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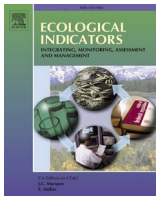

Influence of microplastic-associated biofilms on the bioavailability of a mixture of cadmium and benzo[a]pyrene by the analysis of biomarker gene expression in larval zebrafish

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ABSTRACT

Microplastics (MPs, 1 μm – 5 mm) in aquatic environments undergo complex weathering transformations such as those induced by microbial colonization and biofilm formation, that affect their ability to interact with environmental contaminants (co-contaminants). In this study, the microbial composition of MP biofilms and its influence on the sorption and bioavailability of two co-contaminants with different physicochemistry, benzo[a]pyrene (B[a]P) and cadmium (Cd) in a mixture, were assessed. Aqueous-phase bioavailability was measured by assessment of biomarker gene expression for these toxicants (cytochrome P450 1A, *cyp1a,* and metallothionein 2, *mt2*, for B[a]P and Cd respectively) in larval zebrafish, *Danio rerio*. Significant induction of cyp1a and mt2 gene expression ($p < 0.05$) was observed after exposure to the mixture of Cd, B[a]P and MPs compared to joint exposure with individual contaminants and MPs. Significant changes in bioavailability for *mt2* biomarker ($p < 0.001$) resulted after exposure to a Cd and B[a]P mixture with MPs compared to the same exposure without MPs. Biofilms significantly reduced bioavailability of B[a]P (*cyp1a* gene expression ($p < 0.01$)) but not Cd (*mt2* gene expression) in the mixture with Cd and B[a]P (HDPE + BF + B[a]P + Cd) compared to the same treatment without biofilm (HDPE + B[a]P + Cd). Thus, compared to Cd, the biofilm could provide additional interactions with B[a]P, and new specific active sites on the MPs surface, that reduced B[a]P bioavailability. Additionally, the biofilm microbial community included hydrocarbon-degrading bacteria able to metabolize hydrophobic chemicals. These data indicated that in a mixture of co-contaminants, the biofilm selectively influenced their bioavailability and that the microbial composition of MPs biofilm may have played a key role in reducing B[a]P bioavailability. The results of this study highlight how in a complex exposure scenario characterized by a mixture of different co-contaminants, the polymer and chemical properties and micro-surroundings of the organisms may affect contaminants' bioavailability and/or exposure.

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1. Introduction

The contamination of microplastics (MPs) in the environment is continuous and persistent, posing a concern for potential adverse effects on biota. Many investigations on global pollution of aquatic systems with MPs have focused on the potential harm to marine organisms resulting from MP interactions with exogenous contaminants (termed here "co-contaminants"). Once released into aquatic environments, MPs are likely to encounter a plethora of chemical contaminants, including persistent organic pollutants (POPs), trace metals, and pharmaceuticals, that can sorb to the plastic surface (Khan et al., 2021, Khan et al., 2022). Since co-contaminants on MP surfaces can reach concentrations up to a million times higher than surrounding environment (Mato et al., 2001; Ziccardi et al., 2016), the widespread ingestion of MPs across the different trophic levels can present a concern about their potential role as a vector of harmful chemicals to organisms. At present, the "MP vector effect" has been the objective of many investigations. However, there is a lack of consensus within the scientific community on the nature or the relevance of the MPs to increasing the exposure of organisms to plastic-associated chemicals (Gassel and Rochman, 2019; Koelmans et al., 2022). Some laboratory-based assessments reported that the mechanisms through which MPs interact with substances might affect their bioaccessibility and consequent bioaccumulation and bioavailability to organisms (Trevisan et al., 2019; Zhang et al., 2020). The bioaccessibility and bioavailability of co-contaminants that interact with MPs are likely to relate to the physicochemical properties of the MPs, contaminants, and the medium where the interaction occurs (Fred-Ahmadu et al., 2020). Various studies reported that organic co-contaminants, due to their hydrophobicity, show stronger sorption affinities to MPs and, consequently, are less likely to desorb (Teuten et al., 2009; Bakir et al., 2012; Wang et al., 2020). For example, benzo [a] pyrene (B[a]P), a five-ring polycyclic aromatic hydrocarbon (PAH), due to its highly lipophilic nature, has been shown to strongly sorb to MPs (Liu et al., 2016; Batel et al., 2018; Martínez-Álvarez et al., 2022). While the evidence concerning B[a]P desorption from MPs is limited to artificial in vitro gut environments where the desorbed fraction was<10% of the concentration of sorbed PAH on MPs (Ito et al., 2022). In contrast, the sorption to MPs of inorganic co-contaminants, such as metals, can be driven by a different sorption mechanism ascribable to the chemical specification and the formation of free cation species in the medium (Tourinho et al., 2019). Free cations are likely to react with polar regions of plastic surfaces by electrostatic interactions, which represent one of the main sorption mechanisms for metal ions (Liao and Yang, 2020). Studies on the sorption of Cd to HDPE revealed a higher sorption capacity for Cd and a high desorption rate with varying sorption conditions. Based on the different physicochemical properties of these two co-contaminants, the sorption processes may vary unpredictably, mainly when a mixture of them in the environment occurs. Mixed chemicals may sorb onto MPs simultaneously or at different times, affecting the sorption capacities, co-contaminants' bioaccessibility, and consequently, bioavailability to organisms (Bakir et al., 2012). To date, most experimental studies have mainly evaluated a single contaminant's sorption/desorption event in isolation. However, as in realistic aquatic scenarios, toxicants are seldom present as individual chemicals, the assessment of the sorption behaviour and multiple interactions involving more contaminants simultaneously on MPs represents a critical factor in predicting MPs' environmental risks.

Moreover, the several environmental transformations that MPs undergo in the natural environment may further affect how MPs interact with the surrounding aquatic environment, enhancing the complexity of the role of MPs in co-contaminant transfer. (Zhang et al., 2021; Amaral-Zettler et al., 2020). Several studies have demonstrated that the surface of MPs acts as an anthropogenic substrate for phylogenetically and functionally distinct communities of microorganisms called "biofilm" or "epiplastic communities" (Reisser et al., 2014; Zettler et al., 2013). As more studies investigate the role of this organic layer as a reservoir of environmental contaminants, there are indications that the physical and chemical MPs-surface properties are changed after the attachment of biofilms (Shabbir et al., 2020; Tu et al., 2020). Changes in these properties could therefore influence the sorption behaviour of MPs and microplastics-associated co-contaminants bioavailability. The sorption behaviours of metal ions and organic pollutants on microplastics have been investigated in various studies (Guo and Wang, 2019; Velzeboer et al., 2014; Wang et al., 2021) from which, overall, a consensus has emerged that biofilms increase MP surface area as well as changing functional groups and hydrophobicity of plastic system can enhance the sorption capacity of pollutants to MPs (Guan et al., 2020; Wang et al., 2021).

Recently, laboratory research has revealed that biofilm-covered MPs exhibit a marked enhancement in the sorption of perfluorooctane sulfonate (PFOS) and lead (Pb) compared to virgin ones (Bhagwat et al., 2021), although the relationship between the sorption amount and composition of microorganisms in biofilm has not been investigated. Despite accumulating evidence of several biofilm-mediated processes, the information on how the microplastic-associated biofilm and its microbial composition influence co-contaminant mixtures sorption are still at began, and especially, to date, knowledge of the sorption of multiclass of contaminants on MPs as well as how they are affected by biofilm is very limited (Zhang et al., 2022).

In this study, we selected high-density polyethylene MPs (HDPE-MPs) to evaluate the role of biofilms on co-contaminant sorption by MPs. Target chemicals with different physico-chemistry characteristics, cadmium (Cd) and B[a]P, were selected. Their sorption on the surface of MPs was investigated by evaluating changes in co-contaminant (aquous phase) bioavailability using a multi-biomarkers approach able to provide a broader view of the sensitivity of a given biological matrix to multiple contaminants (Iftikhar et al., 2022). We hypothesized that the presence of biofilms on HDPE-MPs would influence their capacity to sorb Cd and BaP under environmentally relevant concentrations and mixture conditions, modifying fluxes of these plastic-associated contaminants and consequently their bioavailability in larval zebrafish (*D. rerio*). Therefore, the goals of this study were as follows: 1) to investigate the influence of biofilms on sorption of B[a]P and Cd (aquous phase), alone or 2) in a mixture by assessing their bioavailability and measuring changes in expression of biomarker gene transcripts for these substances, cytochrome P450 1A (*cyp1a*), and metallothionein 2 (*mt2*), respectively; 3) to analyze the structure and function of the microbial community constituting the biofilm on MPs surfaces used for exposure tests through a metagenomic characterization. The present study will help us better understand the potential environmental and ecological risk of MPs especially in relation to the co-contaminant transfer.

2. Materials and methods

2.1. Experimental fish

Brood stock zebrafish (wild type line – WIK) were maintained in an aquarium facility at Heriot-Watt University, Edinburgh, UK, and fish welfare regulations from both Heriot-Watt and the UK Home Office were followed for all experimental procedures. Adult fish were kept in recirculating tank systems with a 12:12 light/dark photoperiod and temperature between 27 and 29 °C. The water used in all experiments and for culturing stock fish (also used in the aquarium) was prepared following OECD guidelines (Test 201; Organization for Economic Co-operation and Development 2011), adding to reverse osmosis purified water reagent grade salts. The final concentrations of salts in the water were: 0.294 mg L^{-1} CaCl2·2H2O, 0.123 mg L^{-1} MgSO4, 7H2O, 0.0647 mg L^{-1} NaHCO3, 0.0057 mg L^{-1} KCl and the water was at pH 8.0. Water quality was routinely monitored (e.g., for total ammonia, nitrate, and nitrite) and part-refreshed twice weekly or more frequently if required. Fish were fed newly hatched brine shrimp *Artemia spp*. and a commercial zebrafish diet (ZM Fish Food, ZM Systems, Winchester, UK) daily. To

obtain larvae for experiments, pairs of zebrafish (one male and one female) were separated from stock tanks and gently transferred to 1 L breeding tanks (Mbk Installations Ltd, Nottingham, UK) fitted with a partition to separate the fish overnight. The following morning, the water in the breeding tanks was refreshed, the partitions were removed, and the fish proceeded to spawn. Approximately 60 min later, the adult fish were transferred back to stock tanks. All embryos from multiple spawning pairs were pooled and distributed between Petri dishes at a density of approximately 50 embryos per dish. Zebrafish were left to develop until 72 h post-fertilization (hpf), and hatched larvae were used in experiments. The duration of fish exposure was 24 h, and all larvae were 96 hpf when they were sacrificed at the end of the experiments. All fish were obtained from the HWU Zebrafish Research Facility that has complete approval from the UK Home Office. All experiments were conducted in the facility, and all fish used in experiments were used in accordance with the UK Home Office and under Home Office approved protocols stipulated by the Project License (PL Number P70BAC026) held by the Principal Investigator (TB Henry). All fish used in experimentation were aged <120 hpf.

2.2. Microplastic model and preparation of stock solutions

This work was carried out using high-density polyethylene (HDPE, indicated here HDPE-MPs) originated from the HDPE originated from The Dow Chemical Company, Michigan, USA. The microplastic particles were sieved to obtain a plastic fraction of approximately 200 μm for use in experiments. The particle size distribution was calculated using ImageJ software (Schindelin et al., 2012) and the sizes of n = 30 HDPE particles were 297.9 ± 51.6 μm (Boyle et al., 2020). The use of HDPE was selected in this study because it is one of the most commonly found polymers in the aquatic environment (Lu et al., 2022) and has a high sorption capacity for hydrophobic organic chemicals (Lee et al., 2014; Ziccardi et al., 2016). The HDPE concentration was set at 500 mg L^{-1} for all experiments according to previous MP co-contaminant sorption studies (Bakir et al., 2012; Sleight et al., 2017; Boyle et al., 2020). Stock solutions of B[a]P (Sigma-Aldrich, DE) and Cadmium Chloride (CdCl2, Sigma-Aldrich, DE) were prepared before addition to experimental aquaria to achieve the nominal target concentrations. The B[a]P stock solution was prepared in dimethyl sulfoxide (DMSO, ≥ 99.5%, Sigma-Aldrich, DE) due to its low solubility in water. The Cd stock solution was prepared in ultrapure water (Milli-Q, Merck Millipore, UK) at 50 and 10 mg L^{-1} concentrations, respectively. The final concentrations of DMSO in test solutions did not exceed 0.01% (% v / v) (Kais et al., 2013), so we did not expect a toxicity effect of DMSO in the study organism. The concentration of B[a]P (5 μg L^{-1}) was selected based on previous data that suggested a 10-fold increase in expression of *cyp1a* at this concentration after 24-h exposure of zebrafish larvae at 72 hpf. The Cd concentration (0.24 mg L^{-1}) was selected according to preliminary studies that assessed concentration relations between Cd exposure and larval zebrafish gene expression.

2.3. Experimental design

Two series of experiments were carried out to investigate the influence of biofilm on the sorption of B[a]P (both alone and in a mix with Cd) on HDPE-MPs. In particular, the activity of well-known biomarkers [Cytochrome P450 1A (*cyp1a*), metallothionein (*mt2*)] for the evaluation of B[a]P and Cd bioavailability, respectively, in larval zebrafish with and without biofilm formation on HDPE-MPs was assessed (Fig. 1). Each experiment included three replicate beakers (the experimental replicates, $n = 3$ treatment^{-1}) and 20 larvae in each beaker. Positive co-contaminant control treatment (no HDPE-MPs) as well as negative controls: (fish water), vehicle (DMSO) and HDPE-MPs treatments (with or without biofilm) were included in each experiment.

2.4. Biofilm formation

For biofilm formation, 20 mg of HDPE-MPs were weighed and added directly into each ($n = 11$, for experiment with only B[a]P or Cd, $n = 13$, for experiment with B[a]P and Cd in a mix) clear 20 mL borosilicate vial with a screw top, containing fish medium (20 mL), directly collected from the reservoir of the zebrafish recirculating system. Before collection, bioballs (the substrate of the biofilter of the water system) in the box were agitated and water was collected and filtered two times through filters with different sizes (125 μm and 100 μm) to avoid larger detritus and then pipetted into each experimental vial. Vials were externally wrapped with aluminum foil to protect the biofilm-forming treatments from light and prevent algal growth, and then placed on the roller system and incubated at 27 ± 1 °C for 6 days (Fig. 1). After incubation with media, HDPE-MPs were collected by filtration (100 μm

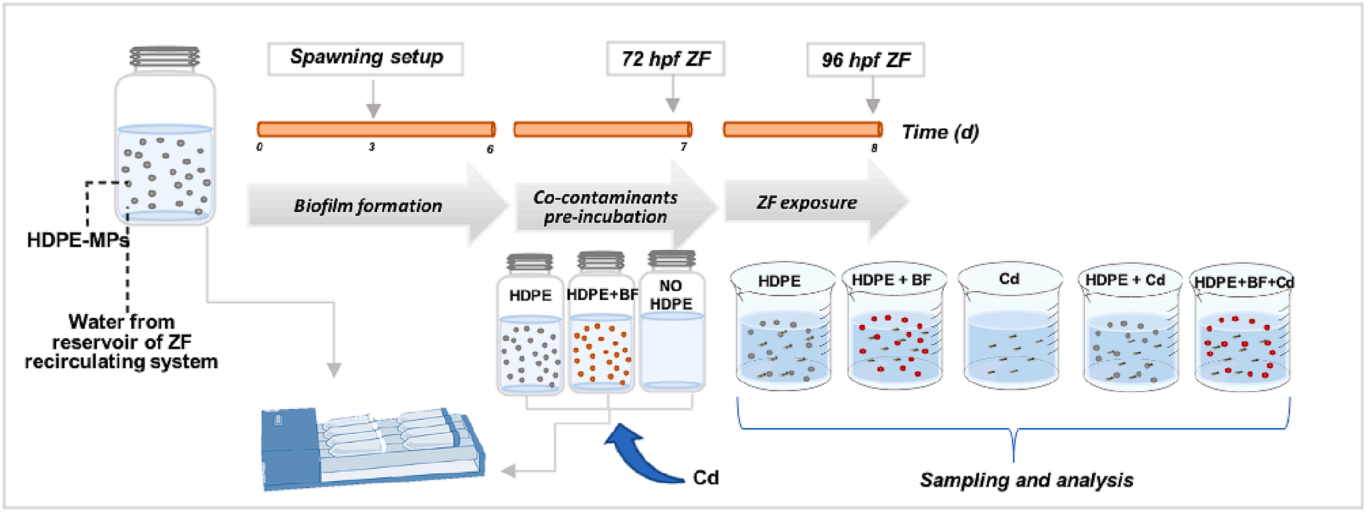

Fig. 1. Experimental design and timeline. In the figure are shown only the vials related to individual treatment group with Cd. The experimental design represented in figure was also followed for the mixture treatment group (Cd/B[a]P). Time "day 0" indicates the start of biofilm formation, which lasted 6 days. Co-contaminant pre-incubation was carried out for 24 h, and finally, zebrafish larvae (ZF at 72 hpf) were exposed for 24 h to the different treatments groups generated: HDPE-MPs (without BF), HDPE + BF, Cd, HDPE + Cd, HDPE + BF + Cd. Controls containing ZF water, and DMSO, are not shown in figure but have been included in the experimental design as described in the text. Time (d) refers to days number. The full experimental steps took 8 days, at the end of which zebrafish samples were collected.

Celltric® filters) and transferred into new vials containing 20 mL of filtered ZF media. Of the vials ($n = 24$) used in the biofilm formation step, for each experiment $n = 3$ vials have been used as control HDPE-MPs with biofilm for the following test exposures, and $n = 3$ vials have been used for metagenomic analysis to evaluate the microbial communities of the biofilms attached on HDPE- MPs (HDPE-MPs + BF). Finally, the remaining vials ($n = 15$) were pre-incubated with co-contaminants.

2.5. Co-contaminants pre-incubation

B[a]P and Cd (alone or in a mix) have been pre-incubated with HDPE-MPs for 24 h before exposure to zebrafish. However, pre-incubation concerned all treatment groups, including 1) HDPE-MPs without biofilm (HDPE-MPs), 2) HDPE-MPs with biofilm (HDPE-MPs + BF), and 3) positive controls (i.e., B[a]P without HDPE- MPs) (Fig. 1). The co-contaminants pre-incubation for HDPE-MPs without biofilm group was obtained by weighing 20 mg of HDPE-MPs into each ($n = 15$ for each treatment group – B[a]P and Cd alone and a mixture of them) clear borosilicate vials with screw top and adding 20 mL of filtered zebrafish media (pristine, i.e., prepared separately and not from fish tanks). Then, 4 μL of B[a]P and 960 μL of Cd stock solutions (50 mg L^{-1} of B[a]P in DMSO, ≥ 99.5%, Sigma-Aldrich; 10 mg L^{-1} of Cd) have been solved to reach 10 μg L^{-1} and 0.48 mg L^{-1}, respectively. For HDPE-MPs + BF group, pre-incubation was obtained by adding co-contaminants to the vials ($n = 15$) containing 20 mL of filtered ZF media and HDPE-MP with biofilm, generated as previously described in section 2.4. Whilst, pre-incubation for positive controls was get by pipetting 20 mL of filtered ZF water into each vials ($n = 15$) and finally adding co-contaminants at the fixed concentrations. All treatment groups were placed in a roller system for 24 h and then used for the zebrafish exposure experiments.

2.6. Zebrafish exposure

For each exposure group, spiked solutions (20 mL) were poured into pre-acid washed (5% HNO3) 50 mL glass beakers (Fig. 1). After bringing the volume to 40 mL with pristine filtered zebrafish media, zebrafish larvae at 72 hpf were gently collected from the Petri dishes with a transfer pipette and added to each exposure group (Table 1). Each beaker ($n = 3$ treatment^{-1}) contained 20 larvae. After 24 h of exposure, larvae within each beaker were pooled together (one replicate:one pool) into a microcentrifuge tube, the exposure water in excess was removed with a pipette tip and the tubes containing larvae were then frozen at − 80 °C until RNA extraction (section 2.7). No developmental toxicity, and no treatment dependent mortality of zebrafish was observed during all exposure tests. For all experimental design phases, observers remained blinded to treatment/control group assignments. All observations and data were obtained in a blinded fashion using samples masked.

Table 1
Exposure and experimental set-up used in this study. Details of concentrations used in single and mixture exposures.

Exposure group	Contaminant nominal concentration	Treatment type
Fish media		No Pre-incubation (Negative-control)
DMSO	0.01% (v / v)	No Pre-incubation (DMSO-control)
HDPE	500 mg L^{-1}	No Pre-incubation (MPs-control)
HDPE + BF		Biofilm formation 6 d (Biofilm-control)
Cd	0.24 mg L^{-1}	No Pre-incubation (Cd-control)
B[a]P	5 μg L^{-1}	No Pre-incubation (B[a]P-control)
Cd + B[a]P	0.24 mg L^{-1} + 5 μg L^{-1}	No Pre-incubation (Cd/B[a]P-control)
HDPE + Cd	500 mg L^{-1} + 0.24 mg L^{-1}	Pre-incubation 24 h
HDPE + B[a]P	500 mg L^{-1} + 5 μg L^{-1}	Pre-incubation 24 h
HDPE + Cd + B[a]P	500 mg L^{-1} + 0.24 mg L^{-1} + 5 μg L^{-1}	Pre-incubation 24 h
HDPE + BF + Cd	500 mg L^{-1} + 0.24 mg L^{-1}	Biofilm formation 6 d + Pre-incubation 24 h
HDPE + BF + B[a]P	500 mg L^{-1} + 5 μg L^{-1}	Biofilm formation 6 d + Pre-incubation 24 h
HDPE + BF + Cd + B[a]P	500 mg L^{-1} + 0.24 mg L^{-1} + 5 μg L^{-1}	Biofilm formation 6 d + Pre-incubation 24 h

2.7. Gene expression analysis

After sample (n = 20 zebrafish larvae per replicate) homogenization with a motor-driven hand homogenizer (Sigma-Aldrich, UK), total RNA was extracted from the pooled larvae in each sample using a commercial kit (RNeasy Mini Plus Kit, Qiagen, UK), following the manufacturer instructions. Treatment with DNase (15 min, Qiagen, UK) was used to eliminate any genomic DNA contamination, and 30 μL of RNase/DNase-free water was added to the final elution of RNA. The concentration and quality of RNA were evaluated spectrophotometrically through NanoDrop (ND-1000, Thermo Fisher Scientific, UK). Each sample was diluted to 100 ng μL^{-1} total RNA, and cDNA was synthesized using 2 μg RNA in 10 μL reaction according to the manufacturer's instructions (Precision nanoScript 2 Reverse Transcriptional Kit, Primer Design, UK). cDNA was stored at − 20 °C prior to gene expression analysis.

Primers for amplification of zebrafish transcripts of cytochrome p450 1a *(cyp1a), metallothionein 2 (mt2),* and *β-actin* (Table 2) have been previously described in Sleight et al., 2017; Henry et al., 2009; Henry et al., 2013. Quantitative Polymerase Chain Reaction (qPCR) was carried out in 20 μL of total volume for each real-time PCR reaction. cDNA was diluted with nuclease-free water (1:25) and mixed with SYBR Green qPCR mastermix (PrecisionPLUS qPCR MasterMix Primer Design, UK) and 300 nM gene-specific primers. Fluorescence was detected over 40 cycles using a PCR machine (StepOne Real-Time PCR System, Applied Biosystems, Warrington, UK) with the following conditions: 2 min of initial enzyme activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and primer-specific annealing at 60 °C for 1 min. All reactions were repeated in triplicate with appropriate no-template controls included in each run. Dissociation analysis was performed within the qPCR run to verify the specificity of the primer pair used.

The efficiency (between 90 and 110%) of the reaction was checked through an evaluation of the slope of the standard curve prepared for each gene transcript for which 10-fold serial dilutions of cDNA were tested. Relative quantification of mRNA transcripts was calculated by comparative C_T method that considers normalization of the change in expression of the gene of interest to that of an internal housekeeping gene. Since no differences in the expression of *β-actin* were observed between treatment and control, including vehicle (DMSO) control, this gene transcript was considered appropriate as an internal reference for all experiments. Data analysis was performed in normalized values to the expression of control treatment groups, subtracting the C_T value of *cyp1a* or *mt2* from that of *β-actin* in the same sample (ΔC_T). The $\Delta\Delta C_T$ value was obtained by subtracting the mean ΔC_T of control groups and the ΔC_T of each sample in all treatment groups. The gene expression fold change was calculated using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001).

2.8. Metagenomic analysis

DNA from microplastic particles was extracted with the Powersoil DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, United States) according to the manufacturer's recommendations. The total nucleic acid extractions from two biofilm samples were quantified using a Qubit HS (high sensitivity) dsDNA kit to prepare the samples for the metagenome sequencing library. The library preparation was achieved

Table 2
Gene-specific primers for cytochrome P450 1a (*cyp1a*), metallothionein 2 (*mt2*), and *β-actin* reference gene used in early life-stage zebrafish. Reference sequence numbers from NCBI and amplicon length in base pair (bp).

Gene	Accession #	Primer	Nucleotide sequence (5′ – 3′)	Amplicon (bp)
cyp1a	NM_131879.2	Forward	AGGACAACATCAGAGACATCACCG	174
		Reverse	GATAGACAACCGCCCAGGACAGAG	
mt2	NM_001131053.2	Forward	TGTTCTCAATCTTGTCTGTTTAATG	108
		Reverse	CATCTCGTGATAGTCTTATTTGC	
β-actin	NM_131031.1	Forward	ACA CAG CCA TGG ATG AGG AAA TCG	138
		Reverse	TCA CTC CCT GAT GTC TGG GTC GT	

using the Swift Biosciences Accel-NGS 2S Plus DNA Kit, per the manufacturer's instructions. Using a Covaris S220 ultrasonicator, fragments were sheared to ~ 450 bp before adding barcodes using the Swift Biosciences 2S dual indexing kit. The subsequent libraries were checked for fragment length using a Bioanalyser DNA 7500 chip, and quantification was performed using a picrogreen fluorescence assay. Libraries were then standardized to 4 nM as verified by qPCR (Kapa Biosystems Library Quantification kit, Applied Biosciences). An equimolar pool of all samples was generated and sequenced on Illumina HiSeq 2500 rapid runs (10–11 pM: V2 rapid sequencing chemistry), yielding reads of 251 bp paired-end sequences. The metagenomic sequence analyses were done using Shotgun sequencing reads, assessed for quality, and adapters were removed using the Trim Galore package (v0.6.4_dev). Low-quality ends were removed from sequences with a Phred score lower than 20. Paired reads were merged using PandaSeq (v2.11), using default settings. The resulting sequences were randomly sub-sampled, which resulted in 2.2 million reads per sample. Metagenome merged reads were de-novo assembled by passing the merged reads into megahit (v1.2.9), thus generating *meta*-contigs. Taxonomic identification of the adapter trimmed reads was performed by comparison to the Maxikraken2 database 38, using the Kraken2 package (v2.0.8_beta) and visualized as Sankey plots using the Pavian Package (Breitwieser, 2016) in R (v1.2.0). Gene annotation of the Meta-contigs was conducted using Prokka (1.13), and these were examined for functional identification using Microbe annotator (v2.0.5) (Seemann, 2014; Ruiz-Perez et al., 2021). All metagenomic sequences generated in this study are available from the NCBI repository under Bioproject PRJNA909009.

2.9. Statistical analysis and data handling

All data presented are means ± standard deviation (SD), and all statistical analyses were performed using R software v. 3.5.0 (R Core Team, 2018). Bioavailability data were tested for normality and homogeneity of variances using the Shapiro-Wilk and Leven's tests, respectively. Statistical differences between datasets were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (for a *p-value* < 0.05, differences were considered significant) to make multiple comparisons.

3. Results

3.1. Sorption of b[a]p or Cd on HDPE-MPs and bioavailability in zebrafish larvae

Significant changes in *cyp1a* and *mt2* expression ($p < 0.001$) were observed in zebrafish larvae after exposure to B[a]P or Cd, respectively, compared to unexposed controls. The sorption of contaminants onto all MPs tested in the present study is shown by reduced *cyp1a* or *mt2* expression in zebrafish larvae exposed to different conditions. Compared to B[a]P exposure without MPs (positive control with only B[a]P – 8.1-fold), mRNA expression of the *cyp1a* gene was significantly reduced (up to 2.4-fold) ($p < 0.05$) when HDPE-MPs were present into the medium (Fig. 2 a). Considering the change in the expression of *cyp1a* in zebrafish larvae exposed at the same concentration of B[a]P, the presence of MPs reduced the bioavailability of B[a]P by up to 70%.

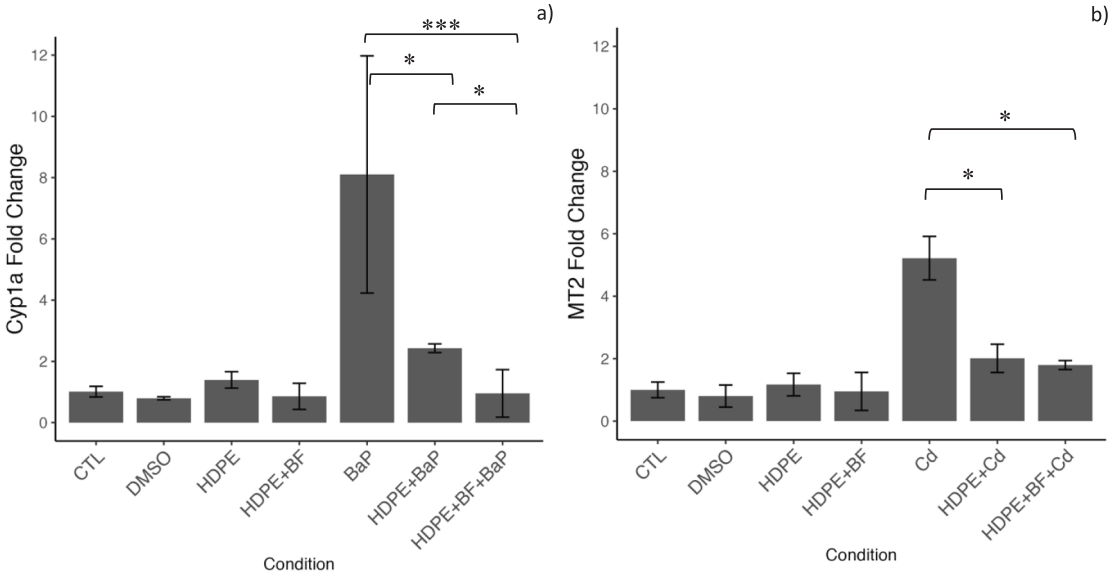

Fig. 2. Relative fold change (FC) in gene expression of a) *cyp1a* and b) *mt2* in ZF larvae exposed to 5 μg L^{-1} of B[a]P and 0.24 mg L^{-1} of Cadmium, respectively, in water, after 24 h pre-equilibrium with 20 mg HDPE-MPs (HDPE + B[a]P or HDPE + Cd), and after 24 h pre-equilibrium with 20 mg HDPE-MPs with 6 days biofilm formation (HDPE + BF + B[a]P or HDPE + BF + Cd). Concentration of HDPE-MPs in suspensions was 500 mg L^{-1}. Expression was normalized to control larvae (CTL) consisting of aquarium ZF media (no HDPE-MPs or contaminant was added). Data are presented as means ± SD (n = 3). Significance differences between exposure groups are indicated by asterisk above error bars (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; one-way ANOVAs with Tukey's test).

However, zebrafish larvae exposed to HDPE-MPs with biofilm attached on their surface showed a reduction of B[a]P bioavailability by up to 88% and a *cyp1a* gene expression more significantly reduced ($p < 0.001$) than B[a]P exposure without MPs. A similar trend was observed for Cd exposure, where the reduction in Cd bioavailability in zebrafish larvae co-exposed to HDPE-MPs and Cd was 61%. Here, a significant decrease in *mt2* mRNA expression ($p < 0.05$) compared to a positive control (only Cd without HDPE-MPs) was found (Fig. 2 b). Similarly, co-exposure to HDPE-MPs with an associated biofilm and Cd resulted in a decrease of Cd bioavailability, as emerged by the significant reduction of *mt2* mRNA expression ($p < 0.05$) found than where there was no HDPE-MPs (only Cd control). For both individual contaminants tested, no significant change in the expression of *cyp1a* or *mt2* in zebrafish larvae exposed to HDPE-MP alone or HDPE with biofilm attached was observed ($p > 0.05$).

3.2. Sorption of b[a]p and Cd mixture on HDPE-MPs and bioavailability in zebrafish larvae

The co-exposure of B[a]P and Cd was evaluated about each single toxicant exposure used as positive controls by assessing the expression levels of biomarker gene transcripts for these toxicants (*cyp1a* and *mt2*, respectively) in larval zebrafish at 72 hpf. However, although *cyp1a* and *mt2* were commonly employed as specific biomarkers of organic xenobiotics (Batel et al., 2016; Sleight et al., 2017) and metals exposures (Henry et al., 2013), respectively, a cross analysis between both specific biomarkers for the mixtures of these contaminants was required. The expression of biomarker genes (*cyp1a* and *mt2*) related to each individual chemical treatment (B[a]P and Cd, respectively) was comparable to what was observed previously (Fig. 2 a and b; Fig. 3 a and b). Considering *cyp1a* gene expression (Fig. 3 a), for both individual contaminants, *cyp1a* mRNA expression was significantly reduced in presence of HDPE-MPs (HDPE + B[a]P, $p < 0.001$; HDPE + Cd, $p < 0.05$ respectively), if compared to respective positive controls (treatments without HDPE-MPs). Based on changes in the expression of *cyp1a,* the presence of HDPE-MPs reduced the bioavailability by up to 57% for Cd. While, a significant increase of *cyp1a* expression ($p < 0.05$, 3.79-fold) was detected for combinatorial exposure to HDPE-MPs with biofilm and Cd (HDPE + BF + Cd) than to same exposure without biofilm (HDPE + Cd, 1.59-fold). Co-exposure with Cd and B[a]P led to a significant reduction of *cyp1a* expression (3.18-fold, $p < 0.05$) in comparison to individual treatment with B[a]P (7.79-fold) (Fig. 3 a). Unlike the reduction of the expression level of *cyp1a* biomarker observed for each contaminant associated with HDPE-MPs (HDPE + Cd and HDPE + B[a]P) than related positive controls (only Cd and B[a]P, respectively), no relevant change in bioavailability was observed for the mixture (HDPE + Cd + B[a]P) compared to the mixture without MPs. Finally, a significative difference for treatment groups that differed for the presence of biofilm (HDPE + BF + B[a]P + Cd and HDPE + Cd + B[a]P) emerged. In particular, a significative reduction in *cyp1a* expression ($p < 0.01$) was observed for the mixture in presence of biofilm (HDPE + BF + B[a]P + Cd, 1.46-fold) if compared to the same treatment without biofilm (HDPE + B[a]P + Cd, 4.43-fold) or to single exposure with Cd, HDPE-MPs and biofilm (HDPE + BF + Cd, 3.-fold). Instead, no change in bioavailability was observed from the comparison between single exposure with B[a]P and the mixture Cd/B[a]P in presence of HDPE-MPs and biofilm (HDPE + BF + B[a]P and HDPE + BF + B[a]P + Cd).

No change in *mt2* mRNA expression was measured in zebrafish larvae exposed to single B[a]P treatment, while a strong induction of biomarker expression (4.98-fold) resulted after Cd treatment (Fig. 3 b). However, a significant reduction ($p < 0.01$) was found after Cd/B[a]P co-exposure if compared with individual treatment with Cd. For all treatments involving individual exposure to B[a]P (HDPE + B[a]P and HDPE + BF + B[a]P), no differences were detected in comparison to negative and positive control. In contrast, for co-exposure with B[a]P and Cd in combination with HDPE-MPs (HDPE + Cd + B[a]P), an induction of *mt2* transcript comparable to individual Cd treatment

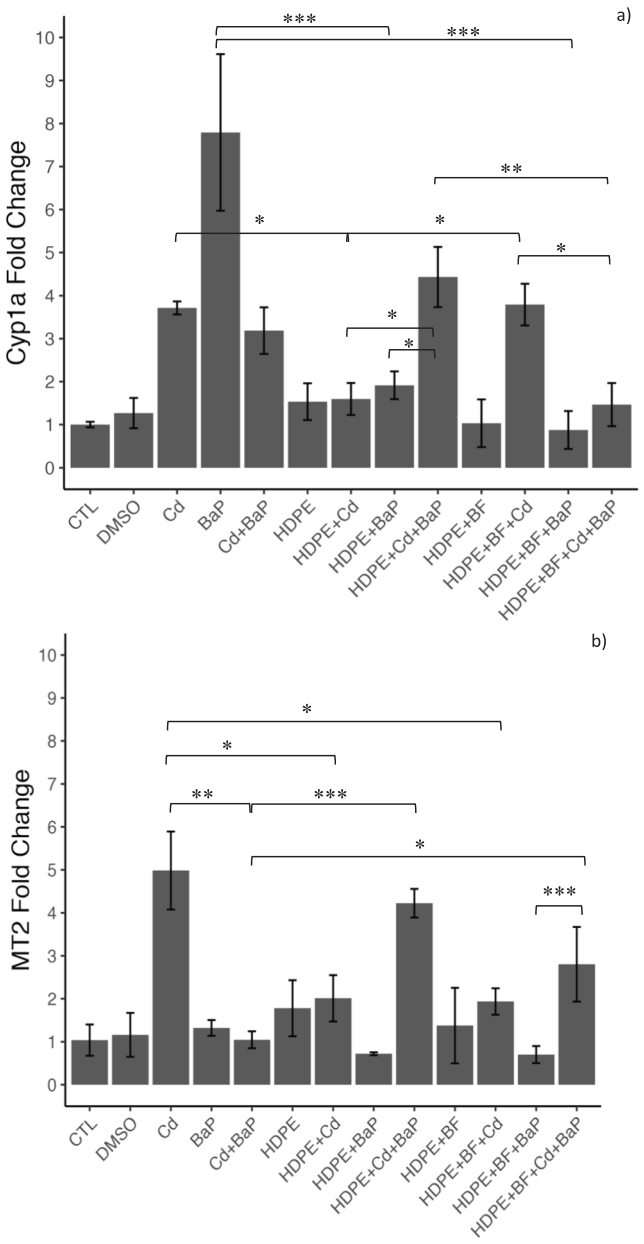

Fig. 3. Relative fold change (FC) in gene expression of a) *cyp1a* and b) *mt2* in ZF larvae exposed to 5 μg L^{-1} of B[a]P or 0.24 mg L^{-1} of Cd or a combination of B[a]P and Cd, in media, after 24 h pre-equilibration with 20 mg HDPE-MPs (HDPE + B[a]P or HDPE + Cd or HDPE + B[a]P + Cd), and after 24 h pre-equilibration with 20 mg HDPE-MPs with 6 days biofilm attached (HDPE + BF + B[a]P or HDPE + BF + Cd or HDPE + BF + B[a]P + Cd). Concentration of HDPE-MPs in suspensions was 500 mg L^{-1}. Expression was normalized to control larvae (CTL) consisting of aquarium ZF media (no HDPE-MPs or contaminant was added). Data were presented as means ± SD (n = 3). Significance differences between exposure groups are indicated by asterisk above error bars (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; one-way ANOVAs with Tukey's test).

(positive control) was observed. Significant induction of *mt2* mRNA expression ($p < 0.05$) also emerged from Cd/B[a]P treatment in the presence of HDPE-MPs and biofilm (HDPE + BF + Cd + B[a]P, 2.8-fold) than positive control of mixture (Cd + B[a]P). However, such induction was reduced compared to that detected after exposure to the mixture of contaminants with HDPE-MPs but without biofilm (HDPE + Cd + B[a]P, 4.2-fold). Finally, from the comparison between treatment groups that differed for Cd presence (such as HDPE + BF + B[a]P and HDPE + BF + Cd + B[a]P), a significant induction was observed ($p < 0.001$) (Fig. 3 b).

3.3. Taxonomic analysis and metabolic profiling of biofilm lining the HDPE-MPs

Taxonomic assignment of shotgun metagenomic merged reads revealed that the biofilm lining the HDPE-MPs (HDPE + BF) was mainly composed of Bacteria (95.6%). In contrast, only a small fraction of reads were associated with Eukaryota (2.2%), Archaea (1.9%), and Viruses (0.2%) (Fig. 4 a). The microbial community was composed of the majority of the phylum *Proteobacteria* (77%), followed by *Actinobacteria* (15.8%), *FCB-Bacteria* (2.2%), *PVC-Bacteria* (1.8%), *Nitrospirae* (1.2%) and *Firmicutes* (1.1%), which appeared less represented (Fig. 2.6b). The phyla with < 1% of total reads were grouped as Others (0.6%), while Unclassified represented only 0.04%. Three classes of *Proteobacteria* (*Alpha, Beta, and Gamma*) were identified, but among them, the order of *Burkholderiales,* belonging to *Betaproteobacteria* class, was the most represented (Fig. 4 c). In contrast, the taxonomic characterization related to the *Actinobacteria* phylum highlighted *Actinobacteria* as the most predominant class, with *Corynebacteriales* as the most abundant order (Fig. 4 d). Also, the Sankey diagram related to the microbial community composition based on relative species abundance for the two samples analyzed showed that *Betaproteobacteria* and *Actinobacteria* were the most numerically abundant group in the community (Figure S1).

Metabolic pathway prediction through MicrobeAnnotator (Figure S2) clustered the majority of reads in the "Biosynthesis of other secondary metabolites, "Drug resistance", "Enediyne biosynthesis", "Macrolide biosynthesis" and "Type II polyketide biosynthesis". Note the high similarity in metabolic pathways exhibited by both genomes analyzed. To analyze the metabolic potential of the microbial community, in the heatmap showing module completeness for specific pathways, the completeness level of all modules recovered is identical or almost identical for the examined genomes (Figure S3). Barplots of modules with completeness above 80% grouped by the category (pathway) showed a high similarity in metabolic pathways by genomes analyzed. No differences were evident due to overall similar metabolic potential encoded.

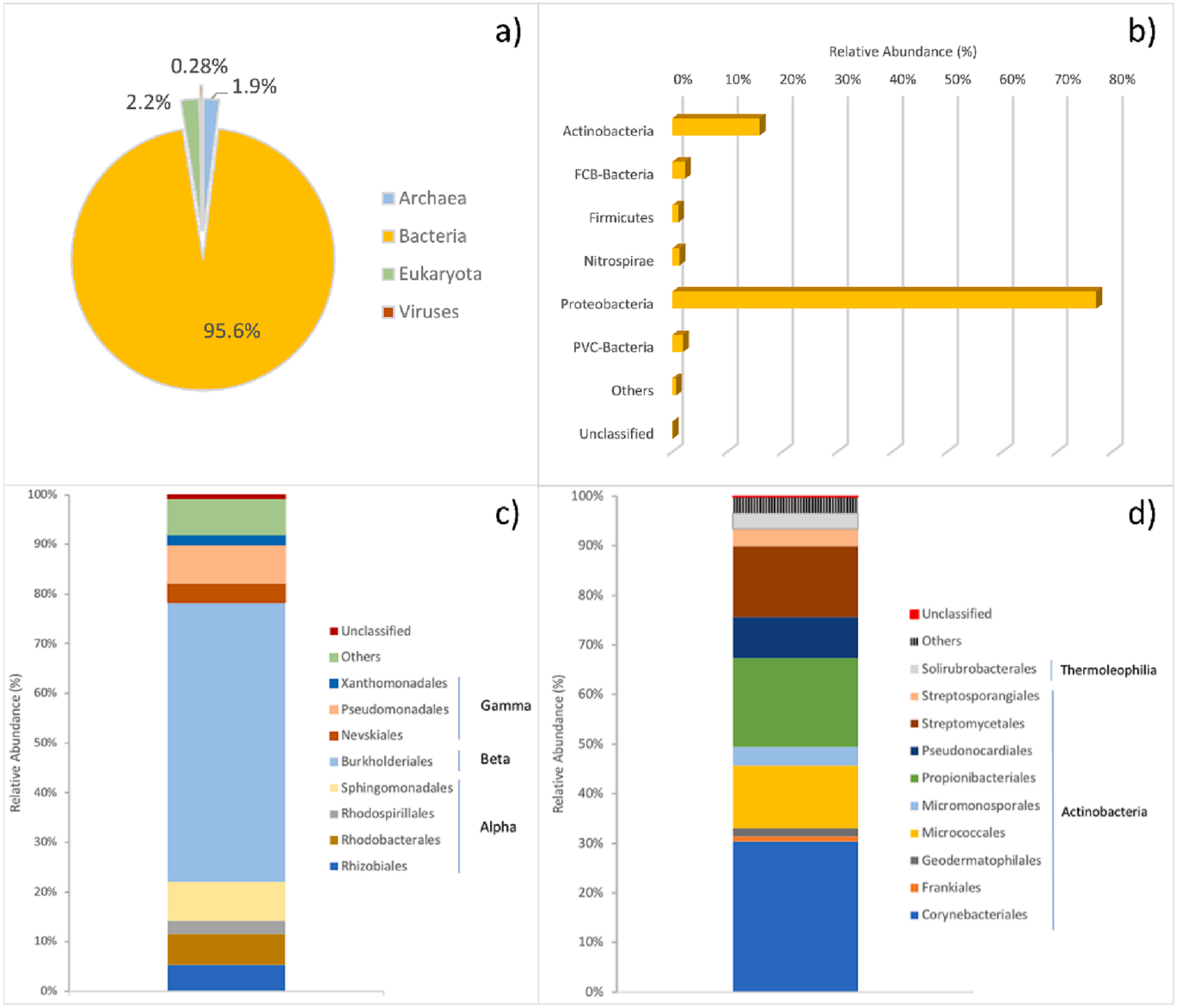

Fig. 4. Taxonomic profiles of the biofilm lining the HDPE-MPs (HDPE + BF). a) Composition at domain level obtained by shotgun analysis; b) Bacterial composition at phylum level inferred from shotgun analysis. For lower values (<1%), the sum of the relative percentages of phyla and orders is indicated as "Others". Relative composition related to the predominant phyla *Proteobacteria* c) and *Actinobacteria* d) showed as Relative Abundance (%) and classified by class and order levels. Only the percentage values>1% of the total reads obtained considered as specific phyla b) or orders c, d) in the figures.

4. Discussion

In this study, the microbial composition of microplastic-associated biofilms and their influence on the sorption by MPs of two different chemicals, Cd and B[a]P, under individual and mixture conditions, was investigated by evaluating changes in co-contaminant bioavailability in larval zebrafish with a multi-biomarkers approach. Results provided evidence that MP-associated biofilms selectively influenced co-contaminant bioavailability to zebrafish larvae when substances were in a mixture and that there is a relationship between sorption behaviour and the composition of microorganisms in biofilms.

We have observed that the individual exposure to B[a]P and Cd, each with HDPE-MPs, resulted in a lower co-contaminant bioavailability to zebrafish larvae compared to the treatment group of each individual co-contaminant where there were no MPs, indicating that a sorption process of each contaminant on MPs has occurred. The MPs and their characteristics (i.e., surface topography or surface area charge) are expected to be the same for all contaminant exposures. Although zebrafish co-exposure to MPs and co-contaminants resulted in similar B[a]P and Cd bioavailability patterns, based on gene expression data, the presence of MPs reduced B[a]P and Cd bioavailability by up to 70% and 61%, respectively. Thus, B[a]P appeared more strongly associated with the HDPE-MPs than Cd. As described in Sleight et al., (2017), differences in response profiles of different specific biomarker genes may be related either to differences in our ability to detect changes in bioavailability of each co-contaminant, or differences in physico-chemistry characteristics of the latter. Therefore, the different bioavailability detected could be ascribable to differences in contaminants physico-chemistry that influence sorption by MPs. In addition, the concentrations of B[a]P and Cd were different (5 μg L^{-1} and 0.24 mg L^{-1}, respectively), and each specific biomarker gene could show differences in response profile (i.e., expression of *cyp1a* may be more sensitive than the expression of mt2 or simply, B[a]P may be more toxic than Cd). Thus, the greater sorption of B[a]P to MPs indicated by lower relative bioavailability (*cyp1a* expression) may be associated with a lower concentration of B[a]P present compared to Cd. The stronger association of B[a]P with MPs compared to Cd may depend on polymer and contaminants' physico-chemistry properties. Since most microplastic polymers found in the environment are hydrophobic structures, the hydrophobic interactions between non-polar (or slightly polar) molecules to the non-polar MP surface are considered one of the main mechanisms governing the sorption of hydrophobic organic chemicals to MPs (Wang et al., 2015; Tourinho et al., 2019). Although plastics are generally considered inert, medium conditions and/or plastic additives can alter their surface, leading to electrostatic interactions (Liao and Yang, 2020). It is known that the MPs' surface can become negatively charged due to the pH of point of zero charge (pHpzc) being lower than most environmental pH (Liu et al., 2018; Xu et al., 2018; Zhang et al., 2018). As a result, the negative net charges on the MP surface are likely to attract positively charged species (Tourinho et al., 2019). So, overall, electrostatic interactions may have led to Cd association with MPs, albeit a predominant effect of hydrophobic interactions on electrostatic interactions could be hypothesized.

A lower co-contaminant bioavailability to zebrafish larvae resulted after short-term B[a]P and Cd sorption to MPs with an associated biofilm. However, as B[a]P appeared more strongly associated with HDPE-MPs with biofilm than Cd, this result seemed to be in contrast with previous reports, where the development of biofilms in aquatic environments enhanced metal sorption and stabilization capacity onto microplastics (Wang et al., 2021; Guan et al., 2020). Over time, as biofilms continuously accrue to the surface of plastic, the sorption of metals is likely continually enhanced due to alteration of the physical and chemical properties of plastic surfaces. (Tien and Chen, 2013). Different studies reported that the presence of biofilms generally increases the charge, roughness, porosity, and hydrophilicity of the plastic surface (Artham et al., 2009, Wang et al., 2020; Rummel et al., 2017). As a consequence, the presence of hydrophilic groups that are associated with a lower hydrophobicity limits the enrichment of organic molecules (i.e., B[a]P) through hydrophobic interactions (Liu et al., 2019). Here, biofilm formation occurring in 6 days was not enough to physically alter the plastic's surface. However, the stronger sorption to MPs of B[a]P compared to Cd could be related to additional interactions (such as Van der Waals forces, π-π, and hydrogen bonding) promoting the sorption of organic contaminants besides hydrophobic dominating interaction. This suggests that the bioavailability of co-contaminants sorbed to MPs cannot be predicted by co-contaminant physico-chemistry alone. Our results indicate that the sorption is influenced by sorptive biofilm properties able to promote additional interactions between sorbent (microplastic polymer) and sorbate (chemical contaminant) and to generate additional active sites for sorption of B[a]P that, consequently, reduce its bioavailability in aqueous phase.

The presence of several contaminants simultaneously in the aquatic environment can produce interactive effects between toxicants resulting in changes in their bioavailability compared to individual exposure. It has been shown that several contaminants, usually present simultaneously in the aquatic environment, may produce either neutralizing, additive, or synergistic effects in biota, thus resulting in variable degrees of toxicity (Di Natale et al., 2019; Ragusa et al., 2017). The short-term interactive results of Cd and B[a]P co-exposure were generally in accordance with previous studies (van den Hurk et al., 2000; Costa et al., 2010; Risso-De Faverney et al., 1999). Simultaneous co-exposure to these contaminants suppressed the induction of *cyp1a* gene expression caused by B[a]P exposure alone (Sorrentino et al., 2005). As in the present study, inhibitory influences on fish MT responses have been recorded by several other investigators after B[a]P and Cd mixed exposure (Sandvik et al., 1997). These results suggest that the co-occurrence of different stressors, which simultaneously trigger different pathways, may result in gene expression profiles of *cyp1a* and *mt2* slightly related to those activated by a single contaminant at a time. Moreover, regulatory mechanisms can act differentially in protecting larvae from increased oxidative stress activated by a single contaminant (Di Natale et al., 2022).

The evaluation of co-contaminant sorption by MPs in exposure mixtures through a multi-biomarkers approach permitted evaluation changes in co-contaminant bioavailability from a broader point of view in relation to a specific exposure condition. For instance, in the B[a]P and Cd mixed exposure with MPs (HDPE + Cd + B[a]P) different bioavailability patterns between *cyp1a* and *mt2* emerged. Indeed, although the expression of *cyp1a* did not highlight a reduction in co-contaminants bioavailability compared to the positive control (Cd + B[a]P), an opposite pattern was observed for both co-contaminants' bioavailability by assessment of *mt2* expression. This opposite trend, consisting of *mt2* transcript induction, could most likely be due to the co-contaminants' different sorption capacity. The chemical fraction analysis related to both contaminants sorbed to HDPE microparticles was not used in this study due to difficulty detecting such low concentrations. It was not the objective of this study to investigate the toxicity nor bioaccumulation of B[a]P, Cd, and MPs or mixture toxicity of these substances. However, previous studies have demonstrated that HDPE tended to accumulate lesser concentration of metals (including Cd) than other plastic types (Rochman et al., 2014). Therefore, based on the bioavailability data of this study, B[a]P may be more strongly associated with the MPs than Cd in mixture exposure. The lower sorption of Cd to MPs indicated by the higher relative bioavailability (*mt2* expression) may be a consequence of a competition mechanism that occurred between both co-contaminants. Thus, by competing with metal ions for the adsorbing sites, the organic contaminant (i.e., B[a]P) may reduce plastic polymer sorption sites available for Cd interactions. Some studies focused on the evaluation of competitive sorption of different co-contaminants by MPs suggested that in general the solute with the greater sorption affinity for a given sorbent can be strongly competitive against the solute having lower sorption affinity (Bakir et al., 2012; Yu and Huang, 2005). Thus, the competitive sorption behavior of B[a]P

onto Cd can be explained by its higher affinity for plastics. This suggests that the short-term co-presence of different contaminants simultaneously may influence their bioavailability to larval zebrafish and that the fate and transport of co-contaminants such as Cd could be affected in the presence of other organic contaminants in aquatic system, thus representing a greater potential concern in the marine environment.

The multi-biomarkers approach focused on the study of the bioavailability pattern that arose from simultaneous exposure to both co-contaminants and MP-associated biofilm, allowed to provide new knowledge about the sorption of different types of contaminants by MPs as well as how their sorption behaviour is affected by biofilm. The reduced co-contaminants bioavailability shown after exposure to Cd and B[a]P mixture in presence of MP-associated biofilm, led to a reduced co-contaminants bioavailability compared to treatment groups without biofilm, as emerged by *cyp1a* and *mt2* gene expression data. Strong sorption of both contaminants by MPs may be suggested, given the lower co-contaminants bioavailability detected by a reduction in *cyp1a* expression. However, the high *mt2* induction observed may likely be due to the weak association of Cd by MP compared to B[a]P. Thus, in fish larvae co-exposed to the mixture of Cd and B[a]P, biofilm presence could promote B[a]P sorption, reducing the availability of sorption sites for Cd interactions. As result, a higher Cd bioavailability may have occurred. Additionally, although in individual Cd exposure, the presence of an associated biofilm determined a reduction in Cd bioavailability to larval zebrafish, the co-occurrence of both contaminants differentially influenced its bioavailability. Therefore, consistent with other findings related on the study of influence of biofilms on the adsorption of different pollutants onto MPs, although MP-associated biofilm enhanced or decreased sorption compared with biofilm-absent MPs, the sorption on MPs of different class of contaminants is mainly governed by the co-contaminants physicochemical properties whose determine their final sorption behavior and mechanisms on MPs (Zhang et al., 2022; He et al., 2022). Therefore, the data herein presented suggested that when plastic materials are exposed to different mixtures of chemical pollutants, the interactions between them may result in patterns of bioavailability not overlapping those arose by a single contaminant. This means that contaminated MPs might accumulate higher amounts of several contaminants from polluted areas but, due to biofilm presence that continuously accrues to the surface of plastic over time, could become potentially bioavailable to aquatic animals in clear areas.

Further analyses with the high-precision chemical determination of concentrations of adhering chemicals will assist in concluding whether contaminants' bioavailability through microplastics poses a significant exposure pathway to biota in natural environments. Longer exposure times would be required to investigate the long-term effect of biofilm and the bioavailability of these contaminants. Moreover, since the different plastic surfaces may have various sorption capacities for different metal ions (Li et al., 2019), an assessment of the interactive effects of B[a]P with other metals on the HDPE-MPs matrix could be important.

The characterization of the MP-associated biofilm used in this study carried out through shotgun metagenome sequencing showed the massive prevalence of organisms from the group of Bacteria. Consistent with previous studies (Vaksmaa et al., 2021; Basili et al., 2020; Turrini et al., 2020), *Proteobacteria* and *Actinobacteria* are the predominant phyla, both comprising bacteria with high diversity in relation to the number of classes and orders. Among the microbial community, we identified several genera previously described to include hydrocarbon-degrading bacteria (HCB), dominantly belonging to the *Burkholderiales* order, some of which were abundant, reaching around 4% of all sequencing reads. These included *Mycolicibacterium*, *Nocardioides*, *Streptomyces*, *Nitrospira*, *Aquabacterium*, *Acidovorax*, *Variovorax*, *Solimonas*, *Perlucidibaca*, *Pseudomonas*, *Polaromonas*, *Limnohabitans*, *Hydrogenophaga*, *Sphingobium,* and *Sphingomonas*. HCB use linear, branched, or aromatic hydrocarbons as the sole energy and carbon source and are often found in oil reservoirs, oil seeps, or oil spills (Joye et al., 2014), contaminated sediments (Kimes et al., 2013) and coal beds (Beckmann et al., 2019), but they are also found ubiquitously in the marine environment (Yakimov et al., 2007; Erni-Cassola et al., 2020; Thompson et al., 2020). HCB comprises > 175 genera (Prince et al., 2010) and features enzymes such as mono- and dioxygenases or peroxidases (Brzeszcz and Kaszycki, 2018). In principle, these might be able to attack the primary polymer structure of plastic and/or further degrade daughter products generated through weathering of the primary polymers (Waymana and Niemann, 2021). Previous studies have found HCB on different polymer types (Zettler et al., 2013; Oberbeckmann et al., 2016; Erni-Cassola et al., 2020) as well as a higher abundance of them in the early stages of biofilm formation (Dussud et al., 2018; Erni-Cassola et al., 2020). Microbial degradation of PAHs depends on various environmental conditions, such as nutrients, number, and kind of microorganisms, nature as well as the chemical property of the PAH being degraded. Identifying abundant hydrocarbon degraders leads to considering their potential role within biofilm in influencing benzo[a]pyrene bioavailability to zebrafish larvae. The metabolic predictions inferred from shotgun metagenomic data grouped the metabolic modules with completeness above 80% in biosynthesis pathways, specifically "Biosynthesis of other secondary metabolites, "Drug resistance", "Enediyne biosynthesis", "Macrolide biosynthesis" and "Type II polyketide biosynthesis." Other modules with completeness above 50% highlighted clustered in "aromatic degradation" pathway. Overall, this metabolic information could support the hypothesis that the biofilm microbial composition on HDPE-MPs included microbial organisms able to metabolize chemical compounds, such as hydrophobic hydrocarbons. This result suggested that biofilm's microbial (Archaea, Bacteria, and Fungi) diversity in addition to influencing the co-contaminant sorption process, may exhibit a capacity for the biodegradation of polycyclic aromatic hydrocarbons sorbed on MPs. Thus, consistent with Jin et al. (2020), microplastics' co-contaminant transfer role may essentially be determined by interactions between attached contaminants and microbial composition, which are further related to bacterial activity and contaminant characteristic. Therefore, although the microbial composition of HDPE-MPs biofilm may have enhanced B[a]P sorption in a simultaneous exposure with Cd, consequently reducing B[a]P bioavailability, colonizing microorganisms can degrade MP-sorbed polycyclic aromatic hydrocarbons (PAHs) generating degradation intermediates that in turn may lead to simultaneous variations of MP-attached contaminants and microorganisms.

5. Conclusion

The results of this study highlight how in complex exposure scenarios characterized by a mixture of different co-contaminants, the MP-associated biofilm selectively influenced co-contaminants bioavailability to zebrafish larvae. The applied exposure scenarios highlighted that multilateral and additional factors such as a mixture of different contaminants, polymer and chemical properties, and micro-surroundings of the organisms may affect contaminants' bioavailability. Here, the effects on bioavailability are attributed only to bioavailable B[a]P and Cd fractions released from the MPs and not the particles themselves since only early-life stage and not free-feeding zebrafish were considered. This allowed us to evaluate the influence of biofilm on co-contaminants bioavailability, disregarding the possibility of having environmentally unrealistic exposure concentration able to induce any morphological, physiological, or developmental disturbances. The multi-biomarker approach proved useful in evaluating changes in co-contaminant bioavailability from a dual point of view for a specific chemical. Based on the bioavailability data of this study, the influence of biofilm in promoting B[a]P sorption and Cd bioavailability have been demonstrated, suggesting that, in the presence of other organic contaminants, the transfer of inorganic co-contaminants (i.e. Cd) to the aquatic environment may be highly affected, thus

representing a potential concern for the marine environment. The microbial community forming biofilm may play a pivotal role in affecting co-contaminants' bioavailability as a wide range of microorganisms within a biofilm are able to degrade hydrophobic organic sorbed compounds by serving as a nutrient source. Thus, although the development of biofilm enhanced B[a]P sorption in a short-time simultaneous exposure with Cd, changes in plastic's physicochemical properties, generation of PAHs biodegradation intermediates and desorption process biofilm-induced may occur over time, differentially influencing co-contaminants bioavailability with unpredictable ecotoxicological effects in the long term. In this context, to predict a more realistic scenario for environmental risk assessment of MP, the influence on uptake and release of different chemicals into aquatic phase metabolic microbial pathway-mediated need to be also considered. Additional research on colonization processes and relationships between the sorption behavior of different pollutants, and of different mixtures of them are needed to fully understand the role of biofilms in contaminant transfer from microplastics to the environment.

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

The original contributions presented in the study are publicly available. The datasets concerning biomarker expression are available via: Di Natale et al., 2023 on IMIS of Flanders Marine Institute (VLIZ).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.110369.

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