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ABSTRACT

Oceanic plastic pollution is of major concern to marine organisms, especially filter feeders. However, limited is known about the toxic effects of the weathered microplastics instead of the pristine ones. This study evaluates the effects of weathered polystyrene microplastic on a filter-feeder amphioxus under starvation conditions via its exposure to the microplastics previously deployed in the natural seawater allowing for the development of a mature biofilm (so-called plastisphere). The study focused on the integration of physiological, histological, biochemical, molecular, and microbiota impacts on amphioxus. Overall, specific alterations in gene expression of marker genes were observed to be associated with oxidative stresses and immune systems. Negligible impacts were observed on antioxidant biochemical activities and gut microbiota of amphioxus, while we highlighted the potential transfer of 12 bacterial taxa from the plastisphere to the amphioxus gut microbiota. Moreover, the classical perturbation of body shape detected in control animals under starvation conditions (a slim and curved body) but not for amphioxus exposed to microplastic, indicates that the microorganisms colonizing plastics could serve as a nutrient source for this filter-feeder, commitment with the elevated proportions of goblet cell-like structures after the microplastic exposure. The multidisciplinary approach developed in this study underlined the trait of microplastics that acted as vectors for transporting microorganisms from the plastisphere to amphioxus.

1. Introduction

Microplastics (<5 mm, MP) entering the ocean from rivers are estimated to be several million metric tons per year (Weiss et al., 2021), resulting in several thousand billion pieces in the world upper ocean (Isobe et al., 2021). The highest MP accumulation were particularly observed for surface waters of the semi-enclosed Mediterranean sea and subtropical gyres (Eriksen et al., 2014; Cózar et al., 2015). The sediments of littoral and subtidal zones, as well as deep sea ocean are also contaminated by MP, with decreasing concentrations when moving away from the shore (Harris, 2020). The MP concentrations vary from 0 to 27 pieces per kilogram of sediment (MP/kg) in the Baltic Sea (Graca et al., 2017) to 72–116 MP/kg in the North Sea (Claessens et al., 2011), and reach 160–380 MP/kg in the Mediterranean Sea (Abidli et al., 2018).

A large number of evidence of adverse biological impacts caused by MP pollution were identified in recent years (Wright et al., 2013; Wesch et al., 2016). This includes (i) plastic ingestion and gut blockage leading to starvation, (ii) the release of toxic additives, (iii) disturbances in energy metabolism, (iv) the induction of inflammation, (v) introduction of

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pathogenic agents colonizing plastic (Anbumani and Kakkar, 2018; Bowley et al., 2021; Heinlaan et al., 2020). The MP exposure is considerable for the suspensivorous filter-feeding species since they filter large volume of water (Galgani, 2013). Most of the studies so far focused on mussels and oysters due to their exploitation in aquaculture, and their use in pollution survey programs (Goldberg et al., 1978). For example, *Mytilus edulis* and *Crassostrea gigas* were able to ingest and retain MP, with 0.37 MP/g and 0.47 MP/g found in their tissues respectively, leading to direct effects on their physiology depending on the size and shape of the microplastics (Van Cauwenberghe and Janssen, 2014). Indeed, *Mytilus edulis* suffered from toxic effects when exposed to MP at both the molecular (Green et al., 2019) and cellular levels (Moos et al., 2012). Furthermore, a DNA microarray study recorded a negative impact of MP on *Mytilus galloprovincialis* gene expression (Avio et al., 2015). In a recent review, either neutral or negative effects were observed (but never positive) on fish and aquatic invertebrates, with the strongest effects observed on lower trophic level organisms that serve as important linchpins for food web structure (Foley et al., 2018).

So far, except for some studies on mussels and corals (Chapron et al., 2018; Li et al., 2020), almost all the toxicity tests were carried out with pristine MP that were never exposed to the marine environment, which could lead to divergences compared to natural conditions. Indeed, a plastic piece entering the seawater is immediately colonized by microorganisms that form a biofilm on its surface, the so-called "plastisphere" (Zettler et al., 2013; Dussud et al., 2018). Microorganisms of the plastisphere are significantly distinct from the surrounding seawater or organic particles, including a wealth of unknown species and potential pathogens (Bowley et al., 2021; Kirstein et al., 2016; Dussud et al., 2018). Few studies have investigated the possible influence of the plastisphere on the gut microbiota of the host (Lu et al., 2019) and even less studies have proven the possibility of bacterial pathogen transfer from the plastisphere to the animal host (Bowley et al., 2021). Given the emergence of the holobiont concept since the early 1990s (Margulis, 1990); the extent to which the plastisphere influences the microbiota of the animal host that accidentally ingests plastic pieces and the resulting impact on its health need to be investigated.

While a few species of marine animals have been studied in the context of potential toxicity of MP, increasing the number of filter-feeding species in this kind of study is extremely important in order to broaden our understanding of the potential impact of MP on marine biodiversity. Herein, amphioxus (*Branchiostoma lanceolatum)* was used as a model organism. It is a filter-feeding marine invertebrate, which is one of the closest relatives of vertebrates, that burrows in the sand in tropical or temperate waters close to the seashore (from 0.5 m to 40 m deep) around the world (Bertrand and Escriva, 2011). Thus, the aims of this study were 1) to homogenize the gut microbial community of amphioxus before the toxicity test under different feeding conditions and 2) to perform the toxicity test based on the condition obtained from the first objective under various microplastic concentrations (0, 50, 500, 5000 particles/L). After the exposures of amphioxus to microplastics, we hypothesized that microplastic ingestion could result in a toxic effect, which could be observed at the morphological, biochemical and gene expression level, as well as physical damage or modification of subcellular components. Secondly, we hypothesized that the effect could be dose-dependent. Lastly, we hypothesized that the microplastic ingestion could result in gut microbial dysbiosis and bacterial transfer. Physiological traits as body size and shape changes and fecal excretion, histopathological and biochemical tests as superoxide dismutase and catalase activities, together with gene expression assays were assessed to evaluate the impact of microplastics on amphioxus adults. The potential transfer of microorganisms from the plastisphere to the gut microbiota of amphioxus has also been tested.

2. Materials and methods:

2.1. MP preparation and incubation in seawater

Polystyrene plastic was used in this study since it represented one of the most detected plastic materials in the coastal sediment (Pagter et al., 2020; Tata et al., 2020). Polystyrene microbeads (106–125 μm, Polysciences) were cryo-grinded (SPEX sample Prep) and sieved to recover irregular MP with a diameter ranging from 50 to 100 μm, corresponding to the size range of food particles that can enter through the mouth of amphioxus (Ruppert et al., 2000). Granulometry analysis was used to characterize the size distribution using a laser diffraction particle size analyzer (Malvern Mastersizer 2000 model with a Scirocco 2000 module).

Microplastics were immersed on September 2019 into 2 L glass tanks (Verres Vagner, France), in which natural seawater was continually renewed (20 mL/min) by direct pumping at 14 m depth in the bay of Banyuls-sur-Mer close to the SOLA observatory station (42°29′300N–03°08′700E, NW Mediterranean Sea, France). Microplastics were immersed for a minimum of one month to allow the development of a mature biofilm (Dussud et al., 2018). The formation of a plastisphere was visualized by confocal microscopy (TCS SP8 confocal laser scanning microscope, Leica, Germany), as previously described (Cheng et al., 2021).

2.2. Amphioxus exposure to four MP concentrations (0, 50, 500, 5000 particles/L)

Amphioxus individuals were collected using a shovel and a sieve at a depth of 0.5–1 m in coastal sandy sediment on October 2019 close to the Argelès-sur-Mer (France), where was previously described (Fuentes et al., 2004). A total of 665 adults with a size of 3.7 ± 0.3 cm were distributed into 19 identical tanks (10 L) filled with 2 L filtered seawater (0.2 μm, Sterivex, Dominique Dutscher) with 35 individuals per tank. Four tanks were used to test amphioxus conditions before MP exposure, 12 tanks for the toxicity test, and the last 4 tanks for the quantification of MP ingestion per individual. Tanks were maintained at 17 °C, placed in a dark closet, and illuminated from above with a 12/12 h light/dark rhythm.

The MP used for exposure were directly sampled from glass tanks to maintain the survivorship of living organisms from the plastisphere (see section 2.1), and its suspension in seawater was maintained with a continuous bubbling flow. According to the amount of MP commonly detected in coastal areas (Harris, 2020), their concentrations were adjusted at 0, 50, 500, and 5000 particles/L (designated as Ctrl, low, middle and high condition) (See Supporting Information for the counting method). During the 16 days' MP exposure, all amphioxus were under starvation conditions. Filtered seawater (0.2 μm, Sterivex, Dominique Dutscher) was changed every two days by transferring the animals to clean tanks, and colonized MP (see section 2.1 for its preparation) were re-added into the tanks after each seawater change. In the meantime, a portion of MP was stored at −80 °C to characterize the microbial community on the plastisphere before amphioxus exposure to MP.

2.3. Amphioxus sampling before and after MP exposure

Sampling of amphioxus individuals was performed in triplicates just before exposure to MP (*i.e.*, control animals, designated as *D0*) and also after 16 days of MP exposure. Dissection was undertaken after paralyzing the amphioxus (a more detailed method as described in the Supporting Information). Five guts or hepatic caecum samples were pooled for biochemical or gene expression tests, and one individual from each tank was used to analyze the gut microbiota. After collection, samples were snap-frozen in liquid nitrogen before storage at −80 °C. For histopathological tests, duplicates of gut and hepatic tissues were

fixed in with fixatives (see section 2.6) before storage at 4 ℃ (Fig. 1).

2.4. MP ingestion and quantification

After 7 days of fasting before MP exposure, 4 tanks were used to evaluate the amount of MP ingested per individual in null, low, middle and high microplastic concentrations. Exposure time to MP was limited to 2 h, according to preliminary experiments showing a mean of 2.6 h (SD = 1 h, n = 5) of intestinal transit (from MP ingestion to egestion) for amphioxus with 3.2 cm body length (SD = 0.33 cm, n = 5). As expected, the larger the size of the amphioxus, the longer the time required for the MP to be egested. After MP exposure for 2 h with the MP concentrations as mentioned above, 5 random individuals were sampled from each tank, distributed into 5 Petri dishes containing 0.2 μm filtered seawater (Sterivex, Dominique Dutscher), and incubated overnight in the dark at 17 °C in order to allow all the ingested MP to be egested. The feces collected in each Petri dish were lysed with proteinase K for 1 h at 55 °C (0.2 mg/mL, as in the previous study (Dussud et al., 2018), Sigma-Aldrich) and the resulting mix was transferred on a 0.22 μm PTFE membrane filter (Merck Millipore). MP extracted from amphioxus feces were counted under the epifluorescent microscope (Olympus AX-70 PROVIS) after staining with Nile Red for 30 min in the dark (0.01 mg/mL, Sigma-Aldrich).

2.5. Measurement of amphioxus body size and shape changes

Photographs were taken from the top of each tank at the beginning and at the end of the 16 days experiment in the four conditions (0, 50, 500, 5000 particles/L). Amphioxus body lengths were measured with the ImageJ software (Panasonic DMC-FZ300) and by considering the straight-line distance between the anterior and posterior tip of each individual. The amphioxus body shape was expressed by the tangent intersection angle of the anterior and posterior tip of the amphioxus.

2.6. Histopathological observations

For both, fresh amphioxus collected from the wild and animals after 16 days of experiment, two fixatives (paraformaldehyde and glutaraldehyde, VWR) were used to fix their gut and hepatic tissues (He et al., 2018). Duplicate samples were imbedded into epoxy resin, sectioned, and observed using a transmission electron microscope (TEM, Hitachi H7500). Percentage of goblet cells was expressed by their number divided by the number of total examined cells (all the intestinal epithelial cells observed under the microscope).

2.7. Biochemical analysis

Enzymatic biomarkers of antioxidant responses were measured in the gut and hepatic tissues using superoxide dismutase (SOD) and catalase (CAT) assay kits (Sigma-Aldrich and Abcam, respectively). According to the manufacturer's instructions, the measurements were performed in triplicates with some modifications. Briefly, gut or hepatic tissues were disrupted and homogenized in PBS buffer with sterile stainless steel beads using a TissueLyser (Qiagen) at 30 Hz for 2 min, and

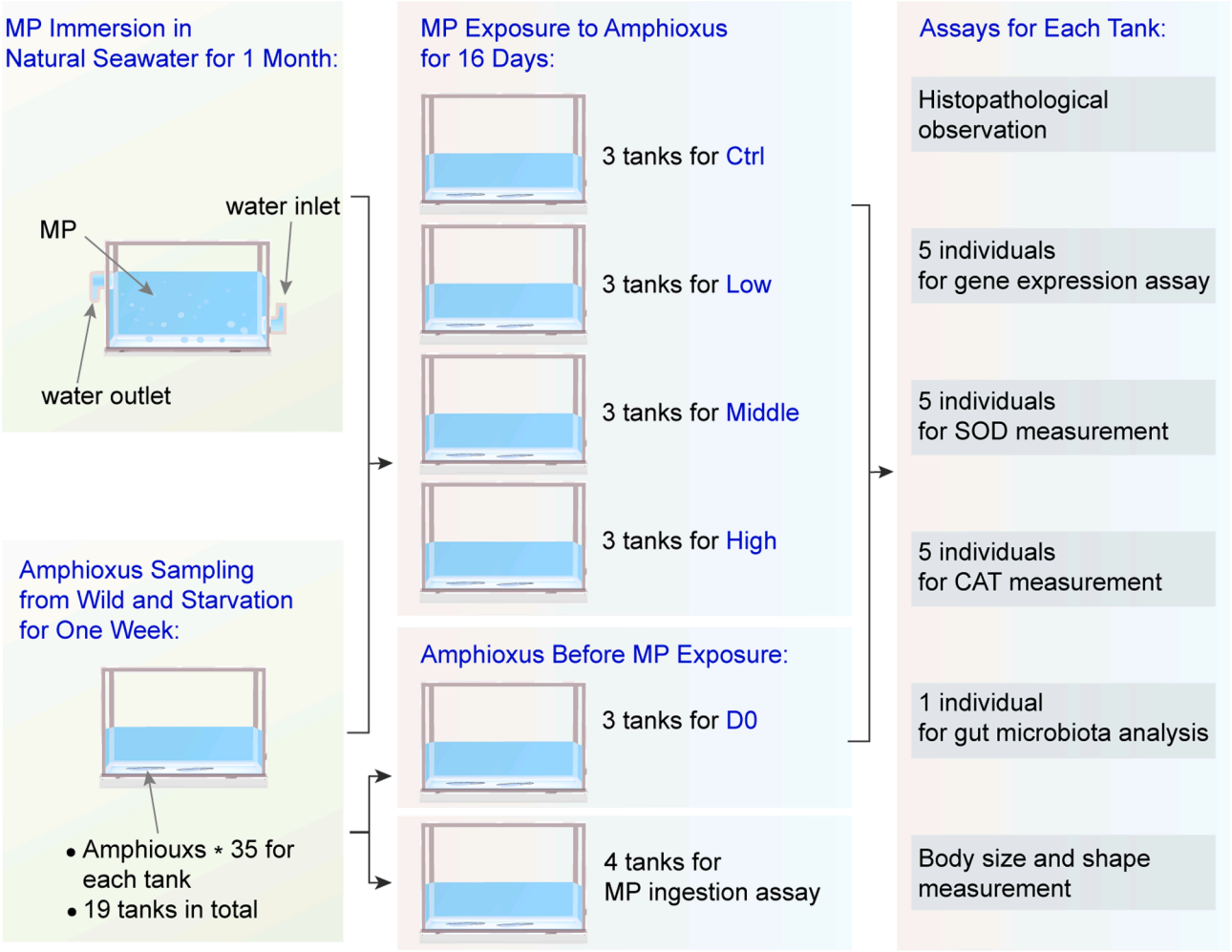

Fig. 1. Schematic presentation of the experiment processes.

then centrifuged for 5 min at 13,000 g. Supernatants were aliquoted for the biochemical test and protein quantification. Protein content of samples was determined using the Bradford method (Bradford, 1976). The enzyme activity was expressed as the relative activity to the respective control.

2.8. Gene expression

Triplicate RNA samples were extracted from gut and hepatic caecum tissues using the RNeasy Mini Kit, following manufacturer's instruction (Qiagen). RNA quality and concentration were verified with DeNovix, RiboGreen dye (Invitrogen) using Victor3™ (Perkin Elmer) and also Agilent 2100 (Agilent) bioanalyzers. The Nanostring technology was applied using molecular barcodes and microscopic imaging to detect and quantify up to several hundred transcripts in hybridization reaction (Kulkarni, 2011). A total of 59 genes were selected to target the immune system, stress response, apoptosis and housekeeping, based on the literature (Huang et al., 2011; Zhang et al., 2016) and online databases (UCSC database (Karolchik et al., 2003), Amphiencode database (Marlétaz et al., 2018), JGI database (Putnam et al., 2008), and LanceletDB database (You et al., 2019). The probes were synthesized by Nanostring Technologies, and further hybridized with the extracted RNA samples by nCounter Analysis System from CRCT platform (Toulouse, France). After the raw data were generated, background and housekeeping gene normalization were carried out with the *advance analysis* module of nSolver software (Nanostring).

2.9. DNA sequencing of microorganisms living on plastics and the gut microbiota

Genomic DNA from MP and gut tissues was extracted using classical phenol–chloroform protocol (Ghiglione et al., 2005). After PCR amplification of the V4-V5 region of the 16S rRNA gene (515F-Y and 926-R primers) (Parada et al., 2016), next generation sequencing was performed on Illumina MiSeq platform (Genoscope, France). Processing of 16S rRNA sequences was performed with DADA2 pipeline (Callahan et al., 2016) using R 3.6.1 version (Bunn and Korpela, 2008). Briefly, the primers were trimmed off before error correction and denoising step, and paired reads were merged (average length from 364 to 378 bp). All singletons and chimeras were removed for the merged reads. The amplicon sequence variants (ASVs) were assigned according to the SILVA release v. 128 database (Quast et al., 2013). Eukaryote, chloroplast, mitochondria and archaea sequences were removed and all the samples were re-sampled to the same depth (1012 tags per sample) by using phyloseq R package (McMurdie and Phyloseq, 2013). The α-diversity indexes (Chao1 richness, Pielou evenness and Shannon diversity) were calculated with Microbiome R package (Lahti et al., 2017). Taxonomy composition was analyzed with web-based platform of MicrobiomeAnalyst (Chong et al., 2020). Relative abundance of ASVs (>5% for each library) within each condition was averaged, and visualized by heatmap with online tool of ClustVis (Metsalu and Vilo, 2015). In addition, the eukaryotic sequences (without paired merge) from the MP samples were analyzed with the procedure mentioned above. All the SSU rRNA data are available in the NCBI SRA repository (accession number PRJNA732566).

2.10. Statistical analysis

For the gene expression assays, student t-tests were conducted on pairwise comparisons on the data for each gene, which was normalized with internal standard followed by the geometric mean of the housekeeping genes, and *p*-value was normalized with the method from Benjamini & Yekutieli (nSolver software) (Benjamini and Yekutieli, 2001). Linear regression analysis was used to evaluate the relation between the differentially expressed genes and MP concentrations.

Unweighted-pair group method with arithmetic (UPGMA) dendrogram based on Bray-Curtis similarities was used for visualization of 16S rRNA-based beta-diversity (PRIMER 6). A similarity profile test (SIMPROF, PRIMER 6) was performed on the null hypothesis that a specific sub-cluster can be recreated by permuting the entry species and samples. The significant branch was used as a prerequisite for defining bacterial cluster. Permutational multivariate analysis of variance (PERMANOVA) analyses were performed with the Bray-Curtis similarity on 16S rRNA data to detect factor's effect (999 permutations). The contribution of each species to the dissimilarity between two groups were performed with similarity percentage analysis (SIMPER, PRIMER 6). Other statistical analyses were performed with one way or multiway ANOVA in R software, Tukey's tests were used for post-hoc analyses.

3. Results

3.1. Starvation as a prerequisite for gut microbiota homogeneity between amphioxus individuals

Illumina MiSeq sequencing was performed on genomic DNA extracted from the gut of sets of 10 amphioxus individuals from each condition, the *wild* (collected from natural environment), *starved* (one week starvation) and *fed* (fed with algae for one week and then starved for another week) (See the material part of Supporting Information). Finally, 3 individuals from *wild*, 8 from *starved* and 6 from the *fed* conditions were used for analyses, the remaining samples were excluded due to insufficient sequencing depth (less than the 1012 tags per sample chosen after resampling). We found that the bacterial communities from the *starved* condition only formed one cluster, with 69% dissimilarity percentage in this group (SIMPER analysis) compared to the *wild* and *fed* conditions (82% and 80%, respectively) (Figure S1). Some bacterial taxa of the *wild* and *fed* conditions have been found within the *starved* cluster, but not the contrary. The homogeneity of the samples from the *starved* condition was confirmed at the taxonomic level (Figure S1). Because the *starved* condition reached the most homogeneous gut microbiota among amphioxus individuals, it has been selected as the suitable condition for further toxicity experiments (see above).

3.2. MP suspension, exposure, and ingestion

Granulometry analysis showed a Gaussian distribution of the polystyrene MP between 50 μm and 100 μm, around a pick at 63 μm (Fig. S2A). A bubbling flow at 35 mL/sec was optimal in our conditions to ensure that more than 50% of MP were continuously suspended in the seawater (Fig. S2B), and all the amphioxus were settled on the bottom of tanks in this condition. MP concentrations were adjusted to 5000, 500, 50 and 0 particles/L (corresponding to 0.42, 0.042, 0.0042, and 0 mg/L, respectively, according to calculation from granulometry results) designated as *High*, *Middle*, *Low* and *Ctrl* conditions hereafter, respectively. Besides, amphioxus counterparts before MP exposure (*D0*) were also sampled in order to perform the comparison before and after MP exposure. All conditions were performed in triplicates and under starvation conditions.

Amphioxus were exposed to MP in the *High*, *Middle*, *Low* and *Ctrl* conditions to quantify MP ingestion. We measured the number of MP ingested per individual for *High*, *Middle*, and *Low* conditions, and we showed that 19 ± 14, 9 ± 6, 2 ± 3, and 0 ± 0 particles were ingested, respectively, in each condition for 2 h. Pairwise comparison showed significant differences among these conditions (Tukey test, $p < 0.05$) (Fig. 2A).

3.3. Amphioxus morbidity and body size change after MP exposure

No mortality was observed during the 16 days of MP exposure. Integrity of oral cirri and metapleural folds was visually checked as an indicator of health condition. We observed signs of morbidity for 3 (SD = 1) out of the 35 individuals for the triplicates exposed to high MP

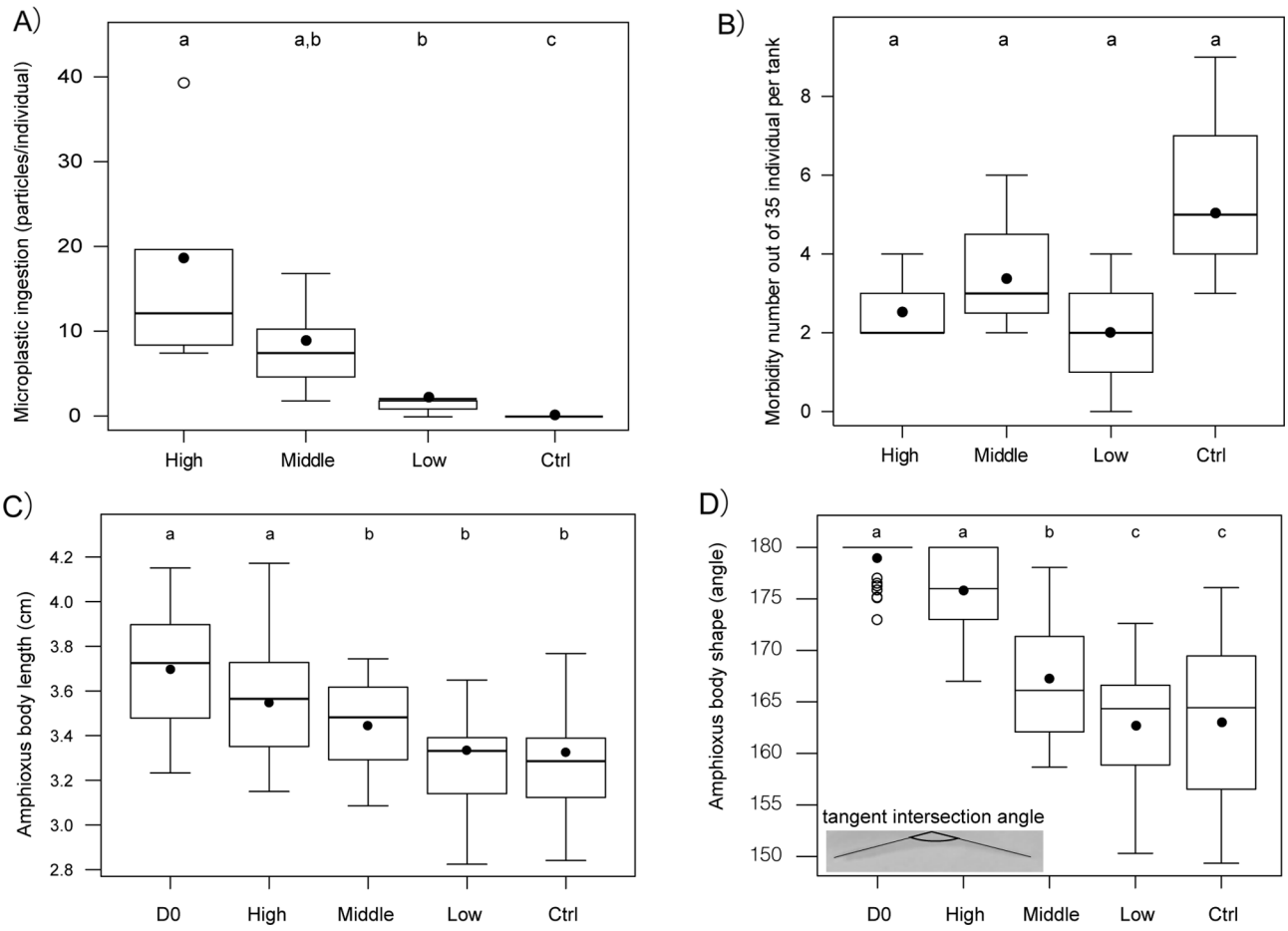

Fig. 2. Variation of MP ingestion (A), amphioxus morbidity (B) body length (C) and amphioxus body shape (D). *D0* corresponds to amphioxus condition before MP exposure (Day 0). The boxes represent the first and third quartiles, heavy horizontal lines inside the boxes indicate the median value, filled circles indicate the mean value and unfilled circle indicate outliers. Label "a", "b", and "c" in the panel indicate significant differences. The number of microplastic ingestion was quantified after 2 h of MP exposure (A), whereas the rest of the parameters (B, C, and D) were detected after 16 days of MP exposure, and the first and third quartiles were overlapped with the heavy horizontal line for the condition *D0* in panel D.

concentration (*High*); 4 (SD = 2) morbid individuals exposed to middle MP concentration (*Middle*); 2 (SD = 2) morbid individuals exposed to low MP concentration (*Low*); 6 (SD = 3) morbid individuals exposed to low MP concentration (*Ctrl*). There were no significant differences among conditions (Tukey test, $p > 0.05$) (Fig. 2B).

Amphioxus body size was measured before (*D0*) and after MP exposure. A significant difference was found between animals in the presence of pre-colonized MP and the control amphioxus that were fasting during the 16 days of the experiment. Significant longer body sizes of amphioxus were measured for *D0* and *High* conditions (3.70 cm and 3.55 cm, respectively) compared to *Middle*, *Low* and *Ctrl* conditions (3.44 cm, 3.30 and 3.27 cm) (Tukey test, $p < 0.05$) (Fig. 2C). Indeed, the starved amphioxus in control conditions changed from straight line shapes at D0 to curved shapes after 16 days without any input of nutrients. The tangent intersection angle was measured as a proxy for the body shape of the amphioxus (Fig. 2D). Significant larger angles were measured for *D0* and *High* conditions (178.93 and 175.69, respectively) compared to *Middle* (167.11), and *Low* and *Ctrl* conditions (162.82 and 163.05, respectively) (Tukey test, $p < 0.05$) (Fig. 2D). A considerable ratio of amphioxus exposed to MP during 16 days conserved their straight-line shape (as the normal form of wild animals), indicative of healthy animals (Figure S3).

3.4. Histopathological observations

Transmission electron microscopy was performed on hepatic caecum samples, since the integrity of the gut samples is extremely difficult to keep following dissection. In contrast to our initial expectation, we did not observe physical damage to the epithelial cells. Phagosomes and goblet cell-like structures were found in the amphioxus hepatic tissue (Figure S4). Goblet cells are mucus-producing cells. An increase in goblet cell number is indicative here of a gut protective reaction to MP exposure. Goblet cells were counted after analyzing a minimum of 100 epithelial cells of each sample. We found that goblet cells represented 2.8 ± 2.8% of the epithelial cells in animals directly caught from the wild (see section 3.1 for the condition). After 16 days, no goblet cells were detected in the amphioxus condition without MP exposure, while it represented (0.28 ± 0.19%), (8.5 ± 12%) and (2.5 ± 3.5%) for the duplicates of amphioxus exposed to low, middle and high MP concentrations, respectively. The results showed an enrichment of the mean percentage of goblet cells after microplastic exposure when compared to the one without microplastic exposure (*Ctrl*) ($p < 0.05$).

3.5. Biochemical tests

The biochemical activities of *SOD* and *CAT* were measured from triplicates of gut and hepatic caecum samples. Relative *SOD* activity of *Gut-D0, Gut-Low, Gut-Middle and Gut-High* were 48 ± 15%, 42 ± 10%, 60 ± 24 %, and 46 ± 26%, respectively, as compared with *Gut-Ctrl,* while no significant differences were found in pairwise comparisons. For the hepatic *SOD* activity, a significant difference was only found between the amphioxus before (*Hepatic-D0*) and after MP exposure at high MP

concentration (*Hepatic-High*), with relative activity of 41 ± 7% and 141 ± 58% as compared to *Ctrl*, respectively (Figure S5).

We also found no significant difference in the *CAT* activity on the gut and hepatic samples among the conditions.

3.6. Gene expression

To examine stress responses caused by MP exposure at the gene expression level, we selected 59 genes for RNA hybridization using Nanostring Technology: 26 genes associated to the immune response (adaptor, effector, complementary system, and oxidative burst), 20

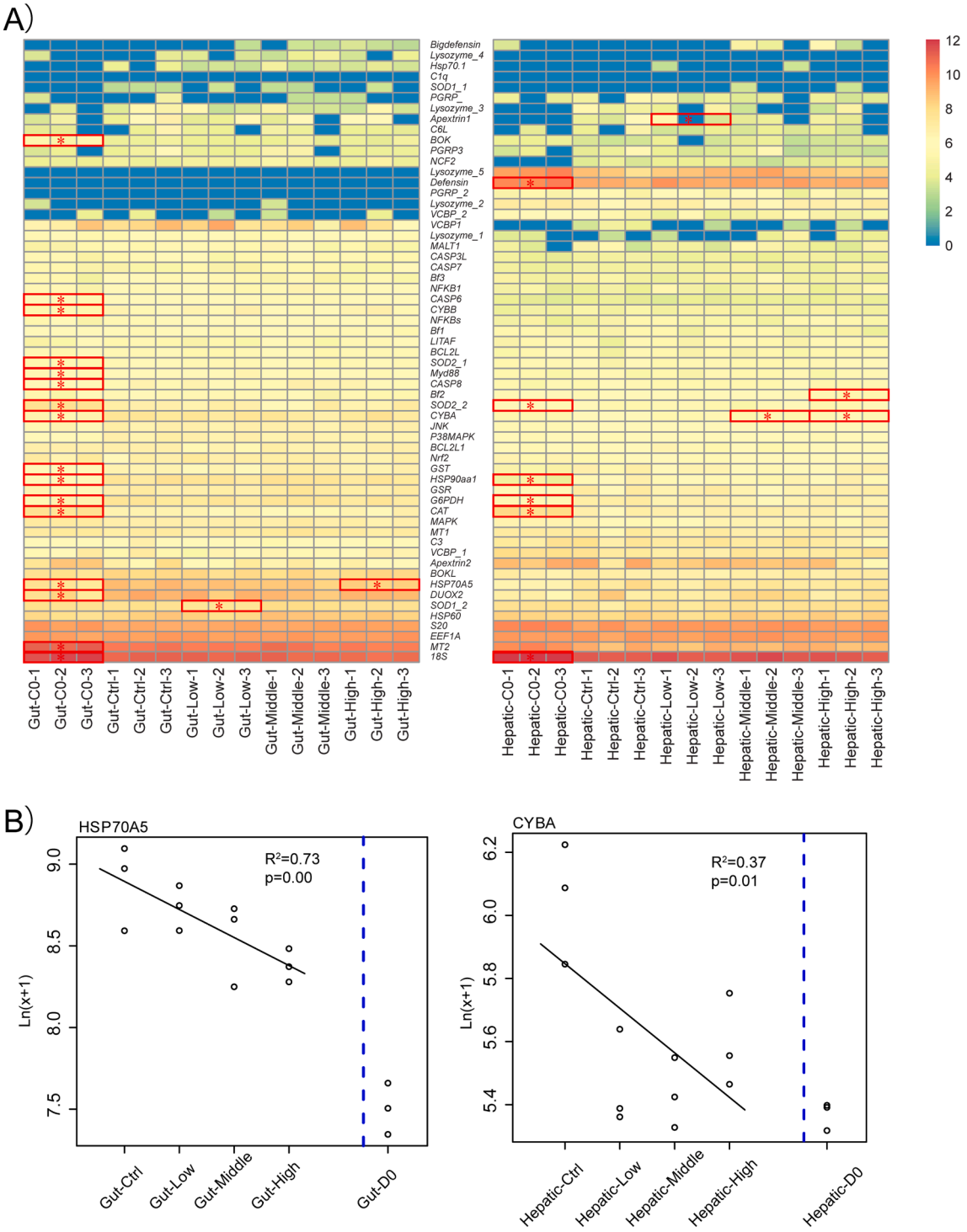

Fig. 3. Gene expressions on triplicate gut and hepatic tissues before (*D0*) and after exposure to *High, Middle, Low* or null (control, *Ctrl*) concentrations of MP. Heatmap (A) was used to display the 59 selected genes related to the immune system, oxidative response, and apoptosis. Significant adjusted *p*-value ($p < 0.05$) are marked within rectangles, gene names are labelled in the center of the figure. The rectangle indicates a significant difference in the condition as compared to *Ctrl*. The color key (A) and the Y-axis (B) represent the logarithmic transformation of normalized gene expression data. The significant correspondences between MP concentration and gene expression levels were detected for the genes *HSP70A5* and *CYBA* (B) using linear regression. Gene full name, functional category, and gene sequences are described in Table S1-S2.

genes associated to stress response (stress induction and antioxidative stress), 9 genes associated to apoptosis, and 4 housekeeping genes (Table S1-S2). Among the whole gene set, the expression of immune gene *C1q* was the only one not detected for both the gut and the hepatic caecum tissue.

Firstly, we explored the differential expression for each gene between before (*D0*) and after starvation (*Ctrl*). More genes were significantly expressed in the gut sample (16 genes) compared to hepatic samples (6 genes) (Fig. 3A), suggesting that the gut could be more sensitive for the selected genes compared to the hepatic caecum under starvation conditions. Among the significantly up-regulated genes in the gut sample, the highest expression level was observed for heat shock protein 70 (*HSP70A5*) among the genes affiliated to stress response (Fig. 3A, Table S1), and upregulations were also found for gene oxidase 2 (*DUOX2*) and cytochrome *b* alpha gene (*CYBA*) for the genes affiliated to the immune system. In terms of hepatic samples, *HSP90aa1* was significantly up-regulated after starvation.

Secondly, we also compared gene expression between the conditions without (*Ctrl*) and with MP exposure (*Low*, *Middle* and *High* condition) after the 16 days' treatments. For gut samples, the gene expression of *SOD1_2* and *HSP70A5* significantly decreased in amphioxus when exposed to low and high MP concentrations, respectively (*Gut-Low*, *Gut-High* in Fig. 3A). For hepatic samples, a significant decrease was observed for 3 genes: *Apextrin1* for amphioxus exposed to low MP concentration (*Hepatic-Low*), the immune gene *Bf2* for amphioxus exposed to high MP concentration (*Hepatic-High*), and *CYBA* for amphioxus exposed to middle and high MP concentrations (*Hepatic-Middle* and *Hepatic-High* in Fig. 3A).

Lastly, we explored the five significantly differentially expressed genes for their expression dependency on MP concentration using linear regression. Gene expression of *HSP70A5* ($R^2 = 0.73$, $p < 0.01$) from the gut and *CYBA* ($R^2 = 0.37$, $p = 0.01$) from hepatic caecum samples showed an expression significantly negatively correlated with the MP exposure concentrations (Fig. 3B).

3.7. Microbial pre-colonization on MP and impact on gut microbiota

After one month of immersion in natural seawater, we observed

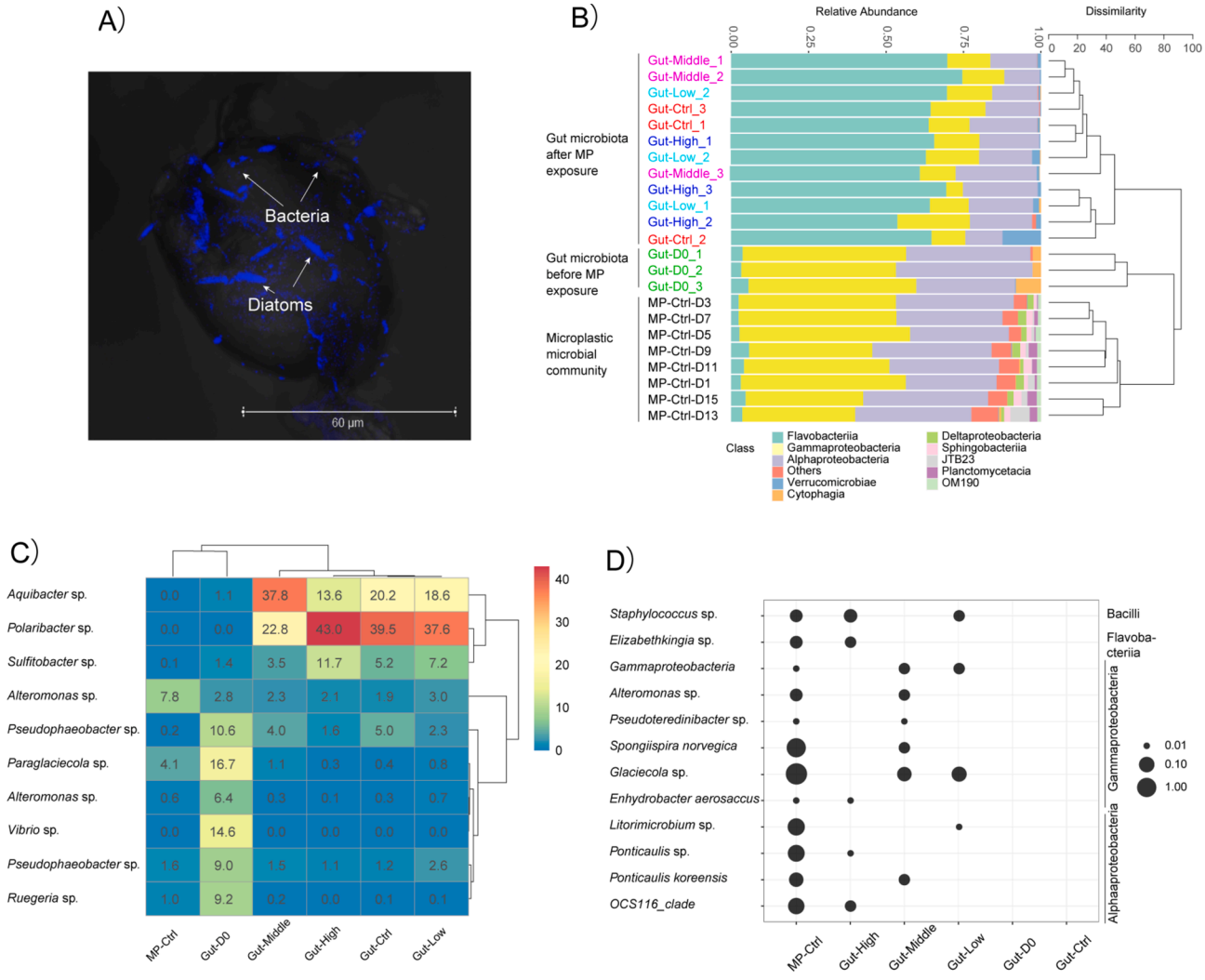

Fig. 4. Microbial life associated to MP after a minimum of one-month pre-colonization in natural seawater (*MP-Ctrl*, sampled every 2 days) and gut microbiota at the beginning (*Gut-D0*) and at the end of the 16 days experiment without (*Gut-Ctrl*) or with *Low, Middle* and *High* concentration of pre-colonized MP (*Gut-Low, Gut-Middle, Gut-High*, respectively). (A) Confocal microscopic observation illustrating the concomitant presence of bacteria and diatoms on *MP-Ctrl* samples. (B) UPGMA dendrogram based on Bray–Curtis dissimilarities between 16S rRNA-based sequencing profiles (right) and cumulative bar charts comparing taxonomic relative abundances (left). (C) Heatmap of the main ASVs with relative abundance over 5% in each condition. Rows and columns are clustered using correlation distance and average linkage, color key represents relative average abundance. (D) Bubble plot based on the presence-absence of bacterial taxonomic groups (genus level on the left, class level on the right) to evaluate the potential bacterial transfer from the MP samples to gut microbiota, bubble size represents the average relative abundance for each condition.

through confocal microscopy the presence of the bacterial-like and diatom-like morphotypes that formed a biofilm on MP, *i.e.* the plastisphere (Fig. 4A).

UPGMA dendrogram based on Bray-Curtis similarities between 16S rRNA-based community structure revealed 3 main clusters (SIMPROF test, $p < 0.05$) (Fig. 4B), including: (1) the bacterial community associated to the plastisphere in natural seawater during the 16 days of the experiment (*MP-Ctrl* sampled every 2 days), (2) the gut microbiota of triplicate amphioxus individuals before MP exposure, and (3) the gut microbiota of triplicate amphioxus individuals without (*Gut-Ctrl*) or with ingestion of pre-colonized MP in *Low*, *Middle* and *High* concentrations (*Gut-Low, Gut-Middle, Gut-High,* respectively). The differences for the three sub-cluster were confirmed by the taxonomic affiliation of the bacterial groups, with *Flavobacteriia* and *Gammaproteobacteria* dominating the gut microbiota after MP exposure (65 ± 5% and 18 ± 4% of the total bacterial community, respectively), *Gammaproteobacteria* and *Alphaproteobacteria* dominating the gut microbiota before MP exposure (*Gut-D0,* 52 ± 21% and 39 ± 6%, respectively) and their evolution in seawater during the course of the 16 days experiment (*MP-Ctrl*, 46 ± 7% and 36 ± 3%, respectively). Interestingly, we could not find a separate sub-cluster separating the *Gut-Ctrl* from the *Gut-Low, Gut-Middle, Gut-High*, with higher dissimilarity between triplicate samples than within each condition. PERMANOVA results confirmed that there was no significant difference for the pairwise comparison of the 4 conditions after 16 days of MP exposure ($p > 0.05$). From the plastisphere, we found that the diatom *Pseudo-Nitzschia* sp. dominated the ASV tags (84 ± 10%) affiliated to eukaryotes (only 4 ± 2% of the total tags), confirming the parallel observation using confocal microscopy (Fig. 4A).

We also found that MP had no significant impact on the α-diversity indexes (Chao1 richness, Pielou evenness and Shannon diversity) of gut microbiota, when comparing gut microbiota of amphioxus without (*Gut-Ctrl*) and with ingestion of pre-colonized MP at different concentration (*Gut-Low*, *Gut-Middle*, *Gut-High*) (Tukey test, $p > 0.05$, Figure S6). We found that 3 ASVs of *Polaribacter* sp., *Aquibacter* sp. and *Sulfitobacter* sp. dominated the gut microbiota of amphioxus in the 4 conditions after 16 days of MP exposure, with the highest abundance reaching 43.0%, 37.8%, and 11.7%, respectively (Fig. 4C). An even distribution of the dominant groups was found for amphioxus condition before MP exposure (*Gut-D0),* including *Pseudophaeobacter* sp., *Paraglaciecola* sp., *Alteromonas* sp., *Vibrio* sp., and *Ruegeria* sp. (Fig. 4C).

Finally, we explored the potential of bacterial transfer from the plastisphere to the gut microbiota. Identification of the transferred ASVs was defined as present on the pre-colonized MP (*MP-Ctrl*) and on one of the conditions after MP exposure (*Gut-Low*, *Gut-Middle* and *Gut-High*), but absent on the samples before MP exposure (*Gut-D0*) and also absent on the samples without MP exposure after 16 days (*Gut-Ctrl*). We found a potential transfer of 12 ASVs from the plastisphere to the gut microbiota.

All of these ASVs belonged to the rare biosphere, with an average abundance of<0.1% (Fig. 4D). Interestingly, we found that the Enterobacteria *Staphylococcus* sp. was transferred in both *Gut-Low* and *Gut-High* groups, when *Glaciecola* sp. and an undetermined Gammaproteobacteria were transferred in both *Gut-Low* and *Gut-Middle* groups. Specific transfers were also found in relation to the MP concentration: *Litorimicrobium* sp. for the *Gut-Low* group; *Alteromonas* sp., *Pseudoteredinibacter* sp., *Spongiispira norvegica* and *Ponticaulis koreensis* for the *Gut-Middle* group; *Elizabethkingia* sp., *Enhydrobacter aerosaccus*, *Ponticaulis* sp. and ASV of OCS_116 clade for the *Gut-High* group (Fig. 4D).

4. Discussion

4.1. Physiological, histopathological, biochemical, and genetic impacts of MP ingestion

The ubiquitous presence of plastics in the ocean attracted great attention in the last decades, with adverse toxic effects reported for a wide range of marine species (Wright et al., 2013). Before the toxicity tests, the MP were immersed for 1 month in natural seawater in order to mimic a mature biofilm (tens of thousands of bacterial counts per square millimeter), as it is classically found on plastics in natural conditions (Cheng et al., 2021). Here, we found that amphioxus indeed could ingest the MP from seawater with its filter-feeding lifestyle (Figure S7), and we showed a limited effect of the presence of MP on the benthic filter-feeder amphioxus compared to starved control conditions. The observations of body length changes coupled with the presence of numerous specific bacteria and diatoms on MP allowed us to deduce that starved amphioxus could take advantage of the biofilm growing on MP for the maintenance of the body size and shape (Fig. 2, Figure S3). It is well known that this filter feeder uses microbes and marine detritus as food sources (Chen et al., 2008). This result suggests that the digestive system, and especially the ileocolonic ring used for twisting ingested food, could use the biofilm growing on MP (Urata et al., 2007). It also opens new routes for further studies to consider MP as a potential vector of nutrients for other marine organisms feeding on bacteria or diatoms (*e.g.*, dinoflagellates, copepods) which have been shown to ingest MP from the environment (Su et al., 2020; Cole et al., 2013).

We also observed that ingestion of MP with their biofilms was related to intestinal changes in the host. In this study, TEM observations showed that goblet cells were never found in the control condition (starving and without MP exposure), while they were observed after MP ingestion. The presence of goblet cells was previously observed after MP ingestion in the European sea bass (*Dicentrarchus labrax*), suggesting a physical defense strategy (Mallik et al., 2021). However, these observations do not allow us to conclude that MP have a deleterious effect on the health of filter-feeding amphioxus, which always secretes mucus to trap food particles and facilitate their transport.

In parallel, observations of anti-oxidative enzyme activities and of the modification of marker gene expression confirmed the limited effects of MP exposure on amphioxus. First, there were no significant differences in gene expression for the 9 apoptosis genes after MP exposure. Only 2 out of the 24 selected immune system genes, *Bf2* and *CYBA* (*Hepatic-High*) were significantly down-regulated in animals exposed to MP (Fig. 3A). The expression of *CYBA* was shown to be negatively correlated to the MP concentration (Fig. 3B). *CYBA* is an immune gene expressed in the epithelial cell of the hepatic caecum and participates in the phagocytic respiratory burst, a process that releases reactive oxygen species to destroy internalized microbes (Stasia, 2016; Yang et al., 2014). Our results hence suggest that amphioxus might be more susceptible to microbial attack under starvation conditions without MP exposure. Two out of 20 oxidative response genes, *HSP70A5* (*Hepatic-High*) and *SOD1_2* from (*Hepatic-High*) were significantly down-regulated after MP exposure (Fig. 3A). The *HSP70A5* was demonstrated to be located in the endoplasmic reticulum lumen. It is a member of molecular chaperones of the HSP70 family, and its function is to assist the folding and assembly of unfolded polypeptides (Gething, 1999). Importantly, *HSP70A5* is an essential component of the signal transduction under stress conditions (such as heat shock, oxidative stress, and nutrient deprivation) (Borges et al., 2012,; Otero et al., 2010; Wang et al., 2017), and it serves as a diagnostic biomarker for health states (Gething, 1999). The *HSP70A5* gene expression decreased significantly with the increase of MP exposure concentration, suggesting that amphioxus were less stressed with the highest MP concentration, thus reinforcing the conclusion of nutrient acquisition from the biofilm growing on MP. This effect contrasts with the classically deleterious effect on animals reported in other toxicity studies that used pristine MP (Yang et al., 2014; Gething, 1999). The biochemical results were in agreement with these results. For instance, the amphioxus without MP exposure showed higher average SOD and CAT activity in the gut.

4.2. Impact on gut microbiota

So far, only limited studies have evaluated the influence of MP on the gut microbiota, most of which used pristine plastics, mainly on the

mouse or zebrafish model, as shown from previous review papers (Fackelmann, 2019; Hirt and Body-Malapel, 2020; Li, 2022).

Based on 16S rRNA sequencing data, we found that MP did not significantly induce a modification of the amphioxus gut microbiota. The dominant bacterial ASVs in amphioxus gut microbiota were shared among high, middle and low concentration of added MP conditions, and with the *Ctrl* condition (without MP), confirming the absence of MP impact in our experimental setting. *Sulfitobacter* sp., *Aquibacter* sp. and *Polaribacter* sp. prevailed in gut microbiota both without (*Gut-Ctrl*) and with MP exposure (*Gut-Low*, *Gut-Middle*, *Gut-High*). These genera were not limited to amphioxus gut microbiota, and are also found in the gut microbiota of mussel, salmon, and sea cucumber (Ciric et al., 2018; Zhao et al., 2019; Auguste et al., 2019). *Sulfitobacter* sp. is known to produce Vitamin B1, B7 and B12, the synthesis of which was often lacking in a high proportion of bacterial species, phytoplankton and vertebrates (McWilliams, 2011; Luo and Moran, 2014). Therefore, this bacterial clade might form a potential mutualism relationship with amphioxus. Moreover, *Sulfitobacter* sp. is also capable to produce antibiotic materials of tropodithietic acid (TDA), which might enhance its survival strategy (Sharifah and Eguchi, 2012). *Aquibacter* sp. and *Polaribacter* sp., as members of Bacteroidetes, can break down high molecular weight materials (particularly polysaccharides and proteins) as primary carbon and energy sources, and get additional energy source from the intestine, which might explain their dominance in the intestine (Zhao et al., 2019; Auguste et al., 2019).

Interestingly, a total of 12 ASVs were found to be transferred from the MP biofilms to the gut microbiota of amphioxus. One ASV affiliated to *Staphylococcus* sp. was part of this transfer, whereas it represented only 0.02% of the total ASVs in the plastisphere. *Staphylococcus* sp. is known as a pathogen for amphioxus, challenging its immune system (Huang et al., 2007). Other harmful bacteria species were previously identified on MP, which could hence act as vector of harmful species, including putative pathogens of animals and humans (*Vibrio* sp.), corals (*Halofolliculina* spp.), fishes (*Tenacibaculum* sp.) (Bowley et al., 2021; Jacquin et al., 2019). However, all these studies were limited by the 16S rRNA sequencing approach that did not allow the analysis of bacterial virulence. Further studies are thus needed before displaying alarmist conclusions on the risk of MP as a vector of active and harmful pathogens for amphioxus.

4.3. Experimental precautions and future perspectives

Our experimental design has taken into account the classical criticisms on studies evaluating the toxic effect of MP (Phuong et al., 2016). First, a realistic concentration gradient (0, 50, 500, 5000 particles/L) was used in this study, corresponding to what is usually found in the natural environment (Li et al., 2020; Phuong et al., 2016). Second, we used irregular shape MP, which is more representative of the larger proportion of secondary MP in the environment. Third, we considered MP pre-colonized by natural microorganisms during more than one month, a period necessary to form a mature biofilm as commonly observed in the natural environment (Jacquin et al., 2019; Amaral-Zettler et al., 2020). Previous studies highlighted that a period of 15 days to one month of pre-colonization is necessary to reach a very diverse and stable mature biofilm in the marine environment (Odobel et al., 2021; Jacquin et al., 2021), thus rendering experiments with pristine MP less relevant (Sharifah and Eguchi, 2012). Furthermore, one of the originalities of our study was to set the conditions to homogenize the gut microbiota diversity within amphioxus individuals, by one-week starvation, before running toxicity tests. It was also suggested as an important process in profiling physiological changes in toxicity tests to improve the ecological relevant and statistical robustness (Melvin et al., 2017). Finally, for each seawater change, the seawater was filtrated with 0.22 μm sterile filter, as a result of this, we could exclude the impact of nutrient source and bacterial community from the seawater.

5. Conclusion

In this study, amphioxus was used as a model organism to evaluate its responses to MP in the context of constant exposure of marine organisms to MP under starvation conditions with a set of comprehensive approaches. The results were contrasted with our initial hypothesis that MP would be detrimental to amphioxus. We found that MP had limited toxic effects on amphioxus after 16 days of exposure using MP with a size range of 50–100 μm. These negligible negative effects were supported by the biochemical and gene expression assays. Observation of body size and shape change unveiled that the plastisphere potentially served as a nutrient source to amphioxus. In detail, the gene expression of specific biomarkers such as *HSP70A5* and *CYBA* were negatively correlated to the MP concentration, suggesting that the plastisphere at high MP concentration provided more energy to the amphioxus, which in turn, exhibited less stress. We did not observe any significant impact of MP exposure on the gut microbial community structure. However, a potential bacterial transfer was observed from the plastisphere to the amphioxus, and the transfers were independent of the ASV abundance in the plastisphere. To fully uncover the MP impact on amphioxus, other factors such as exposure duration, MP size range, or amphioxus at different developmental stages should be considered in the future. We highlight that nanoplastics (<1 μm) might induce an adverse impact on amphioxus due to their endocytosis behavior. These results suggest that amphioxus might be used as a promising bioindicator of microplastic pollution.

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CRediT authorship contribution statement

Jingguang Cheng: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Anne-Leila Meistertzheim:** Conceptualization, Writing – review & editing. **David Leistenschneider:** Investigation. **Lena Philip:** Investigation. **Justine Jacquin:** Investigation. **Marie-Line Escande:** Investigation. **Valérie Barbe:** Writing – review & editing. **Alexandra ter Halle:** Investigation. **Leila Chapron:** Validation. **Franck Lartaud:** Validation. **Stéphanie Bertrand:** Conceptualization, Investigation, Formal analysis, Validation, Writing – review & editing. **Hector Escriva:** Conceptualization, Validation, Supervision, Funding acquisition. **Jean-François Ghiglione:** Conceptualization, Validation, Writing – original draft, Supervision, 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107750.

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