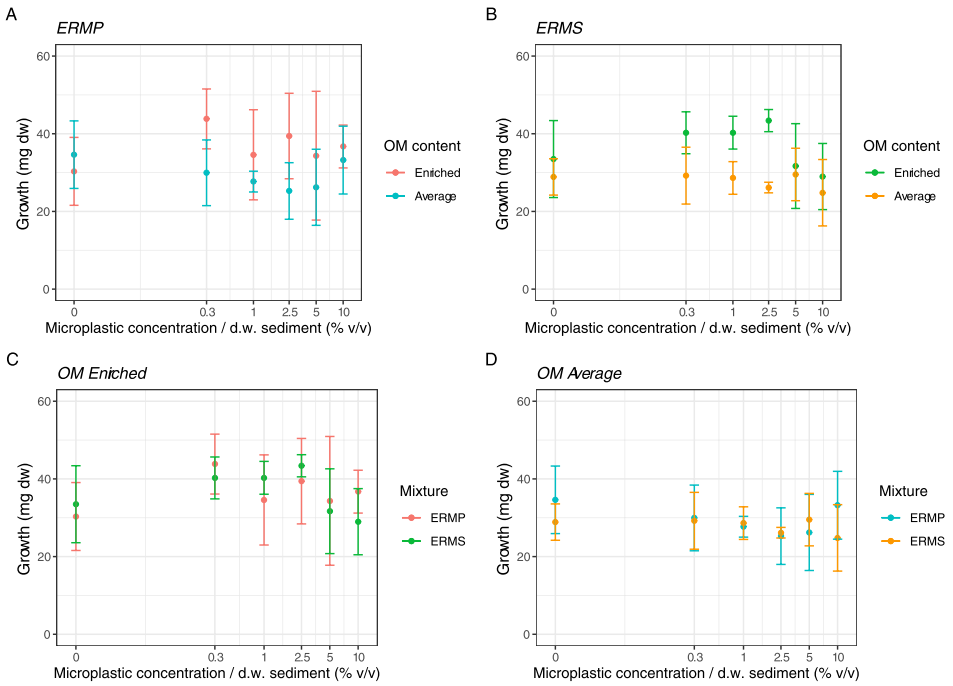

Fig. 2. Mean growth mg dw (± s.d.) of *L. variegatus* after chronic exposure to ERMP or ERMS in sediment with enriched versus average OM sediment content. Note that concentrations are on a log scale, additionally the zero concentration has been converted to 0.01 to allow plotting on the log scale.

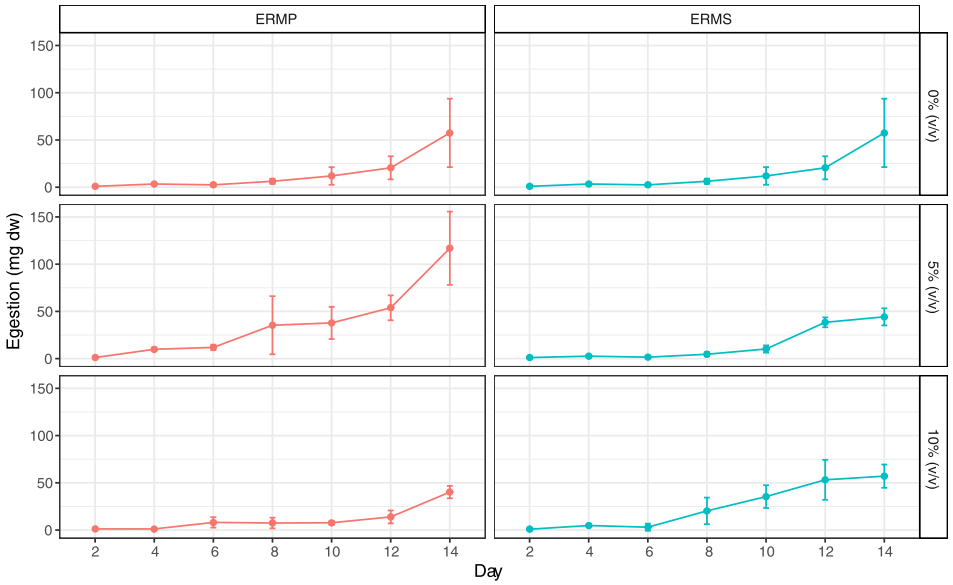

Fig. 3. Egestion of faecal pellets (mg dw) after exposure of *L. variegatus* to ERMP or ERMS over 14 days.

the control and the concentrations tested (Tukey HSD, $p = 0.310$, $p = 0.999$) (Fig. 3; Table S10).

4. General discussion and recommendations

In this study, we introduce a novel approach for making environmentally relevant mixtures of particles within the microplastic size range of 1 to 5000 µm. We provide a recipe for making polydisperse mixtures of microplastic and natural sand and clay particles with a focus on achieving size similarity among them. The chosen size range determines if the particles can be ingested, and by expressing exposure using a particle volume-based concentration ratio instead of using mass or particle number concentration, hypothesized effect mechanisms like that of food dilution can potentially be tested.

This study shows that it is possible to conduct effects tests on environmentally relevant, heterogeneous particle mixtures, here ERMP and ERMS, while adhering to strict QA/QC criteria [7]. There was no difference between the effects of inert ERMP or ERMS particles on the reproduction and growth of *L. variegatus*. This result differs from what has been found in recent meta analyses comparing effects of microplastic to those of natural particles, which generally suggest that microplastics are slightly more toxic than natural particles [36,12,8]. However, these meta analyses have their limitations. They involve the comparison of data from different studies, each carrying considerable

uncertainties, resulting in reduced statistical rigor compared to experiments where all experimental conditions are held constant, as in our study. Published studies often involve particles that differ significantly in terms of polydispersity, compared to the particles tested in our study. Moreover, the particles used in those studies may contain chemicals, with unknown chemical identities and concentrations that vary between experiments. Consequently, differences in toxicity stemming from chemical contaminants are erroneously attributed to inherent microplastics characteristics. In essence, the toxicity of chemicals primarily pertains to the hazard assessment of those chemicals, and should be considered separately from the assessment of particle effects [21,37,38]. Together with the use of inappropriate metrics, these factors complicate comparisons [8,12].

Only for ERMP with enriched OM content we find an adverse effect on the reproduction of *L. variegatus*, higher than the highest dose. However, this is not an environmentally relevant concentration [39-41], or a reliable threshold concentration, and does not alter the fact that the direct comparison between ERMP and ERMS showed no difference. Furthermore, this effect threshold is considerably higher than we found in our previous study ($EC_{50} = 2.51 \pm 0.44$ % d.w). As the experimental set ups were almost identical, this indicates that the repeatability of tests to detect adverse effect induced by microplastic particles may be limited, possibly due to biological variability and the relatively small effect size detected previously.

We did observe a difference in egestion rates between the diverse microplastics and diverse mineral particles tested. Interestingly, when exposed to the same particle volume concentration in the sediment for the two mixtures, *L. variegatus*, albeit only at the 5 % (v/v), showed an increase of egestion when exposed to ERMP compared to ERMS. This suggests that in order to acquire the same amount of nutrition, *L. variegatus* is spending more energy. This is in accordance with the findings of Silva et al. [33], who found that PE-MPs induced depletion of energy reserves [33]. Notably, this difference in effect diminished at the highest concentrations tested 10 % (v/v). Although the sediment was thoroughly homogenized before the start of the experiment, it can be speculated that during the experiments, the bioturbation of the blackworms caused aggregation of microplastics preventing it to become bioavailable. Here the use of a polydisperse mixture increases environmental relevance and gives insight into behavior of particles as a mixture. One factor explaining the response variables more substantially than the different mixtures tested, is the OM content in the sediment. This variable explains significant differences in both the reproduction and weight of *L. variegatus*, highlighting its importance when designing microplastic testing experiments.

5. Conclusion

This study introduced a novel method for creating environmentally realistic mixtures of microplastics (ERMP) with a degree of polydispersity similar to natural microplastics. The effects of these ERMP mixtures and mineral particle mixtures (ERMS) on the reproduction, growth, and egestion of *L. variegatus* were assessed over a 28-day exposure period. The water quality remained consistent across treatments, confirming adequate testing. No significant differences in reproduction or growth were observed between ERMP and ERMS, suggesting neither material was more toxic than the other, even at the highest tested dose (10 % v/v).

The study also showed that egestion rates were higher for ERMP than for ERMS at 5 % (v/v) concentration, suggesting that *L. variegatus* might expend more energy processing microplastics than mineral particles. At the highest concentration (10 % (v/v)), these differences diminished, possibly due to microplastic aggregation reducing bioavailability.

An EC_{50} value of 13.68 ± 5.54 % (v/v) was observed for ERMP with enriched OM content. Organic matter (OM) content was a key factor driving differences in reproduction and growth, highlighting the importance of food quality in microplastic effect studies.

Future research with ERMP should further investigate the role of OM content and particle aggregation in driving toxicity and energy expenditure. Expanding the scope to include more diverse ecosystems and particle types will enhance the environmental relevance of microplastic toxicity assessments.

Environmental implication

Whether hazardous microplastics cause different effects than inert natural particles is a key question in microplastics research. After all, if inert natural particles are equally hazardous as microplastics, we need to assess risks for plastic and natural particles simultaneously. Here, we provide a pioneering method that allows the creation of mixtures containing microplastics and non-polymer particles with an environmentally realistic level of polydispersity. We demonstrate that neither of these particle types causes effects on a sensitive invertebrate species, at high concentrations of up to 10 %. This finding supports the premise of equal effects between the two types of particles.

Declaration of generative AI and AI assisted technologies in the writing process

Statement: During the preparation of this work the author(s) used chatGPT in order to give suggestions on text in order to improve readability and writing style. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Albert A. Koelmans: Writing – review & editing, Supervision, Methodology, Conceptualization. **Xinyi Xie:** Investigation. **Vera Nyangoma de Ruijter:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136538.

Data availability

Data will be made available on request.

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