Accumulation of nylon microplastics and polybrominated diphenyl ethers and effects on gut microbial community of Chironomus sancticaroli

Accumulation of nylon microplastics and polybrominated diphenyl ethers and effects on gut microbial community of *Chironomus sancticaroli*

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Abstract

Microplastics (MP) are emerging contaminants with the capacity to bind and transport hydrophobic organic compounds of environmental concern, such as polybrominated diphenyl ethers (PBDEs). The aim of this study was to investigate the ingestion of nylon (polyamide) MP alone and when associated with PBDEs and their effects on *Chironomus sancticaroli* larvae survival and microbiome structure. Survival, PBDE uptake and microbial community composition were measured in fourth instar larvae exposed for 96 h to BDEs- 47, 99, 100 and 153 in the presence and absence of 1% w/w MP in sediment. Microbiome community structures were determined through high throughput sequencing of 16S small subunit ribosomal RNA gene (16S rRNA). Initial experiments showed that larvae ingested MP faster at 0.5% w/w MP, while depuration was more efficient at 1% w/w MP, although retention of MP was seen even after 168 h depuration. No mortality was observed as a result of PBDEs and MP exposure. MP had a negative effect on PBDE concentration within larvae (η^2=0.94) and a smaller negative effect on sediment concentrations (η^2=0.48). In all samples, microbial communities were dominated by Alphaproteobacteria, Betaproteobacteria, Actinobacteria and Gammaproteobacteria. Bacterial alpha diversity was not significantly affected by PBDEs or MP exposure. However, the abundance of discrete bacterial taxa was more sensitive to MP (X^2=45.81, p=0.02), and PBDE exposure. Our results highlight that *C. sancticaroli* is resilient to acute exposures to MP and PBDEs, but that MP can influence bacterial microbiome structure even after short-term acute exposure.

Keywords: Polyamide, microbiome, midge, freshwater, Contaminants of emerging concern

1. Introduction

Chironomid larvae are widely distributed in aquatic ecosystems, where they play significant roles in sediment bioturbation, organic matter cycling and in aquatic food-webs. As sediment dwelling organisms, chironomid species will frequently come into close contact with a range of pollutants, including as mixtures (Laws et al., 2016; Pérez-Fuentetaja et al., 2015). Consequently, because of their importance and potential exposure to pollutant through sediment, as well as their amenability to laboratory rearing, chironomids have become widely used for ecotoxicological assessment (OECD/OCDE, 2010; Osmulski and Leyko, 1986; Qi et al., 2015). The chironomid species most commonly used in ecotoxicological testing are *Chironomus tentans* or *C. riparius*. However, these species may not be representative for habitats in areas such as Latin America for which they are not native. For these regions, alternative species such as *Chironomus sancticaroli* (Strixino & Strixino, 1981) which is endemic to Latin America (Armitage et al., 1995; Trivinho-Strixino, 2011) may be more suitable for ecotoxicological studies.

As sediment feeders, chironomids ingest a range of food items including small debris, leaf fragments, algal and fungal cells. This cosmopolitan diet means that when sediments become contaminated with both particulate pollutants and chemicals sorbed to the surfaces of particles, these pollutants can be taken up. Work in freshwater habitats has identified the widespread presence of microplastics (MP) of a range of polymer types and sizes within sediments (Browne et al., 2011; Derraik, 2002; Duis and Coors, 2016). MP are generally defined as solid synthetic organic polymer particles with size less than 5 mm (Betts, 2008). MP have been found globally across a range of habitats, including in South America (Alfonso et al., 2020; Barletta et al., 2019; Pazos et al., 2018). They may be released to the environment either as primary particles in consumer products (Fendall

84 and Sewell, 2009) or secondary particles resulting from the fragmentation of macroplastic
85 debris (Stefani et al., 2014).

86 Given the near ubiquitous presence of MP in sediments, there has been a growing
87 concern on the potential impact of these heterogeneous class of pollutants on organisms.
88 The ingestion of microplastics by has been demonstrated in a wide range of freshwater
89 invertebrate species including the amphipods *Gammarus fossarum* (Blarer and
90 Burkhardt-Holm, 2016) and *Hyallela azteca* (Au et al., 2015), the cladoceran *Daphnia*
91 *magna* (Rehse et al., 2016) and insects from the orders Ephemeroptera (mayfly) and
92 Trichoptera (caddisfly) (Windsor et al., 2019). Demonstrated effects of MP on sediment-
93 dwelling species include impacts on survival, growth, and reproduction (Silva et al.,
94 2019; Stanković et al., 2020; Ziajahromi et al., 2018). Additionally, microplastic particles
95 have the potential to cotransport organic compounds into the organism (Bakir et al., 2016;
96 Rainieri et al., 2018; Zarfl and Matthies, 2010), with hydrophobic organic pollutants such
97 as polybrominated diphenylethers (PBDEs) often highlighted (Hirai et al., 2011; Xu et
98 al., 2019). PBDEs are commonly used as flame retardants and are highly hydrophobic
99 chemicals. Hence, they have the potential to bind to the surfaces of microplastics,
100 changing their bioavailability and uptake.

101 The capacity for MP to impact on the gut microbiome has already been
102 established in invertebrate species including: *Folsomia candida* (Collembola) (Ju et al.,
103 2019; D. Zhu et al., 2018), *Apis mellifera* L. (honeybee) (K Wang et al., 2021),
104 *Enchytraeus crypticus* (Oligochaeta) (B.-K. Zhu et al., 2018) and *Metaphire guillelmi*
105 (earthworm) (Cheng et al., 2021). Yet, the impact of mixed microplastic- PBDE pollution
106 events has yet to be widely studied. Recent work by Horton et al (2020), suggested that
107 the impact of such MP mixed pollution events was subtle in a larger sediment dwelling
108 snail species *Lymnaea stagnalis*. However, whether these patterns hold true for much

smaller cosmopolitan species such as *Chironomus sancticaroli* warrants further investigation.

Here we report a study in which the Latin American species *C. sancticaroli* have been exposed to microplastics and PDBE congeners, both separately and in combination. Through the joint exposure of MP with a range of PBDE congeners with different log Kows (octanol-water partition coefficient) ranging from 6.81 (BDE-47) to 7.9 (BDE-153) (Braekevelt et al. 2003). We examined the impact of microplastic and PBDE exposure to *Chironomus sancticaroli* and its microbial gut microbiome community, to determine how microplastic-PBDE interactions affect PBDE uptake, and the chironomid gut microbiome.

We hypothesised that chironomids would rapidly ingest microplastics, that would then be retained within the gut before latter egestion. This rapid ingestion in turn leads us to hypothesise that exposure to microplastics would reduce PBDE bioavailability as a result of strong binding to microplastics within sediment, and thus reduce the accumulation and microbiome effects of PBDEs compared to exposure to PBDEs alone.

2. Materials and Methods

2.1 Microplastic particle preparation

Nylon 6 powder (particles < 50 µm with a mean size of 13–19 µm, measured using a Coulter Counter (Multisizer 3, Beckman, USA), density 1.13 g cm^{-3}) was purchased from Goodfellow, UK. The powder was soaked in Nile Red solution (8 µg mL^{-1} in 80:20 methanol: water solution) to provide a fluorescent label that would allow the detection of particles within the chironomid gut. After labelling, the carrier solvent was evaporated at room temperature for approx. 24 h with occasional mixing until the powder was

completely dry. Particles were then rinsed in deionised water to remove any unbound dye, filtered onto 1.2 μm Whatman GF/C glass microfiber filter papers (GE Healthcare Life Sciences, UK) and redried at 60°C. Experimental treatments consisted of exposure in sediment either with or without microplastics (1% nylon powder by mass). Microplastic-spiked sediments were prepared by mixing 0.8 g of the labelled nylon powder with quartz sand (Sigma Aldrich) and making up to 80 g,

2.2 Experimental organism

Larvae of *C. sancticaroli* were obtained from the colony of the Laboratory of Morphology and Physiology of Culicidae and Chironomidae (LaMFiC[2]) at the Federal University of Paraná. The colony was kept under 25 ± 2°C, 80% relative humidity and photophase: scotophase (12:12) in aerated aquaria following the protocol of Maier et al. (1990). Voucher specimens of this colony (249269 to 249276) are in the Entomology Museum Padre Jesus Santiago Moure of the Zoology Department at the Federal University of Parana (DZUP).

Masses of eggs freshly laid from the colony were transferred to trays containing reconstituted water with 1.2 mg L^{-1} hydrated $CaSO_4$, 0.08 mg L^{-1} KCl, 2.44 mg L^{-1} $MgSO_4 \cdot 7H_2O$, and 1.92 mg L^{-1} Na_2CO_3, conductivity of 160 μS cm^{-1}, pH 7.2 and hardness 16 mg L^{-1} (US EPA 2000). Larvae were maintained with constant aeration and fed TetraMin® fish food three times per week until they reached the fourth instar.

2.3 Ingestion study

A small preliminary study was conducted to quantitatively assess whether chironomid larvae were able to ingest and egest the nylon microplastics. Chironomids were exposed to two concentrations of microplastics in sediment and one control (0%,

0.5% and 1% by mass dry weight fluorescently labelled nylon powder). Ten chironomids were exposed per vessel, with six replicates per treatment. Exposures ran for 48 hours, with sacrificial samples taken at 6 h, 24 h and 48 h (one individual per replicate). At each time point, individuals were imaged at 40X magnification using a Leica epifluorescence microscope. After 48 h, all remaining individuals were transferred to clean sediment (0% microplastics) and depuration allowed to occur for 168 h. As before, sacrificial samples were taken at 6 h, 24 h, 48 h and 168 h for fluorescence microscopy to assess the gut clearance of microplastics. The mean surface area of larvae containing microplastic was calculated at each of the evaluation time points (n=6 per treatment). The measurement of the areas was made by calibrating the scale and transforming images to 8-bit. The threshold was adjusted, the area to be analyzed was selected and then the area showing fluorescence was measured using ImageJ program (version 1.53a) (Rasband, 2012).

Comparison of the fluorescence areas of larvae were conducted using a three-way ANOVA to determine the effect of intake / depuration periods, microplastic concentration (0.5 % and 1 %) and time points (6 h, 24 h and 48 h). 168 h samples were imaged where available, but were excluded from the following analyses due to limited survival of larvae to this point. Residual analysis was performed to test for the assumptions of the three-way ANOVA. Shapiro-Wilk's normality test and Levene's test were used to assess normality and homogeneity of variances. Interaction effects were checked by pairwise comparison using Bonferroni correction. Data were analyzed under R environment (v.1.3.1093) using package *tidyverse, ggpubr* and *rstatix* (R Core Team, 2017).

2.4 PBDE and microplastic exposure setup

A standard PBDE mixture was used to create the spiked sediments, containing the congeners BDE-47 (CAS No 5436-43-1), BDE-99 (CAS No 60348-60-9), BDE-100

(CAS No. 189084-64-8) and BDE-153 (CAS No. 0868631-49-2) in ethyl acetate (Method 527 PBDE Mixture, LGC Standards, Teddington, UK). A serial dilution was prepared in ethyl acetate and spiked into sediments (with/without microplastics) to give nominal concentrations of 94, 188, 375, 750, 1500 and 3000, ng g^{-1}, in addition to ethyl acetate and blank controls. For each treatment, 1 mL of each diluted stock was added to 80 g of sand substrate with or without added microplastics and stirred for 3 mins using a glass rod. Mixed sediment batches were divided between 6 replicate vessels per treatment (13 g per vessel) with the additional substrate saved for analysis. Following dosing, the vessels were left under a fume hood for 2 days with occasional agitation to ensure complete evaporation of the solvent.

All bioassays were carried out in glass vessels containing 13 g of test substrate, covered with 50 mL of reconstituted water. Each replicate vessel contained 15 larvae. Bioassays were conducted in a Bio-Oxygen Demand (BOD) chamber under 25 ± 2°C, 80% relative humidity and photophase: scotophase (12:12) with aeration lines for 96 h. At the end of the exposure, surviving larvae were collected during the photophase. Of the six replicates, three were preserved for PBDE analysis in tissues; wet weight was measured then samples were lyophilized using a Wizard 2.0 freeze drying machine (SP Scientific, New York, USA). Each of the remaining three replicates was split in half, with 7 individuals per replicate frozen for archiving and later analysis, and the remaining individuals per replicate preserved in 1 mL RNA later (Qiagen, Hilden, Germany) in a sterile 2 mL cryovial for nucleic acid extraction. The chironomids were not depurated prior to analysis and so retained their gut content.

2.5 Chironomid microbiome DNA extraction, sequencing, and bioinformatics

To remove surface contaminants, larvae were rinsed in phosphate buffered saline. DNA was extracted from whole organisms using the DNeasy Blood and tissue kit (QIAGEN) under the manufacturers recommended protocol for tissue samples. Approximately 20-30 ng of template DNA was amplified using Q5 High Fidelity Polymerase (New England Biolabs, Hitchin, UK), each with a unique barcode-primer combination (Kozich et al, 2013). Amplification conditions consisted of 25 cycles and initial 30s, 98 ºC denaturation step, followed by annealing phase of 30s at 53 ºC, and a final extension step lasting 90s at 72 ºC. Primer sequence was based on the universal bacterial primer sequence combination 341F and 806R, producing amplicons of ~550 bp spanning the V3-V4 hypervariable regions of the gene encoding 16S small subunit ribosomal RNA (herein, 16S rRNA). PCR products were normalised using Sequalprep normalisation plates (Invitrogen, Carlsbad, USA) and the resultant amplicon library sequenced at a concentration of 5.4 pmol L^{-1} with a 0.6 pmol L^{-1} addition of Illumina generated PhiX control library. Sequencing was performed on an Illumina MiSeq platform using V3 chemistry (Illumina Inc., San Diego, CA, USA).

Sequenced paired-end reads were analysed using an in-house bioinformatics pipeline fully outlined in Newbold et al (2017). Briefly, paired-end reads were joined using PEAR (Zhang et al., 2014), quality filtered using FastX tools (Hannon, *http://hannonlab.cshl.edu*) and chimeras removed with ChimeraSlayer (Haas et al., 2011). Resultant non-chimeric sequences were clustered into operational taxonomic units (OTUs) at the 97% identity cut-off through the application of UCLUST (Edgar, 2010) in the QIIME package (Caporaso et al., 2010), and putative taxonomy assigned using the Greengenes database release 13_2 (McDonald et al., 2012). The raw sequence data reported in this study have been deposited in the European Nucleotide Archive under

study accession number PRJEB27672 (ERP109787). Individual simple accession numbers ERS2599813: ERS2599860.

Analyses of the 16S SSU rRNA microbiome sequences data were carried out in R environment (v.4.0.3) (R Core Team, 2017) using the *Vegan* v2.5-7 (Oksanen et al., 2020) and *phyloseq* v1.37 packages (McMurdie et al., 2013). Taxonomic abundance was visualized in heat trees after removing low abundance counts (less than five) and transformed data to a proportion. A Wilcoxon rank-sum test was used to test for differences between the median abundances of each taxon of larvae from treatments with and without microplastic. To compare the effect of the different nominal PBDE concentrations, heat tree matrices, one for each pairwise comparison were done. The phylogenetic trees were plotted with the log2 ratio of median proportions using *Metacoder* package (Foster et al., 2017).

To account for unequal sequencing depth diversity of bacterial communities were assessed after rarefaction of the 802 OTUs at 90% of the minimum sample depth in the dataset. Rarefied matrix has 489 sequencies. Chao1 index was used to characterize the sample richness, and the Shannon index to describe the sample evenness. Differences of alpha diversity were verified using Wilcoxon rank-sum test (Mann-Whitney) and resulting *p*-values from pair-wise comparison were adjusted by Bonferroni-Holm method. The difference in phylum and class level of relative abundances as an effect of PBDE concentrations and microplastic presence or absence was measured after removing rare taxa. Beta diversity was visualized by a Principal Coordinate Analysis (PCoA) keeping only those OTUs that were detected at least four times in four out of total samples and converted to relative abundances. Bray-Curtis dissimilarity and UniFrac distances were used to measure how many taxes are shared among samples. Bray-Curtis dissimilarity maximizes the pairwise distance between individual samples (Bray and

Curtis, 1957) and UniFrac distances consider the OTU abundance (weighted) and the presence or absence of low OTU abundances (unweighted) (Lozupone et al., 2011). Differences in bacterial structure across samples were determined using permutational multivariate analysis of variance (PERMANOVA) by means of adonis function; additionally, a multivariate homogeneity test of group dispersion analysis using the betadisper function was carried out (Oksanen, 2015). Pairwise comparisons using Tukey's HSD test was performed when significant measures of dispersion were observed.

Differential abundance was analysed through estimating log2 fold changes of bacterial abundance using DESeq2 package (Love et al., 2014). Wald *z* test was measured to infer the significance of the log2 fold changes. Differences with p-value < 0.05 of log2 fold changes were considered statistically significant. The formula supplied to create DESeq object was PBDEs concentrations as blocking factor and microplastic as comparison variable. Data was filtered keeping OTUs having more than a total sum of 5 reads in all samples.

2.6 Chemical analysis

Prepared freeze-dried tissues were weighed accurately, dried with anhydrous sodium sulphate, and then spiked with ^{13}C labelled standards for BDE-47, BDE-99, BDE-100, BDE-153 (Cambridge Isotope Laboratories, Andover, Massachusetts) at concentrations of 50 pg uL^{-1}. Subsequently, the samples were Soxhlet extracted for 16 h in dichloromethane. Lipid content was determined gravimetrically using a 15 mL aliquot of each extract. The remaining sample was dried in a rotary evaporator and the solvent exchanged to hexane. Lipids were removed using a two-step clean-up process. Initially, extract was cleaned using a 23 mm ID column packed with 15 g acidified silica (2:1 by weight activated silica gel: concentrated sulphuric acid) with samples eluted using 300

mL of hexane. These extracts were then evaporated to a <1 mL sample volume under a stream of nitrogen. Secondary clean-up was performed by gel permeation chromatography using a 20 mm ID column packed with 12 g biobeads. Samples were eluted with a 1:1 v/v of hexane: dichloromethane. The collected fraction was evaporated under nitrogen before being transferred to a GC vial containing 25 μL of keeper solution of dodecane plus internal standards $13C_{12}$ labelled PBDE-77 and PBDE-138 (Cambridge Isotope Laboratories, Andover, Massachusetts). The final extract derived from each sample was analysed by Thermo-Finnigan Trace Gas Chromatography Mass Spectrometry (GC-MS) in electron ionisation mode fitted with a ThermoQuest AS2000 autosampler and using a 30 m CPSIL-8 CB pesticide column (0.25 mm diameter, 0.12 mm internal diameter) and calibrated using seven PBDE standards in a linear range from 2.5 to 250 pg μL^{-1} with analysis for BDE-47, BDE-99, BDE-100, and BDE-153. Resource limitations meant that it was only possible to analyse one sediment concentration measurement per treatment.

2.7 Data analyses

The effect of each PBDE congener (BDE-47, BDE-99, BDE-100 and BDE-153), their nominal concentration (99, 188, 375, 1500, and 3000 ng g^{-1}) and microplastics (presence and absence) on the content of PBDE in sediment and larvae, after log transformation, was evaluated separately using a linear model *lm* and least-squares means (LSmeans) (Lenth, 2016) for post hoc comparisons using *multicomp* package (Hothorn et al., 2008) with a significance level of 0.05. Comparisons were performed considering PBDE congener, nominal PBDE concentrations and presence or absence of microplastic as independent variables. PBDE concentration in sediment and larvae was the response variable of each model.

299 To determine whether PBDE nominal concentration and microplastic influenced
300 the PBDE concentration in larvae and sediment, a MANOVA was performed with the
301 nominal sediment concentrations and microplastics as independent variables, and
302 measured concentration of larvae and sediment as the dependent variables. The
303 significance was measured considering the Pillais's Trace criterion. Significant
304 MANOVA was followed up by univariate one-way ANOVA test and differences between
305 groups were determinate by Tukey honest significant difference (HSD) post-hoc test.
306 Analyses were run under R environment (v.1.3.1093) (R Core Team, 2017).

307 **3. Results**

308 **3.1 Ingestion of microplastics**

309 Uptake of the labelled microplastics through ingestion by the chironomids was
310 observed (Fig. 1). The mean area of larvae (n= 6) containing microplastic particles
311 significantly reduced during the depuration period indicating particle egestion ($F_{1,}$
312 $_{54}$=19.35, p <0.05). The mean area measured in larvae during the intake period ranged
313 from 2 to 4.7 mm^2 while during the depuration period ranged from 0.5 to 2.8 mm^2.

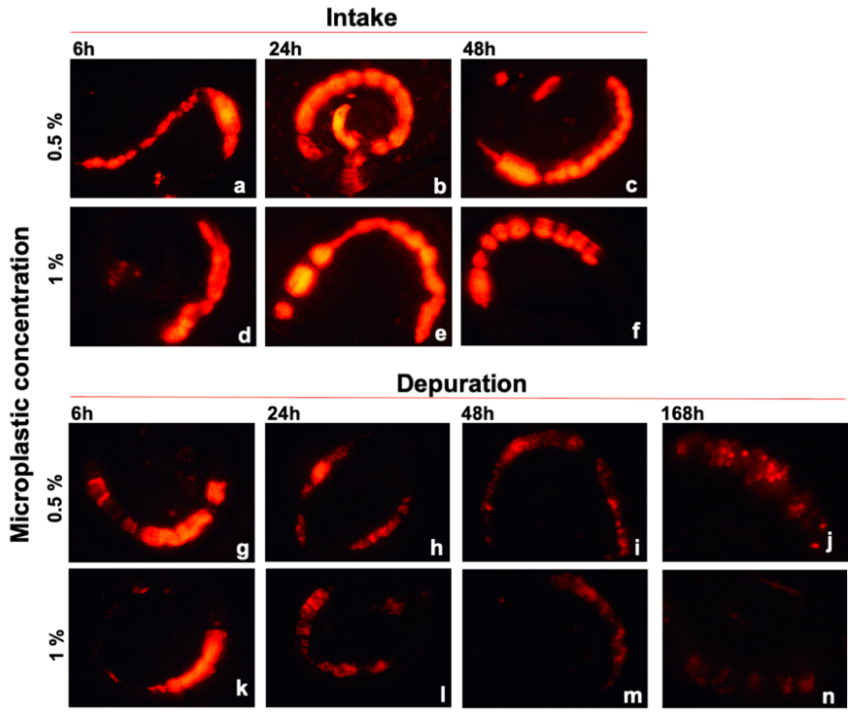
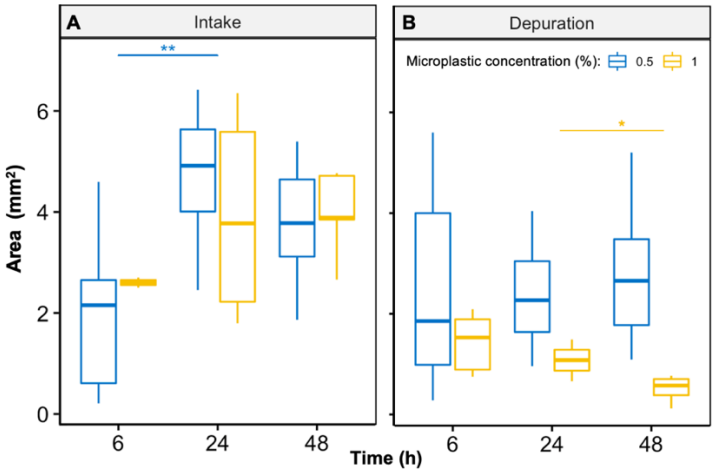


Fig. 1. Fluorescence images of *Chironomus sancticaroli* larvae during the exposure (intake) (from **a** to **f**) and depuration (from **g** to **n**) periods for labelled microplastics at 0.5 % w/w and 1 % w/w. Time points for each period: 6 h, 24 h, 48 h and 168 h. Analysis was performed with an epifluorescence microscope (Leica®; model DMLS2) under 40x magnification

The fluorescence area measured in larvae differed between the exposure (different ingestion) and depuration periods ($F_{1, 54}$=19.36, p<0.05). Fluorescence also fluorescence significantly differed according to microplastic exposure concentration ($F_{1, 54}$=4.72, p=0.03). Furthermore, interaction term of the uptake / depuration period and microplastics concentration was also significant ($F_{1, 54}$=4.17, p=0.04) indicating that the difference with exposure time was concentration dependent). This interaction was also significant for each of the evaluated time points ($F_{2, 54}$=4.10, p=0.02).

Exposure time in the ingestion phase significantly affected measured larval fluorescence ($F_{2, 50}$=6.20, p =0.004). Fluorescence was significantly lower at 6 h than the other evaluated points in the 0.5 % w/w treatment. In contrast, the ingestion of

329 microplastics by larvae in the 1% microplastic treatment showed no significant
330 differences between evaluation times ($p > 0.05$) (Fig. 2.A).



331

332 **Fig. 2**. Internal microplastic area (mm^2) measured in *Chironomus sancticaroli* larvae
333 during the (**A**) intake and (**B**) depuration periods at a given time points (6 h, 24 h and 48 h intake).
334 Sediment microplastic concentrations were 0.5 % and 1 %. ANOVA $F_{2, 54} = 4.11$, $p = 0.022$.
335 *Indicates significant differences at the $p < 0.05$ level based on post-hoc pairwise comparisons
336 with a Bonferroni adjustment.

337 For the depuration period, the effect of time on measured fluorescence was
338 exposure concentration dependent ($F_{1, 50}=8.62$, $p = 0.005$) (Fig. 2.B). For example, at the
339 48 h timepoint, larvae exposed to 1% microplastic showed significantly less microplastic
340 associated fluorescence for those exposed to the 0.5 % treatment loss by 48 h was not
341 significant. Indeed for the higher concentration treatment, no significant variation of
342 fluorescence areas was found between any sampling times ($F_{2, 50}=0.09$, $p = 0.90$) (Fig.
343 2.B). Insufficient chironomids survived until 168 h (possibly due to the lack of supplied
344 food) to allow robust quantitative analysis of retention over this longer time-scale.
345 However, image analysis of surviving larvae did indicate microplastic retention up to this
346 time point (Fig. 1).

3.2 Treatment effects of survival and PBDE uptake

There were no significant effects on larvae survival over 96 h following exposure to PBDEs across the range of tested concentrations, with or without microplastics. This mean that organisms could be collected and analysed for PBDE from all treatment across the full exposure range.

The planned nominal and actual measured concentrations of BDE-47, BDE-99, BDE-100 and BDE-153 in sediment in the presence and absence of microplastics are presented in Table 1. The control sample showed the presence of trace levels of the measured PBDEs. For the remaining treatments, measured PBDE concentrations in sediment were consistently lower than nominal value. Differences between nominal and measured values were highest at the highest nominal concentrations and were greatest in treatment with microplastics, indicating that microplastics (in some way) influence sediment PBDEs concentrations (Supplementary materials - Fig. 1). Nominal compared to measured concentration differences were highest for the low molecular weight congeners (e.g. PBDE-47) compared to higher weight PBDEs (e.g. PBDE-153) both in the presence and absence of microplastics.

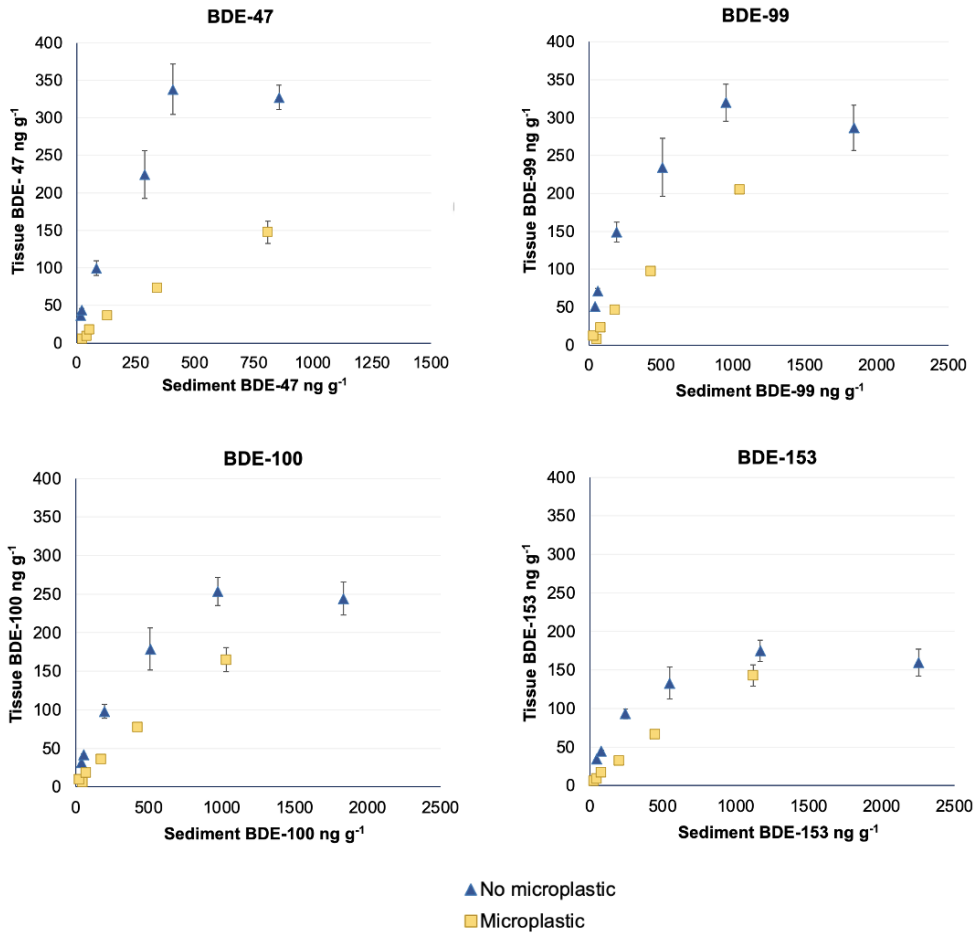
The reasons underlying the difference may be complex and a full analysis was outside of the scope and available resources for this project. However, it is possible these difference may relate to aspects such as losses during dosing and solvent venting, or issues with the efficiency of the extraction method (especially for higher weight PBDEs in the presence of plastics). For ease of communication of results to mean that similar exposure levels can be referred to in the same way, treatments are discussed in respect of planned nominal values.

371

		BDE-47	BDE-99	BDE-100	BDE-153
		Concentration (ng g^{-1})			
	Nominal	Measured			
Without microplastics	Water control	0.5	0.1	0.1	0.1
	Solvent control	1.1	0.3	0.3	0.1
	94	17.4	43.2	39.5	45.1
	188	22.1	59.0	53.4	76.3
	375	83.6	192.3	196.5	243.5
	750	288.3	508.6	509.2	546.2
	1500	406.8	950.3	973.0	1166.5
	3000	855.8	1840.4	1832.4	2252.2
With microplastics	Water control	9.5	1.1	0.7	0.1
	Solvent control	2.7	2.4	1.3	0.1
	94	23.4	30.5	24.7	26.7
	188	41.5	53.1	46.2	45.0
	375	53.1	78.7	70.2	77.7
	750	129.9	178.5	172.2	198.9
	1500	339.8	429.9	421.7	447.1
	3000	808.0	1045.4	1029.7	1118.2

372 **Table 1**. Nominal and measured concentrations of BDE congeners in sediment exposure
373 (ng g^{-1}). Measured concentrations represent one single measurement per BDE congener per
374 nominal concentration.

375 PBDE measurements in larvae demonstrated that microplastics significantly
376 reduced PBDE concentrations in chironomid tissues for all congeners at all
377 concentrations ($p< 0.01$) (Fig. 3; Supplementary materials - Fig. 2). The results of
378 interaction of microplastic with each PBDE congener in larvae displayed similar least
379 square mean (LSM) values considering the presence of microplastics and 47-, 100- and
380 153-BDE ($p>0.05$) (Supplementary materials - Fig. 1).



381

382 **Fig. 3.** Measured PBDE concentrations in sediment, compared to the concentration within
383 *C. sancticaroli* larvae, for each BDE congener, in the presence (Microplastic) and absence of
384 microplastic (No microplastic).

385 Significant multivariate main effects on measured tissue PBDE concentration
386 were found for both the nominal PBDE concentration (Pillai's Trace=1.192, $F_{10,\ 72}$ =
387 10.625, p <0.01) and for the presence or absence of microplastics (Pillai's Trace= 0.944,
388 $F_{2,\ 35}$ = 297.674, p <0.01). A significant interaction term between nominal PBDE
389 concentration and presence or absence of microplastics was also noticed (Pillai's
390 Trace=0.981, $F_{10,\ 72}$ = 6.939, p <0.01) indicating that the extent of the effects of
391 microplastic on tissue accumulation is concentration dependent.

392 **3.3 Microbiome data**

Of the fourteen identified phyla Proteobacteria, Bacteroidetes and Actinobacteria dominated the community. Within these phyla the most abundant classes were Alphaproteobacteria, Betaproteobacteria, Actinobacteria and Gammaproteobacteria from a total of 45 present in the chironomid microbiome community.

There was no significant effect (p >0.05) of PBDE concentration or MP on *C. sancticaroli* larvae bacterial community diversity when using the Shannon or Chao 1 indices (Fig. 4). However, significant changes in the community abundance were detected at class level (X^2=45.81, p=0.02).

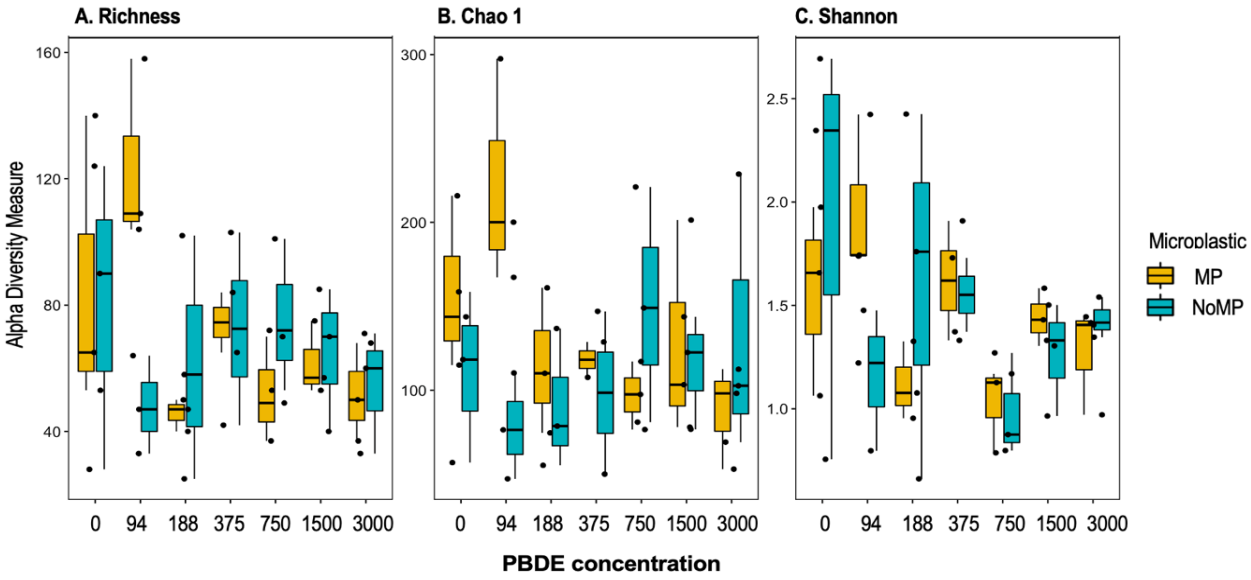


Fig. 4. Species richness (**A**) and Chao 1 (**B**) and Shannon indices (**C**) alpha diversity for bacterial community in *C. sancticaroli* exposed to PBDEs in the presence (MP) and absence of microplastics (NoMP).

Chironomid microbiome composition and the relative abundance of bacteria in the presence or absence of MP are presented as a heat tree (Fig. 5). Nodes illustrate taxonomic levels, and the relationship between those levels represented by the branches. Taxa colored gray appear equally in larvae exposed to PBDEs with the presence and

absence of microplastics, taxa colored in blue are more abundant in larvae exposed to PBDEs without microplastics and those in yellow are more represented in larvae exposed to PBDEs associated with microplastics. Variation of bacterial community of *C. sancticaroli* larvae considering the different PBDEs concentrations are presented in Supplementary materials - Fig. 3.

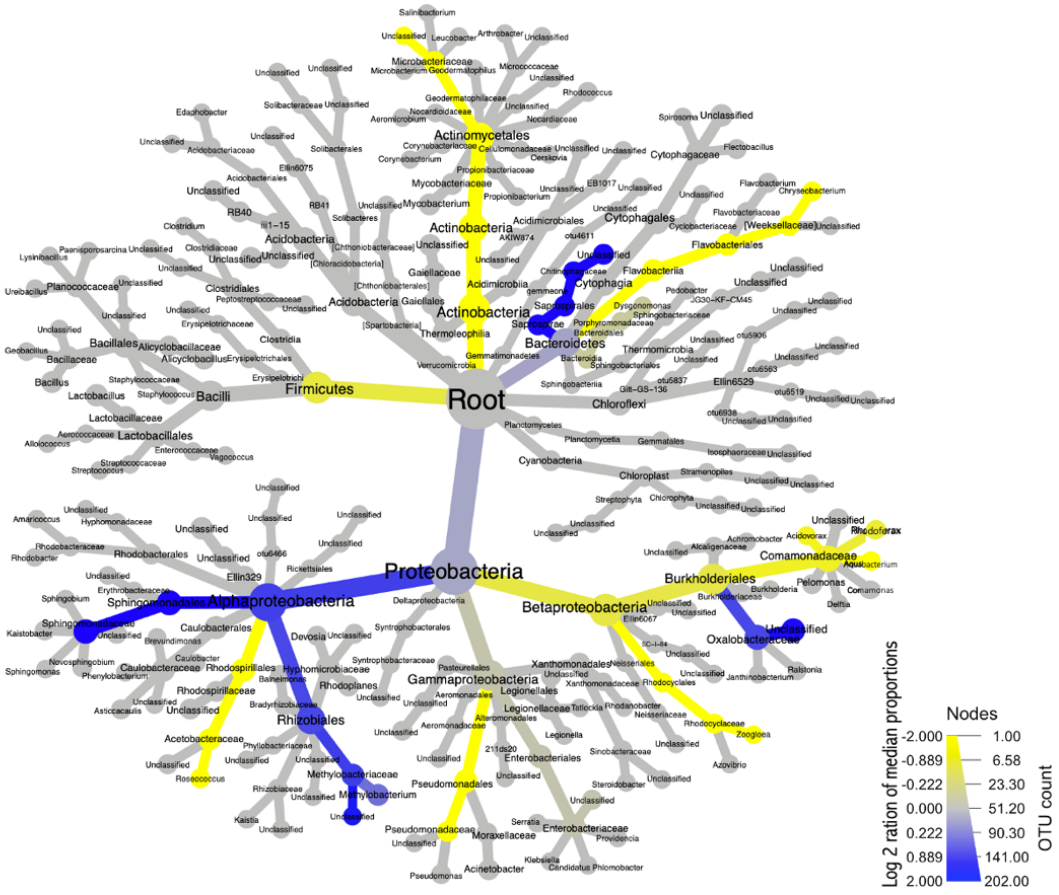


Fig. 5. Heat tree showing the taxonomic differences between bacterial community of *Chironomus sancticaroli* larvae after exposure to PBDE mixtures in the presence and absence of microplastics. Colour gradients represent the difference in log2 ratio of median proportions between microplastic treatments. Blue nodes are taxa more enriched in larvae treated without microplastics, yellow nodes are taxa more enriched in larvae treated with microplastics and grey nodes represent taxa equally present in larvae of both treatments.

Unweighted UniFrac distance based on species presence and absence indicated that exposure to MP was significantly related to microbiota variation (R^2=0.15, p=0.001). Further, when using species abundance information PBDE concentrations also contributed to microbiome variation when considering weighted UniFrac distance (R^2=0.23, p=0.027) and Bray-Curtis dissimilarities (R^2=0.22, p=0.049). Interestingly, the interaction between microplastics and concentrations of PBDE mixture did not influence the bacterial structure using any of the studied metrics (p>0.05). The differences in the composition of the microbiome attributed to PBDEs concentrations, thus, had a local instead of a dispersion effect (F=1.31, p=0.31).

A principal coordinates analysis indicated that principal coordinates 1 and 2 explain, respectively, 43.3% and 19.1% of the variance in Bray-Curtis dissimilarity and 19.9% and 17.6% in unweighted Unifrac distance (Fig. 6). The only significant differences observed were between larvae exposed to 750 ng g^{-1} of the PBDEs mixture and control (p=0.048); and larvae exposed to 750 and 188 ng g^{-1} (p=0.036) considering the post hoc Tukey's HSD (Fig. 6A). There were no significant differences observed in communities from the remaining treatments.

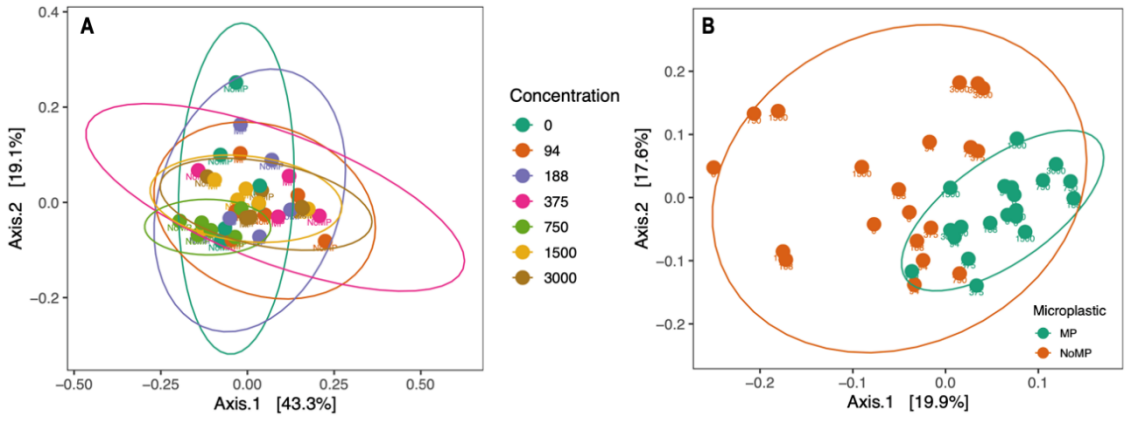


Fig. 6. Principal coordinates analysis (PCoA) of bacterial communities with (**A**) Bray-Curtis dissimilarity and (**B**). Unweighted UniFrac distance in *C. sancticaroli* larvae associated to the mixture of PBDEs at concentrations ranging from 0 to 3000 ng g^{-1} and presence (MP) and absence of microplastics (NoMP).

The difference in microbiome structure between larvae exposed to PBDEs in the presence and absence of microplastic was based on effects for 11 of 177 OTUs. Rhodospirillales (Acetobacteraceae), Actinomycetales (Microbacteriaceae), Burkholderiales (Comamonadaceae: *Aquabacterium*), Aeromonadales (Aeromonadaceae), Flavobacteriales (Weeksellaceae: *Chryseobacterium*) and Pseudomonadales (Pseudomonadaceae: *Pseudomonas*) showed higher abundances in larvae exposed to PBDEs mixtures with microplastics, whereas Bacillales (Planococcaceae: *Lysinibacillus*), Caulobacterales (Caulocacteracear), Pseudomonadales (Moraxellaceae: *Acinetobacter*), Sphingobacteriales (Spohingobacteriaceae: *Pedobacter*) and Rhizobiales (Methylobacteriaceae) orders were more abundant in larvae exposed to PBDEs mixture without microplastics. The remaining 165 OTUs were common for both groups (Fig. 7.A).

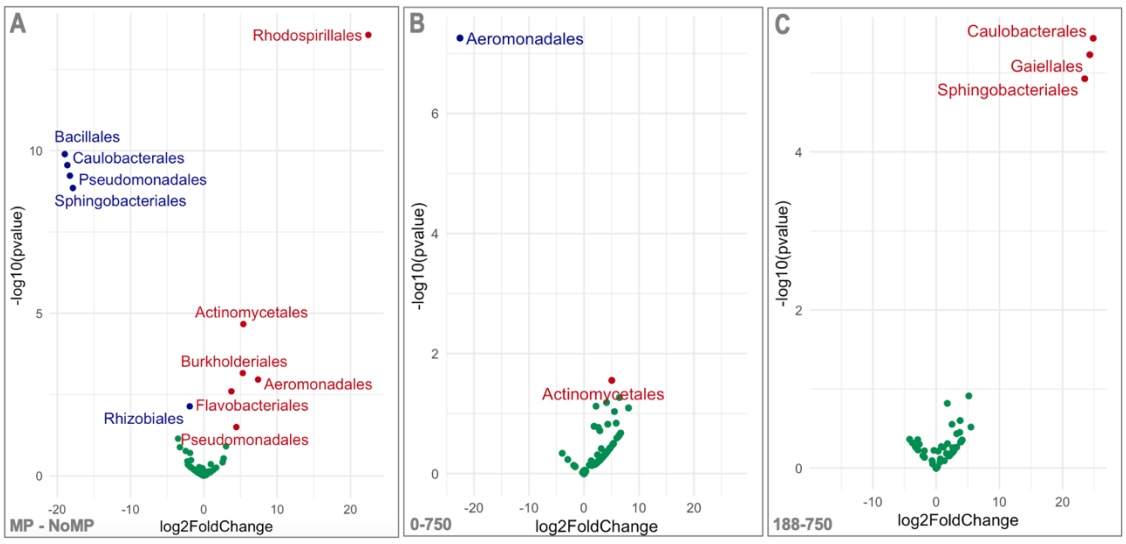


Fig. 7. Volcano plot showing the comparison between the log2 fold change and log10 *p* value for OTUs from the bacterial microbiome of *Chironomidae sancticaroli* larvae exposed to **(A)** microplastic and no microplastic, **(B)** 0 and 750 ng g^{-1} total PBDE and **(C)** 188 and 750 ng g^{-1} total PBDE groups. Red dots depict OTUs abundant in microplastic, 0 and 188 ng g^{-1} groups and blue ones are more abundant no microplastic and 750 ng g^{-1} groups. Green dots represent order features either common between groups or classified as insignificant.

Considering PBDE exposure, bacterial abundance was influenced in larvae exposed with 750 ng g^{-1} when compared to the control, and in larvae exposed with 188 ng g^{-1} when compared to those of 750 ng g^{-1}. Order Actinomycetales was higher in larvae exposed to 750 ng g^{-1}, while Aeromonadales were higher in the controls. When comparing the 188 ng g^{-1} and 750 ng g^{-1} treatments, Caulobacteriales, Giellales and Sphingobacteriales abundance were all greater at the higher PBDEs concentration. No other taxa showed a significant difference from any other comparisons of PBDEs concentrations.

4. Discussion

Ingestion and depuration studies showed that *C. sancticaroli* larvae were able to ingest nylon microplastics. During the depuration phase, ingested microplastics were egested, although this took places over days rather than within the first few hours of transfer to sediment. The extent of ingestion of microplastics was not significantly influenced by the sediment concentration of microplastics. However, larvae exposed to 0.5 % microplastics in sediment retained more particles than individuals exposed to 1 % during the depuration period (Fig. 2). Given similar levels of ingestion at the different exposure concentrations, it is not clear what the biological basis was of this result. However, it does imply that initial exposure concentration can influence the degree to which microplastics are retained within the digestive tract.

482 At the end of the 48 h depuration period, all assessed chironomids still retained
483 some of the labelled microplastics in their gut lumen. Insufficient chironomids survived
484 until 168 h to allow the collection of usable data for robust quantitative analysis of
485 retention over this longer timescale, however, those surviving larvae that could be assess
486 did show retention of MP up to this longer time point. This is far beyond the expected
487 timescale of retention and suggests that microplastics can be retained significantly longer
488 than regular food items.

489 It has previously been suggested for other species within the family Chironomidae
490 such as *Corynoneura scutellata*, that gut residence time (of ingested algae) can be as short
491 as 10-12 minutes (Kesler, 1981). However, invertebrates have been shown to retain
492 microplastics for longer than other particulate matter or natural food (Hurley et al., 2017;
493 Wright et al., 2013). Our results would also suggest this is the case for chironomids. Gut
494 transit time is likely to relate to food composition and quality, with lower quality food
495 retained for longer to enable effective extraction of nutrients (Davies, 1975). Given the
496 minimal nutritional value of microplastic, this may go some way to explaining the
497 observed long retention times, especially in the absence of the supply of additional food,
498 which was the case in this experiment.

499 The presence of MP in sediment caused a significant reduction in the measured
500 PBDE concentration in larvae across all studied congeners. This result is in contrast to
501 the results of our parallel study on the effects of the presence of microplastics on *Lymnaea*
502 *stagnalis* tissue concentrations for the same PBDE congeners. The study in snails
503 indicated no effect of microplastic co-exposure on tissue concentrations except for BDE-
504 47, for which the body burden was significantly reduced (Horton et al., 2020). Here we
505 found microplastics significantly reduced all congeners in chironomid tissues, with BDE-
506 153 being most strongly affected (Supplementary materials - Fig. 2). This highest

suppression of BDE-153 potentially supports a role for hydrophobicity, and thus binding, to the decrease in reduced bioaccumulation resulting from microplastic co-exposure, as this congener has the highest log Kow (7.9) (Braekevelt et al. 2003).

Proteobacteria were the most dominant phylum found in the *C. sancticaroli* microbiome (Sela et al., 2020), consistent with previous findings for other insects (Castillo et al., 2020; Jones et al., 2013; Lim and Ab Majid, 2021). Both the presence of microplastics and PBDE concentration were found to affect the *C. sancticaroli* bacterial microbiome. Exposure to microplastics had the greatest effects with members of the families Comamonadaceae (*Aquabacterium),* Weeksellaceae (*Chryseobacteriym*), Pseudomonadaceae (*Pseudomonas*), Acetobacteraceae, Microbacteriaceae, and Aeromondaceae, showed a significant increase in their abundances in *C. sancticaroli* larvae when microplastics were present.

It has been shown that 25% of the total composition of the chironomid larval bacterial community is made up of species with the capacity to transform toxic compounds (Senderovich and Halpern, 2013). It is recognized that members of the Aeromonadaceae, in addition to playing a role degrading chironomids egg masses (Senderovich and Halpern, 2012), can also protect larvae from the toxic effect of xenobiotics (Laviad and Halpern, 2016; Senderovich and Halpern, 2013). In our study there was an increased abundance of Aeromonadaceae when *C. sancticaroli* larvae were exposed to nylon microplastics alone, and when associated with the PBDEs, while Pseudomonadaceae were increased in the presence of microplastics. The increase in the relative abundance of Aeromonadaceae and Pseudomonadaceae in the presence of microplastics has also been observed in the earthworm *Metaphire guillelmi* exposed to polypropylene microplastics in soil (Cheng et al., 2021).

Furthermore, the abundance of these families has been associated with freshwater biofilms (Gong et al., 2019; Jiang et al., 2018). Increase of these taxa following microplastics exposure may point to bacteria from this group being preferentially associated with the surface of the added nylon materials that are then taken up into the gut via ingestion. A reduction in the abundance of Aeromonadaceae has also been noted in the gut of the land snails *Achatina fulica* as an effect of the ingestion of polystyrene microplastics indicating that the interactions of this taxon with microplastic and subsequent impacts on its presence in microbiomes may be species and context dependent (Song et al., 2020).

The greater presence of Pseudomonaceae in the larval microbiome may be directly linked to the capacity of this taxa to interact with the surface of the added nylon particles. *Pseudomonas* have been found to be enriched in sediments with high microplastic load. The increase of *Pseudomonas* in microplastic polluted sediment may be related to the capacity of this genus to degrade some polymer types, in addition to other complex substrates (Halpern et al., 2009; Ru et al., 2020; Senderovich and Halpern, 2013; Tu et al., 2021). Similarly, the abundance of Pseudomonadaceae in seawater and biofilms has been directly correlated with the presence of microplastics (Tu et al., 2020; Ye et al., 2021). Despite *Pseudomonas* having the ability to biodegrade PBDEs once they are a source of carbon and energy for bacteria development (Huang et al., 2012; Lv et al., 2016; Xin et al., 2014), in our study, *Pseudomonas* abundance was not significantly altered by the enrichment of substrates with PBDEs. As absolute concentration of added microplastic and PBDE concentrations differ greatly this may explain why microplastics, but not PBDEs seemingly had no effect on this genus.

An increase in the abundance of Acetobacteraceae was seen in the current studies for larvae exposed to microplastics. A similar enrichment of the bacterial microbiome has

556 also been observed in adult honey bees (*Apis mellifera* L.) following microplastic
557 exposure (Wang et al., 2021). The enrichment of these commensal bacteria, common in
558 the intestine of some insects, may be associated with their participation in immunity (Roh
559 et al., 2008; Ryu et al., 2008). Compounds such as neonicotinoids and hydrocarbons have
560 also been shown to increase the abundance of Acetobacteraceae and other families of
561 Rhodospirillales order in zebrafish *Danio rerio* (Luo et al., 2021) and soils, respectively
562 (Abbasian et al., 2016). This suggests that these taxa may be able to respond to, and
563 utilise, a range of different complex carbon sources, including potentially those associated
564 with added microplastics.

565 The increase in Microbacteriaceae (Actinomycetales) observed in larvae is
566 consistent with current knowledge, as Actinomycetales is part of the community of
567 bacteria previously observed to colonize microplastics (Wu et al., 2020). This evidence
568 of colonisation has now, however, always been associated with an increase in the
569 presence of these bacterial species in species microbiomes. For example, this family was
570 reduced in the gut of juvenile guppy *Poecilia reticulata* on exposure to MP (Huang et al.,
571 2020).

572 The high abundance of *Aquabacterium* (Burkholderiales: Comamonadaceae),
573 increased in the presence of microplastics, agrees with other reports that indicate the
574 importance of this genus in substrates where microplastics are present (Kelly et al., 2020;
575 McCormick et al., 2016; Ogonowski et al., 2018), including biofilms (Kalmbach et al.,
576 2000). The abundance of *Aquabacterium* in larvae of *Chironomus transvaalensis* has
577 been demonstrated to decrease following species exposure to other xenobiotics such as
578 metals (Laviad-Shitrit et al., 2021). Increases here, suggest a specific interaction with the
579 nylon polymer of microplastic surface that supports ingestion and retention in the
580 microbiome.

An enrichment of a nosocomial pathogen, *Chryseobacterium* (Flavobacteriales: Weeksellaceae), was observed in larvae exposed to sediment with microplastics. An increase in *Chryseobacterium* has also been shown previous studies of the bacterial communities associated with the surfaces of plastic (the "plastisphere") (Galafassi et al., 2021; Gong et al., 2019; Wu et al., 2019). Bacteria belonging to this genus have the potential to degrade plastics (Hou et al., 2021), and have also been linked to the biotransformation of PBDEs (Yu et al., 2019). However, enrichment of this taxon in the presence of plastics is not always the case. For example, this genus was depleted in larval zebrafish as effect of polyethylene microplastics exposure (Zhao et al., 2021).

Even though the genus *Lysinibacillus* (Bacillales: Planococcaceae) has been known as an effective biodegradation organism (Esmaeili et al., 2013; Jeon et al., 2021), in the current study its abundance was outstanding in larvae belonging to the treatment without microplastics. Despite the interaction of microplastics with PBDEs not having been statistically significant, the increase in the abundance of this genus may be associated with the presence of PBDEs in larvae due to its ability to degrade PBDEs, as previously described (Deng et al., 2011). Similarly, the greater abundance of *Acinetobacter* (Pseudomonadales: Moraxellaceae) observed in larvae of treatment without microplastics, could be related to the presence of PBDEs, due to its recognized ability to remove bromine from BDE-44 and BDE-153, as described in sediments (Pan et al., 2018; G. Wang et al., 2021); Other results, different from ours, have shown a high abundance of this genus in polypropylene microplastics (Kelly et al., 2021; Mughini-Gras et al., 2021; Tavşanoğlu et al., 2020).

This is the first time that bacteria belonging to Caulobacteraceae family and *Pedobacter* genus have been shown to respond to PBDE exposure. PBDEs. Although Caulobacteraceae belongs to the main families present in biofilms (Tu et al., 2020) and is

606 part of bacterial community that degrades plastics (Nguyen et al., 2021), our study
607 showed that its abundance was increased primary by the presence of PBDEs rather than
608 the addition of microplastics. Likewise, *Pedobacter* that has been associated with the
609 degradation of cellulose (López-Mondéjar et al., 2016) and polychlorinated biphenyls
610 (PCBs), compounds structurally similar to the PBDEs (Šrédlová et al., 2020), it is also
611 described for the first time associated with the presence of PBDEs.

612 The intermediate concentrations of PBDEs evaluated in this study showed
613 significant increase in the abundance of Shingobacteriales: *Chryseobacterium* and
614 Gaiellales: Gaiellaceae. The greater abundance of *Chryseobacterium* may be related to
615 its ability to degrade BDEs as mentioned by other authors (Shih et al., 2012; Yu et al.,
616 2019); and Gaiellaceae, which is commonly found in soils (Araujo et al., 2020; Luciana
617 and Milton, 2014), only its relationship with the presence of other environmental
618 pollutants such as bisphenols has been documented (Zaborowska et al., 2020).

619 Despite microplastics influencing the content of PBDEs in the sediment and in the
620 larvae, this was not apparent effect of this interaction on the structure of the. *C.*
621 *sancticaroli* larval bacterial microbiome. Independent effects of both microplastics and
622 PBDE exposures on larval bacterial community composition were found. Microplastics
623 have been widely reported to affect bacterial communities and this pattern was observed
624 here. It is also known that some PBDE congeners cause biochemical and molecular
625 alterations to species when present at sufficient concentrations (Palacio-Cortés et al.,
626 2017). However, here we only identified effects on bacterial abundance in larvae exposed
627 to one of the PBDE mixture concentrations (750 ng g^{-1}). This is, perhaps, surprising given
628 the high nominal concentrations to which the larvae were exposed, and the expectation
629 that microbiomes would shift in response to the presence of PBDEs.

5. Conclusions

The gut microbial community plays a key role in promoting the insect's survival, and therefore perturbations as a result of anthropogenic contaminants has the capacity to influence this. As far as we are aware, this is the first study presenting the effect of microplastics associated with PBDEs on bacterial communities in chironomid larvae. Our results provide novel insights into toxicological effects of the studied contaminants.

Microplastics were ingested by *C. sancticaroli* larvae and were retained within the body for > 168 h. However, this exposure had no effect on survival. Accumulation of PBDEs in *C. sancticaroli* tissue was significantly affected by microplastics, presumably because microplastic-PBDE interactions reduced PBDE bioavailability and, hence, uptake of PBDEs into the tissues. The accumulation of different congeners in the body tissues was affected by the presence of microplastics differently, with higher brominated (and thus more highly hydrophobic) congeners more significantly affected potentially due to stronger binding to microplastics.

Microplastics significantly affected microbiome community composition. However contrary to expectations, PBDEs had only minimal effects on the microbiome, both in the presence and absence of microplastics. As microplastics had a stronger effect on the microbiome, this suggests that the provision of new habitat (and potentially a resource substrate) was more important than any toxicological chemical effects of PBDEs on the structure of the chironomid larval microbiome.

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