Accumulation of polybrominated diphenyl ethers and microbiome response in the great pond snail Lymnaea stagnalis with exposure to nylon (polyamide) microplastics

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Accumulation of polybrominated diphenyl ethers and microbiome response in the great pond snail *Lymnaea stagnalis* with exposure to nylon (polyamide) microplastics

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Abstract

Microplastics attract widespread attention, including for their potential to transport toxic chemicals in the form of plasticisers and associated hydrophobic organic chemicals, such as polybrominated diphenyl ethers (PBDEs). The aims of this study were to investigate how nylon (polyamide) microplastics may affect PBDE accumulation in snails, and the acute effects of nylon particles and PBDEs on survival, weight change and inherent microbiome diversity and community composition of the pond snail *Lymnaea stagnalis*. Snails were exposed for 96 hours to BDEs-47, 99, 100 and 153 in the presence and absence of 1% w/w nylon microplastics in quartz sand sediment. No mortality was observed over the exposure period. Snails not exposed to microplastics lost significantly more weight compared to those exposed to microplastics. Increasing PBDE concentration in the sediment resulted in an increased PBDE body burden in the snails, however microplastics did not significantly influence total PBDE uptake. Based on individual congeners, uptake of BDE 47 by snails was significantly reduced in the presence of microplastics. The diversity and composition of the snail microbiome was not significantly altered by the presence of PBDEs nor by the microplastics, singly or combined. Significant effects on a few individual operational taxonomic units (OTUs) occurred when comparing the highest PBDE concentration with the control treatment, but in the absence of microplastics only. Overall within these acute experiments, only subtle effects on weight loss and slight microbiome alterations occurred. These results therefore highlight that *L. stagnalis* are resilient to acute exposures to microplastics and PBDEs, and that microplastics are unlikely to influence HOC accumulation or the microbiome of this species over short timescales.

1. Introduction

Microplastics are a widely-recognised pollutant. The impacts of microplastics on biota and ecosystems, and their interactions with other environmental pollutants under various environmental conditions, are highly uncertain and existing studies have produced contradictory results (see discussions of expert committee summarised in the report published by SAPEA (2019)). Due to the high affinity of microplastic surfaces for hydrophobic organic chemicals (HOCs), there is potential for particles to sorb HOCs (Hirai et al., 2011; Karapanagioti et al., 2011; Rochman et al., 2013b), which may lead to elevated or reduced bioaccumulation of HOCs by organisms that ingest these microplastics (Bakir et al., 2016; Besseling et al., 2013; Rochman et al., 2013a). However, other studies have not found clear evidence for microplastics altering bioaccumulation or toxicity of HOCs (Ašmonaitė et al., 2018; Beiras and Tato, 2019; Besseling et al., 2017; Horton et al., 2018). The question therefore remains as to whether microplastics will significantly alter the impacts of HOCs on organisms.

Within the group of HOCs, polybrominated diphenyl ethers (PBDEs), brominated hydrocarbons commonly used as flame-retardants, are one of the priority pollutant groups. They are found widely throughout the environment (Guan et al., 2007; Hassanin et al., 2004), including in riverine sediments (up to 16088 ng g^{-1} dry weight total PBDEs in riverbank sediment in China (Luo et al., 2007)). Although the majority of PBDE effects have been reported on mammals (He et al., 2011; Ji et al., 2011), PBDEs have been shown to have a range of toxic effects on aquatic invertebrates including neurotoxicity, genotoxicity and endocrine disrupting properties (Breitholtz and Wollenberger, 2003; Díaz-Jaramillo et al., 2016).

As with other persistent organic pollutants, due to their relatively high log Kow, PBDEs sorb to particulate and organic matter within the environment, and to fatty tissues of organisms where they can bioaccumulate (Rahman et al., 2001). Where microplastics and PBDEs occur together, there is the likelihood of interactions. One environmental study found microplastics from the Central Pacific Gyre had surface concentrations of PBDEs up to 9900 ng g^{-1} (Hirai et al., 2011). Teuten et al. (2009) found lower concentrations of up to 57 ng g^{-1} from microplastics at the same location, however both these studies suggest the potential for such interactions to influence organism exposure. While some of these

measured concentrations likely derive from flame-retardant additives rather than sorbed chemicals, the hydrophobic nature of PBDEs and widespread presence of PBDEs suggests that a proportion is sorbed from the environment (Hirai et al., 2011; Mizukawa et al., 2009). Chua et al. (2014) and Rochman et al. (2013a) have shown that the presence of microplastics within experimental systems can lead to increased body burdens of PBDEs in amphipods and fish, with the type and concentration of microplastics affecting the dynamics of bioaccumulation. Microplastics can also change the way in which different PBDE congeners are accumulated, with higher brominated congeners more likely to be accumulated when microplastics are present (Chua et al., 2014).

The gut microbiome is important for nutrition, metabolic function and immunity, with perturbations to the microbial community understood to have implications for organism health and fitness (Licht and Bahl, 2018; Zhu et al., 2018a). A number of studies have been carried out to determine the effects of PBDEs on the gut microbiome of various organisms. Chen et al. (2018) investigated the effects of BDE-71 on the gut microbiome of zebrafish, finding that, in the presence of BDE-71, bacterial diversity was significantly reduced, and bacterial metabolic functioning was altered in a 7-day exposure. Li et al. (2018) showed BDEs-47 and 99 to significantly affect the gut microbial diversity of mice, leading to up- and down-regulation of 45 bacterial OTUs (5-day exposure), while Wang et al. (2018) also found BDE-47 to also lead to a significant reduction in mouse gut microbial diversity and an alteration in the community structure (21-day exposure). Studies have shown that microplastics can similarly alter the gut microbiome of both vertebrates (Jin et al., 2018; Lu et al., 2018) and invertebrates (Zhu et al., 2018a; Zhu et al., 2018b). These studies clearly show that microbiome alterations, expressed as species richness and diversity, are a sensitive endpoint responding to HOC and microplastic exposure, even over short timescales. Therefore, microbiome analysis together with host fitness could provide a fast screening tool for assessing the effects of combined HOCs and microplastics during acute exposures.

The aim of this study was to investigate the effects of microplastics and PBDEs, individually and in combination, on the accumulation, physiology and microbiome of the great pond snail *Lymnaea stagnalis* (Linnaeus 1758). Molluscs have been shown to bioaccumulate organic chemicals (and metals) as they lack the oxidase systems to metabolise xenobiotic substances (Geyer et al., 1982). These traits

make them well suited as a test organism for investigating organic pollutant accumulation (Amorim et al., 2019). Although microplastics and PBDEs have been shown to individually alter the gut microbiome of organisms once ingested, no studies to date have investigated the effects of co-exposure to these pollutants with respect to microbiome responses. We hypothesise that increasing PBDE sediment concentrations will lead to significant changes in the microbiome community (diversity and composition) and that the presence of microplastics will reduce this effect through strong binding of PBDEs, making them less bioavailable to microbiota within the gut. We also hypothesise that the presence of microplastics will reduce PBDE accumulation in the snail.

2. Materials and methods

2.1. Organisms

Adult *Lymnaea stagnalis* were obtained from Blades Biological, UK, and were acclimatised for one week under laboratory conditions prior to the exposure. Cultures were maintained and exposure studies carried out using ISO artificial freshwater as recommended by the OECD for *L. stagnalis* (OECD, 2016). An air pump with an air stone was provided for system oxygenation. Stock cultures and exposures were maintained at 20°C with a 16:8 h light:dark cycle. Snails in culture were fed well-washed iceberg lettuce *ad libitum*.

2.2. Microplastic particles

Nylon 6 powder (mono-constituent substance, density 1.13 g cm^{-3}) was purchased from Goodfellow (Huntingdon, UK). This powder consisted of heterogeneous fragments <50 μm, with a mean size of 13-19 μm, measured using a Coulter Counter (Multisizer 3, Beckman, USA) and had been stained with Nile Red dye.

2.3. PBDEs

125 Method 527 PBDE Mixture was purchased from LGC Standards (Teddington, UK). This mixture
126 contained BDE- 47, 99, 100, 153 and PBB- 153 (PBB-153 was not considered or measured throughout
127 this study), each at a concentration of 500 μg ml^{-1} in ethyl acetate. With respectively log Kows of 6.81,
128 7.32, 7.24, and 7.9, these BDEs were all highly hydrophobic. These congeners are commonly detected
129 within aquatic organisms and have a high propensity for bioaccumulation (Hirai et al., 2011;
130 Shanmuganathan et al., 2011). A serial dilution was carried out in ethyl acetate in order to provide the
131 ultimate concentrations of each BDE congener in sediment of 3000, 1500, 750, 375, 188 and 94 ng g^{-1}.
132 These concentrations were chosen to reflect concentrations found within freshwater sediments (Luo et
133 al., 2007; Sellström et al., 1998; Yin et al., 2017).

134

135 **2.4. Experimental setup**

136 All experimental treatments (PBDEs and controls) consisted of either sediment without added
137 microplastics, or sediment with added microplastics (1% nylon powder by sediment mass, *i.e.* 10 g kg^{-1}).
138 This is a higher microplastic concentration than has previously been found in freshwater sediments,
139 for example up to 71 mg kg^{-1} found in the Antuã River, Portugal (Rodrigues et al., 2018), and 1 g kg^{-1}
140 found by Klein et al. (2015) along the river Rhine in Germany. This concentration was therefore not
141 chosen to represent environmental relevance, but to enable maximum binding of PBDEs to a large
142 available microplastic surface area, with the intention of assessing how this may influence
143 bioaccumulation in a controlled and heavily contaminated exposure. Each microplastic treatment was
144 prepared by weighing 0.8 g nylon powder and mixing with white quartz sand (SiO_2, particle size 210-
145 300 μm, Sigma-Aldrich, Poole, UK) to make up to 80 g. All treatments were also run without
146 microplastics, in which case only 80 g quartz sand was weighed into a vessel. For each PBDE treatment,
147 1 ml of each diluted PBDE stock was added to the 80 g quartz sand substrate (with or without
148 microplastics, hereafter referred to as 'sediment') and stirred for 2 minutes 30 seconds using a glass
149 rod. This bulk mixture was divided between six replicate 100 ml glass exposure vessels (13 g per
150 vessel). A solvent carrier was used for spiking the PBDEs into the sediment, therefore an ethyl acetate
151 solvent control was also set up (1.25 % ethyl acetate in sediment) by carrying out this procedure with

ethyl acetate only. Following dosing, the vessels were left under a fume hood for two days with occasional agitation to ensure complete evaporation of the solvent. Blank controls containing no PBDEs or solvent were also set up both with and without microplastics, but without the need for solvent evaporation. This gave a total of 96 vessels to include blank controls (6 replicates), solvent controls (6 replicates) and PBDE treatments (6 concentrations x 6 replicates) without microplastics, and the same treatments with 1% microplastics.

To prevent suspension of nylon particles due to water surface tension, a small spray bottle of ISO test water was used to spray eight times onto the surface of the dry sediment. 100 ml of ISO test water was then gently introduced to the vessel and the water surface sprayed another seven times to break the water surface tension and allow any floating nylon particles to sink (15 sprays total). Vessels were left to equilibrate for 48 hours prior to introducing the organisms.

One snail was exposed per vessel. Before being added to the test vessels, each snail was rinsed in ISO test water and the shell gently rubbed with a gloved finger to remove any faeces/algae present and patted dry with a tissue. Each snail was weighed and length of shell measured; only snails > 25mm were used in the bioassays at which size all individuals can be expected to be mature (Coeurdassier et al., 2004; Zonneveld and Kooijman, 1989).

During exposures, jars were covered with Parafilm® to prevent escape of snails, pierced 10 times to allow for oxygenation. Exposures ran for 96 hours. No food was provided during test exposures in order to run as a simple an exposure as possible, to avoid complex organic interactions which may confound interpretation of the processes occurring. Snails were observed daily to check for mortality. At the end of the exposure, snails were removed from the water, washed in DI water, patted dry with tissue and weighed. Snails were euthanised and preserved: of the six replicate snails for each treatment, three were preserved for microbiome analysis (directly placed into ethanol) and three for tissue PBDE concentration analysis (immediately frozen at -80°C). Snails were not depurated before weighing or preservation as it was decided that analysing organisms with a full gut would give a more natural representation of environmental exposure and associated internal concentration. The overlying water from the exposure vessels was poured away and sediments were dried in a temperature-controlled

chamber at 25°C until dry (approx. 2 days). Sediment PBDE concentrations were measured in the dried samples at the end of the experiment.

2.5. Chemical analysis

Half of a snail was thawed, removed from the shell and dissected lengthways to obtain a representative sample of the whole body. This tissue was then weighed, ground with sand and dried with anhydrous sodium sulphate. The remaining half snail was refrozen and stored for fluorescence microscopy, to verify microplastic ingestion. Each sample (snail/sediment) was spiked with labelled recovery standards (^{13}C BDE 47, ^{13}C BDE 126 and ^{13}C BDE 153; Cambridge Isotope Laboratories) and soxhlet extracted in dichloromethane (DCM) for 16 h. A small portion of the extract was evaporated to zero volume and the lipid content was determined gravimetrically. The remaining extract was cleaned using automated size exclusion chromatography followed by deactivated (5% deionised water; w/w) alumina column.

The clean extract, was then spiked with labelled internal standards (BDE 77and ^{13}C BDE 138; Cambridge Isotope Laboratories) and 100 µl of sample was injected into a GC-MS (Agilent) with programmable temperature vaporization (PTV) inlet. The PTV injector was kept at 55°C for 0.45 min, and heated to 325°C at a rate of 700°C min^{-1} and kept at 325°C for 5 min. Then the temperature was reduced to 315°C min^{-1} at a rate of 10°C min^{-1}.The GC-MS had a 25 m HT8 column (0.22 mm internal diameter and 0.25 µm film thickness, SGE Milton Keynes, UK) and the carrier gas was helium (2.0 ml min^{-1}). The temperature programme was: isothermal at 80°C for 2.4 min, 25°C min^{-1} to 200°C, 5°C min^{-1} to 315°C and was held at 315°C for 9.8 min. Residues were quantified using internal standard method and also calibration curves of the standard PBDEs (Cambridge Isotope Laboratories) and were recovery corrected. The mean recoveries were: ^{13}C BDE 47- 85%, ^{13}C BDE 126 – 105% and ^{13}C BDE 153- 96% and the LOD was 0.109 ng g^{-1} wet weight.

2.6. Ingestion of microplastics

The snail tissue remaining following the chemical analysis was analysed using a fluorescence microscope (Olympus BX41 microscope with an Olympus U-LH100HG 100W mercury lamp using the green filter of the Cy3 (Olympus U-M39004) filter cube, with Olympus analySIS software) to image and qualitatively verify ingestion of microplastics by the snails.

2.7. Microbiome analysis

2.7.1. DNA extraction and sequencing

DNA was extracted from three snails per treatment (whole snail excluding shell) following the protocol described in the SI. Sample DNA required an additional cleaning step through the application of Genomic DNA Clean & Concentrator kit (Zymo research, USA) under the manufacturer's recommended protocol. Resultant DNA was quantified using the nanodrop 8000 UV-Vis spectrophotometer (ThermoFisher scientific, USA).

Approximately 40 ng of template DNA was amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, Hitchin, UK) each with a unique dual-index barcode primer combination (Kozich et al., 2013). Individual PCR reactions employed 25 cycles of an initial 30 s, 98°C denaturation step, followed by an annealing phase for 30 s at 53°C, and a final extension step lasting 90 s at 72°C. Primers were based upon the universal primer sequence 341F and 806R (Takahashi et al., 2014). An amplicon library consisting of ~550 bp amplicons spanning the V3-V4 hypervariable regions of encoding for the 16S small subunit ribosomal RNA gene (16S rRNA), was sequenced at a concentration of 6 pM with a 10% addition of control phiX DNA, on an Illumina MiSeq platform using V3 chemistry (Illumina Inc., San Diego, CA, USA).

2.7.2. Bioinformatics analysis

Sequenced paired-end reads were joined using VSEARCH (Rognes et al., 2016), quality filtered using FASTX tools (hannonlab.cshl.edu), length filtered with the minimum length of 300 bp, presence of

PhiX and adapters were checked and removed with BBTools (jgi.doe.gov/data-and-tools/bbtools/), and chimeras were identified and removed with VSEARCH_UCHIME_REF (Rognes et al., 2016) using Greengenes Release 13_5 (at 97%) (DeSantis et al., 2006). Singletons were removed and the resulting sequences were clustered into operational taxonomic units (OTUs) with VSEARCH_CLUSTER (Rognes et al., 2016) at 97% sequence identity (Tindall et al., 2010). Representative sequences for each OTU were taxonomically assigned by RDP Classifier with the bootstrap threshold of 0.8 or greater (Wang et al., 2007) using the Greengenes Release 13_5 (full) (DeSantis et al., 2006) as the reference. Unless stated otherwise, default parameters were used for the steps listed. The raw sequence data reported in this study have been deposited in the European Nucleotide Archive under study accession number PRJEB27672 (ERP109787).

2.8. Statistical analysis

2.8.1. Chemistry data

Sediment concentration and snail body concentration data were log transformed for normality. As only one sediment concentration was measured per treatment, it was assumed that each of the three snails analysed per treatment was exposed to this measured concentration. To compare the concentrations of PBDEs in sediment and organisms with and without microplastics, only treatments with added PBDEs were included in the analyses of chemical data (i.e. no control treatments) as the control treatments showed very low or non-detected values which could not be log-transformed. Two-way ANOVAs were carried out for each BDE congener, and the total PBDEs, to determine the relationship between snail tissue concentration, the concentration of PBDEs in the sediment and the presence of microplastics (R statistical software).

2.8.2. Snail weight data

A two-way ANOVA was conducted considering the effects on snail weight change of PBDE concentration and presence of microplastics as factors, and also their interaction.

255

256 **2.8.3. Microbiome data**

257 After quality filtering, a total of 2626755 sequences remained. One sample was removed from the
258 analysis due to low sequencing efficiency (<6000 sequences). Rarefaction curves were used to ensure
259 the sample depth represented the full community. To account for uneven sequencing depth (inherent in
260 NGS platforms) samples were normalized to lowest sequence depth using the rarefy_even_depth
261 function in the R package 'Phyloseq V 1.22.3' (McMurdie and Holmes, 2013). For simplicity, for
262 microbiome analysis with respect to PBDE concentration, nominal PBDE concentrations were used. In
263 order to assess any subtle changes, communities were subdivided into 'core' OTUs (occurring in >50%
264 of samples, at an abundance of >2%) and 'non-core' (all other community members), using the function
265 'prevalence' in the R package 'microbiome' (McMurdie and Holmes, 2013). Analyses were firstly
266 carried out on the whole community and subsequently on the subdivided core and non-core
267 communities.

268 To visualise the relationship between 16 rRNA sequence-based community profiles from different
269 treatments, nonmetric multidimensional scaling (NMDS) was performed using the 'metaMDS'
270 function, based on dissimilarities calculated using the Bray–Curtis index. Additionally, bacterial
271 diversity were assessed using Fishers log series [alpha], as this is largely unaffected by sample sizes >
272 1000 (Magurran, 2004). Differences in bacterial diversity for each PBDE compound and nominal PBDE
273 concentration were tested through the multiple Kruskal-Wallis (H) test, a test which does not assume
274 data normality, using the function 'kruskalmc' in R package 'Pgirmess' version 1.6.9 (Giraudoux et al.,
275 2018). An additional Kruskal-Wallis test was run to determine whether there were differences in
276 microbiome diversity between control and solvent control treatments (Fig. 3). Similarity percentages
277 breakdown procedure (SIMPER) was used to infer the importance of community members within
278 treatments (Clarke, 1993) and again Kruskal-Wallis was used to test significance. Finally, the effect of
279 PBDE concentration, presence of microplastics and their interaction upon community dissimilarity was
280 assessed using the Bray–Curtis index through Permutational Multivariate Analysis of Variance
281 (PERMANOVA, using the 'ADONIS' function in R package 'Vegan' v2.0-10 (Anderson, 2001;
282 Oksanen et al., 2013)). Taxonomic composition was plotted using the R package 'ggplot2' (Wickham,

283 2016). For each treatment, relative abundances per treatment were calculated to account for unequal
284 sampling, taking into account the combined data of the three replicates (Figs. 5, S4 and S5).
285

286 ## 3. Results

287 ### 3.1. Concentration of PBDEs in the presence and absence of microplastics

288 The control treatments (no PBDEs, with and without microplastics) contained trace concentrations of
289 PBDEs in some instances, although most (overall 73%) were below the detection limit of 0.108 ng g^{-1}.
290 The concentrations of different PBDE congeners in relation to its nominal concentrations varied
291 between 41% and 74% (Table 1). When considering all congeners and concentrations both with and
292 without microplastics, measured sediment concentrations overall were 54% of the nominal
293 concentration. PBDEs were present within sediment at statistically significantly comparable
294 concentrations regardless of the presence or absence of microplastics ($p > 0.05$, ANOVA).
295

296 Table 1. Nominal and measured sediment concentrations for each BDE congener, for all PBDE
297 treatments with and without microplastics. Sediment PBDE concentrations were measured at the end
298 of the experiment (one replicate per treatment).

		Measured sediment concentration (ng g^{-1})				
	Nominal concentration (ng g^{-1}, per BDE)	**BDE 47**	**BDE 100**	**BDE 99**	**BDE 153**	**Total BDEs**
	0	0	0	0	0	0
	0 (solvent control)	0	0	0	0	0
	94	34.42	42.67	66.63	88.41	232.12
Without	188	46.80	67.44	108.19	109.85	332.27
microplastics	375	61.92	117.09	191.70	215.99	586.70
	750	233.24	332.20	499.45	456.24	1521.14
	1500	341.34	494.64	805.90	906.48	2548.36

	3000	1776.98	1765.79	2681.38	2290.35	8514.49
	0	0	0	0	0.41	0.41
	0 (solvent control)	0	0	6.93	0.54	7.47
	94	50.77	39.42	68.30	58.05	216.54
	188	56.24	50.58	78.46	81.65	266.92
With	375	170.47	141.11	209.81	197.16	718.55
microplastics	750	326.44	281.85	408.44	367.71	1384.44
	1500	825.13	667.41	978.23	832.01	3302.77
	3000	1449.14	1242.28	1804.11	1593.52	6089.05

The (measured) sediment PBDE concentrations significantly affected PBDE uptake by snails, for all PBDEs independently and combined, higher sediment concentrations resulted in a significantly higher snail body burden ($p < 0.01$, ANOVA, Fig. 1). BDE 47 was the only PBDE congener that showed a significant effect of microplastics on the uptake of PBDEs by snails, with microplastics leading to a significantly lower body burden ($p < 0.01$, ANOVA, Fig. 1). There were no significant interactions between the concentration of PBDEs within the sediment and the presence of microplastics for any of the congeners ($p > 0.05$, two-way ANOVA).

3.2. Survival and weight change

There was 100% survival throughout the exposure. A significant difference was observed in snail wet weight change between microplastic and non-microplastic treatments, with non-microplastic treatments losing significantly more weight on average (0.11 ± 0.13 g) than microplastic treatments (0.03 ± 0.12 g) (two-way ANOVA, $p < 0.01$, Fig. 2). Concentration of PBDE had no effect on weight change (two-way ANOVA, $p > 0.05$) and there was no interaction between PBDEs and microplastics (two-way ANOVA, $p > 0.05$).

3.3. Ingestion of microplastics

Using fluorescence microscopy, ingestion of microplastics was evident within all snails exposed to microplastics (Fig. S1 D-I). Microplastics could be observed both on the surface of the sample (Fig. S1, G and I) and behind membranes (i.e. within organs, Figs. S1. D, F and H). Based on the way the samples were prepared and analysed, it is not possible to quantitatively analyse ingestion, nor to identify the specific locations where microplastics were found or accumulated. However, ingestion could be qualitatively confirmed.

3.4. Microbiome data

3.4.1. Control treatments

Using a multiple-comparison Kruskal-Wallis test, there were no significant differences in microbial diversity (Fisher's Log alpha) between blank controls and solvent controls, nor between control treatments with and without microplastics (Fig. 3. $p > 0.05$, Kruskal-Wallis). This highlights that there was no effect of the solvent control, or of microplastics alone (in the absence of PBDEs), on snail microbiome structure.

3.4.2. Community composition and diversity

Community diversity assessed by Fisher's log alpha (Fig. 4) showed no significant differences between different PBDE concentrations (all $p < 0.05$, multiple Kruskal-Wallis, H). However, although not significant, it should be noted that diversity does appear to appear to be lower at higher PBDE concentrations when microplastics are absent, while the diversity of communities in treatments with microplastics appear largely unaffected by PBDE concentration (Fig. 4). This decline in diversity pattern could be related to a loss in richness (count) of non-core OTUs in treatment with high concentrations of PBDE, in the absence of microplastics (Fig. 4 and Fig. S2). Permutational Multivariate Analysis of Variance showed no significant effect on community dissimilarity based on

PBDE concentration, presence of microplastics and their interactions ($p > 0.05$ for all comparisons, Fig. S3).

3.4.3. Taxonomic microbiome composition

The greatest number of 16S rRNA gene sequences within the *L. stagnalis* microbiome were found to be from the Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Flavobacteria and Bacilli (Fig. S4) irrespective of treatment. The most dominant order across all treatments are the Enterobacterales (Fig. 5), and within that order the genus *Klebsiella* (Fig. S5). When comparing individual OTUs in the controls vs the highest PBDE concentration, similarity of percentage (SIMPER) analysis shows that in the absence of microplastics there was a significantly higher relative abundance of OTUs 5512 and 4432 (both identified as belonging to the Enterobacteriaceae) in the highest PBDE concentration treatment (Table S1). There was also a significant reduction in OTU 8733 (identified as belonging to the Flavobacteriaceae), in the highest dose treatment compared to the control (Kruskal-Wallis test $P < 0.05$, df =1). In contrast, no significant differences were observed in individual relative OTU abundance when microplastics were present (Table S1). Some orders are present only in PBDE treatments, notably sulfate-reducing bacteria (Desulfobacterales and Syntrophobacterales).

4. Discussion

4.1. The snail microbiome

The individuals used in this test were taken from the field and acclimated for this test, therefore the microbial data acquired here is likely representative of biological variability within wild *L. stagnalis.* Laboratory conditions and the lack of food throughout the exposure would likely have led to changes over time, but this would not have been expected to lead to individual or treatment-specific differences. While the microbiome analysis in this study considered the whole microbiome of the snail, it is expected that the majority of OTUs derive from the gut bacterial community. This is highlighted in the

366 communities across all treatments being dominated by Enterobacterales (Fig. 5), a common order
367 comprising gut bacteria (Hu et al., 2018). For example, *Klebsiella*, the most dominant genus observed
368 here within the family Enterobacteriacae (Fig. S5), is a polysaccharide degrader linked to the presence
369 of cellulase (Imran et al., 2016). The *L. stagnalis* core microbiome in this study appears to be similar to
370 other freshwater snails and associated habitats including Enterobacterales, Flavobacterales and
371 Bacillales corresponding to lactic acid production (food fermentation) and cellulose degradation
372 (Béguin, 1990; Dar et al., 2017; Hu et al., 2018).

373

374 **4.2. PBDE accumulation and effects on microbiome**

375 All sediment PBDE concentrations were lower than the nominal concentrations when measured at the
376 end of the exposures. The half-lives of BDEs-47, 99, 100 and 153 are all estimated to be approximately
377 14,400 hours in sediment (Wania and Dugani, 2003), therefore degradation over the experimental time
378 period is not likely to have been a significant factor leading to the discrepancies between nominal and
379 measured concentrations observed here (estimated 0.3% loss due to degradation over 96 hours based
380 on a half-life of 14,400 hours). Some loss of PBDEs may have occurred as a result of volatilisation
381 during the solvent evaporation step, and some may also have bound to the walls of the glass exposure
382 vessels.

383 BDE 47 has the lowest log Kow, which would indicate a greater (although still low) partitioning into
384 the water phase than for the other more hydrophobic PDBEs. In a marine study, Mizukawa et al. (2009)
385 found that proportionally, higher brominated BDE congeners (BDE 209) associated most strongly with
386 sediments, while the composition within overlying seawater was dominated by lower brominated
387 congeners (predominantly BDE 47, but also including BDEs 99 and 100). In our study, BDE 47
388 followed by BDE 99 accumulated most in the snail tissue, with higher internal concentrations compared
389 to the other congeners (Fig. 1). This corresponds with evidence which shows that BDEs 47 and 99 are
390 the most bioavailable PBDE congeners, due to a lower molecular weight and smaller molecules than

391 higher brominated congeners (Liang et al., 2010; Mizukawa et al., 2009; Watanabe and Sakai, 2003;
392 Zhang et al., 2016).

393 There were a number of sulfate reducing bacteria observed within snails exposed to PBDEs (most
394 notably Desulfobacterales and Syntrophobacterales, Fig. 5), bacteria also recognised to be associated
395 with the debromination of PBDEs (Zhao et al., 2018). These bacteria have not commonly been
396 described in relation to other freshwater snail species (Hu et al., 2018) and were not present within the
397 controls. Burkholderiales, one of the dominant orders found within these snails across all treatments,
398 are also associated with PBDE degradation, especially lower brominated congeners (Robrock et al.,
399 2009).

400 PBDE concentration had no significant influence on the microbiome, a result which is in contrast to
401 other studies which found that PBDEs affected bacterial community composition and diversity in
402 sediments and within guts, with changes being congener-dependent (Li et al., 2018; Wang et al., 2018;
403 Yen et al., 2009). We therefore reject the starting hypothesis that increasing PBDE sediment
404 concentrations lead to significant structural changes in the microbiome community over an acute
405 timescale. The difference between this and other studies is likely to be because these represent different
406 exposure scenarios (via food or water), plus other studies have usual run for longer time periods, and
407 have generally used much higher PBDE concentrations (e.g. $\mu g\ g^{-1}$ concentrations in food)

408

409 **4.3. Effects of microplastics on snail physiology and microbiome**

410 There was no effect of any exposure condition on survival. Microplastics did subtly affect the wet
411 weight of the snails. In general, the weight of all snails declined throughout the experiment, likely due
412 to the lack of food within the exposure. However, this decline was less pronounced in snails exposed to
413 microplastics (average 0.03 g weight decline in microplastic-exposed snails, compared to average 0.11
414 g decline in non microplastic-exposed snails, Fig. 4). The reasons for this difference are not clear; most
415 microplastic exposure studies observe a more pronounced weight decline in exposed organisms
416 (Besseling et al., 2013; Zhu et al., 2018a).

The lack of significant influence of microplastics on the microbiome (Fig. 3) is in contrast to other studies on the microbiome response in invertebrates (Zhu et al., 2018a; Zhu et al., 2018b). For example, Zhu et al. (2018b) found a significant increase in the family Bacillaceae within collembolan guts following exposure to microplastics, while our analysis found the order Bacillales to be present in both the microplastic and non-microplastic treatments (Fig. 5). Many gut bacteria are derived from, and influenced by, ingested material, therefore feeding behaviour is likely to have a significant influence on the gut microbiome (Turnbaugh et al., 2009; Zhu et al., 2018b). It was chosen not to feed the snails during the acute exposure, and hence any alterations within the microbiome community could be ascribed solely to the microplastic, PBDEs and their interaction. Despite the lack of significance of microplastics alone, the microbiome analysis suggests that microplastics can subtly influence PBDE impacts on the microbiome. For example, while not significant, there appears to be a tendency for the diversity of non-core bacteria to be lower at higher PBDE concentrations in the absence of microplastics, a trend which is not evident when microplastics were present (Fig. 4, Fig. S2). Microplastics also appear to slightly reduce variability between individuals within the microplastic controls compared to non-microplastic controls i.e. 'reference' gut conditions (Figs. 3 and 4). Within natural conditions, a higher microbial diversity between individuals may be beneficial for populations, increasing resilience to perturbation (Heiman and Greenway, 2016; Lozupone et al., 2012).

4.4. Influence of microplastics and PBDE co-exposure on accumulation and microbiome

Microplastics did not influence sediment PBDE concentrations. This result was expected as the microplastics were not removed from the sediment samples before analysis, therefore during analysis, PBDEs were likely to have been extracted from both the sediment and microplastics simultaneously. The concentrations of PBDEs within the sediment significantly affected the amount of PBDEs taken up within the snail, in line with the expected relationship between external exposure concentration and snail body burden.

Given that snails were not depurated before chemical analysis of the whole body, this analysis took into account any chemicals present within the gut content, in addition to those in snail tissues. Microplastics did not influence the uptake of BDEs-99, 100, 153, nor PBDE uptake as a whole. Therefore these PBDEs were equally available regardless of the presence of microplastics and our hypothesis was not supported. This is in contrast to previous studies carried out on microplastic and PBDE interactions, where microplastics have been shown to enhance uptake of PBDEs into fish tissue (Rochman et al., 2013a) and amphipods (Chua et al., 2014). This could be a result of different polymer types used, different exposure conditions (e.g. temperature, pH) and also the susceptibility of the organism to accumulate or metabolise hydrophobic chemicals.

Previous studies have shown that PBDEs can transfer from microplastics into body tissues (Chua et al., 2014; Rochman et al., 2013a). Hence, the concentrations measured here are likely to be a combination of both gut content and tissue concentrations, especially as fluorescence analysis showed that the nylon particles were ingested by snails (Fig. S1). PBDEs entering tissues are unlikely to be taken up only by ingestion of contaminated particles, as the foot of the snail will be exposed to the sediment-based PBDEs by direct contact with the sediment, and to aqueous phase PBDEs through contact with the water phase (Bakir et al., 2016). To allow uptake into tissues, desorption of the chemical from the sediment (or microplastic) surface, whether externally or within the gut, is needed as a prelude to uptake. While it is anticipated that the main route of exposure to PBDEs was via the sediment (either dermally or via ingestion) (Mizukawa et al., 2009), aqueous phase uptake may also be important and the precise nature of exposure may also vary dependent on the behaviour of the BDE congener: BDE 47 was the only PBDE whose concentration in snails was significantly reduced in the presence of microplastics. BDE 47 is the congener with the lowest log Kow at 6.81, which would be expected to sorb the least strongly to particles (both microplastics and sediment) compared to the other congeners (although it is still highly hydrophobic). This reduced binding affinity could have led to greater BDE 47 partitioning into the water phase in the absence of microplastics, facilitating uptake. The presence of microplastics may have increased the partitioning of BDE 47 to sediment through the addition of a further surface

468 binding phase with a high affinity for HOCs, thus reducing BDE 47 in the more bioavailable water
469 phase, resulting in reduced bioavailability and uptake (Fig. 1).

470 While microplastics can sorb chemicals, other media (e.g. organic matter, sediment) may also
471 accumulate HOCs and therefore should be also be taken into account when considering pathways for
472 exposure and bioavailability (Bakir et al., 2016; Koelmans et al., 2016). Further, if considering trophic
473 transfer, the interactions with the sediment also indicate the importance of measuring organisms with a
474 full gut, as we did within this study (rather than depurated organisms as is usually the case in chemical
475 bioaccumulation studies), given that PBDEs associated with the gut content may also be bioavailable.

476 No consistent significant differences were observed in snail microbiome community diversity in
477 response to either the microplastic or PBDE treatments, although a trend for reduced diversity at high
478 PDBE concentrations in the absence of microplastics was suggested, which warrants further
479 investigation. Hence, our hypothesis of chemical effects on the snail microbiome, influenced by
480 microplastics, was not supported over the short exposure timescale used. When investigating the
481 differences in abundance of specific OTUs, significant differences were seen in the abundance of
482 Enterobacteriaceae and Flavobacteriaceae between the control and high PBDE concentration, only
483 when microplastics were absent (Table S1). Enterobacterales can be induced to bloom within the gut
484 under conditions of stress, for example inflammatory responses produced by the gut immune system
485 (Stecher et al., 2012), which may explain their increase in the presence of high PBDE concentrations.
486 Flavobacterales have been associated with polymer degradation (Mergaert and Swings, 1996; Nogales
487 et al., 2011) and have been commonly found associated with marine plastic debris (Bryant et al., 2016;
488 Oberbeckmann et al., 2018) which could explain their decline in the absence of microplastics (combined
489 with high PBDE concentrations), although it is not possible to link those characteristics directly to this
490 study. The fact that these results were seen only in the absence of microplastics suggests that
491 microplastics may be buffering the effects of PBDEs on the microbiota, although only subtly.

492

493 **4.5. Long term implications and outlook**

Short and long-term exposure are likely to lead to very different microbial community responses, therefore acute exposures can provide information on initial responses to perturbation that would be not be observed during chronic tests (Shade et al., 2012). There is evidence to suggest that microbiomes will respond very quickly to perturbations, for example a study by Yen et al. (2009) found that BDEs 153 and 154 rapidly and irreversibly changed the bacterial community within sediment (within 24 hours). Studies which have found significant changes in organism microbiomes following invertebrate exposure to microplastics usually run for longer timescales, e.g. enchytraeids exposed for seven days (Zhu et al., 2018a) and collembolans exposed for 56 days (Zhu et al., 2018b).

It is recognised that given the simplicity of this exposure, this does not well represent the complexity of an environmental exposure. However, the data here improve our understanding of the interactions between microplastics PBDEs and organisms within a simple controlled exposure, where any effects will be easier to observe and interpret than under complex realistic environmental conditions. The subtle variations in response of the snail microbiome to microplastic exposure, PBDE exposure and co-exposure over a 96-hour exposure indicated that these stressors do affect the structure of the gut community. However, overall response to aspects such as overall diversity were not evident to the same extent as for studies with other species conducted over longer exposure times. These results, therefore, highlight the complexity of responses of organisms to microplastics and organic chemicals, and show the importance of carrying out further studies to understand the interaction between microplastics and HOCs and their influence on organisms in a variety of exposure scenarios and time-scales.

5. Conclusions

Microplastics did not affect survival of the snails. The weight of all snails generally declined throughout the exposure period, however, this decline was less in snails exposed to microplastics. An increased concentration of PBDE in the sediment led to an increased body burden within the snails, however microplastics did not significantly influence this uptake when considering all PBDE congeners overall. BDE 47 was the only congener influenced by the presence of microplastics, leading to a significantly

reduced internal concentration in the presence of microplastics. Overall, the diversity and composition of the snail microbiome was not significantly altered by the presence of PBDEs or microplastics, or both combined. However, when considering individual OTUs, significant effects on individual responses were found that can be functionally linked to the exposure of snails to the PBDEs added, a result only observed in the absence of microplastics. This suggests that microplastics influence how PBDEs will impact on specific OTUs. In summary, these results suggest that microplastics and PBDEs have a limited effect both individually and when combined on HOC accumulation and the microbiome of *Lymnaea stagnalis* within an acute exposure. However the subtle effects seen highlight the importance of carrying out further studies to better understand the mechanisms causing the interaction between microplastics and HOCs given that these relationships may become more pronounced over extended time-scales.

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736

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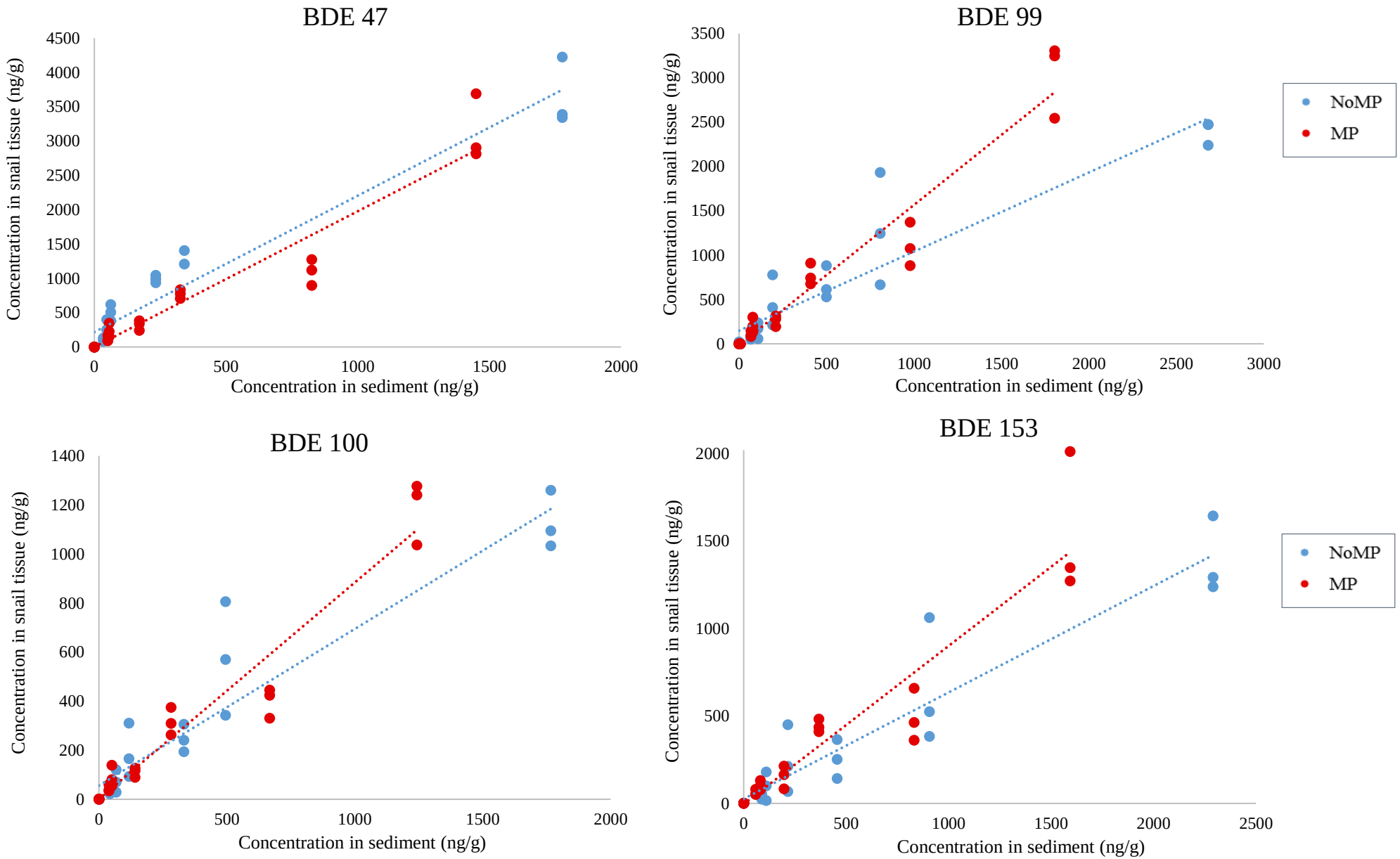
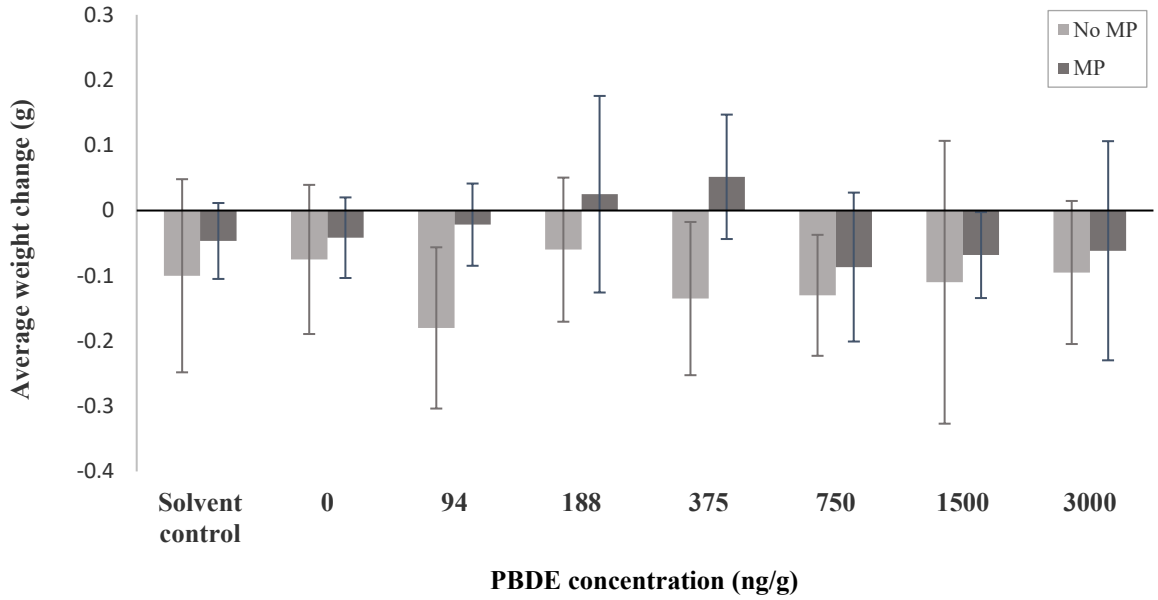
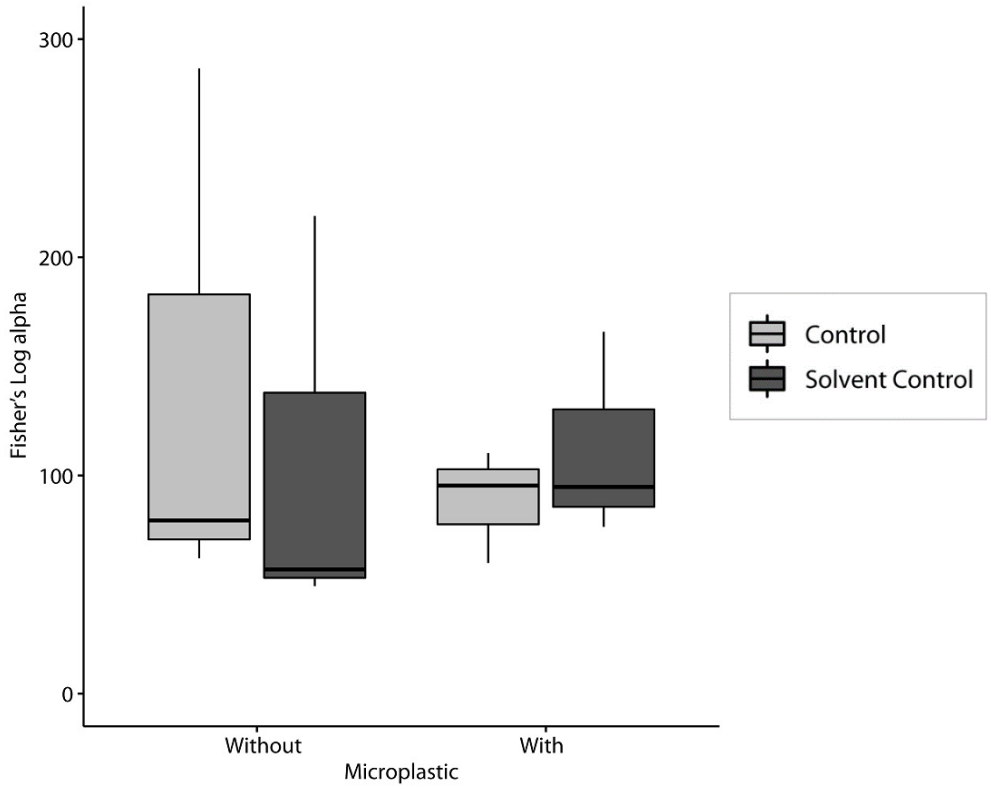
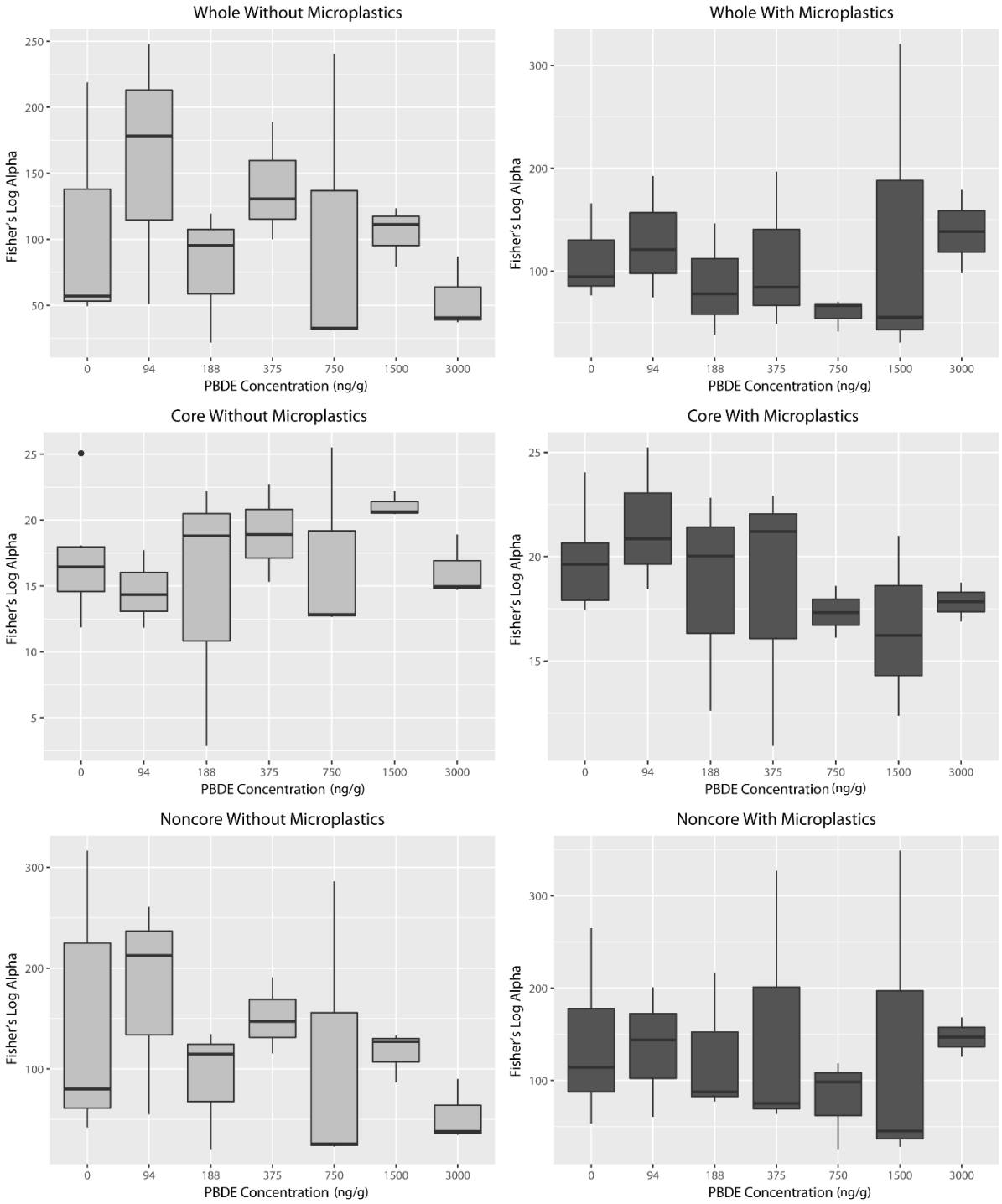
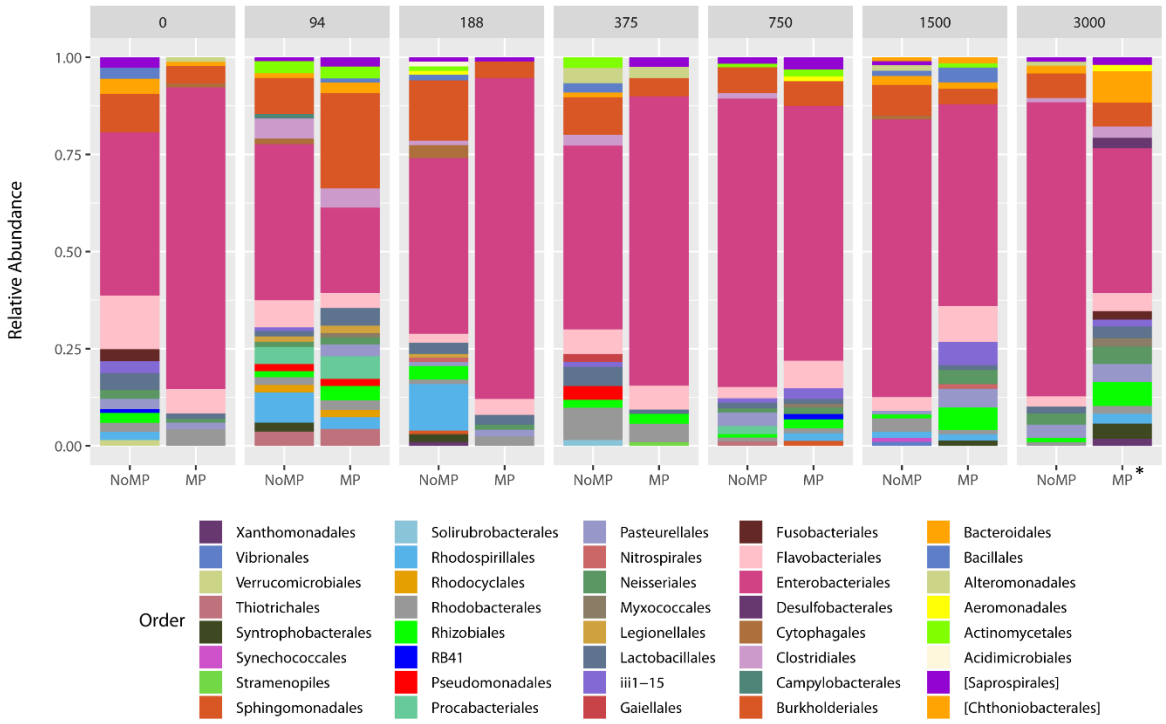

BDE 47
Concentration in snail tissue (ng/g)
Concentration in sediment (ng/g)
BDE 99
Concentration in snail tissue (ng/g)
Concentration in sediment (ng/g)
NoMP
MP
BDE 100
Concentration in snail tissue (ng/g)
Concentration in sediment (ng/g)
BDE 153
Concentration in snail tissue (ng/g)
Concentration in sediment (ng/g)
NoMP
MP


0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
Average weight change (g)
No MP
MP
Solvent control
0
94
188
375
750
1500
3000
PBDE concentration (ng/g)


300
200
100
0
Fisher's Log alpha
Without
With
Microplastic
Control
Solvent Control


Whole Without Microplastics
Fisher's Log Alpha
250
200
150
100
50
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)
Whole With Microplastics
Fisher's Log Alpha
300
200
100
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)
Core Without Microplastics
Fisher's Log Alpha
25
20
15
10
5
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)
Core With Microplastics
Fisher's Log Alpha
25
20
15
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)
Noncore Without Microplastics
Fisher's Log Alpha
300
200
100
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)
Noncore With Microplastics
Fisher's Log Alpha
300
200
100
0
94
188
375
750
1500
3000
PBDE Concentration (ng/g)


0
94
188
375
750
1500
3000
Relative Abundance
1.00
0.75
0.50
0.25
0.00
NoMP
MP
*
Order
Xanthomonadales
Vibrionales
Verrucomicrobiales
Thiotrichales
Syntrophobacterales
Synechococcales
Stramenopiles
Sphingomonadales
Solirubrobacterales
Rhodospirillales
Rhodocyclales
Rhodobacterales
Rhizobiales
RB41
Pseudomonadales
Procabacteriales
Pasteurellales
Nitrospirales
Neisseriales
Myxococcales
Legionellales
Lactobacillales
iii1−15
Gaiellales
Fusobacteriales
Flavobacteriales
Enterobacteriales
Desulfobacterales
Cytophagales
Clostridiales
Campylobacterales
Burkholderiales
Bacteroidales
Bacillales
Alteromonadales
Aeromonadales
Actinomycetales
Acidimicrobiales
[Saprospirales]
[Chthoniobacterales]

738 **Figure captions**

739 Fig. 1. Measured PBDE concentrations in sediment, compared to the concentration within snails, for
740 each BDE congener, with and without microplastics. 'No MP' = without microplastics, 'MP' = with
741 microplastics. Fitted lines represent linear regression.

742

743 Fig. 2. Average snail wet weight change for all PBDE treatments (nominal concentration in ng g^{-1} within
744 sediment) with and without microplastics, based on weight difference between 0 and 96 hours exposure.
745 'No MP' = without microplastics, 'MP' = with microplastics. Error bars represent standard deviation
746 (n = 6).

747

748 Fig. 3: Comparison of changes in microbial community diversity of the snails (Fisher's Log alpha) in
749 the blank and solvent controls, with and without microplastics. The boxes denote interquartile ranges
750 (IQR) with the median as a black line. Whiskers represent lowest and highest values within 1.5 IQR (n
751 = 3).

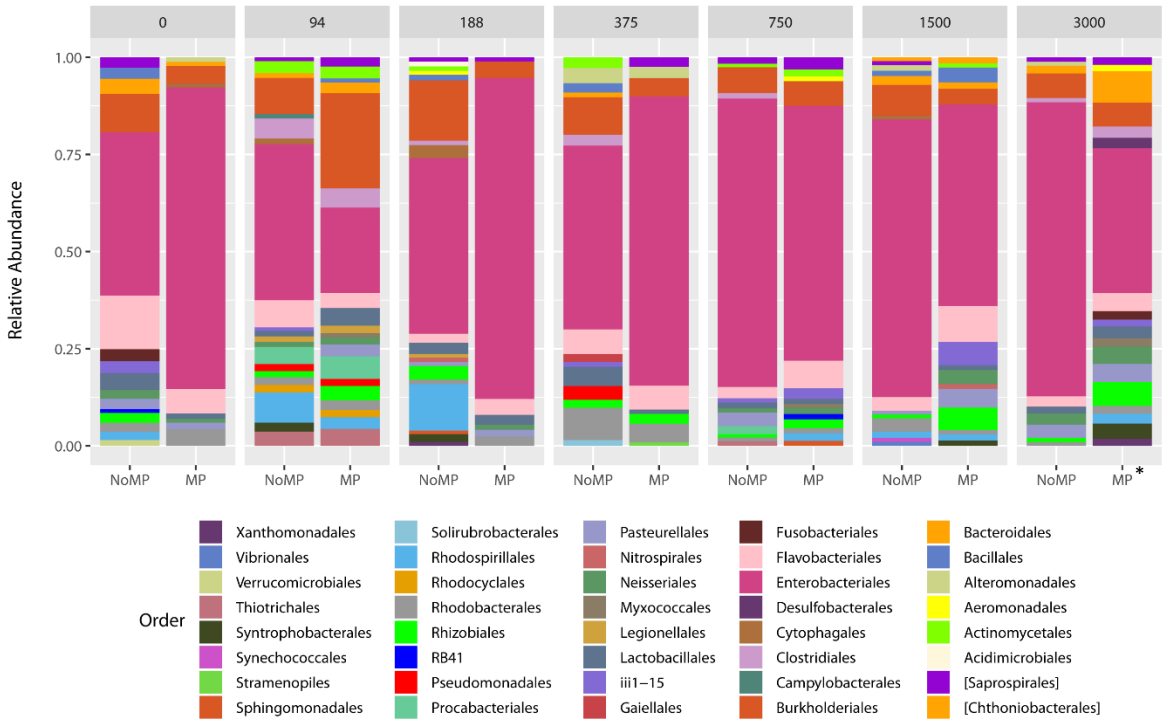
752

753 Fig. 4. Boxplots to show differences in microbial diversity in snails exposed to PBDEs in the absence
754 and presence of microplastics, showing whole data for the whole microbial community, then subsequent
755 separation into 'core' and 'non-core' community. The boxes denote interquartile ranges (IQR) with the
756 median as a black line. Whiskers represent lowest and highest values within 1.5 IQR (n = 3).

757

758 Fig. 5. Order composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with
759 and without microplastics (present/absent). Relative abundance was calculated as rarefied number of
760 sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3)
761 are plotted on Y axis. For ease of representation taxa of an abundance of <0.02 (2%) from an individual

762 sample were excluded. Each column represents a PBDE concentration (0-3000 ng g^{-1}). *Note one
763 sample was removed from this treatment due to inefficient sequencing, therefore N=2.


0
94
188
375
750
1500
3000
Relative Abundance
1.00
0.75
0.50
0.25
0.00
NoMP
MP
*
Order
Xanthomonadales
Vibrionales
Verrucomicrobiales
Thiotrichales
Syntrophobacterales
Synechococcales
Stramenopiles
Sphingomonadales
Solirubrobacterales
Rhodospirillales
Rhodocyclales
Rhodobacterales
Rhizobiales
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Pseudomonadales
Procabacteriales
Pasteurellales
Nitrospirales
Neisseriales
Myxococcales
Legionellales
Lactobacillales
iii1−15
Gaiellales
Fusobacteriales
Flavobacteriales
Enterobacteriales
Desulfobacterales
Cytophagales
Clostridiales
Campylobacterales
Burkholderiales
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