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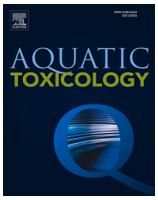

Effects of plasticizer Di(2-ethylhexyl) phthalate (DEHP) on the microbiome of the marine sponge *Hymeniacidon heliophila*

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ABSTRACT

Marine pollution research in the last 15 years focused on an emerging anthropogenic contaminant: plastic debris and more specifically, microplastics. Since, not only its physical impacts on marine invertebrates were studied, but also its additives. Phthalate, a plasticizer commonly found in the ocean and known endocrine disruptor was already observed in different aquatic invertebrates, but few is known about its presence and possible effects in Porifera physiology. Our study aimed to analyze potential shifts in *Hymeniacidon heliophila* (Desmosponge) microbiome after exposure to Di(2-ethylhexyl) phthalate (DEHP), the most common phthalate found in the ocean, in three different doses for 4 and 24 h. Results indicate that alpha diversity had significantly changed between control and exposed organisms but not in all multicomparisons. Microbial community structure changed after exposure as well although most abundant phyla did not vary along the experiment. The core microbiome between control and each exposed organisms contained the vast majority of total ASVs and a few ASVs were exclusive to each experimental group. After DEHP exposure, microbial classes had significant changes and species with phthalate degradation enzymes were identified in a specifically dose dependent manner pointing to a possible bacterial consortium responsible for the phthalate degradation. The bacterial detoxification activity may lead to *H. heliophila* resistance during DEHP exposure in polluted environmental conditions.

1. Introduction

Plastic pollution has been in the center of environmental research for the last 15 years (Iroegbu et al., 2021). Starting from the 1950′s a rising global production of polymers and a mismanagement of its disposal and recycling led to increasing accumulation of plastic in beaches and oceans worldwide (Iroegbu et al., 2021). Apart from the particulate and visual pollution, these materials can also carry chemical treats. During their fabrication, additives are incorporated into its structure to give different characteristics such as colors, UV and fire resistance and malleability (Iroegbu et al., 2021) These properties are necessary to allow their use in different all-day objects such as plastic wraps and innumerous products, from food packaging to domestic appliances (Fierens et al., 2012). Plastic can enter the ocean as macroplastics (plastic fragments larger than 5 mm) or microplastics (particles of less than 5 mm). Microplastics may arrive in the oceans mainly by two different pathways. Those called primary microplastics come from eventual losses during maritime transportation of raw industrial material known as pellets (Karlsson et al., 2018). The second pathway is by degradation of macroplastics already present in the ocean by the action of waves, sun, and other natural phenomena, forming then secondary microplastics.

Among the chemical additives that plastics receive during the manufacture, the most common are the phthalates. These compounds are part of a large chemical family, responsible for giving plastic polymers its malleability and found most in polyvinyl chloride (PVC) and polyethylene. They are known as endocrine disruptors (Chen et al., 2014) and can cause the down regulating of hormone production and affect the reproductive system of humans and other animals such as fishes (Chen et al., 2014), mussels (Gu et al., 2021), polychaetas (Lu et al., 2017), corals (Saliu et al. 2019) and others (Herrero et al., 2015; Marlatt et al., 2022). From all phthalates, the Di(2-ethylhexyl) phthalate (DEHP), was the first synthesized and used in plastic polymers from the 1950′s to the present, being still the most common additive nowadays. With the growth of marine plastic pollution in the last decades, DEHP has arrived in the oceans in increasing levels (Zhang et al., 2019), coming from the leaching of discarded plastics debris and is now an

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important marine chemical pollutant (Koelmans et al., 2013)

The problems of plastics and some of its additives in the oceans have been studied from some time now, mostly in vertebrate species (Ugwu et al., 2021). However, little information is available about the effects of chemical pollution on marine invertebrates, in special sponges (phylum Porifera). These animals are important filter-feeding organisms, with high filtering rates (35 ml.min−1.cm−3 of sponge. Weisz et al., 2008).

Their worldwide benthonic distribution and bioaccumulation capacities make them one good model organism as an indicator for pollutants such as microplastics and its additives (Celis- Hernández et al., 2021; Orani et al., 2022). Few studies tried to investigate the possible intake of microplastics and associated compounds by sponges (Fallon and Freeman, 2021; Giametti and Finelli, 2021). The results indicated the ingestion of microplastics, but also the presence and accumulation of plastic-derived chemical compounds in sponge tissues, with phthalates and specifically DEHP found in all samples analyzed. However, to the best of our knowledge, there are no studies addressing the effects that microplastics pollution and its chemical additives can have on the physiology of marine sponges.

Sponges are well known to live a symbiotic relationship with microbial communities, which can comprise around 40% of total body weight of some species (Vacelet and Donadey, 1977; Webster and Taylor, 2012). This association is quite specific, and the microbiota perform several important physiological roles for their host, such as digestion (Vacelet and Duport, 2004), the production of chemical defenses (Garate et al. 2015) or the detoxification of noxious compounds (Perez et al., 2002; Pita et al., 2018). The relations are finely regulated and even minor changes in the environment are reflected in the holobiont (Pita et al., 2018), Hardoim et al. in press)

Recent experiments in our research group showed that exposure to environmental concentrations of DEPH affect the sponge *Hymeniacidon heliophila* (Ascer et al., 2023, *in prep.*). This sponge is common in the Brazilian southwest coast, with large individuals growing in the intertidal zone close to urban areas. The sponge occurs [[and reproduces]] all year round, reaching a relatively higher biomass in summer. This species is long-lived (Batista et al., 2014. Environm Sci Poll Res 21:5785) and some individuals are being (re)collected at the same points for two decades at different times of the year (Custódio, pers. obs. The sponges are never removed entirely, and what is left can regrow). It was found that when exposed to DEHP the expansion-contraction cycle of the organism is altered, but the animal can recover the normal functions after few hours. In view of the role played by the microbiota in sponges, this study investigates how the *Hymeniacidon heliophila* microbiome respond to an acute exposure to DEHP, drawing possible answers to the sponge behavior to the chemical compound observed previously.

2. Material and methods

2.1. Sponge sampling and maintenance

Samples of the desmosponge *Hymeniacidon heliophila* Wilson, 1911 (Suberitida, Halichondriidae) were collected in the north coast of São Paulo state in the intertidal rocky shore of Praia do Araçá (23° 48′ 46.696″ S 45° 24′ 31.306″ W), São Sebastião (Brazil, southwestern Atlantic) in November 2019. The epibionts were removed with tweezers, the superficial sediment washed with seawater and the animals placed in 2 L glass flasks filled with seawater, placed in cooling boxes and transported (*c.* 30 min) to the laboratory at the Centro de Biologia Marinha (CEBIMar – University of São Paulo). In the laboratory, the samples were maintained in 80 L aquarium with continuous seawater flow. For field control, pieces from the inner part (choanosome) of the *H. heliophila* were preserved directly in RNAlater (QIAGEN) at 4 °C overnight and then transferred to −20 °C. All samples were transferred in 1 L glass flasks stored in cooling boxes (18 °C) and transported (*c.* 3 h) to the Laboratory of Cellular Biology of Aquatic Invertebrates - USP in São Paulo city and maintained in 80 L aquariums with biological filter until use

2.2. Experimental design and exposure to DEHP

Before exposure to DEHP, the individuals of *H. heliophila* were acclimatized for seven days in 80 L aquarium filled with seawater. The aquaria were maintained under natural dark-light cycles with uninterrupted aeration, biological filters and in a controlled temperature room, allowing stabilization of physical and chemical water parameters (pH 8.2 and 24 °C). During acclimation, each 80 L aquaria had eight different animals with three or four functional papillae each. For each experiment, nine different organisms were exposed to three different concentrations of DEHP (QUIMESP), hereafter called Low (0.9 µL/L), Medium (9.0 µL/L) and High (90 µl/L). The DEHP concentration in the environment can vary significantly depending on the location, from as low as 60.69 $ng.L^{-1}$ on the Tropical Western Pacific Ocean (Zhang et al., 2019), to 42.52 $\mu g.L^{-1}$ in Jiaozhou Bay (Xu et al. 2021). The ones used in our study were defined based on maximum DEHP environmental concentrations found in seawater (Martinnen et al. 2003) and proportional lower doses. All animals were maintained in 2 L aquaria with aeration at constant light and 24 °C, and only sponges with opened osculum and a visible perioscular membrane were used in the experiments. This ensured that the specimens were filtering, thus with the aquiferous system organized and fully functional. Three sponges were collected from each treatment after 4 h (T4) and 24 h (T24) of exposure and all 27 samples (six from each DEHP treatment, six from control and three from field control) were preserved directly in RNAlater (QIAGEN) at 4 °C overnight and transferred to −20 °C until the DNA extraction (Fig. 1). The exposure times were determined by preliminary experiments (data not shown) which showed that DEHP blocked sponge contractions during 2 to 8 h. As such, the aim of chosen times is to allow: 1) the microbiome observation during acute DEHP impact (4 h) and 2) enough time for microbiome stabilization (24 h). All seawater and animals used in the experiments were obtained from the same area, a touristic region with variable human presence and boat trafficking during the year. To the best of our knowledge no survey was conducted in the area to measure phthalate abundance and its variation due to seasons, weather and/or tides. All the animals and media used here are from the same area and exposed to the same environmental conditions. Thus, we assume that the data from experiments represent the effects of acute exposure to DEHP

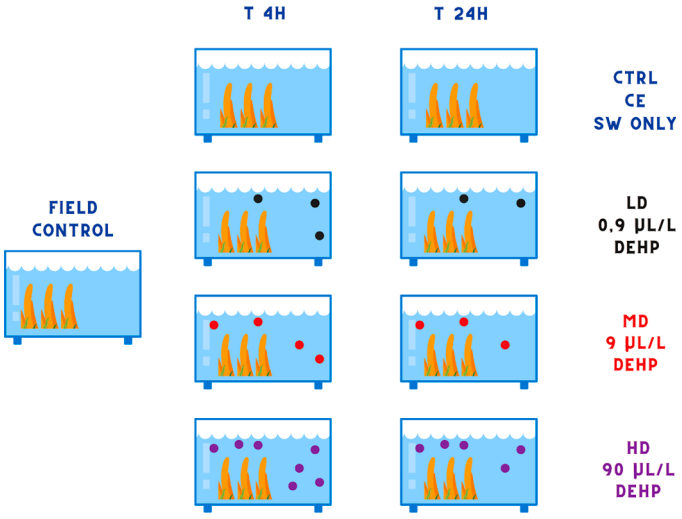

Fig. 1. DEHP exposure experimental design. Samples were named accordingly to the DEHP dose and time of sampling. CE: Control Experiment; SW: Seawater; LD, MD, HD: Low, Medium and High Dose Experiments, respectively; T4H: 4 h of exposure and T24H: 24 h of exposure.

2.3. DNA extraction

Total DNA from sponge's choanosome (0.25 g approximately) from all samples was extracted using the DNeasy PowerSoil DNA Isolation Kit (QIAGEN) following manufacturer's instructions.

2.4. 16S rDNA Illumina sequencing

The primer pair 515F-806R was used to amplify the V4-region of the 16S rRNA gene of Bacteria and Archaea using the extracted DNA (Apprill et al., 2015; Parada et al., 2016) and aliquots were sequenced at the NGS Soluções Genômicas (Piracicaba, SP. Brazil). The reaction mixture (25 μL) was prepared with 2.5 μL of template DNA (20 ng/μL), 0.20 nM of both primers and 2X PCRBio Ultra Mix (PCRBiosystems). The thermal cycle was set as following: 3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Final extension was performed for 10 min at 72 °C (Hardoim et al., 2021). The amplicons obtained were subjected to Illumina sequencing using MiSeq platform.

2.5. Analysis of sequencing data

2.5.1. Data processing

FastQC was used for the initial quality check of the sequences (Wingett and Andrews, 2018). Sequence data were then quality-filtered and trimmed using Trimmomatic version 0.36 (Bolger et al., 2014) when the average quality dropped below 25 in a sliding window of 4 bp. USEARCH version 11.0.667 (Edgar, 2013) was used to merge reads and quality-filter sequences, eliminating those with <230 or >300 nucleotides, with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into unique sequences (Amplicon Sequence Variants, ASV) using the UNOISE3 algorithm (Edgar, 2016) implemented in USEARCH. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME with the Genome Taxonomy Database (GTDB, https://gtdb.ecogenomic.org, Parks et al. 2020). The ASVs were classified using the BLCA algorithm against GTDB (Gao et al., 2017) and the Greengenes 13_5 taxonomy (McDonald et al., 2011). The latter was used to identify and remove mitochondria and chloroplast sequences. All 16S rRNA datasets generated in this study were deposited in the Sequence Read Archive at the National Centre for Biotechnology Information (NCBI, Altschul et al., 1997) under the accession number PRJNA1083510

2.5.2. Community metrics

The non-metric multidimensional scaling (nMDS) was used to detect patterns of Bray-Curtis dissimilarities in community structure at ASV-level using vegan package v. 2.5-6 (Oksanen et al., 2019; R Core Team, 2022). Permutational multivariate analysis of variance (PERMANOVA) was used to assess differences among all 27 samples. *P*-values of PERMANOVA were calculated using 10.000 permutations. Generalized linear models (GLM) were separately fitted to each ASV using negative binomial distribution with the R package mvabund (Wang et al., 2021), given the mean-variance relationship observed. The resulting sum of likelihood ratio statistics and statistical significance was evaluated with analysis of variance (ANOVA). After this initial assessment, replicates from the field control were distinct from any other sample in the dataset and thus they were removed from all further analysis. Barchart plots were generated for the 30 most abundant bacterial and archaeal phyla and the 20 most abundant classes.

An ASV table normalized to 2485 sequence reads per sample was used to determine the richness (CHAO and ACE), diversity (Shannon and inverse Simpson) and evenness (Pielou's evenness) metrics using the R package vegan v. 2.5–698 and a $p < 0.05$ was considered statistically significant. The multiple comparisons of means were performed with Tukey contrasts in the R package multicomp version 1.4-19 (Hothorn et al., 2008; R Core Team, 2022).

The Venn diagram was constructed to determine the number of ASVs specifically assigned to each treatment and time and common to all treatments and times using the online tool InteractiVenn (available in http://www.interactivenn.net/). Here all the comparisons were done between the control from the experiment and one treatment dose. In this study, the core microbiome was defined as ASVs present at the start and end of the experiment in 100% of control and treatment organisms analyzed.

3. Results

3.1. Analysis of the 16S rRNA gene

A total of 2,311,565 sequence reads for the V4-region of the 16S rRNA gene were acquired and examined with USEARCH-UNOISE3 including field control samples that were removed from the subsequent analyses after preliminary non-metric multidimensional scaling (nMDS) showed them as outliers. After quality control, removal of chimera, singletons, mitochondria and chloroplast, 661,781 16S rRNA gene sequences were assigned to 2498 ASVs and then further analyzed. All analysis were performed without field sample reads. For the alpha diversity, the dataset was rarefied resulting in a total of 475.362 sequences that were assigned to 2485 ASVs (Fig. 2).

3.2. Bacterial and archaeal alpha diversity

ANOVA's results from Shannon, inverse Simpson, CHAO, ACE and Pielou's evenness indexes were statistically significant (Fig. 3 Table SI 1). The highest CHAO richness and ACE index values observed was for MD4 (1791 ± 63 and 1813 ± 73, respectively), while lower values were observed for both indexes in CE4 group (1212 ± 224 and 1219 ± 252, respectively). Observed ASVs analysis showed higher values in the high dose experiment (HD4: 1491 ± 120 and HD24: 1421 ± 154) and lower ones after four hours of low and medium doses exposure (LD4: 297 ± 14 and MD4: 203 ± 41).

For Shannon diversity index, the higher and lower values were found in HD4 (5.1 ± 0.13) and CE24 (4.2 ± 0.16), respectively. Same results were observed for inverse Simpson and Pielou's evenness indexes, where HD4 had the highest value (38.8 ± 4.5 and 0.691 ± 0.011, respectively) and CE24 the lower one (19.5 ± 2.6 and 0.529 ± 0.025, respectively).

Multicomparison analysis of ANOVA's results (Table SI 2) indicated that for the low dose, differences were detected for Shannon index between LD24 and CE24, while for medium dose, statistically differences

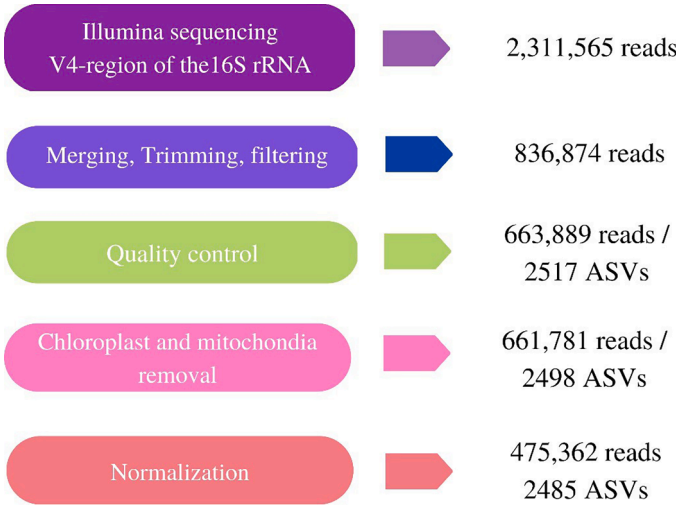

Fig. 2. Steps of the processing of the 16S rRNA gene analyses and the corresponding number of sequences reads and ASVs in each step.

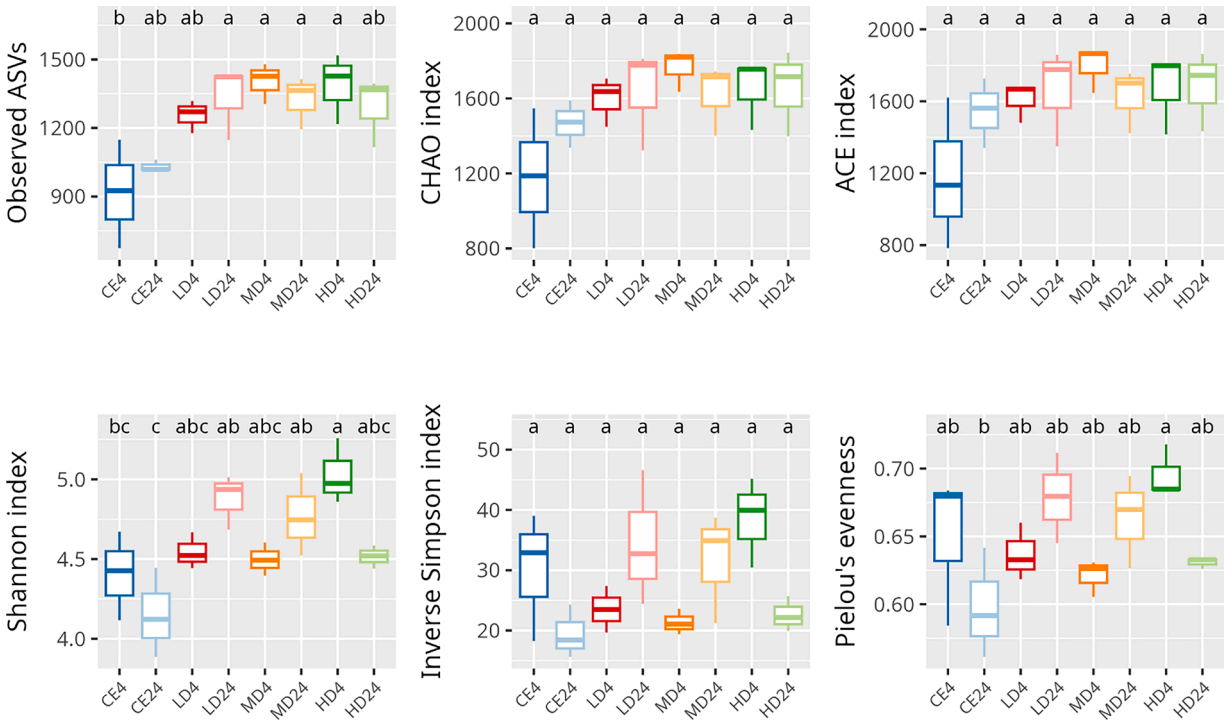

Fig. 3. Alpha-diversity indexes for all observed metrics.

were found in observed ASVs between MD24 and CE4, and between MD4 and CE4. While for high doses, statistically differences were observed in Shannon, inverse Simpson and Pielou's evenness indexes between HD4 and CE24, while between HD4 and CE4, only Shannon index was significant.

3.3. Comparison of community structure

Overall, 30 bacterial and archaeal phyla were detected among all samples (Fig 4A, Table 1). The most diverse community was present in CE24 and MD4 with 29 phyla each, followed by CE4, HD4 and MD24 with 28 phyla each. The LD24 had 27 phyla, and HD24 and LD4 had the lowest diverse community with 26 phyla. The most abundant phyla in all samples were *Proteobacteria* (60.7% in average), *Bacteria* (Unclassified, 17.9%), *Firmicutes* (9.31%) and *Bacteroidota* (6.5%) (Figs. 4A and 5).

At the class level, 65 were registered among replicates (Table 2). The most diverse community was found in MD24 samples with 64 classes, followed by HD4 with 62 classes, HD24 and LD24, each with 61 classes: LD4 with 59 classes, CE4 and MD4 each with 58 classes, and finally CE24 with 57 classes. Results were compiled for classes with average abundance over 0.2%. The most dominant classes in average were *Gammaproteobacteria* (36.7%), *Bacteroidia* (24.1%), *Alphaproteobacteria* (11.7%)

Bacteria (Unclassified, 10.1%), *Cyanobacteriia* (6.6%), *Actinomycetia* (1.7%),

Verrucomicrobiae (1.3%) and *Bacilli* (1%) (Fig.4B, Fig. 6 and Table 2)

The nMDS analysis based on Bray–Curtis distances revealed four different groups in each dose experiment. Always clustering together replicates from the same experimental group. For the three experiments, all CE24 replicates clustered together as well as CE24. LD4, LD24, MD4, MD24, HD4 and HD24 replicates followed the same pattern indicating that there were small differences between replicates and that each cluster was different from each other. CE24 replicates group was the one which differentiate the most from the others since it is the most distanced one in every experimental scenario. For the other three groups formed in each experiment, the ones exposed to DEHP (HD4, HD24, MD4, MD24, LD4 and LD24) were near to each other and to CE24, showing more similarities in microbiome composition (Fig. 7).

3.4. Specificities and commonalities: shared and exclusive ASVs

Exclusives and common ASV's from the three different experiments were analyzed by Venn diagram (Fig. 8). Results showed that the majority of ASVs were common between control and exposed organisms (from 1230 to 1262), while exclusive ASVs ranged from 17 to 87.

In the low dose, core ASVs between control and treated *H. heliophila* was composed by *Proteobacteria* (62.98%), with predominance of *Neptuniibacter pectenicola* (*Gammaproteobacteria*) and Firmicutes (10.4%) composed predominantly by *Bacillus_J thermoamylovorans* (*Bacilli*) (Table SI 3). Exclusives control ASVs abundance changed as well during experiment. At the start and end of the experiment results showed predominance of *Proteobacteria*, but the predominant class changed.

Gammaproteobacteria such as *Cronobacter sakazakii* and *Steroidobacterales Steroidobacter denitrificans* counted for almost 40% of total exclusive ASVs in CE4 while in CE24 *Alphaproteobacteria* from *Sphingomonadaceae* family *Altererythrobacter_E sp003610805* had almost the third of the phylum reads in CE24. Regarding exposed sponge microbiome, LD4 results indicate predominance of Proteobacteria (61.9%), especially *Alphaproteobacteria* such as *Arboricoccus pini* and *Geminicoccus roseus* (*Geminicoccales),* and *Cyanobacteria* (12%) with important presence of *Planktothricoides sp001276715*. At the end of the experiment, LD24 results still indicate predominance of *Proteobacteria* (30%) but *alpha- and gamma-proteobacteria* classes had similar abundance (12%) with important representation of *Gammaproteobacteria Cronobacter malonaticus* and *UBA5109 sp002414385*.

In medium as in low doses experiments (Table SI 4), microbiome core had as predominant ASVs, *Proteobacteria* with 78.7% of abundance, with *Alphaproteobacteria* such as *Novosphingobium sp002556635* representing 74% of total ASVs, followed by *Firmicutes* with 17.6% and predominance of *Anoxybacillus_B vitaminiphilus*. Exclusives ASVs from control experiment show differences between start and end of experiment: *Proteobacteria* counted for 54.3 and 41,30% respectively (with increase of *Gammaproteobacteria* in CE4 - *Cronobacter sakazakii* - and CE24 -

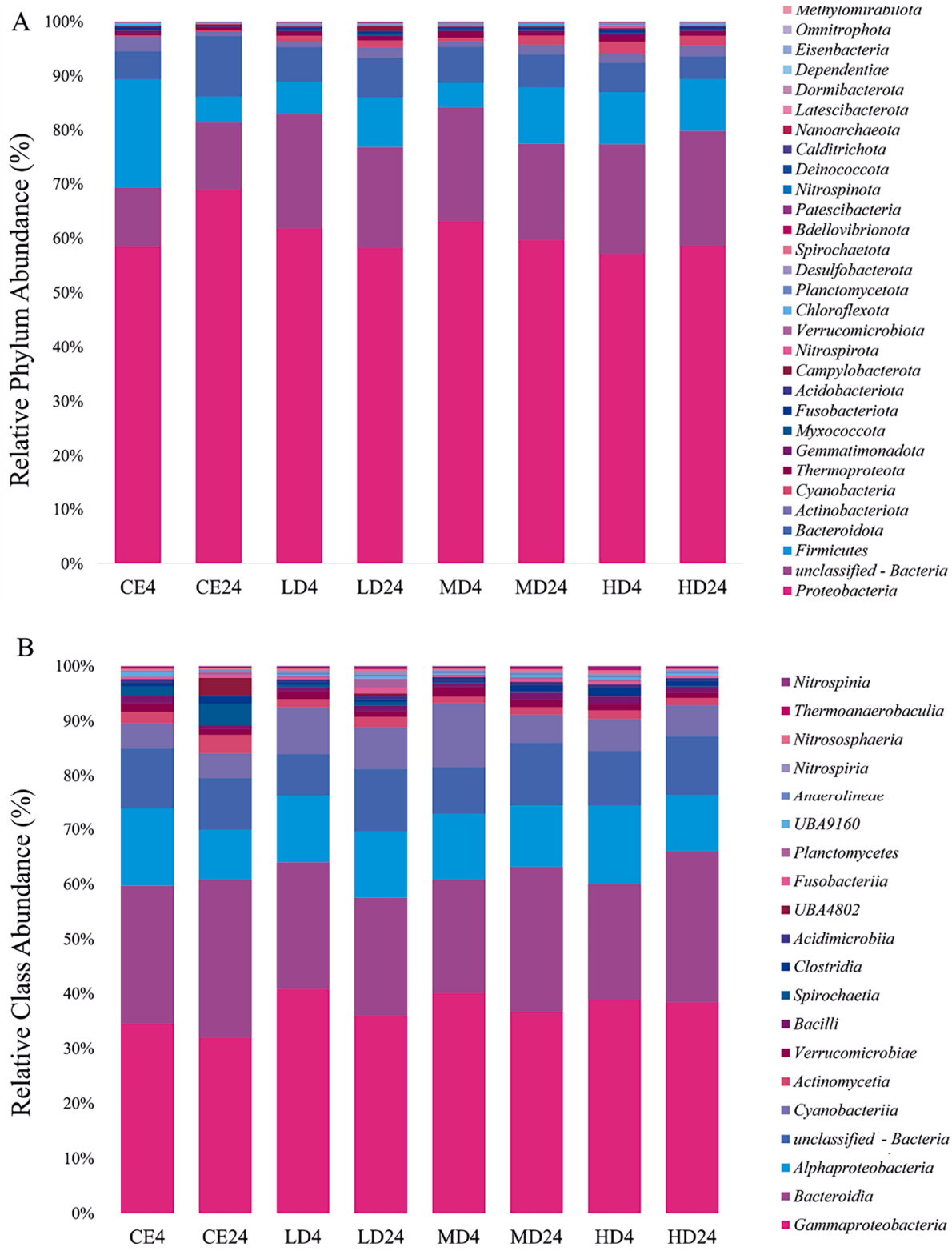

Fig. 4. Phylum- (A) and class-level (B) prokaryotic community composition associated with *Hymeniacidon heliophila* submitted to DEHP exposures. Control experiment at 4 h (CE4) and 24 h (CE24), low dose experiment at 4 (LD4) and 24 h (LD24), medium dose experiment at 4 (MD4) and 24 h (MD24), high dose experiment at 4 (HD4) and 24 h (HD24).

Bacterioplanes sanyensis). Interestingly Bacteriodota had an important grow in abundance after 24 h of exposure and *Aquimarina sp003443675* counted for 45% os all CE24 reads. Exclusives ASVs from DEHP treated sponges were dominated by *Proteobacteria* and *Cyanobacteria*. Sponges treated for 4 h with DEHP had more *Alpha-* than *Gamma- proteobacteria*, with *Inquilinus limosus_C* responsible for 21.6% of total ASVs in MD4. Meanwhile *Oleiphilus messinensis* was the most abundant at the end of experiment, representing 21% of total reads in MD24 and shifting the predominance from *Alpha-* to *Gamma-proteobacteria* at the end of the experiments.

Table 1
Phylum found in *H. heliophila* microbiome exposed to different concentrations of DEHP at start and end of experiment (%).

Phylum	CE4	CE24	LD4	LD24	MD4	MD24	HD4	HD24
Proteobacteria	58,59	68,9	61,81	58,17	63,15	59,65	57,13	58,66
unclassified - Bacteria	10,69	12,54	21,15	18,6	21,05	17,72	20,14	21,18
Firmicutes	20,11	4,76	5,95	9,34	4,55	10,57	9,74	9,62
Bacteroidota	5,14	11,16	6,38	7,27	6,6	6,02	5,4	4,1
Actinobacteriota	2,74	0,81	1,14	1,88	1,03	1,83	1,66	2,05
Cyanobacteria	0,22	0,29	0,98	1,29	0,72	1,69	2,29	1,81
Thermoproteota	0,3	0,44	0,63	0,65	1	0,49	0,98	0,63
Gemmatimonadota	0,59	0,17	0,28	0,31	0,26	0,4	0,38	0,37
Myxococcota	0,12	0,13	0,31	0,34	0,37	0,22	0,33	0,26
Fusobacteriota	0,45	0,1	0,16	0,24	0,1	0,25	0,31	0,27
Acidobacteriota	0,09	0,14	0,24	0,26	0,21	0,21	0,33	0,2
Campylobacterota	0,19	0,07	0,08	0,82	0,04	0,11	0,14	0,05
Nitrospirota	0,08	0,06	0,23	0,14	0,21	0,08	0,28	0,14
Verrucomicrobiota	0,09	0,07	0,17	0,13	0,2	0,14	0,2	0,13
Chloroflexota	0,28	0,1	0,09	0,09	0,08	0,18	0,11	0,11
Planctomycetota	0,05	0,04	0,11	0,15	0,14	0,18	0,19	0,14
Desulfobacterota	0,07	0,03	0,07	0,08	0,1	0,07	0,1	0,08
Spirochaetota	0,06	0,06	0,04	0,03	0,02	0,06	0,08	0,09
Bdellovibrionota	0,03	0,02	0,04	0,06	0,06	0,03	0,03	0,02
Patescibacteria	0,03	0,04	0,06	0,04	0,01	0,03	0,04	0,01
Nitrospinota	0,01	0,02	0,03	0,03	0,05	0,02	0,06	0,03
Deinococcota	0,02	0,01	0,02	0,03	0,02	0,03	0,03	0,03
Calditrichota	0	0	0	0,02	0	0,01	0,01	0,01
Nanoarchaeota	0,01	0,01	0,01	0,01	0,01	0	0,01	0
Latescibacterota	0	0	0,01	0	0,01	0,01	0,01	0
Dormibacterota	0	0	0	0,02	0	0	0	0,01
Dependentiae	0	0	0	0	0	0,01	0,01	0
Eisenbacteria	0	0,01	0	0,01	0	0	0	0
Omnitrophota	0	0	0	0	0	0	0,02	0
Methylomirabilota	0,01	0	0	0	0	0	0	0

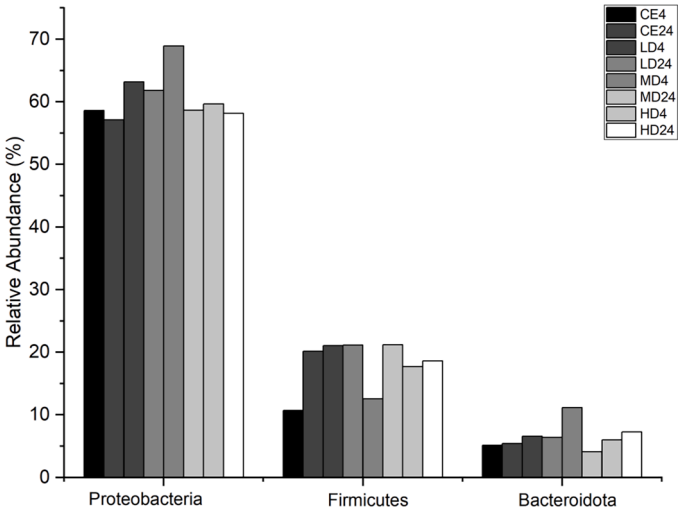

Fig. 5. Relative abundance of dominant phyla for control and exposed groups. Control experiment at 4 h (CE4) and 24 h (CE24), low dose experiment at 4 (LD4) and 24 h (LD24), medium dose experiment at 4 (MD4) and 24 h (MD24), high dose experiment at 4 (HD4) and 24 h (HD24).

Differently of both low and medium doses, the high dose experiment had changes in bacteria phylum abundance for the core microbiome and exclusives (Tab SI 5) during the 24 h of experiment: In core microbiome, *Actinobacteria* ASVs were more abundant (69.1%), where *Ilumatobacter_A coccineus* and *Desertimonas flava* (*Acidimicrobiales*) were dominant followed by *Proteobacteria* (25.6%) represented mainly by *Alphaproteobacteria* from *Rhodospirillales* (*Haematospirillum jordaniae*) and *Rhodobacterales* (*Roseovarius indicus*) classes. As for exclusive ASVs, the control results showed that initially sponges had more *Proteobacteria* (48,26%) especially *Gammaproteobacteria* such *Enterobacterales* (*Cronobacter sakazakii*) and *Steroidobacterales* (*Steroidobacter denitrificans*) than *Bacteroidota* (22.9%). However, at the end of the experiment the order changed and *Bacteroidota* was predominant (46.7%) with the presence predominantly of *Bacteroidia* class type *(UBA9320 sp002746305*) while *Proteobacteria* counted for 23% of exclusive ASVs. For treated sponges, initially *Proteobacteria* was predominant (61%) specially the *Gammaproteobacteria Pelagibaculum spongiae,* followed by *Bacteroidota* species such as *Cytophagales BBD-1991-12 sp001657315*. At the end of the high dose experiments, *Proteobacteria* continued to be the most abundant, especially *Gammaproteobacteria Cronobacter malonaticus*, but *Bacteriodota* phylum was replaced by Cyanobacteria (16%) with predominance of *Crocosphaera sp002172675*. If core microbiomes from control and exposed groups are compared, we can observe that minor representatives' phyla are lost after exposure. Phyla such *Bdellovibrionota, Calditrichota*, and *Campylobacterota* are not represented in core of exposed organisms (Table 3, Fig.9)

4. Discussion

Marine invertebrates are known to host a highly diverse and unique set of microbionts, normally distinct to those found in their surrounding environment. These unique microbiome-host systems, or holobionts, are being studied to understand microbial functions in protection, survival, and fitness of different marine species such as crustaceans, corals and sponges (Bourne et al., 2009; Taylor et al., 2007; Yang et al., 2016) in a constantly changing habitat.

To the best of our knowledge, our results are the first one showing the dose- dependent impact of plastic additives in a sponge's microbiome. *Hymeniacidon heliophila* is a common sponge found mostly in intertidal rocky shores of polluted regions from the coast of Florida (USA) to southeast coast of Brazil. For its habitat preferences, *H. heliophila* is constantly exposed to anthropogenic pollutants such as heavy metals, persistent organic pollutants and most recently, plastic and its additives (Giametti and Finelli, 2021). Recently, sponges had been defined as an important anthropogenic pollution bioindicator, specially microplastics (Girard et al., 2021) and plastic additives had been quantified in sponges' tissues (Giametti and Finelli, 2021)

Table 2
Twenty most abundant classes found in *H. heliophila* microbiome exposed to differe concentration of DEHP at start and end of experiment (%).

Class	CE4	CE24	LD4	LD24	MD4	MD24	HD4	HD24
Gammaproteobacteria	34,1	31,8	40,3	35,4	39,6	36,2	38,2	38
Bacteroidia	25	28,7	22,9	21,3	20,5	26	20,9	27,3
Alphaproteobacteria	13,9	9	12	11,9	11,8	11	14,1	10,2
unclassified - Bacteria	11,1	9,6	7,7	11,5	8,6	11,5	10	10,7
Cyanobacteriia	4,5	4,5	8,4	7,5	11,5	5,1	5,7	5,6
Actinomycetia	2,1	3,3	1,4	1,9	1,1	1,3	1,5	1,3
Verrucomicrobiae	1,6	1,1	1,5	1	1,7	1,3	1,1	1
Bacilli	1,3	0,7	0,8	1	0,8	1,2	1,4	1,1
Spirochaetia	1,7	3,9	0,4	0,6	0,1	0,3	0,2	0,1
Clostridia	0,6	1,2	0,4	0,5	0,2	1	1,5	0,8
Acidimicrobiia	0,6	0,2	0,5	0,6	0,7	0,6	0,6	0,6
UBA4802	0	3,2	0	0,5	0	0	0	0
Fusobacteriia	0,3	0,6	0,3	1	0,2	0,5	0,5	0,3
Planctomycetes	0,2	0,5	0,3	1,6	0,3	0,3	0,3	0,2
UBA9160	0,7	0,2	0,2	0,4	0,2	0,4	0,4	0,3
Anaerolineae	0,2	0,3	0,4	0,4	0,4	0,3	0,4	0,4
Nitrospiria	0,2	0,1	0,4	0,7	0,2	0,4	0,4	0,3
Nitrososphaeria	0,3	0,3	0,3	0,3	0,3	0,3	0,6	0,2
Thermoanaerobaculia	0,4	0,2	0,2	0,3	0,3	0,4	0,3	0,3
Nitrospinia	0,1	0,1	0,3	0,2	0,2	0,2	0,5	0,2

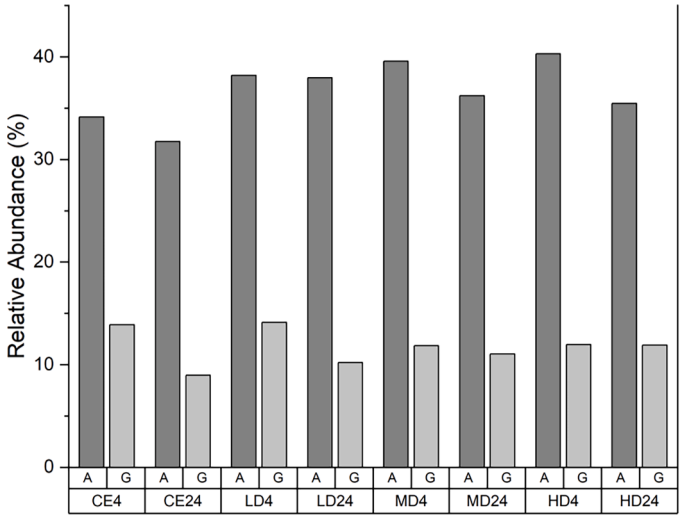

Fig. 6. Relative abundance of *alpha- and gamma-proteobacteria* in control and exposed groups. Control experiment at 4 h (CE4) and 24 h (CE24), low dose experiment at 4 (LD4) and 24 h (LD24), medium dose experiment at 4 (MD4) and 24 h (MD24), high dose experiment at 4 (HD4) and 24 h (HD24). A: *Alphaproteobacteria*, G: *Gammaproteobacteria*.

highlighting the importance of studying its effects in these animals. Sponges were exposed to three different concentrations of DEHP, a commonly used plastic additive often detected in seawater, and sampled after initial exposure. The results showed that all alpha diversity indexes were different, indicating that richness and diversity of the prokaryotic communities responded to the pollutant exposure. Richness indexes such as ACE and CHAO followed the same patterns of distribution, showing that exposure to DEHP increased the number of species in all groups already after 4 h of exposure. These levels did not seem to vary between 4 and 24 h of exposure, even though indexes were higher compared with the start of the experiment. Slightly increase in diversity was observed with Shannon index, especially between control and the high dose groups. *Hymeniacidon heliophila* microbiome had an increase in diversity after DEHP exposure but it did not change during the following 24 h. These changes in the microbiome after DEHP exposure were observed in vertebrate organisms such as the Asian carp species *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* (Kolb et al., 2019), the zebrafish *Danio rerio* (Jin et al., 2018a) and especially in humans (Claus et al., 2016). In general, the exposure led to dysbiosis and positive or negative effects on different diversity indexes, such as CHAO, ACE, and Shannon (Varg et al., 2022). The variation at phylum- and class-levels was performed to elucidate the shifts in the H. heliophila microbiome after DEHP exposure and whether these were beneficial or not to the sponge. The results about community structure indicated that among 30 microbial phyla, *Proteobacteria, Firmicutes* and *Bacteroidota* were dominant, which is in accordance with the literature (Pita et al., 2018). *Proteobacteria* is the most abundant phylum found in symbiosis with Porifera (Bibi et al., 2020; Lo Giudice and Rizzo, 2018; Pita et al., 2018; Nascimento-Silva et al., 2023) and is known to perform different beneficial function to the hosts such as nitrogen fixation (Wilkinson and Fay, 1979) and defense mechanisms (Berde et al. 2024). This phylum abundance had increased during the experiment in control and low doses of DEHP. As the variation was observed in the control group, total Proteobacteria increase may be due to changes associated to the aquarium conditions. These changes were already observed in other aquarium species and seem to be linked to changes in nutrient availability and/or light exposure (Webster and Taylor, 2012). Nevertheless, at class level, *Alpha- and Gamma-proteobacteria* were the most abundant ones in our results. In all experimental groups *Alphaproteobacteria* were less abundant than *Gammaproteobacteria*. When variation of abundance is analyzed, in control and low doses, both classes decreased between 4 and 24 h of exposure, indicating that the smaller concentration of DEHP (0,9 μL/L) did not affect the abundance of *Alphaproteobacteria* in *H. heliophila*. These results corroborate the lack of effects of the lower dose in the contraction pattern of this species observed by Ascer et al. if, as such they hypothesize, microbiome might be able to degrade the DEHP and thus influence the recovery of contraction after the exposure to toxic compounds.

In medium and high doses, even if *Gammaproteobacteria* do apparently have the same decrease as the other experimental groups, *Alphaproteobacteria* maintained the abundance at 4 and 24 h of exposure. The *Alphaproteobacteria* families *Rhodobacteraceae* and *Enterobacterales*, found enriched in medium and lower doses are known to be initial colonizers of plastic debris in the ocean (Roager and Sonnenschein, 2019) and can degrade phthalates (Boll et al., 2020a; Lamraoui et al., 2020). Bacteria belonging to the genus *Cronobacter*, present in all sponges exposed to medium and high doses, were recently described as responsible for DEHP degradation after isolation from polluted soil in Algeria (Lamraoui et al., 2020). The maintenance in the abundance of *Alphaproteobacteria* and the enrichment of *Cronobacter spp*. in samples after 4 and 24 h of exposure to medium and high doses of DEHP indicates that this group may play an important role in *H. heliophila*

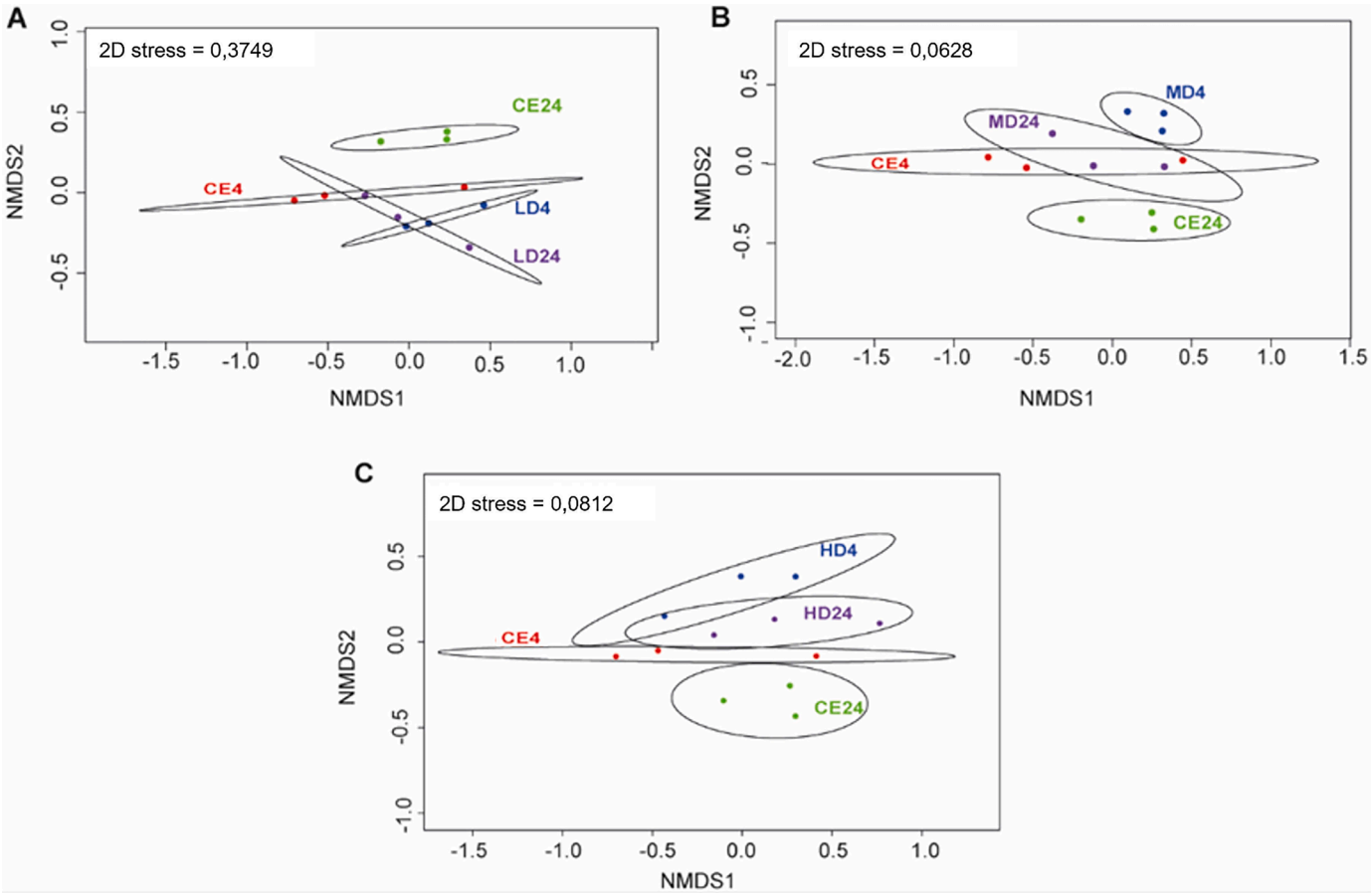

Fig. 7. Non-metric multidimensional scaling (nMDS) plots. nMDS based on Bray–Curtis distances calculated from the normalized ASV table for each treatment against control sponges (A) LD, (B) MD and (C) HD. Control experiment at 4 h (CE4) and 24 h (CE24), low dose experiment at 4 (LD4) and 24 h (LD24), medium dose experiment at 4 (MD4) and 24 h (MD24), high dose experiment at 4 (HD4) and 24 h (HD24).

survival. It is possible that these bacteria (and others) might be degrading the compound that act as a disruptor of the sponge contraction (Ascer et al. 2023b), thus enabling the recovery of the host.

The second most abundant group in the holobiome was *Firmicutes*, and all exposed sponges showed higher abundance of this phylum compared to the controls. Even if this phylum may be enriched in exposed groups, the variation between 4 and 24 h in low dose samples followed control patterns and an increased abundance was observed. However, this pattern changed in medium and high doses, and *Firmicutes* abundance decreased during the same period. Increase in *Firmicutes* after DEHP exposure may be linked to the number of *Bacillus spp*. a bacterium genus also known do degrade phthalate such as *B. subtilis* (Quan et al., 2005), *B. aquimaris* (Aromolaran et al., 2020) and *B. thuringiensis* (Surhio et al., 2014), among others. Their presence seems to be important at the initial 4 h exposure, when their abundance increase. After 4 h until 24 h we observed a decrease in *Firmicutes* indicating that their capacity of degradation was possibly surpassed by other bacteria such as the *Alphaproteobacteria.Bacteroidota* showed a marked decrease when sponges were initially exposed to DEHP. Nevertheless after 24 h of exposure, its abundance increased again in the medium and high doses groups. This phylum was already described as a phthalate decomposer under aerobic conditions (Zhu et al., 2020) and our results indicated that DEHP might trigger *Bacteroidota* species to degrade phthalate after 4 h of exposure.

As the phylum abundance distributions indicated, the lower dose used in this experiment was not enough to make important shifts in the microbiome. There is a significant overlap between LD4 and LD24 communities and both control microbiome. Yet, differences in exposed and control increased in a dose-dependent manner. In medium doses, microbial symbionts in exposed sponges revealed fewer similarities with the control and between 4 and 24 h of exposure. Only MD24 and CE24 showed community overlap. For the highest dose, the nMDs plot indicated no overlap of procaryotic communities between control and exposed groups. This indicates that even if environmental conditions (*viz.* aquaria) were able to change the microbial community in control group, these changes were not the same as the ones elicited by the exposure to phthalate.

Alterations in microbial structure are commonly observed in different aquatic invertebrates exposed to known toxic compounds. The crab *Eriocheir sinensis* exposed to the pesticide imidacloprid caused changes in microbiota in a dose dependent manner, leading to loss of important functions played by the symbionts in the host such as lipid metabolism, digestion and immune defenses (Hong et al., 2020). Exposure to per- and poly-fluoroalkyl substances (PFAS) leaded to significant changes in the microbiome of Manila's clam (*Ruditapes philippinarum*) (Bernardini et al., 2021) The same effects can be observed with plasticizers. A recent review indicates that dose-dependent microbiome response to phthalate can vary among species (Joshi and Bhatt, 2023). However, these studies use predominantly vertebrates, especially mammals. The lack of investigations on the effects of this pollutant exposure to aquatic invertebrate's microbiome does not allow further conclusions without specific establishing each bacteria function.

When analyzing exclusive or core microbiomes, the similarities between controls and exposed animals at 4 and 24 h include ASVs common to all groups. The core microbiome between control and low dose encompassed 1230 ASVs, while between control and medium, and control and high doses contained 1262 and 1237 ASVs, respectively, accounting for almost half of all ASVs described in the dataset. In

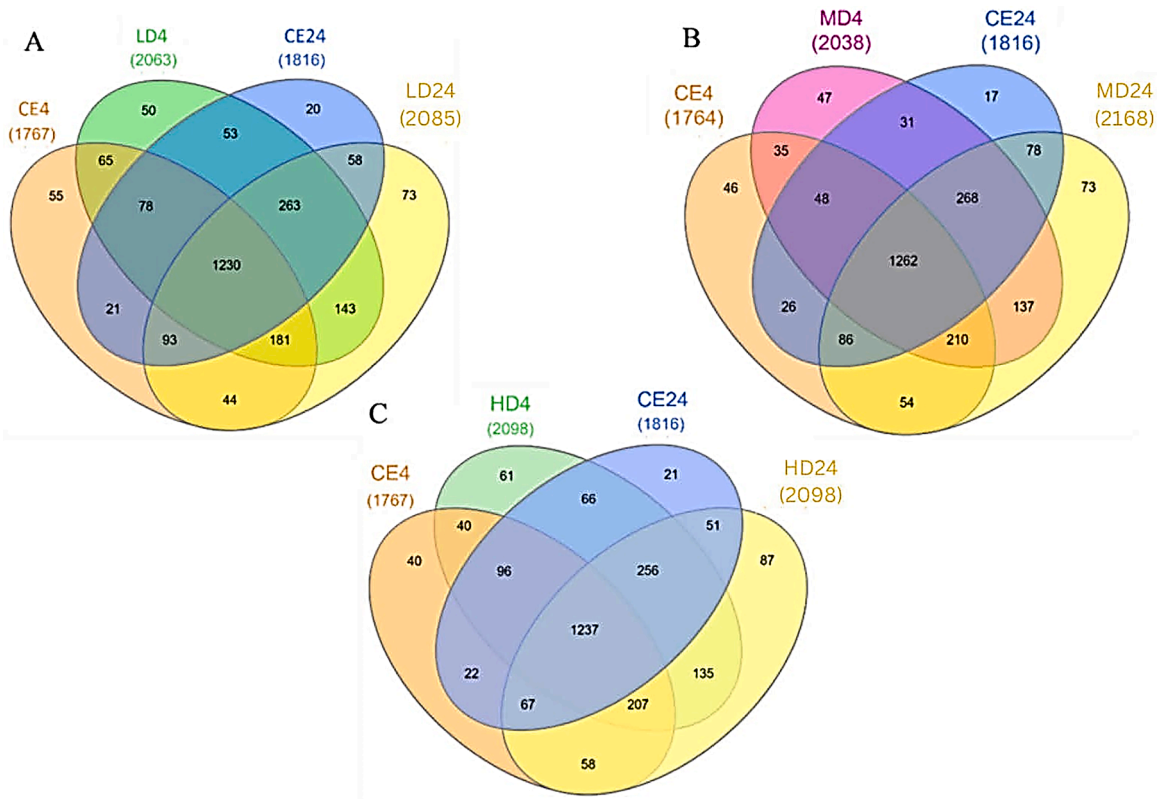

Fig. 8. Venn diagrams for **A** - LD against Control Experiment, **B** MD against Control Experiment and **C** – HD against Control Experiment. Numbers in the graphs indicate how many ASVs were found in each group. Control experiment at 4 h (CE4) and 24 h (CE24), low dose experiment at 4 (LD4) and 24 h (LD24), medium dose experiment at 4 (MD4) and 24 h (MD24), high dose experiment at 4 (HD4) and 24 h (HD24).

Table 3
Class relative abundance in Control and Exposed ASVs cores. *Proteobacteria*, the most abundant one, was represented by its two major classes in bold (%).

Phylum	Control	Exposed
Acidobacteriota	1,47631068	0
Actinobacteriota	2,912621359	1,62601626
Bacteroidota	14,0776699	7,317073171
Bdellovibrionota	0,485436893	0
Calditrichota	0,485436893	0
Campylobacterota	0,970873786	0
Cyanobacteria	3,398058252	0
Desulfobacterota	0,485436893	0
Firmicutes	4,368932039	7,317073171
Fusobacteriota	2,912621359	0
Gemmatimonadota	1,45631068	0,81300813
Myxococcota_A	0,485436893	0
Nitrospirota	0,485436893	0
Planctomycetota	0,485436893	0
Alphaproteobacteria	**19,90291262**	**37,39837398**
Gammaproteobacteria	**44,66019417**	**45,52845528**

general, the majority of core ASVs from the three doses belonged to *Proteobacteria*, an expected result since the majority of the sponge microbiome belongs to this phylum. The notable exception comes from the high dose, in which the core ASVs are mainly from the *Actinobacteriota.* Members of this phylum were found in landfill leachates after phthalate addition, but its abundance decreased after higher doses were added (Huang et al., 2021). This showed that *Actinobacteriota* are sensible to high doses of phthalates and might explain why they were not observed exclusively associated with exposed animals in all doses used in this study.

Analysis of exclusive ASVs from exposed groups could help identify possible phyla and classes of the sponge microbiota able to perform phthalate detoxification during *H. heliophila* exposure. In all treatments, *Proteobacteria* was predominant in exclusive ASVs over other phyla. If considered exclusive ASVs from low and medium doses we can observe that in both, after 4 h of DEHP exposure, *Bacteroidota* was replaced by *Cyanobacteria*. According to KEGG database (Kanehisa and Goto, 2000), *Planktothricoides* sp001276715 and *Crocosphaera sp002172675* present in low and medium doses, respectively, have enzymes able to degrade phthalate, such as dehydrogenase, deoxigenase, esterases, hydrolases, decarboxylases, and CoA ligases, which could help*H. heliophila* to initially deal with the compound. After 24 h of exposure, in low and medium doses, *Cyanobacteria* was still present and enriched (from 10 to 20% in average) but the predominance of species changed, with *Pleurocapsa* spp. becoming more abundant. Also, according to KEGG, this specie has pathways to metabolize polycyclic aromatic hydrocarbons as the other bacteria noted before, and possibly is involved in a second phase of DEHP degradation. When analyzing the high dose, we observed that after 4 h of exposure, there is no evidence of *Cyanobacteria* species among the most abundant ones, but *Bacteroidota* are present and accounting for almost 8% of total exclusive ASVs. Two different species were mostly detected: *Cytophagales* and *Arenibacter,* both with phthalate degradation pathways metabolisms according to KEGG. *Arenibacter* had been observed in floating plastic debris enriched specially in low density polyethylene (LDPE) and polyethylene terephthalate (PET) polymers and is considered potential player in plastic degradation (Delacuvellerie et al., 2019). However, *Cytophagales* were not described with plastic or additives degradation capacities but is known to be able to grow in contaminated regions (Labadie et al., 2021). The most abundant ASVs at the end of the high dose experiment did not contain *Bacteroidota.* Instead, *Cyanobacteria* replaced it as the second most abundant phylum with special participation of *Crocosphaera* sp. once again and *Myxosarcina* spp. The last one might be a novelty regarding its degradation capacities since there are no comprehensive studies about it.

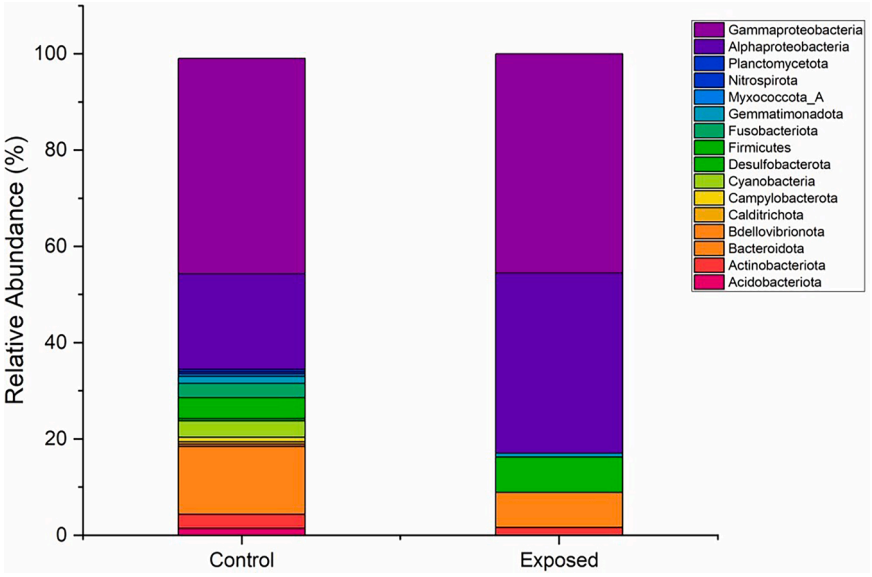

Fig. 9. Changes in core microbiome phyla abundance during DEHP exposure.

Most bacteria found enriched after 4 and 24 h of DEHP exposure not belonging to Proteobacteria had some level of aromatic hydrocarbon degradation tools. This suggests the possibility that a bacterial consortium maintained by the sponge to detoxify the phthalate and other toxic compounds could revert the effects in its physiology as observed before (Ascer et al. 2023, *in prep*).

Results of core bacteria among all samples indicated that, from 2485 ASVs obtained, 206 identified ASVs were considered control core microbiome, with 100% of presence in all control samples. The principal phyla present in this control core microbiome were *Proteobacteria*, followed by *Bacteroidota* and *Firmicutes*. Other ASVs detected were composed by minor phyla. When exposed groups were considered for core microbiome, major phyla abundance was retrieved (*Proteobacteria* 82.9 %, *Bacteroidota* and *Firmicutes*, 7.3%, each one). Surprisingly in exposed core microbiome no evidence of less represented control core microbiome phyla was detected. Results indicate that phthalate exposure had an important impact in exposed core microbiome, reducing minor represented phyla diversity. Nevertheless, when all 24 samples were analyzed, the core microbiome contained 135 ASVs especially those with high relative abundance. Shifts in microbiome after microplastic exposure were observed in a few recent studies. Microplastic particles were able to cause dysbiosis in zebrafish intestinal microbiome (Jin et al., 2018b; Qiao et al., 2019) and in its larvae (Wan et al., 2019).

5. Conclusion

Our results indicate that there is a possible bacteria consortium composed by exclusive and core ASVs, mostly from *Proteobacteria, Cyanobacteria* and *Bacteroidota*. These are already present in *H. heliophila* microbiome, and could act differentially, depending on the concentration and time of exposure to DEHP. The mechanisms of bacterial degradation of phthalates are well studied and can be performed in aerobic and anaerobic conditions, with the activation of dioxygenases, decarboxylases, and dehydrogenases (Boll et al., 2020b) and these enzymes are already detected in the bacterial species found here. Each one of those enzymes participate in a different step of phthalate degradation, highlighting the importance of different bacterial strains to allow the sponge to survive to high doses of phthalate exposure. As observed before by Ascer et al., *H. heliophila* contraction pattern is arrested after exposure to medium and high doses of DEHP but was able to recover contraction after a variable amount of time. This recovery may be linked to the stable presence of the 135 ASVs in 100% of *H. heliophila* exposed and control samples. Although the sponge core microbiome was more diversified before DEHP exposure, minor abundance phyla were not maintained after the contact with the toxic compound. This evidence a probable microbial selection, prioritizing those phyla which presence will help the sponge maintain its vital physiological functions, while exclusives ASVs will help degrade DEHP. The lack of studies of the effects of plastic additives (or any other pollutant) in sponge tissues and microbiome prevent other conclusions based on the available literature. Longer exposure times and metagenomics analysis could indicate further metabolic and detoxification features in *H. heliophila* microbiome during DEHP exposure. Future analysis may shine a light over specific functions of described exclusive bacteria. By species enrichment quantification, isolation, and degradation tests of each one described in this study will allow to recreate the activity succession of the bacterial consortium during phthalate degradation.

Permits

Sampling was performed under the scientific collection permits A097B99 issued by Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, 61460–2 issued by Sistema de Autorização e Informação sobre Biodiversidade do Instituto Chico Mendes de Conservação da Biodiversidade, both from Ministry of the Environment and 260108–001.161/2013 issued by Instituto Florestal, Secretaria do Meio Ambiente do Estado de São Paulo.

CRediT authorship contribution statement

Liv Goldstein Ascer: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Data curation. **Gabriel Nascimento-Silva:** Writing – review & editing, Visualization, Validation, Software. **Cristiane Cassiolato Pires Hardoim:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Márcio Reis Custódio:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107025.

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