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1 Microplastics in freshwater sediments impact the role
2 of a main bioturbator in ecosystem functioning

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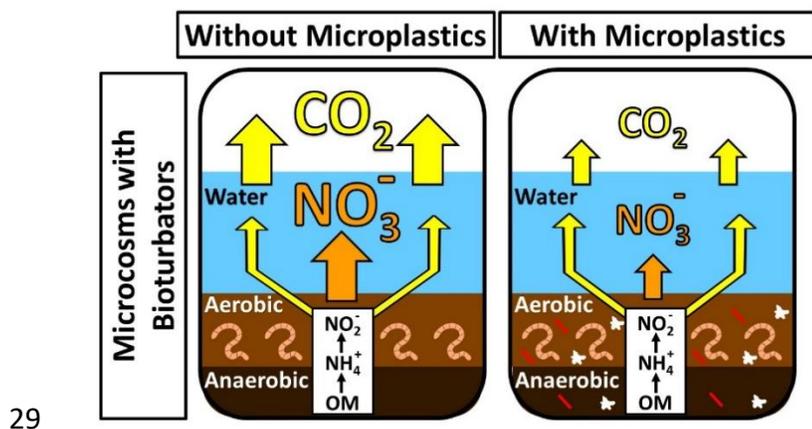
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12 **ABSTRACT**

13 While microplastic transport, fate and effects have been a focus of studies globally, the
14 consequences of their presence on ecosystem functioning have not received the same attention.
15 With increasing evidence of the accumulation of microplastics at sediment-water interfaces there
16 is a need to assess their impacts on ecosystem engineers, also known as bioturbators, which have
17 direct and indirect effects on ecosystem health. This study investigated the impact of microplastics

18 on the bioturbator *Tubifex tubifex* alongside any effects on the biogeochemical processes at the
19 sediment-water interface. Bioturbators were exposed to four sediment microplastic concentrations:
20 0, 700, 7000 and 70000 particles kg⁻¹ sediment dry weight. Though no mortality was present, a
21 significant response to oxidative stress was detected in *tubificid* worms after exposure to medium
22 microplastic concentration (7000 particles kg⁻¹ sediment dry weight). This was accompanied by a
23 reduction in worm bioturbation activities assessed by their ability to rework sediment and to
24 stimulate exchange water fluxes at the sediment-water interface. Consequently, the contributions
25 of tubificid worms on organic matter mineralization and nutrient fluxes were significantly reduced
26 in the presence of microplastics. This study demonstrated that environmentally realistic
27 microplastic concentrations had impact on biogeochemical processes at the sediment-water
28 interface by reducing the bioturbation activities of tubificid worms.



30 **KEYWORDS**

31 Microcosms, tubificid worms, exposure, sediment-water interface, biogeochemical fluxes

32 **SYNOPSIS**

33 Lethal and sub-lethal endpoints for microplastics toxicity tests should not cease at the level of
34 organisms, they must include the impacts of contaminants on ecosystem functioning.

35 INTRODUCTION

36 Despite the global attempts to manage plastic waste, humanity is still far from confronting the
37 plastic crisis.^{1,2} Even with efforts to reduce plastic pollution, the amount of mismanaged plastic
38 waste expected to be released in aquatic ecosystems by 2030 may reach 53 million metric tons
39 (Mt), twice the amount estimated for 2016.³ Microplastics (plastic particles ≤ 5 mm in size)⁴ are
40 part of this plastic waste, and are transported to the ocean via coastal runoff and river discharge.^{5,6}
41 Once in the river environment, depending on their density,⁷ microplastics can float in the water
42 column, or sink into sediment beds⁸⁻¹⁰ where they can be temporary trapped.¹¹ Microplastic
43 concentrations estimated in European riverbed sediments range from 18 up to 75000 particles kg⁻¹
44 dry weight,^{12,13} with the majority of particles detected being polyethylene, polypropylene, and
45 polystyrene polymer types¹⁴ in a wide range of sizes and shapes (fragments, fibers, and spherical
46 forms).^{12,14,15}

47 The sediment-water interface is a hotspot of biological activity in streams and rivers, which play
48 a fundamental role in ecosystem functioning through biogeochemical processes involved in carbon
49 (C) and nitrogen (N) cycles.¹⁶⁻¹⁸ Although these biogeochemical processes are driven mainly by
50 microbial activities,^{17,19,20} benthic invertebrates acting as bioturbators can significantly influence
51 these microbial processes²¹ by sediment reworking, biogenic structure building (e.g., burrows),
52 and bioirrigation (i.e., the action of benthic organisms flushing their burrows with overlying
53 water). These processes can modify pore water chemistry, which will in turn have consequences
54 on microbial communities and associated biogeochemical processes.²²⁻²⁴ Chironomid larvae
55 (Diptera, Chironomidae) and tubificid worms (Oligochaeta, Tubificidae) have been identified as
56 important bioturbators in freshwater ecosystems.²⁵⁻²⁷ Despite this recognized importance of
57 bioturbators on organic matter processing and nutrient recycling at sediment-water interfaces of

58 freshwater ecosystems, the consequences of microplastics exposure of sediments on these
59 bioturbation-driven processes remain unexplored.

60 Bioturbators like tubificid worms are deposit feeders that can be exposed to microplastics trapped
61 in river bed sediments.²⁸⁻³³ Yet, it is still unclear how this exposure may impact their physiology,
62 and activities in sediments. Although there has been little research on this topic, a reduction in the
63 survival rates of deposit feeding organisms like *Chironomus tepperi*³⁴ and *Caenorhabditis*
64 *elegans*³⁵ exposed to microplastics has been previously reported. In contrast, other exposure
65 studies did not find any adverse effects on growth and survival.³⁶⁻³⁸ In aquatic benthic organisms,
66 it has been shown that microplastics could significantly decrease energy reserves,^{39,40} increase
67 lipid peroxidation,⁴¹ trigger oxidative enzymes,⁴² and generate neurotoxicity.³⁸ However, it has
68 also been reported that exposing deposit feeders to microplastics did not affect their energy
69 storage³² nor cause any oxidative damage.³⁷ The feeding activity of benthic macro-organisms
70 could also be reduced by the presence of microplastics in sediments,⁴³ although this is also not
71 universally accepted and may vary with respect to microplastic concentration, size, shape, polymer
72 type, exposure time and species sensitivity itself.^{32,39}

73 The present study aims to fill this gap by investigating the impacts microplastic exposure may have
74 on the tubificid worm *Tubifex tubifex* and on the ecosystem services they offer, by assessing
75 changes to survival, physiological state and bioturbation activity.⁴⁴⁻⁴⁶ Laboratory experiments
76 were conducted in microcosms at four sediment microplastic concentrations. Health (survival,
77 energetic reserves and oxidative stress) of tubificid worms and their potential consequences on
78 bioturbation activities (sediment reworking and bioirrigation rate) and ecological processes
79 (nutrient and CO₂ fluxes) at the sediment-water interface were assessed.

80 MATERIALS AND METHODS

81 Collection and characterization of sediments used

82 Sediments were collected using a metal shovel and metal buckets from the streambed of the Lone
83 des Pêcheurs, a dead arm of the Rhône river in the South-east of France (45°48'41.1"N, 5°6'1.6"E).
84 Collected sediments were sieved (< 3.6 mm) to exclude any large debris and homogenized in a
85 large glass container using a metal scoop. Sediments were then stored at – 20°C to kill any macro-
86 organisms present. In addition, sediment samples were analyzed to determine their particle size
87 distribution, total organic carbon (TOC) and total nitrogen (TN) content. Particle size distribution
88 was obtained using laser granulometer (Mastersizer, 2000, Malvern Instrument) as reported by
89 Gette-Bouvarot et al. (2014).⁴⁷ Three measurements (i.e. three replicates) were carried out for the
90 sediment after ultrasound treatment (50–60 Hz, 1 min) to eliminate non-stable particle
91 aggregates.⁴⁷ TOC and TN contents were determined by high-temperature *in situ* combustion on
92 pre-acidified (i.e., HCL 2 mol/L) dry samples (60°C, 48hr) for three replicates of 15 mg each,
93 using an elemental analyzer (FlashEA, Thermo Electron Corporation). The microplastic content
94 of the sediment was assessed following an approach modified from those of Frei et al. (2019),⁴⁸.
95 Briefly, after drying at 50 °C for 24 hours, a sediment sample of 50 g was suspended in zinc
96 chloride (ZnCl₂) solution (density 1.7 g cm⁻³) for 24 hours to collect microplastic particles and
97 organic components which were later degraded with 30% H₂O₂ in the presence of 0.05 M Fe²⁺
98 (i.e., aqueous). The remaining material was then stained with Nile Red solvent,^{49,50} filtered, and
99 microplastics > 20 µm sizes were quantified under Nikon SMZ1270 fluorescent stereo microscope.
100 (Table 1).

101 **Table 1** Physical characteristics of the sediment (mean ± SD, n = 3).

Measurements		
Grain size distribution (%)	64 μm < Sand < 2 mm	35.9 \pm 5.8
	2 μm < Silt < 64 μm	60.2 \pm 2.9
	Clay < 2 μm	3.9 \pm 0.3
Organic matter composition (% sediment dry mass)	Total organic carbon	2.4 \pm 0.10
	Total nitrogen	0.2 \pm 0.02
Microplastic concentration	3140 particles / Kg dry weight of sediments	

102

103 **Microplastics preparation**

104 A heterogeneous mixture of two types of microplastic fragments (polystyrene (PS) and polyamide
105 (PA)) and one type of microplastic fibers (PA) was used in this study. The PS particles were made-
106 in-house by cutting cuvettes (VWR) into small pieces and then ground in a Retsch ball mill MM
107 400 at 30 Hz for 30 s with liquid nitrogen. The resulting powder was sieved to exclude polystyrene
108 fragments > 1000 μm using a stainless steel sieve mesh. To obtain multiple shapes of PA particles,
109 fibers (L = 500 μm , W = 15 μm) were obtained from Flock Depot (Stuttgart, Germany) and pellets
110 were sourced from Resinex Ltd (High Wycombe, United Kingdom). The pellets were subsequently
111 frozen at -80°C for 72hr and ground using a Fritsch Pulverisette 0 ball mill with liquid nitrogen
112 for 20 minutes. The resulting PA powder was size fractionated by stainless steel sieves to exclude
113 PA fragments > 1000 μm . Prior to their use in the controlled experiments, the particle size
114 distribution of PS and PA particles (i.e., both fragments and fibers) was measured by laser
115 granulometry with the same method and equipment than those used previously to measure
116 sediment grain size distribution (Supplementary Figure 1). The size and morphological features of
117 microplastic particles were investigated with a fluorescent stereo microscope (Nikon SMZ1270)
118 without fluorescence under the brightfield mode. Overall, the microplastic particles used in the
119 experiment presented a wide variety of shapes and sizes varying from 15 to 1500 μm

120 (Supplementary Figure 2), including particles that could be easily ingested by tubificid worms (<
121 63 μm)²⁸.

122 **Bioturbation model organisms**

123 The tubificid worm, *Tubifex tubifex* was chosen to investigate the impacts of microplastics on
124 freshwater bioturbators as this species has a key role in the biogeochemistry and ecology of
125 freshwater benthic habitats,⁴⁵ and due to its wide use in measuring the ecotoxicity of sediments.^{28,51}
126 Tubificid worms were bought from a commercial breeder (GREBIL & Fils, Arry, France) and
127 acclimatized to experimental conditions (i.e., temperature of 20°C, sediments collected from Lone
128 des Pêcheurs, aerated synthetic water, and under a 16:8 h light: dark cycle) for two weeks prior to
129 the start of the experiment.

130 **Experimental design and preparation**

131 A complete randomized block design was used to test the impacts of the addition of four
132 microplastic concentrations (0 particles/kg sediment dry weight (DW) (control); 700 particles/kg
133 sediment DW (low); 7000 particles/kg sediment DW (medium); 70000 particles/kg sediment DW
134 (high)) on microcosms with or without tubificid worms. By measuring the interaction between
135 microplastic treatments and worm presence, this experimental design aimed to evaluate whether
136 the presence of microplastics impacted the role of tubificid worms in sediments compared with the
137 direct impact of microplastics on microorganisms (treatments without worms). The four
138 microplastic treatments were chosen based on reports of actual environmental realistic
139 concentrations reported from Europe to China.^{12,14,52} These investigations indicated the presence
140 of hundreds to thousands of plastic particles per kilogram of dry sediment, with the greatest
141 concentration of microplastics discovered in the riverbeds of the Irwell River, with up to 75,000
142 particles per kilogram of dry sediment.¹²

143 A total of eight treatments (4 microplastic concentrations * 2 worm conditions) was tested with
144 five replicates per treatment ($N_{\text{total}} = 40$). Each replicate was made up of a 2 L glass bottle (internal
145 diameter: 13 cm, height: 25 cm) filled with 8 cm of sediment (i.e., 0.738 kg DW) (Supplementary
146 Figure 3) previously prepared by mixing microplastic particles with sediments to obtain the four
147 desired concentrations. The mixing process took place one week before filling the 2 L glass bottles
148 by using one glass container (L = 50 cm, W = 20 cm, H = 25 cm) per microplastic treatments (4
149 glass containers were used for the initial preparation of the 4 tested microplastic concentrations,
150 Supplementary Table 1). After a week of homogeneous mixing of sediment with the desired
151 concentration of microplastics (for each treatment, microplastics and sediments were thoroughly
152 mixed in a glass container for 30 min every day during 1 week using a stainless steel spoon),
153 sediments were transferred to 2 L glass bottles (i.e. microcosms), with 10 glass bottles per
154 microplastic treatment. By homogeneously mixing sediment and microplastics, we used a
155 simplified distribution of microplastics in sediments compared with heterogeneous distributions
156 of microplastics in freshwater environments,^{7,48} but it was the best way to control the exposure of
157 tubificid worms to microplastic particles. After sediment addition, 10 cm of overlying synthetic
158 freshwater ($96 \text{ mg L}^{-1} \text{ NaHCO}_3$, $39.4 \text{ mg L}^{-1} \text{ CaSO}_4 \cdot 2\text{H}_2\text{O}$, $60 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $4 \text{ mg L}^{-1} \text{ KCl}$,
159 and $6.4 \text{ mg L}^{-1} (\text{CH}_3\text{CO}_2)_2\text{Ca} \cdot \text{H}_2\text{O}$; pH = 7.5)⁵³ was added to each microcosm. Synthetic water
160 was gently added to each microcosm to avoid any sediment resuspension. Microcosms were
161 aerated to maintain a dissolved oxygen (DO) concentration between 7.5 and 8.5 mg L^{-1} in the water
162 column throughout the full duration of the experiment. The experiment was conducted at a constant
163 room temperature of 20°C under a 16:8 h light: dark cycle. After microcosm preparation, 180
164 individuals of tubificid worms having lengths ranging between 1 and 3 cm were introduced into

165 each microcosm dedicated to treatments with worms (half of the microcosms), representing a
166 density of 13,274 individuals per m² commonly observed in the braided arms of the Rhône river.⁵⁴

167 Then, the experiment started for 77 days, an experimental duration which was long enough to
168 observe long-term impacts on tubificid worms without having a depletion in sedimentary organic
169 matter that could impair food availability for tubificid worms.

170 During the last four weeks of the experiment, the bioturbation activities (surface sediment
171 reworking and bioirrigation rate) of worms and biogeochemical processes (CO₂, CH₄, and nutrient
172 fluxes) were assessed to evaluate the influences of microplastic contaminated sediments on
173 individuals and associated ecosystem processes. At the end of the experiment (i.e., day 77),
174 microcosms were dismantled and sediments were sieved on a 250 µm mesh sieve to recover
175 tubificid worms. After collection, tubificid worms were counted to determine their survival rate in
176 each microplastic treatment. Surviving worms were collected with each worm being examined to
177 see whether it moves or not. Then worms from each microcosm were separated in two groups: a
178 first group of around 130 individuals was used to evaluate the physiological state of worms and a
179 second group of around 50 individuals was used to determine the quantity of microplastics ingested
180 by worms. In microcosms where less than 180 individuals were alive, individuals were divided
181 into two groups while maintaining the same ratio of division.

182 To avoid contamination with microplastics from laboratory equipment, we used glass material for
183 handling of particles, manipulating tubificid worms and for exposure experiments. Materials were
184 covered to reduce airborne contamination.

185 **Exposure impact analysis**

186 ***Physiological analyses***

187 The first group of collected worms (130 individuals per microcosm) were kept for 48hr in glass
188 beakers filled with 0.7 μm filtered synthetic water for depuration. Afterwards, tubificid worms
189 were freeze-dried and weighed collectively. These freeze-dried 130 individuals were then divided
190 randomly into four small subgroups, each subgroup being used to perform a given physiological
191 analysis (2 analyses associated with energy body stores and 2 analyses associated with oxidative
192 stress). Energy body stores were determined in the whole animal by extracting and measuring the
193 amount of triglycerides (Sigma Aldrich, T2449 and F6428) and glycogen (Sigma Aldrich, G3293)
194 as described in Hervant et al. (1999) and Salin et al. (2010).^{55,56} Two markers of oxidative stress
195 were measured: malondialdehyde (MDA) content which is a biomarker of lipid peroxidation
196 (LPO) and the antioxidant enzyme superoxide dismutase (SOD) activity which is induced by the
197 formation of reactive oxygen species (ROS). The thiobarbituric acid (TBARS) assay (Cayman
198 chemicals, 10009050) was used to determine the MDA content⁵⁷ and the SOD activity (Cayman
199 chemicals, 706002) was estimated according to Flohe (1984) and Lawniczak et al. (2013).^{57,58}
200 MDA contents were expressed in nanomoles (nmol) of MDA mg^{-1} dry mass, whereas SOD
201 catalytic activities were expressed as U g^{-1} dry mass, where one unit was defined as the quantity
202 of enzyme that reduced 50% of oxidized cytochrome c (which is oxidized under superoxide
203 production).

204 ***Microplastics ingestion analysis***

205 The second group of collected worms (50 individuals combined per microcosm) were cleaned
206 from external debris using vacuum filtered ultrapure (VFUP) water (i.e., filtered through Whatman
207 GF/F filters (0.7 μm)) without any depuration phase. Then, microplastics were extracted from
208 tubificid worms according to Hurley et al. (2017) with some modifications.²⁸ Fresh tubificid
209 worms were weighed and placed into glass tubes previously washed three times with VFUP water.

210 Worms were digested with 30% H₂O₂ in the presence of 0.05 M Fe²⁺ (i.e., aqueous) catalyst
211 solution at an ambient room temperature for 24hr. After digestion, samples were washed with
212 VFUP water then filtered through Whatman GF/F filters (0.7 µm). The number of microplastics
213 were determined using a Nikon SMZ1270 fluorescent stereo microscope after staining with Nile
214 Red of 5 µm mL⁻¹ as final concentration.^{49,50} The stereo microscope was fitted with Intensilight C-
215 HGFIL Lamp (130w) with a 0.75 X objective lens. All measurements were done at an overall
216 magnification of 15 X. Three procedural blanks were prepared simultaneously. These blanks were
217 assumed to indicate background microplastic contamination, and as such, blank values were
218 subtracted from the final results.

219 ***Sediment reworking analysis***

220 As microplastic contamination has been shown to reduce the egestion of fecal pellets by the marine
221 worm *A. marina*,⁵⁹ we also expected that microplastic exposure could reduce the production of
222 fecal pellets at the sediment surface by tubificid worms. To assess this potential effect, the impact
223 of microplastic exposure was measured on the surface sediment reworking (SSR) process induced
224 by tubificid worms during 5 days in the last week of the experiment (from day 70 to day 74,
225 Supplementary Figure 4) using the method of De Nadaï-Monoury et al. (2013).⁶⁰ For each
226 microcosm, a 2 mm thick layer of a particulate tracer (white sand of Fontainebleau) was uniformly
227 distributed at the surface of the sediment seven days before the end of the experiment.
228 Translocation of the tracer due to the fecal deposition by tubificid worms was monitored by taking
229 pictures every 12 hours for five days. Pictures were analyzed using ImageJ software.⁶¹ Automatic
230 pixel counts were performed based on colors (sandy tracer was white whereas fecal pellets
231 appeared in dark, Supplementary Figure 5) to estimate the surface covered by tracer. The
232 remaining area covered by fecal pellets was calculated as the difference between total surface area

233 of microcosms minus surface occupied by the tracer. SSR rate was calculated and expressed in %
234 of area recovered by fecal pellets per hour.

235 ***Bioirrigation activity***

236 A dissolved conservative tracer (i.e., KCl) was added to the water column (as the methodology
237 used by Mermillod-Blondin et al. (2004)⁶² with Br⁻ as conservative tracer) to quantify water fluxes
238 from the water column to sediment. This measurement was done for 1 day, two weeks before the
239 end of the experiment (from day 63 to day 64, Supplementary Figure 4), by monitoring the
240 concentration shift over time of the dissolved tracer. More precisely, for each microcosm, water
241 was replaced with synthetic freshwater enriched with KCl to obtain a concentration of 37 mg L⁻¹
242 of Cl⁻, a concentration which was not a stress for *Tubifex tubifex*,⁶³ and commonly measured in
243 freshwater environments.⁶⁴ Using acid-washed 100 ml syringes, water samples were collected
244 immediately after water column replacement and 1 day after for each microcosm. Water samples
245 were filtered through Whatman GF/F filters (0.7 µm) and Cl⁻ concentration were analyzed using a
246 sequential analyzer based on colorimetric methods (Smartchem 200, AMS Alliance, France). The
247 decrease in Cl⁻ concentrations between the two days was used to evaluate the water fluxes at the
248 sediment-water interface in each microcosm. Based on changes in Cl⁻ concentrations and the water
249 volume in microcosms, these fluxes were expressed in L of water exchanged per day and m⁻².

250 ***Biogeochemical analysis***

251 The influence of microplastic contamination on biogeochemical processes (organic matter
252 mineralization and nutrient cycling) was estimated by measuring the CO₂ and CH₄ fluxes and the
253 fluxes of nutrients (N-NH₄⁺, N-NO₃⁻, N-NO₂⁻ and P-PO₄³⁻) at the sediment-water interface for each
254 microcosm.^{27,65}

255 CO₂ and CH₄ fluxes were measured for 5 days according to Pigneret et al. (2016),²⁷ three weeks
256 before the end of the experiment (from day 56 to day 60, Supplementary Figure 4). Each
257 microcosm was tightly sealed with a lid connected to a pump and a greenhouse gas analyzer
258 (G2201i PICARRO). CO₂ and CH₄ fluxes were measured by monitoring the increase in
259 concentrations every 2 hr during 10 hr at 20 °C for each microcosm. Then, CO₂ and CH₄ fluxes
260 were expressed in mmol per hour and m² of sediment-water interface.

261 For nutrient fluxes, 2/3 of the water column was replaced in each microcosm four weeks before
262 the end of the experiment (from day 49 to day 53, Supplementary Figure 4). For five days, water
263 samples (100 ml) were collected in the water column of each microcosm at daily intervals. Water
264 samples were taken using acid-washed 100 ml syringes, filtered through Whatman GF/F filters
265 (0.7 μm), and analyzed for nutrient concentrations within 24hr using a sequential analyzer based
266 on colorimetric methods (Smartchem 200, AMS Alliance, France). The fluxes of N-NH₄⁺, N-NO₃⁻
267 , N-NO₂⁻ and P-PO₄³⁻ at the sediment-water interface were calculated from the linear changes over
268 time of the concentration of each nutrient in the water column. Then, nutrient fluxes were
269 expressed in mg per day and m² of sediment-water interface. Positive fluxes indicated an efflux of
270 nutrients from sediments to the water column whereas negative fluxes indicated an influx of
271 nutrients from the water column to sediments.

272 **Statistical Analyses**

273 For the three physiological variables measured on tubificid worms (glycogen content, triglyceride
274 content, SOD activity), the influence of microplastic treatments (control, low, medium, and high)
275 was tested using one-way analyses of variance (1-way ANOVAs) with microplastic concentrations
276 as the fixed factor. A one-way ANOVA was also used to test the influence of microplastic
277 treatments on the surface sediment reworking rate due to tubificid worms. For chloride, nutrient

278 fluxes, CO₂ and CH₄ fluxes, the effects of microplastic treatments, tubificid worm treatment and
279 their interactions were tested using two-way analyses of variance (2-ways ANOVA) with
280 microplastic concentrations and tubificid worms as fixed factors. When 2-ways ANOVA revealed
281 significant differences among treatments (i.e., $p < 0.05$), Tukey's post hoc tests were performed to
282 evaluate which treatments significantly differed. For all variables, the normality and the
283 homoscedasticity of the residues were verified using the Shapiro-Wilk's test and the Bartlett's test,
284 respectively. As for survival rates and MDA contents the requirements for normality and
285 homoscedasticity were not met, non-parametric Kruskal-Wallis test were performed. All statistical
286 analyses were done with the RStudio software.⁶⁶

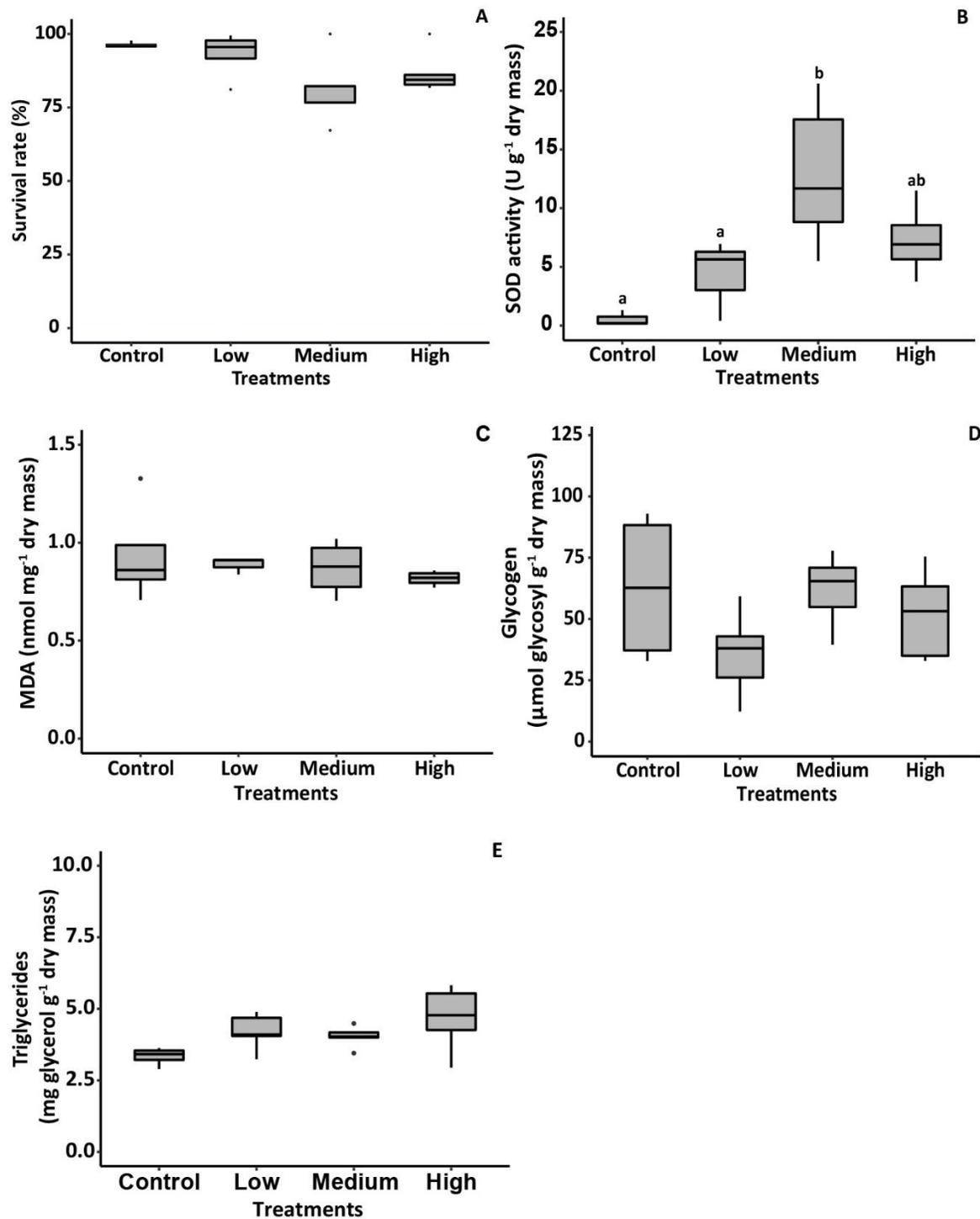
287 **RESULTS AND DISCUSSION**

288 **Survival and physiology of *Tubifex tubifex***

289 After 77 days of exposure, the survival rates of tubificid worms were higher than 75% in all
290 microplastic treatments (Figure 1A). Survival rate was not significantly affected by microplastic
291 concentrations (Kruskal–Wallis test, $H(3) = 3.8$, $p = 0.28$) although it tended to decrease for
292 individuals exposed to the medium microplastic concentration in comparison with the control
293 treatment. Interestingly, this trend was associated with a significant increase in SOD activity in
294 tubificid worms exposed to the medium microplastic concentrations (Figure 1B, 1-way ANOVA,
295 $p < 0.05$) with a mean value of $12.8 \pm 6.2 \text{ U g}^{-1}$ dry mass which was 16-fold higher than in the
296 control treatment ($0.8 \pm 1.9 \text{ U g}^{-1}$ dry mass). These differences in SOD activity among microplastic
297 treatments were not associated with significant changes in MDA concentrations among tubificid
298 worms exposed to different microplastic treatments (Figure 1C, Kruskal–Wallis test, $H(3) = 2.49$,
299 $p = 0.48$). Observed increase in SOD activity showed that tubificid worms exposed to microplastics
300 produced antioxidant defense enzymes to catalyze the dismutation of superoxide into molecular

301 oxygen and hydrogen peroxide.^{27,67} Thus, the physiology of tubificid worms was influenced by
302 reactive oxygen species (ROS) generated by microplastic exposure, especially for the medium
303 microplastic concentration. In general, the presence of ROS causes lipid peroxidation (LPO)
304 damage that leads to an increase in MDA which is a by-product of LPO.⁶⁸ Nevertheless, the lack
305 of significant increase in MDA concentrations in tubificid worms despite an increased production
306 of SOD enzymes indicated that tubificid worms succeeded in neutralizing both ROS production
307 and oxidative damage to maintain their homeostasis (a mechanism described by Trestrail et al.
308 (2020)).⁶⁹ This mechanism has never been detected in invertebrates but has been already observed
309 in the freshwater fish *Oreochromis niloticus* by Ding et al. (2018).⁷⁰ These authors found that the
310 presence of polystyrene microplastics significantly increased SOD activity in the fish without
311 affecting its MDA content.⁷⁰ Thus, in the present experiment, it is likely that oxidative stress
312 provoked by microplastic exposure was efficiently managed by antioxidative defenses of tubificid
313 worms, preventing cellular damages caused by high lipid peroxidation levels.⁶⁹

314 In addition, the presence of microplastics at different concentrations did not significantly affect
315 energy reserves in tubificid worms (Figure 1D, E, 1-way ANOVAs, $p = 0.2$ for glycogen and $p =$
316 0.1 for triglyceride concentration). These findings are in agreement with Van Cauwenberghe et al.
317 (2015) who observed that polystyrene microspheres had no significant impact on the energy
318 reserves of the lugworm *Arenicola marina* (deposit feeder) or the blue mussel *Mytilus edulis* (filter
319 feeder).³² Theoretically, the antioxidative defenses activated in tubificid worms when exposed to
320 microplastics should have generated a significant consumption of energy reserves, leading to lower
321 stocks of glycogen and triglycerides in worms exposed to medium microplastic concentrations.^{71,72}
322 However, the present study indicated that tubificid worms did not rely on their energy stores to
323 manage oxidative stress induced by microplastics exposure.



324

325

326

327 **Figure 1.** Survival rates (A), superoxide dismutase activity (B), malondialdehyde concentration
 328 (C), concentration of glycogen (D), and triglyceride (E) of *Tubifex tubifex* after seventy-seven days
 329 of microplastic exposure in sediments. For each treatment, data are presented as means \pm S.D. (n

330 = 5). Different letters indicate significant differences among microplastic treatments (Tukey test
331 at $p \leq 0.05$). The absence of letters indicates no significant difference among treatments (Tukey
332 test at $p \geq 0.05$).

333 **Ingestion of microplastics**

334 Although the microplastic mixture contained particles that could be easily ingested by tubificid
335 worms ($< 63 \mu\text{m}$)²⁸ and despite the presence of high microplastic concentrations in sediments (up
336 to 70000 particles/kg DW), no significant numbers of microplastic particles (neither fragments nor
337 fibres) were detected by fluorescence microscopy in the 50 organisms digested with Fenton reagent
338 at the end of the experiment (Supplementary Table 2). This lack of ingested microplastics could
339 partly explain the lack of observed effects on energy reserves in tubificid worms. For example,
340 Wright et al. (2013) reported that longer gut residence time of ingested microplastics was
341 positively correlated with the consumption of energy reserves in marine worms (*Arenicola*
342 *marina*).⁴⁰ As we did not detect microplastics particles in tubificid worms tissues, tubificid worms
343 were more likely able to exclude the microplastics when feeding on sediments, thereby avoiding a
344 large oxidative stress and the high energy expenditure associated with defense against oxidative
345 damages. This interpretation is supported by Rodriguez et al. (2001) who showed that tubificid
346 worms could selectively feed on fine organic rich particles in sediment.⁷³ Selective feeding
347 excluding microplastic particles has been also reported by other aquatic deposit feeders as
348 polychaetes of the genus *Marenzelleria*⁷⁴ Therefore, the observed elevation in SOD activity in
349 tubificid worms exposed to microplastics could be the result of external physical damage induced
350 by plastic particles rather than due to ingestion.⁷⁵ It is also worth noting that the physiological
351 impacts induced by microplastic exposure on biota have been reported to be species-specific.³⁹ In
352 the present study, it can be concluded that the benthic species *T. tubifex* was relatively tolerant to

353 the microplastic exposure applied, explaining the low impact on MDA and worm survival. Such
354 conclusion was consistent with other field studies that have reported that *T. tubifex* is one of the
355 most pollution-resistant species living in freshwater environments.^{76,77}

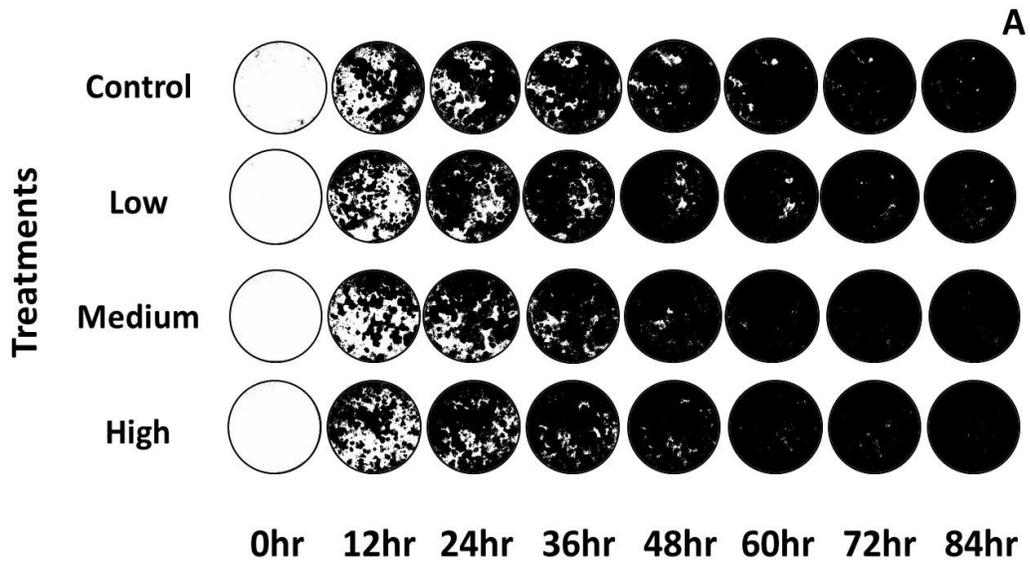
356 **Bioturbation activities of *Tubifex tubifex***

357 While surface sediment reworking (SSR) was absent in the treatments without tubificid worms,
358 SSR rapidly occurred after the addition of the white Fontainebleau sand (non-fluorescent tracer)
359 in microcosms with *Tubifex tubifex* (Figure 2A). The mean surface reworked by tubificid worms
360 exceeded 50% of microcosm surface for all treatments after 12hr (Figure 2B). The surface of
361 sediment reworked increased strongly within the first 12hr to reach values higher than 50% (Figure
362 2A). As most of the sediment surface was already reworked within these first 12hr, SSR rates
363 decreased after this time (Figure 2B). For this reason, only the areas occupied by fecal pellets
364 within the first 12hr were used to compare treatments (Figure 2C). Although statistical analyses
365 were only marginally significant (1-way ANOVA, $0.05 < p < 0.1$), SSR due to fecal pellet
366 deposition by worms tended to decrease with the increasing concentration of microplastics added
367 to sediments. After 12hr of tubificid worm bioturbation, the SSR was $60.9 \pm 5\%$ in the treatment
368 without microplastic (control) and was reduced by around 12% in the treatment with the highest
369 microplastic concentration.

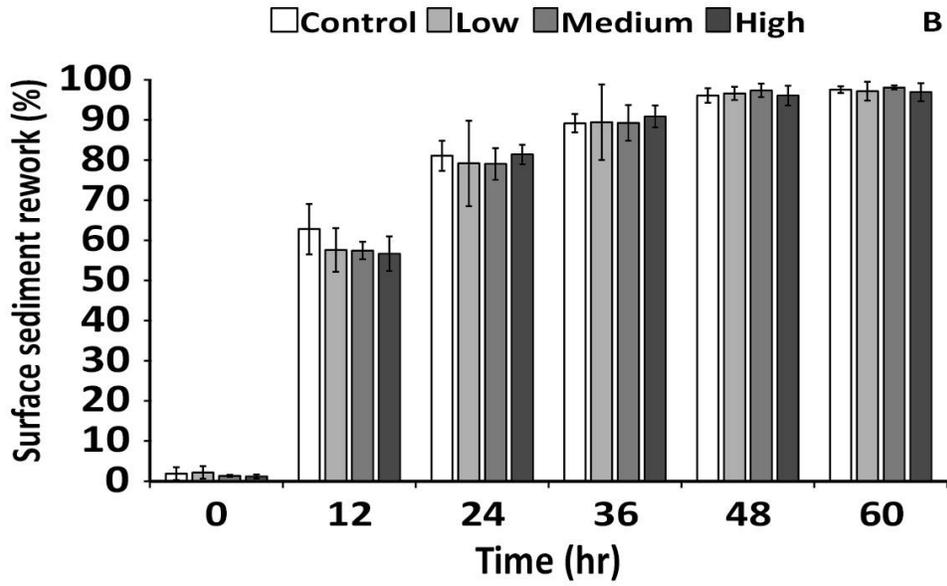
370 Tubificid worms significantly increased the water flux between water and sediments in the
371 microcosms (2-way ANOVA, worm effect, $p < 0.05$) regardless of the microplastic treatment (2-
372 way ANOVA, interaction between worm treatments and microplastic treatments, $p = 0.8$). For all
373 microplastic treatments, the exchange of Cl^- measured at the sediment-water interface was around
374 two-fold higher in treatments with tubificid worms than in treatments without worms (Figure 2D).
375 Nevertheless, it is worth noting that no significant difference in water fluxes was detected between

376 treatments with and without worms at medium microplastic concentrations (Tukey's test, $p = 0.1$)
377 whereas significant differences between treatments with and without worms were measured for all
378 other microplastic concentration treatments (Tukey's tests, $p < 0.05$).

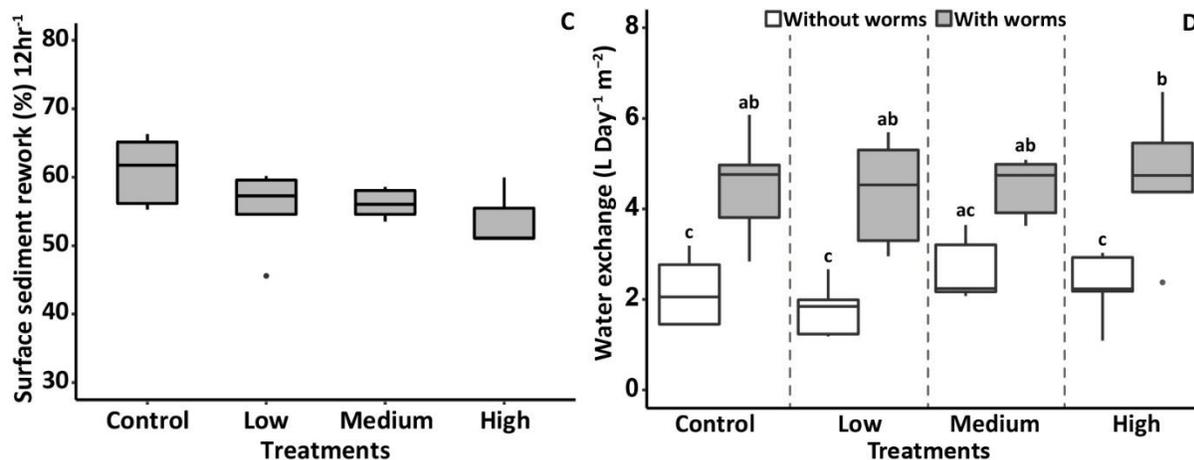
379 SSR measurements indicate that the presence of microplastics tended to decrease the feeding
380 activity and the deposition of fecal pellets by tubificid worms at the sediment surface. In contrast,
381 the bio-irrigation process generated by tubificid worms and assessed by water flux measurements
382 was not strongly affected by microplastic treatments. Thus, microplastic contamination did not
383 affect similarly bioirrigation and feeding activities of tubificid worm, highlighting the complex
384 influences of microplastic contaminations on bioturbators. Remarkably, the different impacts of
385 microplastics on bioirrigation and sediment reworking processes could have different effects on
386 ecosystem functioning because these two bioturbation processes are known to differently stimulate
387 nutrient fluxes and mineralization processes in sediments.^{26,78-80}



388



389



390
 391 **Figure 2.** Sediment surface images taken from four replicates of each microplastics treated
 392 microcosm in the presence of tubificid worms. White pixels represent the white Fontainebleau
 393 sand tracer disappearing within 84hr through the addition of fecal pellets (A). Surface sediment
 394 reworking within 60 hr (B), percentage of surface sediment reworking in first 12hr (C) and
 395 bioirrigation (D). For each treatment, data are presented as means \pm S.D. (n = 5). Different letters
 396 indicate significant differences among treatments (Tukey test at $p \leq 0.05$). The absence of letters
 397 indicates no significant difference among treatments (Tukey test at $p \geq 0.05$).

398 **Biogeochemical Fluxes**

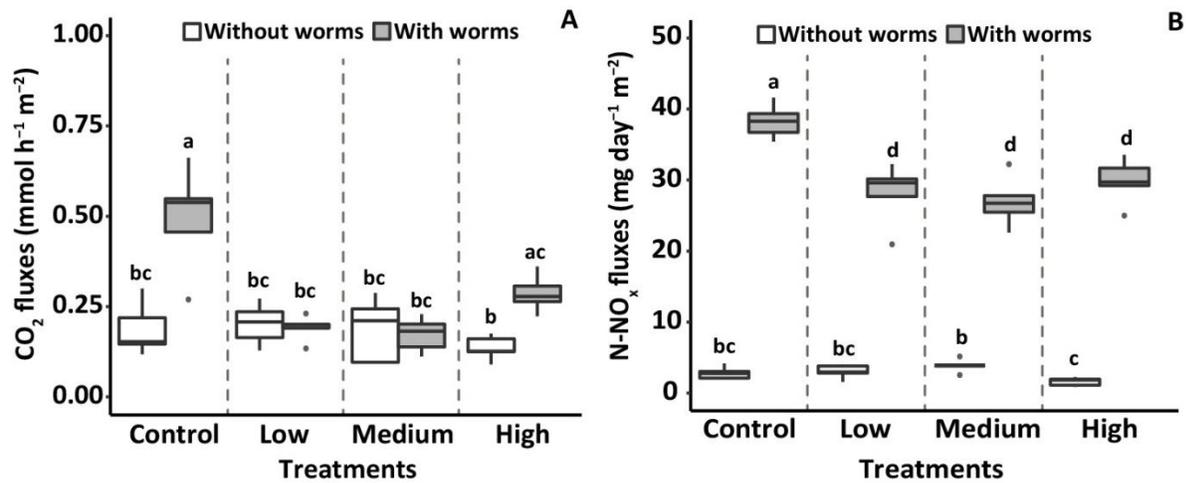
399 No significant CH₄ fluxes were measured from all microcosms, whereas significant CO₂ fluxes
 400 were calculated from linear increases of CO₂ concentration over time (Supplementary Figure 6A,
 401 and 6B). Overall, tubificid worms significantly increased CO₂ fluxes (Figure 3A, 2-way ANOVA,
 402 worm effect, $p < 0.05$) depending on microplastic concentrations in sediments (2-way ANOVA,
 403 statistical interaction between worm treatments and microplastic treatments, $p < 0.05$). CO₂ fluxes
 404 were around 2.5-fold higher with worms ($0.47 \text{ mmol h}^{-1} \text{ m}^{-2} \pm 0.2$) than without worms (0.19 mmol
 405 $\text{h}^{-1} \text{ m}^{-2} \pm 0.1$) in the control treatment (Figure 3A, Tukey's HSD, $p < 0.05$). In contrast, CO₂ fluxes
 406 were similar between control microcosms and microcosms with worms in the presence of low and

407 medium microplastic concentrations (Figure 3A, Tukey's HSD, $p > 0.5$). Surprisingly, this
408 inhibition of the effects of tubificid worms on CO_2 fluxes was not observed in the treatment with
409 the highest microplastic concentration where tubificid worms stimulated by two-fold the CO_2
410 fluxes emitted from microcosms (Figure 3A, Tukey's HSD, $p < 0.05$). It is also worth noting that
411 microplastic contamination did not have a direct effect on microbial production of CO_2 as CO_2
412 fluxes were comparable among the four microplastic treatments without tubificid worms (Figure
413 3A, Tukey's HSD, $p > 0.05$).

414 Nutrient flux measurements did not report any linear increase in N-NH_4^+ and P-PO_4^{3-}
415 concentrations with incubation time, indicating no significant fluxes of these two nutrients from
416 sediments to water column (Supplementary Figure 6C, and 6D). The absence of N-NH_4^+ and P-
417 PO_4^{3-} fluxes associated with the lack of measured CH_4 flux (associated with methanogenesis
418 processes) indicate that no strong anoxic conditions occurred in the sediment used for the
419 experiment. More precisely, aerobic conditions in sediments probably inhibited the fluxes of PO_4^{3-}
420 released to the water column by facilitating the adsorption of P with Fe(OH)_3 and Mn(OH)_2 .²⁶ Such
421 aerobic conditions could also have minimized the release rates of NH_4^+ from sediments by
422 facilitating a rapid nitrification of NH_4^+ produced by organic matter mineralization into N-NO_3^-
423 .⁸¹ In line with this facilitated nitrification process, N-NO_x ($\text{N-NO}_3^- + \text{N-NO}_2^-$) concentrations
424 linearly and significantly increased during the incubation, indicating a significant release rate of
425 NO_3^- from sediments to the water column (Supplementary Figure 6E, and 6F). Overall, tubificid
426 worms significantly increased the NO_x fluxes from sediments to the water column in microcosms
427 (Figure 3B, 2-way ANOVA, worm effect, $p < 0.05$). However, this stimulation of NO_x fluxes due
428 to the presence of worms was significantly reduced in presence of microplastics at low, medium
429 and high concentrations (Figure 3B, 2-way ANOVA, interaction "worm*MPs" effect, $p < 0.05$,

430 Tukey's HSD post-hoc tests, $p < 0.05$). As observed for CO_2 fluxes, NO_x fluxes were comparable
431 among the four microplastic treatments without tubificid worms (Figure 3B, Tukey's HSD, $p >$
432 0.05), suggesting no direct influence of microplastics on microbial processes involved in N
433 cycling.

434 These results clearly highlighted that microplastic exposure significantly reduced the contribution
435 of tubificid worms on organic matter processing and nutrient cycling at the sediment-water
436 interface. To our knowledge, this is the first evidence of an adverse impact of microplastics not
437 only on organisms, but also on their contributions to ecosystem functioning.



438
439 **Figure 3.** CO_2 (A) and N-NO_x ($\text{N-NO}_3^- + \text{N-NO}_2^-$) fluxes (B) measured in the water columns. For
440 each treatment, data presented as means \pm S.D. ($n = 5$). Different letters mean significant
441 differences among treatments (Tukey test at $p \leq 0.05$).

442 Main conclusions and perspectives

443 The present study highlighted a significant impact of microplastic exposure on bioturbation-driven
444 processes (organic matter mineralization and nutrient fluxes). These effects were probably
445 associated with a reduction of bioturbation activity of tubificid worms monitored by fecal pellet

446 production at the sediment-water interface. However, the obtained results were only marginally
447 significant because more than 50% of the sediment surface area was already covered by fecal
448 pellets at the first time of analysis (12hr). Under these conditions, it was difficult to observe
449 significant results among treatments. Thus, for future experiments, monitoring fecal pellet
450 production at higher frequencies (every 3hr for example) would permit more pertinent calculations
451 of fecal pellet deposition rates. Furthermore, the use of luminophore tracer distribution in the
452 sediment column at the end of the experiment⁸²⁻⁸⁴ or techniques such as computed tomography
453 (CT) scanning⁸⁵ could be promising approach to evaluate more precisely the impacts of
454 microplastic exposure on bioturbation process.

455 Interestingly, we also found that the effects of microplastics on the stimulation of biogeochemical
456 processes by worms were not positively correlated with microplastic concentrations. First, the
457 positive effects of tubificid worms on CO₂ fluxes were totally inhibited at the lowest concentration
458 of microplastics in sediments. Similarly, the role of tubificid worms in N-NO_x fluxes from
459 sediment to water column was also impacted in the presence of low microplastic concentrations.
460 These results indicate that concentrations exceeding 700 particles/kg sediment DW and observed
461 in a wide range of freshwater environments^{12,14,48,86} may damage the role played by bioturbators
462 on ecosystem functioning. Second, medium microplastic concentrations (7000 particles/kg
463 sediment DW) induced stronger adverse impacts on tubificid worm physiology (higher SOD
464 activity) and activities (worm effect on CO₂ fluxes) than the highest concentration of microplastics
465 in sediments (70000 particles/kg sediment DW). This surprising result could be due to the ability
466 of tubificid worms to better detect and then to avoid microplastic particles at their highest
467 concentration (may be because they are less embedded with sediment particles than for lower
468 concentrations). This mechanism of microplastic avoidance has been already reported in other

469 invertebrate species which were able detect the physical presence of plastic particles and/or
470 chemical cues associated with microplastics.^{75,87} Under these conditions, high concentrations of
471 microplastic particles might have modified the burrowing behavior of tubificid worms (as
472 suggested by change in fecal pellet production) and the gallery network produced by worms in
473 sediments. For example, it has been largely observed that contaminants could influence the
474 structure of biogenic structures produced by bioturbators in sediments^{27,84} and soils.⁸⁸ Therefore,
475 change in gallery network would have modulated the solute fluxes at the sediment-water interface
476 by modifying the exchange area between sediments and surface water,⁸⁹ impacting the
477 biogeochemical processes occurring in sediments.⁹⁰ Further experiments based on precise
478 quantifications of tubificid worm activities in sediments (creation of biogenic structure, sediment
479 reworking using luminophores) are needed to decrypt these complex interactions among
480 microplastic contaminations, tubificid worm bioturbation and biogeochemical processes at the
481 sediment-water interface of freshwater ecosystems.

482

483 **SUPPORTING INFORMATION**

484 Additional information is provided on microplastic characterization, microcosm setup, workflow
485 for parameter analysis, sediment surface images and ImageJ analysis, gas and nutrient fluxes, the
486 number of microplastics added to the sediment, and the number of microplastics extracted from
487 worms.

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503

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