Biomarker responses in zebrafish (*Danio rerio*) following long-term exposure to microplastic-associated chlorpyrifos and benzo(k)fluoranthene

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Abstract

Continuously increasing plastic production causes a constant accumulation of microplastic particles (MPs) in the aquatic environment, especially in industrialized and urbanized areas with elevated wastewater discharges. This coincides with the release of persistent organic pollutants (polycyclic aromatic hydrocarbons (PAHs), pesticides) entering limnic ecosystems. Although the assessment of potential effects of environmental pollutants sorbed to MPs under chronic exposure scenarios seems vital, data on potential hazards and risk by combined exposure to pollutants and microplastics for aquatic vertebrates is still limited. Therefore, zebrafish (*Danio rerio*) were exposed over 21 days to the organophosphate insecticide chlorpyrifos (CPF; 10 and 100 ng/L) and the PAH benzo(k)fluoranthene (BkF; 0.78 and 50 µg/L) either dissolved directly in water or sorbed to different MPs (irregular polystyrene, spherical polymethyl methacrylate; ≤ 100 µm), where CPF was sorbed to polystyrene MPs and BkF was sorbed to polymethyl methacrylate MPs. Contaminant sorption to MPs and leaching were documented using GC-EI-MS; potential accumulation was studied in cryosections of the gastrointestinal tract. Enzymatic biomarkers and biotransformation were measured in liver and brain. Overall, exposure to non-contaminated MPs did not induce any adverse effects. Results of fluorescence tracking, CYP1A modulation by BkF as well as changes in acetylcholinesterase activity (AChE) by CPF were less pronounced when contaminants were sorbed to MPs, indicating reduced bioavailability of pollutants. Overall, following exposure to waterborne BkF, only minor amounts of parent BkF and biotransformation products were detected in zebrafish liver. Even high loads of MPs and sorbed contaminants did not induce adverse effects in zebrafish; thus, the potential threat of MPs as vectors for contaminant transfer in limnic ecosystems can be considered limited.

1. Introduction

Microplastic particles (MPs) have been detected ubiquitously at variable amounts in virtually every compartment of aquatic ecosystems (Alimi et al., 2017; Hidalgo-Ruz et al., 2012; Klein et al., 2015; Triebskorn et al., 2019). The exponential increase in plastic production and inadequate waste management are leading to millions of tons of plastic debris (Geyer et al., 2017; Jambeck et al., 2015; PlasticsEurope, 2018), which is rapidly discharged into aquatic systems as macro- or microplastics (GESAMP, 2015; Geyer et al., 2017). As a consequence, not only MPs themselves might pose a risk to aquatic organisms, but also environmental pollutants unintentionally sorbed onto these particles from the surrounding environment. Thus, the binding of e.g., polycyclic aromatic hydrocarbons (PAHs) and pesticides to MPs in aquatic ecosystems may be unintentionally promoted. Sorption of complex contaminant mixtures to MPs prior to release into aquatic systems has already been investigated in several studies, indicating a potential transport of sorbed contaminants into aquatic organisms *via* ingestion (Chen et al., 2019; Pannetier et al., 2019a; Rochman et al., 2013a).

The occurrence of the PAH benzo(k)fluoranthene (BkF) in aquatic ecosystems is well documented and the contaminant evidentially adsorbs to MPs (2 ng/g - 119 µg/g plastic; Pannetier et al., 2019a; Rochman et al., 2013a; Wang et al., 2020), it was chosen in the present study as a model contaminant. Another environmentally relevant contaminant is the organophosphate insecticide chlorpyrifos (CPF), which has been detected in surface waters at concentrations of 1 - 46 ng/L, with the global application being focused on fruit and vegetable treatment (EFSA, 2019; Ensminger et al., 2011; Tousova et al., 2017; Witczak et al., 2018).

Since both model contaminants (BkF, CPF) regularly occur in surface water and given the emergence of MP hotspots, interactions between these compounds and MPs in aquatic ecosystems are likely. Irregular fragments of the packaging material polystyrene (PS) and spherical beads of the polymethyl methacrylate (PMMA) from automotive and computer industries (PlasticsEurope, 2018) were used in the present study. Both types of MPs have repeatedly been documented in environmental samples (Hermsen et al., 2017; Klein et al., 2015; Koelmans et al., 2019). Two different shapes of MPs were chosen to represent the two largest fractions of detected MPs in surface waters (Klein et al., 2015).

Previous studies reported contradictory effects of MPs at different biological levels (Ašmonaite et al., 2018; Jovanović et al., 2018; Karami et al., 2017; Khan et al., 2015; Pannetier et al., 2020; Rochman et al., 2013b), hence, it seems vital to investigate the consistency of biological responses to the exposure with MPs under scenarios more representative of environmental conditions, i.e., prolonged and continuous exposure scenarios with chronic exposure levels (Santana et al., 2018).

The aim of the present study was, therefore, to consolidate the evidence for the transfer of hazardous organic pollutants by MPs and for potential adverse effects in limnic vertebrates. To this end, adult zebrafish (*Danio rerio*) were chronically exposed to low and high concentrations of BkF (0.78 µg/L and 50 µg/L) and CPF (10 ng/L and 100 ng/L) either dissolved in water or sorbed to PMMA or PS (≤ 100 µm), respectively. Adsorption and fate of the contaminants were documented *via* gas chromatography with an electron ionization source (GC-EI-MS) and MP uptake and BkF transfer were investigated microscopically in gastrointestinal cryosections. As the elevated lipophilicity of BkF and CPF (log K_{OW} 6.11 and 4.96, respectively) suggests an increased potential for accumulation and bioconcentration in tissues of *Danio rerio* (Barranco et al., 2017; El-Amrani et al., 2012), ultra performance liquid chromatography coupled with fluorescence detection and mass spectrometry (UPLC-FLD/MS) was used to measure tissue concentrations and to identify potential formation of metabolites in target organs in zebrafish. Biological effects were assessed by selected enzymatic biomarkers (AChE activity, CYP1A induction). Modulation of the activity of the biotransformation enzyme 7-ethoxyresorufin-*O*-deethylase (EROD) in liver homogenates derived from model organisms such as zebrafish (*Danio rerio*) can be used to determine the catalytic activity as a measure of the rate of CYP1A-mediated changes in metabolic activities after exposure to both MP-associated BkF and BkF freely dissolved in water (Hanslik et al., 2020).

Effects of most organophosphate insecticides are based on the inhibition of the membrane-bound enzyme acetylcholinesterase (AChE; Rodríguez-Fuentes et al., 2015). CPF leads to enzyme inhibition by covalent binding to serine -OH groups and subsequent accumulation of acetylcholine within the synaptic cleft, and finally results in impaired neurotransmission (Colovic et al., 2013; Russom et al., 2014). Various studies have shown organophosphate insecticides to not only be metabolized by cytochrome P450 enzymes, but also to potentially inhibit biotransformation enzymes (Kais et al., 2017;

Rodríguez-Fuentes et al., 2015). Therefore, samples from BkF and CPF exposure were also analyzed with regard to CYP450 activity in the EROD assay (Kais et al., 2018, 2017).

2. Materials and Methods

2.1 Materials and Chemicals

All chemical reagents were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany). Stock solutions were prepared for BkF (2 g/L in DMSO in a pre-saturated glass vial) and CPF (3 g/L in DMSO), covered in aluminum foil, stored at 4 °C. Otherwise, all solutions were prepared directly prior to use.

Two different types of microplastic (≤ 100 µm) were used for the experiments with an initial concentration of 1×10^6 particles/L. For experiments with BkF, unstained spherical beads of PMMA (density 1.19 g/cm³; Goodfellow, Cambridge, England) were applied at 0.016 g/L. For experiments with CPF, PS (density 1.04 g/cm³; INEOS Styrolution, Frankfurt, Germany) stained for red fluorescence (0.05 % Hostasol Red 5B) was used at a concentration of 0.16 mg/L.

2.2 Zebrafish (*Danio rerio*) husbandry and contaminant exposure

Adult wild-type zebrafish (*Danio rerio*) aged 18 - 24 months from the "Westaquarium" strain were obtained from the fish facility at the Aquatic Ecology and Toxicology Group. Fish breeding and maintenance have been licensed by regional animal welfare authorities under reference 35e9185.64/BH Braunbeck. All experiments with adult zebrafish were specifically licensed by the animal welfare committee of the Regional Council of Karlsruhe, Germany (35-9185.81/G-122/15). For general information on fish breeding and maintenance condition, see Lammer et al. (2009).

For exposure to CPF and BkF, fish were held in 5 and 10 L glass aquaria, respectively, at a 14:10 h light:dark cycle in active-carbon-filtered natural tap water (pH 8.1 ± 0.05) at 25 ± 0.5 °C. Exposure followed a semi-static scenario with 50 % daily water exchange, which guaranteed that ammonia, nitrite and nitrate concentrations were kept below threshold values (5 mg/L, 1 mg/L and 140 mg/L,

117 respectively; Lawrence, 2011; Matthews et al., 2002), see Supplemental Information 1. In the case of
118 the BkF positive controls, water was exchanged twice daily to prevent BkF loss through sorption to
119 aquaria glass walls.

120 Fish were fed at a daily ration of 1 % of wet weight with TetraMin™ flakes (Tetra, Melle, Germany),
121 distributed on two feedings. Particular care was taken to prevent adsorption of contaminants to residu-
122 al feces particles and MP accumulation by scraping surfaces before water exchange and allowing par-
123 ticles and feces to settle (approx. 60 min) before siphoning the remnants twice.

124 The experimental setup and treatment groups were identical for BkF and CPF: (1) one negative control
125 (without both MP and substance); (2) one control with non-contaminated, pristine MPs; (3) positive
126 controls with 0.78 µg/L and 50 µg/L waterborne BkF or 10 ng/L and 100 ng/L waterborne CPF (sub-
127 stance-only control); and (4) two treatment groups (with BkF or CPF sorbed to MPs at two different
128 loading concentrations). For the latter treatment groups, MP suspensions with either 0.78 µg/L and 50
129 µg/ BkF sorbed to PMMA or 10 ng/L and 100 ng/L CPF sorbed to PS were freshly prepared 48 h be-
130 fore application. Concentrations always refer to nominal values unless stated otherwise.

131 For BkF experiments, groups comprised 21 randomly allocated zebrafish, resulting in a total of 252
132 zebrafish (male:female ratio of 56:46), with an average wet weight of 0.37 ± 0.05 g and 3.1 ± 0.2 cm
133 total body length (snout to tail tip). For CPF experiments, each exposure group consisted of 15
134 zebrafish, resulting in a total of 180 zebrafish (male:female ratio of 65:35), with an average wet weight
135 of 0.29 ± 0.08 g and 3.4 ± 0.2 cm body length. For animal welfare reasons exposures were conducted
136 in duplicates only. During the daily examination of groups, none of the zebrafish displayed any symp-
137 toms of stress or disease; no specimen died during the experimental time course.

138

139 **2.3 Loading of MPs with benzo(k)fluoranthene and chlorpyrifos and water analysis during**
140 **experimental exposure**

141 For the treatment groups with either 0.78 µg/L or 50 µg/L BkF sorbed to PMMA, a dispersion of
142 PMMA and dissolved BkF (from 2 g/L BkF stock) in 50 ml aqua bidest (final DMSO concentration of
143 0.005 %) was incubated at room temperature for two days on a Certomat S-II orbital shaker at 100

144 rpm (Sartorius Stedim Biotech, Göttingen, Germany) and subsequently filtered over 0.2 µm Whatman
145 Puradisc cellulose acetate filter (GE Healthcare, Solingen, Germany). BkF-loaded MPs were washed
146 with 4× 10 ml aqua bidest and resuspended by backwashing the filter with 4× 10 ml tap water. Nega-
147 tive controls with pristine MP were treated likewise (for details, see Hanslik et al., 2020). Incubation
148 of PS with 10 ng/L and 100 ng/L CPF was carried out in the same manner as that for BkF (final
149 DMSO concentration of 0.01 %). The particle size distribution of PMMA and PS (≤ 100 µm) is given
150 in Supplementary Information 2 (Fig. SI 1). Prepared MP suspensions were subsequently added to the
151 according treatment groups.

152 To analyze the incubation process, aqueous phases and particle suspensions during all steps (incuba-
153 tion, washing, resuspension) were collected and measured separately in triplicates using GC-EI-MS.
154 Erlenmeyer flasks used for MP incubation were pre-incubated overnight with BkF and CPF to mini-
155 mize sorption losses. BkF samples were extracted by adding 1.1 ml *n*-hexane, vortexed for 2 min and
156 frozen (-20 °C) to facilitate phase separation. Phenanthrene was used as an internal standard for BkF
157 samples. For CPF measurements, samples were spiked with atrazine as internal standard, dried under a
158 stream of nitrogen, and resuspended in acetone/ethyl acetate (1:1 v/v). For both approaches, the extrac-
159 tion solvent was filtered through 0.2 µm syringe filters and transferred to glass vials for subsequent
160 measurements. Further details on measurement parameters are given in Supplementary Information 1
161 (section 1.1, 1.2).

162 During the experiments, water samples were taken two hours the after daily water exchange and after
163 24 hours at the end of each experiment (on days 14 and 21 for BkF and CPF, respectively) to deter-
164 mine potential leaching of sorbed substances by GC-EI-MS (Tab. SI 2).

165

166 **2.4 Sampling and preservation of zebrafish tissues**

167 For both experiments, tissue samples were taken after 3, 7, 14, and 21 days. After three days of expo-
168 sure, groups were sampled for enzymatic assays (EROD, AChE) only. After 7, 14, and 21 days, sam-
169 ples were collected for all endpoints investigated. After euthanasia with 1.5 M tricaine (ethyl-3-
170 aminobenzoate; MS 222), fish were measured, weighed and dissected. The entire gastrointestinal tract

was immersed in modified Davidson's fixative (Mulisch and Welsch, 2015) and stored at 4 °C for 3 - 5 days before proceeding with embedding for cryosectioning. Immediately after euthanasia, whole liver samples for EROD assays and whole brain samples for AChE activity measurements were frozen in liquid nitrogen and stored at -80 °C until further analysis. Samples for tissue analysis of BkF (liver) and CPF (brain) were collected after 21 days of continuous exposure and treated identically.

2.5 Cryosectioning and confocal laser scanning microscopy (CLSM) of gastrointestinal tracts of zebrafish

Based on the autofluorescence of BkF at 403 nm (Rivera-Figueroa et al., 2004), MP-associated transfer and possible accumulation of BkF within the intestine of *Danio rerio* was investigated in unstained cryosections of the gastrointestinal tract, using a Nikon Eclipse 90i/C1 confocal laser scanning microscope (CLSM, Nikon, Duesseldorf, Germany). To avoid extraction of BkF by dehydration with ethanol, cryosectioning was preferred to paraffin embedding. See Supplementary Information 4 for processing details.

In addition to brightfield images, fluorescence images (10× magnification) of optical sections were recorded in individual confocal images (λ_{EX} = 405 nm, λ_{EM} = 432 - 467 nm) and processed with FIJI (Preibisch et al., 2009; Schindelin et al., 2012). Fluorescence was measured in stitched confocal images as mean pixel intensities (sum of foreground intensities/number of foreground pixels) above the background pixel signal (Hanslik et al., 2020). Results were normalized to the background fluorescence of control samples. Fractions of liver tissue, chyme or fluorescent particles within the chyme were excluded from the analysis (see Supplementary Information 4).

Gastrointestinal tracts from CPF experiments were processed identically to BkF samples to verify MP uptake *via* ingestion and to detect possible physiological effects of PS particles during passage through the intestinal tract. The fluorescent PS particles were tracked by epifluorescence excitation (λ_{EX} = 540 - 580 nm, λ_{EM} = 600 - 660 nm, exposure time 60 ms, Nikon Eclipse 90i, Nikon, Germany).

2.6 Hepatic 7-ethoxy-resorufin-*O*-deethylase (EROD) assay

Frozen liver samples were processed and extracted based on protocols by Örn et al. (1998) and Batel et al. (2016) using a TissueLyser II (Qiagen, Hilden, Germany). Samples were measured in triplicates, and emitted resorufin signals were compared to a resorufin standard curve (0.018 - 2.4 µM) prepared in phosphate buffer (15 mM $NaH_2PO_4 \times H_2O$, 68 mM $Na_2HPO_4 \times 2\ H_2O$, 1.2 mM $MgCl_2^- \times 6\ H_2O$; pH 7.4). EROD activity was measured over 20 min in crude lysates using a GENios plate reader (λ_{EX} = 544 nm, λ_{EM} = 590 nm; Tecan, Crailsheim, Germany).

Protein contents were measured according to Lowry et al. (1951) with the DC™ Protein Assay kit from Bio-Rad (Feldkirchen, Germany). In case the protein yield was ≤ 0.2 mg/ml, samples were excluded from further analysis. Results were expressed in relation to protein contents as picomoles of resorufin produced per milligram of protein and per minute (pmol/mg of protein/min).

2.7 Acetylcholinesterase activity measurements

AChE activity was measured in brain samples of zebrafish based on protocols by Kais et al. (2015) and Küster (2005). See Supplementary Information 5 for processing details. Enzyme kinetics were determined in a 96-well plate (TPP, Trasadingen, Switzerland) at 415 nm over 10 min using a GENios plate reader (Tecan, Crailsheim, Germany) and changes in optical density over time and protein content (ΔOD/min/mg of protein) were recorded. AChE activity levels were standardized with respect to the protein contents and compared to control samples. Protein contents were measured according to Lowry et al. (1951) with the DC™ Protein Assay kit from Bio-Rad.

2.8 Accumulation and biotransformation of benzo(k)fluoranthene and chlorpyrifos in liver and brain tissues of zebrafish

Frozen tissue samples were extracted in 200 µl acetonitrile for 30 min in an ultrasonic bath. After a centrifugation step, the supernatant was used directly for analysis with an ACQUITY ultra performance liquid chromatography coupled with a fluorescence detector for BkF analysis (λ_{EX} = 240 nm,

223 λ_{EM} = 420 nm) and coupled to a Xevo G2-XS high-resolution mass spectrometer (HRMS) equipped
224 with an electrospray ionization source (Waters, Eschborn, Germany). The limit of detection (LoD) was
225 0.1 ng/ml for BkF and 1 ng/ml by UPLC/MS detection for CPF; amounts detected were referenced to
226 individual sample weight. MarkerLynx (Waters, Eschborn, Germany) and a combined non-
227 target/suspect screening were used for identification of possible transformation products based on the
228 approach described by Kühnert et al. (2017), see Supplementary Information 6.
229
230 **2.9 Data analysis**
231 Throughout the experiments, no significant differences were found between replicates of each treat-
232 ment and therefore, data from replicate treatments were combined. For statistical evaluation, data were
233 tested for normality and equal variances. To identify statistically significant differences (* $p < 0.05$, **
234 $p < 0.01$, *** $p < 0.001$) between treatment groups *versus* the control group at the corresponding sam-
235 pling timepoint (3, 7, 14 or 21 d), either parametric ANOVA followed by Holm-Sidak *post-hoc* test
236 (AChE activity) or non-parametric Kruskal-Wallis analysis followed by Dunn's *post-hoc* test (BkF
237 signal in the intestine of *Danio rerio* and EROD assay) were performed using SigmaPlot v. 13 (Systat
238 Software, Erkrath, Germany). Outliers were identified with Grubbs test (accounting for 1.53 % of the
239 data), and results were expressed as mean values (MV) ± standard error of the mean (SEM).
240
241 **3. Results**
242 **3.1 Microplastic loading with benzo(k)fluoranthene and chlorpyrifos and water analysis**
243 Quantifying the final amount of BkF and CPF sorbed to MP particles is crucial to assess the potential
244 amount being transferred to zebrafish. For BkF, GC-EI-MS measurements have proven that up to 80
245 % of BkF was sorbed to spherical PMMA particles and less than 10 % remained in the aqueous phase
246 after 24 h of incubation (Tab. SI 3; Hanslik et al., 2020). The sorption capacity of irregular PS parti-
247 cles for chlorpyrifos was reduced, if compared to that of PMMA for BkF, since only approximately 60
248 % of CPF was sorbed to PS particles, possibly due to the lower log K_{OW} of 4.96 (BkF log K_{OW} 6.11).
249 As a consequence, up to 40 % of CPF was present in the aqueous phase after incubation with PS parti-

cles (Tab. SI 3). However, since all vials for incubation were pre-saturated, a loss of CPF due to binding to glassware could be considered negligible.

The analytical measurements from samples taken during the experimental exposure after 2 h as well as after 24 h indicated only minor amounts of BkF (0.1 - 0.2 ng/L) and CPF (< 0.02 ng/L) in groups treated with contaminant-loaded MPs (Tab. 1). Thus, over 24 h exposure in glass aquaria under experimental conditions, only minor leaching took place. Therefore, in our study, the main route of uptake by zebrafish exposed to contaminant-loaded MPs was through ingestion of BKF- or CPF-sorbed MPs.

Tab. 1. Concentrations of benzo(k)fluoranthene (BkF) and chlorpyrifos (CPF) measured in water samples from experimental groups (treated with either BkF sorbed to PMMA-MPs or CPF sorbed to PS-MPs) by GC/EI-MS. Data refer to 1 - 3 L of extracted water using solid phase extraction, or a calculated amount of approximately 6.4 mg of microplastic separated by filtration from water samples. n. a. = not assessed. Results ± % relative standard deviation, LoD (Limit of Detection) for BkF ≤ 1 ng/ml, and CPF ≤ 20 ng/ml.

	BkF-sorbed PMMA		**CPF-sorbed PS**	
Nominal concentration	0.78 µg/L	50 µg/L	10 ng/L	100 ng/L
Water samples [ng/L]	0.1 ± 10.1 %	0.2 ± 3.9 %	< 0.02	< 0.02
Filter [ng/mg microplastic]	4.1 ± 3.1 %	17.9 ± 6.2 %	n. a.	n. a.

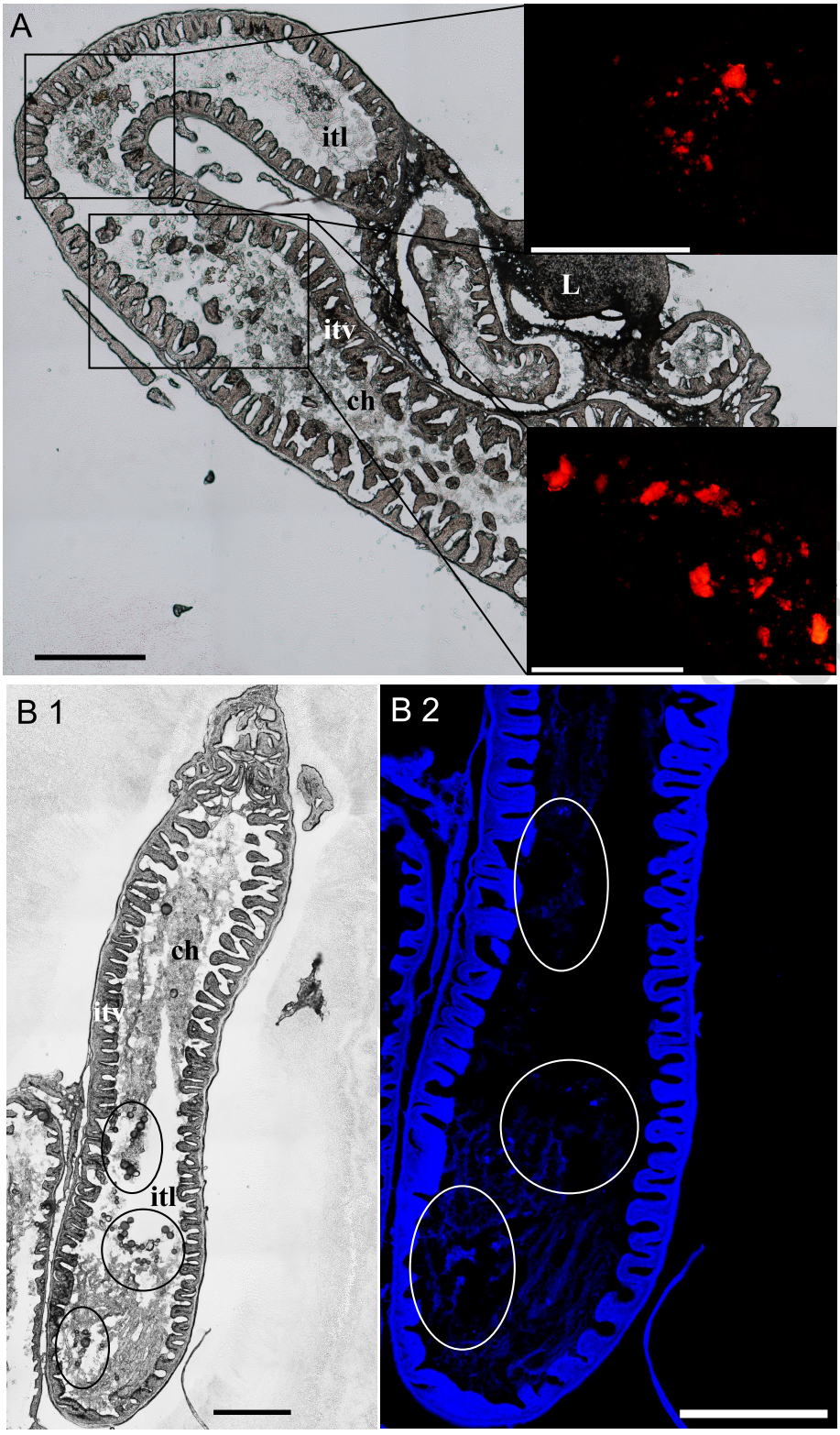
3.2 Long-term exposure of zebrafish to MP-sorbed chlorpyrifos and benzo(k)fluoranthene

3.2.1 Microplastic uptake by zebrafish

Over the entire exposure period of 21 days, zebrafish readily ingested MPs in all groups, including controls (Fig. 1). PS could be identified by epifluorescence excitation (540 - 580 nm) of the red dye (Hostasol Red 5B; see insert Fig. 1A), but no leaching of the dye could be observed since no red fluorescence signal was detected apart from PS particles. The irregularly shaped PS-MPs could exclusively be identified within the intestinal lumen; there was no intervillous or intercellular localization of

271 particles, neither uptake into the intestinal epithelium nor translocation to tissues beyond the intestinal
272 lining.

273 BkF-sorbed PMMA could easily be identified within the chyme due to its spherical shape (Fig. 1B 1).
274 In addition, BkF was detected by CLSM at 340 - 380 nm excitation wavelength due to the blue fluo-
275 rescence signal (circles in Fig. 1B 2). As for the PS-MPs, there was neither uptake of PMMA-MPs
276 into the intestinal epithelium, nor translocation to tissues beyond.



277

278 **Fig. 1.** Unstained sections of the gastrointestinal tract of zebrafish (*Danio rerio*) exposed for 21 days to MPs
279 sorbed with either chlorpyrifos (Fig. 1A) or benzo(k)fluoranthene (Fig. 1B). (**A**) Irregularly shaped PS micro-
280 plastic particles detected by red epifluorescence of the dye Hostasol Red 5B (inserts; excitation filter = 540 - 580
281 nm, emission filter = 600 - 660 nm, exposure time 60 ms). (**B 1**) Spherical PMMA microplastic particles (black
282 circles) in the lumen of zebrafish intestine. (**B 2**) Enlarged CLSM image of blue BkF-related fluorescence of

PMMA-MPs in intestinal lumen (white circles; excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm). Note, that the intestinal tissue exhibits a natural background fluorescence at the respective wavelength. L = liver, itl = intestinal lumen, itv = intestinal villi, ch = chyme, scale bars = 500 µm (Nikon Eclipse 90i/C1, Nikon, Duesseldorf, Germany).

3.2.2 Fluorescence tracking of benzo(k)fluoranthene in zebrafish intestinal tissues

Alterations of fluorescence signals after exposure to waterborne BkF and MP-associated BkF in the intestinal tissue of zebrafish were investigated in unstained cryosections after 7, 14 and 21 days of exposure (Fig. 2A). All samples exhibited a natural background fluorescence, which remained stable over the exposure period. If compared to corresponding negative controls, only zebrafish exposed to 50 µg/L waterborne BkF (positive controls) showed a 5-fold increase in fluorescence after 7 days and a 2.5-fold increase after 14 days ($p < 0.05$ and $p < 0.01$ respectively). After 21 days of exposure, fluorescence intensity in the intestines of positive controls had decreased to the level of negative control. Zebrafish groups treated with BkF sorbed to PMMA did not show any significant changes in fluorescence intensity and remained stable over time.

3.2.3 Hepatic EROD activity in zebrafish

After 3, 7, 14, and 21 days of exposure to either BkF or CPF, samples were analyzed for induction of hepatic cytochrome P450 activity (Fig. 2B). Similar to intestinal fluorescence signals after BkF exposure, CYP1A activity in zebrafish was significantly increased over the exposure period only in the positive control with 50 µg/L BkF (after 7 - 14 days, $p < 0.01$; after 3 and 21 days, $p < 0.001$). Treatments with sorbed BkF did not alter CYP1A activity in zebrafish since EROD levels varied between 20 - 50 pmol resorufin × mg^{-1} protein × min^{-1} over time. Induction patterns for both treatments and the positive control with 0.78 µg/L BkF were similar to the MP control (10 - 50 pmol resorufin × mg^{-1} protein × min^{-1}). Pristine PMMA did not alter the hepatic EROD activity over the experimental period.

Exposure to CPF induced an increase in CYP1A activity in *Danio rerio* compared to the control. Both treatments with PS-sorbed CPF induced similar alterations like waterborne CPF and CYP1A activity was significantly increased ($p < 0.05$; $p < 0.01$) in both exposure scenarios, as seen by a two- to three-fold increase after three days of approximately 20 pmol resorufin × mg^{-1} protein × min^{-1}, to seven days of exposure (56 - 60 pmol resorufin × mg^{-1} protein × min^{-1}) when compared to basal control levels. Another two-fold increase was observed after 14 days (90 - 140 pmol resorufin × mg^{-1} protein × min^{-1}), except for the positive control (10 ng/L), where a delayed three-fold increase was detected after 21 days. However, based on EROD induction levels, no differentiation could be made between low and high concentrations of CPF (10 ng/L; 100 ng/L) or the application form (waterborne *versus* sorbed to MPs). Similar to PMMA, pristine PS did not alter the EROD induction pattern over 21 days.

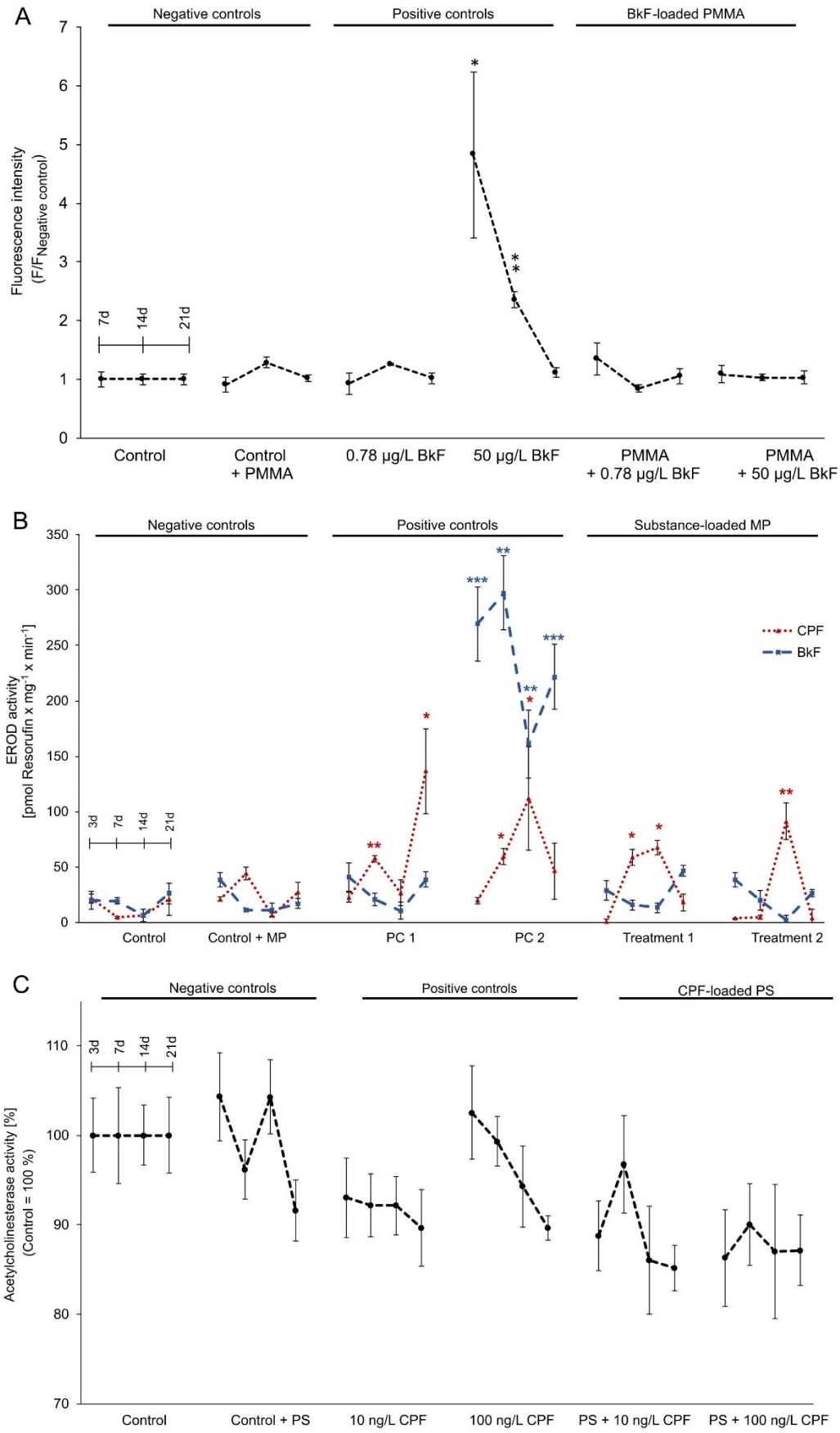
3.2.4 Acetylcholinesterase activity in zebrafish brain samples

In line with findings from various developmental stages in zebrafish (Richendrfer and Creton, 2015; Rodríguez-Fuentes et al., 2015; Yen et al., 2011), acetylcholinesterase activity in brain samples of zebrafish showed a trend to decline, following the 21 days of exposure to environmentally relevant concentrations of CPF in all treatments as compared to the control (Fig. 2C). However, low and high concentrations of waterborne and MP-sorbed CPF failed to reach significance due to elevated standard deviations within treatment groups.

3.2.5 Uptake of benzo(k)fluoranthene and chlorpyrifos and investigation of biotransformation products in zebrafish

Tissue samples were analyzed after 21 days of continuous exposure. A distinct fluorescence BkF peak was detected at 11.58 min by UPLC-FLD only in one out of three samples upon exposure to 50 µg/L waterborne BkF (positive controls; Fig. 3A). Overall detectable amounts of parent BkF in liver tissues varied between replicate samples from 0.03 - 3.12 ng (limit of quantification ≤ 0.01 ng) and due to these overall low amounts, UPLC-HRMS analysis did not allow to clearly identify biotransformation products of BkF (Fig. 3B). Thus, no distinct discrimination between types of biotransformation prod-

ucts (Phase-I, Phase-II and functional groups) could be made. However, metabolites with increased polarity, which were detected earlier than parent BkF at 11.58 min (Fig. 3A), can be assumed to originate from phase-II of biotransformation (i.e., glucuronide, sulfate conjugation). Exposure to environmentally relevant concentrations of both waterborne and MP-associated CPF (10 ng/L or 100 ng/L) did not lead to detectable amounts of chlorpyrifos or potential biotransformation products in brain tissues of zebrafish (LoQ ≤ 1.0 ng).



341

342 **Fig. 2.** Development of (**A**) BkF-related fluorescence in intestinal epithelia, (**B**) hepatic EROD activity and (**C**)

343 acetylcholinesterase activity in brain of zebrafish (*Danio rerio*) exposed for 21 days to benzo(k)fluoranthene

(BkF) and chlorpyrifos (CPF) *via* the water phase (positive controls) or sorbed to PMMA (PMMA + 0.78 µg/L BkF or PMMA + 50 µg/L BkF) and PS (PS + 10 ng/L CPF or PS + 100 ng/L CPF) particles. As controls, zebrafish were either exposed to clean water (negative control) or water with pristine MPs (Control + PMMA/PS). (**A**) BkF-related fluorescence in intestinal epithelia (relative to negative controls). Only exposure to 50 µg/L waterborne BkF showed a significant increase, whereas PMMA-sorbed BkF failed to induce any change (n = 6; exception at PMMA + 0.78 µg/L BkF after 7 days with n = 5; * $p < 0.05$, ** $p < 0.01$; Kruskal-Wallis analysis, Dunn's *post-hoc*). (**B**) EROD activity in livers of zebrafish exposed to BkF (blue, n = 5 - 10) only showed a significant increase as compared to negative controls after exposure to high concentrations of water-borne BkF, whereas CPF (red, n = 3 - 6) induced a transient increase at lower concentrations of waterborne CPF as well as PS-sorbed CPF (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; colors of asterisks indicate assignment to corresponding exposure scenario; Kruskal-Wallis, Dunn's *post-hoc*). (**C**) Acetylcholinesterase activity in brain samples from zebrafish (expressed as % of the negative controls) showed no significant alterations in either exposure scenario over 21 days of exposure (n = 6, exception at 100 ng/L CPF after 21 days with n = 5; note that activity axis starts at 70 % for better visibility; ANOVA, Holm-Sidak *post-hoc*). N = 2; data are given as means ± SEM.

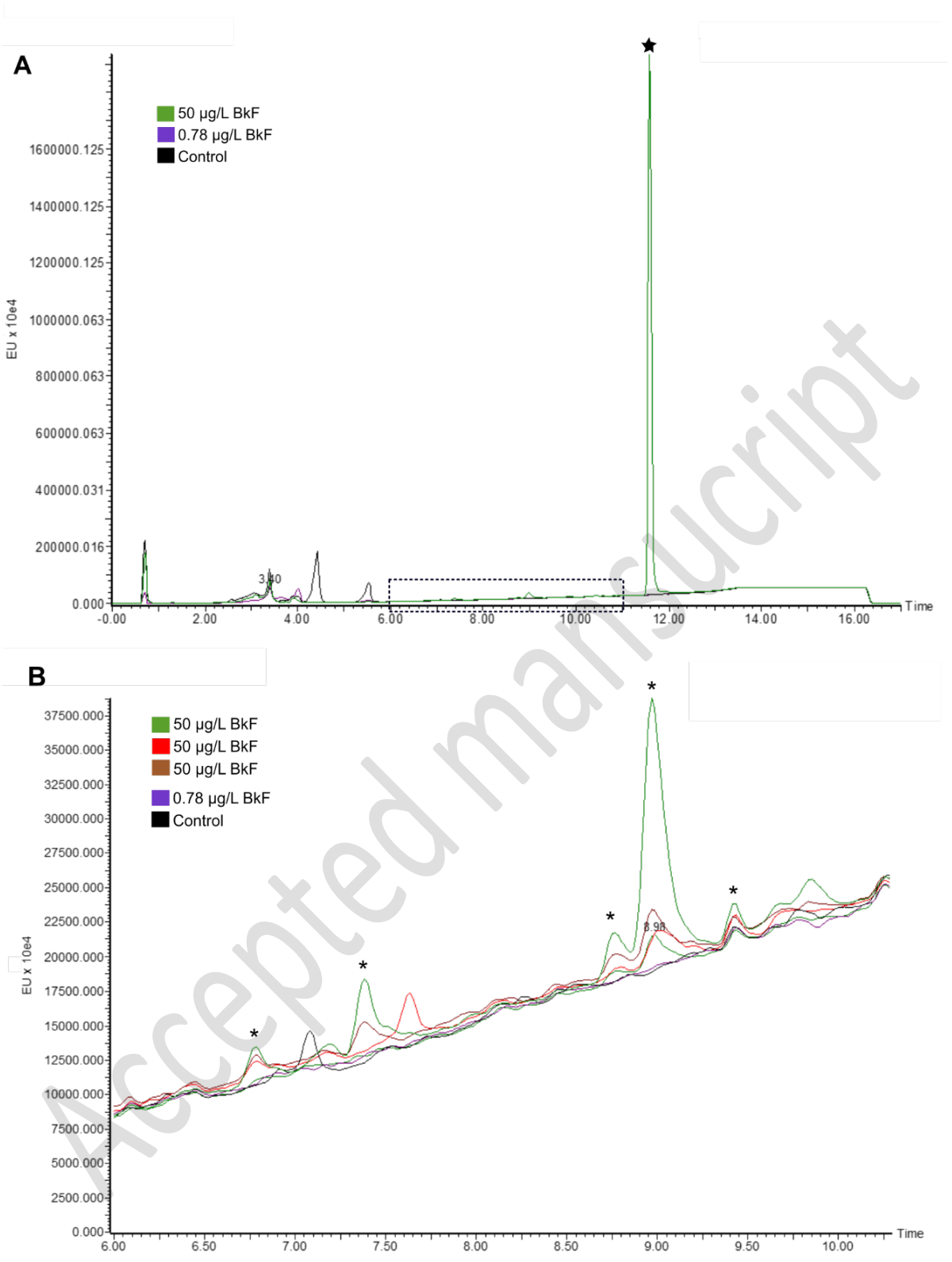


Fig. 3. Chromatogram of benzo(k)fluoranthene (BkF) measured by ultra performance liquid chromatography coupled with fluorescence detection (UPLC-FLD) and its biotransformation products in liver extracts of zebrafish (*Danio rerio*) after 21 days of continuous exposure. (**A**) Distinct BkF peak (★) at 11.58 min in one sample from exposure to 50 µg/L waterborne BkF (green). The dashed box between 6.0 - 11.0 min is enlarged in

(**B**), demonstrating similar peaks (*) of biotransformation products from samples exposed to waterborne 50 µg/L BkF (green, red, brown).

4. Discussion

4.1 Sorption behavior of benzo(k)fluoranthene and chlorpyrifos and water analysis

GC-EI-MS measurements indicated that up to 80 % of BkF adsorbed to PMMA during the loading procedure and similar sorption rates were documented for CPF and PS-MPs (up to 60 %; Tab. SI 3). Here, 0.6 - 0.7 mg of BkF were sorbed to 1 g PMMA particles, whereas in environmental samples much lower amounts of PAHs have been documented on marine plastic samples (24 µg PAHs/g plastic) from the North Pacific Subtropical Gyre or MPs deposited on beaches (Chen et al., 2019; Pannetier et al., 2019a; Yeo et al., 2017). Our results were consistent with data reported in other studies under laboratory conditions, as similar sorption rates of approximately 80 % have been documented for PE particles and CPF in filtered seawater after 2 h of incubation (Bellas and Gil, 2020; Garrido et al., 2019). Comparable results were reported in another lab study for freshwater conditions, where 72 h of incubation of CPF and different types of MPs resulted in sorption rates of up to 60 % (Allen et al., 2018), proving that the pollutant load on MPs could be significantly increased under laboratory conditions compared to contaminant loads on MPs detected in environmental samples.

However, water analysis of samples taken during experimental exposure in this study revealed that desorption of both BkF and CPF from MPs into the water phase was negligible (Tab. 1). Here, the distribution of contaminants between the MPs, the aqueous phase, and the organic material (i. e., fish, faeces, and food remnants) was altered, and BkF could sorb mainly to the organic components compared to the sorption/desorption determined in clear water (see Tab. SI 3), thereby reducing the detectable amount of BkF in the aqueous phase, as also discussed by Tourinho et al. (2019). These findings are comparable with minor desorption rates for PAHs from littoral plastic debris into seawater of 12 % only (León et al., 2018). As a consequence, in our study, exposure *via* ingestion of pollutant-sorbed MPs was the most relevant exposure route for zebrafish.

391 Desorption processes of microplastic-sorbed pollutants in multivariate ecosystems and organisms are
392 not yet entirely understood, since the variety of plastic materials and environmentally relevant contam-
393 inants is infinite. Though, the ingestion of MPs facilitates an alternative route of exposure and evi-
394 dence has been shown that ingested microplastics have the potential to cause physical and chemical
395 harm (Wright et al., 2013; Jovanović, 2017).

396

397 **4.2 Microplastic particle uptake by zebrafish**

398 Successful uptake of MPs into the intestinal lumen could unequivocally be confirmed for both types of
399 pristine and contaminated MPs by visual detection with CLSM/epifluorescence and brightfield imag-
400 es. These findings are in line with the reports for a variety of taxa in both aquatic and terrestrial eco-
401 systems as well as under laboratory conditions (Bour et al., 2018; Choi et al., 2018; Jabeen et al.,
402 2018; Karami et al., 2017; Karlsson et al., 2017; Lehtiniemi et al., 2018; Santana et al., 2018; Weber et
403 al., 2018). In none of the experimental groups, physical impairment of tissue integrity or translocation
404 across epithelial lining of the intestinal tract could be detected. Furthermore, exposure to both pristine
405 PMMA-MPs and PS-MPs did not induce alterations in EROD activity, AChE activity or the fluores-
406 cence signal, which is in line with results for exposure to pristine PE, LDPE, PVC, PS particles (Batel
407 et al., 2018; Cormier et al., 2019; Jovanović et al., 2018; Mazurais et al., 2015; Rainieri et al., 2018).

408 Yet, even effects upon ingestion of pristine, non-contaminated MPs on fish are inconsistent and dis-
409 cussed controversially. Whereas, some studies did not detect any adverse effects (Batel et al., 2020;
410 Jovanović et al., 2018; Karami et al., 2017; Khan et al., 2015; Mazurais et al., 2015; Santana et al.,
411 2017) other studies reported physiological impairments and biochemical modulations in target organs
412 (Batel et al., 2018; Choi et al., 2018; Cormier et al., 2019; Espinosa et al., 2017; Jabeen et al., 2018;
413 Karami et al., 2016; Lei et al., 2018). In fish, short-term effects of MP-associated contaminants were
414 investigated either by intentional loading of MPs under laboratory conditions (Batel et al., 2018, 2016;
415 Cormier et al., 2019; Khan et al., 2015; Rochman et al., 2014) or by application of MPs recovered
416 from environmental samples or conditioned by pre-exposure in natural environments (Ašmonaite et
417 al., 2018; Pannetier et al., 2020; Rochman et al., 2014, 2013a). In contrast, data on chronic exposure to
418 MPs loaded with chemical pollutants are very limited; even more so, results reported varied consider-

ably but all indicated a potential transfer of pollutants sorbed to MPs to aquatic vertebrates (Capó et al., 2021; Cormier et al., 2021; Qiu et al., 2020).

In the present study, elevated MP amounts of 1×10^6 MP particles/L were used as a worst-case scenario, since other studies failed to detect MPs in vertebrate consumers when applying lower, more environmentally relevant, amounts of MPs (Grigorakis et al., 2017; Santana et al., 2017). In a similar study, Triebskorn et al. (2019) described ingestion of MPs of different size, shape, color, and polymer type after exposure *via* both the aqueous media or in association with food particles. The influence of particle size regarding potential toxic effects in Japanese medaka (*Oryzias latipes*) was shown to increase with decreasing size (Liu et al., 2021), which is clearly linked to sorption kinetics based on surface-to-volume ratios (Heinrich et al., 2020; Menéndez-Pedriza and Jaumot, 2020). This suggests that MPs may alter the bioavailability by shifting the uptake route from water to dietary exposure (Khan et al., 2015). However, the risk of ingesting contaminated MPs is not necessarily higher than the risk of feeding on contaminated natural prey (Bakir et al., 2016; Koelmans et al., 2016), since the contaminant load on MPs recovered from the aquatic environment may certainly be much lower, as demonstrated for dioxin-like chemicals (Chen et al., 2019) and for PAHs (Pannetier et al., 2019a; Yeo et al., 2017), if compared to contaminant loads in prey accumulated from the water phase (Diepens and Koelmans, 2018; Jovanović, 2017; Ziccardi et al., 2016).

4.3 Effects of benzo(k)fluoranthene in zebrafish

The fluorescence signal of BkF in the intestinal tissue of zebrafish was significantly increased only after exposure to 50 µg/L waterborne BkF ($p < 0.01$). Similar holds true for the induction of hepatic EROD activity (Figs. 2A/B), presumably due to an increased metabolic activity and detoxification of BkF (Billiard et al., 2008; Whyte et al., 2000). Encoding for CYP450 enzyme as one of the main detoxification phase-I enzymes (Whyte et al., 2000), *cyp1a* is particularly evident in the liver. However, UPLC-FLD measurements of BkF in liver tissues revealed only minor amounts of parent BkF (0.03 - 3.12 ng BkF/sample) and biotransformation products could only be detected after exposure to waterborne 50 µg/L BkF (positive control). This is in line with previous studies that even low amounts BkF

446 metabolites have the potential for CYP1 induction exceeding those of parent BkF (Bucheli and Fent,
447 1995; Spink et al., 2008). Further proving that translocation of MP-sorbed BkF from intestinal epithe-
448 lia to the liver was negligible, and thus, bioavailability of BkF for zebrafish. In addition, the metabolic
449 capacity of the gut of teleost fish has been previously demonstrated (Sarasquete and Segner, 2000),
450 and, thus, detoxification and excretion of BkF and BkF metabolites *via* the gut might have reduced the
451 contaminant load already before being metabolized in the liver.

452 Even though MP uptake was confirmed visually in cryosections of the gastrointestinal tract of *Danio*
453 *rerio* (Fig. 1C), there was no evident translocation of PMMA-sorbed BkF. Therefore, levels of hepatic
454 CYP1A induction remained low in all treatment groups with MP (Fig. 2B). Studies on the desorption
455 of various hydrophobic organic contaminants (such as polychlorinated biphenyls) in artificial gut flu-
456 ids have shown that bioavailability of substances sorbed to MPs can vary over a wide range
457 (Mohamed Nor and Koelmans, 2019) and that the uptake of pollutants by ingestion of pollutant-loaded
458 MPs may be negligible (Lee et al., 2019).

459 Similar effects were investigated in studies with marine mussels and copepods, were MP-associated
460 PAHs were not translocated *via* ingested particles to organisms, and thus, failed to increase tissue con-
461 centrations of the sorbed model pollutants (Bartonitz et al., 2020; Magara et al., 2018; Paul-Pont et al.,
462 2016; Sørensen et al., 2020). Likewise, the translocation of metals sorbed to PE particles was overall
463 significantly reduced within zebrafish, as compared to waterborne exposure (Khan et al., 2015), indi-
464 cating diminished bioavailability and reduced desorption. Further, it can be assumed that BkF follows
465 different detoxification pathways via gills, the gut and liver, thereby decreasing EROD activity
466 (Sarasquete and Segner, 2000).

467 Water analyses documented free BkF concentrations between 0.1 - 0.2 ng/L in the aqueous phase
468 (Tab. 1), indicating that the concentration of desorbed BkF was probably too low to induce effects
469 through aqueous exposure. The reduced uptake of contaminants might be due to impaired bioavailabil-
470 ity of BkF resulting from high binding affinities to MPs (Sleight et al., 2017), indicating competitive
471 binding of contaminants between MPs and organisms (Heinrich and Braunbeck, 2020). Negligible
472 desorption rates of BkF as measured during our experimental exposure (see Tab. 1) support the as-
473 sumption that ingestion of BkF-sorbed MPs was the major route of uptake by zebrafish.

Data on chronic MP exposure (with or without sorbed contaminants) in adult fish were inconclusive. As shown in zebrafish by Rainieri et al. (2018), a diet supplemented with organic pollutants and metals sorbed to LDPE did not induce alterations in intestinal EROD activity. In contrast, *cyp1a1* gene expression in liver samples was significantly upregulated after three weeks of exposure (Rainieri et al., 2018). Another study investigated the effects of 80 days feeding of European seabass (*Dicentrarchus labrax*) with diets spiked with environmentally relevant amounts polychlorinated biphenyls, polybrominated biphenyl ethers and methyl mercury sorbed to MPs (Granby et al., 2018). Bioavailability and gene expression levels were altered up to 40 days of exposure; however, midway through the exposure period, gene expression patterns of *cyp1a1* for detoxification and *il1b* for immune response in livers of seabass reverted to control levels, indicating only minor effects on liver detoxification mechanisms. In contrast, exposure of adult male zebrafish to pristine 100 µg/L PS microplastics (5 µm), over 21 days, already altered gene expression patterns related to hepatic glycolipid metabolism (Zhao et al., 2020). Still, the authors detected a significant decrease in growth of fish exposed to MP, which could not be documented in our study over the exposure period of 21 days.

Short residence time of MPs in the gastrointestinal tract is an essential aspect concerning desorption processes from MPs (Mohamed Nor and Koelmans, 2019; Uber et al., 2019), which may result in a reduced contaminant uptake, even if ingestion of contaminated MPs takes place (Batel et al., 2016; Khan et al., 2015; Pannetier et al., 2020). Since the passage of MPs through the digestive tract is a transitory process restricted to a few hours, the potential for bioaccumulation and biomagnification is further reduced (Grigorakis et al., 2017; Güven et al., 2017; Rainieri et al., 2018). In addition, less prominent effects in adult zebrafish as compared to larval stages could be due to different sensitivity as a function of a more effective biotransformation in, e.g., much larger livers (Pannetier et al., 2020, 2019b).

None of the studies mentioned above followed EROD induction over time, and thus, may have underestimated temporal variations. As shown in the present study, sampling time may influence results, especially after prolonged exposure periods, when compensatory reactions of the test organisms might occur (Bucheli and Fent, 1995; Rainieri et al., 2018). Therefore, these incremental measurements of

501 EROD activity give more in-depth information and further emphasize the diminished risk of MPs as a
502 vector for environmental contaminants.

503

504 **4.4 Effects of chlorpyrifos in zebrafish**

505 Experimental exposure to waterborne CPF and PS-sorbed CPF over 21 days only induced significant
506 upregulation in EROD activity from day 7 onwards, which is in accordance with another study in ju-
507 venile carp (*Cyprinus carpio*) exposed for 40 days, where exposure to 1.2 µg/L CPF significantly in-
508 duced EROD activity in liver tissue and increased CYP450 mRNA levels (Xing et al., 2014). In the
509 present study, after 21 days of exposure to 100 ng/l waterborne CPF and both PS-associated CPF
510 treatments, EROD activity reverted to basal levels as compared to the controls. Similar results were
511 obtained for earthworms (*Aporrectodea caliginosa*) exposed to CPF, where CYP450 induction was
512 less pronounced after 21 days of exposure to high CPF concentrations (10 mg/kg dry soil), if com-
513 pared to short-term exposure over 3 days to low CPF concentrations (0.51 mg/kg dry soil; Sanchez-
514 Hernandez et al., 2014).

515 Analogous to CPF, other insecticides with a thiophosphate backbone like diazinon and parathion can
516 be metabolized by CYP450 into respective -oxon forms (Yen et al., 2011). Therefore, biotransfor-
517 mation by CYP450 can cause an indirect effect on acetylcholinesterase activity by accelerating the
518 biotransformation into a more potent AChE inhibitor like CPF-oxon (Binelli et al., 2006; Rodríguez-
519 Fuentes et al., 2015). In the present study, no measurable amounts of parent CPF or biotransformation
520 products were detected in brain tissues above the limit of detection of 0.2 ng/sample, indicating no
521 distinct accumulation of CPF in zebrafish. Thus, since EROD activity decreased to basal levels at the
522 end of the exposure period, this effect was likely driven by either an efficient degradation and excre-
523 tion after 21 days, once CYP1A enzymes were induced and metabolization was activated (Bucheli and
524 Fent, 1995; Whyte et al., 2000) or – as shown for AChE and EROD activity levels from literature
525 evaluated by Wu et al. (2005) – adaptation to prolonged toxicant exposure might occur.

Investigating the metabolic influence of organophosphorus insecticides in combination with neurotoxicological markers such as AChE activity provides more specific insights into biochemical interactions. However, only a trend towards a reduced AChE activity became evident in our study, since the increase in EROD activity in positive controls and MP treatment groups after 7 days is opposed only by a statistically non-significant reduction of AChE activity (Figs. 2B/C). Most likely, since only environmentally relevant concentrations of CPF were applied in this study (10 ng/L; 100 ng/L), effects were less pronounced, as no parent CPF or biotransformation products of CPF could be detected in brain tissue of zebrafish by UPLC-HRMS measurements. In addition, as verified by analytical measurements, no freely dissolved CPF was present in the water phase of treatment groups exposed to CPF-sorbed MPs, and thus, bioavailability of CPF was diminished.

Overall, the trend towards AChE inhibition by waterborne CPF in zebrafish were similar with findings from other studies, indicating impairment of neurotransmitter metabolism, gene transcription and protein levels as well as neuro-behavioral alterations (Gómez-Canela et al., 2017; Özdemir et al., 2018; Richendrfer and Creton, 2015), even though concentrations in older studies exceeded environmental levels by several orders of magnitude. In contrast, earlier studies with MP-sorbed CPF gave opposing results, since CPF-loaded HDPE (1 µg/L) led to significantly higher acute toxicity in the marine copepod *Acartia tonsa* (survival, feeding, egg production, recruitment), if compared to dissolved CPF (Bellas and Gil, 2020). Acute insecticide toxicity of deltamethrin and dimethoate to *Daphnia magna* was not affected by the presence of small PS spheres (300×10^6 particles/L), regardless of their chemical binding affinities (Horton et al., 2018). In our study, the analytical quantification failed to detect measurable concentrations of CPF in the water phase of experimental groups, proving that CPF did not significantly desorb from PS particles (Tab. 1). It should be noted that the binding affinity of CPF to the irregular PS particles was lower compared BkF and PMMA particles (log K_{OW} 6.11; 4.96, respectively), and therefore, CPF could have desorbed from the PS particles under altered conditions more easily (e.g., in the intestine; Bejarano et al., 2003). Nevertheless, the applied concentrations of 10 and 100 ng/L CPF slightly altered CYP450 activity, but were too low to cause accumulation in zebrafish brain tissues.

In the present study, we could demonstrate that CPF sorbed to MPs in environmentally relevant concentrations induced only minor effects in adult zebrafish, which was most likely due to reduced bioavailability. No persistent adverse effects were induced in zebrafish by chronic exposure to clean MPs and even high amounts of contaminated MPs (1×10^6 particles/L) failed to promote persistent significant changes in biochemical markers or bioaccumulation of both contaminants in liver and brain tissues. Although, the sorption of contaminants on MPs to environmental samples was previously validated (Pannetier et al., 2017; Rochman et al., 2013a), data on chronic MP exposure, with or without sorbed contaminants, are nevertheless scarce and require further investigation. With regard to the ubiquitous presence of insecticides, PAHs and MPs in surface waters, the contaminant fluxes accumulating in aquatic ecosystems from contaminated MPs might be considered negligible (Besseling et al., 2019; Koelmans et al., 2016; Triebskorn et al., 2019).

5. Conclusions

The present study documented that prolonged exposure of adult zebrafish to different MPs sorbed with either BkF or CPF at environmentally relevant concentrations did not induce significant adverse effects in the investigated biomarkers. This study provides further evidence that the vector hypothesis for MPs in aquatic ecosystems may be rejected as MPs represent an insignificant fraction of potential binding sites for pollutants and are therefore probably less relevant in aquatic ecosystems. In contrast, when considering biological effects of even smaller particles such as nanoparticles originating from MP degradation, a paradigm shift is likely and harmful impact of nanoparticles on biota is more likely, and thus, requires further investigation.

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583

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