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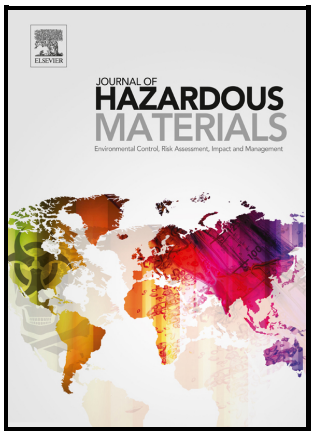

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Martin van der Plas, Tom A.P. Nederstigt, Krijn B. Trimbos, Martina G. Vijver

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Size-dependent impacts from polystyrene micro- and nanoplastics on freshwater invertebrates: a mesocosm study combining environmental DNA metabarcoding and morphological identification

Martin van der Plas[a*], Tom A.P. Nederstigt[a], Krijn B. Trimbos[a], Martina G. Vijver[a]

[a] Institute of Environmental Sciences, University of Leiden, Leiden, The Netherlands

ORCID

0009-0004-0166-2951 (M. van der Plas)
0000-0002-4692-1214 (T. A. P. Nederstigt)
0000-0001-5280-6434 (K. B. Trimbos)
0000-0003-2999-1605 (M. G. Vijver)

*Corresponding author: m.van.der.plas@cml.leidenuniv.nl

Abstract

Micro- and nanoplastics (MNPs) are widespread in freshwater ecosystems worldwide. While numerous studies have demonstrated that exposure to MNPs may induce adverse effects at the level of individual organisms, their potential long-term impacts at the level of communities remain poorly understood. The current study comprised a 14-week outdoor mesocosm experiment in which naturally established communities of freshwater invertebrates were exposed to spherical europium-doped polystyrene (PS) particles (15 µm and 150 nm) at concentrations resembling the higher end of those reported for natural surface waters. Community-level responses were assessed by morphological identification of macroinvertebrates and emerging insects, and by environmental DNA (eDNA) metabarcoding. Morphological assessments revealed no significant treatment effects. However, eDNA metabarcoding revealed subtle but significant responses, including a temporary reduction in macroinvertebrate taxa richness and distinct community shifts in the 15 µm treatment within two weeks after exposure. These impacts were transient, with communities recovering before the end of the experiment, despite water column MNP concentrations remaining at ~50% of nominally applied concentrations. Our results indicate transient impacts of PS MNPs under the tested conditions, and thereby highlight the suitability of eDNA metabarcoding as a tool for detecting higher-tier impacts from stressors to which community-level responses may be subtle.

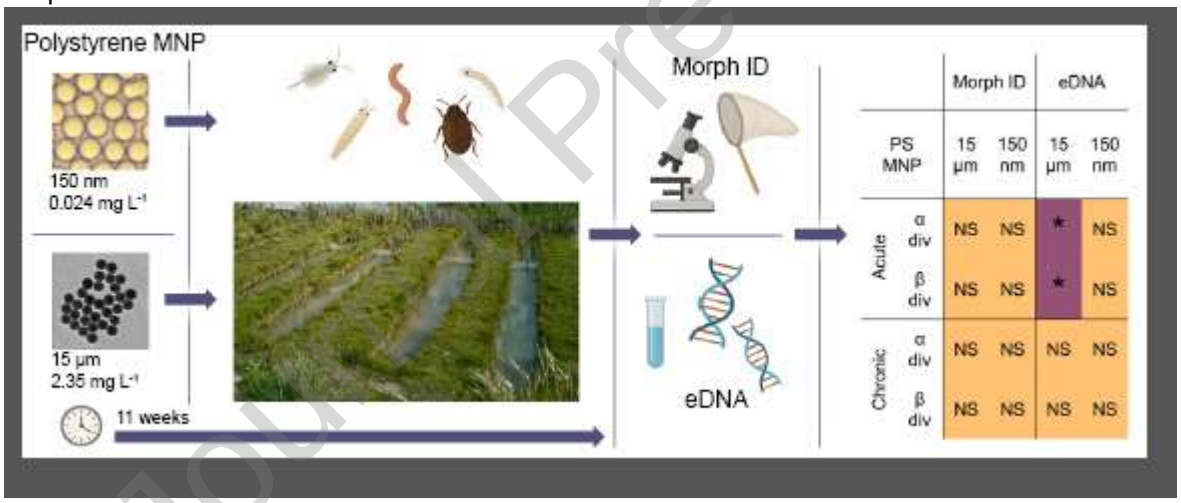
Environmental implications

Micro- and nanoplastics are emerging contaminants in freshwater ecosystems. Our findings indicate that environmentally relevant concentrations of polystyrene particles can induce acute, transient changes in invertebrate community composition. These results highlight that even low-level, persistent plastic pollution can alter community composition. Moreover, our study demonstrates that environmental DNA metabarcoding is a sensitive tool for early detection of subtle community impacts. These insights are valuable for environmental monitoring, risk assessment, and the development of mitigation strategies for plastic pollution in freshwater ecosystems.

Keywords

Ecotoxicology; Aquatic ecosystems; Ecosystem-level responses; Community composition; Emerging insects

Graphical abstract



1. Introduction

Freshwater ecosystems are regarded as major sinks of plastic waste, and numerous studies over the past years have demonstrated that various size classes of plastics are ubiquitously present in lakes (Nava et al., 2023), ponds (Brooks et al., 2023), rivers (van Emmerik & Schwarz, 2020), streams (Leterme et al., 2023), and wetlands (Qian et al., 2021) around the world. Even in cases where sources of plastic emissions may be identified and effectively abated, the fragmentation of macroplastics already present in freshwater environments is likely to result in the unstoppable generation of micro- and nanoplastics (MNPs) over the coming decades (Liro et al., 2023). Findings from laboratory-based studies to date have provided ample indication that MNPs can adversely affect a variety of processes and life history traits in aquatic organisms, such as locomotion (Chen et al., 2017; De Felice et al., 2019),

growth and body size (Besseling et al., 2014; De Felice et al., 2019; Eltemsah & Bøhn, 2019; Lei et al., 2018), reproduction (Besseling et al., 2014; De Felice et al., 2019; Lei et al., 2018), larval development (Besseling et al., 2014; De Marco et al., 2022), mortality (Eltemsah & Bøhn, 2019; Lei et al., 2018), energy production (Silva et al., 2021) and predator avoidance behavior (Ferreira et al., 2023).

However, natural environments are generally more complex than laboratory settings, leading to considerable uncertainty about how such individual-level effects may scale up to impact populations and communities under real-world conditions (Castro-Castellon et al., 2022). Organisms in natural freshwaters may for example be able to mitigate exposure by active and passive avoidance (Araújo et al., 2016), have the opportunity to escape predators due to an availability of more diverse sheltering sites (Araújo et al., 2020), or simply be more strictly limited in terms of population growth by processes other than those considered in laboratory studies, such as interspecific interactions. Conversely, subtle effects at the level of individuals may be exacerbated in natural environments due to interactions with other prevalent forms of stress, whether these are naturally occurring (Silva et al., 2022) or derived from anthropogenic sources (Xiang et al., 2022). Such complexity necessitates a shift toward higher-tier, community-level studies that better reflect ecological realities and capture the potential cascading effects of MNP exposure (de Ruijter et al., 2025; Lenz et al., 2016; Thompson et al., 2024).

A growing number of recent studies have sought to address these complexities by employing higher-tier experimental designs. The literature presents a spectrum of findings: although numerous studies have documented minimal or negligible effects of MNP exposure on freshwater communities (Klasios et al., 2024; Marchant et al., 2023; Martínez Rodríguez et al., 2023), some have identified more substantial responses. For example, Redondo-Hasselerharm et al. (2020) observed significant reductions in macroinvertebrate diversity following 15 months of exposure, being that these effects were largely driven by a single species. Moreover, these effects were not evident after three months of exposure, indicating long term effects of the treatments. Yıldız et al. (2022) documented short-term alterations in emergence behavior among Chironomidae. In this study, effects were only observed in the first seven days of exposure. Collectively, these heterogeneous and at times conflicting findings underscore the persistent uncertainty regarding the ecological consequences of micro- and nanoplastic (MNP) exposure on freshwater invertebrate assemblages.

Higher-tier assessments of community level responses to stressors typically takes place through mesocosm studies from which organisms are (sub)sampled, identified, and counted, a process which commonly is both time consuming and labor intensive (Macaulay et al., 2025). Because of this, the number of samples and sampling frequency is limited. Moreover, the targeted communities are often limited due to required expertise and time constraints. In recent years, environmental DNA (eDNA) metabarcoding has gained traction as an alternative tool for assessing community-level responses to environmental stressors (van der Plas et al., 2025). Compared to morphology-based

approaches, eDNA metabarcoding is a minimally invasive approach that provides high-resolution insights into (shifts in) community composition, while simultaneously reducing the labor intensity and taxonomic expertise typically required for sampling and taxonomic identification (Fediajevaite et al., 2021). In the context of experimental ecotoxicology, this could enable frequent and in-depth monitoring of community structure over time, which potentially gives way to the early identification of ecological responses to low-level or chronic stress, which could prove especially useful in plastic research due to the expected chronic exposure over the coming decades.

The current study aimed to assess long-term population- and community-level impacts of polystyrene MNPs on aquatic invertebrates, applying several complementary methods. We employed outdoor lentic mesocosms in which pioneer-stage species assemblages (i.e., early successive stage, as mesocosms are fully emptied and cleaned after each experiment)–including both microbial and macrofauna communities–were established naturally over winter and spring. Mesocosm were treated with 150 nm and 15 µm spherically-shaped $Eu(tta)_3$-doped polystyrene (PS-Eu) particles, which allowed for sensitive and accurate measurements of water column concentrations of MNPs throughout the experiment. Community-level responses were assessed by (1) sampling and morphological identification of macroinvertebrates, to determine impacts on taxon abundances, (2) monitoring of insect emergence, to assess potential life-history or developmental impacts of exposure, and (3) environmental DNA (eDNA) metabarcoding, to allow the surveying of a broader range of taxa—including macroinvertebrates as well as small-bodied or cryptic organisms that are often overlooked when using morphological methods, as well as a higher temporal coverage. Together, these complementary methods allowed for a higher-tier evaluation of community-level responses to MNPs in freshwater ecosystems.

2. Methods

2.1 Experimental design

The experiment was conducted in the outdoor facility 'The Living Lab' in Leiden, the Netherlands, from June until September 2021. This period was chosen to coincide with peak macroinvertebrate activity in temperate freshwater ecosystems and to encompass key life stages (larval, juvenile, adult) that emerge between early summer and autumn (Hill et al., 2016; Verberk et al., 2008). A total of 24 experimental mesocosms was used, each with a volume of ~850 L (dimensions: 5 x 0.5-0.6 x 0.3 m). Mesocosms resembled typical characteristics of agricultural drainage ditches, with natural sand- and clay-based sediments, with clay being the dominant substrate (see the graphical abstract for a section of the mesocosm set-up). Prior to the application of treatments, each mesocosm was connected to an adjacent waterbody to allow for the natural establishment of species assemblages, where a mesh (1 cm diameter) prevented larger organisms such as fish and amphibians

to enter the system. One month prior to the experiment, each mesocosm was disconnected from the adjacent waterbody using acrylic sheets to prevent further exchange of biota, water and applied treatments during the experiment. The experiment was set up in a block design, with 8 mesocosm serving as controls, 8 mesocosm exposed to 15 µm polystyrene (PS) particles and 8 mesocosm exposed to 150 nm PS particles (see Sections 2.2-2.5). Physicochemical water quality parameters (i.e., temperature, pH, dissolved oxygen, conductivity, chlorophyll A and turbidity) were monitored weekly throughout the experiment using a Hach HQ40d multimeter (Hach Ltd., Colorado, USA) and an AquaFluor® handheld fluorometer (Turner Designs, Inc., San Jose, USA), and results are presented in the supplementary information (SI Fig. S1).

2.2 Preparation and *in vitro* characterization of PS-Eu particles

Treatments consisted of spherically-shaped Eu(tta)$_3$-doped polystyrene (PS-Eu) particles with nominal diameters of 150 nm and 15 µm. Polystyrene is among the most commonly detected polymer types in surface waters worldwide, but accurate quantification of low concentrations of polystyrene MNPs in complex environmental matrices remains challenging (Materić et al., 2022; Xu et al., 2022). Metal-doped MNPs, such as those used in the current study, provide a means to circumvent challenges in this regard by enabling sensitive and accurate quantification based on the detection of the metal entrapped within the polymer matrix (Mitrano et al., 2019). The PS-Eu particles used in the current experiment were synthesized via emulsion polymerization and a combined swelling-diffusion technique according to Luo et al. (2022).

Hydrodynamic sizes, (poly)dispersity indices, and zeta potential of pristine 150 nm PS-Eu particles were measured in suspensions prepared Milli-Q water (Milipore Milli-Q reference A+ system, Waters-Millipore Corporation, Milford, MA, USA) using a Malvern Zetasizer Ultra (Malvern, Malvern, UK), and were 162.8 ± 1.7 nm, 0.02 ± 0.01, and -27.7 ± 1.3 mV, respectively (mean ± standard deviation, n = 10). Since hydrodynamic size analysis of micrometric particles can produce inaccurate estimates (Caputo et al., 2021), pristine particle sizes of 15 µm PS-Eu particles were assessed in separate aliquots (n = 5) of stock suspensions by regular light microscopy, and determined at 13.2 ± 0.2 µm (n = 20). Particle morphology was determined by light- (15 µm particles) and transmission electron microscopy (150 nm particles) (TEM JEOL 1010, Japan Electron Optics Laboratory, Mitaka Tokyo), and particles were found to be spherical in shape and homogenous in size (SI Fig. S2).

2.3 Extraction and analysis of Eu(tta)$_3$ from PS-particles

Incorporation of Eu(tta)$_3$ into the polystyrene matrix was quantified from gravimetrically determined dilution ranges of PS-Eu stock suspensions prepared in duplicate in MiliQ water (100mg L^{-1} – 0.1 mg L^{-1} / 5.3×10^{13} – 5.3 × 10^{10} particles L^{-1} for 150 nm particles and 100 mg L^{-1} – 1 mg L^{-1} / 5.3 × 10^{7} – 5.3 × 10^{5} particles L^{-1} for 15 µm particles). Prior to analysis via inductively-coupled plasma mass

spectrometry (ICP-MS, PerkinElmer NexION 300D, PerkinElmer, Waltham, Massachusetts, United States), 1 mL of sample volume was digested in 2 mL 8:1 analytical grade HNO_3 : H_2SO_4 mixture (v/v) at 70 °C for 24 h. Eu concentrations in PS-Eu suspensions were found to increase linearly (R^2 = 0.99 for 150 nm particles and R^2 = 0.98 for 15 µm particles) with gravimetrically determined particle mass, indicating a homogeneous integration of $Eu(tta)_3$ across PS particles. Mass percentages of Eu in PS particles were 1.13 ± 0.1% and 0.4 ± 0.1% (mean ± standard deviation) for 150 nm and 15 µm particles, respectively.

Method validation was performed in medium from the experimental site, along gravimetrically determined dilution ranges of PS-Eu stock suspensions prepared in duplicate (100 mg L^{-1} – 0.1 mg L^{-1} / 5.3×10^{13} – 5.3×10^{10} particles L^{-1} for 150 nm particles and 100 mg L^{-1} – 0.1 mg L^{-1} / 5.3×10^{7} – 5.3×10^{5} particles L^{-1} for 15 µm particles). Limits of detection and quantification (LoD and LoQ) were calculated as 3.3σ/S and 10σ/S (with σ denoting the standard deviation from 6 matrix control replicates, and S denoting the slope of the calibration curve), and determined at 0.008 (LoD) and 0.009 (LoQ) mg PS-Eu L^{-1} for 150 nm particles, and 0.07 (LoD) and 0.27 (LoQ) mg PS-Eu L^{-1} for 15 µm particles (SI Fig. S3).

2.4 *In situ* characterization of PS-Eu particles and Eu release tests

Aggregation and sedimentation rates of 150 nm PS-Eu particles were assessed in water from the experimental site through dynamic light scattering (DLS, using a Malvern Zetasizer Ultra as described in Section 2.2). Water was pre-filtered over 0.45 µm cellulose filters (Millipore, Massachusetts, USA) to reduce background noise and suspensions were prepared at concentrations of 1 mg L^{-1} (n = 5) to enable adequate detection. Sedimentation rates were estimated based on derived count rates, which provides a measure of scattering intensity and decreases as particles settle out of suspension. Upon incubation, hydrodynamic diameters immediately showed a small increase relative to pristine particle sizes (236.2 ± 4.4 nm), indicative of (hetero)aggregation (SI Fig. S4). Derived count rates decreased by ~35 % within 24 h of incubation, after which suspensions stabilized in terms of all measured parameters.

Release of Eu from PS-Eu particles was assessed via dialysis in suspensions prepared in water from the experimental site. Particle suspensions were prepared at concentrations of 350 g L^{-1} for 150 nm particles and 1 g L^{-1} for 15 µm particles to enable quantification of low release rates. Incubation took place at 20 °C in pre-hydrated Pur-A-Lyzer™ Mini dialysis tubes with a molecular weight cut-off of 12-14 kDa (Sigma-Aldrich, Burlington, Massachusetts, United States), and samples were collected after one, seven, 11, 18 and 28 days of incubation. Sample preparation and analysis was performed as described in Section 2.3. Release rates (expressed % of total Eu present in particle suspensions) remained < 4 % for 150 nm particles and < 2 % for 15 µm (SI Fig. S5).

2.5 Treatment application and measurements in test systems

Nominal treatment concentrations were 0.024 mg L^{-1} (1.26×10^{10} particles L^{-1}) for 150 nm particles and 2.35 mg L^{-1} (1.26×10^{6} particles L^{-1}) for 15 µm particles, resulting in an equivalent particle surface area being applied for both particles sizes (i.e., 8.8×10^{14} nm^2 L^{-1}). Treatment concentrations for 150 nm particles were based on the higher end of reported concentrations of polystyrene nanoplastics in natural freshwaters (0 - 0.024 mg L^{-1}) by Materić et al., (2022), and concentrations for 15 µm particles were aligned bases on equivalent surface areas. To ensure applications resulted in a spatially homogeneous distribution of treatments across the test systems, stock suspensions were prepared in 10 L of demineralized water which were stirred gently and evenly across the surface of each mesocosm immediately after preparation. 350 mL spatially integrated water samples were collected throughout the experimental period, of which a 50 mL subsample was stored for analysis of PS-Eu concentrations. Sample preparation and analysis was performed as described in Section 2.3.

2.6 Macroinvertebrate sampling

Macroinvertebrates were sampled two weeks prior to treatment application, and at two- and eleven weeks after treatment application. To minimize disturbance and enable repeated sampling from the same mesocosms, macroinvertebrates were sampled in ~170 L sections (i.e., one running meter per sample, and ~20% of the total volume per mesocosm), isolated from the far end of each mesocosm using a stainless steel sheet. Subsections were sampled using a dip net fitted with a 150 µm mesh to capture pelagic macroinvertebrates. Sweeping was continued until no additional specimens were collected. Subsequently, the upper 3–5 cm layer of sediment was retrieved and sieved over a 500 µm stainless steel mesh to collect benthic macroinvertebrates. All collected specimens were sorted into unique morphospecies, counted and identified on-site to the lowest taxonomic level feasible using an inhouse identification guide. Subsampling was performed for highly abundant taxa, and total abundances were back transformed to the total sample volume prior to analysis. Organisms were returned to their original mesocosm after identification.

2.7 Emerging insect sampling

Emerging (aquatic) insects were collected using pyramid-shaped emergence traps constructed from stainless steel (dimensions: 60 × 60 × 74 cm; depth × width × height), covered with white insect netting (300 holes/cm^2). Traps were placed in the center of each mesocosm, where they remained until the end of the experiment, and lower ends were submerged in the water layer by ~1-2 cm to prevent escape from trapped specimens. A collection bottle containing 70% ethanol was attached to an opening at the top of each trap to capture emerging insects, following a design adapted from Cadmus et al. (2016) and described in detail in Barmentlo et al. (2021). Collection bottles were retrieved and replaced with fresh ethanol at two-week intervals for a total of 12 weeks, starting two weeks post-treatment.

Collected insects were transferred to CTAB buffer and stored at -20 °C until morphological identification. All specimens were identified to the order level, and Coleoptera and Diptera were identified to family level, following identification or field guides by Chinery (2010), Cranston (2004) and Oosterbroek & Jong (2012).

2.8 eDNA sampling and extraction

Water samples for eDNA extraction were collected every week for the first seven weeks, starting one week before the treatment application, and every other week for the remaining seven weeks, totaling to ten sampling moments. Each sample consisted of 500 mL of water, and was collected as 50 mL subsamples distributed homogeneously across the length, width and depth of each mesocosm, aiming to collect DNA from the full aquatic invertebrate community. Samples were collected using sterilized 50 mL syringes and stored in Nalgene bottles at 4 °C until filtration. eDNA was concentrated onto 0.45 µm polyethersulfone (PES) filters (Sterlitech Corporation), loaded on Nalgene filter units (Thermo Fisher, Waltham, Massachusetts, United States) connected to a vacuum pump (filtered volume per sample: 300 mL). Filtration took place on-site and within hours after samples were collected. A negative field control was collected by filtering sterile water alongside the actual environmental samples. Filters were stored in CTAB at -20°C until DNA extraction. All materials were sterilized in between steps using diluted bleach, and were subsequently rinsed with demineralized water. DNA extraction took place one year after collection in a dedicated DNA lab and was performed using the CTAB protocol from Turner et al. (2014), modified by (Beentjes et al., 2021). No additional post-extraction clean-up steps were needed. Extraction controls were included for each sampling week by omitting the initial step of the extraction protocol and proceeding with a sterile water sample instead. Two positive control samples were also added to the total set of samples, being a DNA extract from *Palaemon serratus* and *Scoloplos armiger*, obtained through Naturalis Biodiversity Center (Leiden, the Netherlands). These were used in the downstream process to assess contamination and tag-jumping. (see section 2.9). Final extracts were stored at -20 °C until library preparation.

2.9 Library preparation

The COI marker was used to amplify invertebrate DNA, using the mICOIintF and jgHCO2198 primers (Leray et al., 2013). A two-step PCR protocol was employed to generate dual-indexed Illumina amplicon libraries using primers extended with 5’ Illumina adapter sequences. PCRs contained environmental samples (eDNA), negative field, extraction and PCR samples and positive control samples. The first PCR was performed in triplicate to minimize PCR bias. The PCR reaction was carried out in a 20 µL volume containing 10 µL of 2× TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Thermo Fisher Scientific), 1 µL of each primer at a concentration of 10 µM, 3 µL of template DNA, and nuclease-free water to reach the final volume. A touchdown PCR protocol was used to

improve specificity, following the protocol from (Leray et al., 2013). Initial denaturation happened at 95°C for 10 minutes, followed by 16 cycles of 95°C for 15 seconds, 62°C for 30 seconds with a decrement of 1°C per cycle, and 72°C for one minute. This was followed by an additional 24 cycles at 95°C for 15 seconds, 46°C for 30 seconds, and 72°C for one minute, with a final extension at 72°C for five minutes.

After the first PCR, amplification products were visualized using an Invitrogen E-Gel 96 2% SYBR Safe gel (Thermo Fisher Scientific) to assess amplification success. PCR products were then pooled and purified using 0.9× NucleoMag NGS Clean-up and Size Selection beads (Macherey-Nagel). The second PCR was performed using the same reaction mix and cycling protocol for each marker, with a unique set of Illumina IDT10 indexes (Integrated DNA Technologies, Coralville, Iowa, United States) for each sample. The PCR reaction was carried out in a 20 µL volume containing 10 µL of 2× TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Thermo Fisher Scientific), 1.5 µL of each index primer, 2 µL of template DNA, and nuclease-free water to reach the final volume. The thermal cycling conditions consisted of an initial denaturation at 95°C for three minutes, followed by 10 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for one minute, with a final extension at 72°C for five minutes. The indexed amplicon libraries were assessed using a TapeStation system 4200 (Agilent Technologies, California, United States) with a D1000 ScreenTape Assay to verify fragment size and quality. Libraries were then equimolarly pooled using an OT-2 liquid-handling robot (Opentrons, New York, United States). A final bead-based clean-up was performed before sequencing. Sequencing was carried out on an Illumina NovaSeq 6000 SP (250 PE flow cell) platform by Macrogen Europe (Amsterdam, the Netherlands).

2.10 Bioinformatics

Raw sequencing data were processed using QIIME 2 2023.7 (Bolyen et al., 2019). Reads were demultiplexed, and primer and adapter sequences were removed using *qiime cutadapt trim-paired* (Martin, 2011), allowing a maximum error rate of 0.2 and requiring a minimum overlap of 5 bp. Reads lacking primer sequences were discarded. Subsequent denoising, merging, chimera removal, and amplicon sequence variant (ASV) inference were performed using *qiime dada2 denoise-paired*, with sequences truncated at 225 bp in both forward and reverse directions (Callahan et al., 2016). Taxonomic classification was conducted using a Naïve Bayes classifier trained on the MIDORI2 COI reference database (GB264 release; Leray et al., 2022). The classifier was trained with the *qiime feature-classifier fit-classifier-naive-bayes* command (Bokulich et al., 2018; Pedregosa et al., 2011). As COI is a highly variable marker, it often shows substantial intraspecific diversity, which can inflate diversity estimates when using unclustered ASVs. Clustering into operational taxonomic units (OTUs), or molecular OTUs (MOTUs), provides a more appropriate resolution for species-level ecological and monitoring studies in eukaryotes (Antich et al., 2021). Therefore, ASV's were subsequently clustered

into operational taxonomic units (OTUs) using *qiime feature-classifier classify-consensus-vsearch* (even though ASVs offer high- resolution data), with a sequence identity threshold of 90% and a minimum consensus of 70% (Rognes et al., 2016).

OTUs were retained for downstream analysis if they were classified to at least the phylum level and belonged to invertebrate taxa. Because the initial classification yielded a relatively low proportion of confident taxonomic assignments, a second round of taxonomic assignment was performed using BLASTn against the full NCBI GenBank nucleotide (nt) database (Sayers et al., 2025). The top ten hits for each OTU were retrieved and used for lowest common ancestor (LCA) analysis. To reduce computational load, this second classification step was applied only after preliminary filtering had reduced the number of OTUs to a manageable subset (SI table S1). To minimize the influence of tag-jumping, normalization was performed based on the positive control samples (Rodriguez-Martinez et al., 2023). Specifically, the highest relative read abundance of the positive control OTUs in environmental samples was determined and used as a threshold (0.0542% RRA): OTUs with read abundances below this threshold within any sample were removed from that sample (i.e., set to zero reads). To further reduce false positives stemming from laboratory or field contamination, OTUs with the highest read count in any negative control sample were removed from the entire dataset (Drake et al., 2022). Additionally, OTUs detected in field control samples were excluded if their read counts exceeded 10% of the maximum read count observed in environmental samples. As an additional conservative filtering step, any OTU that occurred only once within a treatment-timepoint combination was set to zero for that combination. Rarefaction curves were generated using the *vegan* package in R (Oksanen et al., 2022; R Team, 2022) to assess sequencing depth and determine an appropriate rarefaction threshold, which was then applied. The rarefied dataset was used for diversity analyses.

OTU read data were converted to binary occurrence (i.e., presence-absence) data prior to analysis, assuming that read abundance does not reliably reflect true organismal abundance in invertebrate eDNA datasets. The obtained dataset showed relatively low OTU counts per sample and high variability among replicates. To reduce stochastic variation in OTU detection, minimize false negatives, and improve the reliability of observed community patterns, sampling timepoints were grouped in pairs. This resulted in one pre-treatment and four post-treatment composite timepoints. The resulting dataset was split into a macroinvertebrate and microinvertebrate dataset (the latter including zooplankton and smaller taxa), allowing assessment of potential MNP effects on distinct size-based community assemblages. Statistical analyses were performed on the full dataset as well as on both subsets separately.

2.11 Data analysis

Treatment effects on invertebrate communities were evaluated using multiple diversity metrics. For morphology-based macroinvertebrate data, alpha diversity was assessed using taxonomic richness, total abundance, and Shannon–Wiener diversity. Beta diversity was calculated using both Bray–Curtis and Sørensen dissimilarity indices. Additionally, Bray–Curtis dissimilarity was computed on $\log_{10}$-transformed abundance data to reduce the dominance of highly abundant taxa and to better capture more subtle treatment effects. Emerging insect responses were assessed based on cumulative abundance data. Abundances were $\log_{10}$-transformed prior to analysis. Given the low abundances obtained across taxa, only data for the Chironomidae family were analyzed statistically. For eDNA-based assessments, OTU richness and Sørensen dissimilarity were used to characterize community-level effects. No abundance-based analyses (e.g., total read counts, Shannon–Wiener diversity, Bray–Curtis dissimilarity) were conducted for eDNA data, as COI metabarcoding read abundances are known to correlate poorly with actual abundances (Elbrecht & Leese, 2015; Shelton et al., 2023). Potential impacts at the level of individual OTUs were assessed by calculating changes in OTU occupancy (i.e., the number of mesocosms in which an OTU was detected) across mesocosms within treatment groups over time (Δ occurrence).

Effects of treatments and time on alpha diversity metrics—including cumulative abundance of emerging insects—were analyzed using generalized additive models (GAMs) assessed with the *gam* function from the *mgcv* package (Wood, 2017). Treatment was included as a fixed effect, while time was modeled using a smoothing function with a basis dimension (k) set to match the number of sampling moments (i.e., three for the morphology data, six for the emerging insect data and five for the eDNA data). To account for repeated measures across experimental replicates, mesocosm identity was included as a random effect using a random smooth (s(mesocosm_ID, bs = "re")). Models were fitted using restricted maximum likelihood (REML). Post hoc comparisons were conducted based on estimated marginal means, using the *emmeans* package (Lenth, 2024). Beta diversity responses to treatment and time were assessed through PERMANOVAs, using the *adonis2* function from the *vegan* package (Oksanen et al., 2022), incorporating mesocosm identity as a random effect. Post hoc pairwise comparisons were performed separately for each treatment and timepoint using the *pairwise.adonis2* function (Martinez Arbizu, 2017). Beta dispersion was evaluated using the *betadisper* function (*vegan* package), followed by Tukey's Honest Significant Difference (HSD) test for multiple comparisons. For all assessments containing multiple testing, *p*-values were adjusted using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Pre-treatment differences between communities in alpha diversity were tested with ANOVAs and beta diversity with PERMANOVAs to ensure communities were not significantly different from the start. All statistical analyses were performed in R 4.5.1 (R Core Team,

2024). Results were considered statistically significant at $p < 0.05$, and where p-values were adjusted for multiple testing, these were reported as such.

3. Results

3.1 Fate of PS-Eu MNPs in experimental system

PS-Eu concentrations in water samples collected immediately after treatment application were 2.47 ± 0.17 and 0.02 ± 0.01 mg PS-Eu L^{-1} for 150 nm and 15 µm particles, respectively (Fig. 1). These concentrations correspond to $1.32 \times 10^6 \pm 9.3 \times 10^4$ and $1.16 \times 10^{10} \pm 9.3 \times 10^8$ particles L^{-1}, and 105 ± 7.2 and 91 ± 7.5 % of nominally applied concentrations. PS-Eu concentrations initially showed a decline in both treatments over a period of 3 weeks, with reductions being most rapid and pronounced in 15 µm treatments. Water column concentrations subsequently fluctuated throughout the remainder of the experiment, likely due to a combination of sedimentation and resuspension processes driven by evapotranspiration, biological transport, and sample collection. Overall, 15 µm treatments showed a more substantial reduction from nominally applied treatment concentrations than 150 nm treatments, likely due to higher natural sedimentation rates associated with larger particle size. Time-weighted average concentrations were 1.08 ± 0.10 mg PS-Eu L^{-1} for 150 nm and 0.02 ± 0.01 mg PS-Eu L^{-1} for 15 µm particles.

3.2 Morphology-based assessment of macroinvertebrate responses

A total of 73,152 invertebrate specimens were collected over the course of the experiment, representing 64 morphospecies (SI Fig. S6A). Arthropods comprised the majority of collected invertebrates, which in terms of taxonomic diversity were dominated by Coleoptera (beetles, 16 morphospecies), Hemiptera (true bugs, 11 morphospecies), and Diptera (true flies, 11 morphospecies). Analysis of alpha diversity metrics revealed no significant differences between either treatment and controls on overall abundance ($F_{(2, 21)} = 0.371$, $p = 0.695$, $\eta^2 = 0.03$), species richness ($F_{(2, 21)} = 1.748$, $p = 0.199$, $\eta^2 = 0.14$), or Shannon–Wiener diversity ($F_{(2, 21)} = 0.705$, $p = 0.505$, $\eta^2 = 0.06$) prior to the start of the experiment (Fig. 2A–C). Beta diversity metrics in turn also showed no significant differences in abundance- (Bray–Curtis: $R^2 = 0.036$, $F_{(2, 21)} = 0.395$, $p = 0.957$), $\log_{10}$-transformed abundance- (log Bray–Curtis: $R^2 = 0.065$, $F_{(2, 21)} = 0.733$, $p = 0.845$), or incidence-based measures (Sørensen: $R^2 = 0.062$, $F_{(2, 21)} = 0.696$, $p = 0.839$) of community dissimilarity before treatments were applied (Fig. 2E–G).

Although the full GAM model indicated a near-significant difference in richness in the 15 µm treatment compared to the control (estimated difference: 2.17 ± 0.96 (mean ± SE), t(60.8) = 2.26, $p = 0.083$, 95% CI: −0.20 to 4.53), none of the pairwise contrasts were statistically significant ($p > 0.05$ for all; Fig. 2A–C). Macroinvertebrate abundances showed a clear positive trend over the course of the experiment, indicating a temporal effect (estimated df = 1.00, F = 14.07, $p < 0.001$), but no significant differences were detected between treatments ($p > 0.05$ for all contrasts; Fig. 2B). Macroinvertebrate

communities displayed significant dissimilarity for conventional Bray–Curtis, Bray–Curtis on log_{10}-transformed data and Sørensen indexes between treatments and controls, and over time (all $p = 0.001$), but no significant interaction effects were observed in this regard (Sørensen: $R^2 = 0.024$, $F_{(2, 66)} = 0.98$, $p = 0.467$; Bray–Curtis: $R^2 = 0.019$, $F_{(2, 66)} = 0.73$, $p = 0.567$; log-Bray–Curtis: $R^2 = 0.021$, $F_{(2, 66)} = 0.92$, $p = 0.499$; Fig. 2E–G). When analyzed per timepoint, Sørensen dissimilarity in the treatment groups showed a diverging trend from controls at two weeks ($R^2 = 0.130$, $F_{(2, 21)} = 1.574$, $p = 0.085$) and eleven weeks post-treatment ($R^2 = 0.141$, $F_{(2, 21)} = 1.730$, $p = 0.072$), although these differences were not statistically significant (Fig 2E). Further examination of community composition revealed that, at two weeks after treatment application, beta dispersion (i.e., within-treatment community dissimilarity) in both 150 nm and 15 µm treatments was significantly higher than in controls, with similar effect sizes (150 nm vs. control: difference = −0.122, 95% CI = −0.228 to −0.016, $p = 0.022$; 15 µm vs. control: difference = −0.110, 95% CI = −0.217 to −0.004, $p = 0.040$; see SI Fig. S7). No significant difference in this regard was detected between the two treatments themselves (difference = −0.012, 95% CI = −0.118 to 0.094, $p = 0.957$).

3.3 Responses in insect emergence

A total of 4,568 insects were collected and identified across 144 samples, with 4,361 belonging to Chironomidae (non-biting midges). The cumulative abundance of emerged Chironomidae differed significantly between treatments, with both the 15 µm and 150 nm treatments showing higher overall emergence rates than controls (back-transformed estimated means: control = 80.6, 15 µm = 120.6, 150 nm = 212.6; $p = 0.036$ for both contrasts; Fig. 2D). However, this difference was driven primarily by a single replicate in the 15 µm treatments, and two replicates in the 150 nm treatments, each of which exhibited a > 15-fold higher abundance than the treatment median. Analyses based on data where these replicates were excluded yielded no significant differences in emergence rates between treatments.

3.4 eDNA-based assessment of responses

Sequencing yielded a total of 175,853,857 raw reads, from which 116,035,452 high-quality reads were retained following filtering and denoising, resulting in 31,528 unique ASVs. Subsequent correction for potential contamination, based on control samples, reduced the dataset to 2,351 ASVs. This corresponded to a 21.7% decrease in total reads, which were then clustered into 1,197 OTUs. Rarefaction curves for all samples reached a plateau at ~7000 reads (SI Fig. S8). After subsequent filtering and data preparation steps, 121 OTUs were retained for analysis (SI Fig. S6B). The filtered dataset was subsequently divided into one dataset for macroinvertebrates and one for microinvertebrates (predominantly zooplankton). The macroinvertebrate subset included all OTUs assigned to the phyla Annelida, Cnidaria, Malacostraca, and Mollusca, as well as the classes Arachnida

and Insecta, totaling to 48 OTUs. The microinvertebrate subset comprised all OTUs from the phyla Gastrotricha and Rotifera, and the classes Branchiopoda, Hexanauplia, and Ostracoda, totaling to 59 OTUs. OTUs from Arthropoda that were not assigned to a lower taxonomic level were included only in the full dataset. A detailed overview of sequencing and filtering results can be found in the supplementary information (SI Table S1).

For all three datasets, OTU richness differed significantly over time across all treatments (GAM smooth term for time in full model: edf = 1.00, $F = 7.05$, $p = 0.009$; macroinvertebrate model: edf = 2.97, $F = 4.21$, $p = 0.004$; micro-invertebrate model: edf = 3.54, $F = 16.05$, $p < 0.001$). An overall decline in richness across treatments was observed over the course of the experiment for the full invertebrate and micro-invertebrate datasets (Fig. 3A and 3C), whereas the macroinvertebrate dataset showed an initial decrease followed by a return to pre-treatment levels (Fig. 3B). No significant differences in OTU richness between communities were observed before- or after treatment application for any of the data sets (full model: $F_{(2, 21)} = 0.947$, $p = 0.404$, $\eta^2 = 0.08$; macroinvertebrate model: $F_{(2, 21)} = 1.421$, $p = 0.264$, $\eta^2 = 0.12$; micro-invertebrate model: $F_{(2, 21)} = 1.854$, $p = 0.181$, $\eta^2 = 0.15$). However, a decline in OTU richness was evident in the 15 µm treatment at the first post-treatment timepoint, which was apparent in both the full dataset (Fig. 3A) and macroinvertebrate dataset (Fig. 3B). While this decline was non-significant for the full dataset (95% CI = −0.25 to 12.00, $p = 0.070$), it was significant for the macroinvertebrate subset, with values at one and two weeks after treatment being on average 3.63 points lower than one and zero weeks before treatment (95% CI = 0.32 to 6.93, $p = 0.021$).

Community composition (assessed via Sørensen dissimilarity) showed significant variation over time and between treatments (Fig. 3D–F). PERMANOVA results for all three datasets revealed significant effects of treatments and time on all invertebrates collectively (Treatment: $R^2 = 0.034$, $F_{(2, 105)} = 2.48$, $p = 0.001$; Time: $R^2 = 0.177$, $F_{(4, 105)} = 6.52$, $p = 0.001$), and macroinvertebrates (Treatment: $R^2 = 0.028$, $F_{(2, 105)} = 1.98$, $p = 0.001$; Time: $R^2 = 0.150$, $F_{(4, 105)} = 5.30$, $p = 0.001$) and microinvertebrates individually (Treatment: $R^2 = 0.036$, $F_{(2, 105)} = 2.66$, $p = 0.001$; Time: $R^2 = 0.186$, $F_{(4, 105)} = 1.25$, $p = 0.001$). Treatments effect were furthermore found to interact significantly with time for each assessed group (all invertebrates: $R^2 = 0.075$, $F_{(8, 105)} = 1.37$, $p = 0.001$; macroinvertebrates: $R^2 = 0.078$, $F_{(8, 105)} = 1.37$, $p = 0.001$; microinvertebrates: $R^2 = 0.068$, $F_{(8, 105)} = 1.25$, $p = 0.013$).

When datasets were analyzed separately by treatment, all groups showed significant temporal shifts in community composition ($R^2 = 0.23$ to 0.29, $F_{(4, 35)} = 2.58$ to 3.51, $p = 0.003$ to 0.002). PERMANOVAs conducted per timepoint indicated significant differences between communities at week one and two after treatment (T1+T2) in all three datasets, and at week five and seven after treatment (T5+T7) in the full dataset. Additionally, significant differences were already present before adding treatments (T-1+T0) in both the full dataset and the micro-invertebrate subset (see Fig. 3D–F for statistical output of PERMANOVA models per timepoint). Pairwise comparisons revealed a

significant difference in community composition between the controls and the 15 µm treatments at one and two weeks post treatment (R^2 = 0.150, $F_{(1, 14)}$ = 2.48, p = 0.030), as well as between the 15 µm and 150 nm treatments at five and seven weeks post treatment (R^2 = 0.121, $F_{(1, 14)}$ = 1.94, p = 0.038) in the full dataset. None of the other pairwise comparisons returned significant differences. Analyses of beta dispersion revealed no significant differences between treatments and control at any of the assessed timepoints (SI Fig. S9A-C), indicating that observed differences in community composition were not due to variation in within-group dispersion.

Relative changes to pre-treatment conditions in occurrences of individual OTUs (SI Fig. S10) and OTUs clustered per taxonomic group (Fig. 4) revealed a predominant decrease in Annelida OTUs in 15 µm treatments directly after treatment application compared to the controls. Within the same timeframe, Gastrotricha occurrences showed a stronger decrease in both treatments in comparison to controls as well. However, prior to treatment application, Gastrotricha already showed occurrences in the mesocosms assigned to the 15 µm and 150 nm treatments that respectively were 1.7 and 1.9 times higher than those in controls.

4. Discussion

The current study examined effects of 15 µm and 150 nm spherical polystyrene (PS) MNPs on freshwater invertebrate communities by integrating morphological identification, monitoring of insect emergence, and environmental DNA (eDNA) metabarcoding. Our goal was to assess long-term population- and community-level impacts of polystyrene MNPs on aquatic invertebrates under ecologically realistic conditions. Our findings show that MNP exposure led to small but statistically significant shifts in community composition, primarily detected through eDNA-based analysis. Interestingly, these changes were observed only in the 15 µm treatment group. However, the community-level response appeared diffuse, with no clear effects on specific taxa, suggesting a subtle and widespread alteration of the invertebrate assemblage rather than targeted impacts.

Neither morphological macroinvertebrate identification nor insect emergence data revealed significant effects of 15 µm and 150 nm treatments. This outcome aligns with several previous studies reporting limited or no ecological responses to MNPs under environmentally realistic conditions (e.g., Klasios et al., 2024; Martínez Rodríguez et al., 2023; Stanković et al., 2022). Other studies have however reported adverse effects on individual organisms at similar or slightly higher concentrations, including morphological changes (Yıldız et al., 2022), altered reproductive output (Cheng et al., 2023) and reduced mobility (Suwaki et al., 2020). Moreover, ingestion of MNPs has been frequently documented across freshwater invertebrate taxa, including in studies conducted under realistic exposure conditions (Langenfeld et al., 2024; Mora-Teddy et al., 2024; Silva, Machado, Campos, Rodrigues, et al., 2022). The findings from the current study suggest that while such processes may indeed be considered reliable

measures of exposure and stress responses at the (sub)-individual level, their propagation to population- and community-level responses under currently realistic environmental concentrations may be minimal, at least for pristine, spherical PS particles in the sizes and concentrations tested in our study. It is however known that different shapes and sizes can have different effects on organisms (Junaid et al., 2023). In this regard, further higher-tiered assessment of impacts from different MNPs (i.e., in terms of size, shape, and composition), and on a wider variety of taxa and conditions, may be considered a critical step in obtaining a more comprehensive understanding of the implications of MNPs in aquatic ecosystems, especially when conducted in parallel with assessments at lower levels.

In contrast to morphology-based approaches, eDNA analyses revealed small but statistically significant shifts in total invertebrate and macroinvertebrate alpha and beta diversity, as well as in microinvertebrate beta diversity, exposed to 15 µm PS particles. The observed effect did not persist, implying that communities either adapted to the exposure or stabilized over time. Since a considerable fraction of PS particles remained suspended in the system throughout the experiment, a reduction in exposure alone is unlikely to explain the diminishing response. The observed reduction of treatment effects is more likely due to biological processes than to a substantial decrease in exposure. Additionally, changing environmental conditions may have influenced the community's response (Moyo, 2022). For instance, the gradual decline in water temperature over the course of the experiment could have lowered metabolic demands, potentially buffering the physiological stress associated with ingesting non-nutritive particles. Conversely, in warmer conditions, such stress might have been amplified due to increased energy requirements and possible nutritional dilution (Kratina et al., 2019). Nonetheless, overall observed patterns were diffuse and could not be attributed to trends of specific taxa, indicating a broad community-level response. Notably, these shifts were minor and temporal, which renders an evaluation of their ecological relevance challenging.

Our results show that effects were observed only for the larger 15 µm particles and not for the 150 nm particles. It is important to note that the two particle treatments were standardized by surface area rather than by mass or particle number. This approach was chosen to facilitate comparison while avoiding unrealistically large differences between treatments—such as those that would arise if normalization were based solely on particle number (resulting in strongly diverging total volumes and masses) or on mass (resulting in strongly diverging particle numbers and with that the chance for organisms to interact with the particles)—and to account for detection limits. However, this design inherently resulted in the 15 µm treatment containing a substantially greater total particle volume and mass (approximately tenfold higher) than the 150 nm treatment. Scherer et al. (2017) showed that MNP ingestion in freshwater invertebrates is size dependent (both determined by particle size and organism size). However, since the observed responses in our study were diffuse and could not be attributed to specific taxa, assuming ingestion as the primary route of effect explaining the difference

in response between the particles sizes remains speculative. If nutritional dilution contributed to the observed responses, as discussed in the previous paragraph, this difference in total particle mass could have played a role. If that is true, the dose then alters the response, as has been shown by Amariei et al. (2022), who explicitly quantified trade-offs between harmful food dilution (microplastics reducing the calorie/nutrient intake) and possible benefits if biofilms grow on plastics (providing some nutrition) in Daphnia magna. It should be noted that speculating on these mechanisms remains hypothetical, as the current results do not allow clear attribution of either the route or the nature of the observed effects.

One of the main advantages of eDNA metabarcoding in community assessments is its high sensitivity, which can help detect a broader range of taxa than obtained through morphological identification. In particular, eDNA enables the detection of small-bodied and cryptic taxa (e.g., Gastrotricha, Rotifera) that are often missed in morphological surveys, and allows for the resolution of closely related species into unique OTUs without the need for expert taxonomic skills (Keck et al., 2017). This can improve taxonomic resolution and increase the likelihood of picking up sensitive species. In this study, however, the number of macroinvertebrate taxa detected was similar between both methods. This suggests that, although eDNA may still have captured more sensitive taxa, the difference is not solely due to a higher overall sensitivity. Another factor to consider is that eDNA allows for more frequent sampling with relatively little additional effort or disturbance to the system. This enabled us to achieve higher temporal resolution than was feasible using morphological identification. Notably, the most pronounced shifts in community composition based on eDNA occurred within the first two weeks after treatment, suggesting that the denser sampling schedule may have contributed to capturing short-term responses that might otherwise have gone undetected. It should be noted that beta diversity models based on eDNA indicated a significant overall treatment effect even at the pre-treatment stage, suggesting that communities were not fully comparable before exposure. Nonetheless, pairwise PERMANOVA tests at this timepoint revealed no significant differences between specific treatments. In contrast, significant pairwise differences emerged post-treatment between the control and 15 µm treatment, and between both treatment groups, indicating a divergence in community composition following exposure. While no strong overall treatment effects were detected, these results highlight the value of eDNA as a sensitive tool for detecting subtle ecological responses under realistic conditions, aligning with the findings of Van der Plas et al. (2025).

Despite the relatively long duration of our experiment, there remains a possibility that longer-term, cumulative, or transgenerational effects may still have gone undetected. This was for instance shown in the study by Redondo-Hasselerharm et al. (2020), although concentrations where effects were observed were higher than those applied in our study. Taxa with longer life cycles or seasonal phenology, such as many macroinvertebrates, may experience exposure for durations that are several-

fold longer than those under which effects were assessed in the current experiment, and this in turn may amplify ultimate effects of stress responses (Haegerbaeumer et al., 2019). Moreover, conventional polymers are highly persistent in the environment, and for many organisms, exposure is likely to take place over numerous consecutive generations (Junaid et al., 2023). For compounds and materials exhibiting such properties, short-term assessments which focus only on part of an organism's life cycle may hold little relevance to realistic exposure conditions, and long-term and multi-generational studies may be considered a more suitable approach towards understanding their ultimate environmental impacts (Nederstigt et al., 2022). Notably, however, impacts in the current experiment were observed at one to two weeks after application of the 15 µm PS treatment, suggesting that initial response to MNP exposure may also hold some degree of relevance, an observation shared by (Yıldız et al., 2022). Any long-term significance of the short-term effects observed in this study cannot be determined based on the current dataset. While the transient nature of the effects suggests limited long-term impact, the possibility of delayed effects emerging over extended time intervals cannot be entirely excluded.

Our outdoor mesocosm experiment was designed to reflect the ecological characteristics of small stagnant ditches, incorporating natural habitat structure, seasonal fluctuations, and realistic community assembly under outdoor conditions. These shallow ditch ecosystems are common in agricultural landscapes and are considered ecologically relevant under the EU Water Framework Directive (WFD 2000/60, EC, 2000). While we observed subtle biological responses to MNP exposure within this context, it is important to acknowledge that different water types may exhibit different exposure dynamics and (community-level) responses (Guo et al., 2024). Therefore, it is uncertain whether the low levels of impact observed here is representative of broader, water type-specific community sensitivities. Additionally, the plastics used in this study were pristine, spherical polystyrene particles without additives. In contrast, environmental plastic pollution is typically more heterogeneous, consisting of irregularly shaped fragments with varying polymer compositions, chemical additives, and adsorbed contaminants (Osman et al., 2023), all of which may modulate ecological effects. As such, the impacts observed here should be viewed as conservative estimates of MNP toxicity on ditch ecosystems.

5. Conclusions & Outlook

The results from the current study provide no evidence that exposure to MNPs at concentrations representative of the higher end of those reported in surface waters cause persistent effects on the structure of freshwater invertebrate communities. However, eDNA metabarcoding revealed subtle, short-term shifts in community composition that were not detected by morphological assessments. These findings underscore the sensitivity of eDNA-based methods for detecting early or minor ecological responses, which may be particularly valuable in higher-tier risk assessments of MNPs.

To this end, we note that the multi-facetted nature of plastic pollution in natural environments, characterized by complex mixtures consisting of different polymers and their additives, as well as varying particle shapes and sizes, remains a key obstacle for acquiring a comprehensive understanding of the environmental risks associated with MNPs. Addressing this complexity in higher-tier experimental designs is especially challenging due to practical limitations in treatment diversity, replication, and sampling frequency. In this context, our findings suggest that eDNA metabarcoding constitutes a promising tool for addressing these challenges by enabling high-resolution monitoring of community responses, while simultaneously reducing sampling effort.

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

During the preparation of this work the author(s) used ChatGPT in order to check and improve language and grammar. 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.

References

Amariei, G., Rosal, R., Fernández-Piñas, F., & Koelmans, A. A. (2022). Negative food dilution and positive biofilm carrier effects of microplastic ingestion by D. magna cause tipping points at the population level. *Environmental Pollution*, *294*(September 2021). https://doi.org/10.1016/j.envpol.2021.118622

Antich, A., Palacin, C., Wangensteen, O. S., & Turon, X. (2021). To denoise or to cluster, that is not the question: optimizing pipelines for COI metabarcoding and metaphylogeography. *BMC Bioinformatics*, *22*(1), 1–24. https://doi.org/10.1186/s12859-021-04115-6

Araújo, C. V. M., Moreira-Santos, M., & Ribeiro, R. (2016). Active and passive spatial avoidance by aquatic organisms from environmental stressors: A complementary perspective and a critical review. *Environment International*, *92–93*, 405–415. https://doi.org/10.1016/j.envint.2016.04.031

Araújo, C. V. M., Pereira, K. C., Sparaventi, E., González-Ortegón, E., & Blasco, J. (2020). Contamination may induce behavioural plasticity in the habitat selection by shrimps: A cost-benefits balance involving contamination, shelter and predation. *Environmental Pollution*, *263*. https://doi.org/10.1016/j.envpol.2020.114545

Barmentlo, S. H., Schrama, M., de Snoo, G. R., van Bodegom, P. M., van Nieuwenhuijzen, A., & Vijver, M. G. (2021). Experimental evidence for neonicotinoid driven decline in aquatic emerging insects. *Proceedings of the National Academy of Sciences*, *118*(44), 2–9. https://doi.org/10.1073/pnas.2105692118

Beentjes, K. K., Barmentlo, S. H., Cieraad, E., Schilthuizen, M., van der Hoorn, B. B., Speksnijder, A. G. C. L. C. L., & Trimbos, K. B. (2021). Environmental DNA metabarcoding reveals comparable responses to agricultural stressors on different trophic levels of a freshwater community. *Molecular Ecology*, *31*(5), 1430–1443. https://doi.org/10.1111/mec.16326

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate : A practical and powerful approach to multiple testing author (s): Yoav Benjamini and Yosef Hochberg Source : Journal of the Royal Statistical Society . Series B (Methodological), Vol . 57 , No . 1 (1995), Publi. *Journal of the Royal Statistical Society*, *57*(1), 289–300.

Besseling, E., Wang, B., Lürling, M., & Koelmans, A. A. (2014). Nanoplastic affects growth of S. obliquus and reproduction of D. magna. *Environmental Science and Technology*, *48*(20), 12336–12343. https://doi.org/10.1021/es503001d

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 1–17. https://doi.org/10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Brooks, J. M., Stewart, C. J., Haberstroh, C. J., & Arias, M. E. (2023). Characteristics and fate of plastic pollution in urban stormwater ponds. *Environmental Pollution*, *320*(January), 121052. https://doi.org/10.1016/j.envpol.2023.121052

Cadmus, P., Pomeranz, J. P. F., & Kraus, J. M. (2016). Low-cost floating emergence net and bottle trap: comparison of two designs. *Journal of Freshwater Ecology*, *31*(4), 653–658.

https://doi.org/10.1080/02705060.2016.1217944

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Caputo, F., Vogel, R., Savage, J., Vella, G., Law, A., Della Camera, G., Hannon, G., Peacock, B., Mehn, D., Ponti, J., Geiss, O., Aubert, D., Prina-Mello, A., & Calzolai, L. (2021). Measuring particle size distribution and mass concentration of nanoplastics and microplastics: addressing some analytical challenges in the sub-micron size range. *Journal of Colloid and Interface Science*, *588*, 401–417. https://doi.org/10.1016/j.jcis.2020.12.039

Castro-Castellon, A. T., Horton, A. A., Hughes, J. M. R., Rampley, C., Jeffers, E. S., Bussi, G., & Whitehead, P. (2022). Ecotoxicity of microplastics to freshwater biota: Considering exposure and hazard across trophic levels. *Science of the Total Environment*, *816*, 151638. https://doi.org/10.1016/j.scitotenv.2021.151638

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., & Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the Total Environment*, *584–585*, 1022–1031. https://doi.org/10.1016/j.scitotenv.2017.01.156

Cheng, S., Jessica, Yoshikawa, K., & Cross, J. S. (2023). Effects of nano/microplastics on the growth and reproduction of the microalgae, bacteria, fungi, and Daphnia magna in the microcosms. *Environmental Technology and Innovation*, *31*, 103211. https://doi.org/10.1016/j.eti.2023.103211

Chinery, M. (2010). *de Nieuwe Insectengids*. Tirion Natuur.

Cranston, P. S. (2004). Insecta : Diptera , Chironomidae. In *The freshwater invertebrates of the Malaysia region* (pp. 710–734).

De Felice, B., Sabatini, V., Antenucci, S., Gattoni, G., Santo, N., Bacchetta, R., Ortenzi, M. A., & Parolini, M. (2019). Polystyrene microplastics ingestion induced behavioral effects to the cladoceran Daphnia magna. *Chemosphere*, *231*, 423–431. https://doi.org/10.1016/j.chemosphere.2019.05.115

De Marco, G., Conti, G. O., Giannetto, A., Cappello, T., Galati, M., Iaria, C., Pulvirenti, E., Capparucci, F., Mauceri, A., Ferrante, M., & Maisano, M. (2022). Embryotoxicity of polystyrene microplastics in zebrafish Danio rerio. *Environmental Research*, *208*(December 2021), 112552. https://doi.org/10.1016/j.envres.2021.112552

de Ruijter, V. N., Redondo-Hasselerharm, P. E., & Koelmans, A. A. (2025). A brief history of microplastics effect testing: Guidance and prospect. *Environmental Pollution*, *368*(October 2024), 125711. https://doi.org/10.1016/j.envpol.2025.125711

Drake, L. E., Cuff, J. P., Young, R. E., Marchbank, A., Chadwick, E. A., & Symondson, W. O. C. (2022). An assessment of minimum sequence copy thresholds for identifying and reducing the prevalence of artefacts in dietary metabarcoding data. *Methods in Ecology and Evolution*, *13*(3), 694–710. https://doi.org/10.1111/2041-210X.13780

Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS ONE*, *10*(7), 1–16. https://doi.org/10.1371/journal.pone.0130324

Eltemsah, Y. S., & Bøhn, T. (2019). Acute and chronic effects of polystyrene microplastics on juvenile and adult Daphnia magna. *Environmental Pollution*, *254*, 112919.

https://doi.org/10.1016/j.envpol.2019.07.087

European Commission. (2000). Directive 2000/60. *Journal of the European Communities*, *327*, 1–72.

Fediajevaite, J., Priestley, V., Arnold, R., & Savolainen, V. (2021). Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*, *11*, 4803–4815. https://doi.org/10.1002/ece3.7382

Ferreira, C. S. S., Venâncio, C., & Oliveira, M. (2023). Nanoplastics and biota behaviour: Known effects, environmental relevance, and research needs. *TrAC - Trends in Analytical Chemistry*, *165*. https://doi.org/10.1016/j.trac.2023.117129

Guo, M., Noori, R., & Abolfathi, S. (2024). Microplastics in freshwater systems: Dynamic behaviour and transport processes. *Resources, Conservation and Recycling*, *205*(April), 107578. https://doi.org/10.1016/j.resconrec.2024.107578

Haegerbaeumer, A., Mueller, M. T., Fueser, H., & Traunspurger, W. (2019). Impacts of micro- and nano-sized plastic particles on benthic invertebrates: A literature review and gap analysis. *Frontiers in Environmental Science*, *7*(FEB). https://doi.org/10.3389/fenvs.2019.00017

Hill, M. J., Sayer, C. D., & Wood, P. J. (2016). When is the best time to sample aquatic macroinvertebrates in ponds for biodiversity assessment? *Environmental Monitoring and Assessment*, *188*(3), 1–11. https://doi.org/10.1007/s10661-016-5178-6

Junaid, M., Liu, S., Chen, G., Liao, H., & Wang, J. (2023). Transgenerational impacts of micro(nano)plastics in the aquatic and terrestrial environment. *Journal of Hazardous Materials*, *443*(PB), 130274. https://doi.org/10.1016/j.jhazmat.2022.130274

Keck, F., Vasselon, V., Tapolczai, K., Rimet, F., & Bouchez, A. (2017). Freshwater biomonitoring in the Information Age. *Frontiers in Ecology and the Environment*, *15*(5), 266–274. https://doi.org/10.1002/fee.1490

Klasios, N., Kim, J. O., & Tseng, M. (2024). No Effect of Realistic Concentrations of Polyester Microplastic Fibers on Freshwater Zooplankton Communities. *Environmental Toxicology and Chemistry*, *43*(2), 418–428. https://doi.org/10.1002/etc.5797

Kratina, P., Watts, T. J., Green, D. S., Kordas, R. L., & O'Gorman, E. J. (2019). Interactive effects of warming and microplastics on metabolism but not feeding rates of a key freshwater detritivore. *Environmental Pollution*, *255*, 113259. https://doi.org/10.1016/j.envpol.2019.113259

Langenfeld, D., Bucci, K., Veneruzzo, C., McNamee, R., Gao, G., Rochman, C. M., Rennie, M. D., Hoffman, M. J., Orihel, D. M., Provencher, J. F., Higgins, S. N., & Paterson, M. J. (2024). Microplastics at Environmentally Relevant Concentrations Had Minimal Impacts on Pelagic Zooplankton Communities in a Large In-Lake Mesocosm Experiment. *Environmental Science and Technology*, *58*(43), 19419–19428. https://doi.org/10.1021/acs.est.4c05327

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *Science of the Total Environment*, *619–620*, 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103

Lenth, R. (2024). *emmeans: Estimated marginal means, aka least-squares means (Version 1.10.0) [R package]. CRAN.* https://cran.r-project.org/package=emmeans

Lenz, R., Enders, K., & Nielsen, T. G. (2016). Microplastic exposure studies should be environmentally realistic. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(29), E4121–E4122. https://doi.org/10.1073/pnas.1606615113

Leray, M., Knowlton, N., & Machida, R. J. (2022). MIDORI2: A collection of quality controlled, preformatted, and regularly updated reference databases for taxonomic assignment of eukaryotic mitochondrial sequences. *Environmental DNA*, *4*(4), 894–907. https://doi.org/10.1002/edn3.303

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 1–14. https://doi.org/10.1186/1742-9994-10-34

Leterme, S. C., Tuuri, E. M., Drummond, W. J., Jones, R., & Gascooke, J. R. (2023). Microplastics in urban freshwater streams in Adelaide, Australia: A source of plastic pollution in the Gulf St Vincent. *Science of the Total Environment*, *856*(May 2022), 158672. https://doi.org/10.1016/j.scitotenv.2022.158672

Liro, M., Zielonka, A., & van Emmerik, T. H. M. (2023). Macroplastic fragmentation in rivers. *Environment International*, *180*(September), 108186. https://doi.org/10.1016/j.envint.2023.108186

Luo, Y., Li, L., Feng, Y., Li, R., Yang, J., Peijnenburg, W. J. G. M., & Tu, C. (2022). Quantitative tracing of uptake and transport of submicrometre plastics in crop plants using lanthanide chelates as a dual-functional tracer. *Nature Nanotechnology*, *17*(4), 424–431. https://doi.org/10.1038/s41565-021-01063-3

Macaulay, S. J., Jeppesen, E., Riebesell, U., Nejstgaard, J. C., Berger, S. A., Lewandowska, A. M., Rico, A., Kefford, B. J., Vad, C. F., Costello, D. M., Wang, H., Pimentel, I. M., Ramos, J. B. e, González, J., Spilling, K., Domis, L. de S., Boersma, M., Stockenreiter, M., Meerhoff, M., … Piggott, J. J. (2025). Addressing grand ecological challenges in aquatic ecosystems: how can mesocosms be used to advance solutions? *OIKOS*, *e11020*, 1–23. https://doi.org/10.1111/oik.11020

Marchant, D. J., Martínez Rodríguez, A., Francelle, P., Jones, J. I., & Kratina, P. (2023). Contrasting the effects of microplastic types, concentrations and nutrient enrichment on freshwater communities and ecosystem functioning. *Ecotoxicology and Environmental Safety*, *255*(September 2022), 114834. https://doi.org/10.1016/j.ecoenv.2023.114834

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal2*, *17*(1), pp-10. https://doi.org/doi:10.14806/ej.17.1.200

Martinez Arbizu, P. (2017). *pairwiseAdonis: Pairwise Multilevel Comparison using Adonis* (p. R package version 0.4.1).

Martínez Rodríguez, A., Marchant, D. J., Francelle, P., Kratina, P., & Jones, J. I. (2023). Nutrient enrichment mediates the effect of biodegradable and conventional microplastics on macroinvertebrate communities. *Environmental Pollution*, *337*(June), 122511. https://doi.org/10.1016/j.envpol.2023.122511

Materić, D., Peacock, M., Dean, J., Futter, M., Maximov, T., Moldan, F., Röckmann, T., & Holzinger, R. (2022). Presence of nanoplastics in rural and remote surface waters. *Environmental Research Letters*, *17*(5). https://doi.org/10.1088/1748-9326/ac68f7

Mitrano, D. M., Beltzung, A., Frehland, S., Schmiedgruber, M., Cingolani, A., & Schmidt, F. (2019). Synthesis of metal-doped nanoplastics and their utility to investigate fate and behaviour in complex environmental systems. *Nature Nanotechnology*, *14*(4), 362–368. https://doi.org/10.1038/s41565-018-0360-3

Mora-Teddy, A. K., Closs, G. P., & Matthaei, C. D. (2024). Microplastics and riverine

macroinvertebrate communities in a multiple-stressor context: A mesocosm approach. *Science of the Total Environment*, *951*(August), 175456. https://doi.org/10.1016/j.scitotenv.2024.175456

Moyo, S. (2022). An enigma: A meta-analysis reveals the effect of ubiquitous microplastics on different taxa in aquatic systems. *Frontiers in Environmental Science*, *10*(September), 1–17. https://doi.org/10.3389/fenvs.2022.999349

Nava, V., Chandra, S., Aherne, J., Alfonso, M. B., Antão-Geraldes, A. M., Attermeyer, K., Bao, R., Bartrons, M., Berger, S. A., Biernaczyk, M., Bissen, R., Brookes, J. D., Brown, D., Cañedo-Argüelles, M., Canle, M., Capelli, C., Carballeira, R., Cereijo, J. L., Chawchai, S., … Leoni, B. (2023). Plastic debris in lakes and reservoirs. *Nature*, *619*(7969), 317–322. https://doi.org/10.1038/s41586-023-06168-4

Nederstigt, T. A. P., Peijnenburg, W. J. G. M., Blom, R., & Vijver, M. G. (2022). Correlation analysis of single- and multigenerational endpoints in Daphnia magna toxicity tests: A case-study using TiO2 nanoparticles. *Ecotoxicology and Environmental Safety*, *241*(February), 113792. https://doi.org/10.1016/j.ecoenv.2022.113792

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., … Weedon, J. (2022). *vegan: Community Ecology Package* (R package version 2.6-4). https://cran.r-project.org/package=vegan

Oosterbroek, P., & Jong, H. de. (2012). *De Europese families van muggen en vliegen (Diptera). Determinatie, diagnose, biologie.* KNNV Uitgeverij.

Osman, A. I., Hosny, M., Eltaweil, A. S., Omar, S., Elgarahy, A. M., Farghali, M., Yap, P. S., Wu, Y. S., Nagandran, S., Batumalaie, K., Gopinath, S. C. B., John, O. D., Sekar, M., Saikia, T., Karunanithi, P., Hatta, M. H. M., & Akinyede, K. A. (2023). Microplastic sources, formation, toxicity and remediation: a review. In *Environmental Chemistry Letters* (Vol. 21, Issue 4). Springer International Publishing. https://doi.org/10.1007/s10311-023-01593-3

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D., Brucher, M., Perrot, M., & Duchesnay, É. (2011). Scikit-learn: Machine Learning in Python. *Journal OfMachine Learning Research*, *12*, 2825–2830. https://doi.org/10.4018/978-1-5225-9902-9.ch008

Qian, J., Tang, S., Wang, P., Lu, B., Li, K., Jin, W., & He, X. (2021). From source to sink: Review and prospects of microplastics in wetland ecosystems. *Science of the Total Environment*, *758*, 143633. https://doi.org/10.1016/j.scitotenv.2020.143633

R Core Team. (2024). *R: A Language and Environment for Statistical Computing* (Version 4.5.1).

Redondo-Hasselerharm, P. E., Gort, G., Peeters, E. T. H. M., & Koelmans, A. A. (2020). Nano- And microplastics affect the composition of freshwater benthic communities in the long term. *Science Advances*, *6*(5). https://doi.org/10.1126/sciadv.aay4054

Rodriguez-Martinez, S., Klaminder, J., Morlock, M. A., Dalén, L., & Huang, D. Y. T. (2023). The topological nature of tag jumping in environmental DNA metabarcoding studies. *Molecular Ecology Resources*, *23*(3), 621–631. https://doi.org/10.1111/1755-0998.13745

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *2016*(10), 1–22. https://doi.org/10.7717/peerj.2584

Sayers, E., Beck, J., Bolton, E., Brister, J., Chan, J., Connor, R., Feldgarden, M., Fine, A., Funk, K., Hoffman, J., Kannan, S., Kelly, C., Klimke, W., Kim, S., Lathrop, S., Marchler-Bauer, A., Murphy,

T., O'Sullivan, C., Schmieder, E., … Pruitt, K. (2025). Database resources of the National Center for Biotechnology Information in 2025. *Nucleic Acids Research*, *53*, 20–29.

Scherer, C., Brennholt, N., Reifferscheid, G., & Wagner, M. (2017). Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. *Scientific Reports*, *7*(1), 1–9. https://doi.org/10.1038/s41598-017-17191-7

Shelton, A. O., Gold, Z. J., Jensen, A. J., D'Agnese, E., Andruszkiewicz Allan, E., Van Cise, A., Gallego, R., Ramón-Laca, A., Garber-Yonts, M., Parsons, K., & Kelly, R. P. (2023). Toward quantitative metabarcoding. *Ecology*, *104*(2), 1–18. https://doi.org/10.1002/ecy.3906

Silva, C. J. M., Machado, A. L., Campos, D., Rodrigues, A. C. M., Patrício Silva, A. L., Soares, A. M. V. M., & Pestana, J. L. T. (2022). Microplastics in freshwater sediments: Effects on benthic invertebrate communities and ecosystem functioning assessed in artificial streams. *Science of the Total Environment*, *804*. https://doi.org/10.1016/j.scitotenv.2021.150118

Silva, C. J. M., Machado, A. L., Campos, D., Soares, A. M. V. M., & Pestana, J. L. T. (2022). Combined effects of polyethylene microplastics and natural stressors on Chironomus riparius life-history traits. *Environmental Research*, *213*(May). https://doi.org/10.1016/j.envres.2022.113641

Silva, C. J. M., Patrício Silva, A. L., Campos, D., Machado, A. L., Pestana, J. L. T., & Gravato, C. (2021). Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by Chironomus riparius (Diptera) larvae. *Journal of Hazardous Materials*, *402*(August 2020). https://doi.org/10.1016/j.jhazmat.2020.123775

Simon N. Wood. (2017). *Generalized Additive Models An Introduction with R SECOND EDITION CHAPMAN & HALL/CRC Texts in Statistical Science Series Series Editors Statistical eory: A Concise Introduction Practical Multivariate Analysis, Fifth Edition Interpreting Data: A First Cour*. 1–496. https://doi.org/10.1201/9781315370279

Stanković, J., Milošević, D., Jovanović, B., Savić-Zdravković, D., Petrović, A., Raković, M., Stanković, N., & Stojković Piperac, M. (2022). In Situ Effects of a Microplastic Mixture on the Community Structure of Benthic Macroinvertebrates in a Freshwater Pond. *Environmental Toxicology and Chemistry*, *41*(4), 888–895. https://doi.org/10.1002/etc.5119

Suwaki, C. H., De-La-Cruz, L. T., & Lopes, R. M. (2020). Impacts of microplastics on the swimming behavior of the copepod temora turbinata (Dana, 1849). *Fluids*, *5*(3). https://doi.org/10.3390/fluids5030103

Team, R. C. (2022). *R: A language and environment for statistical computing*.

Thompson, R. C., Courtene-Jones, W., Boucher, J., Pahl, S., Raubenheimer, K., & Koelmans, A. A. (2024). Twenty years of microplastic pollution research-what have we learned? *Science (New York, N.Y.)*, *386*(6720), eadl2746. https://doi.org/10.1126/science.adl2746

Turner, C. R., Miller, D. J., Coyne, K. J., & Corush, J. (2014). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (hypophthalmichthys spp.). *PLoS ONE*, *9*(12), 1–20. https://doi.org/10.1371/journal.pone.0114329

van der Plas, M., Trimbos, K. B., Bosker, T., & Vijver, M. G. (2025). eDNA-based approaches advance ecotoxicology: Insights and best practices from eDNA metabarcoding studies in evaluating stress-induced aquatic (macro-) invertebrate community composition. *Ecological Indicators*, *172*(February 2024), 113269. https://doi.org/10.1016/j.ecolind.2025.113269

van Emmerik, T., & Schwarz, A. (2020). Plastic debris in rivers. *Wiley Interdisciplinary Reviews: Water*, *7*(1), 1–24. https://doi.org/10.1002/wat2.1398

Verberk, W. C. E. P., Siepel, H., & Esselink, H. (2008). Life-history strategies in freshwater macroinvertebrates. *Freshwater Biology*, *53*(9), 1722–1738. https://doi.org/10.1111/j.1365-2427.2008.02035.x

Xiang, Y., Jiang, L., Zhou, Y., Luo, Z., Zhi, D., Yang, J., & Lam, S. S. (2022). Microplastics and environmental pollutants: Key interaction and toxicology in aquatic and soil environments. *Journal of Hazardous Materials*, *422*(June 2021), 126843. https://doi.org/10.1016/j.jhazmat.2021.126843

Xu, Y., Ou, Q., Jiao, M., Liu, G., & Van Der Hoek, J. P. (2022). Identification and Quantification of Nanoplastics in Surface Water and Groundwater by Pyrolysis Gas Chromatography-Mass Spectrometry. *Environmental Science and Technology*, *56*(8), 4988–4997. https://doi.org/10.1021/acs.est.1c07377

Yıldız, D., Yalçın, G., Jovanović, B., Boukal, D. S., Vebrová, L., Riha, D., Stanković, J., Savić-Zdraković, D., Metin, M., Akyürek, Y. N., Balkanlı, D., Filiz, N., Milošević, D., Feuchtmayr, H., Richardson, J. A., & Beklioğlu, M. (2022). Effects of a microplastic mixture differ across trophic levels and taxa in a freshwater food web: In situ mesocosm experiment. *Science of the Total Environment*, *836*(April). https://doi.org/10.1016/j.scitotenv.2022.155407

Figure captions

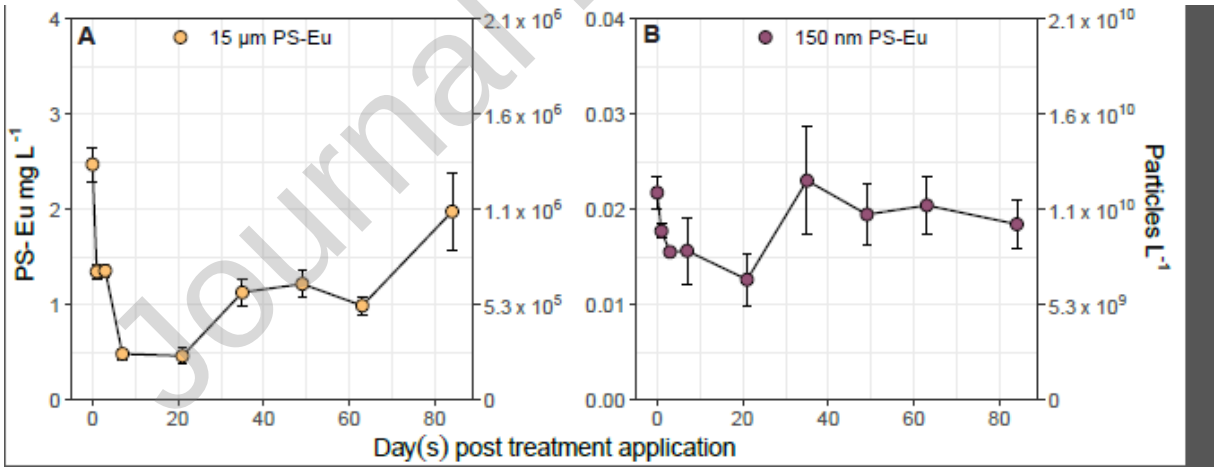

Figure 1. Water column concentrations (mean ± standard error, *n* = 7) of 15 µm (**A**) and 150 nm (**B**) PS-Eu particles over the course of the experiment. Nominal treatment concentrations were 0.024 mg L^{-1} (1.26 × 10^{10} particles L^{-1}) for 150 nm particles and 2.35 mg L^{-1} (1.26 × 10^{6} particles L^{-1}) for 15 µm particles.

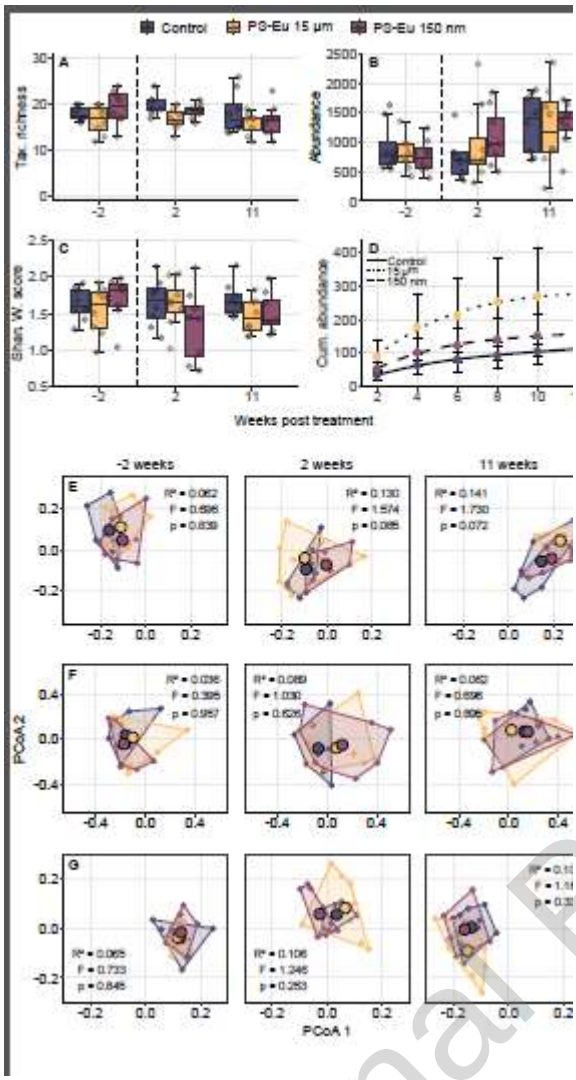

Figure 2. Morphology-based macroinvertebrate community composition metrics, i.e., taxonomic richness (**A**), total abundance (**B**), Shannon–Weiner index scores (**C**), $\log_{10}$-transformed cumulative emerging insect abundance (**D**), Sørensen dissimilarity (**E**), Bray–Curtis dissimilarity (**F**), and Bray–Curtis dissimilarity based on $\log_{10}$-transformed data (**G**). Note that the axes of figures for different indices are on different scales.

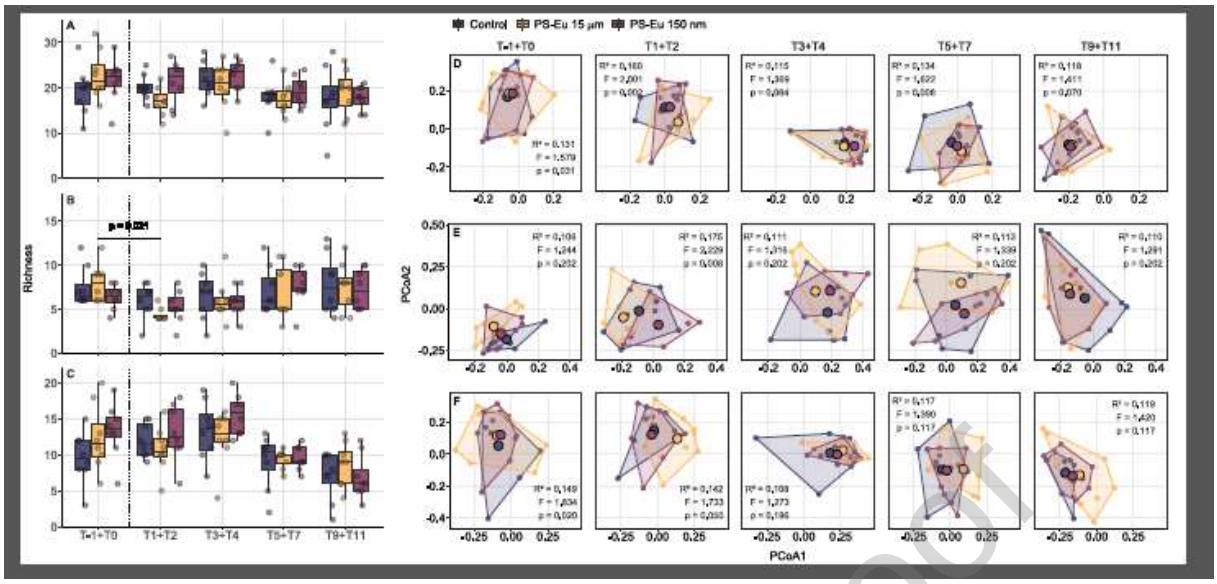

Figure 3. eDNA-based community composition metrics, presented per combined timepoint. **A-C**: OTU richness per treatment over time for all invertebrate data (**A**), macroinvertebrate data (**B**) and microinvertebrate data (**C**). The dotted line indicates the moment of treatment application. **D-F**: Sørensen dissimilarity based on all invertebrate data (**D**), macroinvertebrate data (**E**) and microinvertebrate data (**F**). Note that the axes of different figures are on different scales.

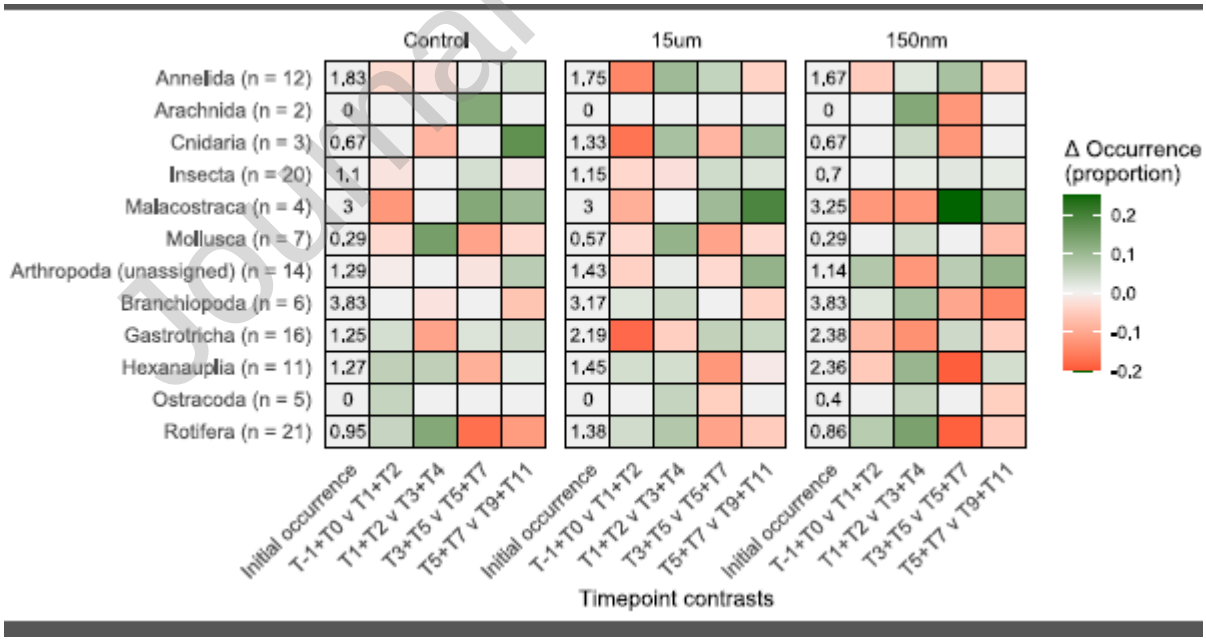

Figure 4. Heatmap showing the change in average occurrence of OTUs per taxonomic group, per treatment, and per (combined) timepoint contrast (i.e., compared to the previous timepoint). The total number of observed OTUs per taxa are given between brackets. The first column within each treatment shows the initial average occurrence of OTUs at the pre-treatment timepoint. Values indicated by the

color scale represent the change in average number of mesocosms (out of 8) where an OTU was present. A value of 1 indicates that an OTU appeared in all 8 mesocosms (from being absent before), while -1 indicates it disappeared from all mesocosms. For clarity, the color scale is limited to the observed range of changes (from −0.2 to 0.25), rather than the theoretical maximum range (−1 to 1).

Environmental implications

Micro- and nanoplastics are emerging contaminants in freshwater ecosystems. Our findings indicate that environmentally relevant concentrations of polystyrene particles can induce acute, transient changes in invertebrate community composition. These results highlight that even low-level, persistent plastic pollution can alter community composition. Moreover, our study demonstrates that environmental DNA metabarcoding is a sensitive tool for early detection of subtle community impacts. These insights are valuable for environmental monitoring, risk assessment, and the development of mitigation strategies for plastic pollution in freshwater ecosystems.

Declaration of interests

☐ 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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Martina Vijver reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Long-term community-level impacts of polystyrene MNPs were assessed in freshwater mesocosms.
- Treatment concentrations resembled higher end of those reported for natural surface waters.
- Morphological assessments provided no indication of community-level impacts.
- eDNA metabarcoding showed subtle, transient shifts in community composition.