Towards a greater understanding of the presence, fate and ecological effects of microplastics in the freshwater environment

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CHAPTER 6

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Accepted for publication in Ecotoxicology and Environmental Safety

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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., 2013d), 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., 2013c). 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)). 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 had surface concentrations of PBDEs up to 9900 ng g^{-1} (Hirai et al., 2011), suggesting the potential for such interactions to influence organism exposure. Chua et al. (2014) and Rochman et al. (2013c) 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. (2018a) 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 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*. No food was provided during test exposures. Preliminary experiments showed *L. stagnalis* to ingest and egest the nylon microplastics used for this study (personal observation).

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 previously stained with Nile Red dye.

2.3. PBDEs

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

2.4. Experimental setup

Experimental treatments consisted of either microplastics (1% nylon powder by sediment mass) or sediment without added microplastics. Microplastic treatments were prepared by weighing 0.8 g nylon powder and mixing with white quartz sand (SiO_2, particle size 210-300 µm, Sigma-Aldrich, Poole, UK) to make up to 80 g. For each treatment, 1 ml of each diluted

PBDE stock was added to the 80 g quartz sand substrate (with or without microplastics, hereafter referred to as 'sediment') and stirred for 2 minutes 30 seconds using a glass rod. This bulk mixture was divided between six replicate 100 ml glass exposure vessels (13 g per vessel). As a solvent carrier was used for spiking the PBDEs into the sediment, an ethyl acetate 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 control treatments were made by mixing nylon powder and quartz sand using the same procedure, but without the need for solvent evaporation.

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.

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. 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. 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 of the 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 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.

2.8.3. Microbiome data

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

'microbiome' (McMurdie and Holmes, 2013). Analyses were firstly carried out on the whole community and subsequently on the subdivided core and non-core communities.

To visualise the relationship between 16 rRNA sequence-based community profiles from different treatments, nonmetric multidimensional scaling (NMDS) was performed using the 'metaMDS' function, based on dissimilarities calculated using the Bray–Curtis index. Additionally, bacterial diversity were assessed using Fishers log series [alpha], as this is largely unaffected by sample sizes > 1000 (Magurran, 2004). Differences in bacterial diversity for each PBDE compound and nominal PBDE concentration were tested through the multiple Kruskal-Wallis (H) test, a test which does not assume data normality, using the function 'kruskalmc' in R package 'Pgirmess' version 1.6.9 (Giraudoux et al., 2018). An additional Kruskal-Wallis test was run to determine whether there were differences in microbiome diversity between control and solvent control treatments (Fig. 3). Similarity percentages breakdown procedure (SIMPER) was used to infer the importance of community members within treatments (Clarke, 1993) and again Kruskal-Wallis was used to test significance. Finally, the effect of PBDE concentration, presence of microplastics and their interaction upon community dissimilarity was assessed using the Bray–Curtis index through Permutational Multivariate Analysis of Variance (PERMANOVA, using the 'ADONIS' function in R package 'Vegan' v2.0-10 (Anderson, 2001; Oksanen et al., 2013)). Taxonomic composition was plotted using the R package 'ggplot2' (Wickham, 2016). For each treatment, relative abundances per treatment were calculated to account for unequal sampling, taking into account the combined data of the three replicates (Figs. 5, S4 and S5).

3. Results

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

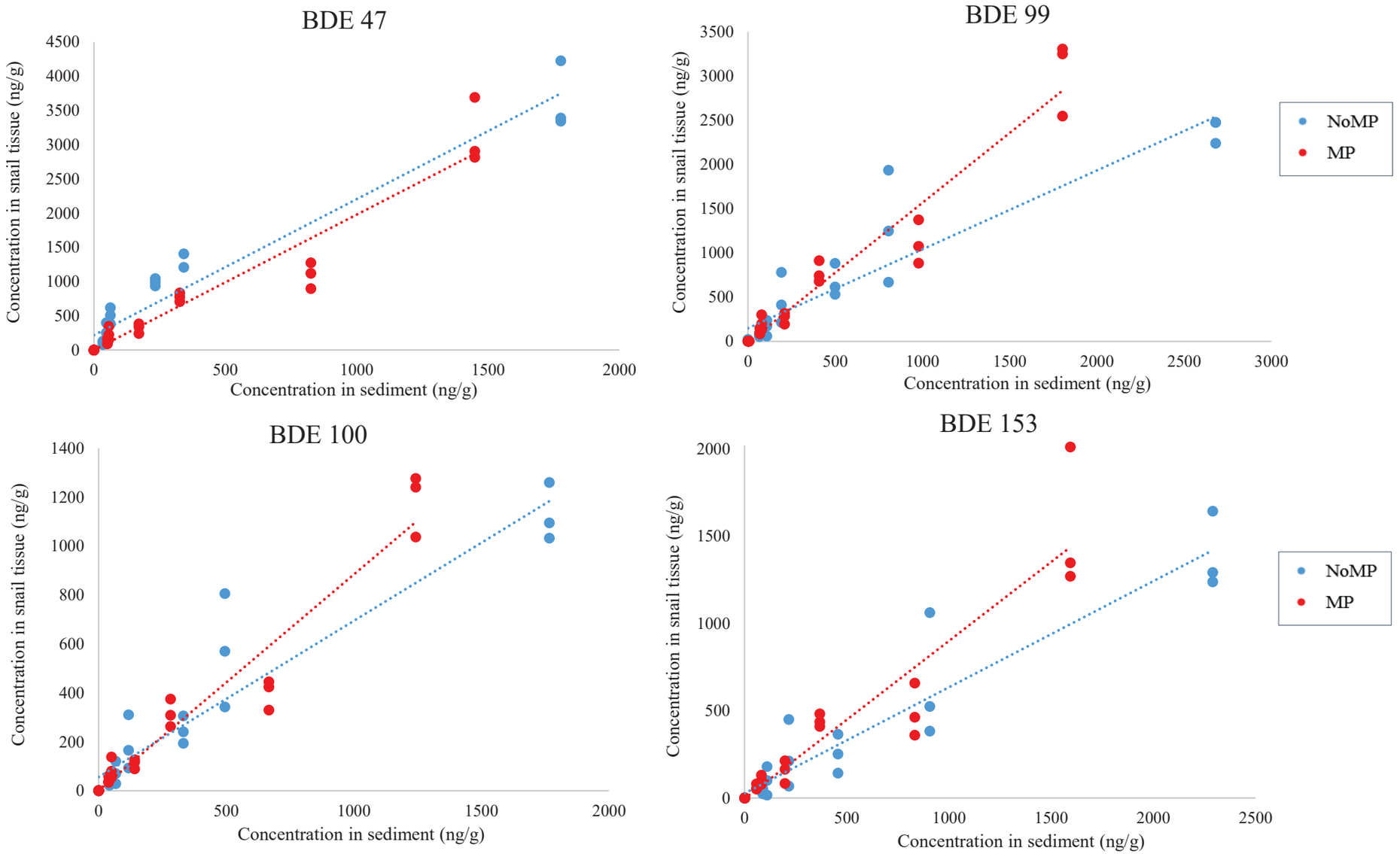
The control treatments (no PBDEs, with and without microplastics) contained trace concentrations of PBDEs in some instances, although most (overall 73%) were below the detection limit of 0.108 ng g^{-1}. The concentrations of different PBDE congeners in relation to its nominal concentrations varied between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

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).

Table 1. Nominal and measured sediment concentrations for each BDE congener, for all PBDE treatments with and without microplastics. Sediment PBDE concentrations were measured at the end 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**
Without microplastics	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
	188	46.80	67.44	108.19	109.85	332.27
	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
With microplastics	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
	375	170.47	141.11	209.81	197.16	718.55
	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

Fig. 1. Measured PBDE concentrations in sediment, compared to the concentration within snails, for each BDE congener, with and without microplastics. 'No MP' = without microplastics, 'MP' = with microplastics.



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, $p > 0.05$).

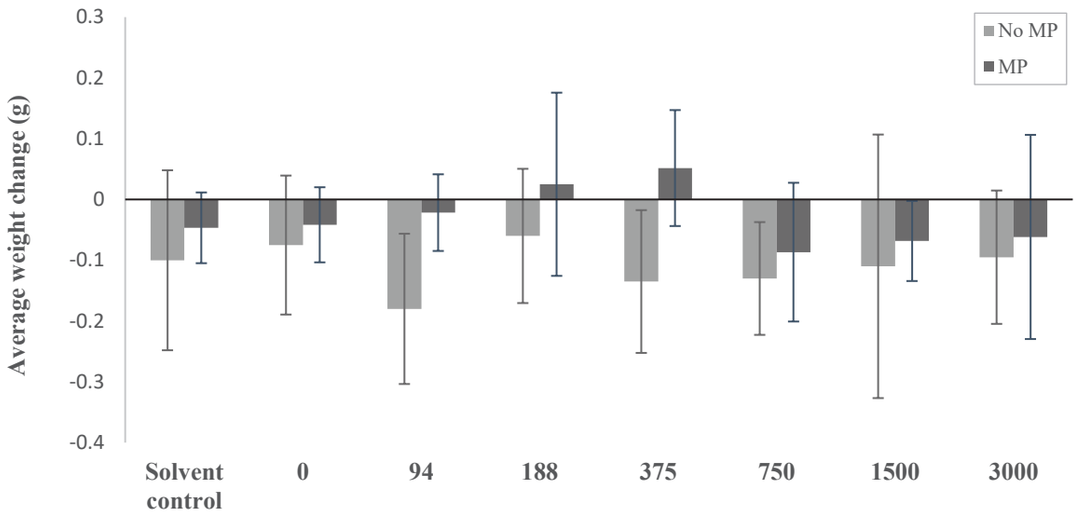


Fig. 2. Average snail wet weight change for all PBDE treatments (nominal concentration in ng g^{-1} within sediment) with and without microplastics, based on weight difference between 0 and 96 hours exposure. 'No MP' = without microplastics, 'MP' = with microplastics.

3.3. Ingestion of microplastics

Ingestion of microplastics was qualitatively confirmed using fluorescence microscopy (Fig. S1). Microplastics are clearly visible within the tissues of the snails, 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, microplastics were visibly present within all snails exposed to microplastics (Fig. S1 D-I).

3.3. Microbiome data

3.3.1. 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.

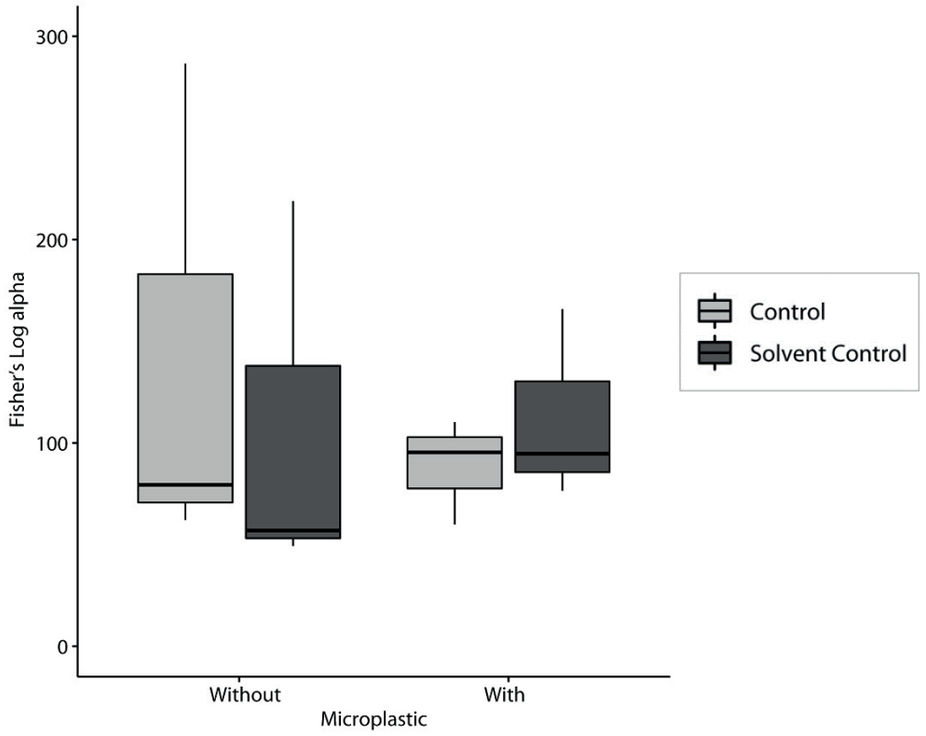


Fig. 3: Comparison of changes in microbial community diversity of the snails (Fisher's Log alpha) in the blank and solvent controls, with and without microplastics.

3.3.2. 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 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).

The visualisation of the community composition in the NMDS suggested some clustering at high PBDE concentrations in the absence of microplastics (Fig. S3). However, the Permutational Multivariate Analysis of Variance showed that none of this clustering was significant ($p > 0.05$ for all comparisons). Replicate variability was high and possibly rendered this non-significance. Attempting to introduce more stringent criteria to determine core OTUs did not change the results.

3.3.3. 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).

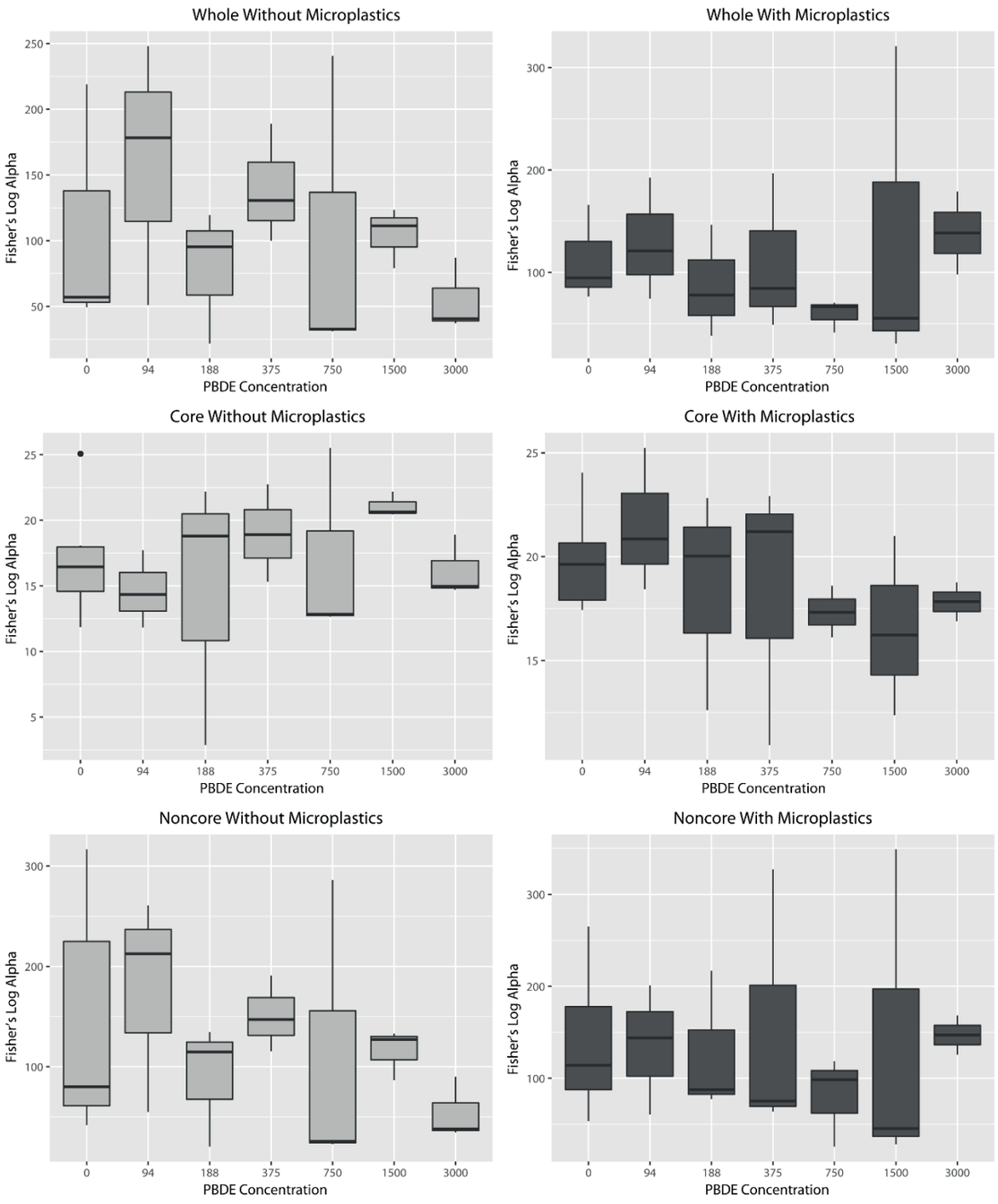


Fig. 4. Boxplots to show differences in microbial diversity in snails exposed to PBDEs in the absence and presence of microplastics, showing whole data for the whole microbial community, then subsequent separation into 'core' and 'non-core' community.

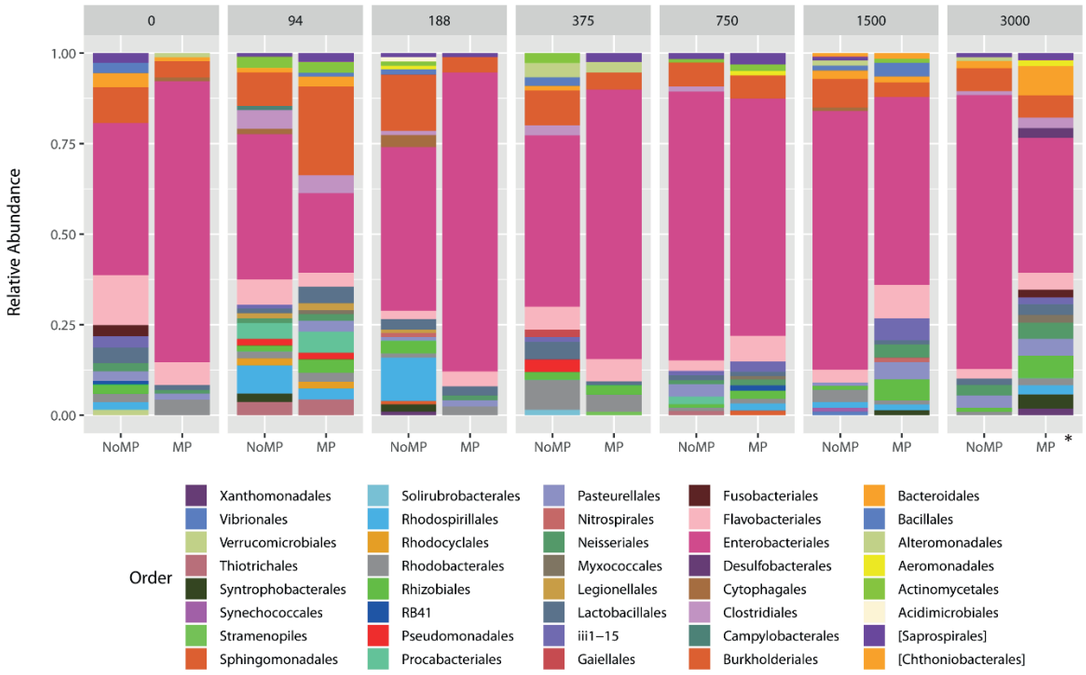


Fig. 5. Order composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3)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

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.* 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 communities across all treatments being dominated by Enterobacterales (Fig. 5), a common order comprising gut bacteria (Hu et al., 2018). For example, *Klebsiella*, the most dominant genus observed here within the family Enterobacteriacae (Fig. S5), is a polysaccharide degrader linked to the presence of cellulase (Imran et al., 2016). The *L. stagnalis* core microbiome in this study appears to be similar to other freshwater snails and

associated habitats including Enterobacterales, Flavobacterales and Bacillales corresponding to lactic acid production (food fermentation) and cellulose degradation (Béguin, 1990; Dar et al., 2017; Hu et al., 2018).

4.2. PBDE accumulation and effects on microbiome

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

BDE 47 has the lowest log Kow, which would indicate a greater (although still low) partitioning into the water phase than for the other more hydrophobic PDBEs. In a marine study, Mizukawa et al. (2009) found that proportionally, higher brominated BDE congeners (BDE 209) associated most strongly with sediments, while the composition within overlying seawater was dominated by lower brominated congeners (predominantly BDE 47, but also including BDEs 99 and 100). In our study, BDE 47 followed by BDE 99 accumulated most in the snails, with higher internal concentrations compared to the other congeners (Fig. 1). This corresponds with evidence which shows that BDEs 47 and 99 are the most bioavailable PBDE congeners, due to a lower molecular weight and smaller molecules than higher brominated congeners (Liang et al., 2010; Mizukawa et al., 2009; Watanabe and Sakai, 2003; Zhang et al., 2016).

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

PBDE concentration had no significant influence on the microbiome, a result which is in contrast to other studies which found that PBDEs affected bacterial community composition and diversity in sediments and within guts, with changes being congener-dependent (Li et al., 2018; Wang et al., 2018a; Yen et al., 2009). This difference is likely to be because these studies represent different exposure scenarios (via food or water) and also generally used much higher PBDE concentrations (e.g. $\mu g\ g^{-1}$ concentrations in food), although Chen et al. (2018) found significant microbiome community shift in zebrafish exposed to just 5 ng L^{-1} in water. We therefore reject the starting hypothesis that increasing PBDE sediment concentrations lead to significant structural changes in the microbiome community over an acute timescale.

4.3. Effects of microplastics on snail physiology and microbiome

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

Previous studies have shown that PBDEs can transfer from microplastics into body tissues (Chua et al., 2014; Rochman et al., 2013c). Hence, the concentrations measured here are indeed likely to be a combination of both gut content and tissue concentrations, especially as our preliminary studies have shown that the nylon particles are ingested by snails (personal observation.). 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 binding phase with a high affinity for HOCs, thus reducing BDE 47 in the more bioavailable water phase, resulting in reduced bioavailability and uptake (Fig. 1).

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

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

of microplastics suggests that microplastics may be buffering the effects of PBDEs on the microbiota, although only subtly.

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).

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 lower 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.

Acknowledgements

This study was funded by the Newton Fund, grant number UKNE/N00065X/1 awarded to the Universidade Federal do Paraná, Brazil and the Centre for Ecology and Hydrology, UK, and Conselho Nacional das Fundações Estaduais de Amparo à Pesquisa - Fundação Araucária, grant number 45191.460.30251.29012015. We would like to thank Alex Walton for characterising the nylon particles.

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CHAPTER 6

SUPPLEMENTARY INFORMATION

DNA extraction procedure

Three snails were analysed per treatment. Frozen snails were defrosted, removed from the shells, then rinsed in phosphate buffered saline prior to DNA extraction. In order to capture the entire snail microbiome, DNA was extracted from a whole snail through the application of both chemical and enzymatic lysis. Per 250 mg of snail tissue 100 µl of lysis buffer (10 ppmL 1 M Tris pH7.5, 1 ppmL 0.5 M EDTA, 2 ppmL 10% SDS and 4 ppmL 5 M NaCl, made up in molecular grade water) and 20 µl proteinase K solution (20 mg ml^{-1}) was added. Tissue was ground and mixed with a disposable polypropylene tissue pestle and handheld tissue grinder. To ensure complete cell lysis, samples were incubated at 37°C overnight. When samples were fully lysed, proteins were removed through the addition of 600 µl 5M NaCl (per 250 mg of snail weight), mixed well through vortexing and allowed to precipitate for 10 mins at room temperature. 700 µl of lysate was moved to a clean tube and centrifuged at 20000 x g for 10 minutes. Supernatant was transferred to a new tube, mixed by inversion and DNA precipitated through the addition of 650 µl absolute ethanol. DNA was pelleted through centrifugation at 20000 x g for 10 minutes. Ethanol was removed and pelleted DNA was cleaned using 400 µl 70% ethanol. Pellet was centrifuged again at 20000 x g for 2 minutes and ethanol aspirated. Pelleted DNA was air dried to remove residual ethanol and resuspended in 500 µl molecular grade water. Sample DNA required an additional cleaning step performed through the application of Genomic DNA Clean & Concentrator kit (Zymo research) under the manufacturer's recommended protocol. Resultant DNA was quantified using the nanodrop 8000 UV-Vis spectrophotometer (ThermoFisher scientific).

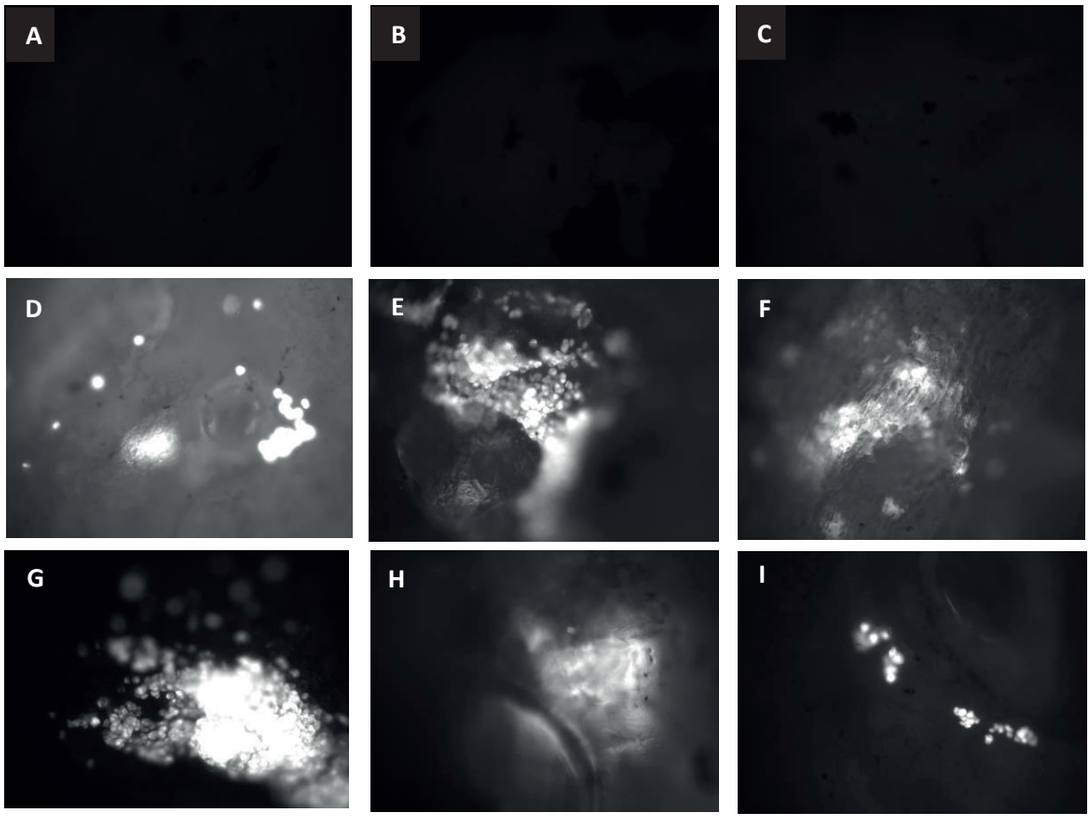

Fig S1. Microscope images showing fluorescently labelled nylon particles within the guts of snails: blank controls (no microplastics) = A-C, microplastic controls = D-F and 3000 ng g^{-1} PBDEs with microplastics = G-I.

Table S1. The OTUs which show the most change in abundance between the highest nominal PBDE concentration (3000) and control treatment, without microplastics (a) or with microplastics (b). Significance at the P > 0.05 level was determined through the application of a Kruskal Wallis test. Significant OTUs are highlighted with an asterisk. Taxonomic identity is given at the highest resolution, using a 97% threshold. ^abundance based on rarefied values

(a) Without Microplastics

	Average^	Standard deviation	ratio	Average^ in 3000 ng g^{-1}	Average^ in solvent control	Cumulative sum	Kruskal-Wallis chi-squared	df	P value	Taxonomic Identity
OTU5512*	0.02	0.02	1.48	357.33	74.67	0.28	3.86	1	0.0495*	Enterobacteriaceae
OTU4432*	0.02	0.01	1.48	355.67	81	0.32	3.86	1	0.0495*	Enterobacteriaceae
OTU32	0.10	0.06	1.79	2851.33	1621.67	0.18	2.33	1	0.1266	Enterobacteriaceae (*Klebsiella*)
OTU2245	0.01	0.01	0.96	84	35	0.42	0.05	1	0.8273	Enterobacteriaceae (*Serratia marcescens*)
OTU8733*	0.03	0.02	1.27	146.67	547	0.24	3.86	1	0.0495*	Flavobacteriaceae
OTU12263	0.01	0.02	0.72	16.33	154	0.36	1.19	1	0.2752	Leptotrichiaceae (*Streptobacillus moniliformis*)
OTU3412	0.01	0.01	1.69	170.67	172.67	0.34	0.05	1	0.8273	Pasteurellales
OTU16390	0.01	0.01	0.93	15	119.33	0.41	2.33	1	0.1266	Rhodospirillaceae
OTU10409	0.01	0.01	1.15	98.33	172	0.39	0.43	1	0.512	Bacteroidales
OTU3010	0.01	0.01	1.45	144.67	124.67	0.38	0.05	1	0.8273	Neisseriaceae

(b) With Microplastics

	Average^	Standard deviation	ratio	Average^ in 3000 ng g^{-1}	Average^ in solvent control	Cumulative sum	Kruskal-Wallis chi-squared	df	P value	Taxonomic Identity
OTU5512	0.02	0.01	1.18	140.5	252.67	0.29	0	1	1	Enterobacteriaceae
OTU4432	0.01	0.01	1.16	118	230.67	0.31	0.33	1	0.5637	Enterobacteriaceae
OTU32	0.19	0.09	2.07	892.5	3265.67	0.26	3	1	0.0833	Enterobacteriaceae (*Klebsiella*)
OTU2245	0.01	0.01	1.03	218	53.67	0.33	0	1	1	Enterobacteriaceae (*Serratia marcescens*)
OTU8733	0.01	0	3.03	172.5	140.33	0.35	0	1	1	Flavobacteriaceae
OTU12263	0.01	0	1.87	87.5	10	0.43	3	1	0.0833	Leptotrichiaceae (*Streptobacillus moniliformis*)
OTU3010	0.01	0.01	1.12	197.5	69	0.37	0.33	1	0.5637	Neisseriaceae
OTU3412	0.01	0.01	1.2	214.5	120.67	0.39	0.33	1	0.5637	Pasteurellales
OTU16395	0.01	0.01	1.07	103.5	19	0.41	0	1	1	Rhizobiaceae
OTU10409	0.01	0.01	1.5	212	69.67	0.40	3	1	0.0833	Bacteroidales
OTU999	0.02	0.01	1.18	140.5	252.67	0.29	0	1	0.2207	Desulfobacteraceae

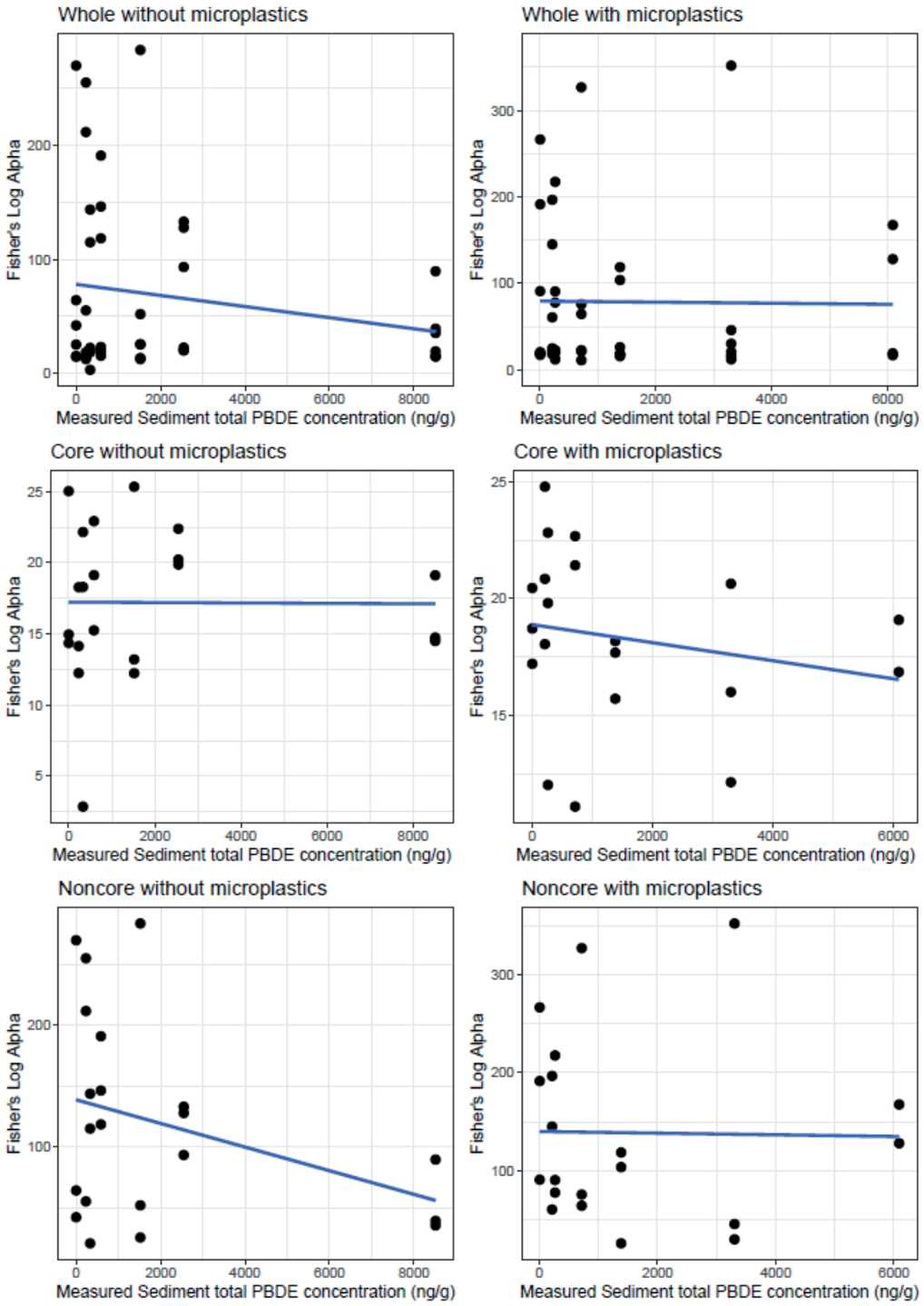

Fig S2. Linear regression of Fisher's log alpha diversity in relation to total sediment PBDE concentration (all congeners combined). Each data point represents an individual snail. Only one sediment concentration value was measured per treatment, therefore these regression lines are to provide a visual representation of the data only.

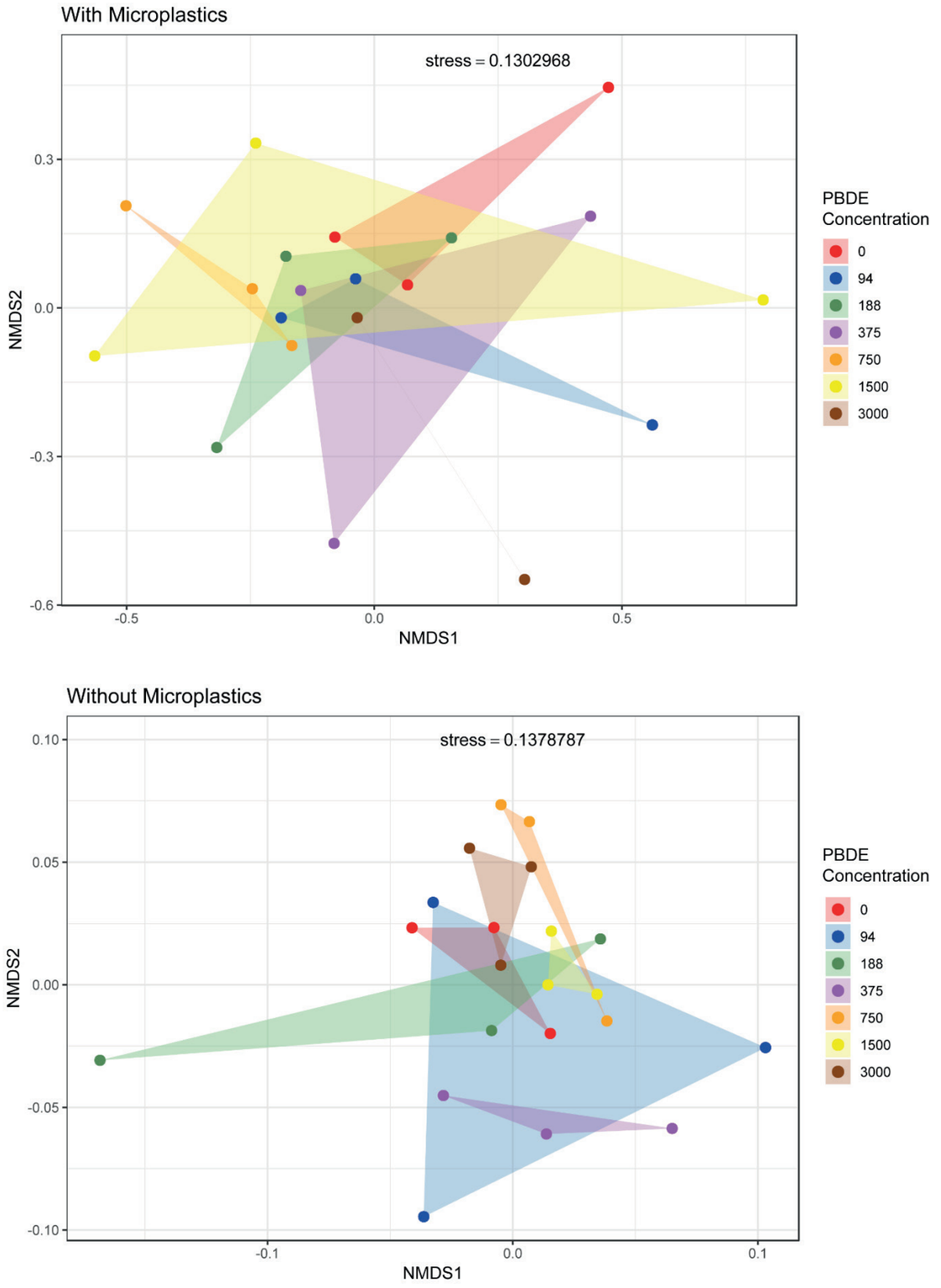

Fig S3. NMDS plots showing community dissimilarity for each PBDE treatment, with or without microplastics.

Fig. S4. Class composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3) are plotted on Y axis. For ease of representation classes of an abundance of <0.02 (2%) from an individual sample were excluded. *Note one sample was removed from this treatment due to inefficient sequencing, therefore *N*=2.

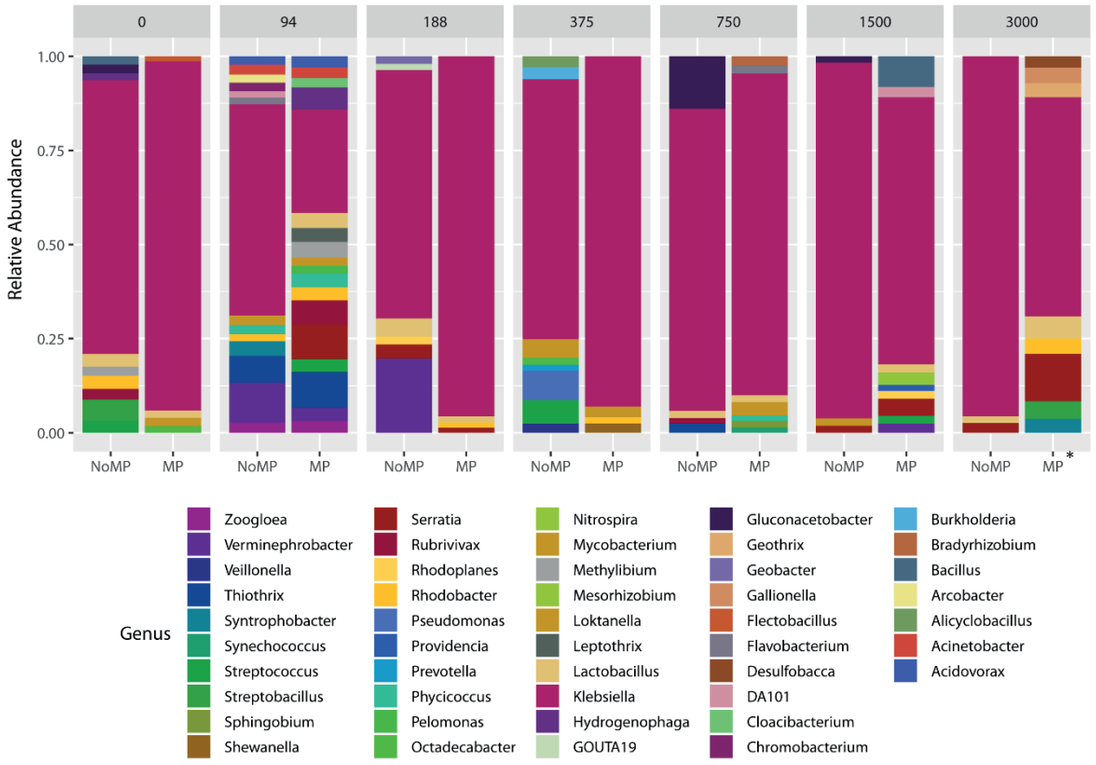

Fig S5. Genus composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3) are plotted on Y axis. For ease of representation genera of an abundance of <0.03 (3%) from an individual sample were excluded. *Note one sample was removed from this treatment due to inefficient sequencing, therefore *N*=2.