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Long-term exposure of a free-living freshwater micro- and meiobenthos community to microplastic mixtures in microcosms

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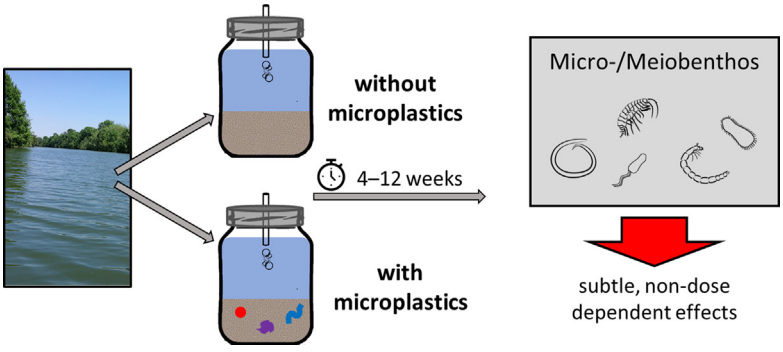
HIGHLIGHTS

- First microcosm study on the effects of MPs on freshwater micro -and meiobenthos
- No effects on the abundance flagellates and ciliates (microbenthos)
- Significant effects of MPs on meiobenthic organisms
- No decrease of the pollution-sensitive NemaSPEAR[%]-index in the presence of MPs
- Microcosm experiments are able to reveal subtle, indirect effects of MPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics in a wide range of shapes and polymer types (MPs; <5 mm) accumulate in freshwater sediments, where they may pose an environmental threat to sediment-dwelling micro- and meiobenthos. To date, the effects of MPs on those organisms have mostly been studied in single-species experiments exposed to high particle concentrations. By contrast, there have been few investigations of the effects resulting from the long-term exposure of natural communities to environmental relevant MPs. This research gap was addressed in the present study. A microcosm experiment was conducted to examine the impact of a mixture of MPs of varying polymer composition, shape, and size (50% polystyrene (PS) beads: 1-μm diameter; 37% polyethylene terephthalate (PET) fragments: 32 × 21 μm in size, and 13% polyamide (PA) fibers 104 × 15 μm in size; % based on the total particle number) provided at two concentrations (low: 4.11×10^5 MPs/kg sediment dw and high: 4.11×10^7 MPs/kg sediment dw) and two exposure durations (4 and 12 weeks) on a micro- and meiobenthic community collected from a freshwater sediment. MPs exposure did not alter the abundance of protozoa (ciliates and flagellates) as well as the abundance and biomass of meiobenthic organisms (nematodes, rotifers, oligochaetes, gastrotrichs, nauplii), whereas the abundance and biomass of harpacticoid copepods was affected. Neither nematode species diversity (species richness, Shannon-Wiener index, and evenness) nor the NemaSPEAR[%]-index (pollution-sensitive index based on freshwater nematodes) changed in response to the MPs. However, changes in the structure of the meiobenthic and nematode community in the presence of environmentally relevant MPs mixtures cannot be excluded, such that microcosms experiments may be of value in detecting subtle, indirect effects of MPs.

1. Introduction

Microplastics (MPs; <5 mm) pollution poses a threat to ecosystems worldwide (Silva et al., 2018). After entering aquatic environments, MPs undergo biofouling/sedimentation, which alters their specific densities

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(e.g., Galloway et al., 2017; Leiser et al., 2020) such that even MPs made up low-density polymer types (Hidalgo-Ruz et al., 2012), including polyethylene (PE) and polypropylene (PP), can sink down to the sediment. Indeed, MPs concentrations in riverine sediments can be up to 600,000-fold higher than in the water phase (Scherer et al., 2020). In a recent study of the Elbe River, the average and maximal concentrations of MPs (125–5000 μm) was 2080 and 14,100 particles/kg sediment (Scherer et al., 2020). Reliable data on particles <125 μm are currently lacking, partly because no uniformly standardized sample protocols are used limiting the comparability of different studies (Lindeque et al., 2020; Nguyen et al., 2019). However, it can be assumed that numbers for smaller particles (1–125 μm) are two to three orders of magnitude higher (Lenz et al., 2016). MPs in the sediment in general are also characterized by a larger diversity of polymer types than in the water (e.g., Horton et al., 2017; Klein et al., 2015; Scherer et al., 2020) and are mainly present as spheres, fibers, and fragments (Blair et al., 2019; Hurley et al., 2018; Tibbetts et al., 2018). Sediments thus represent a sink for MPs (Cera et al., 2020) and in turn a greater potential risk for sediment-dwelling benthic invertebrates (Frei et al., 2019; Haegerbaeumer et al., 2019a; Walkinshaw et al., 2020).

Due to their high structural and functional diversity and abundance (Giere, 2009), meiobenthos (or meiofauna; defined by their body size, that pass through a 500-μm mesh but are retained on a 44-μm mesh; Giere, 2009) occupy an important role in benthic food webs, as they connect lower and higher trophic levels (e.g., Majdi and Traunspurger, 2015). Changes in meiobenthic communities, such as in response to pollution, can thus be expected to impact the freshwater environment overall and nematodes in particular, as they comprise up to 90% of the meiobenthic community (e.g., Traunspurger et al., 2020; Traunspurger, 2021).

Benthic organisms (ciliates, flagellates, rotifers, annelids, nematodes, crustaceans) are able to ingest MPs of different sizes, shapes, and polymer types (reviews, e.g., by Adam et al., 2019; Scherer et al., 2018; Triebskorn et al., 2019; studies, e.g., by Fueser et al., 2019; Fueser et al., 2020a, 2020b). However, despite the ecological relevance of meiobenthic organisms in MPs research (Giere, 2019), the ecotoxicological effects of MPs on nematodes have scarcely been investigated (Haegerbaeumer et al., 2019a). Most studies on the effects of MPs on nematodes made use of the model organism *Caenorhabditis elegans* and examined toxicity endpoints such as oxidative stress, mobility, and survival (reviewed by Bhagat et al., 2021; Hu et al., 2020), but large deviations in the effect levels were determined (Bhagat et al., 2021). For example, in water and sediments, a 50% inhibition of *C. elegans* reproduction was observed after 96 h at PS bead concentrations of 0.57 mg 1-μm PS beads/ml (Mueller et al., 2020b) and 4.8–11.3 mg 1-μm PS beads/g sediment dry weight (dw) (Höss et al., 2022 in press). Moreover, long-term multigenerational tests (21-day exposure) assessing the population growth of *C. elegans* showed a 100-fold higher susceptibility (Mueller et al., 2020a). Whether species-specific population growth responses translate into a shift in nematode species composition under more realistic exposure scenarios remains to be tested using model ecosystems (Prokić et al., 2021; de Sá et al., 2018; Stanković et al., 2021).

Model ecosystems are appropriate tools for higher-tier risk assessments of chemicals in sediments (Diepens et al., 2017; EFSA Panel on Plant Protection Products and their Residues (PPR), 2013). They include the use of microcosms containing samples from natural ecosystems but with the tests run under controlled conditions (Brock et al., 2015). Compared to standardized single-species tests, the main advantage of microcosm studies in environmental risk assessments is that intra- and interspecific interactions within a community can be investigated over the long-term (Brock et al., 2015). With this approach, both direct and indirect effects of the substances of interest at the population and community level can be taken into account (van den Brink et al., 2009). Up to now, microcosms have been used in studies of the effects of different substances on freshwater meiobenthos, especially nematodes (summarized in Höss, 2021).

The impacts of MPs on benthic invertebrates (meio- and macrofauna) and benthic ecosystem functioning have been addressed in only a few studies, conducted in outdoor (Lin et al., 2020; Redondo-Hasselerharm et al., 2020; Stanković et al., 2021) and laboratory (Huang et al., 2021; López-Rojo et al., 2020; Silva et al., 2022; Wakkaf et al., 2020; You et al., 2020) microcosm set-ups, but none have included protozoa. Although the comparability of those studies is limited, given their differences with respect to the studied environment (marine, freshwater, soil) and polymer type (single polymers: PE, polyethylene terephthalate [PET], polyvinyl chloride [PVC], PS, or polymer mixtures), deleterious effects of MPs were observed for several endpoints. For example, these included changes in leaf litter composition and an increase in the mortality of the detritivore caddisfly *Sericostoma pyrenaicum* after exposure to 10-μm PS microspheres (at 1 μg PS microspheres/ml; López-Rojo et al., 2020). However, MPs at a concentration of 0.05–50 g/kg sediment dw were shown to exert both positive and negative effects on recolonization by macroinvertebrates (Redondo-Hasselerharm et al., 2020). The effects of a realistic MPs composition in terms of size and shape on a native benthic meiobenthic community sampled from a freshwater sediment has yet to be analyzed in long-term experiments conducted under controlled conditions.

To fill this research gap, we examined the impact of a mixture of MPs, differing in their shape and polymer type (50% PS beads, 37% PET fragments, 13% polyamide [PA] fibers) and provided at low (4.11×10^5 MPs number or 9.68 mg MPs per kg sediment) and high (4.11×10^7 MPs number or 968 mg MPs per kg sediment) concentrations, on the micro- and meiobenthic communities in a freshwater sediment. The microcosms were filled with field-collected sediment that included the native fauna and were subsequently spiked with the MPs mixtures. After 4 and 12 weeks, protozoans (flagellates and ciliates) and meiobenthic invertebrates (nematodes, rotifers, oligochaetes, harpacticoid copepods, gastrotrichs and nauplii) were sampled and their abundance and biomass were measured. Nematodes, as the most abundant group, were identified to the species level, which allowed their structural and functional diversity as well as a specific pollution index to be determined.

Previous ecotoxicological studies obtained conflicting results on the sensitivity of benthic invertebrates, especially nematodes, to MPs (Haegerbaeumer et al., 2019a). Our interest here was to monitor the response of micro- and meiobenthic communities to MPs under a realistic, long-term exposure scenario. We hypothesized that direct toxic effects on protozoa and meiobenthos were unlikely but that MPs interference with food consumption (Rauchschwalbe et al., 2021) would result in indirect food web effects evidenced as changes in community structure.

2. Material and methods

2.1. Collecting site and site properties

Sediment containing the indigenous meiobenthic community was collected in June 2020 from the Lippe stream (51°41′21.4″N 7°49′37.6″E), located in the city of Hamm in North Rhine-Westphalia, Germany. The flow velocity was 0.125 ± 0.05 m/s (n = 4), as measured with a hand-held velocity meter (Schiltknecht MC20). The sediment of the Lippe is fine-grained, with 4.2% clay (>2 μm), 28.4% fine silt (2–20 μm), 29.0% coarse silt (20–63 μm), and 38.4% sand (63–2000 μm).

Sediment (0.566 l per microcosm; in total about 20–30 l) with its indigenous invertebrates was collected using a stainless-steel grab sampler and was carefully put into a metal tub, from which the top sediment layers of 5 cm were then taken into another metal tub. Additionally, stream water was collected from the same site to be used as the overlying water in the microcosms. The sediment and the stream water were transported to the laboratory and stored in a metal tub at 20 °C until further use. The stream water was filtered through a 10-μm mesh before storage. One month before the start of the experiment, sediment and stream water were allowed to acclimatize to the laboratory conditions.

2.2. Polymers, shapes and sizes of applied microplastics

The shapes and composition of the MPs used in the mixture were chosen according to a field study on MPs occurrence in sediments of the Rhine

River (Klein et al., 2015). In that study, the smallest size fraction in the sediments consisted of 50% beads, 37% fragments, and 13% fibers, based on particle numbers. Three polymer types were chosen (PS, PET, PA), as they are among the polymer types most widely used in industry and in consumer products (PlasticsEurope, 2020). The choice of high-density polymers ensured that the MPs remained in the sediment (Table 1) (Hidalgo-Ruz et al., 2012).

Fluorescent PS beads with a diameter of 1 μm (Fluoresbrite® yellow-green microspheres, cat.# 17154-10; excitation maxima: 441 nm; emission maxima: 485 nm) were purchased from Polysciences Europe GmbH (Hirschberg, Germany). Nominal bead densities were verified by counting the PS beads in aliquots of defined dilutions of the stock suspensions using a hemocytometer (Neubauer Improved; 0.02-mm chamber depth; Brand GmbH + Co KG, Wertheim, Germany). Actual test concentrations deviated from the nominal concentrations and thus from the manufacturer's specifications by ≤10% for the 1-μm PS beads. The PS beads were assumed to be spherical in shape (Mueller et al., 2020b). The diameters of an aliquot of 100 PS beads were measured using fluorescence microscopy (Zeiss Axio Scope.A1, Jena) (Table 1). The PS bead stock suspension contained residues of the anionic surfactant sodium dodecyl sulfate (SDS, max. 5%). However, we didn't expect any negative effect of SDS on the benthic communities in the microcosms. Due to the dilution in the microcosms, a final concentration of max. 0.000001% SDS (0.01 ppm) could be expected, which is far below any reported toxicity values for SDS (e.g., Romanelli et al., 2004). Moreover, Mueller et al. (2020b) could not detect any inhibitory effects on the reproduction of the nematode *C. elegans* in the leachate of 1-μm PS bead suspensions which were 100,000-fold higher concentrated compared to the highest concentration in these microcoms.

PA microfibers were produced from commercially available fluorescent nylon tights (4798F-orange; PSYWORK-Schwarzlicht), and PET microfragments from fluorescent Mountain Dew PET bottles (PepsiCo, Inc.). The tights were cut into small pieces using a scissor and then crushed in a mortar with distilled water. The water (including small fibers) was then tipped into a beaker. The process was repeated using the remaining material in the mortar. The suspension was filtered through a filter cascade (200, 80, and 10 μm) and the microparticles that remained on the 10-μm sieve were used. PET fragments were produced by shredding a PET bottle using a steel file and then crushing the pieces in a mortar containing distilled water. The resulting liquid suspension was sieved (80 and 35 μm) and the fragments that passed through both sieves were used in the experiment.

The shapes of the MPs were confirmed (Supplementary material; Figs. S1 and S3) and the dimensions (length, width) of 100 PET fragments and PA fibers were measured using fluorescence microscopy (Zeiss Axio Scope.A1, Jena). For the fragments and fibers, the volume was calculated by assuming spherical and cylindrical shapes, respectively (Table 1). The mass of the particles was derived from the calculated volume and the specific density of each material (Table 1). Since the PET fragments and PA fibers were produced in our laboratory, their surface structures were additionally characterized by scanning electron microscopy (Hitachi S-450). The microparticles were prepared by gold-coating (19.3 g/cm^3) for 150 s at 30 mA using a sputter coater (Bal-TEC SCD 005) (layer thickness: 22 nm). The images showed (sharp-edged) PET fragments and porous PA fibers (Supplementary material; Figs. S2 and S4).

The selection of MP concentrations was based on field data for river sediments (MPs fraction 62–200 μm; Klein et al., 2015), while concentrations of smaller particles (1-μm PS beads) were extrapolated using the assumption of 3-dimensional fragmentation of plastic particles (Lenz et al., 2016), thus simulating a worst-case scenario. Finally, this resulted in particle-density-based and mass-based numerical concentrations of 4.11×10^5 MPs/kg sediment dw and 9.68 mg/kg sediment dw for the low concentration (LC), and 4.11×10^7 MPs/kg sediment dw and 968 mg/kg sediment dw for the high concentration (HC), respectively.

2.3. Experimental design (microcosm set-up)

The effects of the MPs on the meiobenthic communities as a whole were monitored in 24 indoor microcosms (glass jars with a volume of 1.7 l and a diameter of 12 cm; not acid washed) over 4 and 12 weeks. The indigenous benthic organisms were allowed a 1-month period of acclimatization (tub with air supply under laboratory conditions). Then the homogenized sediment was added to the microcosms and its meiobenthic community was analyzed (T0). The sediments in the microcosms were spiked with the MPs mixture (described in Section 2.2), which were then gently but thoroughly mixed using a glass rod. Four replicates for every treatment were set up for each sampling date (total $3 \times 4 \times 2 = 24$ microcosms; 12 per sampling date). The microcosms were filled to a depth of 5 cm with homogenized sediment, transferred using a stainless-steel scoop. The filtered stream water was carefully added until a 10-cm water column was visible on top of the sediments.

The experiments were conducted under a light-dark regimen of 12 h light and 12 h dark with a photon flow density of 15 μE/s × m^2 and a constant water temperature of ~20 °C. The glass jars of the microcosms were screwed shut with a lid to prevent excessive evaporative loss and airborne MPs contamination. The microcosms were aerated with oxygen via a tube that was led through a hole in the lid of the microcosms for the entire duration of the experiment; evaporated water was replaced using a 50:50 mixture of deionized water and water collected from the Lippe, to prevent an increase of conductivity due to evaporation.

2.4. Sampling and analysis

For the analysis of biological parameters, whole microcosms were sacrificed. On each sampling date (after 4 and 12 weeks of exposure), four microcosms were chosen randomly from the eight replicates of each treatment. T0 sampling (before application) was carried out from the bulk sample before the sediment was distributed to the microcosms.

2.4.1. Benthic community

2.4.1.1. Protozoa. Samples were obtained from each replicate microcosm by collecting the 5-cm sediment layer using a corer (3.5 cm diameter) and then homogenized with a glass rod. A 1-ml portion was transferred into microtubes and vortexed for 3 s. From the overlying water, a subsample of 7.5 μl was taken, filled up with 92.5 μl deionized water, and transferred to the counting chamber. Protozoans (ciliates and flagellates) were counted using a counting chamber (Nagoette, 0.5-mm depth, 1.25-mm^3 volume). All samples for enumeration were analyzed by microscopy (Zeiss, Scope A1) at 100× magnification. Ciliates and flagellates were detected by their characteristic movements and then counted.

Table 1
Particle characteristics (polymer type, size in μm, volume [10^{-6} mm^3 /particle] and mass [10^{-6} mg/particle]). Mean ± SD.

Parameter	Unit	PS beads	PET fragments	PA fibers
Polymer		Polystyrene	Polyethyleneterephtalate	Polyamide
Density	g/cm^3	1.05	1.38	1.15
Size (length/width)	μm	0.96 ± 0.06	32 ± 28/21 ± 19	104 ± 84/14.5 ± 7.8
Volume	10^{-6} mm^3	0.00048 ± 0.000095	39 ± 110	23 ± 26
Mass	10^{-6} mg	0.0005 ± 0.0001	54 ± 148	27 ± 29

2.4.1.2. Meiobenthos. For the meiobenthic community analysis, three sediment sub-samples were collected from each microcosm using three glass corers (5-cm layer; diameter 3.5 cm; total 29 cm^2 and 144 cm^3 sediment). Sampled sediment was fixed with formalin (4% v/v) and stained with Rose Bengal (300 mg/ml).

Meiobenthic organisms were extracted according to the centrifugal-flotation method of Pfannkuche and Thiel (1988), using silica gel with a specific density of 1.13 g/cm (Ludox TM 50, Sigma-Aldrich, St. Louis, MO, USA). After two rounds of centrifugation, the supernatant of each sample was filtered through a 10-μm sieve. The retained meiobenthic organisms were thoroughly washed with water and then counted under a stereomicroscope (40× magnification; Olympus SZ40, Shinjuku, Tokyo, Japan) by sorting them into major meiobenthic groups and then dividing them into individual size classes. Specifically, meiobenthos can be divided into permanent (nematodes, rotifers, gastrotrichs, tardigrades, ostracods, copepods, nauplii larvae) and temporary (oligochaetes, micro-turbellarians, cladocerans, chironomids) organisms based on their increasing body dimensions up to the macrofaunal size range (Giere, 2009).

The carbon biomass of all meiobenthic organisms was calculated and expressed as the dw (μg C/100 g sediment wet weight [ww]; Supplementary material, Table S1). The calculations were based on the assumption of a carbon content of 45%, a specific gravity of 1.13, and a dw:ww ratio of 0.25 (Feller and Warwick, 1988). The exception was rotifers, for which a specific gravity of 1.0, a dw:ww ratio of 0.21, and a carbon content of 42.7% (McCauley, 1984) were used in the calculations.

2.4.1.3. Nematodes. The first 50 nematodes of each replicate were mounted on slides according to the method of Seinhorst (1959), identified to the species level (at 1250× magnification), and classified into one of five feeding-types based on the morphological attributes of their buccal cavities (Traunspurger, 1997): detritus feeders (mainly bacteria), epistrate feeders (mainly algae), suction feeders (plant/hyphal feeders and omnivorous/predatory feeders) and chewers.

The nematode species number and the Shannon-Wiener index (H_S) on a log10 basis [according to the equation $H_S = -\Sigma P_i \times \log(P_i)$, where P_i is the relative frequency of a species and S the total number of nematode species found] were calculated. The H_S was then used to calculate the Evenness (E) [$E = H_S/\ln(S)$].

In addition to the diversity indices, our analysis included a nematode-specific pollution-sensitive index, the NemaSPEAR[%]-index, which has been shown to negatively and dose-dependently correlate with sediment contamination (Höss et al., 2011, 2017). The NemaSPEAR[%]-index is calculated as the percentage of pollution-sensitive nematode species (Nematode SPEcies At Risk; as defined by Höss et al., 2017) in a certain sample.

$$NemaSPEAR[\%] = 100 \times \frac{\sum log[NemaSPEAR]_{relAb}}{\sum \log[All\ Species]_{rel\ Ab}} \quad (1)$$

where $\log[\text{NemaSPEAR}]_{\text{relAb}}$ and $\log[\text{All Species}]_{\text{relAb}}$ are log-transformed ($\log[x + 1]$) relative abundance of the NemaSPEAR and of all species, respectively.

2.5. Data analysis

2.5.1. Univariate parameters

All data were checked for normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test). A significance level of $p < 0.05$ was set for all comparisons.

Time-related effects (T0, T1-C, T2-C) of univariate parameters (abundance, biomass, indices) were tested using a one-way ANOVA (post-hoc Tukey). A two-way ANOVA (post-hoc: Dunnett's test) with time and treatment as independent factors was performed to analyze significant differences among the control (C) and the two MPs treatments (LC, HC) for each time point for all univariate parameters (abundance, biomass, indices of nematodes, feeding-types of nematodes). For statistical analyses, non-normally distributed data were logarithmically transformed to achieve normal distribution.

2.5.2. Multivariate analysis

Time-related effects on meiobenthic and nematode species composition in the control microcosms were evaluated using non-metric multidimensional scaling (nMDS) using Bray-Curtis similarity data based on the log (x + 1)-transformed abundance data of the meiobenthic taxa and nematode species. A stress value <0.2 is considered acceptable (Clarke and Warwick, 2001). ANOSIM (analysis of similarities) was performed to identify significant differences ($p < 0.05$) between time points. The R-value indicates the strength of the factors (close to 1: high separation; close to 0: no separation). SIMPER (similarity percentages) analysis was performed to evaluate the contribution of each meiobenthic taxon/nematode species to the similarities in community composition. For nMDS, ANOSIM and SIMPER analysis, the PRIMER software was used (version 6.1.5, PRIMER-E, Plymouth, UK).

Treatment-related changes in nematode species composition with time were analyzed by the multivariate Principal Responses Curves (PRCs) method (Van den Brink and Ter Braak, 1999). PRC is a multivariate ordination method based on a redundancy analysis (RDA) to compare the responses of the nematode communities in the MPs treatments over time (Van den Brink and Ter Braak, 1999). Interactions between treatment and time (sampling date) were used as the explanatory variables, whereas time served as a co-variable. A linear combination of variables (changes of log (x + 1)-transformed abundance) was calculated to determine the deviation of each assemblage in a treated microcosm from assemblages in the control microcosms at each sampling date expressed as the first, second or third principal component of the variance explained by treatment differences in time (canonical coefficient; c_{dt}). PRCs were derived by plotting c_{dt} against time. Each treatment was statistically compared to the control by Monte Carlo permutation using the CANOCO software package (Version 4.5) (Ter Braak and Šmilauer, 2002). The corresponding scores (b_K) allowed interpretations of the PRCs at the species level. Specifically, the higher the b_K value of a species, the more the actual response patterns of the taxon were likely to follow the PRCs patterns. Nematode species with negative scores were inferred to follow the opposite pattern, whereas species with scores close to 0 presumably had no response or a response unrelated to the PRCs pattern.

3. Results

3.1. Protozoa

Within the course of the experiment, absolute abundances of ciliates and flagellates considerably changed with time, with a decrease of ciliates within the first four weeks, and a transient increase of flagellates (Table 2). However, a two-way ANOVA revealed significant differences in flagellates' abundances for the factor time ($F = 15.516$, $p < 0.001$), but no significant differences for ciliates ($F = 0.502$, $p < 0.488$). No treatment-related effects over time were observed for flagellates ($F = 0.966$, $p < 0.4$) and ciliates ($F = 0.532$, $p < 0.596$).

3.2. Meiobenthos

3.2.1. Controls

The abundance of the total meiobenthos in the control microcosms stayed quite constant during the whole experiment (one-way ANOVA; $F = 0.159$, $p = 0.856$; Table 2), whereas the total meiobenthic biomass varied considerably but not significantly with time (one-way ANOVA; $F = 1.989$, $p = 0.205$; Table 2). This can be explained by shifts in the composition of meiobenthos (based on the abundance), which consisted of six major meiobenthic groups: nematodes, rotifers, oligochaetes, harpacticoid copepods, gastrotrichs and nauplii (Table 2; Supplementary material, Fig. S5). The nMDS with ANOSIM (global $R = 0.398$, $p = 0.019$; Fig. 1A) revealed a significant difference in meiobenthos composition between the

Table 2
Summary of the mean (± SD) abundance (Ind = individuals) and biomass (only of meiobenthic taxa) of protozoa and meiobenthos in microcosms with different MPs treatments (C = control; LC = low MPs concentration; HC = high MPs concentration) at three time points (T0 = application, T1 = 4 weeks after application, T2 = 12 weeks after application). *Significant difference of LC or HC vs. C (two-way ANOVA, post-hoc: Dunnett's test $p < 0.05$, more information in Table S2).

Taxon	Unit	T0	T1			T2		
			C	LC	HC	C	LC	HC
Ciliates	Ind/g Sed ww	2133 ± 1795	933 ± 1532	933 ± 911	1500 ± 1570	800 ± 689	1067 ± 435	667 ± 800
Flagellates	Ind/g Sed ww	8100 ± 5100	23,000 ± 18,000	23,000 ± 12,000	14,000 ± 7900	6500 ± 1400	8700 ± 2400	8000 ± 1200
Σ Meiobenthos	Ind/100 g Sed ww	720 ± 131	813 ± 206	848 ± 255	957 ± 124	795 ± 338	442 ± 65	497 ± 92
	µg C/100 g Sed ww	553 ± 424	896 ± 929	1037 ± 905	1455 ± 892	165 ± 36	343 ± 115	320 ± 157
Nematodes	Ind/100 g Sed ww	410 ± 92	474 ± 119	500 ± 243	511 ± 55	613 ± 288	284 ± 79	338 ± 62
	µg C/100 g Sed ww	26 ± 13	36 ± 34	53 ± 32	46 ± 21	41 ± 7	32 ± 5	13 ± 16
Rotifers	Ind/100 g Sed ww	175 ± 74	165 ± 55	161 ± 71	191 ± 51	116 ± 81	32 ± 15	56 ± 39
	µg C/100 g Sed ww	$8 \times 10^{-5} \pm 3 \times 10^{-5}$	$7 \times 10^{-5} \pm 3 \times 10^{-5}$	$7 \times 10^{-5} \pm 3 \times 10^{-5}$	$9 \times 10^{-5} \pm 2 \times 10^{-5}$	$5 \times 10^{-5} \pm 4 \times 10^{-5}$	$1 \times 10^{-5} \pm 7 \times 10^{-5}$	$3 \times 10^{-5} \pm 2 \times 10^{-5}$
Oligochaetes	Ind/100 g Sed ww	117 ± 68	164 ± 79	143 ± 61	223 ± 35	38 ± 8	119 ± 72	96 ± 56
	µg C/100 g Sed ww	515 ± 432	858 ± 927	970 ± 937	1400 ± 884	111 ± 40	309 ± 118	295 ± 144
Harpacticoid copepods	Ind/100 g Sed ww	16 ± 11	3 ± 5	40 ± 22 *	26 ± 38	26 ± 16	8 ± 8	7 ± 4
	µg C/100 g Sed ww	10 ± 8	1 ± 2	12 ± 7 *	8 ± 12	11 ± 5	2 ± 2	2 ± 1
Gastrotrichs	Ind/100 g Sed ww	1 ± 2	8 ± 10	3 ± 5	5 ± 10	0 ± 0	0 ± 0	0 ± 0
	µg C/100 g Sed ww	0.09 ± 0.17	0.70 ± 0.86	0.22 ± 0.44	0.44 ± 0.87	0 ± 0	0 ± 0	0 ± 0
Nauplii	Ind/100 g Sed ww	3 ± 4	0 ± 0	3 ± 5	1 ± 3	2 ± 3	0.3 ± 0.5	2 ± 3
	µg C/100 g Sed ww	2 ± 3	0 ± 0	2 ± 3	1 ± 2	1 ± 2	0.2 ± 0.3	1 ± 2

Fig. 1. Non-metric multidimensional scaling (nMDS) plots for relative abundances of (A) meiobenthic groups (nematodes, rotifers, oligochaetes, harpacticoid copepods, gastrotrichs, nauplii) and (B) nematode species in the control microcosms at different sampling dates; T0, T1 and T2 = before, 4 weeks and 12 weeks after MPs application, respectively. Analysis is based on Bray-Curtis similarities of the log (x + 1)-transformed abundance data; significant differences were identified by ANOSIM ($p < 0.05$) between sampling dates, which are shown with different letters. Global R: (A) 0.398; (B) 0.569.

control microcosms after 4 and 12 weeks ($R = 0.771, p = 0.029$), with oligochaetes and harpacticoid copepods contributing most dominantly to these time-related changes (SIMPER analysis: 58%; Table 2).

3.2.2. Microplastic effects

MPs had no significant effects on the abundance (two-way ANOVA; $F = 2.8$, $p = 0.087$) and biomass (two-way ANOVA; $F = 0.286$, $p = 0.755$) of the total meiobenthos during the entire duration of the experiment (Table 2). After 12 weeks of exposure, although not statistically significant, abundance was 37–45% lower while biomass was 2-fold higher in the MPs treatments than in the control (Table 2), which was mainly due to a decreased abundance of oligochaetes in the control microcosms (Supplementary material, Fig. S5), contributing most to the total biomass (Table 2).

Regarding the meiobenthic groups separately, MPs had no influence on the absolute abundances and biomass of nematodes, rotifers, oligochaetes, gastrotrichs and nauplii within the experiment duration, respectively (Table 2; $p > 0.05$; for detailed information on the two-way ANOVA see Table S2 in the Supplementary material). Harpacticoid copepods responded to MPs (two-way ANOVA; $F = 6.454$, $p = 0.008$), with abundance ($q' = 3.368$, $p = 0.006$) as well as biomass ($q' = 3.401$, $p = 0.006$), with a transient increase after 4 weeks under low MPs influence compared to control conditions (Table 2; Supplementary material, Table S2).

Principle response curves revealed significant changes in meiobenthos composition compared to the control in course of the experiment, with time and MPs treatment (including the interaction with time) contributing 15% and 31% to the total variance in meiobenthos composition (Fig. 2). However, the differences were only statistically significant in the microcosms with low MPs concentrations (Monte Carlo permutation test; F-ratio: 3.70, $p < 0.016$). Harpacticoid copepods contributed most dominantly

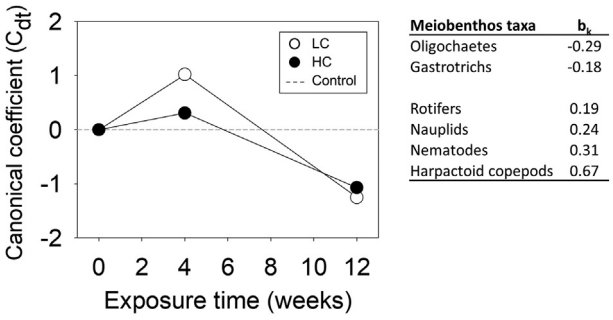


Fig. 2. Principle Response Curves (PRCs) generated from log-transformed abundances (Ind/100 g sediment) of six meiobenthic groups in microcosms treated with a mixture of MPs in two concentrations (LC = low concentration; HC = high concentration); b_K: species scores; sum of all canonical eigenvalues: 0.261; PRCs for the first axis explain 82% of the species-environment relationship.

to these changes in meiobenthos composition (increase after 4 weeks; Fig. 2), confirming the ANOVA results.

3.3. Nematodes

3.3.1. Controls

Deposit-feeding nematodes dominated the control microcosms, making up 80–86% of all nematodes, followed by chewers (predators and omnivorous nematodes: 11–16%), suction feeders (plant, hyphal and omnivorous feeders; 0.5–8%) and epistrate feeders (algae feeders; 0–2%) (Table 3; for absolute abundances see Table S3 in the Supplementary material). In total 26 nematode species were found in the control microcosms over the whole experiment, with each control microcosm hosting 9–12 nematode species (Table 3). The most dominant species was *Daptonema dubium* (47% of all nematode individuals), followed by *Eumonhystera* species (*E. vulgaris*, *E. filiformis*), *Ironus tenuicaudatus* and *Paraplectonema pedunculatum*, each, still representing >5% of all nematodes (Supplementary material, Table S4). Multivariate statistics (nMDS) of nematode species composition revealed significant shifts in the controls with time (global R: 0.26, $p = 0.021$; Fig. 1B). Nematode species composition was significantly different only between controls at T0 and after 4 weeks of MPs exposure (ANOSIM; $R = 0.354$, $p = 0.029$; Fig. 1B). However, these time-related changes in nematode species composition had no consequences for the number of nematode species (one-way ANOVA, number of nematode species: F = 1.606, $p = 0.253$; Table 3) or functional indices (DF: F = 0.461, $p = 0.645$; EF: F = 0.16, $p = 0.855$; S: F = 1.659, $p = 0.244$; Pre + Om: F = 0.572, $p = 0.584$; Table 3), which showed no significant differences between the three sampling dates. But the Shannon-Wiener index and the Evenness increased significantly over time (one-way ANOVA, H_S: F = 5.864, $p = 0.023$; E: F = 11.424, $p = 0.003$; Table 3). The values of the Shannon-Wiener index were significantly higher at 12 weeks than at either the start of the experiment (q = 34.079, $p = 0.043$; Table 3) or after 4 weeks (q = 34.300, $p = 0.034$; Table 3). The values of the Evenness were significantly higher after 12 weeks compared to the start conditions (q = 26.579, $p = 0.003$; Table 3) and after 12 weeks compared to 4 weeks (q = 34.634, $p = 0.024$; Table 3). Moreover, the NemaSPEAR [%]-index changed significantly over time (one-way ANOVA, F = 7.18, $p = 0.014$; Table 3) and was significantly higher after 12 weeks compared to 4 weeks ($t = 3.745$, $p = 0.014$; Table 3).

3.3.2. Microplastic effects

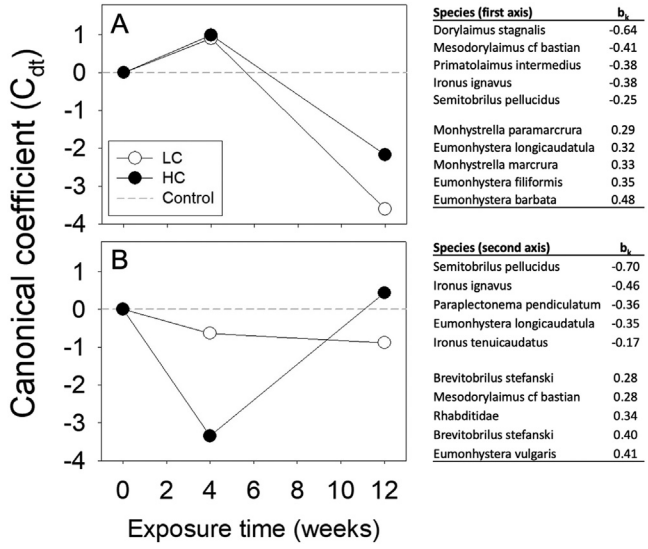
3.3.2.1. Species diversity. The MPs treatments had no effect on nematode species diversity at either 4 or 12 weeks (Table 3). Two-way ANOVA revealed no significant differences between both MPs treatments and the control for the total number of species (F = 3.439, $p = 0.054$; Table 3), the Shannon-Wiener index (F = 2.452, $p = 0.114$; Table 3) and the Evenness (F = 1.199, $p = 0.325$; Table 3).

3.3.2.2. NemaSPEAR[%]-index. The NemaSPEAR[%]-index showed to be relatively high throughout the experiment, with values ranging between a good (30–54%) and a high (>54%) ecological status. This pollution-sensitive nematode index was even higher in the MPs treatments, however, the differences between the treatments were not statistically significant over the whole experimental duration (two-way ANOVA; F = 3.216, $p = 0.064$; Table 3).

3.3.2.3. Nematode species and feeding-type composition. PRCs with Monte-Carlo permutation revealed significant differences in nematode species composition over time between both MPs treatments and the control (Monte Carlo permutation test: $p < 0.05$; Fig. 3), with time and MPs treatment contributing 19% and 31% of the total variance in species composition, respectively. After 4 weeks only the higher MPs concentration affected the nematode community significantly (F-ratio: 2.16, $p = 0.008$), while the communities in the lowly dosed microcosms could not be distinguished from the controls (F-ratio: 0.89, $p = 0.578$). After 12 weeks, nematode species composition significantly responded to the MPs at low (F-ratio: 2.43, $p = 0.004$) and high concentrations (F-ratio: 2.14, $p = 0.01$). While the changes in nematode species composition after 12 weeks clearly appeared in the PRCs of the first axis (Fig. 3A), the changes at 4 weeks became obvious when regarding the PRCs of the second axis, still explaining 30% of the species-environment relationship (Fig. 3B).

Table 3
Diversity indices (number of species [number of species for pooled replicates in parentheses], Shannon-Wiener index, Evenness), feeding-types (DF: detritus, mainly bacterial feeders; EF: epistrate, mainly algae feeders; S: mainly omnivorous suction feeders; Pre + Om: predators and omnivores) and the pollution-sensitive NemaSPEAR[%]-index calculated for nematode communities in the different treatments (C = control, LC = low MPs concentration, HC = high MPs concentration) at the respective sampling dates (T0 = before application, T1 = 4 weeks after application, T2 = 12 weeks after application).

Parameter	T0	T1			T2		
		C	LC	HC	C	LC	HC
Diversity indices							
Number of species	10 ± 2 (21)	9 ± 3 (14)	11 ± 2 (18)	12 ± 1 (20)	12 ± 1 (17)	12 ± 1 (17)	11 ± 1 (16)
Shannon-Wiener	0.63 ± 0.13	0.61 ± 0.15	0.72 ± 0.14	0.82 ± 0.11	0.89 ± 0.09	0.89 ± 0.06	0.86 ± 0.06
Evenness	0.26 ± 0.03	0.29 ± 0.03	0.30 ± 0.04	0.33 ± 0.03	0.36 ± 0.03	0.36 ± 0.02	0.36 ± 0.01
Feeding-types							
DF (%)	79.9 ± 15.0	79.6 ± 9.8	74.1 ± 5.7	72.4 ± 6.1	86.1 ± 5.3	57.0 ± 16.3	73.6 ± 17.2
EF (%)	1.5 ± 1.9	0.0	0.0	1.4 ± 2.9	1.0 ± 1.1	3.5 ± 2.5	3.5 ± 4.7
S (%)	7.6 ± 9.0	4.5 ± 3.0	3.0 ± 2.6	2.5 + 2.5	0.5 ± 1.0	16.6 ± 16.7	10.2 ± 11.6
Pre + Om (%)	11.0 ± 6.0	15.9 ± 8.4	22.9 ± 3.4	23.7 ± 8.2	12.4 ± 5.1	22.9 ± 4.8	12.7 ± 7.3
Pollution index							
NemaSPEAR[%]	43.4 ± 8.1	35.1 ± 8.0	41.9 ± 7.1	57.5 ± 4.2	57.8 ± 9.5	54.5 ± 6.5	61.7 ± 7.6



Species (first axis)	b_k
Dorylaimus stagnalis	-0.64
Mesodorylaimus cf bastian	-0.41
Primatolaimus intermedius	-0.38
Ironus ignavus	-0.38
Semitobrilus pellucidus	-0.25
Monhystrella paramarcrura	0.29
Eumonhystera longicaudatula	0.32
Monhystrella marcrura	0.33
Eumonhystera filiformis	0.35
Eumonhystera barbata	0.48

Species (second axis)	b_k
Semitobrilus pellucidus	-0.70
Ironus ignavus	-0.46
Paraplectonema pendiculatum	-0.36
Eumonhystera longicaudatula	-0.35
Ironus tenuicaudatus	-0.17
Brevitobrilus stefanski	0.28
Mesodorylaimus cf bastian	0.28
Rhabditidae	0.34
Brevitobrilus stefanski	0.40
Eumonhystera vulgaris	0.41

Fig. 3. Principle Response Curves (PRCs) generated from log-transformed abundances (Ind/100 g) of nematode species in microcosms treated with a mixture MPs in two concentrations (LC = low concentration; HC = high concentration); b_K: species scores for the five species with the highest and lowest scores; sum of all canonical eigenvalues: 0.250; (A) PRCs for the first axis (explaining 42% of the species-environment relationship); (B) PRCs for the second axis (explaining 30% of the species-environment relationship).

For the changes in nematode species composition after 12 weeks, PRCs revealed the decreases of the abundances of bacterial-feeding nematodes of the genera *Eumonhystera* and *Monhystrella*, and the simultaneous increase of abundances of omnivorous suction feeders (*Dorylaimus stagnalis* and *Mesodorylaimus* cf. *bastian*) as well as the omnivorous and predatory species *Ironus ignavus* and *Semitobrilus pellucidus* as main drivers (Fig. 3A; Fig. 4). After 4 weeks, also omnivorous and predatory nematodes benefitted from the MPs treatment (only high concentration: *S. pellucidus* and *I. ignavus*), while other species (*Eumonhystera vulgaris* [bacterial feeder] and *Brevitobrilus stefanski* [omnivore]) were negatively affected by the high MPs treatment (Fig. 3B; Fig. 4).

The MPs-induced changes in species composition also affected the composition of feeding-types, with an increase of dominance of suction feeders, omnivores and predators and a subsequent decrease of dominance of bacterial feeders in the MPs-treated microcosms (Table 3). However, the effects on the trophic structure were rarely statistically significant. Only in the lowly concentrated MPs treatment after 12 weeks, the percentage of deposit-feeding nematodes was significantly lower than in the control ($q' = 3.669, p = 0.003$; −29.4%).

4. Discussion

This is the first study to employ microcosms to address the long-term impacts of environmentally relevant MPs mixtures on freshwater micro- and meiobenthic communities. Whereas MPs exposure did not significantly alter the abundance of ciliates and flagellates, the absolute abundance and biomass of harpacticoid copepods increased significantly over time in the lower concentrated MPs treatment compared to the control. However, these effects should be regarded with caution as (1) the subtle effects observed after 12 weeks of MPs exposure did only occur in the lowly dosed microcosms, and not in the microcosms with a 100-times higher MPs concentration, and (2) the differences in meiobenthic community structure originated from time-related changes in the controls (Fig. 1A; Table 2; Supplementary material, Table S2). This observation is consistent with those of previous studies showing that abundances (and thus the biomass) of meiobenthic organisms in freshwater environments may fluctuate, as would be expected from the highly dynamic nature of streams and rivers (e.g., Brüchner-Hüttemann et al., 2020; Majdi et al., 2011; Traunspurger et al., 2015).

In contrast to the subtle, non-significant changes in total nematode abundance and biomass (Table 2), the responses of nematodes species and feeding-type composition to the MPs treatments were clearer. The results of single-species toxicity tests with the nematode *C. elegans* (EC10: 0.87–2.0 mg/g sediment dw; Höss et al., 2022 in press) were consistent with the effects in the highly concentrated microcosms (968 mg/kg = 0.968 mg/g sediment dw). However, the following issues should be carefully considered in interpreting the MPs effects observed in this study: (1) Nematode species composition in the control microcosms showed some variation over time (Fig. 1B; Table 3). Microcosms are dynamic systems which partly reflect time-dependent variability of nematodes in the field (e.g., Brüchner-Hüttemann et al., 2020). Moreover, the set-up of microcosms can cause also stress to the inhabiting fauna, which then partly recovers during the study. Changes in control microcosms in course of the experiment were also observed for soil nematodes (e.g., Höss et al., 2021). (2) Nematode species diversity (described by species richness, Shannon-Wiener index, and Evenness) was not affected by the MPs treatments. (3) The NemaSPEAR[%]-index, an indicator of chemical stress in freshwater nematode communities (Höss et al., 2011), did not decrease in the MPs treatments, always indicating a good to high ecological status (35–62%) as defined by Höss et al. (2017), suggesting no direct chemical stress for the nematode community. Other microcosm studies have shown that nematode communities exposed to toxic chemicals respond with a clear decrease in the NemaSPEAR[%]-index (Haegerbaeumer et al., 2016; Höss et al., 2017).

Our results suggest that the observed shifts in meiobenthos and nematode species composition were not caused by direct adverse effects of the MPs. Instead, they might be attributed to changes in other biotic or abiotic conditions (e.g., shifts in the food web) in the microcosms during the experiment, which had not been measured. This would support our hypothesis that changes in nematode species composition can be explained by a disturbance in food availability. It would also account for the decrease in deposit-feeding nematodes in the MPs treatments after 12 weeks, to the benefit of nematodes of other feeding-types. Studies with *C. elegans* in aqueous medium (Mueller et al., 2020b) and in sediment (Höss et al., 2022 in press) have shown that exposure of the nematode to PS beads of various sizes impedes its consumption of bacteria (Rauchschwalbe et al., 2021), leading to reduced reproduction. In more detail, regardless of the exposure medium (water: Mueller et al., 2020b; sediment: Höss et al., 2022 in press), the bead/bacteria ratio was a good predictor of MPs-toxicity (EC50 of 1-μm PS beads based on beads/bacteria: ~1). In their microcosm study of the effects of iron oxide colloids on freshwater meiobenthic communities, Höss et al. (2015) showed that meiobenthic abundance was strongly dependent on bacterial activity; direct effects of the colloids were detected only sporadically. These results demonstrate the need to consider indirect food web effects and the nutritional conditions in natural exposure scenarios.

Since this is the first study to evaluate the effects of MPs on freshwater micro- and meiobenthos in microcosms, our findings can hardly be compared to published data. However, comparing the results of the present study with data of Stanković et al. (2021), examining the effects of a mixture of MPs on macrozoobenthos in a freshwater pond, revealed that meiobenthos and especially nematodes are more responsive to MPs than macrofauna. Although that study used higher concentrations of MPs (2500 mg/kg sediment dw), the authors showed that MPs did not induce any negative impacts on macrofauna, whether in terms of diversity or species composition as analyzed by multivariate statistics (based on abundance and biomass; Stanković et al., 2021). While studies of freshwater systems are lacking, the effects of MPs on meiobenthos and nematode communities in marine and terrestrial habitats have been studied under realistic conditions (Lin et al., 2020; Wakkaf et al., 2020). The abundance of marine meiobenthos (copepods, polychaetes, nematodes) was significantly lower in the presence of <40-μm PVC spheres at concentrations of 20 and 40 mg/kg sediment dw than in the control (Wakkaf et al., 2020). Moreover, the marine nematode community was significantly less diverse, as determined using the Shannon-Wiener index, in the presence than in the absence of MPs (Wakkaf et al., 2020). A response of the marine nematodes was

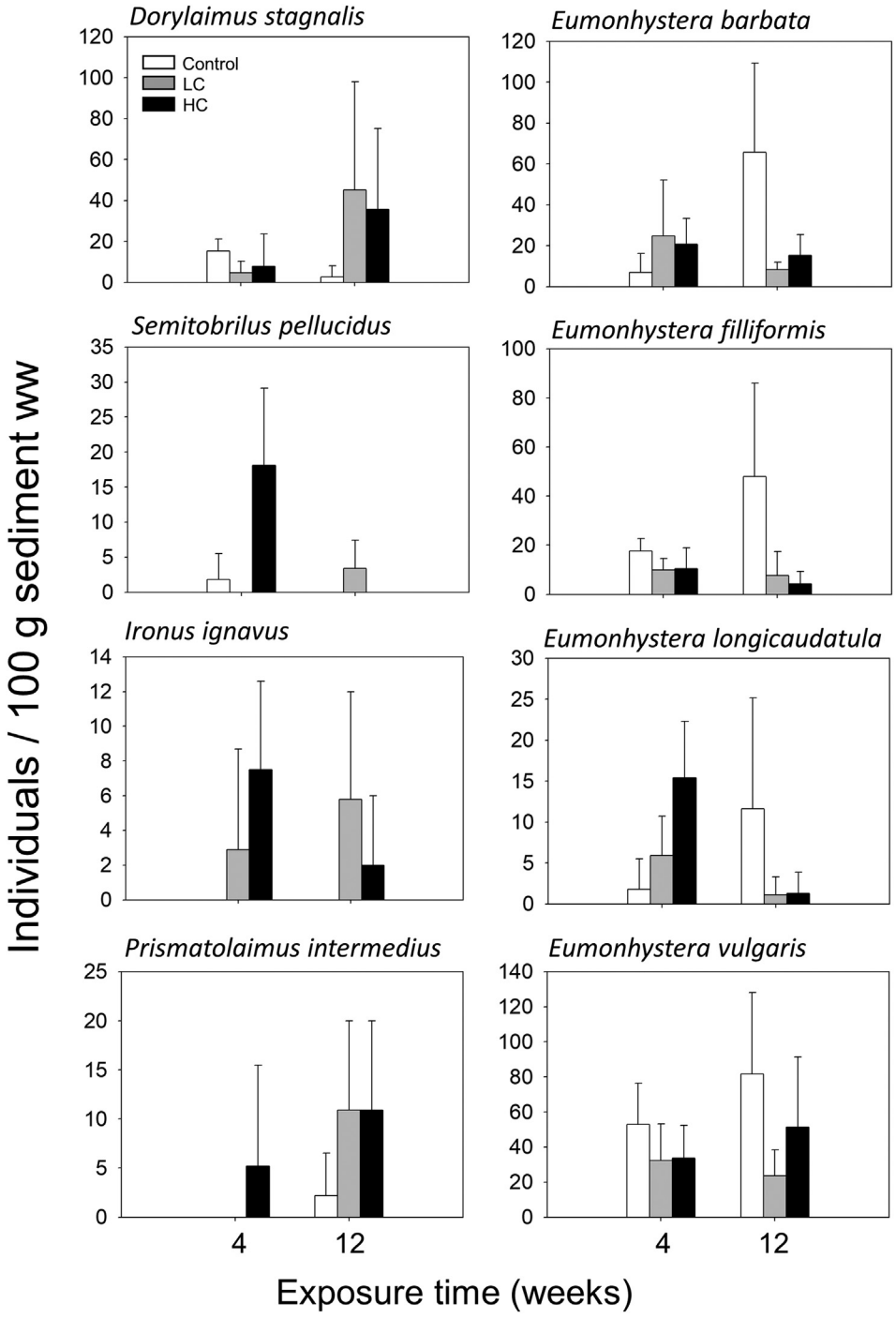


Fig. 4. Abundances (individuals/100 g sediment; mean ± SD) of nematode species with high species scores in PRCs analysis (see Fig. 3); LC and HC indicate low and high MPs concentration, respectively.

observed over a concentration range similar to that used in the lowly concentrated treatment of the present study. In a long-term terrestrial field study (287 days), the total abundance of nematodes was reduced by 15.4–19.7% in the presence of 37-μm low-density PE fragments provided at lower concentrations (11,400–39,000 PE fragments/kg; Lin et al., 2020) than in the present study. Moreover, the trophic structure of the soil nematode community was significantly affected by the PE fragments, with declines in omnivorous and predatory nematodes (Lin et al., 2020). These changes were found to be directly and indirectly (via effects on microarthropod abundance) caused by the PE fragments (Lin et al., 2020). Although in our study there was a slight change in the feeding-type composition of nematodes after 12 weeks, the soil nematodes of the study by Lin et al. (2020) responded at considerably lower concentrations and the negative effects were clearly dose-dependent. The longer exposure duration in that study than in ours (41 vs. 12 weeks) may have contributed to the higher sensitivity of the soil nematodes (Lin et al., 2020). While it is also possible that the ecotoxicological behavior of PE and PS differs, studies of the fungicide fludioxonil have shown similar sensitivities of soil and freshwater nematodes in microcosm experiments (Haegerbaeumer et al., 2019b; Höss et al., 2020).

Finally, no clear conclusions can be made regarding the relevance of the MPs concentrations used in this first freshwater microcosm study. In the past, MPs < 80 μm of freshwater environments could not be detected, because they were mainly sampled with filter mesh sizes of >80 μm (Adam

et al., 2019), therefore potentially underestimating smaller MPs. According to Lindeque et al. (2020), it can be assumed that the MPs concentrations increase with decreasing filter mesh sizes. Therefore, due to technical and sampling limitations as well as still missing uniformly standardized protocols, reliable data about MPs < 125 μm are lacking (Bellasi et al., 2020; Nguyen et al., 2019; Lindeque et al., 2020). However, as MPs are a very heterogeneous class of substances, whether other types of polymers or significantly smaller fragments produce stronger effects on freshwater benthic communities is unclear. Given the complexity of the benthic habitat and food web in freshwaters and the importance of the associated ecosystems (Majdi and Traunspurger, 2015), future experiments should not focus only on the effects of MPs on single organisms but also on complex ecosystem processes (e.g., nutrient cycling; Huang et al., 2021). Longer-term effects (e.g., annual study) should also be considered, as the poor degradability of MPs in freshwater sediments causes their prolonged persistence, which may eventually alter sediment properties.

5. Conclusion and future studies

In conclusion, a mixture of MPs induced effects on the benthic community in freshwater microcosms (abundance and biomass data), at relatively high MPs concentrations (100-fold higher than expected environmental realistic concentrations; based on extrapolations; Lenz et al., 2016). The observed shifts in the meiobenthos and in nematode community structures might be explained by indirect food web effects rather than by direct MPs toxicity. The NemaSPEAR[%]-index was used to interpret the observed shifts in nematode species composition from an ecotoxicological perspective and supported our conclusion that the risks of the applied MPs-mixture for micro- and meiobenthic communities were generally low. Nevertheless, this study showed for the first time that slight changes in freshwater benthic community structure induced by the presence of environmentally relevant composite MPs cannot be excluded.

CRediT authorship contribution statement

Marie-Theres Rauchschwalbe – data curation, writing - original draft, review & editing.

Sebastian Höss – conceptualization, supervision, writing - review & editing.

Arne Haegerbaeumer – conceptualization, methodology, investigation, writing - review & editing.

Walter Traunspurger – conceptualization, investigation, supervision, writing - review & editing.

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. Supplementary data

Additional information on microplastic particle characterization, biomass calculation of meiobenthos and data for meiobenthic organisms and nematode species. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154207.

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