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Beneath the surface: Decoding the impact of *Chironomus riparius* bioturbation on microplastic dispersion in sedimentary matrix

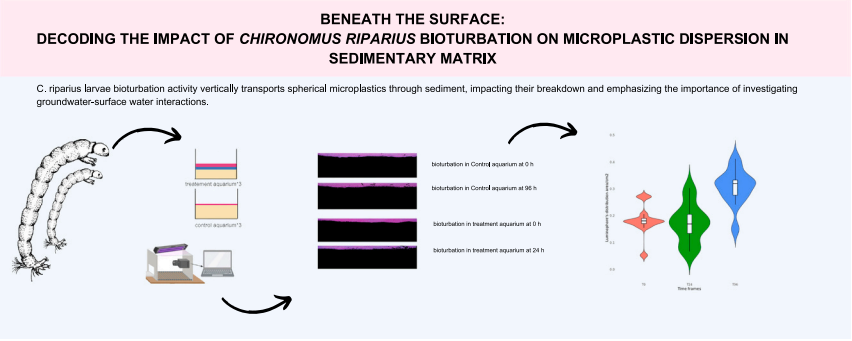
Khouloud Sebteoui [a,*], Djuradj Milošević [b], Jelena Stanković [c], Viktor Baranov [d], Boris Jovanović [e], Stefan Krause [f,g], Zoltán Csabai [a,h]

[a] Department of Hydrobiology, Faculty of Sciences, University of Pécs, Hungary
[b] Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Serbia
[c] Department of Hydrobiology and Water Protection, Institute for Biological Research "Siniša Stanković" National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia
[d] Estación Biológica de Doñana-CSIC / Doñana Biological Station-CSIC, Spain
[e] Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA, USA
[f] School of Geography, Earth and Environmental Sciences, University of Birmingham, United Kingdom
[g] LEHNA- Laboratoire d'ecologie des hydrosystemes naturels et anthropises, University of Lyon, Darwin C & Forel, 3-6 Rue Raphaël Dubois, 69622 Villeurbanne, France
[h] Balaton Limnological Research Institute, Tihany, Hungary

HIGHLIGHTS

- Microplastics are vertically transferred by *Chironomus riparius* larvae bioturbation.
- Microplastic size is different after being ingested by *Chironomus riparius*.
- The presence of microplastics has significant effect on *Chironomus riparius* bioturbation activity.

GRAPHICAL ABSTRACT



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ABSTRACT

A detailed understanding of microplastics (MPs) behaviour in freshwater ecosystems is crucial for a proper ecological assessment. This includes the identification of significant transport pathways and net accumulation zones, considering their inherent, and already proven influence on aquatic ecosystems. Bioavailability of toxic agents is significantly influenced by macroinvertebrates' behaviour, such as bioturbation and burrowing, and their prior exposure history. This study investigates the effect of bioturbation activity of *Chironomus riparius* Meigen, 1804 on the vertical transfer of polyethylene MPs ex-situ. The experimental setup exposes larvae to a scenario of 10× the environmentally relevant high concentration of MPs (80 g m^{-2}). Bioturbation activity was estimated using sediment profile imaging with luminophore tracers. This study demonstrated that spherical MPs are vertically transferred in the sediment due to the bioturbation activity of *C. riparius* larvae and that their

* Corresponding author.
E-mail address: khouloud.sebteoui@gmail.com (K. Sebteoui).

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presence influences the intensity of the bioturbation activity over time. The present findings provide a noteworthy contribution to the understanding of the relationship between ecosystem engineers and the dispersion and accumulation of MPs within freshwater ecosystems.

1. Introduction

Plastic pollution has become a serious and growing environmental crisis (Iroegbu et al., 2021; Krause et al., 2022). Fragmentation of plastic into smaller particles <5 mm in size, referred to as microplastics (MPs), or even smaller nanoplastics (NPs) (Gigault et al., 2016), facilitates their transport, distribution, and bioavailability in freshwater ecosystems, and consequently their toxic effects on aquatic biota. Therefore, a detailed mechanistic understanding of the MPs' lifecycle across aquatic and terrestrial ecosystems is crucial for adequate assessment of their ecological risks. This includes the identification of major transport pathways of MPs and net accumulation zones.

The processes controlling the fate and transport of MPs in aquatic ecosystems are still not understood sufficiently. Aquatic ecosystems contain high concentrations of MPs due to their proximity and connectivity to potential sources such as urban areas and agricultural land (Wang et al., 2021). The environmental fate of MPs depends on their physicochemical properties, size, shape, and density, and may also be influenced by biological processes such as bioturbation, geochemical processes, stream flow velocity, sediment transport, and trophic connections (Atugoda et al., 2021; Conkle et al., 2018; Eerkes-Medrano et al., 2015).

Temporally, MP concentrations in surface water systems have been found to be influenced by meteorological and seasonal dynamics, including unpredictable storm and flood events at shorter time scales but also seasonal variability (Mintenig et al., 2019). Beside these natural processes, human interference can reduce river velocity, allowing MPs to settle and potentially increase their sediment concentration if transported across the water-sediment interface (Ding et al., 2019). Furthermore, recent works (Drummond et al., 2022; Krause et al., 2021; Frei et al., 2019) have highlighted the relevance of hyporheic exchange (facilitated by advective forces) for transporting smaller MP fractions (<100 μm) into streambed sediments.

In addition to physical factors, the transport, accumulation, and ultimate fate of MPs in aquatic environments can be influenced by biological processes. This includes indirect changes to MP transport properties due to biofilm interactions or entrapment in submerged vegetation (Chubarenko et al., 2016), metal absorption, aggregation (Leiser et al., 2020), or the direct action of biological processes such as bioturbation (Koelmans et al., 2014).

Bioturbation in aquatic systems is mainly driven by the activities of invertebrates in the sediment. It represents a natural process that has been defined as “all transport processes carried out by animals that directly or indirectly affect sediment matrices” (Kristensen et al., 2012). In aquatic ecosystems, bioturbation can significantly influence biogeochemical processes, fluxes between water and sediment, and microbial activities (Mermillod-Blondin and Rosenberg, 2016).

Bioturbation is assumed to not only affect the fate of organic matter and nutrients via digestion (Hölker et al., 2015), but also to facilitate the movement and distribution of pollutants across aquatic terrestrial interfaces (Krause et al., 2017, 2022). As a result of the chemistry changes of bioturbated sediments, the sensitivity and behaviour of species (such as bioturbation and burrowing) and their prior exposure history, have a substantial impact on pollutant bioavailability (Chapman, 1999). The research conducted by Wazne et al. (2023) and related investigations collectively underscore the capacity of microplastics to influence bioturbation dynamics by altering the behaviour and activities of pivotal organisms within ecosystems. Species engaged in bioturbation, such as *Tubifex tubifex*, hold a pivotal role in governing sediment nutrient cycling. The introduction of microplastics can induce a decline in the bioturbation activities of these organisms, thereby impacting organic matter mineralization and nutrient fluxes crucial for ecosystem functionality (You et al., 2023). The interplay between microplastics and bioturbating species may precipitate extensive shifts in ecosystem functions, underscoring the substantial repercussions of microplastics on bioturbation and, consequently, overall ecosystem health and operational dynamics.

Chironomid larvae, tubificid worms, and mayfly larvae (Chaffin and Kane, 2010) are benthic aquatic macroinvertebrates that represent crucial bioturbators in freshwater ecosystems. Their bioturbating activity has considerable impact on the availability of nutrients, carbon turnover (Hölker et al., 2015; Baranov et al., 2016a, 2016b), geochemical fluxes between the water column and the sediment, and respiration (Baranov et al., 2016a, 2016b). Non-biting midges (Chironomidae, Diptera) constitute a highly diverse taxon of insects with over 10,000 species estimated world-wide (Ferrington, 2008; Ashe and O'Connor, 2012), and are one of the most abundant macroinvertebrates in fresh and brackish water ecosystems (Armitage et al., 1995). Inhabiting the uppermost layers of sediment, many species within Chironomids (i.e., subfamily Chironominae) act as deposit-feeders of organic matter. Their feeding behaviour is mostly non-selective, determined by bioavailability, and (rarely) selective, based typically on the nutritive value and food type (From and Rasmussen, 1984; Armitage et al., 1995). Chironomid construct and irrigate U-shaped tubes in the upper layers of sediments, which significantly stimulates sediment metabolism, O_2 uptake, and the activity of aerobic heterotrophic bacteria in sediment (Nogaro et al., 2007).

The impact of sediment-associated pollutants on macroinvertebrates' sensitivity and their transport across the sediment-water interface affects bioturbation and its associated ecosystem functions (van der Meer et al., 2017). An efficient method to better understand the precise effects of bioturbation on the vertical transfer of MPs is to use time-lapse sediment profile imaging with tracers. This method uses fluorescent particles called luminophore tracers to track the movement and settling of sediment particles over time. By tracking the changing fluorescence patterns, researchers can gain insights into sediment transport processes and sedimentation rates. This method has been successfully used to observe and track the dynamics at the water-sediment interface (Diaz and Cutter, 2001; Solan et al., 2003).

The impact of bioturbators on the distribution, transfer, and bioaccumulation of MPs in freshwater ecosystems remains largely uncharted territory. Therefore, using the combination of luminophore tracers and sediment coring, the aims of this study are 1) to test the potential toxic effects of MPs on chironomid larvae; 2) to investigate the influence of bioturbation activity on the vertical transfer of MPs in laboratory experiments; 3) to test whether *C. riparius* larvae can contribute to the breakdown of MPs by comparing MP size before and after ingestion; and 4) to assess the effects of MP particles on the magnitude of the bioturbation activity rate to better understand the MP lifecycle and accumulation net-zones in freshwater ecosystems. To that end, this study investigates the following hypothesis: the bioturbation activity rate of different sediment-dwelling fauna increases the vertical transfer of MP particles in the sediment, representing a significant and currently underexplored transport mechanism. This results in MP accumulation in the deeper layers of sediment and cannot be explained by purely physical transport and deposition processes.

2. Material and methods

Tracing the transport of MPs through sediments by bioturbation is

challenging, and generally accepted experimental protocols are still lacking. Fluorescence sediment profile imaging (f-spi) has proven to a be an efficient method to observe organism-sediment interactions, especially in a marine environment, using time-lapse imaging to follow the path of luminophore tracers (Solan et al., 2004) to quantify and define the bioturbation activity.

This approach was adopted and modified in the present experiments to quantify the bioturbation activity of *C. riparius* larvae. It was combined with sediment coring to trace the vertical transfer of the MPs and the time scale of their bioturbation activity. Microplastics were counted for each 1 cm layer of sediment to investigate the vertical transfer of the MPs from the upper to the lower layer of sediment based on an adapted protocol from Lagauzére et al. (2011). The originally described methodology was performed on cylindrical aquariums, using a different sediment type and varied species (*Tubifex tubifex*). Notable alterations included the substitution of cylindrical glass tubes with rectangular-shaped aquariums, a different sediment content and quantity, adjustments in core measurements, and the size and number of sediment slices. In addition, image analysis was used to count particles instead of using the microplate fluorometer methodology. The primary objectives of these adaptations were to optimize the efficiency and accuracy of data collection, and the methodology is highly tailored to the specific experimental design of the presented study.

2.1. Characterization of particles

Fluorescent tracer methods were used to quantify the rate of bioturbation under the MP exposure, using luminophores as a visual marker for imaging bioturbation in the sediment. Luminophores are particles of defined properties that are distinguished by their luminescent properties when excited by a light source. This experiment used inorganic coloured quartz sand coated with pink, fluorescent, inert paint with a size range of 125–200 μm (Glasspebbles UK) as a medium for *Chironomus riparius* Meigen, 1804 larvae.

The MPs used in this study were blue (colour stable: resistant to changes that can be caused by light or chemical factors) polyethylene spheres with a density of 1.08 g/cc, and a size range from 53 to 63 μm (Cospheric BLPMS-1.08). Polyethylene was chosen as it is the most ubiquitous polymer present in the environment (Whiteley et al., 2005). To validate the size of luminophores and MPs, 100 random particles of each were measured under a microscope, a Leica Wild M420 with Zeiss KL1500lcd cold light Olympus SC50 Camera 40×, prior to the experiment.

2.2. Test organisms

C. riparius larvae, a standard model species frequently used in sediment toxicity tests and a good model organism for bioturbation (He et al., 2015), were collected from a population reared in the ecotoxicology laboratory at the Faculty of Science and Mathematics, University of Niš, Serbia. The culture was maintained under controlled conditions with a temperature range of 20.5 ± 0.5 °C, a photoperiod of 16 h (+8 h in the dark), and constant aeration. Chironomid larvae were fed with TetraMin® fish food flake mixture. The identification of the species from the laboratory culture used in the study was confirmed recently by DNA barcoding. The analysed sequences matched with *C. riparius* in GenBank with a percentage of matching of identity of 95 % for 99 cases and 100 % for 1 case (Janakiev et al., 2023).

2.3. OECD sediment spiked toxicity test

To test the potential toxicity of MPs and luminophore particles on *C. riparius* larvae, the sediment spiked toxicity test No. 218 of the Organisation for Economic Co-operation and Development (OECD, 2004) was performed. The setup consisted of three different treatments, luminophores, polyethylene MPs, and the mixture of luminophores and MPs. All treatments and the control had 3 replicates.

MP concentrations were based on a high concentration of MP particles, 80 g/m^2, a scenario proposed in Stanković et al. (2020) and Yıldız et al. (2022), which is 10 times the environmentally relevant concentration. MP concentrations of 0.0043 g cm-2, 0.00324 g cm-2 and 0.00216 g cm-2 were used per beaker. For the luminophores, 3 concentrations were used, calculated based on the mass intended to be used for the imaging experiment. After adapting the calculations to the beaker size, the luminophore masses of 0.0544 g cm-2, 0.0412 g cm-2 and 0.0268 g cm-2 were added. For the mixture, treatment beakers the same MP concentrations were added and covered with a 3 mm layer of luminophores.

All glass beakers had a volume of 370 mL and a diameter of 6 cm. Sterilized coarse sand and a mixture of 50 % tap water and 50 % distilled water were added to the beakers. The sand to water ratios were 1:4, as recommended by the OECD guidelines (OECD, 2004). 20 larvae of *C. riparius* were introduced to each beaker three days after hatching. Following the introduction, larvae were surveyed daily for 10 days, fed regularly every two days with TetraMin, 0.5 g per larvae during the first five days, and 1 g per larvae during the last five days, according to the OECD guidelines (OECD, 2004). Aeration was provided for the full duration of the experiment and the temperature and photoperiod were followed as previously described in the test organism section.

2.4. Preparation of the experimental setup

To test for the influence of bioturbation activity on the vertical transfer of MPs, the experiment was conducted using six rectangular glass containers with a total surface area of 0.016 m^2 per aquarium, a total volume of 0.004 m^3, and a sediment volume of 0.001 m^3.

The aquariums were prepared as follows: three duplicates of the control aquarium containing only 5 cm of fine quartz sand and a 3 mm layer of luminophores (125–200 μm), and three duplicate treatment aquariums containing the same high concentration (80 g m-2) of MPs in a 1 cm thick layer, added on top of the 5 cm layer of fine quartz sand, then covered with a 3 mm layer of luminophores. In each aquarium, 50 larvae were placed, respectively: 10 s instar larvae, 10 fourth instar larvae, and 30 third instar larvae. The widths of the head capsules were used to determine the larval instar based on the method described in (Watts and Pascoe, 2000).

2.5. Imaging methodology

To visualize the bioturbation pattern and quantify the bioturbation activity of the larvae, a time lapse imaging protocol was used for the sediment-profile imaging technique under UV light for fluorescence detection (Solan et al., 2004). Each aquarium was placed on a stable rotating platform and slowly pushed inside a black box equipped with a UV-A lamp (wavelength 315–400 nm) and a camera (Canon EOS 1300D) that was linked to a laptop. Pictures were taken using the Digital Photo Professional 4 software. Pictures were taken from all four sides of the aquariums.

Each aquarium was under the UV light for an average period of 6 min. The process was repeated continuously for 96 h, taking pictures every hour for the first 24 h. Then, to standardize the process, pictures were taken every 2 h for the rest of the experiment. The experiment was halted once the first adults emerged. For the sake of efficiency of method, a time frame of 24 h was considered to analyse the distribution of luminophores.

The images were analysed by ImageJ ver. 1.23y software using a specially designed plugin from the Ocean and Earth Science National Oceanography Centre Southampton, combined with a personalized Python code for photo segmentation and pixel area calculations for more accurate results (excluding the colour reflection from aquarium walls).

The area of the distribution of luminophores was first calculated in pixels, then converted to cm^2 using the method described in Teal et al.

(2009), considering the width of the image in cm, (all images had been resized accordingly to 18 cm). The conversion was made using the following equation:

$$(\text{Width of the image (cm)}/\text{number of pixels})*10000.$$

1 pixel in image = 49.21 μm.

These measurements were applied to a total of 96 images, 12 from each set of aquariums, in the assigned four-time frames of 24-hour intervals.

2.6. Sediment coring

The imaging methodology used only provided information on the distribution of particles along the periphery of the aquariums, lacking precise data regarding the activity within the sediment core. Therefore, to detect the presence of tracers and MPs in different sediment layers, cores of sediment were subsampled, and particles were counted in each layer to investigate the vertical transfer of MPs from the upper to lower layers of sediment.

At the end of the experiment, three PVC tubes with a diameter of 5 cm and a height of 7 cm were inserted into the sediment in each container. Each tube was carefully removed to avoid sediment loss while maintaining the undisturbed structure of the sediment layers, placed vertically on a flat plate, covered with cellophane, and placed in a freezer (−18 °C). After 24 h, the frozen sediment was extracted from the tubes and each core was cut into 5 equal layers of 1 cm each with nylon wire, preserving the original layering of the sediment. Then, particles, luminophores, and MPs were counted manually under a Leica Wild M420 Zeiss KL1500lcd microscope equipped with a cold light Olympus SC50 Camera using 100× magnification for the MPs and 40× for the luminophores.

The particle count was normalized by a factor of 100 to facilitate the analysis and data visualisation. The normalization was carried out as follows:

Microspheres were quantified using a calibration with sediment samples of known microsphere concentrations. This allowed us to have the number of microspheres per layer (n) and the total number within the profile (N). To achieve a specific normalization by 100, the fraction (n/N) of microspheres per layer was determined. Subsequently, the microsphere concentration was normalized by dividing the obtained fraction by 100, resulting in the adjusted concentration formula:

$$C\ normalized = \frac{n}{100 \times z \times A \times N}$$

where z represents the thickness of the sampling layer in centimetres.

A = the core area

2.7. Analysis of ingested particles

At the end of the experiment, 180 larvae were extracted from the containers (the rest of the larvae were damaged during the sediment coring process) and preserved in 70 % ethanol. The process was performed as quickly and efficiently as possible to reduce regurgitation of the stomach content. Afterwards, the chitin and hard tissues were digested using an alkaline protocol: a solution of 10 % KOH (Tsangaris et al., 2021). The samples were incubated at 60 °C for 48 h with no agitation. The prolonged incubation time at a stable elevated temperature delivered optimal results for chitin digestion, and the tissue was completely digested. This facilitated the extraction of the particles for the next step. Particles were isolated using 10 μm pore size cellulose filter paper (Meschery Nagel MN640) and counted using the aforementioned microscope system.

2.8. Statistical and image analyses

Statistical methods were used to assess the different aspects of the study. To analyse the toxicity test results, the Kruskal–Walli's test was applied to assess the difference in mortality rates of larvae between control and treatment. As for the comparison of the size difference between ingested MPs and the ones retrieved from the sediment, the diameters of 100 particles were measured from each medium using ImageJ. Afterwards, Welch's ANOVA was used to investigate the statistical significance of the particles' size differences.

A two-way ANOVA was used to compare luminophore particle distribution between the control and treatment aquariums (2 groups treatment and control) across distinct layers of the sediment (5 groups/layers). Tukey's honest significant difference (HSD) post-hoc tests were carried out for the pairwise comparison between analysed layers. This approach facilitated and simplified the layer-wise examination, enabling the identification of statistically significant differences in particle distribution, thereby elucidating the impact of the treatment on specific depth levels within the aquariums. However, to assess the distribution of microplastic particles in the layers of sediment, we used the Welch ANOVA test.

To calculate the area of distribution of luminophores, Cyberlink Photodirector 365.ink was used for image treatment and images were resized to have standardized dimensions. Afterwards, Python code, provided in the supplementary material, was used to calculate the exact area of distribution of the luminophores based on the pink coloured pixels.

It is noted that except for the mortality rates, which were tested by a nonparametric alternative, all other input parameters fulfilled the assumptions of normality. The number of luminophores across different treatments and layers was analysed by two-way ANOVA, albeit the homogeneity of variances was not met (Levene'Test, $p < 0.05$) since the robustness of analysis of variance (ANOVA) was considered appropriate for univariate analysis given the equal size of samples collected. The assumptions were tested using the Shapiro-Wilk Test for normality and Levene's Test for homogeneity. All the tests were performed in R (R x64 4.1.1).

3. Results

3.1. Toxicity test

Exposure of *C. riparius* larvae to three different treatments, luminophores, polyethylene MPs and the mixture of luminophores and MPs for a duration of 10 days did not result in any significant effects on the mortality rate of chironomid larvae (Kruskal Wallis, $p = 0.1179$).

3.2. Luminophore distribution

Luminophore particles were extracted from all five sediment core layers, with three replicates for control and treatment: from the top (layer 1) to the last layer on the bottom of the aquariums (layer 5) (Fig. 2). The variability of luminophore particles in the sediment was significantly influenced by both the type of treatment (two-way ANOVA $F = 9.76$, $p < 0.01$) and the sediment depth (two-way ANOVA, $F = 3661.29$, $p < 0.001$). The interaction between treatments and different sediment layers were also shown to be significant (two-way ANOVA, $F = 5.56$, $p < 0.01$). While the number of luminophore particles significantly decreased with the increase of sediment depth, the control aquariums had a higher number of particles in the second and third layers, where these differences were the most pronounced (Fig. 2). The differences in number of particles across the layers was significant (Tukey, $p < 0.05$) except between the fourth and fifth layers due to the limited number of particles retrieved from these last 2 layers.

3.3. Image analysis results

Comparing the dispersal area of luminophores for the control setup during the four timeframes, 0 h, 24 h, 48 h and 96 h, revealed no

Table 1
Size of 100 random particles of polyethylene particles (MPs) and luminophores (lumi) (mean ± SD) prior to the experiment, along with size of 100 random particles of polyethylene particles (mean ± SD) from the cored sediment post-experiment (MPs -sed), and particles extracted from the larvae (MPs-ing).

Particles	Length (μm)	Width (μm)	Coefficient of variance %
lumi	144.16 ± 37.8	128.4 ± 36	26.22
MPs	61.6 ± 5.23 (diameter)	–	8.49
MPs-sed	65.81 ± 5.8 (diameter)	–	8.81
MPs-ing	50.02 ± 7.5 (diameter)*	–	14.99

* Indicates significance at the 0.05 level.

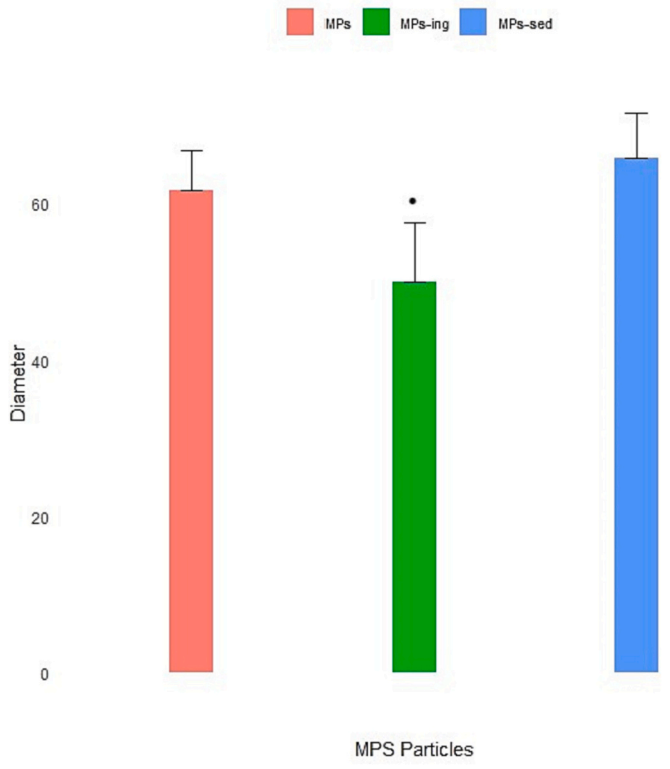

Fig. 1. Histogram illustrating the sizes of 100 randomly selected polyethylene particles (MPs) prior to the experiment, as well as the sizes of 100 randomly selected polyethylene particles post-experiment (MPs-sed) and particles extracted from the larvae (MPs-ing), each represented by their mean and standard deviation.

significant difference ($t = -0.930$, df = 9, $p = 0.4073$, Table S1). When only the treatment setup was considered, the luminophore's dispersal area significantly increased at the end of the experiment (Fig. 3), (two sample paired *t*-test, $t = 4.063$, df = 9, $p = 0.0028$, Table S1). The most significant increase of dispersal area occurred during the first 24 h of the experiment ($p = 0.00179$; Table S1), and then stabilized during the remainder of the experiment without significant fluctuations (Fig. 3). More precisely, there were no significant changes in dispersal areas of luminophores between 24 and 96 h.

When the control was compared with the treatment aquariums, the dispersal areas were substantially higher in the treatment ones. Significant changes in the dispersal areas were observed only at the end of the experiment (96 h; $t = -5.525$, df = 39, $p = 0.00036$, Table S1); (Fig. 4).

3.4. Sediment cores

3.4.1. MPs transfer

Microplastics were detected in all five layers of the extracted sediment cores (Fig. 5). This fact evidences the vertical transfer of MPs due to bioturbation activity of *C. riparius* larvae. Comparing the MP number in the three replica treatments revealed no significant difference (Welch's ANOVA test: $F = 0.03879$, $p = 0.962$, $p > 0.05$), with the highest average number of particles in the top layer, with 331 particle/cm^2.

3.4.2. Size differences in ingested MPs vs MPs from the sediment

One hundred MPs from sediment and from the larvae guts were randomly measured for their diameter size. The comparison revealed a significant difference in the diameter of the spheres (F = 7.634, df = 195.8, $p = 0.0062$). The mean size ± SD of the MPs in the sediment (60.53 ± 5 μm) was higher than the ones from the gut content (59.72 ± 4.57 μm), Table 1. (Fig. 1).

4. Discussion

The focal point of this scientific investigation is unravelling the mechanisms of microplastics (MPs) transfer within the sedimentary matrices in lotic systems. Analyses revealed that the larvae bioturbation activity influenced the vertical distribution of the MPs in the sediment layers. Furthermore, the presence of polyethylene MPs in sediment had a significant effect on the temporal pattern of the benthic organism bioturbation activity, accelerating their movement in the first 24 h of the exposure significantly compared to the rest of the experiment (Fig. 3).

4.1. Vertical distribution of MPs

Vertical transfer of MPs due to bioturbation activity in the terrestrial environment was confirmed in previous studies (e.g., Heinze et al., 2021; Gao et al., 2021). Our study is among the first to investigate the transfer of MPs in freshwater environments, facilitated by chironomid larvae bioturbation. Results corroborated the vertical transfer of the MPs in the testing environment in a relatively short time frame (96 h) and in a depth of 5 cm. Typically, chironomid larvae create a U-shape burrow, to a depth up to 10 to 20 cm, depending on the environmental conditions (Ratte et al., 1997), for *Chironomus riparius* larvae, the depth of bioturbation is relatively shorter, a mean depth of approximatively 4.2 cm (Charbonneau and Hare, 1998), therefore this study is more specific to the first pathways of microplastics in their transfer from the water realm to the shallow layers of the sediment. Previous studies have suggested the potential of bioturbation influencing the transfer of MPs. Lwanga et al. (2017), demonstrated that earthworms can incorporate microplastics from litter into their burrows, highlighting their potential to transport microplastics in soil. Also, Meng et al. (2023), investigated the fragmentation and depolymerization of microplastics in soil and found that soil-dwelling earthworms, such as *Lumbricus terrestris*, could transport and ingest plastic debris in the soil. It was also confirmed in the freshwater environment by the present study, which is among the first to investigate the transfer of microplastic (MP) particles in the sediment through the bioturbation of *Chironomus riparius* larvae.

The interaction between the larvae and microplastic particles in the sediment appeared to be influenced by the feeding behaviour of the former, as well as the size and shape of the microplastic particles Coring of the sediment proved that the distribution of luminophores is significantly different between the second, third, and fourth layer of sediment in the control and treatments (Fig. 4). This might be explained by the selective intake of a specific particle size range by the *C. riparius* larvae. This chironomid species is a non-selective feeder, however, previous studies revealed that their feeding size particles range from 20 to 200 μm (Armitage et al., 1995; Henriques-Oliveira et al., 2003), through a rigorous analysis of the size of their food particles during different season and for their different life stages. These studies provided valuable insights into the diet and feeding behaviour of chironomid larvae. Silva et al. (2019) substantiated the ingestion of polyethylene particles ranging from 32 to 63 μm by *C. riparius* larvae. Building upon this, their subsequent study (Silva et al., 2019) revealed that *Chironomus riparius*

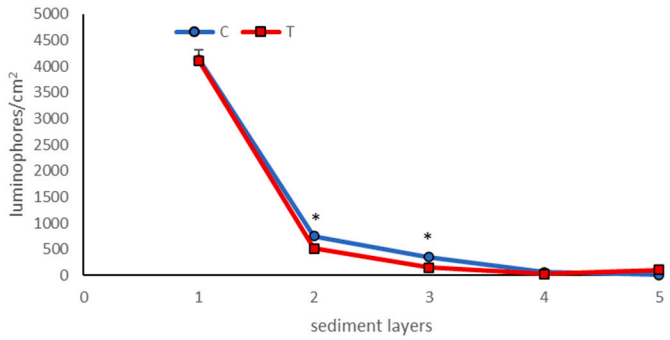

Fig. 2. difference of distribution of luminophore particles between the control and the treatment aquariums emphasizing the effect of presence of microplastics (MPs) on the distribution of luminophores.
Note: * indicates significance at the 0.05 level. /
*C: control; T: treatment.

exhibits a particular susceptibility to accumulate higher number of particles, and this accumulation is notably dependent on the shape and size of the particles. Moreover, the excretion of particles is suggested to be also shape dependent, with a pronounced result that the irregular shaped particles have a tendency of a higher aggregation. Particles that are similar to sediment, with regards to their size and structure, such as luminophores, are easier to exclude from the digestive system of larvae than the MPs. Hence MPs can reside for longer in the digestive system. According to Bervoets and Blust (2003), microplastics have a greater tendency to reside for a longer duration in the fish guts, which explains the contrast of the number of microplastics recovered from the chironomid larvae's digestive track compared to the luminophores particles in our experiment.

4.2. *Luminophores distribution analysis*

Luminophore imaging determined the temporal dynamics of bioturbation in experiments. MPs accumulation caused a decreased bioturbation activity of larvae, which can be revealed from the differences of the luminophore distribution between the control and the treatment conditions (Fig. 2). This can be explained by the temporal pattern of bioturbation in the present study. In fact, the largest area of distribution of the luminophores was measured in the first 24 h of exposure to MPs. During the following 24 h of the exposure, the bioturbation in the treatment aquariums was not significantly different. So, the first contact of the larvae with the MPs resulted in a pronounced feeding activity that contributed to the high reworking capacity in the sediment. According to Scherer et al. (2017) *C. riparius* larvae ingest polystyrene beads in a size range of 10–90 μm, with a frequency of 226 particles per hour. This can explain the speed of reworking during the first 24 h interval. After that, it is a probable explanation that, due to the accumulation of particles in the larvae gut, the process of reworking decreased. According to Setyorini et al. (2021), MPs particles of 50 μ m can accumulate in the digestive system of chironomid larvae and persist throughout all the life stages. This can explain the peak of bioturbation that occurred in the first 24 h of exposure. After that, the high number of ingested MPs may have been responsible for the slowing of larvae activity, thus causing a decrease of the bioturbation rate throughout the rest of the experiment time. The effect of microplastics on the activity of organisms is still not

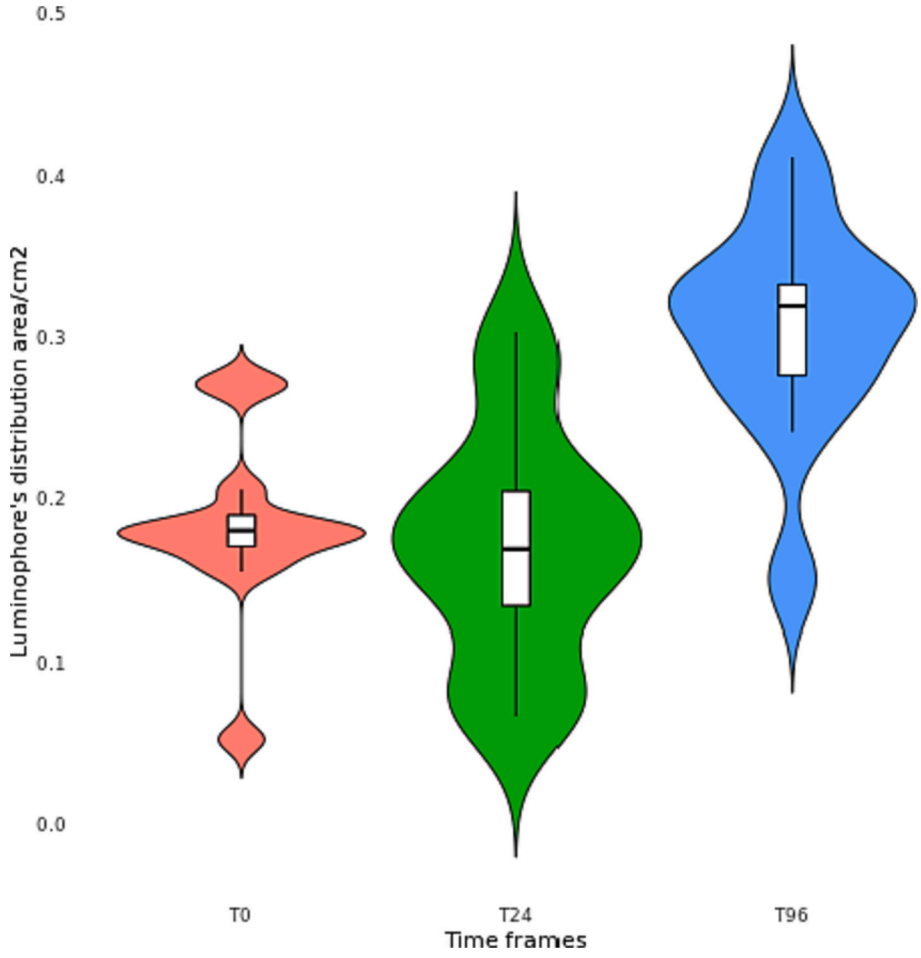

Fig. 3. luminophore's distribution area from 12 sets of images for treatment aquariums (T) throughout the 3 timeframes 0H, 24H, 96H.

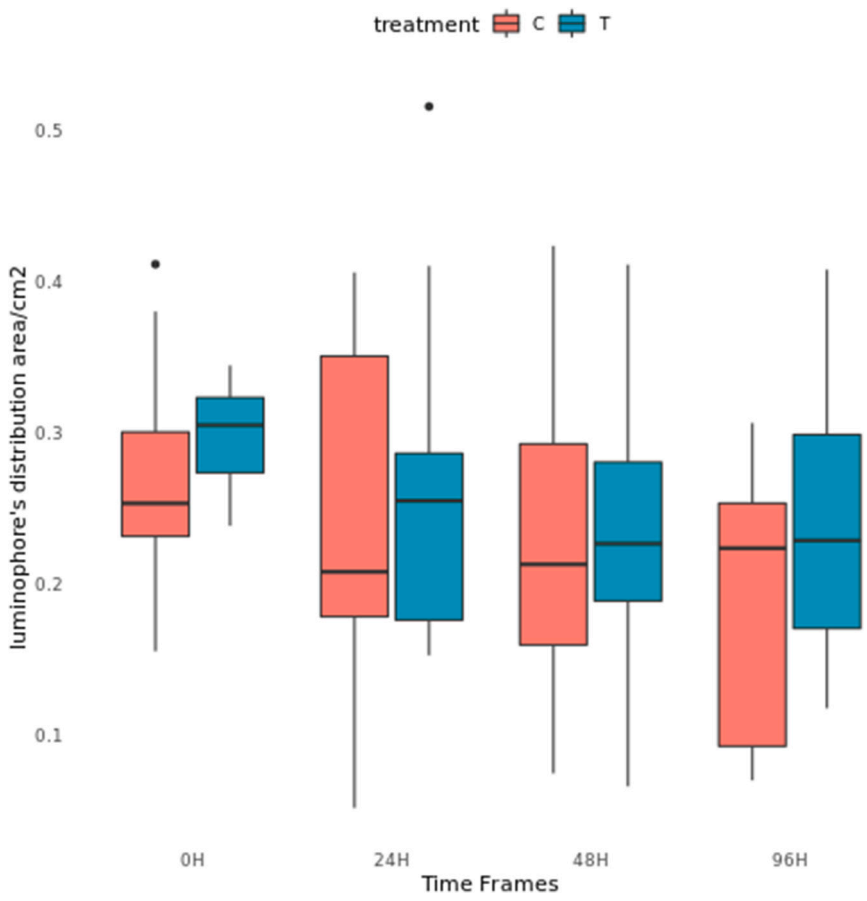

Fig. 4. Luminophore's distribution area from 12 sets of images for each control aquarium (c) and treatment aquariums (T) throughout the 4 timeframes 0H, 24H, 48H, 96H.

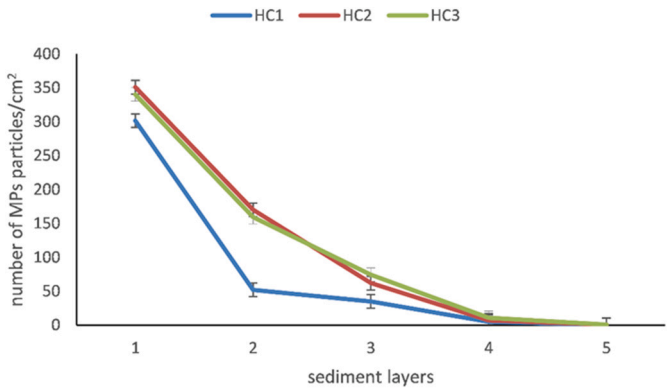

Fig. 5. Microplastics (MPs) distribution in the 5 sediment layers of the 3 replicate of treatment aquariums (HC1, HC2, HC3).
*HC: referring to High Concentration.

well studied, however, an in-depth meta-analysis study done by Sun et al., 2021, revealed that environmentally relevant concentration of microplastics can influence the locomotor activity of aquatic biota, like fish, and Daphnia, by significantly inhibiting their average movement speed. In our study we investigated further the precise temporal pattern of this effect on the chironomid larvae, revealing the heightened activity of the larvae during the first 24 h of exposure, followed by the decrease of their activity for the rest of the experiment time. This pioneering investigation marks one of the first comprehensive examination of the impact of microplastics on the behavioural patterns of freshwater bioturbators, including the temporal sequence in their activities.

4.3. Particle ingestion

The comparison of the diameter size of the ingested polyethylene particles by the larvae, and the particles retrieved from the sediment revealed a significant difference (Fig. 1). The ingested particles are significantly smaller than the particles that were present in the medium at the end of the experiment. MPs ingestion by *Chironomus* sp. has been observed previously (e.g., Silva et al., 2019; Setyorini et al., 2021) However, change in the physical size of the ingested MPs has not been investigated yet. In that regard, present research is a first step towards further investigation of the reduction in size of ingested MPs by chironomids. Thus, this may suggest that chironomids can potentially contribute to the biodegradation of MPs in freshwater environments. Previously, the shredding and degradation of MPs by freshwater benthic macroinvertebrates were confirmed only for caddisfly larvae (Valentine et al., 2022). However, recent research (Janakiev et al., 2023) revealed that the activity of the gut microbiome of *C. riparius*, specifically of *Peribacillus simplex* and *Peribacillus frigoritolerans* can potentially result in MPs digestion. These 2 bacteria showed a remarkable ability of growth especially on Polyethylene. The results presented in this study microplastic particles ingestion by chironomid larvae signifies a crucial entry point into understanding the implications of plastic pollution in aquatic ecosystems. However, the complexity of this ecological interaction suggests the necessity for future investigations.

The investigation into the vertical transfer of microplastic particles, facilitated by *Chironomus riparius* larval bioturbation, represents a significant advancement in the field of microplastic pollution research. This study elucidates the potential impact of *Chironomus riparius* larval bioturbation on the vertical transport of microplastics within freshwater benthic ecosystems. The findings offer valuable insights into the

intricate dynamics between microplastics and freshwater benthic organisms, underscoring the necessity for ongoing investigations to precisely evaluate the potential consequences of larval bioturbation on microplastic particle transfer across diverse environmental contexts.

This research, distinguished by its focused examination of nuanced interactions between microplastics and freshwater benthic organisms, contributes noteworthy insights into the behaviour of these particles in aquatic environments. However, the study is not without limitations, including assumptions about sediment homogeneity, uncertainties regarding bioturbation depth, and potential interference from other environmental factors influencing larval bioturbation behaviour. These constraints not only highlight the distinctive nature of the current study but also serve as pivotal areas for future research, aiming to deepen our understanding of microplastic pollution in freshwater environments.

5. Conclusions

In conclusion, this study on the vertical transfer of microplastic particles through the bioturbation of *Chironomus riparius* larvae represents a groundbreaking contribution to the field of microplastic pollution research. The investigation successfully met its objectives and confirmed the formulated hypotheses. By elucidating the pivotal role of bioturbation by *Chironomus riparius* larvae in the vertical transfer of microplastics within freshwater benthic ecosystems, the research underscores the necessity of incorporating the behaviour of freshwater benthic organisms into assessments of microplastic impact on aquatic environments. Furthermore, the study's revelations regarding the feeding behaviour of *Chironomus riparius* larvae and their selective ingestion of specific particle size ranges offer valuable insights into the intricate interactions between microplastics and freshwater benthic organisms. This emphasizes the imperative for continued research to advance our understanding of microplastic pollution in freshwater environments.

Ethics approval

A study did not require ethics approval.

CRediT authorship contribution statement

Khouloud Sebteoui: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Conceptualization. **Djuradj Milošević:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Conceptualization. **Jelena Stanković:** Writing – review & editing, Methodology, Conceptualization. **Viktor Baranov:** Writing – review & editing, Writing – original draft, Conceptualization. **Boris Jovanović:** Writing – review & editing, Writing – original draft, Conceptualization. **Stefan Krause:** Writing – review & editing, Writing – original draft, Conceptualization. **Zoltán Csabai:** Writing – review & editing, Writing – original draft, Supervision, Resources.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170844.

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