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Microplastics in Limnic Ecosystems

Investigation of Biological Fate and Effects of Microplastic Particles and Associated Contaminants in Zebrafish (*Danio rerio*)

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Previously published data

Unless stated otherwise, the content of the present thesis was created exclusively by me (acquisition, evaluation, visualization and discussion of the data). Contents already published in scientific journals or jointly produced with students are marked accordingly in the corresponding sections.

The following review article was jointly written as a co-author. Only the original version of the sections of the manuscript prepared entirely by me are incorporated in chapter I.3.2.

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The following review article was jointly written as a co-author. Only the sections of the manuscript prepared entirely by me are incorporated in chapter I.4.

Patrick Heinrich, Lisa Hanslik, Nadine Kämmer, Thomas Braunbeck (2020). *The Tox is in the Detail: Technical Fundamentals for Designing, Performing and Interpreting Experiments on Toxicity of Microplastics and Associated Substances.* Environmental Science and Pollution Research. https://doi.org/10.1007/s11356-020-08859-1.

The following manuscript was recently published in Comparative Biochemistry and Physiology – Part C: Toxicology and Pharmacology, and is integrated into Chapter II and IV.

Lisa Hanslik, Carmen Sommer, Sven Huppertsberg, Stefan Dittmar, Thomas P. Knepper, Thomas Braunbeck (2020). *Microplastic-associated trophic transfer of benzo(k)fluoranthene in a limnic food web: Effects in two freshwater invertebrates (Daphnia magna, Chironomus riparius) and zebrafish (Danio rerio).* DOI: 10.1016/j.cbpc.2020.108849. Chapter IV

Further contents of this thesis will be submitted soon for publication based on the following manuscripts:

Lisa Hanslik, Bettina Seiwert, Sven Huppertsberg, Thomas P. Knepper, Thorsten Reemtsma, Thomas Braunbeck. *Biomarker responses in zebrafish (Danio rerio) following long term exposure to microplastic-associated chlorpyrifos and benzo(k)fluoranthene.* Chapter III

Lisa Hanslik, Sven Huppertsberg, Nadine Kämmer, Thomas P. Knepper, Thomas Braunbeck. *Environmental exposure of microplastics in a highly polluted stream in Germany. Analytical quantification and toxicological effects in zebrafish (Danio rerio).* Chapter V

The thesis contains data from the following state examination theses, which were designed and supervised by me, data were collected and analyzed jointly with the students:

Luisa Nuttall (2018): Microplastic as vector for the insecticide cypermethrin and potential effects on the model organism *Danio rerio*.

Carmen Sommer (2019): Mikroplastik-assoziierter Transfer von Benz(k)fluoranthen auf den Zebrabärbling (*Danio rerio*) in einer einfachen Modellnahrungskette.

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Abstract

Given the continually increasing global polymer production, environmental pollution from plastic debris has been widely perceived as an ecological threat with potentially adverse (eco-)toxicological impacts on various species and ecosystems. Especially small fragments of plastic debris measuring $1 \leq 1000$ µm in size – so-called microplastics (MPs) – can pose a risk to biota by physical or chemical hazards due to ingestion, sorption and transfer of anthropogenic environmental pollutants (e.g., pesticides, polycyclic aromatic hydrocarbons, pharmaceuticals). In recent years, there has been extensive research on the impact of streams and rivers as main entry routes for MPs into the marine environment. However, the evidence of adverse effects caused by the intake of MPs and the transfer of contaminants sorbed to MPs into freshwater ecosystems is still not conclusively documented.

Therefore, this thesis addressed the impact of microplastic particles in limnic ecosystems by investigating the biological fate and effects of MPs and associated anthropogenic pollutants on different life stages of zebrafish (*Danio rerio*). To this end, effects of two common environmental pollutants (benzo(k)fluoranthene, chlorpyrifos) and two synthetic polymers (polystyrene, polymethyl methacrylate) were investigated with regard to alterations of complementary biomarkers during acute and chronic exposure, as well as the trophic transfer of MPs and MP-sorbed contaminants from invertebrate organisms to zebrafish and acute toxic effects of MPs pre-exposed in a natural aquatic ecosystem.

Overall, the sorption of both anthropogenic pollutants to MPs could be confirmed under various exposure scenarios. In contrast to recent study results, no adverse effects were observed when zebrafish were exposed to clean, pristine polystyrene and polymethyl methacrylate. The ingestion of MPs was documented in the gastrointestinal tract of zebrafish and did not induce any physiological impairment. The uptake of both pollutants was verified for all investigated species by GC-MS and fluorescence measurements.

In the acute exposure scenario using the fish embryo toxicity test (FET), zebrafish embryos displayed sublethal effects and morphological deformities related to the underlying mode of action of benzo(k)fluoranthene and chlorpyrifos. Exposure to MP-associated contaminants significantly reduced malformations and attenuated biomarker responses of acetylcholine esterase activity and CYP450 induction and hence indicated a reduced bioavailability of both pollutants for zebrafish embryos.

Chronic exposure of adult zebrafish to both types of MPs and pollutants was designed to assess the potential impact of long-term exposure and possible bioaccumulation of these contaminants of emerging concern. However, exposure to MP-sorbed pollutants failed to alter biomarker responses over the prolonged experimental period. In addition, a novel approach was applied to monitor the uptake of benzo(k)fluoranthene in the intestinal epithelium using confocal laser scanning microscopy. However, no significant bioaccumulation or biotransformation of benzo(k)fluoranthene and chlorpyrifos in brain

and liver tissue could be demonstrated using ultra performance liquid chromatography coupled with fluorescence detection and with a high-resolution mass spectrometer (UPLC-FLD/HRMS).

In order to address the possible vector effects of MPs, the transfer of benzo(k)fluoranthene and polymethyl methacrylate particles in a simplified limnic food web were investigated, consisting of zooplankton (*Daphnia magna*), sediment-dwelling invertebrates (*Chironomus riparius* larvae) and zebrafish as high-level predator. However, the trophic transfer of benzo(k)fluoranthene *via* MPs could not be confirmed by a combination of highly sensitive fluorescence tracking using CLSM, hepatic CYP450 induction, and advanced chemical-analytical methods.

To account for the demand for environmentally relevant exposure scenarios and to improve risk assessment of MPs, a mixture of the most commonly used synthetic polymers (polyethylene, polypropylene, polystyrene, polyvinyl chloride) was exposed in a well-monitored surface water body. To evaluate the potential transfer of hazardous pollutants under natural conditions, the toxicity of the MP mixture was assessed in terms of acute toxic effects (FET), potential neurotoxic effects (AChE activity, larval visual motor response test) and effects of dioxin-like substances (EROD assay) using zebrafish embryos. In conclusion, the exposed MP mixture did not elicit significantly different effects than the natural particles from sediment and suspended matter samples.

Eventually, it could only be confirmed that MPs could act as carriers for environmental contaminants following ingestion by various organisms. However, increased deleterious effects of MP-sorbed contaminants on various sensitive life stages of zebrafish could not be corroborated by acute or prolonged exposure, trophic transfer, or natural exposure. The findings suggest that the sorption of pollutants to MPs is more likely to reduce the bioavailability as a result of slow desorption within the organism. Although, MPs offer an alternative exposure route for aquatic organisms *via* ingestion. Even chronic exposure at environmentally relevant concentrations did not induce bioaccumulation of both highly lipophilic substances in zebrafish tissues. Lastly, the sorption behavior of MPs under realistic environmental exposure conditions was more likely to approximate the behavior of natural particles. Consequently, MPs might pose only a limited risk to limnic communities, especially with regard to the comparatively small fraction of plastic particles in freshwater ecosystems compared to the multitude of naturally occurring (a)biotic particles.

Zusammenfassung

Vor dem Hintergrund der stetig steigenden globalen Kunststoffproduktion wird die Umweltverschmutzung durch Plastikabfälle weithin als eine ökologische Bedrohung mit potenziell negativen (öko-)toxikologischen Auswirkungen auf verschiedene Arten und Ökosysteme wahrgenommen. Insbesondere kleine Fragmente von Kunststoffabfällen mit einer Größe von $1 \leq 1000$ µm, sogenannte Mikroplastikpartikel (MP), können ein Risiko für Organismen aufgrund physikalischer oder chemischer Gefährdung durch Aufnahme, Sorption und Übertragung von anthropogenen Umweltschadstoffen (z. B. Pestizide, polyzyklische aromatische Kohlenwasserstoffe, Pharmazeutika) darstellen. In den letzten Jahren wurden vermehrt Untersuchungen über den Einfluss von Gewässern und Flüssen als Haupteintrittspfade für MP in die marine Umwelt durchgeführt. Konsistente Belege für die schädlichen Wirkungen von Mikroplastikpartikeln und die Übertragung von an MP gebundenen Schadstoffen in Süßwasserökosysteme sind jedoch bisher nicht dokumentiert.

Daher befasst sich diese Arbeit mit dem Einfluss von Mikroplastikpartikeln auf limnische Ökosysteme durch die Untersuchung des biologischen Verhaltens und die Auswirkungen von MP und an MP gebundene anthropogene Schadstoffe auf verschiedene, sensitive Lebensstadien von Zebrafischen (*Danio rerio*). Zu diesem Zweck wurden die Auswirkungen von zwei verbreiteten Umweltschadstoffen (Benzo(k)fluoranthen, Chlorpyrifos) und zwei synthetischen Polymeren (Polystyrol, Polymethylmethacrylat) untersucht. Der Fokus wurde dabei auf potenzielle Änderungen verschiedener Biomarker bei akuter und chronischer Exposition sowie den trophischen Transfer von Mikroplastik und MP-gebundenen Schadstoffen gelegt. Zusätzlich wurden für die Beurteilung von umweltrelevanten Auswirkungen die akuten Effekte von MP, welches zuvor in einem natürlichen Gewässer exponiert wurde, untersucht.

Insgesamt konnte die Sorption beider anthropogener Modellschadstoffe an MP unter verschiedenen Expositionsszenarien bestätig werden. Im Gegensatz zu neueren Studienergebnissen wurden bei der Exposition gegenüber sauberem, reinem Polystyrol und Polymethylmethacrylat keine schädlichen Wirkungen beobachtet. Die Aufnahme von MP wurde im Magen-Darm-Trakt von Zebrafischen nachgewiesen und führte zu keiner physiologischen Beeinträchtigung. Die Aufnahme beider Schadstoffe wurde für alle untersuchten Arten mittels GC-MS und Fluoreszenzmessungen verifiziert.

Im akuten Expositionsszenario unter Verwendung des Fischembryo-Toxizitätstests (FET) zeigten Zebrafischembryonen subletale Effekte und morphologische Missbildungen, die mit der zugrunde liegenden Wirkungsweise von Benzo(k)fluoranthen und Chlorpyrifos zusammenhängen. Die Exposition gegenüber MP-gebundenen Kontaminanten reduzierte die Deformationen signifikant und verminderte die Biomarkerreaktionen der Acetylcholinesterase Aktivität und der CYP450 Induktion, was auf eine verminderte Bioverfügbarkeit der Substanzen für Zebrafischembryonen hinweist.

Die chronische Exposition adulter Zebrafische gegenüber beiden Mikroplastikarten und Schadstoffen hatte die Bewertung potenzieller Auswirkungen einer langfristigen Exposition und einer möglichen Bio-

akkumulation dieser problematischen Schadstoffe zum Ziel. Die Exposition gegenüber MP-gebundenen Schadstoffen führte jedoch zu keiner Veränderung in den untersuchten Biomarkern über den Versuchszeitraum. Des Weiteren wurde ein neuartiger Ansatz entwickelt, um die Aufnahme von Benzo(k)-fluoranthen im Darmepithel mittels konfokaler Laser-Scanning-Mikroskopie zu erfassen (CLSM). Es konnte jedoch keine signifikante Bioakkumulation oder Biotransformation von Benzo(k)fluoranthen und Chlorpyrifos im Gehirn- und Lebergewebe mittels Hochleistungsflüssigkeitschromatographie (UPLC) gezeigt werden.

Die Untersuchung möglicher Vektorwirkungen von Mikroplastikpartikeln wurde in einem limnischen Nahrungsnetz, bestehend aus Zooplankton (*Daphnia magna*) und sedimentlebenden Wirbellosen (*Chironomus riparius* Larven) sowie Zebrafischen als Konsumenten der obersten Trophiestufe, durchgeführt. Dabei stand die Übertragung von Benzo(k)fluoranthen und Polymethylmethacrylat-Partikeln im Fokus. Jedoch konnte der trophische Transfer mit einer Kombination aus hochempfindlichen Fluoreszenzmessungen mittels CLSM, hepatischer CYP450 Induktion und chemisch-analytischen Methoden nicht bestätigt werden.

Für eine verbesserte Risikobewertung von Mikroplastikpartikeln bedarf es umweltrelevanten Expositionsszenarien. Deshalb wurde eine Mischung der am häufigsten verwendeten synthetischen Polymere (Polyethylen, Polypropylen, Polystyrol, Polyvinylchlorid) in einem gut überwachten Oberflächenwasserkörper exponiert. Zur Bewertung eines potenziellen Transfers gefährlicher Schadstoffe unter diesen natürlichen Bedingungen wurde die Toxizität des MP-Gemisches in Bezug auf die akute Toxizität (mittels FET), potenzielle neurotoxische Wirkungen (mittels AChE Aktivität, Schwimmverhalten von Zebrafischlarven) und Wirkungen dioxinähnlicher Substanzen (mittels EROD Assay) bewertet. Im Vergleich zu natürlichen Partikeln aus Sediment- und Schwebstoffproben induzierte das exponierte MP-Gemisch jedoch keine signifikant anderen Effekte in Zebrafischembryonen.

Schlussendlich konnte bestätigt werden, dass Mikroplastik als Träger von Umweltkontaminanten fungieren kann. Schädliche Auswirkungen von MP-gebundenen Schadstoffen auf verschiedene empfindliche Lebensstadien von Zebrafischen konnten jedoch weder durch akute oder längere Exposition noch durch trophischen Transfer oder bei natürlicher Exposition bestätigt werden. Die Ergebnisse legen nahe, dass die Bindung von Schadstoffen an MP eher die Bioverfügbarkeit aufgrund der langsamen Desorption im Organismus verringert, obwohl sie einen alternativen Expositionsweg mittels Ingestion für Wasserorganismen bietet. Selbst bei chronischer Exposition in umweltrelevanten Konzentrationen kam es zu keiner Bioakkumulation der beiden lipophilen Substanzen in Zebrafischgeweben. Es ist wahrscheinlich, dass sich das Sorptionsverhalten von MP unter realistischen Umweltexpositionsbedingungen eher dem Verhalten natürlicher Partikel annähert. Folglich stellen Mikroplastikpartikel nur ein begrenztes Risiko für aquatische Lebensgemeinschaften insbesondere im Hinblick auf den vergleichsweise geringen Anteil von Kunststoffpartikeln in Süßwasserökosystemen im Gegensatz zu der Vielzahl natürlich vorkommender (a)biotischer Partikel dar.

I. Introduction

I.1 Plastics in modern society

The global polymer production has increased exponentially since the onset of industrial production in the 1950s (Geyer et al., 2017). Synthetic polymers have profoundly changed our daily lives due to their versatile properties such as malleability, light weight, durability, corrosion resistance, and insulation, resulting in technical advantages, energy savings, and numerous other societal benefits (Andrady and Neal, 2009; Thompson et al., 2009). Synthetic polymers are typically produced by polymerization of monomers derived from fossil hydrocarbons such as ethylene and propylene (Thompson et al., 2009; Geyer et al., 2017), and obtain their beneficial properties from various chemical additives, such as plasticizers, dyes or flame retardants (Teuten et al., 2009).

In 2018, global polymer production reached a volume of 359 million tons, of which 51 % was produced in Asia and 17 % (62 million tons) in the European Union (see Fig. 1). The primary plastic demand included packaging material (40 %) and the building/construction sector (19.8 %), with polypropylene (PP), polyethylene (PE) and polyvinyl chloride (PVC) being the most commonly used polymer types (Geyer et al., 2017; PlasticsEurope, 2019). Single-use plastics (e.g., packaging material), designed for immediate disposal, with a life span of less than one year (Geyer et al., 2017), consequently also represented the main fraction in household and industrial waste, accounting for 17.8 million tons (61 %) of total plastic waste in the EU (PlasticsEurope, 2019). Even though a significant proportion of 32.5 % of the total plastic waste was recycled in the EU in 2019, the majority was recovered thermally and partly recycled for energetic recovery (42.6 %), while the remaining 24.9 % were still deposited in landfills (PlasticsEurope, 2019). Worldwide, about 60 % of the plastic waste is discarded without recycling (Geyer et al., 2017). As none of the commonly used types of plastics is biodegradable, decomposability is rather unlikely, while the potential for accumulation in landfills and the environment due to littering is increasing (Barnes et al., 2009). Consequently, the ubiquitous occurrence of plastic waste in the environment has already been proposed as a geological indicator for the Anthropocene (Zalasiewicz et al., 2016).

The continuously increasing plastic production and deficient waste management lead to an estimated 5 to 12 million tons of plastic debris being disposed of on land that might finally end up in the oceans (Jambeck et al., 2015). Recent estimates also indicate that rivers can transport between 1.2 to 2.4 million tons of plastic debris to seas and oceans per year (Lebreton et al., 2017). Schmidt and co-workers (2017) have shown that land use and population density correlate with environmental plastic pollution, further indicating that rivers are the most important link between global land surface and oceans and could contribute up to 94 % of the total aquatic plastic contamination by the transport of plastic debris. Further studies on the release of plastic debris into the environment from diffuse and point sources are indispensable to assess possible short- and long-term impacts of plastic particles in limnic environments (GESAMP, 2016). In aquatic systems, the useful properties of plastics, in particular their low weight

and longevity, pose a substantial risk as they increase mobility, accumulation, and persistence of plastic particles due to slow and only partial degradation (Barnes et al., 2009; Eubeler et al., 2009; Scherer et al., 2017).

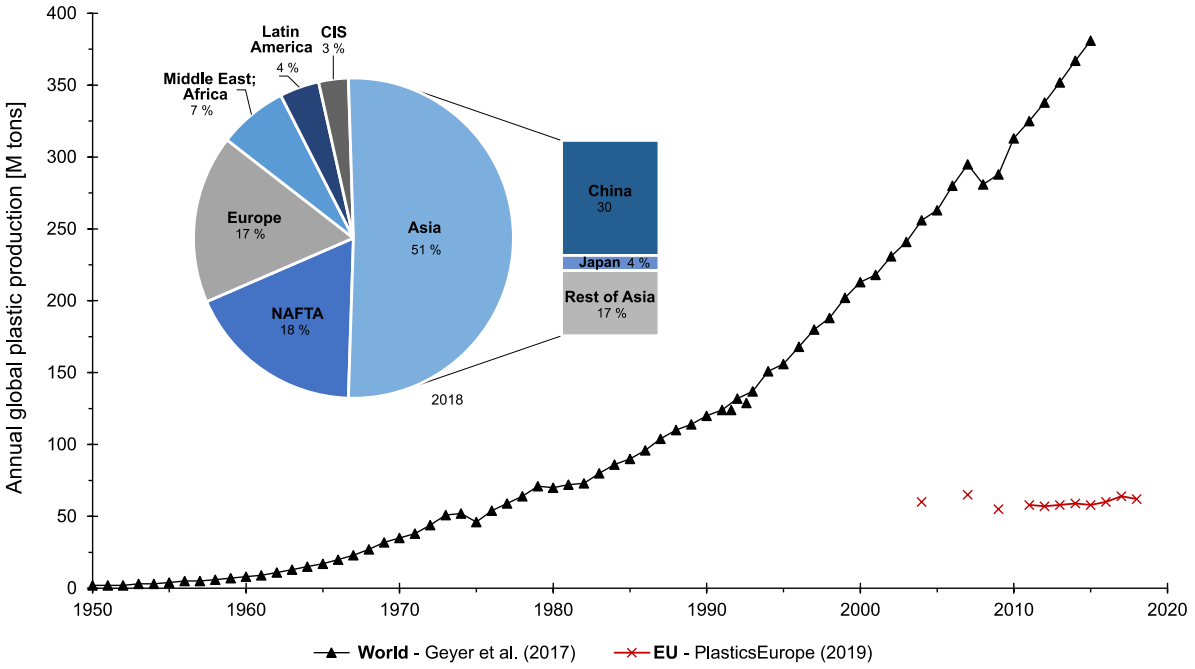

Fig. 1. Continuously increasing global plastic production from 1950 to 2015 (black triangle), and steady European plastic production from 2004 to 2018 (red cross), in million tons. Distribution of global plastic production [%] in 2018 (pie chart), with China as one of the largest producers followed by NAFTA countries (Canada, USA, Mexico) and Europe. CIS = Commonwealth of Independent States. Adapted from Geyer et al. (2017) and PlasticsEurope (2019).

Consequently, the accumulation of plastic debris affects terrestrial, marine and limnic ecosystems (Barnes et al., 2009; Dris et al., 2015). It has been demonstrated that the average size of plastic fragments in the environment seems to be decreasing, whereas the abundance of small plastic debris is continuously increasing due to fragmentation processes (Barnes et al., 2009). At present, synthetic polymer fragments have been detected in planktonic samples (Thompson et al., 2004; Beer et al., 2018), on marine and freshwater shorelines (Browne et al., 2011; Imhof et al., 2013), and in deep-sea sediments (Van Cauwenberghe et al., 2013).

I.2 Microplastics: origin, fate and environmental behavior

Given the omnipresence of plastic debris in the environment, plastic fragments undergo degradation processes resulting in even smaller fragments, ranging from few centimeters to nanometers. Hereafter, as suggested by Hartmann et al. (2019), fragments between 1 µm to 1000 µm in size are therefore defined as microplastic particles (MPs).

MPs comprise manufactured small polymer particles for domestic or industrial applications – so-called primary microplastics – which are used as plastic pellets, raw material or abrasive microbeads, fillers or detergents in the cosmetic industry (Cole et al., 2011; Siegfried et al., 2017) and particles resulting from the degradation and fragmentation of larger plastic items, so-called secondary microplastics. Leading physicochemical factors influencing the formation of secondary MPs are UV radiation (Rabek, 1996), oxidation, hydrolysis (Wagner and Lambert, 2018) and weathering due to salinity, temperature, pH and shear stress by wave action (Jahnke et al., 2017). Further degradation can be promoted by biofilm formation and microbial degradation (Wright et al., 2013; Rummel et al., 2017), with the first evidence of bacterial communities that are capable of degrading plastic particles (Ghosh et al., 2013; Tsiota et al., 2018). However, fragmentation does not end in the micrometer range; thus, further breakdown and formation of nano-sized particles are likely, as the surface area and hence the reactivity of the polymer particles are increasing (Klein et al., 2018; Mattsson et al., 2018). Nevertheless, primary MPs only account for a small proportion of approximately 15 % of plastic debris compared to the amounts of secondary MPs originating from mismanaged plastic waste (Boucher and Friot, 2017).

The abundance of MPs in freshwater environments is highly variable and heterogeneous, and thus comparable to findings from marine environments (Klein et al., 2018). Particle numbers detected in surface waters range from 0.027 particles/m^3 in the Laurentian Great Lakes (Eriksen et al., 2013) up to 18700 particles/m^3 in Amsterdam canal water (Leslie et al., 2017). The river Rhine approximately discharges 191.6 million MPs per day into the North Sea (Mani et al., 2015). Transport behavior and fate are closely related to size, shape, and density of polymer particles (Kooi et al., 2018), and modeling results indicated that micro- and nanoplastics could be retained in riverine systems (Besseling et al., 2017). As documented by Scherer et al. (2020a), the composition of plastic debris in rivers (water column and sediment) reflects the most commonly produced and used polymers (polyethylene, polypropylene, polyvinyl chloride and polystyrene), and it has been demonstrated that the particle load was markedly increased in the vicinity of urbanized or industrialized areas (Alimi et al., 2017; Kooi et al., 2018; Bikker et al., 2020).

Once discharged into the environment, both types of MPs undergo degradation processes (Fig. 2), and thus, pose a risk to biota by either physical or chemical hazard. It was already documented that MPs can be ingested by several aquatic organisms and may transport harmful chemicals or additives to organisms causing endocrine disruption, carcinogenic and mutagenic effects, as summarized in various reviews (Andrady, 2011; Wright et al., 2013; Eerkes-Medrano et al., 2015; Ziccardi et al., 2016; Ivleva et al.,

2017; Anbumani and Kakkar, 2018; Besseling et al., 2019; Botterell et al., 2019; Haegerbaeumer et al., 2019; Triebskorn et al., 2019; Du et al., 2020). Moreover, depending on the polymer type and density, plastic particles with a density ≥ 1 g/cm^3 such as polyethylene terephthalate (PET) and polyvinyl chloride (PVC), polyamide (PA) and polymethyl methacrylate (PMMA), might undergo sedimentation processes and thus accumulate in upper sediment layers (Alomar et al., 2016; Martin et al., 2017). For buoyant MPs with a density ≤ 1 g/cm^3 such as polystyrene (PS), polypropylene (PP) and polyethylene (PE), surface properties and weight have to be altered (e.g., by surface biofilm formation) to induce sinking and sedimentation (Andrady, 2011; Rummel et al., 2017).

However, compared to natural particles, such as algae, clay minerals or detritus, MPs persist and accumulate in the environment over much longer time scales. Therefore, MPs are susceptible to further transport and are available to a wide range of organisms in all compartments of marine and limnic ecosystems (Kooi et al., 2018).

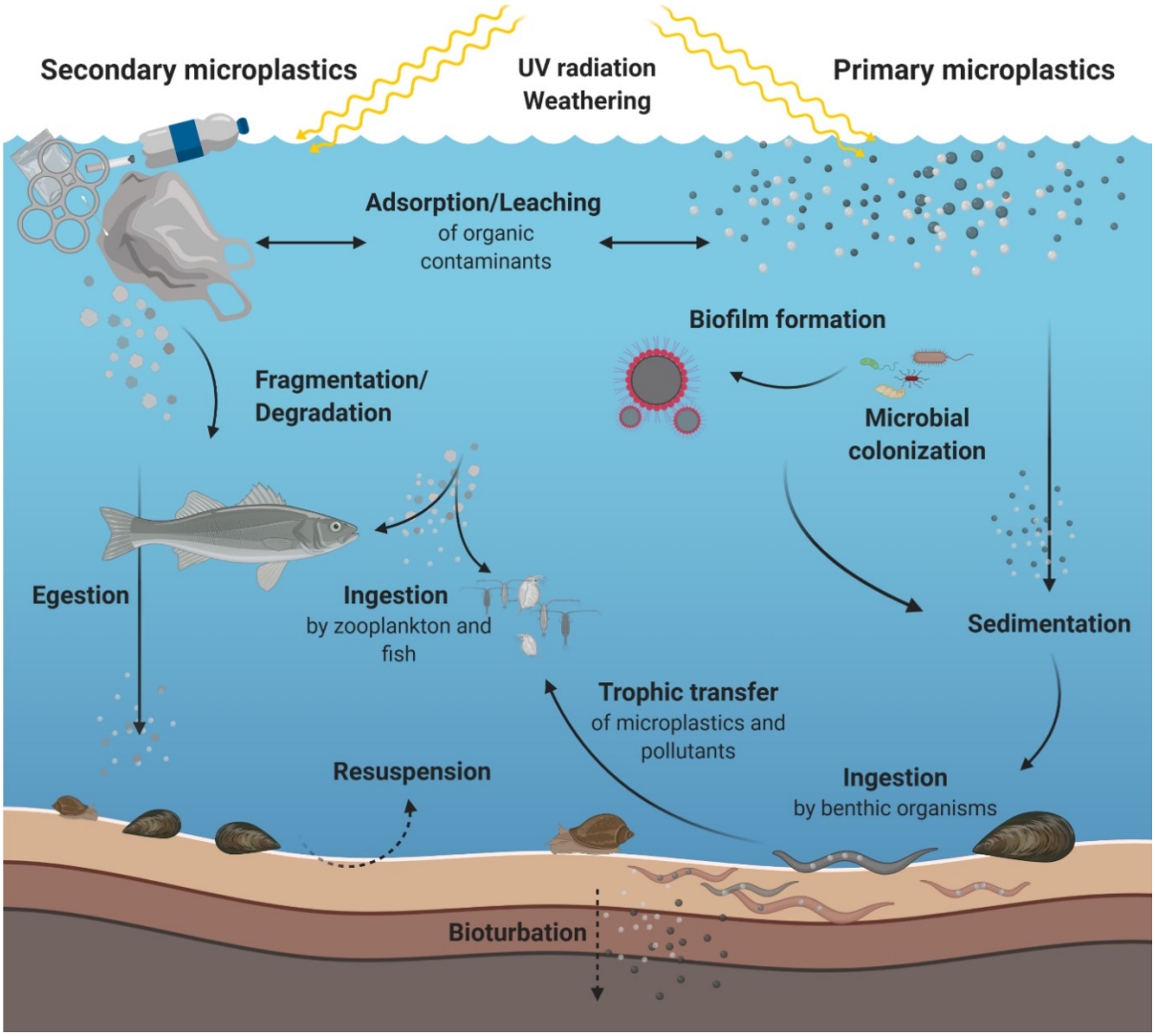

Fig. 2. Possible pathways and interactions of microplastics in aquatic ecosystems. Adapted from Wright et al. (2013).

I.3 Adverse effects of environmental pollutants and microplastics in aquatic ecosystems

Since the majority of all plastics are used and disposed of within days to weeks (Geyer et al., 2017), the terrestrial and freshwater environment are particularly at risk from extensive plastic contamination (Horton et al., 2017; Schmidt et al., 2017). Especially in industrialized and urbanized areas, high wastewater discharge rates can lead to elevated concentrations of particulate and dissolved pollutants in rivers and streams, eventually leading to the sorption of environmentally relevant pollutants to MPs (Eerkes-Medrano et al., 2015; Horton et al., 2017; Schmidt et al., 2017). Thus, the binding of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and pesticides to MPs in aquatic ecosystems may be unintentionally promoted. Hence, environmental contaminants, MPs, and microplastic-sorbed pollutants might be taken up by aquatic organisms, possibly inducing adverse effects at various biological levels and consequently disrupting the ecosystem (Fig. 3).

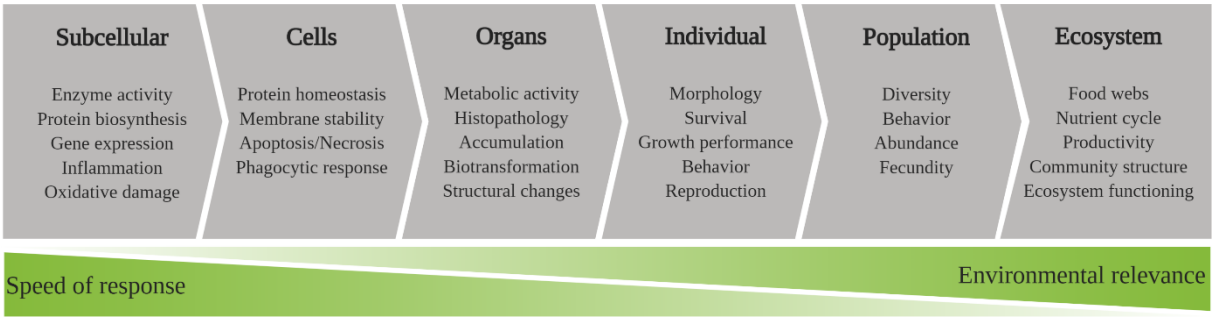

Fig. 3. Potential targets and effects of environmental contaminants and microplastic-associated pollutants across different biological levels, regarding the response pace and environmental relevance. Adapted from Fent (2013).

The sorption of complex contaminant mixtures on MPs from aquatic environments has already been investigated in several studies, indicating a possible transport of sorbed contaminants into aquatic organisms through ingestion (Rochman et al., 2013a; Chen et al., 2019; Pannetier et al., 2019). The successful accumulation of particularly lipophilic organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, polybrominated biphenyl ethers (PBDEs) or metals has already been documented for MPs collected from surface waters, shorelines and beaches or after exposure to wastewater (Rochman et al., 2013a; Ašmonaite et al., 2018; Vedolin et al., 2018; Le Bihanic et al., 2020). Amounts of PCBs and PAHs extracted from collected MPs ranged from 1 ng/g up to 850 ng/g plastic (Rochman et al., 2013a; Rios Mendoza and Jones, 2015; Pannetier et al., 2019a). These findings illustrate that MPs, due to the hydrophobicity of the surface of polymer particles, are capable of accumulating hazardous organic compounds in concentrations several orders of magnitudes higher compared to the surrounding medium (Hüffer and Hofmann, 2016).

Furthermore, the uptake of MPs, fragments and fibers was demonstrated for various marine and freshwater taxa, such as copepods (Cole et al., 2013; Bellas and Gil, 2020; Sørensen et al., 2020), mollusks (Avio et al., 2015; Paul-Pont et al., 2016; Magara et al., 2018; Weber et al., 2020), echinoderms (Beiras

and Tato, 2019; Trifuoggi et al., 2019) and fish (Sanchez et al., 2014; Rochman et al., 2015; Batel et al., 2016, 2018; Hermsen et al., 2017; Choi et al., 2018; Jovanović et al., 2018). MPs can therefore act as a vector for environmental pollutants and thus may provide a new exposure pathway *via* uptake of contaminated particles by aquatic organisms (Teuten et al., 2009; Koelmans et al., 2013).

Possible effects of contaminant transfer by MPs encompass the accumulation of contaminants through desorption of pollutants from MPs or prey upon ingestion (Fig. 4A/B), as demonstrated by Rainieri et al. (2018) for zebrafish and polyethylene MPs sorbed with PCBs and PBDEs; and depuration effects of contaminant body burdens by MPs (Fig. 4C), as documented by Gerdes et al. (2019) for *Daphnia magna* exposed to PCBs and MPs. However, as described previously (Koelmans et al., 2016; Besseling et al., 2019; Triebskorn et al., 2019), the contaminant fluxes accumulating in aquatic ecosystems through ingested MPs might be considered negligible compared to the sorption capacities of various natural particles (clay minerals, black carbon, etc.). Although the sorption of contaminants on MPs in environmental samples has been validated previously (Rochman et al., 2013a; Pannetier et al., 2017; Le Bihanic et al., 2020), the overall relevance of MPs for the transfer of environmental pollutants remains controversial (Koelmans et al., 2016; Wagner and Lambert, 2018).

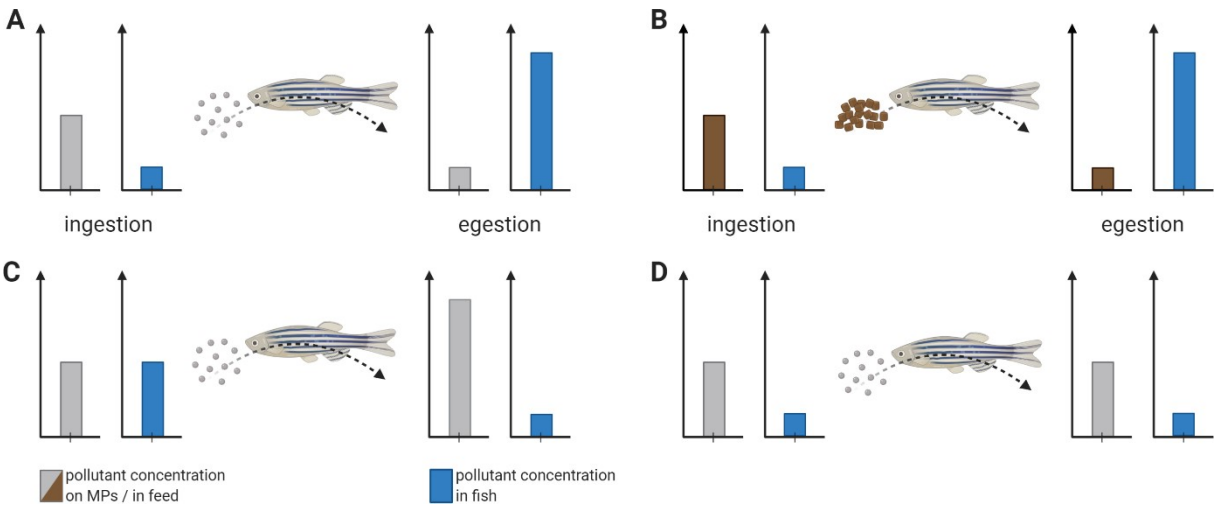

Fig. 4. Possible effects of contaminant transfer *via* different exposure routes. (**A**) Accumulation of contaminants in zebrafish taken up either by microplastic particles (**B**) or natural food. (**C**) Depuration effect by contaminant clearance from zebrafish *via* microplastic particles. (**D**) Passage of microplastic particles without effects. Adapted from Koelmans et al. (2016).

I.3.1 Effects of MPs on aquatic invertebrate organisms

The uptake of MPs has already been demonstrated for various invertebrate species, such as arthropods, echinoderms, annelids, mollusks, algae, and bacteria. An overview of recent studies on the effects of MPs and additional chemical and physical stressors is given in Tab. 1. Cole et al. (2013) stated that in aquatic ecosystems, especially lower trophic levels with non-selective filter-feeding organisms and deposit-feeders are at risk of microplastic exposure. Adverse effects of exposure to MPs resulted in reduced fitness, decreased reproductive performance (Ziajahromi et al., 2017; Redondo-Hasselerharm et al., 2018; Jaikumar et al., 2019; Schür et al., 2020) or increased bioavailability of environmental pollutants sorbed to MPs (Avio et al., 2015; Kim et al., 2017; Bellas and Gil, 2020; Felten et al., 2020), whereby MPs were even likely to alter the ecosystem structure of freshwater sediment bacterial communities (Kleinteich et al., 2018).

Depending on the polymer size and shape, microplastic fragments might be mistaken for prey, as exemplarily documented by Ory et al. (2017), where blue polyethylene fragments were mistakenly ingested by Amberstripe scad (*Decapterus muroadsi*) instead of copepod prey. In contrast, Ogonowski et al. (2018) emphasized that filter and suspension feeders such as daphnids and mussels are able to distinguish between edible and non-edible particles due to their adaptation to feed on mixtures of organic and abiotic particles and therefore concluded that these organisms are not particularly at risk from microplastic pollution (Ogonowski et al., 2018). The deviant results of the tested microplastic polymers, shapes, sizes and particle amounts (see Tab. 1) also indicated that aquatic invertebrates readily ingest and excrete MPs, with retention times ranging from minutes to several hours (Scherer et al., 2017; Nel et al., 2018). Nevertheless, adverse effects were most likely associated with particle numbers and pollutant loads exceeding concentrations of environmental relevance (Rehse et al., 2016; Triebskorn et al., 2019; Schür et al., 2020).

However, aquatic invertebrate species such as water flea neonates (*Daphnia magna*) and benthic larvae of the non-biting midge (*Chironomus riparius*) are valuable prey for vertebrates (fish) at higher trophic levels and could thereby transfer hazardous organic compounds (HOCs) or additives associated with MPs. Depending on the feeding strategy, both invertebrate species are susceptible to microplastic exposure, by either ingesting buoyant or sedimented MPs (Silva et al., 2019). Furthermore, both invertebrates are relevant models for chemical risk assessment under REACH (OECD, 2004, 2012) and in ecological studies (Jemec et al., 2016). Study results on the uptake of pollutants sorbed to MPs vary and are controversially discussed (Teuten et al., 2007; Gouin et al., 2011; Rochman, 2013; Koelmans et al., 2016). Also, the potential of MPs to transport sorbed contaminants across several trophic levels is still insufficiently investigated (Skjolding et al., 2014; Batel et al., 2016; Rochman et al., 2017; Tosetto et al., 2017; Elizalde-Velázquez et al., 2020) and therefore requires further research.

Tab. 1. Studies on the effects of microplastics and additional (chemical) stressors on aquatic invertebrate species (arthropods, echinoderms, annelids, mollusks, algae, and bacteria). Mixture effects indicate possible modulation of contaminant effects (↑↓ up/down regulation) by presence of microplastic particles. Polymer types: polystyrene (PS), polyethylene, low-density/high-density (PE), polypropylene (PP), polyamide (PA), polyvinyl chloride (PVC), polymethyl methacrylate (PMMA), polycarbonate (PC), polyethylene terephthalate (PET). Biomarkers: superoxide dismutase (SOD), catalase (CAT), total glutathione (GSH), glutathione peroxidase (GPx). n.a.= data unavailable.

Species	Polymer	Shape/ Color	Particle size	Test concentration	Additional stressor	Exposure time	Endpoints	(Mixture) Effects	Reference
Chironomus riparius	PE	irregular	32 - 500 µm	1.25 - 20 g/kg	-	28 d	Life-history traits	↓ Emergence, growth	Silva et al., 2019
	PA	irregular	10 - 180 µm	100 mg/kg (1×10^4 particles/kg)	Zinc-salt (Zn)	28 d	Emergence, reproduction, sex ratio, fitness	No effect	Khosrovyan and Kahru, 2020
	PVC	rounded	< 50 µm	5×10^{-4} - 5 g/L	Imidacloprid	28 d	Emergence, mass, development	↑ Toxicity of imidacloprid ↓ Emergence, mass	Scherer et al., 2020b
Daphnia magna	PE	spherical, irregular; fluorescent	2.6, 4.1 µm	10^2 - 10^5 particles/ml	-	21 d	Immobilization, reproduction, growth	↑ Mortality ↓ Reproductive output, growth	Ogonowski et al., 2016
	PS	beads, fluorescent	2 µm	0.1 - 1 mg/L	-	21 d	Immobilization, reproduction, growth	No effect	Rist et al., 2017
	PS, PS-COOH	spherical	200 µm	1 - 30 mg/L	Nickel (Ni)	48 h	Immobilization	↑ Mortality for PS-COOH ↑ Ni accumulation	Kim et al., 2017
	PA	irregular	15 - 20 µm	200 mg/L	Bisphenol A (BPA)	48 h	Immobilization	↓ Toxicity of BPA	Rehse et al., 2018
	PE	spherical, fluorescent	1 - 5 µm	0.02, 0.2 mg/L	-	21 d	Growth, mortality, reproduction	↑ Mortality ↓ Offspring	Pacheco et al., 2018
	PS	spherical	1 µm	3×10^5 particles/ml	Dimethoate; Deltamethrin	72 h	Immobilization	No effect	Horton et al., 2018
	PE	irregular	140 µm	100 mg/L	Landfill leachate, WWTP effluent, river/ spring water	48 h	Immobilization, mortality	No effect	Jemec Kokalj et al., 2019

Tab. 1. continued

Species	Polymer	Shape/ Color	Particle size	Test concentration	Additional stressor	Exposure time	Endpoints	(Mixture) Effects	Reference
Daphnia magna	PS	irregular	< 63 µm	400, 2000, 10000 particles/ml	-	4 × 21 d	Survival, reproduction, growth, offspring fitness	↓ Survival ↓ Reproduction ↓ Growth	Schür et al., 2020
	PE	spherical	1 - 4 µm	1, 10 mg/L	Deltamethrin	21 d	Life-history traits	↓ Fertility, brood, survival ↑ Toxicity	Felten et al., 2020
Ceriodaphnia dubia	PE	spherical, fiber, fluorescent, white	1 - 4 µm	31.25 - 2000 µg/L	-	48 h - 8 d	Survival, growth, reproduction, morphology	↑ Mortality ↓ Offspring ↓ Body size	Ziajahromi et al., 2017
Daphnia magna, Daphnia pulex, Ceriodaphnia dubia	PE	spherical, fluorescent, irregular	1 - 5 µm 1 - 10 µm	10^2 - 10^5 particles /ml	-	7 - 21 d	Reproductive output, length, chronic toxicity	↓ Offspring ↓ Terminal length	Jaikumar et al., 2019
Gammarus pulex, Hyalella azteca, Asellus aquaticus, Sphaerium corneum, Tubifex spp., *Lumbriculus variegatus*	PS	irregular	20 - 500 µm	0.1 - 40 % plastic weight in sediment	-	28 d	Survival, reproduction, growth	↓ Growth (*G. pulex*)	Redondo-Hasselerharm et al., 2018
Gammarus pulex	PMMA	spherical	40.2 µm	0.05 - 105 particles/cm^2	Temperature	24 h	Metabolism, feeding rate	↓ Metabolic rate	Kratina et al., 2019

Tab. 1. continued

Species	Polymer	Shape/ Color	Particle size	Test concentration	Additional stressor	Exposure time	Endpoints	(Mixture) Effects	Reference
Gammarus roeseli	PA	irregular	40 - 63 µm	500 µg/L	Phenanthrene	90 h	Acute toxicity, feeding rate, swimming behavior	↓ Bioavailability of phenanthrene	Bartonitz et al., 2020
Artemia parthenogenetica	PS	spherical, fluorescent	10 µm	1 - 1 × 10^4 particles/ml	-	14 d	Survival, growth, development	No effect	Wang et al., 2019b
Calanus helgolandicus	PS	n. a.	20 µm	75 particles/ml	-	24 h - 9 d	Feeding capacity, reproduction, survival, respiration	↓ Feed ingestion ↓ Egg size ↑ Mortality	Cole et al., 2015
Acartia tonsa	HDPE	irregular	1.4 - 42 µm	0.1 - 100 µg/L	Chlorpyrifos	48 h	Survival, fecundity, feeding, egg viability	↓ Fecundity, Egg viability ↑ Bioavailability, Mortality	Bellas and Gil, 2020
Acartia tonsa, Calanus finmarchicus	PE, PS	spherical	10 - 200 µm	n. a.	Fluoranthene; Phenanthrene	96 h	Acute toxicity	↓ Bioaccumulation ↓ Lethality	Sørensen et al., 2020
Sphaerechinus granularis	PS, PMMA	n. a.	10, 50, 80, 230 µm	0.1 - 10 mg/L	-	72 h	Development, mitotic activity, fertilization rate	↑ Cytogenic abnormalities, deformities ↓ Fertilization	Trifuoggi et al., 2019
Paracentrotus lividus	PE	irregular	5.5 µm	1, 10 mg/L	4-*n*-nonylphenol	48 h	Growth, survival, development	No effect	Beiras and Tato, 2019
Potamopyrgus antipodarum	PA, PC, PS, PET, PVC	irregular	4.6 - 602 µm	30, 70 % of feed	-	8 w	Morphology, embryogenesis, development	No effect	Imhof and Laforsch, 2016
Mytilus edulis, Arenicola marina	PS	spherical	10, 30, 90 µm	110 particles/ml, 110 particles/g	-	14 d	Energy metabolism	No effect	Van Cauwenberghe et al., 2015

Tab. 1. continued

Species	Polymer	Shape/ Color	Particle size	Test concentration	Additional stressor	Exposure time	Endpoints	(Mixture) Effects	Reference
Mytilus galloprovincialis	PE, PS	n. a.	< 100 µm	1.5 g/L	Pyrene	7 d	Gene expression, histopathology, DNA damage, oxidative stress	↑ Bioavailability ↑ DNA damage ↓ Lysosomal membrane stability	Avio et al., 2015
Mytilus spp.	PS	spherical, fluorescent	2, 6 µm	32 µg/L (2000 particles/ml)	Fluoranthene	7 d	Histopathology, oxidative stress, gene expression	↑ Histopathological lesions ↑ SOD, CAT, gene expression	Paul-Pont et al., 2016
Perna	PVC	n. a.	0.1 - 1.0 µm	0.125 g/L (1.1×10^{10} particles/L)	-	90 d	Physiology, mortality, oxidative stress, DNA damage, clearance rate	No effect	Santana et al., 2018
Mytilus edulis	PE	white	10 - 90 µm	100, 1000 particles/ml	Fluoranthene	96 h	Oxidative stress	↓ Bioavailability ↓↑ SOD, CAT, GSH, GPx	Magara et al., 2018
Dreissena polymorpha	PS	irregular, fluorescent	< 63 µm	0 - 10^5 particles/ml	-	14 d	Immune response, oxidative stress, clearance rate, mortality	↓ Oxidative stress	Weber et al., 2020
Isochrysis galbana	PE	spherical, irregular	2 - 6 µm	0.5 - 25 mg/L	Chlorpyrifos	72 h	Growth	↓ Toxicity, bioavailability	Garrido et al., 2019
Sediment bacterial community	PE	spherical, fluorescent	212 - 250 µm	20, 200 mg/g sediment	Phenanthrene, Anthracene	14 d	Microbial community composition	↓ Bioavailability	Kleinteich et al., 2018

I.3.2 Effects of MPs on zebrafish (*Danio rerio*)

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Over the last decade, microplastic fragments were detected in caught wild fish in different regions, including the Mediterranean sea (Anastasopoulou et al., 2013), tropical estuaries in the southwest Atlantic (Possatto et al., 2011), the North Pacific Gyre (Boerger et al., 2010; Davison and Asch, 2011) and highly frequented shipping areas such as the English Channel (Lusher et al., 2013) and the North and Baltic Sea (Rummel et al., 2016; Beer et al., 2018). Microplastics have been documented in 3 % to approximately 40 % of the investigated individuals, whereby fragments and fibers mainly originated from secondary microplastics such as bags, fishing ropes or textiles, consisting of polyethylene, polystyrene, polyamide, and polypropylene (Boerger et al., 2010; Anastasopoulou et al., 2013; Lusher et al., 2013; Rummel et al., 2016).

However, Beer et al. (2018) did not detect a significant increase of microplastic pollution over the last 25 years in plankton samples and digestive tracts of two planktivorous fish species (*Clupea harengus, Sprattus sprattus*) from the Baltic Sea. Compared to the contrasting results of increasingly contaminated plankton samples by Thompson et al. (2004), these findings suggest that the severity of plastic pollution in aquatic ecosystems is linked to the degree of urbanization in the catchment area rather than to the global plastic production rates, as suggested by Jambeck et al. (2015).

Wagner et al. (2014) already stated a lack of literature dealing with the effects of MPs and particle-associated contaminants in freshwater ecosystems. Since then, only a few studies have been published. Studies on the effects of MPs on aquatic vertebrates under controlled laboratory conditions are mainly conducted with zebrafish (*Danio rerio*) as a promising aquatic vertebrate model for medical and toxicological tests (Hill et al., 2005; Braunbeck et al., 2015; Barranco et al., 2017). Documented effects of clean, pristine MPs on different life stages of zebrafish from recent studies are summarized in Tab. 2. In 6 out of 17 studies, no effects on zebrafish were documented (35 %), and the majority of studies were conducted with adult zebrafish. In addition, there is a clear bias towards polystyrene and polypropylene as key synthetic polymers. Upon ingestion, adverse effects of MPs can be induced by physical damage, leading to histopathological alterations such as inflammation, necrosis or damage of the mucosal lining (Lu et al., 2016; Paul-Pont et al., 2016; Lei et al., 2018; Qiao et al., 2019a), reduced feed intake (Cole et al., 2015) and thus reduced growth (Zhao et al., 2020). Furthermore, biochemical modulations (e.g., hepatic stress, endocrine disruption, dysbiosis of the gut microbiome, oxidative stress, etc.) can be

induced either by the transfer of microplastic-sorbed contaminants sorbed from the environment (Rochman et al., 2013b, 2014; Pannetier et al., 2020) or by the intentional loading of MPs with model pollutants under laboratory conditions (Avio et al., 2015; Paul-Pont et al., 2016; Choi et al., 2018; Magara et al., 2018; Cormier et al., 2019; Qiao et al., 2019b).

Recent studies on adverse effects on zebrafish of MPs pre-exposed to environmental pollutants or in co-exposure to dissolved contaminants (e.g., metals, PAHs, PCB, PBDEs, endocrine-disrupting chemicals) are summarized in Tab. 3. Here, only two studies investigated possible effects under environmentally realistic exposure scenarios. One study was conducted by Qiao et al. (2019b), where adult zebrafish were chronically exposed to polystyrene particles, copper, and natural organic matter. The second study from Jemec Kokalj and co-workers (2019) illustrated the acute toxicity of MPs on zebrafish embryos, where polyethylene particles were pre-exposed to effluent from wastewater treatment plants, landfill leachate, and river water. Overall, in only two of ten studies, no effects of MPs on zebrafish have been detected (Jemec Kokalj et al., 2019; Batel et al., 2020). Whereas, the majority of studies documented a reduced bioavailability of the model contaminants and thus a reduction in toxic effects in zebrafish (Khan et al., 2015; Chen et al., 2017; Sleight et al., 2017; Batel et al., 2018). In conclusion, the most recent experimental results still indicate a possible transfer of microplastic-associated contaminants from both, the lower trophic levels to high-level consumers as well as to different tissues and organs in zebrafish (*Danio rerio*), but also illustrate reduced toxicity of co-exposed contaminants and, therefore rather lowering the risk of these pollutants than increasing their harmful potential.

However, the evidence of adverse effects caused by the ingestion of MPs and the transfer of contaminants sorbed to MPs is still not conclusively documented, as shown by recent studies, summarized in Tab. 1 - 3. In consequence, there is a persistent need for further studies on the transfer of environmental pollutants, in particular insecticides and PAHs, through microplastic polymers other than polystyrene and polyethylene, to assess possible adverse effects on invertebrate and vertebrate freshwater species (Wagner et al., 2014; Karami, 2017; Wagner and Lambert, 2018; Triebskorn et al., 2019).

Tab. 2. Studies on the effects of microplastics on different life stages of zebrafish. Effects indicate possible modulation of biomarkers by presence of microplastic (↑↓ up/down regulation). Polymer types: polystyrene (PS), polyethylene, low-density/high-density (PE), polypropylene (PP), polyamide (PA), polyvinyl chloride (PVC). Endpoints: ethoxyresorufin-*O*-deethylase (EROD), acetylcholine esterase (AChE), fish embryo toxicity test (FET), superoxide dismutase (SOD), catalase (CAT), total glutathione (GSH). n.a. = data unavailable. *Taken from Triebskorn et al. (2019) and supplemented with additional data, Copyright © Elsevier 2019*.

Life stage	Polymer	Shape/Color	Particle size	Test concentration	Exposure time	Endpoints	Effects	Reference
adult	PS	fluorescent, clear	70 nm, 5, 20 µm	2.9×10^2 - 1.1×10^{10} particles/L	4 h - 3 w	Oxidative stress, histopathology, metabolomics (liver)	↑ Inflammation ↑ SOD, CAT ↑ Energy metabolism	Lu et al., 2016
adult	PE	fluorescent	1 - 5 µm, 10 - 20 µm	1.2×10^6 particles/L	6 h - 2 w	Histopathology (intestine), EROD (liver)	No effect	Batel et al., 2016
embryo	PS	spherical	45 µm	2×10^4 particles/L	120 h	Larval locomotion, AChE, gene expression, oxidative stress, body length	No effect	Chen et al., 2017
larvae	LDPE	irregular	< 17.6 µm	1.04×10^3 - 1.04×10^5 particles/L	20 d	Condition factor, transcriptomics, histopathology	No effect	Karami et al., 2017
embryo adult	PE	fluorescent	1 - 5 µm, 10 - 20 µm	5×10^6 - 3.2×10^7 particles/L	6 - 24 h	Acute toxicity (FET), EROD (gill)	No effect	Batel et al., 2018
adult	PA, PE, PP, PS, PVC	irregular, spherical (PS)	70 µm, 0.1 - 5.0 µm (PS)	0.001 - 10 mg/L	10 d	Mortality, histopathology (gill, liver, kidney, intestine)	↑ Mortality (10 mg/L PP) ↑ Intestinal damage	Lei et al., 2018
adult	LDPE	rounded	125 - 250 µm	n. a.	3 w	Histopathology, gene expression	No effect	Rainieri et al., 2018
adult	PS	spherical	0.5, 50 µm	100, 1000 µg/L	14 d	Composition of gut microbiome, histopathology, functional gene analysis	↑ Intestinal mucus cells ↓↑ Gut microbiome dysbiosis, gene analysis	Jin et al., 2018
adult	PS	spherical	0.2 - 90 µm	2×10^5 particles/L	2 h	Gene expression (immune status)	↑ Gene expression	Lu et al., 2018

Tab. 2. continued

Life stage	Polymer	Shape/Color	Particle size	Test concentration	Exposure time	Endpoints	Effects	Reference
embryo larvae	PE	spherical, fluorescent	10 - 45 µm	20 mg/L (480 particles/ml)	14 d	Growth, hatching, oxygen consumption rates, transcriptomics	No effect	LeMoine et al., 2018
adult	HDPE	spherical (colored)	10 - 600 µm	2 mg/L	96 h	Behavior, gene expression (*cyp1a, vtg*), physiology	↑ Gene expression (liver) ↑ Abnormal behavior	Mak et al., 2019
embryo larvae	PS	spherical, fluorescent	1 µm	100, 1000 µg/L (1.9×10^6 - 10^7 particles/L)	24 - 120 h	Swimming competence, gene expression, development	↓ Swimming distance, speed ↑ Oxidative stress, inflammation	Qiang and Cheng, 2019
adult	PS PP	bead, fragment, fiber, fluorescent	15 µm, 4 - 40 µm, 20 - 100 µm	10 µg/L (5400 beads/L, 680 fibers/L)	21 d	Composition of gut microbiome, histopathology (intestine), oxidative stress, gene expression	↑ Mucosal damage ↑ Inflammation, SOD ↓↑ Gut microbiome dysbiosis, gene expression	Qiao et al., 2019a
larvae	PS	spherical, fluorescent, clear	5 µm, 50 µm	100, 1000 µg/L	7 d	Composition of gut microbiome, oxidative stress, metabolomics, transcriptomics	↓ CAT, GSH ↓↑ Gut microbiome dysbiosis, metabolomics	Wan et al., 2019
adult	mix of HDPE, PS	irregular	25 - 90 µm	100, 1000 µg/L	20 d	Transcriptomics, behavior, histopathology (liver, gills)	↑ Gene expression (immune response) ↑ Locomotor activity ↓ Tissue integrity	Limonta et al., 2019
embryo larvae	PE	rounded, irregular	38.3 ± 15.6 µm	6.2 - 100 mg/L	144 h	Acute toxicity (FET), morphology	↑ Mortality ↓ Hatching rate ↑ Morphologic deformities	Malafaia et al., 2020
adult	PS	spherical	5 µm	20, 100 µg/L	21 d	Growth, condition factor, transcriptomics, metabolism	↓ Condition factor ↓ Glycolipid metabolism ↓ Metabolic activity	Zhao et al., 2020

Tab. 3. Studies on the effects of microplastics and additional (chemical) stressors on different life stages of zebrafish. Mixture effects indicate possible modulation of contaminant effects (↑↓ up/down regulation) by presence of microplastic particles. Polymer types: polyethylene, low-density/high-density (PE), polystyrene (PS), polyvinyl chloride (PVC). Contaminants: polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS). Endpoints: ethoxyresorufin-*O*-deethylase (EROD), acetylcholine esterase (AChE), fish embryo toxicity test (FET), superoxide dismutase (SOD), malonaldehyde (MDA), metallothionein (MT). n.a. = data unavailable.

Life stage	Polymer	Shape/ Color	Particle size	Test concentration	Additional stressor	Exposure time	Endpoints	Mixture effects	Reference
adult	PE	spherical	19 - 107 µm	10^4/ 10^5/ 10^6 particles/L	Silver (Ag)	4 - 24 h	Bioavailability	↓ Ag bioavailability	Khan et al., 2015
adult	PE	fluorescent	1 - 5 µm, 10 - 20 µm	1.2×10^6 particles/L	Benzo(*a*)pyrene (BaP)	6 h - 2 w	Histopathology, EROD (liver)	↑ Transfer of BaP to intestine	Batel et al., 2016
embryo	PS	spherical	45 µm	2×10^4 particles/L	17 α-ethinylestradiol (EE2)	120 h	Larval locomotion, AChE, gene expression, oxidative stress, body length	↑ Gene expression ↓ EE2 bioavailability	Chen et al., 2017
embryo	uPVC	irregular	200 - 250 µm	400 mg/L	Phenanthrene, 17 α-ethinylestradiol	96 h	Gene expression (*vtg*, *cyp1A*), development	↓ Bioavailability	Sleight et al., 2017
embryo adult	PE	fluorescent	1 - 5 µm, 10 - 20 µm	5×10^6 - 3.2×10^7 particles/L	Benzo(*a*)pyrene	6 - 24 h	Acute toxicity (FET), EROD (gill)	↓ BaP toxicity	Batel et al., 2018
adult	LDPE	rounded	125 - 250 µm	n. a.	PCBs, PBDEs, PFOA, PFOS, Methylmercury	3 w	Histopathology, gene expression	↑ Tissue accumulation, ↑ Gene expression	Rainieri et al., 2018
embryo	PE	n. a.	11 - 13 µm	10, 100 mg/L	Oxybenzone, BaP, PFOS	120 h	Acute toxicity (FET), EROD, gene transcription, behavior	↑ EROD, ↑*cyp1a* gene expression	Cormier et al., 2019
adult	PS	clear, fluorescent	0.1 µm, 20 µm	40 mg/L	Copper (Cu), natural organic matter	14 d	transcriptomics, oxidative stress	↑ MDA, MT ↓ SOD ↑ Cu bioavailability	Qiao et al., 2019b
embryo	PE	irregular	140 ± 80 µm	100 mg/L	Landfill leachate, WWTP effluent, River/spring water	96 h	Acute toxicity (FET), physiology	No effect	Jemec Kokalj et al., 2019
adult	LDPE	n. a.	4 - 6, 125 - 500 µm	1 mg/day	Benzo(*a*)pyrene	21 d	Histopathology, EROD (liver)	No effect	Batel et al., 2020

I.4 Implications for laboratory applications

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Before starting to work with MPs in laboratory studies, preliminary considerations are required to ensure optimal experimental design and mindful use of resources. Without claiming to be exhaustive, it might be important to answer some of the following questions beforehand:

- Is my experiment about possible trophic transfer, or is it just on plain effects of MPs at the cellular, organismal, or behavioral level?
- What is the underlying mechanism of interest: Is it just a proof-of-principle whether the transfer of particle-sorbed pollutants is possible?
- Do I need environmentally relevant particle concentrations, or is the exposure scenario independent of particle size and/or amount?
- Are the shape and the surface properties of my MPs of choice chosen appropriately, as adsorption/desorption is influenced by surface-volume ratios as well as surface functionalization of the polymer itself?
- The composition of the MPs used should be known: Which additives (plasticizer, dyes, protective agent and/or surfactants, etc.) might leach during the experiment?
- Is there a potential for interference between the test substance(s) of interest and the selected type of MPs? Is there a need to run additional controls to obtain conclusive results?
- What about the variability of my experimental results? How many independent replicates do I need to obtain at a reproducible data set?
- Might chronic exposure scenarios be of higher environmental and ecological relevance than acute toxicity test, or is it even necessary to include a co-exposure scenario to simulate environmentally relevant mixture toxicity?
- How about natural reference particles to account for possible “particle only”-related effects?
- Which is the best way of preparing homogenous microplastic suspensions, and might this procedure interfere with the certain characteristics of MPs (surfactants, ozonation and formation of byproducts, shaking, aeration, loss during filtration)?
- How can I keep track of my MPs in the experimental setup and avoid accumulation or loss over the test period?
- Are the MPs of choice compatible with the characteristics, habitat, and ecosystem relevance of the test organism?

Several reviews already criticized the lack of appropriate preliminary considerations in recent MP research (Wagner et al., 2014; Karami, 2017; Triebskorn et al., 2019). In fact, only a few communications address appropriate selection and handling of MPs for ecotoxicological and general aquatic research; rather, they focus on constraints and difficulties of present MP studies as well as the risk of loss and accumulation of MPs during biotic sampling, storage, contamination in the field and laboratory (Karami, 2017; Lusher et al., 2017; Paul-Pont et al., 2018; Eitzen et al., 2019).

Furthermore, there is an upcoming demand for natural reference particles (Ogonowski et al., 2016; Scherer et al., 2017; Paul-Pont et al., 2018) since MPs only represent a very small fraction of inorganic particles in aquatic ecosystems (Koelmans et al., 2016). Yet, no ideal reference particle has been agreed on, since the characteristics of inorganic natural particles like kaolin, clay minerals, quartz sand or glass beads are not in accordance with the various characteristics of MPs. Due to different density properties, natural particles tend to sediment more quickly than most types of MPs and thus are no longer available in the water column.

Aiming at more realistic exposure scenarios, artificial weathering *via* UV radiation, ozone treatment or mechanical crushing might be of interest as well as biofilm formation on MPs. Manipulated MPs might represent environmentally more realistic surface characteristics than primary, spherical and clean MP beads. However, a major limitation for relevant MP research might be the availability of adequate amounts of pre-treated MPs. Thus, a comprehensive characterization of MPs is of utmost importance to obtain conclusive results and ensure reproducibility of experiments. This holds true for the design of any exposure experiment, technical aspects such as optimization of the application of test solution (static, semi-static, flow-through), prevention of MP accumulation by siphoning buoyant particles or avoiding loss of MPs on surfaces due to excessive aeration are of great importance.

When working with MPs, most laboratory materials should be replaced by disposable glassware to prevent plastic-plastic interactions (desorption of substances, loss of MPs). To prevent accumulation of MPs over the test period, test solutions need to be mixed thoroughly by gentle agitation (e.g., pipetting up and down prior to exchange). In order to avoid aggregation of MPs in larger test vessels like aquaria, dispersing test solutions mechanically by small submersible pumps may be beneficial, as described by Karami (2017). Care has to be taken, as the addition of plastic materials within the experimental setup might lead to changes in the sorption equilibria and result in unpredicted particle numbers and/or altered concentrations of the model substance.

I.5 Aims and objectives

Considering the increasing global plastic production, environmental pollution with plastic debris has been widely perceived as an ecological threat with potential adverse (eco-)toxicological impacts on various aquatic species and ecosystems. Extensive knowledge has been gained on the influence of rivers and streams as main entry routes for MPs into marine environments during the last years (Klein et al., 2015; Schmidt et al., 2017; Siegfried et al., 2017). So far, only a few studies have addressed the fate and effects of microplastics at the starting point of microplastic transport pathways: freshwater ecosystems. Therefore, the overall objective of this thesis was to provide further insights into the impact of MPs in limnic ecosystems by providing comprehensive information on the biological fate and effects of MPs and associated anthropogenic pollutants in freshwater fish (Fig. 5).

Due to inconclusive results on the effects of MPs and the possible transfer of environmental pollutants to various aquatic organisms – as summarized in Tab. 1 to 3 – there is need to investigate further: (**1**) the underlying acute toxic effects of MPs and associated anthropogenic pollutants with regard to possible impacts on sensitive developmental stages of fish; (**2**) effects of chronic long-term exposure of fish to MPs and contaminants to approach more realistic environmental exposure scenarios; as well as (**3**) possible vector effects resulting from the trophic transfer of MPs and MP-associated contaminants to vulnerable high-level vertebrate consumers; and (**4**) the adverse effects of MPs on freshwater vertebrates following exposure under natural conditions. Thereby, the assessment of potentially hazardous effects of MPs regarding their impact on freshwater species under controlled laboratory conditions as well as realistic environmental exposure scenarios can be enabled.

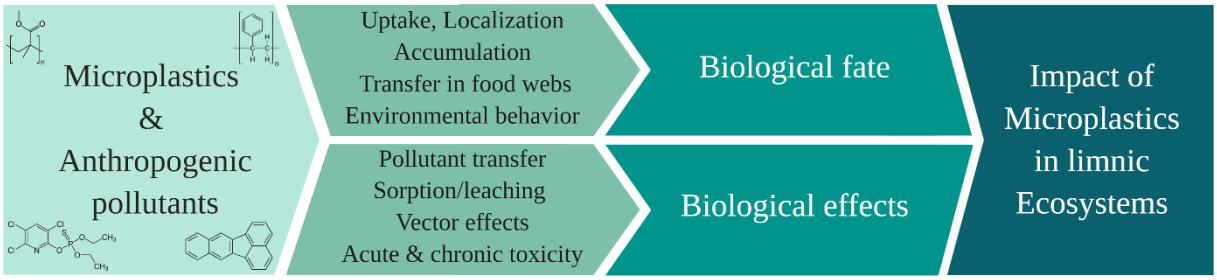

Fig. 5. Research approach for assessing the potential impact of microplastics and microplastic-associated pollutants in limnic ecosystems, using two representative types of microplastics in combination with two model anthropogenic pollutants.

Two common environmental pollutants with different modes of action were selected: The polycyclic aromatic hydrocarbon benzo(k)fluoranthene (BkF), originating from incomplete combustion and pyrolysis processes (e.g., forest fires, exhaust gases, and cigarette smoke; EFSA, 2007), and the organophosphate pesticide chlorpyrifos (CPF), globally applied for fruit and vegetable treatment (EFSA, 2019). Both substances occur in surface waters and are priority pollutants under the European Water Framework Directive (European Parliament and European Council, 2001; Arle et al., 2016; Busch et al., 2016)

and effectively sorb to microplastic debris in the environment (Rochman et al., 2013a; Bellas and Gil, 2020). The increased lipophilicity of benzo(k)fluoranthene and chlorpyrifos (log K_{OW} 6.11 and 4.96, respectively) also indicates an enhanced potential for accumulation and bioconcentration in fish tissues and increased sorption on polymer surfaces (Heinrich et al., 2020). Moreover, two synthetic polymers with different properties in terms of shape and density were selected: Irregular fragments of the thermoplastic polymer polystyrene (PS; $\rho = 1.05$ g/cm^3) and spherical beads of the thermoset polymethyl methacrylate (PMMA; $\rho = 1.19$ g/cm^3). The zebrafish (*Danio rerio*) was chosen as the model organism for all experiments conducted in the scope of this thesis, as it represents the aquatic vertebrate model for medical and toxicological tests and risk assessment (Hill et al., 2005; Embry et al., 2010; Braunbeck et al., 2015). A multidisciplinary approach was applied by coupling the analytical quantification of the selected model pollutants and the assessment of morphological and physiological alterations as well as enzymatic biomarkers and tracking *via* advanced fluorescence microscopy to determine the fate and behavior of the model pollutants and MPs within target organisms and experimental compartments.

To assess the acute toxicity of MPs and both model pollutants, adverse effects on zebrafish embryos between 2 h postfertilization (hpf) and 96 hpf were investigated. Therefore, zebrafish embryos were exposed either to clean, pristine MPs or different concentrations of the dissolved model contaminants as well as to both contaminants sorbed to MPs. Enzymatic biomarkers (ethoxyresorufin-*O*-deethylase assay, acetylcholine esterase activity) were employed to complement findings on morphological alterations observed in the fish embryo toxicity test (FET) and to set concentration thresholds (EC_{10}) for subsequent chronic exposure scenarios. Sorption and leaching of both model pollutants to and from MPs were determined using GC-MS to assess the available amount of BkF and CPF in aquatic exposure scenarios.

In a next step, the potential chronic effects of MPs, BkF, and CPF on adult zebrafish were monitored over 21 days of continuous exposure. Uptake of MPs and contaminant transfer was investigated microscopically in gastrointestinal cryosections of zebrafish. To determine the potential contaminant transfer into zebrafish, selected enzymatic biomarkers such as acetylcholine esterase activity (AChE), EROD activity, tissue accumulation and formation of biotransformation products of BkF and CPF in brain and liver of zebrafish were investigated. These endpoints were chosen according to the mode of action of the applied substances in the respective target organs and progressively measured at several time points to detect temporary changes. Thus, chronic exposure scenarios enhance the environmental relevance of potential adverse effects of MPs and associated contaminants and therefore complement the risk assessment of MPs.

Furthermore, vector effects of MPs, potential biomagnification processes and trophic transfer of BkF *via* MPs were investigated in a two-level limnic food web to investigate the emerging threat of MPs in limnic ecosystems and to enhance the environmental relevance of exposure scenarios. The food web consisting of zooplankton (*Daphnia magna*), sediment-dwelling invertebrates (*Chironomus riparius*

larvae) and zebrafish (*Danio rerio*) as the high-level predator. Furthermore, the amount of BkF sorbed to MPs and taken up by invertebrates has been determined *via* GC-MS and was tracked by fluorescence microscopy. In addition, BkF uptake by zebrafish was quantified by a novel approach of fluorescence measurements in cryosections of the gastrointestinal tract and supplemented with measurements of the induction of cytochrome P450 in liver samples of zebrafish using the EROD assay.

To investigate the effects and sorption behavior of MPs in natural freshwater environments, a mixture of four different MPs (50 % polyethylene, 25 % polypropylene, 15 % polystyrene, 10 % polyvinyl chloride) was exposed in a tributary of the river Rhine (south of Frankfurt/Main, Germany) for 21 days. The analytical quantification of the substances present in the river (e.g., pharmaceuticals, pesticides, drugs and metabolites) and the sorption to MPs were assessed by a target screening using GC/LC-MS. Following the recovery of the MP mixture, adverse effects of the exposed MPs, water samples, suspended matter and sediment samples from the tributary were evaluated concerning acute toxicity (FET), potential neurotoxic effects (AChE activity, larval visual motor response test) and effects of dioxin-like substances (EROD assay) using zebrafish embryos (2 hpf - 120 hpf). The results were to complement the effects of MPs on sensitive life stages of freshwater vertebrates and should provide evidence for the potential transfer of hazardous contaminants under realistic and natural conditions.

The variability of the exposure scenarios allows a comprehensive assessment of the hazard potential of MPs in limnic ecosystems with respect to a possible transfer of MPs and MP-associated contaminants to different aquatic organisms and their effects at different developmental stages of zebrafish. Thus, a decisive contribution can be made to a better understanding of the biological fate and effects of MPs in the aquatic environment.

II. Acute toxicity effects of microplastics and sorbed model contaminants in zebrafish (*Danio rerio*)

II.1 Introduction

The highly variable properties of MPs (e.g., shape, size, composition, density, surface structure, etc.) affect their spatial distribution, biological interactions and fate in our ecosystems and thus pose a risk to invertebrate and vertebrate fauna (Jabeen et al., 2018; Lehtiniemi et al., 2018; Rainieri et al., 2018; Besseling et al., 2019; Franzellitti et al., 2019). Despite the ubiquitous occurrence of MPs in our aquatic environment (Hidalgo-Ruz et al., 2012; Klein et al., 2015; Alimi et al., 2017; Triebskorn et al., 2019), the potential ecological risk of MPs is further exacerbated by unintentional sorption of environmental pollutants (e.g., pesticides and polycyclic aromatic hydrocarbons) and substances introduced during the production process, such as monomers, plasticizers, dyes and flame retardants (Teuten et al., 2009).

Therefore, it is indispensable to investigate possible hazardous effects of MPs and the selected model pollutants to identify possible synergistic effects or enhanced transfer of pollutants by MPs. The polycyclic aromatic hydrocarbon benzo(k)fluoranthene and the organophosphate pesticide chlorpyrifos have been evaluated regarding acute toxic effects on early developmental stages of zebrafish (*Danio rerio*). For this purpose, zebrafish embryos were exposed to either clean, pristine beads of the polymer polymethyl methacrylate (PMMA), various concentrations of dissolved model pollutants, or benzo(k)fluoranthene and chlorpyrifos sorbed to PMMA particles. Different endpoints were considered to determine lethal and sublethal developmental malformations, teratogenic and neurotoxic effects as well as biotransformation and detoxification capacities in zebrafish embryos.

II.1.1 Fish embryo toxicity test (FET)

The fish embryo toxicity test according to OECD TG 236 (2013) has been established as a mandatory component in ecotoxicity testing under the REACH regulation of the European Union (European Parliament and European Council, 2006) for the identification of potential environmental hazards and as a basis for risk assessment of xenobiotics such as plant protection products, pharmaceuticals, personal care products, or environmental pollutants (Lammer et al., 2009; Braunbeck et al., 2015). Testing of microplastics and associated contaminants is therefore considered essential. The pervasive occurrence of MPs in the marine and limnic environment and their potential effects on aquatic fauna are therefore intensely discussed (Wagner et al., 2014; Triebskorn et al., 2019).

The test procedure of the fish embryo toxicity test with zebrafish embryos (≤ 120 hpf) complies with the 3R-principles of Russell and Burch (1959) and the European animal welfare legislation (EU, 2010; Strähle et al., 2012). The FET was applied to document the acute toxicity of benzo(k)fluoranthene (BkF), chlorpyrifos (CPF), pristine MPs, and MP-sorbed BkF/CPF on embryonic stages of zebrafish. As

described before in chapter I.4 (Implications for laboratory applications), minor adjustments had to be made for experiments with MPs to prevent interactions between test materials and MP suspensions as well as unintended losses due to sorption. Furthermore, the integration of additional sublethal endpoints (e.g., formation of edemata, heartbeat, blood circulation, scoliosis, etc.) was favored to investigate further adverse effects regarding chronic responses and teratogenicity (Lammer et al., 2009), since alterations in these endpoints can serve as an early warning for potential downstream effects (Embry et al., 2010).

II.1.2 *In vivo* live-imaging ethoxyresorufin-*O*-deethylase (EROD) assay

Early life stages of zebrafish (oocytes, embryos, and larvae) have already proven the ability to metabolize xenobiotics and exogenous compounds actively (Andreasen et al., 2002; Goldstone et al., 2010; Otte et al., 2010; Loerracher et al., 2020) and, thus, appear to be a particularly sensitive biomarker (Whyte et al., 2000). The cytochrome P450-dependent monooxygenases (CYPs), especially isoforms of the CYP 1 to 4 subfamilies, are essential groups of enzymes for biotransformation and detoxification of xenobiotics (Nebert and Russell, 2002; Guengerich, 2008). Further, the CYP signal transduction pathway is highly conserved in various vertebrate species, including mammals and fish (Whyte et al., 2000; Schlenk et al., 2008).

One of the most commonly used *in vivo* and *in vitro* approaches to assess CYP1A activity is the ethoxyresorufin-*O*-deethylase (EROD) assay, induced by contaminant exposure (Payne, 1976; Whyte et al., 2000; Saad et al., 2016). The inductive response involves the cytosolic aryl hydrocarbon receptor (AhR), where xenobiotics like polycyclic aromatic hydrocarbons (PAHs), pesticides, polychlorinated biphenyls (PCBs), bind as ligands and initiate the transcription of various genes. The protein synthesis is further promoted, and finally, the catalytic activity of CYP1A is increased (Sarasquete and Segner, 2000; Otte et al., 2010). Consequently, the EROD assay can be used to determine catalytic activity as a measure of the rate of CYP1A-mediated deethylation of the substrate (7-ethoxyresorufin) to fluorescent resorufin (Billiard et al., 2006; Vehniäinen et al., 2012; Heinrich et al., 2014). This correlation is an indicator of the present amount of CYP1A in a sample and hence a direct measure of AhR activation (Whyte et al., 2000; Schlenk et al., 2008). The exposure of zebrafish embryos to dissolved BkF (BkF_{AQ}) and BkF-sorbed MPs (BkF_{MP}) might lead to cellular uptake of the polycyclic aromatic hydrocarbon (PAH) BkF. Thus, measuring the activity of the biotransformation enzyme 7-ethoxy-resorufin-*O*-deethylase (EROD) is a sensitive endpoint to determine changes in metabolic activities.

Further development of the EROD assay by Otte et al. (2010) resulted in the *in vivo* EROD live-imaging assay (Boehler et al., 2018; Kais et al., 2018) in zebrafish embryos (96 hpf) by further increasing the sensitivity of the test through the reduction of the experimental exposure period. After three hours of exposure of *Danio rerio* embryos (96 hpf) to the PAH benzo(k)fluoranthene and MP-associated

benzo(k)fluoranthene, the fluorescent resorufin metabolite has accumulated in the liver and gastrointestinal tract of the zebrafish embryo (Andreasen et al., 2002) and can subsequently be measured using fluorescence microscopy. In adult zebrafish, the EROD induction can be measured in different tissues such as liver, gills, and kidney (Otte et al., 2010; Batel et al., 2018). Care has to be taken because endogenous factors such as age, sex, hormone levels, and the physiological condition of experimental fish may influence the responsiveness of the CYP1A system (Sarasquete and Segner, 2000; Whyte et al., 2000; Saad et al., 2016). Several invertebrate species have reduced abilities to metabolize PAHs effectively and may thereby pose a risk for higher trophic levels (Livingstone, 1991; Whyte et al., 2000; Carrasco Navarro et al., 2013). Therefore, assessing the EROD induction in conjunction with additional biomarkers and analytical quantification of contaminants in target organs is intended to provide a robust set of parameters for exposure and risk assessment (Bucheli and Fent, 1995; Whyte et al., 2000).

II.1.3 *In vivo* acetylcholine esterase activity (AChE)

This section includes content, which was produced jointly with Luisa Nuttall.

The membrane-bound enzyme acetylcholine esterase (AChE) is located in post-synaptic membranes in central and neuromuscular synapses of the cholinergic system (Behra et al., 2002). AChE is responsible for the cleavage of the neurotransmitter acetylcholine, the primary neurotransmitter of the cholinergic system, into choline and acetic acid to end cholinergic transmission (Padilla et al., 1999; Scott and Sloman, 2004). The enzyme can be inhibited by various environmental contaminants, including organophosphate pesticides (Küster and Alterburger, 2006; Kais et al., 2015; Rodríguez-Fuentes et al., 2015; Nunes et al., 2018). The irreversible inhibition results in an increased level and duration of neurotransmitter action, leading to impaired neurotransmission and unregulated excitation (Colovic et al., 2013; Russom et al., 2014). Furthermore, the bioactivation of xenobiotics can significantly increase the binding to cholinesterase and thus further enhance AChE inhibition (Schlenk et al., 2008).

AChE activity is well known to be hampered upon exposure to the organophosphate pesticide chlorpyrifos (CPF) at different developmental stages in zebrafish (Yen et al., 2011; Richendrfer and Creton, 2015; Rodríguez-Fuentes et al., 2015). CPF leads to enzyme inhibition by covalent binding to serine -OH groups, and subsequent accumulation of acetylcholine in the synaptic cleft (Colovic et al., 2013). Similar results have been observed in terrestrial and aquatic ecosystems for mixtures of anthropogenic pollutants (Scott and Sloman, 2004; Kais et al., 2015; Massei et al., 2015; Tousova et al., 2017; Amoatey and Baawain, 2019).

The measurement of AChE activity in tissue samples has been established by Ellman et al. (1961), was further adapted to microtiter plates by Guilhermino et al. (1996), with modification by Küster (2005) for use in zebrafish embryos and was optimized in terms of samples size by Kais et al. (2015). As already described by Ellman et al. (1961), the measurement of AChE activity is based on the degradation of

acetylthiocholine to acetate and thiocholine by the enzyme acetylcholine esterase. Thiocholine reacts with dithiobis-nitrobenzoate, and thereby produces a yellow color. Thus, the change in optical density is a direct measure of hydrolysis catalyzed by AChE (Tousova et al., 2017). In *Danio rerio* embryos, AChE activity in whole-body homogenates was determined after continuous exposure over 96 h to different concentrations of chlorpyrifos and CPF sorbed to MPs. In adult zebrafish, however, AChE activity can be measured in various tissues, including muscle, brain, and gills (Clemente et al., 2004; Lopes et al., 2017). Care has to be taken to avoid masking of neurotoxic effects by sublethal toxic effects of CPF. Therefore, the effects of CPF concentrations above the EC_{10}, determined using the FET, have to be interpreted cautiously.

II.2 Materials and methods

II.2.1 Microplastics and chemicals

For acute toxicity experiments, unstained, spherical beads of PMMA (≤ 50 µm, density 1.19 g/cm³, Goodfellow Cambridge Ltd., England) with an initial concentration of 1×10^6 particles/L were applied (0.05 g/L). All chemical reagents were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany). Stock solutions were prepared for benzo(k)fluoranthene (CAS No. 207-08-9, analytical grade ≥ 99 %) at 2 g/L in DMSO in pre-saturated glass vials, covered in aluminum foil, stored at 4 °C and for chlorpyrifos (CAS No. 2921-88-2, analytical grade ≥ 98 %) at 3 g/L in DMSO. Dilution series for both substances were conducted with freshly prepared artificial water (63 mg/L $NaHCO_3$, 5.5 mg/L KCl, 294.0 mg/L $CaCl_2 \times 2\ H_2O$, 123.3 mg/L $MgSO_4$) according to OECD TG 203 (2019). Tested concentrations for BkF ranged from 0.048 µg/L to 200 µg/L (DMSO 0.01 %) and for CPF from 1 µg/L to 800 µg/L (DMSO 0.02 %) and were chosen upon literature data and pre-tests. The artificial water served as a negative control, and the pH was adjusted daily to 7.75 ± 0.02 with sodium hydroxide (NaOH) and hydrogen chloride (HCl) before use. Otherwise, all solutions were directly prepared before use. Measurement of protein contents was carried out with the DC™ Protein Assay kit from Bio-Rad (Feldkirchen, Germany), according to Lowry et al. (1951), using bovine serum albumin (0.125 - 2 mg/ml, in phosphate buffer) as standard.

II.2.2 Loading of PMMA particles with model substances, recovery and sorption validation

This section includes content based on a manuscript (see Chapter IV) written entirely by myself and has currently been published in Comparative Biochemistry and Physiology – Part C: Toxicology and Pharmacology. The content was created jointly with support from Stefan Dittmar and Sven Huppertsberg. Sven Huppertsberg conducted the analytical quantification of BkF and CPF, and Stefan Dittmar

measured the particle recovery and distribution. However, sample preparation and data evaluation were always conducted entirely by me.

For the exposure of zebrafish embryos to BkF or CPF sorbed to MPs, 0.05 g/L PMMA was added to the dilution series of the model substance in 40 ml Aqua bidest, and incubated at room temperature overnight on a Certomat S-II orbital shaker at 100 rpm (Sartorius Stedim Biotech, Göttingen, Germany). After incubation, all suspensions were filtered separately over 0.2 µm Whatman Puradisc cellulose acetate filter (GE Healthcare, Solingen, Germany) using a 10 ml BD Discardit™ II syringe (Becton & Dickinson, Heidelberg, Germany). The PMMA particle suspensions were washed four times with 10 ml Aqua bidest and subsequently resuspended by backwashing the filter with 40 ml artificial water (Fig. 6) to ensure that BkF/CPF originates from the particulate phase only. Negative controls with pristine PMMA were treated likewise, and incubation of CPF suspensions was performed analogously.

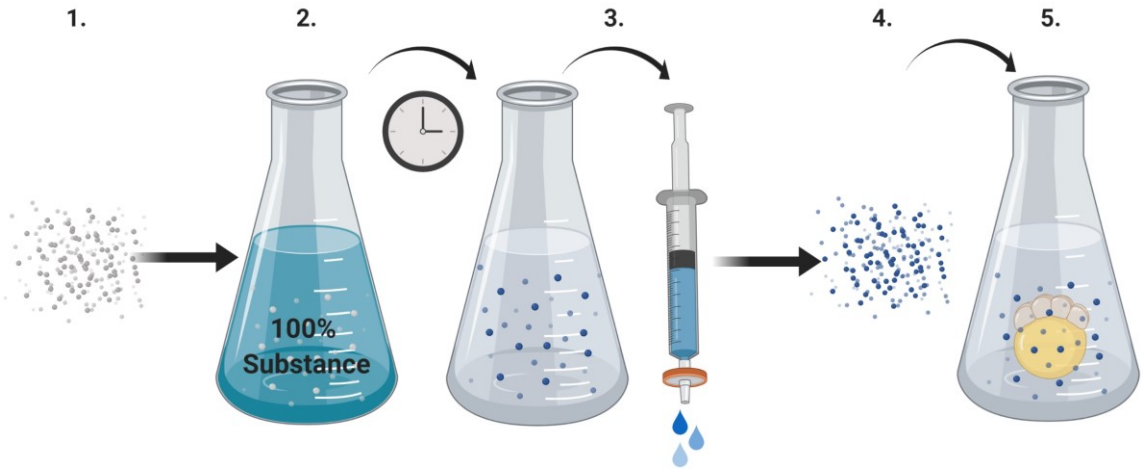

Fig. 6. Process of MP loading with model pollutants. Incubation of pristine MPs in dissolved model substance, overnight for 16 - 24 h on a shaker at 100 rpm. Filtration of the MP suspension with 0.22 µm filter using a 10 ml syringe and washing steps with Aqua bidest to obtain loaded MPs. Elution in artificial water (according to OECD TG 203) and exposure of *Danio rerio* embryos to eluate.

Particle recovery during filtration and resuspension of PMMA particles was examined according to Eitzen et al. (2019). A particle counter (SVSS, PAMAS, Germany) with a laser-diode sensor was used by measuring the light extinction of particles in a size range of 5 to 200 µm. To minimize the number of background particles, initial PMMA suspensions (0.05 g/L) were prepared with ultra-pure water (resistivity > 17 MΩ·cm; ELGA Berkefeld LabWater, Veolia Water Technologies, Celle, Germany). Laboratory glassware was cleaned with distilled water, rinsed twice using ultra-pure water with an intermediate ultrasound treatment step (10 min). Four replicate measurements were performed to determine particle recovery. For each measurement, 75 ml of initial MP suspension were placed in a glass beaker and measured immediately. Subsequently, 5 × 10 ml of the suspension were filtered over a 0.2 µm cellulose acetate filter (reversely pre-rinsed twice with 10 ml of ultra-pure water), before the filter was backflushed 5 × with 10 ml of artificial water. The resuspended particle suspension was collected in a glass

beaker and instantly measured. To account for background particles, three blanks were prepared accordingly, using ultra-pure water instead of initial MP suspension.

Aqueous phases and particle suspensions of all steps (incubation, washing, resuspension) were collected and measured separately in triplicates to verify the successful sorption to MPs. In addition, Erlenmeyer flasks for MP incubation were pre-incubated overnight with BkF and CPF to minimize sorption losses. BkF samples were extracted by adding 1.1 ml *n*-hexane, then vortexed for 2 min and frozen (-20 °C) to facilitate phase separation. Phenanthrene was used as an internal standard for BkF samples. For CPF measurements, samples were spiked with atrazine as internal standard, dried under a stream of nitrogen, and resuspended in acetone/ethyl acetate (1:1). For both approaches, the extraction solvent was filtered through 0.2 µm syringe filters and transferred to glass vials for subsequent measurements. The analytical quantification of BkF and CPF was conducted using gas chromatography coupled to mass spectrometry in 1 µl injection volume (GC-MS, Agilent Technologies, Santa Clara, USA, GC 7890B, column 19091S-433 I HP, MSD 5977B), see Tab. 4 for further details on instrument settings.

Tab. 4. Instrument settings for BkF and CFP sorption validation measurements of particulate and aqueous phases. *Data were produced jointly with Sven Huppertsberg.*

	Benzo(k)fluoranthene				**Chlorpyrifos**				
Instrument settings	GC-MS, 7693 autosampler, GC 7890B, MSD 7977B, Agilent Technologies, Santa Clara, USA				GC-MS, 7693 autosampler, GC 7890B, MSD 7977B, Agilent Technologies, Santa Clara, USA				
	Heater 250 °C; Pressure 64 kPa, Septum purge flow 3 ml/min (He), Temperature MSD Transfer Line 280 °C				Heater 250 °C, Pressure 60 kPa, Septum purge flow 3 ml/min (He), Temperature MSD Transfer Line 280 °C				
Injection volume / type	1 µl / Standard/Split-Splitless Inlet				1 µl / Standard/Split-Splitless Inlet				
Carrier Gas	Helium				Helium				
Column	Agilent 19091S-433I HP-5ms, 30m × 250 µm × 0.25µm				Agilent HP-5ms, 30m × 250 µm × 0.25µm				
Oven program		Rate [°C/min]	Temperature [°C]	Hold time [min]		Rate [°C/min]	Temperature [°C]	Hold time [min]	Run time [min]
	Initial		60	2	Initial		50	0.75	0.75
	Ramp 1	40	325	0	Ramp 1	20	120	0	4.25
	Ramp 2	20	325	2	Ramp 2	2	230	0	77.58
	Post run	0	300	10	Ramp 3	50	290	0	83.58
					Post run		290	10	
Detection	Ion Source EI, MS Source 230°C, MS Quadrupole 150°C				Ion Source EI, MS Source 230 °C, MS Quadrupole 150 °C,				
	SIM Phenanthrene (ISTD) m/z 176(qualifier), m/z 178 (quantifier), BkF m/z 250 (qualifier), m/z 252 (quantifier				SIM Atrazine (ISTD) m/z 200 (qualifier), m/z 215 (quantifier), CPF m/z 199 (qualifier), m/z 197 (quantifier)				
LoD/LoQ	≤ 1ng/ml / ≤ 2.5 ng/ml				≤ 1.5 ng/ml / ≤ 5.0 ng/ml				
Calibration	12-point calibration, 2.5 - 350 ng/ml BkF				5-point calibration, 5 - 50 ng/ml CPF				
Internal standard	100 ng phenanthrene				100 ng atrazine				

II.2.3 Zebrafish *(Danio rerio)* husbandry and egg collection

For all experiments in the scope of the present thesis, adult zebrafish (*Danio rerio*) from the "Westaquarium" strain maintained at the fish facility at the Aquatic Ecology and Toxicology Group at University of Heidelberg (licensed by regional animal welfare authorities, 35–9185.64/BH) were used. Rearing, breeding conditions and egg collection were conducted as described in detail by Lammer et al. (2009) and by OECD TG 236 (2013). In short, healthy, adult zebrafish aged 6 to 24 months were kept in groups (> 1 L water/fish) in a flow-through system with 10 % daily water exchange, to safeguard ammonia, nitrite and nitrate levels below detection limits (5 mg/L, 1 mg/L, 140 mg/L, respectively), under an artificial 14:10 h light:dark regime, at 26.0 ± 1 °C. Fish were fed twice daily with *Artemia* spec. Nauplii (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA) and TetraMin™ flakes (Tetra, Melle, Germany). For spawning, the day before egg collection, groups of male and female zebrafish (sex ratio 2:1) were transferred to spawning chambers. The next morning, eggs were collected, and the fertilization success was confirmed microscopically. Batches of eggs between 4-cell to 16-cell stage (Kimmel et al., 1995), displaying proper development (yolk, germinal disc, intact and clear chorion) were chosen for experimental exposure.

II.2.4 Zebrafish embryo toxicity test

This section includes content from a previously published manuscript that was written by me as a co-author (Heinrich et al., 2020)*; Reprinted with permission from Environmental Science and Pollution Research; Heinrich P., Hanslik L., Kämmer N., Braunbeck T., The Tox is in the Detail: Technical Fundamentals for Designing, Performing and Interpreting Experiments on Toxicity of Microplastics and Associated Substances, Copyright © Springer 2020. The reproduced content was entirely written by myself.*

To determine the acute toxicity and EC_{10} levels of the model contaminants benzo(k)fluoranthene and chlorpyrifos, the fish embryo toxicity test (FET) was performed according to OECD TG 236 (2013). Zebrafish embryos were continuously exposed to either dissolved or MP-sorbed BkF and CPF as early as one hour postfertilization (hpf) up to 96 hpf. For each test, several controls were included: Artificial water served as a negative control, a positive control (4 mg/L of 3,4-dichloroaniline), a solvent control (DMSO 0.01 - 0.02 %) and a microplastic control with clean MPs for MP experiments only. After fertilization was confirmed by microscopic examination, eggs were transferred to 24-well plates (TPP, Trasadingen, Switzerland) with 2 ml of the test solution. All test vessels were pre-incubated and sealed with self-adhesive foil (SealPlate®, EXCEL Scientific, Asbach, Germany) to prevent evaporation and cross-contamination. In tests with MP-associated substances, the 24-well plates were supplemented with HPLC flat-bottom glass vials (neoLab Migge, Heidelberg, Germany) to prevent plastic-plastic interactions between test materials and MP suspensions and the plate was covered with the lid and fixed with

an elastic band instead of self-adhesive foil, to prevent evaporation of test substance (Heinrich et al., 2020). Semi-static exposure with a daily renewal of solutions was performed at 26 ± 1 °C at 16:8 light:dark regime with 20 embryos per treatment. Care was taken to prevent microplastic accumulation by mixing the test solution thoroughly before renewal. Lethal and sublethal effects were documented at 24, 48, 72 and 96 hpf according to OECD TG 236 and Nagel (2002) using a Zeiss Olympus CKX41 microscope with an Axio Cam ICc1 (software Zen lite 2011, Carl Zeiss, Oberkochen, Germany). The experiments were conducted in three independent replicates each (n = 3 × 20), and results were expressed as cumulated effects of individuals in percent at 96 hpf. Tests were considered valid with an effect rate in negative controls < 10 % and > 30 % mortality in positive controls following OECD TG 236.

II.2.5 *In vivo* live-imaging EROD assay

The *in vivo* EROD induction was investigated according to Kais et al. (2018). For the live-imaging assay, ten zebrafish embryos per treatment and replicate (n = 3 × 10) were raised in artificial water until 96 hpf and subsequently exposed to different concentrations of dissolved BkF_{AQ} and BkF_{MP} (0.048 - 50 µg/L) for three hours. Exposure to β-naphthoflavone (75 µg/L) served as a positive control, and exposure to DMSO (0.01 %) served as solvent control. Negative controls were exposed to artificial water only, and MP controls were exposed to pristine MPs in artificial water. Embryos were subsequently washed twice for 5 min in artificial water and incubated for 20 min in 0.6 mg/L 7-ethoxyresorufin. After immobilization with 0.016 % tricaine (MS 222, methane sulphonate), embryos were embedded in 1 % low-melting agarose (SeaKem HGT Agarose, Cambrex BioScience, Rockland, USA) in lateral orientation (left side down) and covered with 0.016 % tricaine solution to maintain viability. Accumulation of the fluorescent resorufin metabolite in the gastrointestinal tract of the zebrafish embryo was subsequently measured using an inverted Nikon Eclipse 90i epifluorescence microscope (λ_{EX} = 560 ± 20 nm, λ_{EM} = 630 ± 30 nm, dichroic beam splitter at 595 nm, Nikon, Duesseldorf, Germany) at 10 × magnification (Nikon Plan Flour water immersion objective NA 0.17, WD 16.0 mm) with a Nikon DS-Ri-1 camera (Fig. 7). Images were acquired and processed with NIS-Elements 4.0 imaging software (Nikon, Duesseldorf, Germany), and signals were normalized to basal background fluorescence signals of the negative controls.

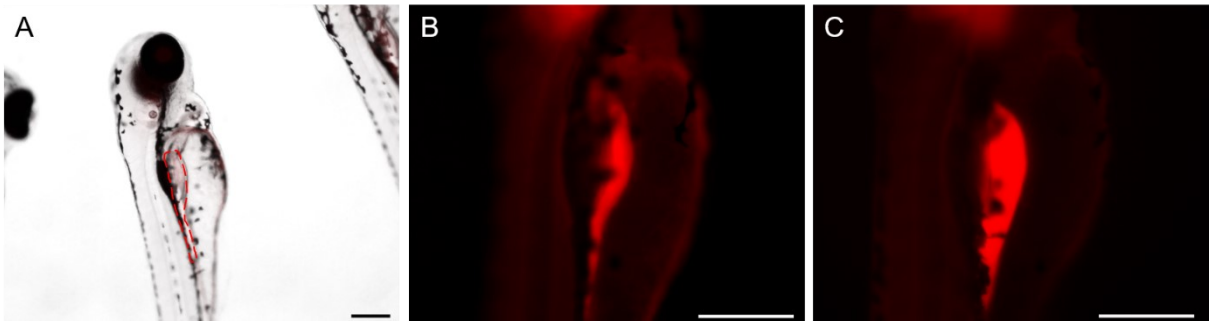

Fig. 7. EROD fluorescence signal in the gastrointestinal tract of zebrafish embryos (96 hpf) after 3 hours of exposure. (**A/B**) Negative control exposed to artificial water, brightfield and red fluorescence image, the gastrointestinal tract is outlined (**A**, red dashed line). (**C**) Zebrafish embryo exposed to 50 µg/L dissolved benzo(k)fluoranthene. Excitation filter = 560 ± 20 nm, emission filter = 630 ± 30 nm, exposure time 40 ms, Nikon Eclipse 90i, scale bars 250 µm.

II.2.6 Acetylcholine esterase activity

This section includes content that was produced jointly with Luisa Nuttall.

The AChE activity was measured according to a protocol established by Kais et al. (2015). In short, 15 zebrafish embryos per treatment were continuously exposed as described for the FET, to incrementally increasing concentrations (1 - 800 µg/L) of dissolved CPF (CPF_{AQ}) and MP-sorbed CPF (CPF_{MP}). A negative control (artificial water), a positive control (2 mg/L of paraoxon-methyl), a solvent control (DMSO 0.02 %) and a microplastic control with pristine MPs were applied if necessary. After 96 hpf, embryos were washed three times for 10 min in artificial water, and excess water was removed before shock freezing in liquid nitrogen. For analysis, pseudoreplicates of 3 × 5 pooled zebrafish per treatment (15 embryos in total per treatment and replicate, n = 3 × 15) were thawed on ice and homogenized in 400 µl ice-cooled phosphate buffer with 0.1 % Triton X-100 using a TissueLyser II for 60 s at 30 Hz (Qiagen, Hilden, Germany). After centrifugation for 15 min at 10.000 ×G (4 °C, Heraeus Multifuge 1 S-R, Thermo Scientific, Schwerte, Germany), the supernatant was transferred and stored on ice for further enzyme kinetic and protein measurements. 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). Phosphate buffer served as a blank and samples were applied in quadruplicates (50 µl), before adding 50 µl phosphate buffer and 100 µl of 0.89 mM 5,5′-dithiobis-2-nitrobenzoic acid. The kinetic reaction was initiated by adding 100 µl of 0.9 mM acetylthiocholine iodide, and changes in optical density and protein content (ΔOD/min/mg of protein) over time were recorded. AChE activity values were compared to control samples and expressed as percent of the negative control. Enzyme activities were standardized to the protein content of each sample, assessed with the DC™ Protein Assay kit from Bio-Rad (Feldkirchen, Germany).

II.2.7 Data analysis

Effective concentrations (EC_{10}) were calculated from FET data based on probit analysis using linear maximum likelihood regression in ToxRat (version 2.10.03, ToxRat Solutions, Alsdorf, Germany). For statistical evaluation, data were tested for normality (Kolmogorov-Smirnov test) and equal variances (Levene's test). To identify statistically significant differences (p-value = 0.05) of treatments compared to the according control, either parametric ANOVA followed by Holm-Sidak *post-hoc* test or nonparametric Kruskal-Wallis analysis, followed by Dunn's *post-hoc* test, were applied. Significant differences between exposure scenarios (with/without MPs) were analyzed by t-test using SigmaPlot 13 (Systat Software, Erkrath, Germany). Results were expressed as mean values (MV) ± standard deviation (SD).

II.3 Results

II.3.1 Microplastic loading, recovery and sorption validation

This section includes content based on a manuscript written entirely by myself and is currently prepared for submission in Ecotoxicology and Environmental Safety: Lisa Hanslik, Bettina Seiwert, Sven Huppertsberg, Thomas P. Knepper, Thorsten Reemtsma, Thomas Braunbeck. Biomarker responses in zebrafish (Danio rerio) following long-term exposure to microplastic-associated chlorpyrifos and benzo(k)fluoranthene (see Chapter III).

Quantifying the final amount of BkF and CPF sorbed to MP particles is crucial to assess the potential amount being transferred to zebrafish. For BkF, measurements with GC-MS have proven that up to 80 % of BkF was sorbed to spherical PMMA particles and less than 10 % remained in the aqueous phase after 24 h of incubation. Up to 20 % of BkF can desorb from BkF-loaded particles over 24 h exposure in clean water and might be taken up further without particle-mediated transfer (Tab. 5).

The sorption capacity of PMMA particles for chlorpyrifos was reduced compared to BkF, as only 50 % of CPF had sorbed to PMMA particles, possibly due to the lower log K_{OW} of 4.96 compared to BkF (log K_{OW} 6.11). Up to 50 % of CPF was still present in the aqueous phase after incubation with PMMA particles over 24 h (Tab. 5). Since all vials for incubation were pre-saturated, a loss of CPF due to sorption to glassware was considered negligible. However, the desorption of CPF within 24 h in Aqua bidest was considerably higher compared to BkF, and it was estimated from actual measurement values that up to 80 % of CPF had leached into the aqueous phase. Increased availability of PS-associated CPF for zebrafish would therefore be conceivable.

Tab. 5. Measured concentrations of benzo(k)fluoranthene and chlorpyrifos sorbed to PMMA using GC-MS. Residues of BkF and CPF were measured directly in the aqueous phase after filtration. Not assessed = n. a. Data given as mean values ± SD. *Data jointly produced with Sven Huppertsberg.*

		Nominal concentration	PMMA	Aqueous phase	Desorption (24 h)
BkF	[µg/L]	50	31.8 ± 8.2	4.2 ± 0.2	7.5 ± 3.4
	[%]	100	63.6 ± 16.3	8.5 ± 0.2	23.6 ± 10.7
CPF	[µg/L]	800	n. a.	420.4 ± 21.0	321.9 ± 16.1
	[%]	100	n. a.	52.6 ± 5.0	-

In addition to the analytical quantification of sorption success, a possible particle loss during the preparation of MP-loaded suspensions due to a necessary filtration step was investigated using a small volume syringe system particle counter (SVSS, PAMAS, Rutesheim, Germany). All measurements were carried out in four replicates. Background particle numbers were determined from blanks for initial PMMA suspensions (< 1 %) and resuspensions (< 10 % in total) and subtracted from each measurement. With respect to counted particles over the entire measuring range of 5 to 200 µm, 38.7 ± 4.3 % of the PMMA particles were recovered in the resuspension (Fig. 8A/B). As all treatments, including the control with pristine PMMA, were treated alike, particle numbers were similarly reduced in all MP exposure scenarios. Deviations occurred for different size fractions. Especially the recovery of larger particles (< 30 µm) showed increased variation due to a lower number of particles detected over a wide size range. Nonetheless, it has been demonstrated that the method of spiking MP particles with dissolved contaminants and filtering the solution to obtain only substance-loaded particles is successful and reproducible.

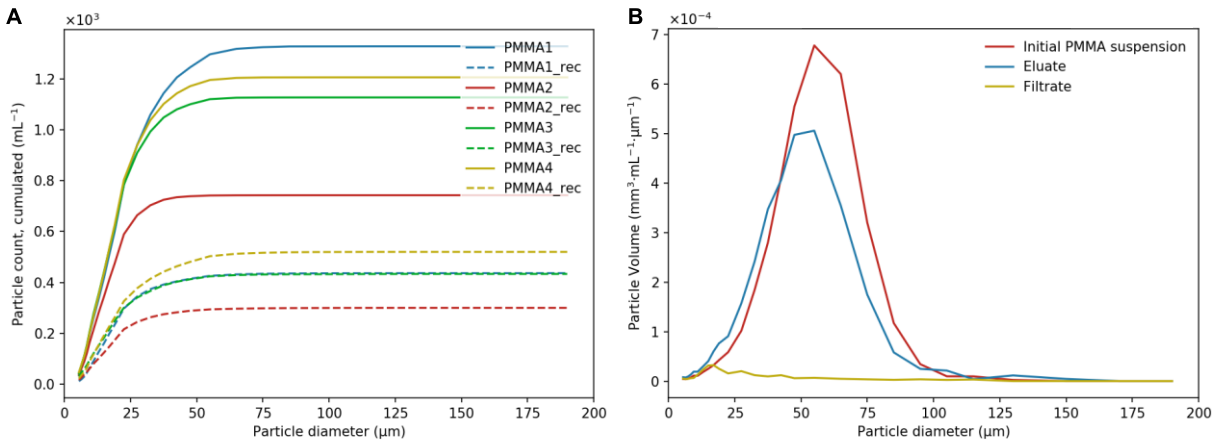

Fig. 8. Results of particle recovery measurements using a small volume syringe system particle counter (SVSS, PAMAS). (**A**) Cumulated particle count from four replicate measurements. Solid line = initial particle suspension, dashed line = recovery of particles in the eluate. (**B**) Distribution of particle volume before and after washing steps. Initial PMMA suspension = before washing/incubation (red), Eluate = after filtration and washing steps (blue), Filtrate = aqueous phase of filtration process (yellow), n = 3. *Data were produced jointly with Stefan Dittmar.*

II.3.2 Zebrafish embryo toxicity test

Continuous exposure of zebrafish embryos to BkF and CPF in either application (dissolved or MP-sorbed) induced only sublethal effects. Overall, effects were observed as early as 48 hpf with increasing emergence over time and concentration. Furthermore, the cumulated number of sublethal effects at 96 hpf in zebrafish (Fig. 9A/10A) was reduced for both substances when zebrafish were exposed to MP-associated substances (EC_{10} BkF_{AQ} = 1.5 µg/L, BkF_{MP} = 17.5 µg/L; EC_{10} CPF_{AQ}= 243.0 µg/L, CPF_{MP} = 785.3 µg/L). Exposure to clean, pristine MP as a secondary control did not induce effects in either scenario. For exposure to BkF, sublethal effects were observed as early as 48 hpf and covered edema (pericardial and yolk sac) and cardiovascular effects (reduced blood circulation, seldom reduced heartbeat). For dissolved BkF_{AQ}, the three highest concentrations (50 µg/L, 100 µg/L, 200 µg/L) induced significant effects in 40 - 50 % of the embryos ($p < 0.01$, $p < 0.001$). In contrast, BkF_{MP} induced significant effects in 20 % of *Danio rerio* embryos ($p < 0.05$) only when exposed to 100 µg/L and 200 µg/L of sorbed BkF (Fig. 9A). At lower concentrations (0.78 - 25 µg/L), the cumulated number of effects was not significantly increased compared to controls, but the number of effects induced by BkF_{MP} was consistently lower. Most prominent differences between exposure to BkF_{MP} and BkF_{AQ} ($p < 0.05$) were observed for 50 µg/L and 100 µg/L using a t-test, indicating 40 % fewer effects in zebrafish embryos exposed to BkF_{MP}.

Exposure to CPF induced different sublethal effects, such as cardiovascular effects as early as 48 hpf (reduced blood circulation, reduced heartbeat) and spinal deformities from 72 hpf onwards (scoliosis and kyphosis). After hatching between 48 to 72 hpf, embryos frequently displayed increased motion activity, eventually leading to tremors and convulsion at later developmental stages. Exposure to CPF_{MP} led to decreased sublethal effects, especially concerning spinal deformities, tremors, and convulsion, which occurred only infrequently. Solely the exposure to the highest concentration of waterborne CPF_{AQ} (800 µg/L) induced significant effects ($p < 0.05$) in 22 % of zebrafish embryos at 96 hpf. In contrast, the number of cumulated effects for exposure to MP-associated BkF ranged between 1 - 12 % (Fig. 10A). However, exposure to either 800 µg/L of dissolved CPF_{AQ} or MP-associated CPF induced significantly more sublethal effects in zebrafish (22 % and 8 % respectively, $p < 0.05$) compared to controls. Similar to BkF exposure scenarios, sublethal effects upon CPF_{MP} exposure (17.5 - 800 µg/L) were reduced by up to 55 % compared to waterborne CPF_{AQ}. Nevertheless, in lower concentrations (1 - 8.75 µg/L), MP-associated CPF induced 1 - 5 % effects, whereas CPF_{AQ} induced only up to 3 % effects in *Danio rerio* embryos. As a result, differences between exposure scenarios were less pronounced.

II.3.3 *In vivo* EROD activity

The investigated concentration range for the EROD activity included low nanogram BkF concentrations (0.048 - 0.195 µg/L) to adapt to the increased sensitivity of cellular responses. Overall, the EROD induction rate was increased with increasing BkF concentrations, whereas neither the solvent control (DMSO 0.01 %) nor exposure to pristine MP (MP control) altered EROD induction rates in zebrafish after three hours of exposure. The overall induction of CYP450 was decreased by up to 60 % when zebrafish were exposed to different concentrations of MP-associated BkF. All exposure scenarios with dissolved BkF_{AQ} resulted in significant CYP450 induction ($p < 0.01$, $p < 0.001$). Levels ranged from a 2.3-fold increase at the lowest concentration of 0.048 µg/L to a 5.4-fold increase after exposure to 50 µg/L (Fig. 9B). The lowest concentration of MP-sorbed BkF (0.048 µg/L) did not increase EROD activity, and only the three highest concentrations tested (3.125 - 50 µg/L) induced significant changes up to a 3.3-fold increase ($p < 0.001$). Between exposure scenarios (BkF_{AQ} compared to BkF_{MP}) significant differences were only detected for 0.048 µg/L ($p < 0.01$), 0.78 µg/L and 50 µg/L ($p < 0.05$ by t-test). Nevertheless, MP-sorbed BkF led to EROD induction rates that were distinctly reduced compared to waterborne BkF_{AQ} exposure scenarios.

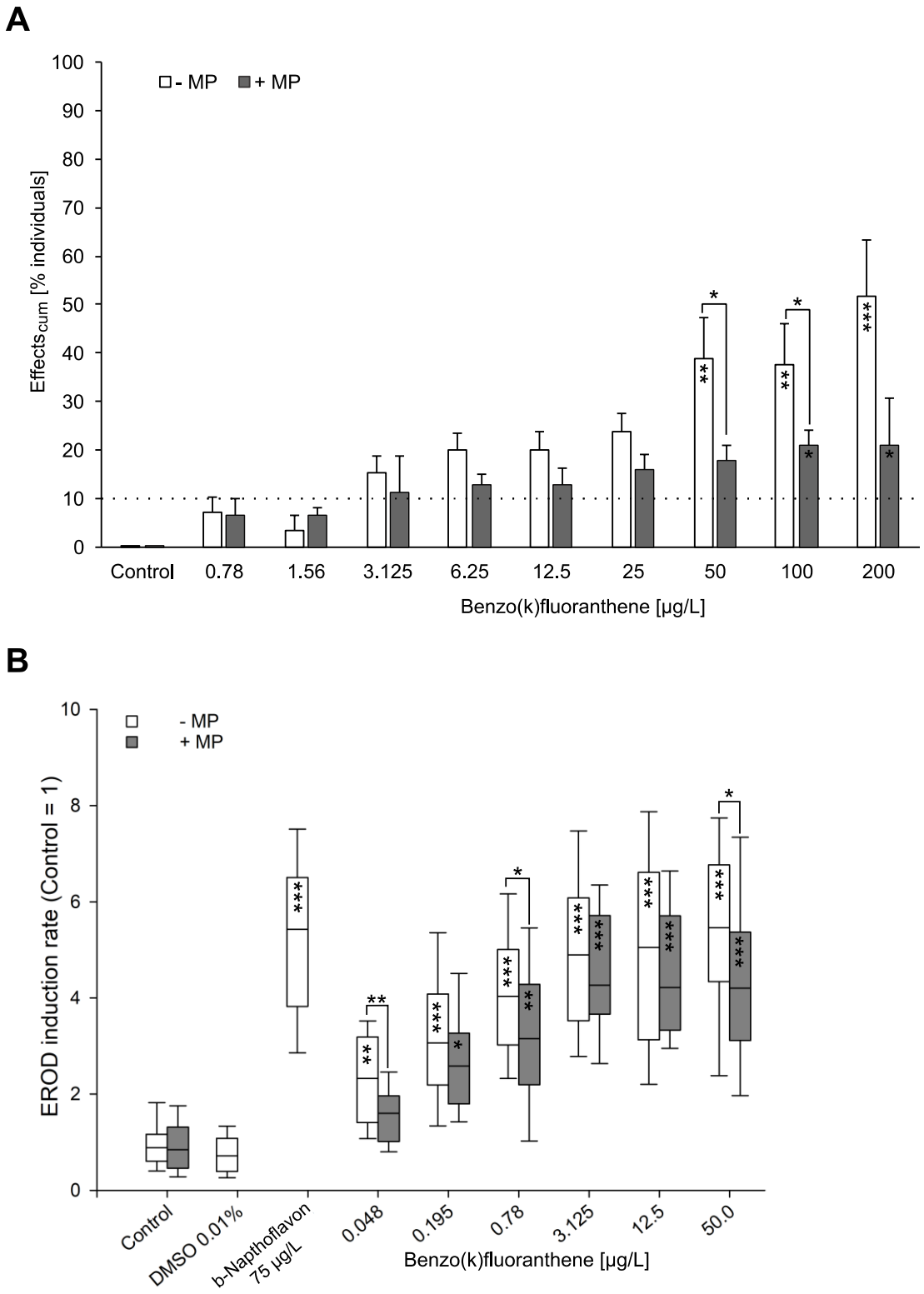

Fig. 9. Results of acute toxicity tests of benzo(k)fluoranthene as dissolved (white bars, - MP) or MP-associated exposure scenario (grey bars, + MP) with *Danio rerio* embryos (96 hpf). (**A**) Cumulated sublethal effects of BkF in zebrafish embryo toxicity test according to OECD TG 236. Dotted line indicates 10 % validity threshold (non-parametric Kruskal-Wallis analysis and Dunn's *post-hoc* and t-test, N = 3, n = 60, MV ± SD). (**B**) EROD induction rate after 3 h acute exposure to BkF, normalized to background fluorescence of control (nonparametric Kruskal-Wallis analysis and Dunn's *post-hoc* and t-test, data are presented as median, whiskers represent 95 % confidence intervals, N = 3, n = 30). Differences between groups (- MP/+ MP) marked with a bracket are significant (t-test, $p < 0.05$). Asterisks within bars/boxes indicate significant differences between treatment and control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

II.3.4 Acetylcholine esterase activity

With increasing CPF concentrations, a decrease in AChE activity was induced in whole-body homogenates of 96 hpf zebrafish embryos. In general, continuous exposure to clean, pristine MP did not alter AChE activity. Overall, after exposure to CPF_{MP}, the reduction of AChE activity was less pronounced compared to CPF_{AQ} exposure (Fig. 10B). Low concentrations (1 - 8.75 µg/L) of either dissolved or MP-sorbed CPF did not reduce AChE activity. In contrast, a pronounced decreasing trend of AChE activity was induced by CPF concentrations ≥ 17.5 µg/L. Significant differences ($p < 0.01$, $p < 0.001$, t-test) between the two exposure scenarios (CPF_{AQ}, CPF_{MP}) were observed for all concentrations between 17.5 µg/L to 800 µg/L. The two highest waterborne CPF_{AQ} concentrations resulted in significant inhibition of AChE activity by up to 70 % ($p < 0.01$). In comparison, 800 µg/L CPF_{MP} resulted only in 50 % of inhibition in *Danio rerio*, indicating a reduced effect rate of 70 % due to MP-associated CPF exposure.

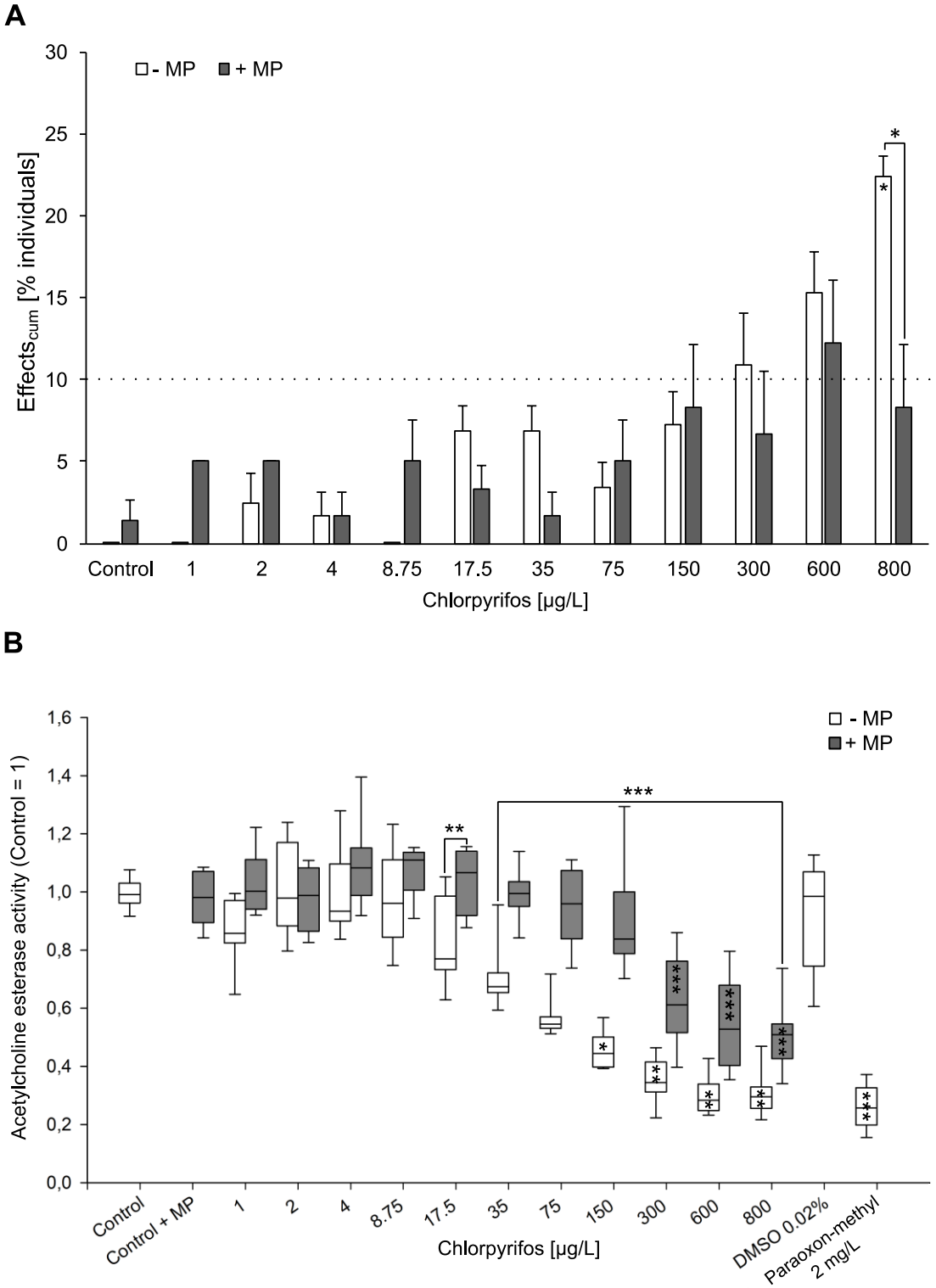

Fig. 10. Results of acute toxicity test of chlorpyrifos as dissolved (white bars, - MP) or MP-associated exposure scenario (grey bars, + MP) with zebrafish embryos (96 hpf). (**A**) Cumulated sublethal effects of CPF in the zebrafish embryo toxicity test according to OECD TG 236. Dotted line indicates 10 % validity threshold (nonparametric Kruskal-Wallis analysis and Dunn's *post-hoc* and t-test, N = 3, n = 60, means ± SD). (**B**) Acetylcholine esterase activity after continuous exposure to CPF for 96 h, expressed as % of the control. Differences between groups (- MP/+ MP) within the bracket are significant (t-test, $p < 0.001$). Asterisks within bars/boxes indicate significant differences between treatment and control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA and Holm-Sidak *post-hoc* and t-test, data are presented as median, whiskers represent 95 % confidence intervals, N = 3, n = 45).

II.4 Discussion

II.4.1 Effects of benzo(k)fluoranthene on zebrafish embryos

Dose-dependent sublethal toxic effects were observed following BkF_{AQ} and BkF_{MP} exposure, with the most prominent physiological malformations comprising edema (pericardial and yolk sac) and cardiovascular effects (reduced blood circulation, seldom reduced heartbeat). These sublethal effect patterns were consistent with findings of several previous studies investigating the acute toxicity of PAHs (from anthropogenic sources) and BkF in early developmental stages of zebrafish (Billiard et al., 2008; Carls et al., 2008; Otte et al., 2010; Incardona et al., 2011; Jung et al., 2013). Another study described identical sublethal effects for *Danio rerio* exposed to 100 - 200 µg/L BkF (Fleming and Di Giulio, 2011) and a similar significantly increased EROD activity ($p < 0.05$) after exposure to 10 µg/L and 100 µg/L BkF. In contrast, the present results demonstrate that a significant EROD induction was already achieved at the lowest concentrations of dissolved BkF_{AQ} (0.048 µg/L). This discrepancy may be due to the 3 h acute exposure of zebrafish embryos (96 hpf) in the present study compared to the prolonged exposure over 72 - 96 h, hence, increased sensitivity of the *in vivo* live-imaging EROD assay as described by Kais et al. (2018). The observed developmental effects in fish embryo toxicity tests (above 10 % effect threshold) at concentrations above 3.125 µg/L BkF might therefore indicate incipient defense and detoxification mechanisms to prevent potential cellular damage.

With increasing concentrations of BkF, however, increasing sublethal developmental effects were induced, as metabolic capacities were most likely exceeded (Perrichon et al., 2016). The results have to be interpreted with respect to EC_{10} concentrations as the upper limit of the exposure range in the EROD assay (BkF_{AQ} = 1.5 µg/L, BkF_{MP} = 17.5 µg/L). Thereby, unspecific systemic effects that might interact with the endpoints investigated, including CYP1A activity and AChE enzyme activity, might be avoided (Kais et al., 2015). The EROD induction rates for waterborne and MP-associated BkF were already significantly increased ($p < 0.001$) below the EC_{10}, indicating increased metabolic activity in zebrafish embryos (Whyte et al., 2000).

Exposure to BkF_{MP} reduced effects by up to 40 % in zebrafish embryo toxicity tests and up to 60 % in EROD assays. These effects may also be due to the reduced availability of particles in exposure scenarios with MP-associated BkF, as approximately 80 % of the initial BkF concentration was available on the PMMA surface after filtration and washing steps. However, effects were reduced to a greater extent and exposure to clean, pristine MPs as additional control excluded additive effects of MPs and contaminants. It seems plausible that MPs served as an alternative sorption surface, based on a partitioning process between different available compartments such as the MP surface and the zebrafish embryo (Dąbrowski, 2001; Koelmans et al., 2013; Heinrich and Braunbeck, 2020).

Due to the strong sorption affinity of BkF, as reflected by the octanol-water partitioning coefficient (log K_{OW} = 6.1 - 6.4), only minor desorption rates of approximately 15 % over 24 h (see Tab. 5) have been

detected. Similar reduced effects were detected in a study with TECAM membranes as passive samplers (Wincent et al., 2015), leading to reduced bioaccumulation and reduced toxicity in adult Japanese medaka (*Oryzias latipes*), documented by hepatic EROD activity. Only a few studies have investigated adverse effects on different developmental stages in fish, induced by exposure to MP-associated PAHs, with highly variable results (Rochman et al., 2013b, 2014; Khan et al., 2015; Sleight et al., 2017; Batel et al., 2018; Cormier et al., 2019). Others observed similar effects in cell lines (Heinrich and Braunbeck, 2020) and emphasized the importance of freely available contaminants in co-exposure scenarios to induce effects in aquatic organisms (Wincent et al., 2015; Sørensen et al., 2020). Conversely, the uptake of MPs resulted in depuration effects rather than the accumulation of contaminants (Koelmans et al., 2013; Diepens et al., 2015; Herzke et al., 2016). As a result, the reduced effect sizes upon BkF_{MP} exposure in the fish embryo tests and the EROD assay were most likely elicited by low amounts of freely available BkF in the aqueous phase. Here, the slow desorption of BkF from MPs compared to BkF_{AQ} exposure yielded only low amounts of BkF in the aqueous phase.

II.4.2 Effects of chlorpyrifos on zebrafish embryos

Similar to BkF exposure, sublethal effects of CPF (log K_{OW} 4.7 - 5.3) in zebrafish embryo toxicity tests were dose-dependent for dissolved and MP-associated exposure scenarios, with cardiovascular effects occurring as early as 48 hpf (reduced blood circulation, reduced heartbeat) and spinal deformities from 72 hpf onwards (scoliosis and kyphosis). These effects were consistent with the results of several other studies, describing adverse morphological effects in larval zebrafish after exposure to 10 - 350 µg/L CPF (Richendrfer et al., 2012; Jin et al., 2015; Jeon et al., 2016; Cao et al., 2018). Compared to BkF, effects were less pronounced with a maximum of cumulated effects at 96 hpf of 22 % after exposure to 800 µg/L dissolved CPF_{AQ}. AChE plays an essential role in neuronal and muscular development, and hence, the survival of zebrafish embryo and larvae (Yen et al., 2011). Therefore, it has been studied extensively as a biomarker for neurotoxicity. Organophosphate pesticides (OPs) such as CPF are known to inhibit AChE enzyme activity (Sledge et al., 2011; Kais et al., 2015). Similar inhibition of AChE activity has been demonstrated in studies using early developmental stages of zebrafish (Yen et al., 2011; Jin et al., 2015; Richendrfer and Creton, 2015; Rodríguez-Fuentes et al., 2015; Jeon et al., 2016). So far, the effects of OPs in combination with microplastic particles have received less attention, although both pollutants (pesticides and MPs) are of emerging concern and known to occur in aquatic ecosystems (Horton et al., 2017; Lohmann, 2017; Wagner and Lambert, 2018).

In contrast to the present results, the literature on interactive effects of MPs and (organophosphate) insecticides is confounding: The presence of high amounts of PS particles (300.000 particles/ml) did not alter the toxicity of deltamethrin (pyrethroid insecticide) and dimethoate (organophosphate insecticide) in terms of survival and mobility of *Daphnia magna* over 72 h (Horton et al., 2018). Whereas Garrido et al. (2019) have shown that CPF loaded polyethylene particles (PE) significantly reduced the impact

on the growth rate of microalgae ($p < 0.05$) compared to waterborne CPF exposure. In another study, PE particles delayed mortality in juvenile common goby (*Pomatoschistus microps*) over 96 h in a co-exposure scenario with pyrene, but also altered AChE activity in the absence of pyrene, indicating neurotoxic effects induced by pristine PE particles (Oliveira et al., 2013). Bellas and Gil (2020) have documented that pristine PE particles did not induce adverse effects in the marine copepod *Acartia tonsa*, even with particle amounts similar to the present thesis (2475 particles/ml versus 1000 particles/ml). Nonetheless, adverse effects of CPF sorbed to PE were increased by up to 80 % compared to waterborne CPF exposure, suggesting synergistic effects of MPs and CPF (Bellas and Gil, 2020). In contrast, in the present thesis, a delay in AChE inhibition has been detected in CPF_{MP} exposure scenarios compared to CPF_{AQ}, indicating a reduced bioavailability of CPF for *Danio rerio* embryos and no additive or synergistic effects of pristine MPs and CPF. As illustrated by GC-MS measurements, CPF readily desorbed from PMMA particles and could have released up to 80 % of the sorbed substance into the aqueous phase but failed to alter biomarker responses to a greater extent. The enhanced desorption of the organophosphate pesticide CPF is in line with results from León et al. (2018), emphasizing that less hydrophobic compounds exhibit faster desorption rates (inverse correlation) than hydrophobic substances with a log $K_{OW} > 5$ such as BkF (log K_{OW} 6.11). The determined sorption capacities of CPF were comparable to results from studies using different types of microplastic polymers, such as polyethylene, polyvinyl chloride, polystyrene, polyethylene terephthalate, and polypropylene (Allen et al., 2018; Garrido et al., 2019; Bellas and Gil, 2020).

It became apparent that results have to be interpreted cautiously since possible secondary effects might conceal neurotoxic effects caused by systemic, sublethal effects of CPF concentrations above the EC_{10} level (Kais et al., 2015). Hence, a distinct inhibition of AChE activity by CPF_{AQ} and CPF_{MP} was detectable even below EC_{10} (CPF_{AQ} = 243.0 µg/L, CPF_{MP} = 785.3 µg/L), as well as significant differences ($p < 0.01$, $p < 0.001$) between both exposure scenarios (dissolved and MP-associated, Fig. 10A/B). Already low amounts of OPs might alter zebrafish behavior without affecting the embryonic development or AChE enzyme activity (Richendrfer and Creton, 2015) and consequently might lead to severe impairment of behavioral patterns at later developmental stages (Levin et al., 2004; Tilton et al., 2011; Jin et al., 2015).

II.5 Conclusions

The effects of BkF and CPF in *Danio rerio* embryos documented by the acute toxicity tests, reflect the known mode of action of PAHs and OPs in aquatic vertebrates and emphasize the necessity to investigate multiple biological endpoints. The exposure to pristine MPs did not induce adverse effects in zebrafish and thus was suitable as additional particle control. No synergistic effects of MP-sorbed contaminants in either biomarker could be demonstrated. A reproducible approach to load MPs with pollutants of interest was established based on surface sorption processes and separation of particulate and aqueous phase *via* filtration. It seems likely that the resulting particle suspensions are more likely to mimic environmental conditions rather than clean MPs, co-exposed with dissolved contaminants under controlled laboratory conditions. As illustrated by GC-MS measurements of BkF and CPF, the sorption behavior of model pollutants to MPs is crucial to evaluate the results adequately.

Furthermore, leaching processes of contaminants from MPs have been identified as a predominant source of bioavailable contaminants for zebrafish embryos. Results of the acute toxicity tests have confirmed the sensitivity of embryonic and larval stages of zebrafish (*Danio rerio*) as a tool to assess the adverse effects of environmental pollutants sorbed to MPs. Sublethal toxic effects induced by BkF and CPF may lead to irreversible alterations at later developmental stages, as the delay in toxic effects of MP-associated pollutants might not be transitory. Consequently, the effects could be deleterious to fish behavior, survival, and recruitment under environmental conditions. Therefore, further in-depth research was conducted using additional biomarkers and endpoints for various and prolonged exposure scenarios with different developmental stages of zebrafish to complement findings from acute toxicity tests. In the following chapters, the effects of MPs were assessed with particular regard to their potential role in limnic ecosystems and further implications for a more comprehensive risk assessment of MPs in the aquatic environment.

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

This chapter is based on a manuscript written entirely by myself and currently prepared for submission in Ecotoxicology and Environmental Safety, including content that was created jointly with Bettina Seiwert and Sven Huppertsberg. Bettina Seiwert conducted the UPLC-FLD/HRMS measurements of BkF and CPF in zebrafish tissues, and Sven Huppertsberg conducted the GC-MS measurements of BkF and CPF in aqueous samples. The corresponding figures are marked. However, experimental conduction, sample preparation, enzymatic assays, fluorescence measurements and data analysis were always conducted entirely by me.

III.1 Abstract

The continuously increasing plastic production promotes rising numbers of microplastic particles (MPs) in the aquatic environment, especially in highly industrialized and urbanized areas with high wastewater discharges. This may coincide with the release of persistent organic pollutants, such as polycyclic aromatic hydrocarbons and insecticides, entering the limnic ecosystems. Data on potential risks for the aquatic vertebrate fauna is still limited and assessing possible effects of environmental pollutants sorbed to MPs under chronic exposure scenarios seems vital. Therefore, we investigated the modulation of different enzymatic biomarkers, tissue accumulation and biotransformation in liver and brain of zebrafish (*Danio rerio*) over 21 days after exposure to the organophosphate insecticide chlorpyrifos (CPF) and the PAH benzo(k)fluoranthene (BkF), sorbed to different MPs (irregular polystyrene, spherical polymethyl methacrylate). MP uptake by zebrafish was documented in cryosections of the gastrointestinal tract. Overall, exposure to pristine MPs did not induce adverse effects in zebrafish. Sorption to MPs and leaching behavior of CPF and BkF were documented using GC-MS. Results of fluorescence tracking, CYP450 induction, and AChE activity were less pronounced when contaminants were sorbed to MPs, indicating a reduced bioavailability. Only minor amounts of parent BkF and BkF biotransformation products were detected in zebrafish liver. Thus, the effects were most likely due to the desorption of substances from ingested MPs. High loads of MPs and sorbed contaminants did not induce significant adverse effects in zebrafish upon chronic exposure, whereby the potential threat of MPs as a vector for contaminant transfer in limnic ecosystems can be considered very limited.

III.2 Introduction

Microplastic particles (MPs) are detected ubiquitously in highly variable amounts in every compartment of aquatic ecosystems (Hidalgo-Ruz et al., 2012; Klein et al., 2015; Alimi et al., 2017; Triebskorn et al., 2019). The exponential increase in plastic production and inadequate waste management are leading to millions of tons of plastic debris (Jambeck et al., 2015; Geyer et al., 2017; PlasticsEurope, 2018) being discharged into aquatic systems as macro- or microplastics (GESAMP, 2015). However, since the majority of all plastics are used and disposed within days to weeks (Geyer et al., 2017), terrestrial and freshwater environments are particularly at risk from extensive pollution (Horton et al., 2017; Schmidt et al., 2017). Nevertheless, comparatively little research has been conducted on the abundance of MPs in freshwater ecosystems (Wagner and Lambert, 2018; Fahrenfeld et al., 2019). Polystyrene (PS) was selected as it represents one of the most used microplastics due to its main application as packaging material, while polymethyl methacrylate (PMMA) has a wide application range in automotive and computer industries (PlasticsEurope, 2018) but has received less investigation so far (Hermsen et al., 2017; Trifuoggi et al., 2019). Nevertheless, both types of MPs were detected in environmental samples (Hermsen et al., 2017; Koelmans et al., 2019). The association of MPs with environmentally relevant pollutants is particularly relevant in aquatic ecosystems since industrialized and urbanized areas with high wastewater discharge rates can lead to elevated concentrations of particulate and dissolved pollutants in rivers and streams (Eerkes-Medrano et al., 2015; Horton et al., 2017; Schmidt et al., 2017). Thus, the binding of polycyclic aromatic hydrocarbons (PAHs) and insecticides to MPs in aquatic ecosystems may be unintentionally promoted. The high variance of MP properties (e.g., shape, size, composition, density, surface structure, etc.) affect spatial distribution, biological interactions and the fate of MPs in ecosystems, thus posing a risk to invertebrate and vertebrate organisms (Jabeen et al., 2018; Lehtiniemi et al., 2018; Rainieri et al., 2018; Redondo-Hasselerharm et al., 2018; Franzellitti et al., 2019).

Not only MPs themselves might pose a risk to aquatic organisms, but also unintentionally sorbed environmental pollutants from the aquatic environment (e.g., insecticides and PAHs), and substances introduced during the production process like monomers, plasticizers, dyes, and flame retardants (Teuten et al., 2009). Sorption of complex contaminant mixtures on MPs prior to aquatic exposure has already been investigated in several studies, indicating a potential transport of sorbed contaminants into aquatic organisms *via* ingestion (Rochman et al., 2013a; Chen et al., 2019; Pannetier et al., 2019a). The PAH benzo(k)fluoranthene (BkF) occurs in quantifiable amounts in aquatic ecosystems and evidentially sorbs to MPs (2 - 25 ng/g plastic, Pannetier et al., 2019a; Rochman et al., 2013a). CYP450 enzymes are involved in the biotransformation of xenobiotics, such as PAHs, within the aryl hydrocarbon receptor (AhR) pathway (Sarasquete and Segner, 2000; Whyte et al., 2000). Hence, measuring the activity of the biotransformation enzyme 7-ethoxy-resorufin-*O*-deethylase (EROD) in liver homogenates is a sensitive endpoint to detect changes in metabolic activities after exposure to MP-associated BkF and dissolved BkF in zebrafish (*Danio rerio*). Environmental concentrations of the organophosphate insecticide chlorpyrifos (CPF) are detected in surface waters (10 - 46 ng/L), as the global application is focused on fruits

and vegetable treatment (Ensminger et al., 2011; Tousova et al., 2017; Witczak et al., 2018; EFSA, 2019). The membrane-bound enzyme acetylcholine esterase (AChE) can be inhibited by various environmental contaminants, including organophosphate insecticides (Rodríguez-Fuentes et al., 2015). CPF leads to enzyme inhibition by covalent binding to serine -OH groups, subsequent accumulation of acetylcholine in the synaptic cleft, and results in impaired neurotransmission (Colovic et al., 2013; Russom et al., 2014). Similarly, studies have shown that organophosphate insecticides can be metabolized by cytochrome P450 enzymes and potentially inhibit biotransformation enzymes (Rodríguez-Fuentes et al., 2015; Kais et al., 2018). Therefore, samples from BkF and CPF exposure were analyzed with regard to CYP450 activity using the EROD assay. The increased 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* (El-Amrani et al., 2012; Barranco et al., 2017). Thus, ultra performance liquid chromatography coupled with fluorescence detection and with mass spectrometry (UPLC-FLD/MS) were used to investigate tissue concentrations and the possible formation of metabolites in target organs after continuous exposure.

However, both contaminants occur in surface waters and are priority pollutants under the European Water Framework Directive (European Parliament and European Council, 2008; Moschet et al., 2014; Arle et al., 2016). Interactions of these compounds and MPs in aquatic ecosystems are therefore likely. Due to the emergence of MP hotspots and underestimated environmental concentrations of hazardous organic pollutants (HOC; Kooi et al., 2016; Besseling et al., 2019), improving future risk assessment of MPs is essential (Santana et al., 2018; Franzellitti et al., 2019; Triebskorn et al., 2019). Therefore, it seems vital to investigate the consistency of biological responses to MPs, considering prolonged, chronic and continuous exposure scenarios (Santana et al., 2018), since previous studies reported contradictory effects of MPs at different biological levels (Rochman et al., 2013b; Khan et al., 2015; Karami et al., 2017; Ašmonaite et al., 2018; Jovanović et al., 2018; Pannetier et al., 2020).

This study aimed to provide further evidence for the transfer of HOCs by MPs and potential adverse effects on limnic vertebrates. Therefore, we assessed the effects of chronic exposure of adult zebrafish (*Danio rerio*) to BkF and CPF sorbed on two environmentally prominent types of MPs (polystyrene, polymethyl methacrylate). Monitoring of both, the sorption behavior and fate of the contaminants were documented *via* GC-MS. MP uptake and BkF transfer in gastrointestinal cryosections were investigated microscopically. In addition, selected enzymatic biomarkers such as AChE activity, CYP1A induction, and tissue accumulation in brain and liver of zebrafish were chosen according to the mode of action of the applied substances in the respective target organs. Results of the incremental measurements of the selected biomarkers, qualify the influence of different types and surfaces of MPs on contaminant transport over a prolonged, continuous exposure scenario.

III.3 Materials and methods

III.3.1 Materials and chemicals

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 Ltd., England) were applied (0.016 g/L). For experiments with CPF, stained (0.05 % Hostasol Red 5B) red fluorescent PS (density 1.04 g/cm³, INEOS Styrolution Group, Frankfurt, Germany) was used (0.16 mg/L PS). All chemical reagents were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany). Measurement of protein contents was carried out with the DC™ Protein Assay kit from Bio-Rad (Feldkirchen, Germany), using bovine serum albumin (BSA) in phosphate buffer as standard. 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 and. Otherwise, all solutions were prepared directly before use.

III.3.2 Zebrafish (*Danio rerio*) husbandry and experimental setup

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. Regional animal welfare authorities have licensed fish breeding under reference 35e9185.64/BH. All experiments with adult zebrafish licensed by the animal welfare committee of the Regional Council of Karlsruhe, Germany (35-9185.81/G-122/15). Fish were held in 5 L glass aquaria for CPF experiments and in 10 L glass aquaria for BkF experiments. Identical maintenance conditions of 25 ± 0.5 °C, pH 8.1, and 14:10 h light:dark cycle were assured for all experimental setups. Semi-static water exchange (50 % daily) safeguarded nitrate, ammonia, and nitrite concentrations below detection limits.

The experimental setup and treatment groups were identical for both substances (BkF/CPF), consisting of one negative control (without MP and substance), one control with pristine MP, two positive controls (substance only) and two treatment groups (with substance sorbed to MP) in two different concentrations each. For the treatment groups, MP suspensions with either 0.78 µg/L and 50 µg/ BkF sorbed to PMMA or 10 ng/L and 100 ng/L CPF sorbed to PS were always prepared 24 h in advance. Fish were fed twice daily 1 % of wet weight with TetraMin™ flakes (Tetra, Melle, Germany). After daily water exchange, substances, and filtered MP suspensions with sorbed substances were added accordingly. Overall, care was taken to prevent MP accumulation by scraping surfaces before water exchange and allowing particles and feces to settle before siphoning the remnants twice.

BkF experiments with PMMA were conducted in 10 L glass aquaria with 21 randomly allocated zebrafish per group, a total of 252 zebrafish (male:female ratio of 56:46 %), measuring 0.37 ± 0.05 g wet weight and 3.1 ± 0.2 cm total body length (snout to tail tip). BkF concentrations for the positive controls

and treatment groups were set to 0.78 µg/L and 50 µg/L. Water was exchanged twice daily in the positive controls (dissolved BkF), to prevent loss through sorption. CPF experiments with red fluorescent PS were carried out in 5 L glass aquaria with 15 randomly allocated zebrafish in each exposure group. A total of 180 zebrafish (male:female ratio of 65:35 %), with an average wet weight of 0.29 ± 0.08 g and 3.4 ± 0.2 cm body length, was used. CPF concentrations for both positive controls and treatment groups were set to 10 ng/L and 100 ng/L.

For animal welfare reasons, the two different experiments were conducted independently in duplicates. During the daily examination of groups, no zebrafish displayed signs of stress or disease symptoms and no specimen died during the experiments. Water samples were analyzed concerning leaching of sorbed substances after 14 days (BkF) and 21 days (CPF) with GC-MS. Further parameters of GC-MS are provided in Annex I.1 (Tab. A1). Concentrations always refer to nominal values unless stated otherwise.

III.3.3 Loading of MPs with benzo(k)fluoranthene and chlorpyrifos and validation of sorption

Sven Huppertsberg investigated the sorption of BkF and CPF on MPs and conducted analytical quantification of BkF and CPF in aqueous samples. Sample preparation and solid-phase extraction were carried out by me.

The particle loading process was carried out according to Hanslik et al. (Hanslik et al., 2020). In short, for the treatment groups with either 0.78 µg/L or 50 µg/L BkF sorbed to PMMA, a dispersion of 0.016 g/L PMMA and dissolved BkF (from 2 g/L BkF stock) in 50 ml Aqua bidest (final DMSO concentration of 0.005 %) was incubated at room temperature for two days, on a Certomat S-II orbital shaker at 100 rpm (Sartorius Stedim Biotech, Göttingen, Germany) and subsequently filtered over 0.2 µm Whatman Puradisc cellulose acetate filter (GE Healthcare, Solingen, Germany). Washed with 4 × 10 ml Aqua bidest and BkF-loaded MPs were resuspended by backwashing the filter with 4 × 10 ml tap water. Negative controls with pristine MP were treated likewise. Incubation of 0.16 mg/L PS with 10 ng/L and 100 ng/L CPF was carried out similarly. The particle size distribution of PMMA and PS (≤ 100 µm) is given in Annex I.2 (Fig. A1).

Aqueous phases and particle suspensions of all steps (incubation, washing, resuspension) were collected and measured separately in triplicates using GC-MS. In addition, Erlenmeyer flasks for MP incubation were pre-incubated overnight with BkF and CPF to minimize sorption losses. BkF samples were extracted by adding 1.1 ml *n*-hexane, then vortexed for 2 min and frozen (-20 °C) to facilitate phase separation. Phenanthrene was used as an internal standard for BkF samples. For CPF measurements, samples were spiked with atrazine as internal standard, dried under a stream of nitrogen, and resuspended in acetone/ethyl acetate (1:1). For both approaches, the extraction solvent was filtered through 0.2 µm syringe filters and transferred to glass vials for subsequent measurements. Analytical quantification was

conducted using GC-MS for CPF and BkF samples. Further details on measurement parameters are given in Annex I.2.

III.3.4 Sampling and preservation of zebrafish tissue

Sampling was identical for both experimental setups. Samples were taken after 3, 7, 14 and 21 days. After three days of exposure, groups were sampled for enzymatic assays (EROD, AChE) only. At all other stages, samples were collected for all endpoints investigated. After euthanasia with 1.5 M tricaine (MS 222, Sigma-Aldrich) and decapitation, 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 until samples had subsided (3 - 5 days) before proceeding with embedding for cryosectioning. Whole liver samples for EROD assay and brain samples for AChE activity measurements were quickly dissected, immediately frozen in liquid nitrogen, and stored at -80 °C until further analysis. Additional samples for tissue analysis of BkF (liver) and CPF (brain) were collected after 21 days of continuous exposure and treated identically.

III.3.5 Cryosectioning of gastrointestinal tracts of zebrafish and confocal laser scanning microscopy (CLSM)

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) as BkF emits fluorescence at 403 nm (Rivera-Figueroa et al., 2004). To avoid extraction of BkF by dehydration with ethanol, cryosectioning was favored over paraffin embedding. Therefore, tissue samples were dehydrated (3 × 5 min in 1 × phosphate-buffered saline) and incubated in 10 % sucrose solution in PBS for 50 min at room temperature. After a subsequent incubation overnight in 30 % sucrose solution in PBS at 4 °C, the samples were incubated with 1:1 sucrose solution (30 %) and Tissue Freezing Medium (1 Leica Biosystems, Germany) for 4 h at room temperature to prevent tearing of the intestinal cavity during the cutting process. Samples were embedded and frozen in Leica Tissue Freezing Medium, 20 µm sections were cut horizontally at -20 °C, using a Leica CM 3050 S cryostat (Leica Biosystems, Nussloch, Germany). Afterward, the sections were transferred to Superfrost Plus™ Adhesion Microscope Slides (Thermo Fisher Scientific, Karlsruhe, Germany), left unstained and stored at 4 °C for a maximum of seven days until microscopic analysis.

Fluorescence was recorded in optical sections in individual confocal images (λ_{EX} = 405 nm, λ_{EM} = 432 - 467 nm) in addition to a brightfield image. All cryosections of each experiment were imaged in brightfield and fluorescence mode with consistent settings at 10 × magnification and processed with

FIJI (Preibisch et al., 2009; Schindelin et al., 2012). Fluorescence was recorded in optical sections 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 background fluorescence of control samples. Possible presence of liver tissue, chyme or fluorescent particles within the chyme were excluded from the analysis (see Annex I.3 for additional information).

Gastrointestinal tracts from CPF experiments were processed identically to BkF samples and investigated at 10 × magnification to verify MP uptake *via* ingestion and to detect possible physiological effects of polystyrene particles during passage through the intestinal tract. The fluorescent PS particles were tracked by epifluorescence excitation in the red fluorescence spectrum (λ_{EX} = 540 - 580 nm, λ_{EM} = 600 - 660 nm, exposure time 60 ms, Nikon Eclipse 90i, Nikon, Germany).

III.3.6 Hepatic 7-ethoxy-resorufin-*O*-deethylase (EROD) assay

Frozen liver samples were thawed on ice and homogenized for 30 s at 30 Hz using a TissueLyser II (Qiagen, Hilden, Germany) in 800 µl ice-cold extraction buffer (15 mM $NaH_2PO_4 \times H_2O$, 68 mM $Na_2HPO_4 \times 2\ H_2O$, 1.2 mM $MgCl_2^- \times 6\ H_2O$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol, pH 7.4) based on protocols by Örn et al. (1998) and Batel et al. (2016). 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). Phosphate buffer as blank (250 µl), duplicate wells of resorufin and homogenized samples in triplicates (50 µl) were added to black 96-well microplates (Brand, Wertheim, Germany). Enzymatic reaction was induced by adding 100 µl of a mix of 6 µM 7-ethoxyresorufin and 10 µM dicoumarol, followed by 100 µl of 0.8 mM NADPH tetrasodium salt (in phosphate buffer). 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 content was measured according to Lowry et al. (1951) with the DC™ Protein Assay kit from Bio-Rad, using BSA (0.125 - 2 mg/ml, in phosphate buffer) as standard. Fluorescence was measured at 690 nm (GENios plate reader) after 20 min incubation at room temperature. In case the protein yield was ≤ 0.2 mg/ml, measurements had to be excluded from the 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).

III.3.7 Acetylcholine esterase activity measurements

AChE activity was measured in whole brain samples of zebrafish based on protocols by Kais et al. (2015) and Küster (2005). Frozen samples were thawed on ice and homogenized for 1 min at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany) in 800 µl of 0.1 M phosphate buffer with 0.1 %

Triton X-100. After centrifugation for 15 min at 10.000 × G (4 °C, Heraeus Multifuge 1 S-R, Thermo Scientific, Schwerte, Germany), the supernatant was transferred and diluted 1:1 with 0.1 M phosphate buffer for further enzyme kinetic measurements and protein determination. 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). Phosphate buffer served as a blank and samples were applied in quadruplicates (50 µl), before adding 50 µl phosphate buffer and 100 µl of 0.89 mM 5,5′-dithiobis-2-nitrobenzoic acid. The kinetic reaction was initiated by adding 100 µl of 0.9 mM acetylthiocholine iodide, and changes in optical density over time and protein content (ΔOD/min/mg of protein) were recorded. AChE activity levels were compared to control samples and expressed as percent of the negative control. Protein content was measured according to Lowry et al. (1951) with the DC™ Protein Assay kit from Bio-Rad and compared to BSA (0.0625 - 1 mg/ml, in phosphate buffer) as a standard. All enzyme activities were standardized with respect to the protein content.

III.3.8 Accumulation and biotransformation of benzo(k)fluoranthene and chlorpyrifos in liver and brain tissue 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, λ_{EM} = 420 nm) and coupled to a Xevo G2-XS high-resolution mass spectrometer (HRMS) equipped with an electrospray ionization source (Waters, Eschborn, Germany). The injection volume was set to 10 µl. UPLC separation was achieved using an ACQUITY UPLC HSS T3 column (100 × 2.1, 1.7 µm, flow rate 0.45 ml/min, column temperature 45 °C). The mobile phase consisted of (A) water (0.1 % formic acid) and (B) acetonitrile (0.1 % formic acid) with the following gradient: 0 - 0.25 min, 2 % B, 12.25 - 15 min, 99 % B, 15.1 - 17 min, 98 % B. Ionization source conditions were as follows: capillary voltage of 0.7 kV in positive ion mode and -1.0 kV in negative ion mode, source temperature 140 °C, and desolvation temperature 550 °C. The sampling cone voltage was set to 20 V and source offset to 50 V. Nitrogen and argon were used as cone and collision gases and the desolvation gas flow was 950 L/h. The mass range was set to m/z 50 to m/z 1200 and the data were recorded in negative and positive centroid mode with 0.15 s scan time. Further information is provided in the Annex I.4. The limit of detection (LoD) was 0.1 ng/ml for BkF and 1 ng/ml by UPLC/MS detection for CPF and detected amounts were referenced to individual sample weight. MarkerLynx (Waters, Eschborn, Germany) was used and a combined non-target/suspect screening was applied to screen for possible transformation products based on the approach described by Kühnert et al. (2017).

III.3.9 Data analysis

No significant differences were found between replicates of each treatment and therefore, data from replicate treatments were combined. For statistical evaluation, data were tested for normality and equal variances. To identify statistically significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between treatment groups versus the control group, either parametric ANOVA followed by Holm-Sidak *post-hoc* test (AChE activity) or nonparametric Kruskal-Wallis analysis followed by Dunn's *post-hoc* test (BkF signal in the intestine of *Danio rerio* and EROD assay) was performed using SigmaPlot 13 (Systat Software, Erkrath, Germany). Outliers were identified with Grubbs test and results were expressed as mean values (MV) ± standard error of the mean (SEM).

III.4 Results

III.4.1 Microplastic loading with benzo(k)fluoranthene and chlorpyrifos

Quantifying the final amount of BkF and CPF sorbed to MP particles is crucial to assess the potential amount being transferred to zebrafish. For BkF, measurements with GC-MS have proven that up to 80 % of BkF was sorbed to spherical PMMA particles and less than 10 % remained in the aqueous phase after 24 h of incubation, as described already in Hanslik et al. (2020). Up to 20 % of BkF can desorb from BkF-loaded particles over 24 h exposure in clean water and might be taken up further without particle-mediated transfer (Tab. 6). The sorption capacity of irregular PS particles for chlorpyrifos was reduced compared to BkF and PMMA particles, as approximately 60 % of CPF was sorbed to PS particles, possibly due to the lower log K_{OW} of 4.96 compared to BkF (log K_{OW} 6.11). Up to 40 % of CPF was still present in the aqueous phase after incubation with PS particles (Tab. 6). However, since all vials for incubation were pre-saturated, a loss of CPF due to binding to glassware was considered negligible. However, the desorption of CPF was higher compared to BkF and after 24 h in Aqua bidest and therefore an estimated amount of approximately 60 - 80 % of CPF was present in the aqueous phase. Therefore, the increased availability of PS-associated CPF for zebrafish could be conceivable.

Tab. 6. Measured concentrations of benzo(k)fluoranthene sorbed to PMMA and chlorpyrifos sorbed to PS using GC-MS. Residues of BkF and CPF were measured directly in the aqueous phase after filtration. Not assessed = n. a. Data given as mean values ± SD. *Data were produced jointly with Sven Huppertsberg.*

		Nominal concentration	MPs	Aqueous phase	Desorption (24 h)
BkF	[µg/L]	50	31.8 ± 8.2	4.2 ± 0.2	7.5 ± 3.4
	[%]	100	63.6 ± 16.3	8.5 ± 0.2	23.6 ± 10.7
CPF	[µg/L]	800	n. a.	341.8 ± 17.1	253.3 ± 12.7
	[%]	100	n. a.	42.7 ± 5.0	-

Results from water analysis indicated only negligible amounts of BkF and CPF in the aqueous phase (< 0.02 - 0.2 ng/L) in treatment groups with loaded MP. Thus, over 24 h exposure in glass aquaria, only minor leaching took place (Annex I.1, Tab. A2), and the main route of uptake by zebrafish in the treatment groups was most likely through ingestion of BKF/CPF-sorbed MPs. Detailed information on water sample preparation and measuring parameters is given in Annex I (Tab. A1).

III.4.2 Long-term exposure of zebrafish to MP-sorbed chlorpyrifos and benzo(k)fluoranthene

III.4.2.1 Microplastic uptake by zebrafish

Over the experimental course of 21 days, zebrafish readily ingested MP (Fig. 11) in all MP groups (control with pristine MP and the treatment groups with sorbed MP). PS was detected by epifluorescence excitation (540 - 580 nm) of the red dye (Hostasol Red 5B). No leaching of the dye was observed since no red fluorescence signal was detected aside from PS particles. In the experiments with BkF, PMMA could be easily identified within the chyme due to its spherical shape (Fig. 11B 1). In addition, BkF was detected by CLSM at 340 - 380 nm excitation wavelength. PMMA sorbed with BkF was detected due to its spherical shape and a blue fluorescence signal, as shown in Fig. 11B 2 (circles). The ingested amounts of MP varied throughout all treatments, as the time of sampling and individual feeding behavior are known to influence excretion rates in fish (Siccardi et al., 2009; Lawrence et al., 2012). In all cryosections, MP was found exclusively in the intestinal lumen and chyme. No intervillous or intercellular particles were detected, and no adverse effects of MPs on tissue integrity have been observed.

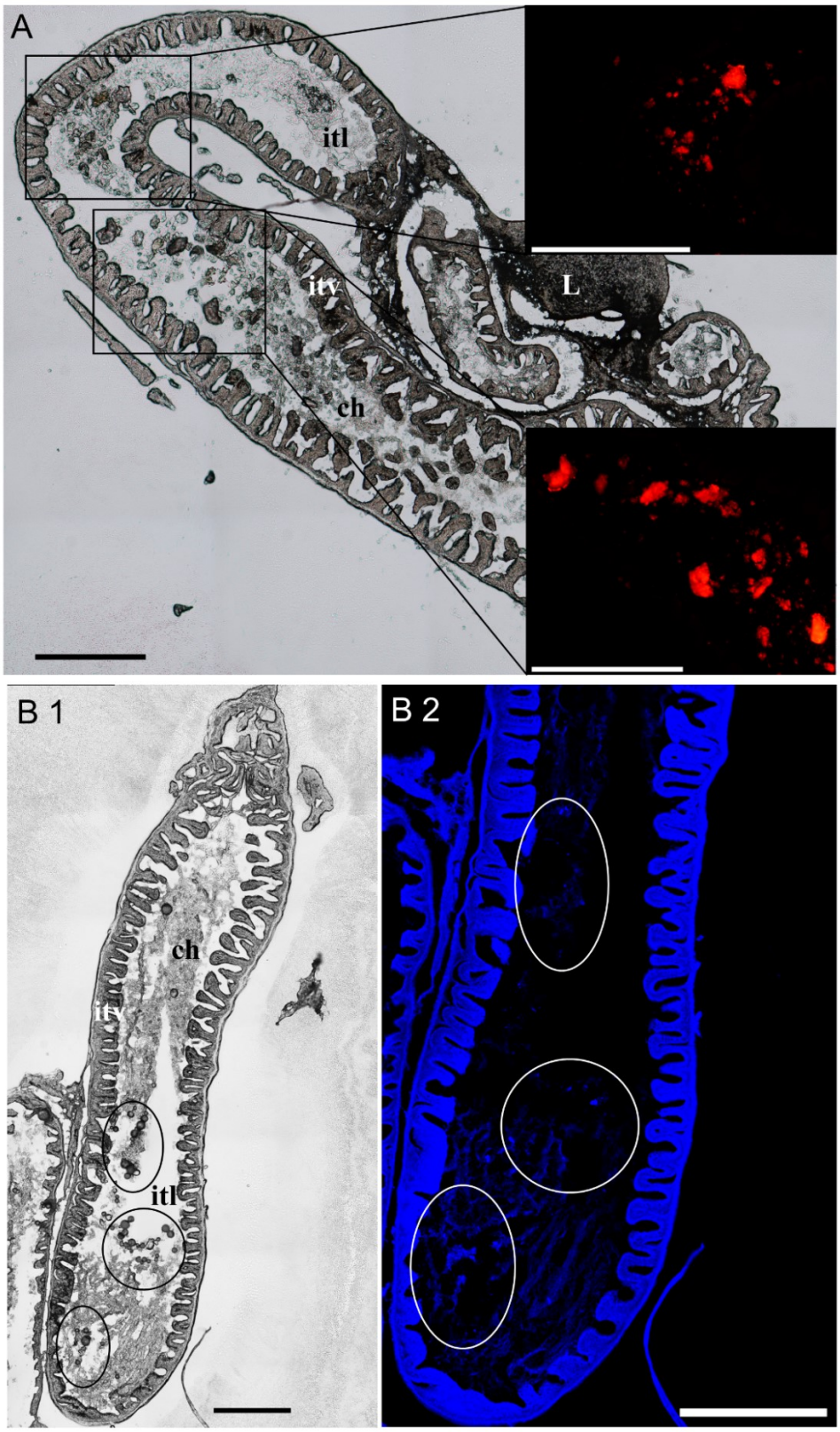

Fig. 11. Gastrointestinal tract sections of zebrafish exposed to MP sorbed with either chlorpyrifos or benzo(k)fluoranthene, after 21 days of exposure. (**A**) Irregular shaped PS particles detected *via* red epifluorescence (excitation filter = 540 - 580 nm, emission filter = 600 - 660 nm, exposure time 60 ms). (**B 1**) Spherical PMMA particles (black circles) in zebrafish intestine. (**B 2**) Enlarged CLSM image of blue BkF fluorescence signal in intestinal tissue (excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm) and BkF-related fluorescence of PMMA particles (white circles). L = liver, itl = intestinal lumen, itv = intestinal villi, ch = chyme, scale bars = 500 µm (Nikon Eclipse 90i/C1, Nikon, Duesseldorf, Germany).

III.4.2.2 Fluorescence tracking of benzo(k)fluoranthene in zebrafish intestinal tissue

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. 12). A comparison of the results with the corresponding negative control from the respective time point revealed no significant uptake of BkF into intestinal tissue. Only the positive control with 50 µg/L waterborne BkF induced a 5-fold fluorescence increase after 7 days and a 2.5-fold increase after 14 days ($p < 0.05$, $p < 0.01$ respectively). After 21 days of exposure, fluorescence intensity within positive control samples decreased to negative control levels. Regardless of the sorbed BkF concentration, treatment groups with BkF sorbed to PMMA did not show any significant changes in fluorescence intensity and remained stable over time.

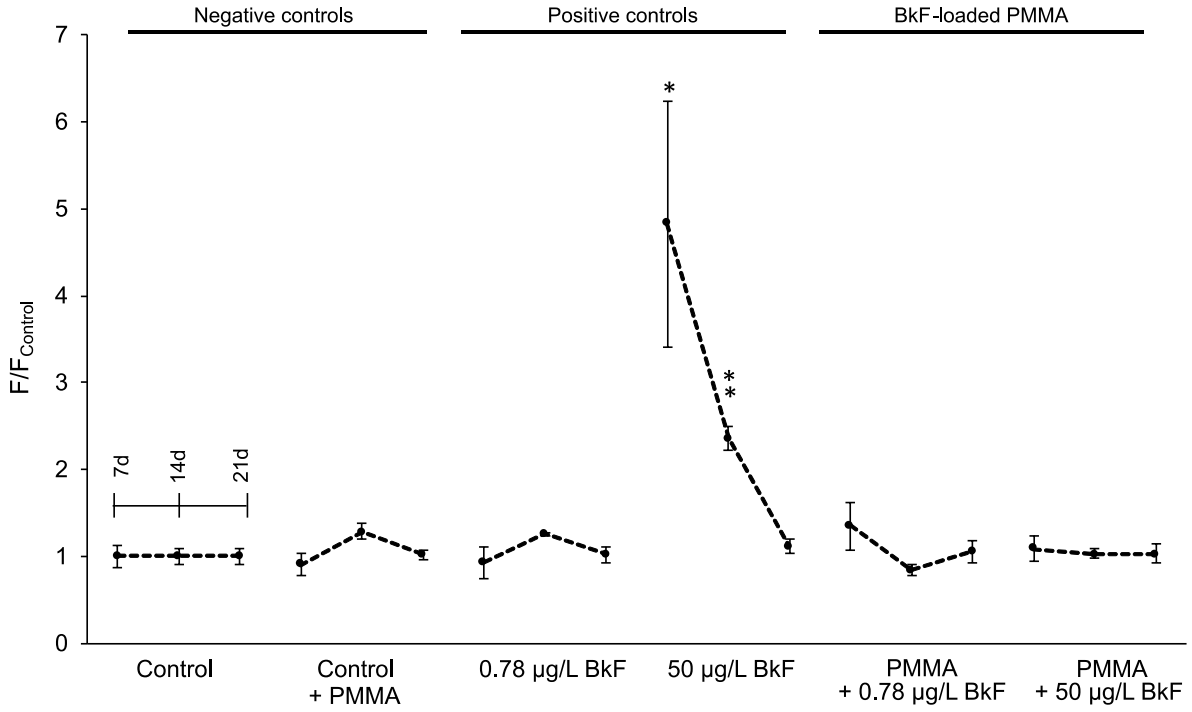

Fig. 12. Intestinal fluorescence signal in Danio rerio over 21 days of exposure to benzo(k)fluoranthene and PMMA. Zebrafish were either exposed to water (Control), water with pristine PMMA (Control + PMMA), positive controls with dissolved substances at two different concentrations, or two different treatments with BkF-associated PMMA. Fluorescence alterations are expressed as relative grey values of the control (water). Kruskal-Wallis analysis, Dunn's post-hoc (* $p < 0.05$, ** $p < 0.01$). n = 6, except for first sampling of PMMA + 0.78 µg/L BkF (n = 5). N = 2, Data are given as means ± SEM.

III.4.2.3 Hepatic EROD activity in zebrafish

Samples were analyzed after 3, 7, 14 and 21 days of exposure to either BkF or CPF (Fig. 13). 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 ($p < 0.01$, $p < 0.001$). 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 during exposure.

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. CYP1A activity was significantly increased in a similar manner ($p < 0.05$, $p < 0.01$) in both treatments. A two- to three-fold increase from basal control levels after three days of approximately 20 pmol resorufin × mg^{-1} × min^{-1}, to seven days of exposure (56 - 60 pmol resorufin × mg^{-1} protein × min^{-1}). And another two-fold increase after 14 days (90 - 140 pmol resorufin × mg^{-1} protein × min^{-1}). However, no differentiation could be made based on EROD induction levels between low and high concentrations of CPF (10 ng/L, 100 ng/L) or the application form (dissolved, sorbed). The induction pattern over 21 days was alike for both positive controls and treatment groups, culminating after 7 - 14 days of exposure and decreasing to basal levels after 21 days.

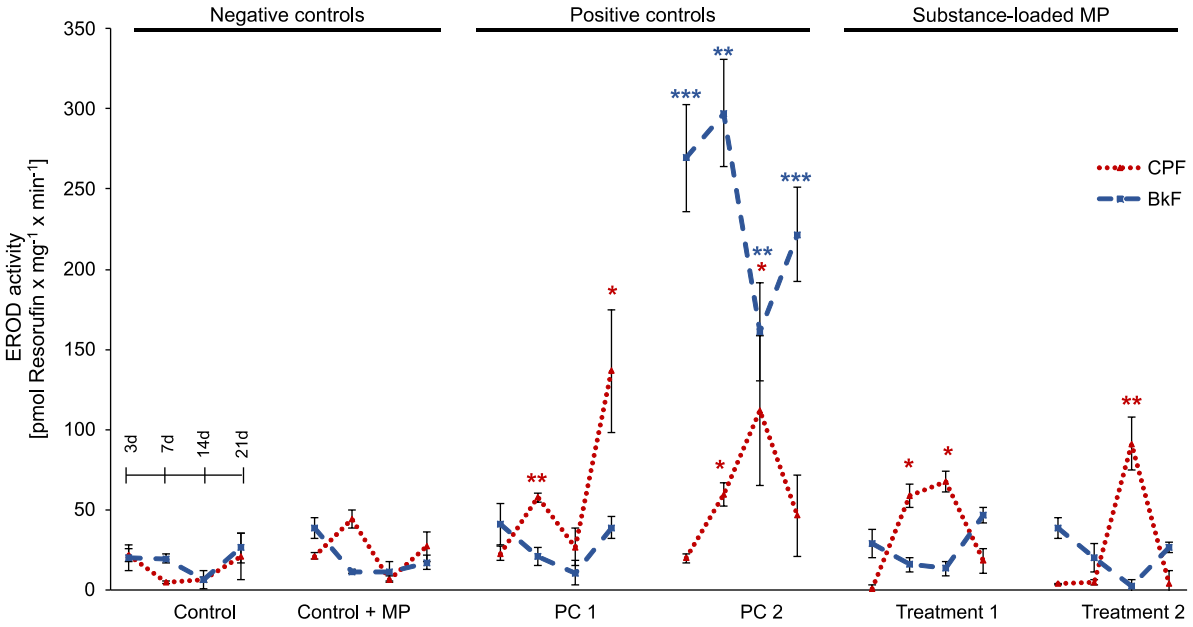

Fig. 13. EROD activity in liver of *Danio rerio* exposed to benzo(k)fluoranthene (blue) or chlorpyrifos (red). Zebrafish were either exposed to water (Control), water with pristine MP (Control + PMMA/PS), positive controls with dissolved substances at two different concentrations (PC 1/2 = 0.78 µg/L, 50 µg/L of BkF or 10 ng/L, 100 ng/L of CPF), or two different treatments with MP-associated CPF/BkF at two different concentrations (Treatment 1/2 = PMMA + 0.78 µg/L BkF or PMMA + 50 µg/L BkF, PS + 10 ng/L CPF or PS + 100 ng/L CPF). Kruskal-Wallis analysis, Dunn's *post-hoc* (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, colors of asterisks indicate assignment to corresponding exposure scenario). BkF: n = 5 - 10, CPF: n = 3 - 6. N = 2, Data are given as means ± SEM.

III.4.2.4 Acetylcholine esterase activity in zebrafish brain samples

Brain samples of zebrafish were analyzed during chronic exposure to environmentally relevant concentrations of CPF (Fig. 14). Acetylcholine esterase activity is well known to be hampered by CPF exposure at different developmental stages in zebrafish (Yen et al., 2011; Richendrfer and Creton, 2015; Rodríguez-Fuentes et al., 2015). Exposure of *Danio rerio* to waterborne CPF and sorbed CPF at both concentrations, lead to decreased AChE activity. Enzyme activity was inhibited by up to 10 % in all CPF treatments, with a maximum inhibition of 15 % after 21 days when zebrafish were exposed to PS sorbed with 10 ng/L CPF. Due to substantial standard deviations within each group, no significant changes in AChE activity were detected even though an overall trend of increasing AChE inhibition was visible over the experimental time course.

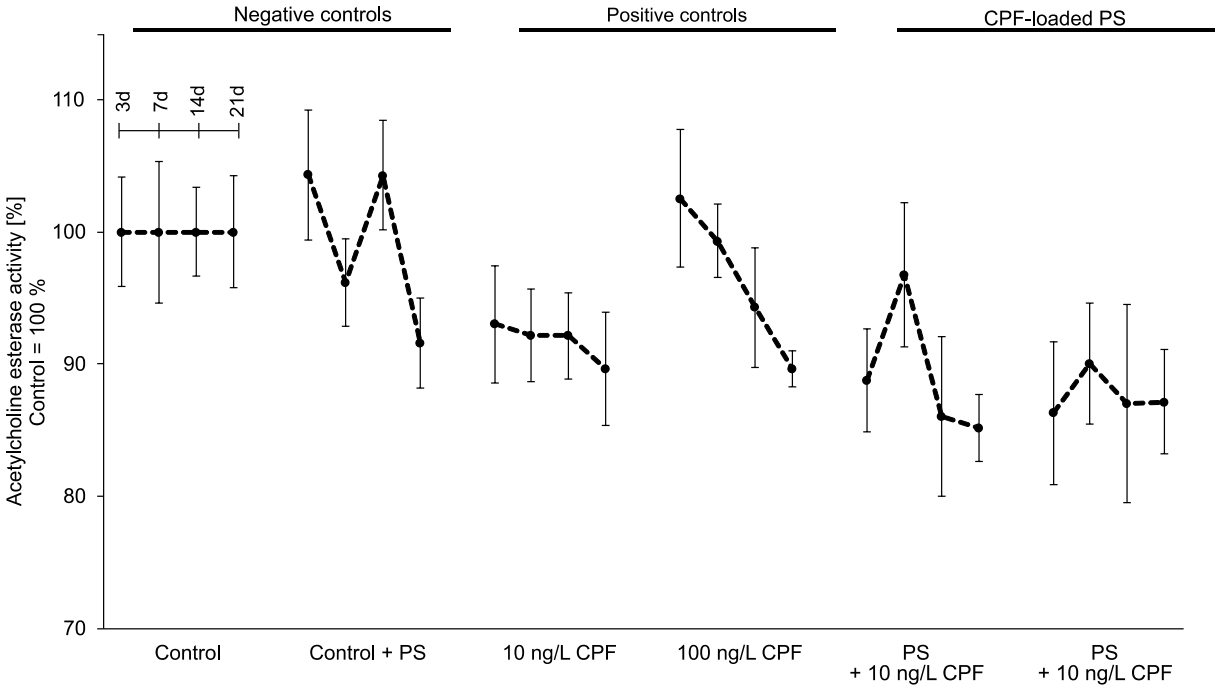

Fig. 14. Acetylcholine esterase activity in zebrafish over 21 days of exposure to chlorpyrifos and PS. Zebrafish were either exposed to water (Control), water with pristine PS (Control + PS), positive controls with dissolved CPF at two different concentrations, or two different treatments with CPF-associated PS at two different concentrations. Activity is expressed as % of the control (water). ANOVA, Holm-Sidak *post-hoc*, n = 6, except for 100 ng/L CPF after 21 days (n = 5). Note that activity axis starts at 70 % for better visibility. N = 2, Data are given as means ± SEM.

III.4.2.5 Accumulation and biotransformation products of benzo(k)fluoranthene and chlorpyrifos 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 sample of the positive controls upon exposure to 50 µg/L waterborne BkF (Fig. 15A). Amounts of BkF in liver tissue varied between replicate samples from 0.03 - 3.12 ng (limit of quantification ≤ 0.01 ng). UPLC-HRMS analysis did not allow to detect or to identify biotransformation products of BkF, due to overall low amounts (Fig. 15B). Thus, no distinct discrimination between types of biotransformation products (Phase-I, Phase-II and functional groups) could be made. However, metabolites with increased polarity, which were detected earlier than BkF at 11.58 min (Fig. 15A), are assumed to originate from phase-II of biotransformation (e.g., 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 tissue of zebrafish (limit of detection of 0.2 ng CPF/sample, limit of quantification ≤ 1.0 ng).

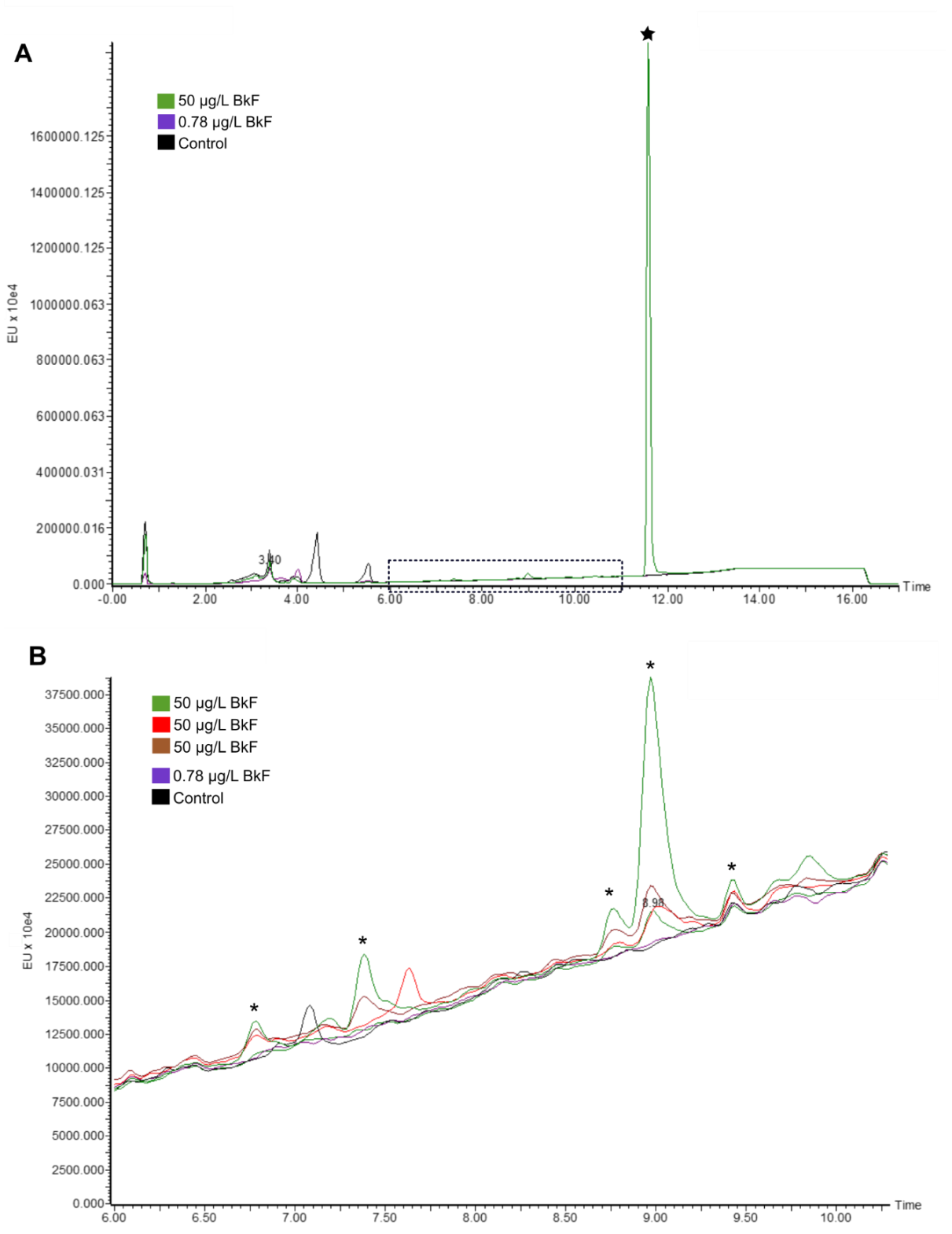

Fig. 15. UPLC-FLD chromatogram of benzo(k)fluoranthene (BkF) and biotransformation products in liver extracts of zebrafish after 21 days of continuous exposure. (**A**) Distinct BkF peak (star) at 11.58 min in one sample from exposure to 50 µg/L waterborne BkF (green). The dashed box between 6.0 - 11.0 min is enlarged in (**B**), demonstrating similar peaks (*) of biotransformation products from samples exposed to waterborne 50 µg/L BkF (green, red, brown). *Data were produced jointly with Bettina Seiwert.*

III.5 Discussion

III.5.1 Sorption of benzo(k)fluoranthene and chlorpyrifos to microplastic particles

GC-MS measurements proved that up to 80 % of BkF adsorbed to PMMA during the pre-incubation (Tab. 6). Here, 0.6 - 0.7 mg of BkF were sorbed to 1 g PMMA particles. In contrast, 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 (Yeo et al., 2017; Chen et al., 2019; Pannetier et al., 2019a). These results show that the pollutant load on MPs could be significantly increased under laboratory conditions compared to environmental exposure scenarios. Furthermore, water analysis documented that desorption of BkF from PMMA into the aqueous phase over 24 h was negligible (Tab. A2). This is in line with findings from littoral plastic debris and desorption rates of only 12 % for PAHs into seawater (León et al., 2018). Therefore, exposure *via* ingestion of loaded MPs is the most relevant exposure route for *Danio rerio* in our study.

Similar sorption rates of up to 60 % of CPF to PS particles were documented by GC-MS. These results were consistent with data reported in other studies, documenting sorption rates for PE particles and CPF of approximately 80 % in filtered seawater after 2 h of incubation were documented (Garrido et al., 2019; Bellas and Gil, 2020). Comparable results were reported for freshwater conditions in another lab study, where sorption of CPF to different types of MP resulted in sorption rates of 60 % after 72 h of incubation (Allen et al., 2018). Here, the analytical quantification of CPF in water samples indicated no measurable amounts of CPF in the aqueous phase; thus, no desorption from PS particles over 24 h (Tab. A2), even though the analytical quantification of the de-/sorption behavior revealed high amounts of CPF in the aqueous phase. These discrepancies might be due to different scenarios: Analytical quantification of desorption rate in Aqua bidest compared to exposure in the experimental setup with zebrafish. The exposure time may have been too short (24 h) to increase CPF concentrations in the aqueous phase (Allen et al., 2018; Seidensticker et al., 2018), but the intended route of exposure was *via* sorption to MPs, and therefore daily water exchange and removal of MPs were necessary. It has been shown that in glass-like polymers such as PS and PMMA (glass transition temperature for PS = 100 °C and PMMA = 105 °C), surface adsorption is the predominant mode of sorption and desorption processes are therefore possible (Hüffer and Hofmann, 2016; Uber et al., 2019).

Nevertheless, desorption processes in multivariate ecosystems and organisms are not yet entirely understood, since the variety of plastics and environmentally relevant contaminants is infinite. Studies on the desorption of hydrophobic organic contaminants and polychlorinated biphenyls (PCBs) in artificial gut fluids have shown, that bioavailability of substances sorbed to MPs can vary over a wide range (Mohamed Nor and Koelmans, 2019) and that uptake of pollutants by ingestion of pollutant-loaded MPs may be negligible (Lee et al., 2019). Though, the ingestion of MPs facilitates an alternative route of exposure and requires further consideration for future risk assessment, as evidence has illustrated that

ingested microplastics have the potential to cause physical and chemical harm (Wright et al., 2013; Jovanović, 2017).

III.5.2 Long-term exposure to benzo(k)fluoranthene and chlorpyrifos

III.5.2.1 Microplastic particle uptake by zebrafish

Successful ingestion of MPs in all treatments with MP (pristine and sorbed) was confirmed by visual detection with either CLSM and brightfield images for BkF experiments or epifluorescence and bright-field images for CPF experiments. Our findings were in line with the reported uptake of MPs for a variety of taxa in aquatic and terrestrial ecosystems as well as under laboratory conditions (Karami et al., 2017; Karlsson et al., 2017; Bour et al., 2018; Choi et al., 2018; Jabeen et al., 2018; Lehtiniemi et al., 2018; Santana et al., 2018; Weber et al., 2018). Here, no physical impairment of tissue integrity was detected for either spherical or irregular shaped MPs.

Still, effects of ingested pristine, clean MPs on fish are controversially discussed and highly variable, as some studies did not detect adverse effects (Khan et al., 2015; Mazurais et al., 2015; Karami et al., 2017; Santana et al., 2017; Jovanović et al., 2018). Whereas, other studies reported physiological impairments and biochemical modulations in target organs (Karami et al., 2016; Espinosa et al., 2017; Choi et al., 2018; Jabeen et al., 2018; Lei et al., 2018). So far, only a few studies investigated the effects of MP-associated contaminants in fish, either by intentional loading under laboratory conditions (Rochman et al., 2014; Khan et al., 2015; Batel et al., 2016, 2018; Cormier et al., 2019) or by application of MPs from environmental samples or pre-exposure in natural environments (Rochman et al., 2013a, 2014; Ašmonaite et al., 2018; Pannetier et al., 2020). However, data of chronic exposure to chemical pollutants sorbed on MPs is still very limited.

Even though in this study the MP amount of 1×10^6 MP particles/L was rather high in the aqueous media, other studies failed to detect MPs in vertebrate consumers when applying lower, more environmentally relevant, amounts of MP (Grigorakis et al., 2017; Santana et al., 2017). Similar to this study, MPs of different size, shape, color and polymer were ingested to a certain extent when applied directly to the aqueous media or offered associated with feed (Triebskorn et al., 2019). This suggests that MPs may alter the bioavailability by changing the uptake route from water to dietary exposure in model fish species (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 concentration on MPs was much lower compared to bioaccumulated substances within the prey (Ziccardi et al., 2016; Jovanović, 2017; Diepens and Koelmans, 2018).

III.5.2.2 Effects of benzo(k)fluoranthene in zebrafish

The fluorescence signal of BkF in the intestinal tissue of zebrafish was significantly increased after exposure to 50 µg/L waterborne BkF ($p < 0.01$), similar to the EROD activity. Overall, effects evoked by exposure to waterborne BkF (0.78 µg/L, 50 µg/L) and BkF sorbed to PMMA were pronounced only slightly (see Fig. 12/13). In the present study, EROD induction was significantly upregulated ($p < 0.01$ and $p < 0.001$) as early as three days of exposure, presumably due to increased metabolic activity and detoxification of BkF (Whyte et al., 2000; Billiard et al., 2008). Encoding for the CYP450 enzyme as one of the main detoxification phase-I enzymes (Whyte et al., 2000), *cyp1a1* is particularly evident in the liver. UPLC-FLD measurements of BkF in liver tissue revealed only minor amounts of parent BkF and biotransformation products only after exposure to waterborne 50 µg/L BkF, indicating the reduced significance of bioaccumulation and biotransformation processes in our study. In contrast, exposure of zebrafish embryos to 5 µg/L benz(*a*)anthracene at early pharyngula stage (26 hpf) already induced significant *cyp1a* gene expression and increased formation of biotransformation products, detected with a similar measurement setup (Kühnert et al., 2017). Exposure to pristine PMMA did not induce alterations in all observed endpoints, which is in line with results for PE, LDPE, PVC and PS particles (Mazurais et al., 2015; Batel et al., 2018; Jovanović et al., 2018; Rainieri et al., 2018; Cormier et al., 2019).

Even though MP uptake was confirmed visually in cryosections of the gastrointestinal tract of *Danio rerio* (Fig. 11B 2), no transfer of PMMA-sorbed BkF was evident as levels of CYP1A induction remained low in treatment groups compared to controls over 21 days of exposure (Fig. 13). Similar effects were investigated in studies with marine mussels and copepods, were MP-associated PAHs were not transferred *via* ingested particles to organisms and did not increase tissue bioaccumulation or bioavailability of the model pollutants (Paul-Pont et al., 2016; Magara et al., 2018; Bartonitz et al., 2020; Sørensen et al., 2020). Likewise, the transfer of metals sorbed to PE particles within zebrafish was overall significantly reduced (Khan et al., 2015), indicating diminished bioavailability and reduced desorption. Further, it can be assumed that BkF may follow different detoxification pathways, thereby decreasing *cyp1a1* activity, as documented in scallops (Pan et al., 2005).

Water analyses documented BkF concentrations between 0.1 - 0.2 ng/L in the aqueous phase, indicating that the concentration of desorbed BkF was probably too low to induce adverse effects through aqueous exposure. The reduced uptake of contaminants might be due to impaired bioavailability of BkF resulting from high binding affinities to MPs (Sleight et al., 2017). These results were consistent with negligible desorption rates of BkF in water over 24 h (Tab. 6) and promoted the ingestion of BkF-sorbed MPs as the primary route of uptake. Further accumulation of BkF in the aqueous phase was not intended in this exposure scenario and therefore prevented by daily water exchange.

Still, data on chronic MP exposure (with or without sorbed contaminants) of adult *Danio rerio* is limited. 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. Whereas in liver samples, *cyp1a1*

gene expression was significantly upregulated after three weeks of exposure (Rainieri et al., 2018). Another study investigated the effects of long-term feeding of European seabass (*Dicentrarchus labrax*) with diets spiked environmentally relevant amounts of contaminants (e.g., polychlorinated biphenyls PCBs, polybrominated biphenyl ethers PBDEs) 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, values reverted to control levels, indicating only minor effects on liver detoxification and lipid distribution. Whereas, exposure of adult male zebrafish to pristine 100 µg/L PS microplastics (5 µm), over 21 days, already induced adverse effects in hepatic glycolipid metabolism at different biochemical and transcriptional levels (Zhao et al., 2020). Still, the authors detected a significant decrease in growth of fish exposed to MP, which was not noticed in our study though feeding impairment may influence metabolic activity.

Short residence time in the gastrointestinal tract is another essential aspect concerning desorption processes from MPs (Mohamed Nor and Koelmans, 2019; Uber et al., 2019) and may result in a reduced contaminant uptake even if ingestion of contaminants sorbed to MP is a novel exposure route (Khan et al., 2015; Pannetier et al., 2020). Furthermore, the passage of MP through the digestive tract is a transitory process, and thus the potential for bioaccumulation and biomagnification is further reduced (Grigorakis et al., 2017; Güven et al., 2017; Rainieri et al., 2018). Reduced effect size and deviations could be due to different sensitivity of adult zebrafish compared to larval stages (Pannetier et al., 2019b, 2020) and sample size. In the present study, the sex-ratio in zebrafish was close to 50 %, equalizing potential effects due to gender differences. Since EROD activity can be subjected to biological variability in either sex (Whyte et al., 2000), this issue may be mitigated in future studies by increased sample size (Saad et al., 2016).

However, none of the studies mentioned above investigated EROD induction patterns at different time points during experimental exposure and thus may underestimate variations over time. The sampling time point may influence results, especially if compensatory reactions of the test organisms might come in to focus (Rainieri et al., 2018), leading to reduced gene activities and thus reduced modulations after prolonged exposure periods (Rochman et al., 2013b; Granby et al., 2018). Therefore, these incremental measurements of EROD activity give more in-depth information and further emphasize the diminished risk of MPs as a vector for environmental contaminants.

III.5.2.3 Effects of chlorpyrifos in zebrafish

Experimental exposure to waterborne CPF and PS-associated CPF (10 ng/L, 100 ng/L) over 21 days only induced significant upregulation in EROD activity from seven days onwards ($p < 0.05$, $p < 0.01$). This was in accordance with another study, were exposure to 1.2 µg/L CPF significantly induced EROD activity and increased CYP450 mRNA levels in liver tissue of juvenile carp over 40 days (Xing et al.,

2014). Effects increase with increasing CPF concentrations as exposure to dissolved CPF (300 µg/L) over 21 days, seemed to disturb hepatic lipid metabolism and gut microbiota dysbiosis of adult male zebrafish (Wang et al., 2019a). Similar results were obtained for invertebrates (Binelli et al., 2006; Sanchez-Hernandez et al., 2014). Analogous to CPF, other insecticides with a thiophosphate backbone like diazinon and parathion can be metabolized by CYP450 into respective -oxon forms (Yen et al., 2011). Therefore, biotransformation by CYP450 can cause an indirect effect on the acetylcholine esterase by accelerating the biotransformation into a more potent AChE inhibitor like CPF-oxon (Binelli et al., 2006; Rodríguez-Fuentes et al., 2015). Here, no measurable amounts of parent CPF or biotransformation products were detected in brain tissue above the limit of detection of 0.2 ng/sample, indicating no distinct accumulation of CPF in zebrafish. Thus, an efficient degradation and excretion after 21 days of exposure might have been likely, since EROD activity levels in groups exposed to waterborne CPF and MP-associated CPF decreased to basal levels at the end of the exposure period as well.

Investigating the metabolic influence of organophosphorus insecticides in combination with neurotoxicological markers such as AChE activity, provide novel insights to biochemical interactions. This was also evident in our study since the increase in EROD activity in positive controls and treatment groups after seven days have been contrasted by a decrease in AChE activity in the corresponding groups (Fig. 13/14). This trend was especially evident in the positive control (100 ng/L CPF) when EROD activity significantly increased ($p < 0.05$) after 7 and 14 days, and AChE activity was inhibited by up to 10 %. Most likely, as 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 were detected in brain tissue of zebrafish by UPLC-HRMS measurements.

Overall, inhibitory effects of waterborne CPF in zebrafish were in line with findings from other studies, indicating impairment of neurotransmitter metabolism, gene transcription and protein levels as well as neuro-behavioral alterations (Richendrfer and Creton, 2015; Gómez-Canela et al., 2017; Özdemir et al., 2018), even though concentrations in previous studies exceeded environmental levels by several orders of magnitude. Whereas, studies with MP-sorbed CPF demonstrated contradictory 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), compared to dissolved CPF (Bellas and Gil, 2020). In comparison, 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 different chemical binding affinities (Horton et al., 2018). The analytical quantification failed to detect measurable concentrations of CPF in the aqueous phase in the present study, proving that CPF did not significantly desorb from PS particles (Annex I, Tab. A2). Most likely, CPF desorbed after ingestion, as CYP450 induction and AChE activity in zebrafish were similarly altered by waterborne CPF and CPF-loaded PS (Fig. 13/14). Since the binding affinity of CPF was lower compared to BkF (log K_{OW} 6.11 and 4.96, respectively), CPF most probably desorbed from the PS particles under altered conditions

(e.g., in the intestine) and was therefore more easily bioavailable (Bejarano et al., 2003), but did not accumulate in zebrafish brain tissue.

With regard to biomarkers, the overall picture is inconsistent, since alterations may be prone to individual variations, like AChE activity, or related to factors other than exposure conditions (Paul-Pont et al., 2016; León et al., 2018; Magara et al., 2018). Previous studies investigated that the developmental stage of the model organism might be of importance. Effects of CPF were pronounced differently in larval and adult zebrafish (Rodríguez-Fuentes et al., 2015), and the time point of exposure at adult stages might as well hamper effects (Sledge et al., 2011), which implies impairments for individual aquatic organisms up to population level. Even though lesions were not evident in our study, adverse effects of MPs can be induced by physical damage through MP ingestion, leading to a modulation of biochemical or enzymatic responses (Avio et al., 2015; Paul-Pont et al., 2016; Magara et al., 2018). As described previously (Koelmans et al., 2016; Besseling et al., 2019; Triebskorn et al., 2019), the contaminant fluxes accumulating in aquatic ecosystems from ingested MPs might be considered negligible. Although the sorption of contaminants on MPs in environmental samples was previously validated (Rochman et al., 2013a; Pannetier et al., 2017), adverse effects on biota were not consistently detected and the overall relevance of MPs to transfer environmental pollutants remains controversial (Magara et al., 2018; Wagner and Lambert, 2018).

III.6 Conclusions

Chronic exposure of zebrafish to MP-sorbed environmental pollutants (e.g., insecticides, PAHs) has been given little consideration so far. In the present study, 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. However, future studies may investigate effects on other developmental stages of zebrafish, closing gaps for risk assessment of MPs. Nevertheless, the vector hypothesis for MPs in aquatic ecosystems may be further rejected as MPs represent an insignificant fraction of potential binding sites for pollutants and are therefore probably irrelevant in aquatic ecosystems. In contrast, when considering the biological effects of nanoparticles originating from MPs, a paradigm shift is likely and requires further investigation.

III.7 Annex I

I.1. Water analysis to determine leaching of benzo(k)fluoranthene (BkF) and chlorpyrifos (CPF) from MPs

Method Parameters

Tab. A1. Solid phase extraction conditions, sample preparation and measuring parameters for benzo(k)fluoranthene (BkF) and chlorpyrifos (CPF) in water samples. *Data were produced jointly with Sven Huppertsberg.*

		Benzo(k)fluoranthene	Chlorpyrifos
Solid phase extraction	**Cartridges**	Chromabond C18ec, 3 ml, 500 mg, Machery-Nagel (Düren, Germany)	OASIS HLB (3cc 60mg), Waters (Eschborn, Germany)
	LOT nr	17.317 (Sorbent 1017/1)	161A38292A
	Conditioning	1 × 3 ml methanol, 1 × 3 ml H_2O (MilliQ)	3 × 2 ml methanol, 4 × 2 ml H_2O (MilliQ)
	Filtration	1 L	β = 10 ng/L = 3 L, β = 100 ng/L = 1 L
	Drying	10 min N_2	30 min N_2
	Elution	3 × 2 ml *n*-hexane, 10 min absorbing, reduction to 1 ml by N_2	3 × 1.5 ml acetone/ethyl acetate (1:1), 10 min absorbing, reduction to 1 ml by N_2
	Filter extraction	30 min ultrasonic-bath in 10 ml *n*-hexane, reduction to 1 ml by N_2	-
GC-MS parameters	**Instrument settings**	GC-MS, 7693 autosampler, GC 7890B, MSD 7977B, Agilent Technologies, Santa Clara, USA	GC-MS, 7693 autosampler, GC 7890B, MSD 7977B, Agilent Technologies, Santa Clara, USA
	Column	Agilent 19091S-433I HP-5ms Inert, 30m × 250 µm × 0.25µm	Agilent HP-5MSI, 30m × 250 µm × 0.25µm
	Injection volume	1 µl	1 µl

Oven program

	Rate [°C/min]	Temperature [°C]	Hold time [min]
Initial		60	2
Ramp 1	40	325	0
Ramp 2	20	325	2
Post run	0	300	10

	Rate [°C/min]	Temperature [°C]	Hold time [min]	Run time [min]
Initial		50	0.75	0.75
Ramp 1	20	120	0	4.25
Ramp 2	15	230	0	77.58
Ramp 3	10	290	0	83.58
Post run		290	10	

Tab. A1. continued

		Benzo(k)fluoranthene	Chlorpyrifos
GC-MS parameters	**Detection**	SIM Phenanthrene (ISTD) m/z 176 (qualifier), m/z 178 (quantifier), Benzo(k)fluoranthene m/z 250 (qualifier), m/z 252 (quantifier)	SIM Atrazine (ISTD) m/z 200 (qualifier), m/z 215 (quantifier), Chlorpyrifos m/z 199 (qualifier), m/z 197 (quantifier)
	LoD/LoQ	≤ 1 ng/ml, ≤ 2.5 ng/ml	≤ 20 ng/ml, ≤ 60 ng/ml
	Calibration	8-point calibration, 0 - 500 ng/ml benzo(k)fluoranthene	6 -point calibration, 0 - 1000 ng/ml chlorpyrifos
	Internal standard	100 ng phenanthrene	100 ng atrazine

Tab. A2. Results of water and filter samples from treatment groups of long-term exposure experiments with benzo(k)fluoranthene-loaded PMMA and chlorpyrifos-loaded PS. Samples were taken two hours after daily water exchange. Results refer to 1 - 3 L of extracted water using solid phase extraction, or approximately 6.4 mg of microplastic separated by filtration from water samples. Not assessed = n. a. Results ± % relative standard deviation, LoD (Limit of Detection) for BkF ≤ 1 ng/ml, and CPF ≤ 20 ng/ml. *Data were produced jointly with Sven Huppertsberg.*

	BkF loaded PMMA		**CPF loaded 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

I.2. Validation of the sorption process of benzo(k)fluoranthene (BkF) and chlorpyrifos (CPF) to MPs using GC-MS

I.2.1 Analytical quantification of benzo(k)fluoranthene sorption to microplastic particles

Instrument settings

Injection Volume: 1 µl

Solvent washes (acetone) 3 × 3 µl pre-injection, 3 × 3 µl post-injection

Injection Type: Standard

Split-Splitless Inlet

Heater 250 °C, Pressure 64 kPa, Septum purge flow 3 ml/min (He)

Temperature MSD Transfer Line 280 °C

Detection

Ion Source Electron ionization (EI), MS Source 230 °C, MS Quadrupole 150 °C, SIM Phenanthrene (ISTD) m/z 176 (qualifier), m/z 178 (quantifier), Benzo(k)fluoranthene m/z 250 (qualifier), m/z 252 (quantifier)

Calibration

12-point calibration was performed between 2.5 - 350 ng/ml, and an evaluation was achieved by an equal weighting of standards. $R^2 = 0.9795$. Limit of Detection (LoD) ≤ 1 ng/ml and Limit of Quantification (LoQ) ≤ 2.5 ng/ml. Quality Control (QC, 100 ng/ml standard) was determined before and after the measurement of the samples and the deviation was smaller than 10 %.

I.2.2 Analytical quantification of chlorpyrifos sorption to microplastic particles

Instrument settings

Injection Volume: 1 µl

Solvent washes (acetone) 3 × 3 µl pre-injection, 3 × 3 µl post-injection

Injection Type: Standard

Split-Splitless Inlet

Heater 250 °C, Pressure 60 kPa, Septum purge flow 3 ml/min (He)

Temperature MSD Transfer Line 280 °C

Detection

Ion Source Electron ionization (EI), MS Source 230 °C, MS Quadrupole 150 °C, SIM Atrazine (ISTD) m/z 200 (qualifier), m/z 215 (quantifier), Chlorpyrifos m/z 199 (qualifier), m/z 197 (quantifier)

Calibration

5-point calibration was performed between 5 - 50 ng/ml, evaluation was achieved by an equal weighting of standards. R^2 = 0.9986. Limit of Detection (LoD) ≤ 1.5 ng/ml and Limit of Quantification (LoQ) ≤ 5.0 ng/ml. Quality Control (QC, 100 ng/ml standard) was determined before and after the measurement of the samples and the deviation was smaller than 10 %.

I.2.3 Particle size distribution of both microplastic particle types applied in experiments

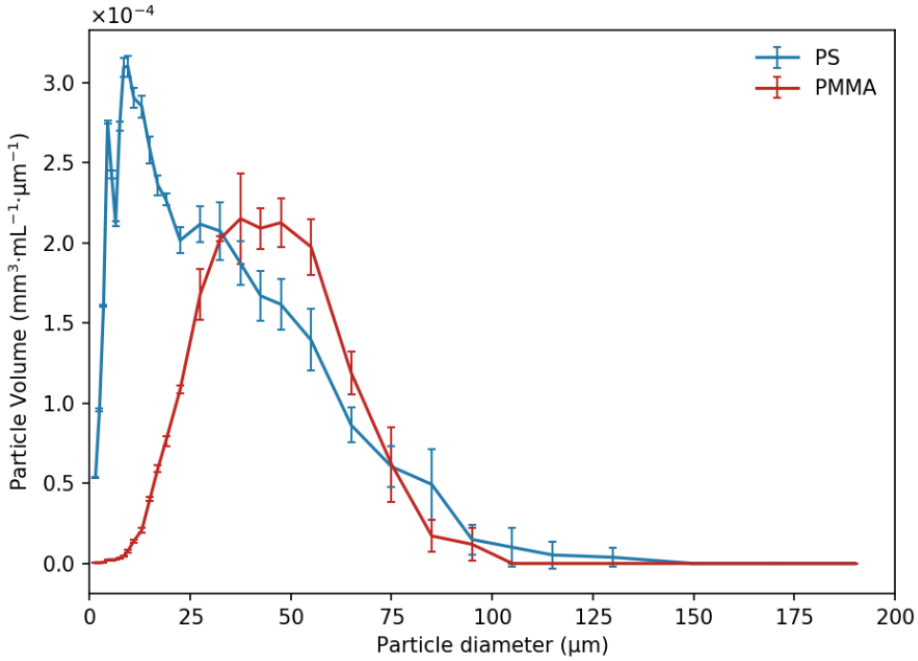

Fig. A1. Blank value corrected particle size distribution of PMMA suspension (n = 3) and PS suspension (n = 6) used in experiments. Spherical PMMA in isopropanol (20 %), irregular PS in Tween (2 mg/L), measured with a particle counter (SVSS, PAMAS, Rutesheim, Germany). *Data were produced jointly with Stefan Dittmar*

I.3. Fluorescence tracking in gastrointestinal cryosections of zebrafish with image data analysis using FIJI

Image Acquisition details for gastrointestinal tract sections

HV: 18, Offset: 0, Laser power: 45, Overlap: 10 %, Pinhole: 3.1 AU, Optical resolution 0.23 µm, Scan speed: ¼, Scan size: 1024

FIJI Plugins used and parameters

Grid/Collection stitching for stitching of single 10 × images to complete image of the intestinal tract with overlap: 5 %, fusion method: linear blending, default parameters (Preibisch et al., 2009).

I.4. UPLC-FLD parameters for determination of benzo(k)fluoranthene (BkF) and BkF transformation products in zebrafish liver

Calibration

6-point calibration was performed between 0.1 - 10 ng/ml BkF, matrix effects were excluded by standard addition of BkF and recovery measurements, LoD ≤ 0.01 ng/ml, LoQ ≤ 0.03 ng/ml

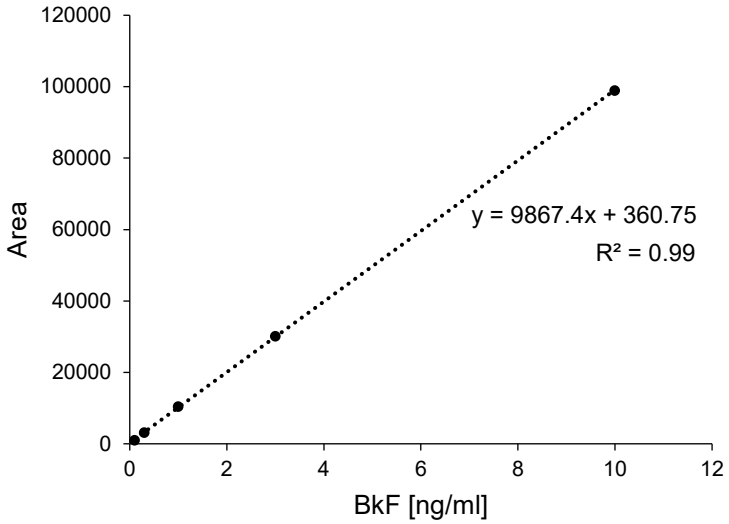

Fig. A2. Calibration curve for benzo(k)fluoranthene measurements *via* UPLC-FLD (ACQUITY UPLC, Xevo XS, HSS T3 column, Waters, Eschborn, Germany). *Data were produced jointly with Bettina Seiwert.*

IV. Microplastic-associated trophic transfer of benzo(k)fluoranthene in a limnic food web: Effects in two freshwater invertebrates (*Daphnia magna, Chironomus riparius*) and zebrafish (*Danio rerio*)

This chapter is based on a manuscript written entirely by me and was recently published in Comparative Biochemistry and Physiology – Part C: Toxicology and Pharmacology (DOI: 10.1016/j.cbpc.2020. 108849) and includes content, which was created jointly with Carmen Sommer, Stefan Dittmar and Sven Huppertsberg. The corresponding figures are marked. Carmen Sommer conducted trophic transfer experiments and CLSM measurements under my supervision. Sven Huppertsberg conducted the analytical quantification of BkF and CPF, and Stefan Dittmar measured the particle recovery and distribution. However, sample preparation, EROD experiments, analytical quantification of invertebrates using GC-MS and data analysis were conducted entirely by me.

IV.1 Abstract

The continuously growing plastic production and incomplete recycling processes open manifold entry routes for microplastic particles (MPs) into the environment. Since knowledge on trophic transfer of contaminants sorbed to MPs is still insufficient for freshwater systems, the transfer of the model pollutant benzo(k)fluoranthene (BkF) sorbed to polymethyl methacrylate (PMMA) particles in a limnic food web was investigated: Two freshwater invertebrates (*Daphnia magna* and *Chironomus riparius* larvae) were selected and either left untreated, exposed to pristine PMMA, PMMA-associated BkF, or exposed to dissolved BkF (BkF_{AQ}). As second-level consumers, zebrafish (*Danio rerio*) were fed twice daily with pre-treated invertebrates over two days. Induction of hepatic cytochrome P450 by BkF was determined as 7-ethoxy-*O*-resorufin deethylase (EROD) activity. Both invertebrate species readily ingested PMMA particles, tracked *via* fluorescence microscopy and accumulated BkF_{AQ}, measured *via* GC-MS. Fluorescence signals in gastrointestinal tracts of zebrafish were quantified with confocal laser scanning microscopy (CLSM). The fluorescence signal in gastrointestinal tracts of zebrafish was not altered, whereas, EROD activity was significantly induced when zebrafish were fed with *Chironomus riparius*, pre-exposed to BkF_{AQ}. Trophic exposure scenarios with BkF sorbed to PMMA did not result in any alterations of investigated endpoints in both invertebrate species and zebrafish compared to controls. Given that BkF amounts were in the low ng-range, as detected by GC-MS, the transport of BkF sorbed to MPs to zebrafish was less effective than direct exposure to waterborne BkF_{AQ}, and the potential threat of trophic transfer of substances such as BkF in limnic food webs may have been overestimated.

IV.2 Introduction

Microplastics (MPs) are plastic particles in a size range of 1 µm to 1 mm (Hartmann et al., 2019) and have become a global concern since they have been detected ubiquitously in the environment and enter rivers and oceans through atmospheric transport, rain, erosion, surface runoff and effluent from wastewater treatment plants. Exponentially increasing plastic production and deficient waste management lead to millions of tons of plastic debris (Jambeck et al., 2015; Geyer et al., 2017; PlasticsEurope, 2018) being discharged as macro- or microplastics into aquatic systems (GESAMP, 2015). Plastic debris accumulation affects terrestrial as well as marine and limnic ecosystems (Barnes et al., 2009; Dris et al., 2015). In aquatic systems, the useful properties of MPs, namely its low weight and longevity, become a major problem as they increase mobility, accumulation, and persistence due to slow and only partial degradation (Barnes et al., 2009; Eubeler et al., 2010; Scherer et al., 2017).

Although recent monitoring studies revealed that MPs could be found universally in different freshwater compartments (Klein et al., 2015; Mani et al., 2015), the number of microplastic studies associated with freshwater ecosystems accounts for less than 4 % of recent scientific publications on MPs (Wagner and Lambert, 2018). Due to their small size and variable properties, MPs are available to a wide range of organisms and can enter the food web. The uptake of MPs has already been documented for various marine and limnic invertebrates and vertebrates (Cole et al., 2013, 2016; Mazurais et al., 2015; Batel et al., 2016, 2018; Ogonowski et al., 2016; Beiras et al., 2018; Bour et al., 2018). This can result in adverse effects such as disturbed food intake (Cole et al., 2011; Besseling et al., 2013; Setälä et al., 2014), translocation of MPs into various tissues (Browne et al., 2008; Avio et al., 2015), inflammation or signs of stress (Oliveira et al., 2013; Rochman et al., 2013b; Lu et al., 2016) or behavioral changes (Tosetto et al., 2016; Rochman et al., 2017).

Especially aquatic invertebrate species such as water flea (*Daphnia magna*) neonates and benthic larvae of the non-biting midge (*Chironomus riparius*) are important species of the freshwater food web as they are valuable prey for vertebrates (fish) at higher trophic levels and may thereby transfer hazardous organic compounds (HOCs) or additives associated with MPs (Lee et al., 2009; Khosrovyan and Kahru, 2020). Detection of MPs in the digestive tract of wild-caught fish (Lusher et al., 2013; Neves et al., 2015) and results from laboratory studies (Cole et al., 2013; Farrell and Nelson, 2013; Setälä et al., 2014) demonstrated that MPs could be ingested from algae as well as macrozooplankton and suggest transfer across trophic levels. However, the polymer particles themselves may not be the only risk to aquatic organisms: Pollutants sorbed from the aquatic environment (e.g., polycyclic aromatic hydrocarbons, PAHs) or substances introduced on purpose during the production process (e.g., monomers, plasticizers, dyes, flame retardants) might pose an additional risk (Teuten et al., 2009).

Since MPs provide additional sorption surfaces for hazardous organic compounds (HOCs), beside naturally occurring (in-)organic particles, they increase the risk for alternative routes of uptake through ingestion by different aquatic organisms. Given that the amount of MPs in aquatic environments is

expected to rise further (GESAMP, 2015), there is a need to investigate interactions between environmentally hazardous contaminants (like PAHs) and MPs (Wagner and Lambert, 2018; Franzellitti et al., 2019; Triebskorn et al., 2019).

Ubiquitous contamination of aquatic environments with PAHs from anthropogenic sources is a known problem as well as their capacity to bioconcentrate (Whyte et al., 2000; Berrojalbiz et al., 2009; Teuten et al., 2009). Adsorption of PAHs to MPs has already been confirmed and has the potential to transfer contaminants to aquatic organisms with partially deleterious effects (Rochman et al., 2013a; Pannetier et al., 2020). In aquatic ecosystems, benzo(k)fluoranthene (BkF) occurs in quantifiable amounts (2 - 25 ng/g plastic) sorbed to MPs (Rochman et al., 2013a; Pannetier et al., 2019a). The potential of MPs to transport sorbed pollutants has frequently been investigated but remains unclear (Teuten et al., 2007; Gouin et al., 2011; Rochman, 2013; Koelmans et al., 2016). Although vector effects of MPs to transfer sorbed contaminants across several trophic levels are no longer undisputed, these are still insufficiently documented. The activity of the biotransformation enzyme 7-ethoxy-resorufin-*O*-deethylase (EROD) in liver homogenates is a sensitive endpoint to illustrate changes in metabolic activities since the trophic transfer of BkF might cause toxic effects in zebrafish (Whyte et al., 2000).

Although various types of plastics are produced globally, the market is dominated by a few classes, namely polyethylene, polypropylene and polystyrene (GESAMP, 2015; PlasticsEurope, 2018), and thus, these are the most abundant polymers found in the environment (Andrady, 2011; Hidalgo-Ruz et al., 2012; Wagner et al., 2014). So far, the binding capacity of polymethyl methacrylate (PMMA) has not been investigated, as PMMA is less abundant in the environment if compared to polyethylene, polypropylene and polystyrene (Geyer et al., 2017). However, PMMA is an important environmental pollutant due to its wide application spectra in automotive and computer industries (PlasticsEurope, 2018). PMMA concentrations may have been underestimated in the aquatic environment, since polymethyl methacrylate and polycarbonates are prone to degradation processes, when natural debris is chemically and thermally removed from environmental samples (Wagner and Lambert, 2018).

Therefore, the present study was designed to assess the combined effects of the model PAH BkF sorbed to PMMA particles along with two trophic levels of a simplified limnic food web, consisting of zooplankton (*Daphnia magna*) and sediment-dwelling invertebrate (*Chironomus riparius* larvae) and zebrafish (*Danio rerio*) as high-level predator. To determine the amounts of BkF transferred to each compartment of the food web, tracking by means of fluorescence microscopy was combined with chemical analysis by GC-MS. In addition, BkF uptake by zebrafish (*Danio rerio*) was quantified by fluorescence measurements in cryosections of the gastrointestinal tract and related to the induction of cytochrome P450 in the liver (EROD assay).

IV.3 Materials and methods

IV.3.1 Materials and chemicals

For all experiments, unstained spherical PMMA beads with a mean diameter of 48 µm, and a density of 1.19 g/cm³ (Goodfellow Cambridge Ltd., Huntingdon, England) were used at an initial concentration of 1×10^6 particles/L (0.05 g/L). All chemical reagents were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany) and Roth (Karlsruhe, Germany) unless stated otherwise. Measurement of protein contents was carried out with the DC™ Protein Assay kit from Bio-Rad (Dreieich, Germany). For BkF, an 8 mM stock solution was prepared in DMSO, in a pre-saturated glass vial, covered in aluminum foil, and stored at 4 °C. All other solutions were prepared immediately before use.

IV.3.2 Animal husbandry

IV.3.2.1 Invertebrate organisms (*Daphnia magna, Chironomus riparius*)

Water flea neonates (*Daphnia magna*, 5 - 6 days) and larvae from the harlequin fly (*Chironomus riparius,* 2nd - 3rd larval stage) were obtained from the Department of Aquatic Ecotoxicology, University of Frankfurt/Main (Germany). Daphnids were cultured in 1 L glass beakers in Elendt M4 medium (Elendt, 1990) at 20 °C at a 16:8 h light:dark cycle and fed with *Scenedesmus obliquus* according to OECD TG 211 (OECD, 2012). Clutches of chironomids were reared in glass beakers with Elendt M4 medium at 20 °C under a constant 14:10 h light:dark cycle. Hatched larvae were fed with grounded TetraMin™ flakes (Tetra, Melle, Germany) in accordance with OECD TG 218 (OECD, 2004).

IV.3.2.2 Zebrafish (*Danio rerio*)

Adult wild-type zebrafish (*Danio rerio*) aged 16 - 24 months from the "Westaquarium" strain were obtained from the fish facility at the Aquatic Ecology and Toxicology Group (Center for Organismal Studies, University of Heidelberg, Germany, licensed by regional animal welfare authorities under 35-9185.64/BH Braunbeck). Animal experimentation with zebrafish was licensed under reference 35-9185.81/G-122/15. Fish were kept in 5 L glass aquaria at 25 ± 1 °C, at a 14:10 h light:dark cycle, with constant aeration and 5-fold water exchange per day. Continuous flow-through conditions guaranteed that nitrate, nitrite, and ammonia concentrations were kept below detection limits to ensure optimal maintenance conditions (Matthews et al., 2002; Lawrence, 2011). Zebrafish in the daphnid food web experiment were 0.27 ± 0.05 g in wet weight (ww) and 3.30 ± 0.24 cm in total body length (snout to tail tip). The fish in the chironomid food web experiments were of comparable weight and size (0.26 ± 0.05 g and 3.14 ± 0.16 cm).

IV.3.3 Loading of MPs with benzo(k)fluoranthene and determination of particle recovery

For the exposure of the invertebrate organisms to particle-associated BkF, a suspension of 1×10^6 PMMA particles/L and 400 µM BkF (from 8 mM BkF stock) in 20 ml Aqua bidest with a final DMSO concentration of 0.005 % was incubated at room temperature overnight on a Certomat® S-II orbital shaker at 100 rpm (Sartorius Stedim Biotech, Goettingen, Germany). After the incubation, all suspensions were filtered over a 0.2 µm Whatman Puradisc cellulose acetate filter (GE Healthcare, Solingen, Germany) using a 10 ml BD Discardit™ II syringe (Becton & Dickinson, Heidelberg, Germany). After washing with 4 × 10 ml Aqua bidest, BkF-loaded PMMA particles were resuspended by backwashing the filter with 20 ml Elendt M4 medium. Negative controls with pristine PMMA were treated likewise. Given concentrations always refer to nominal values if not stated otherwise.

IV.3.3.1 Analytical quantification of benzo(k)fluoranthene sorption to PMMA particles

GC-MS was used to quantify the sorption of BkF to PMMA particles. Aqueous phases and particle suspensions of all steps (incubation, washing, resuspension) were collected and measured separately in triplicates. Erlenmeyer flasks for PMMA incubation were pre-incubated overnight with 200 µM BkF solution to minimize sorption loss. Phenanthrene was used as an internal standard. Samples were extracted by adding 1.1 ml *n*-hexane, then vortexed for 2 min and frozen to facilitate phase separation. The extraction solvent was filtered through 0.2 µm syringe filters and transferred to glass vials for GC-MS measurements (GC 7890B, column 19091S-433 I HP, MSD 5977B, carrier gas helium, Agilent Technologies, Santa Clara, USA). For detailed information on GC-MS parameters, see Annex II.1.

IV.3.3.2 PMMA particle recovery after filtration

Particle recovery during filtration and resuspension of PMMA particles was analyzed according to Eitzen et al. (2019), using a small volume syringe system particle counter (SVSS, PAMAS, Rutesheim, Germany). Particles were measured by light extinction with a laser-diode sensor in a size range of 5 to 200 µm. In order to minimize the number of background particles, initial PMMA suspensions (0.05 g/L) were prepared with ultra-pure water (resistivity > 17 MΩ·cm, ELGA Berkefeld LabWater, Veolia Water Technologies, Celle, Germany). Laboratory glassware was cleaned with distilled water, rinsed twice using ultra-pure water with an intermediate ultrasound treatment step (10 min).

Four replicate measurements were performed to determine particle recovery. For each measurement, 75 ml of initial PMMA suspension was placed in a glass beaker and measured immediately. Subsequently, 5 × 10 ml of the suspension was filtered over an 0.2 µm cellulose acetate filter (reversely pre-rinsed with 2 × 10 ml of ultra-pure water) before the filter was backwashed with 5 × 10 ml of Elendt M4

medium. The resuspension was collected in a glass beaker and instantly measured. To account for background particles, three blanks were prepared accordingly, using ultra-pure water instead of initial PMMA suspension.

IV.3.4 Trophic transfer of PMMA particles

IV.3.4.1 Exposure of *Daphnia magna* and *Chironomus riparius* to PMMA particles

Over the experimental period of 48 hours, zebrafish were fed twice daily either (un-)treated primary consumers or a commercial diet (negative control, TetraMin™ flakes). Overall, for loading invertebrates with MPs, 40 daphnids per zebrafish per day and 80 chironomid larvae per zebrafish per day were incubated for 24 h in advance for each exposure scenario. Untreated invertebrates reared in Elendt M4 medium served as a negative control. Three different exposure scenarios were constructed to expose the pelagic daphnids and benthic chironomid larvae under similar conditions: (**1**) Invertebrates were directly incubated in dissolved 400 µM BkF. (**2**) Invertebrates were exposed to pristine PMMA (1×10^6 particles/L). (**3**) Exposure of invertebrates to PMMA (1×10^6 particles/L), loaded with 400 µM BkF (nominal concentration).

All pre-treatments were carried out in glass beakers (25 ml) with 20 ml Elendt M4 medium covered with a glass lid to prevent airborne particle input and to ensure optimum conditions for particle uptake by chironomids and daphnids. Exposure of invertebrates over 24 h was carried out in two consecutive days, starting one day prior to the beginning of the experiment. Before transfer to fish tanks, specimens were thoroughly rinsed with clean water to remove excess particles and avoid the transfer of waterborne BkF.

For fluorescence measurements, an additional 14 individuals per treatment of each species (per experiment) were imaged with a Nikon Eclipse 90i epifluorescence microscope in brightfield and fluorescence images (4 × magnification, excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm, Nikon, Duesseldorf, Germany) and analyzed with FIJI v.1.52p (Schindelin et al., 2012) to determine the uptake of PMMA, BkF_{AQ} and PMMA-associated BkF after 24 h exposure. To quantify the transferred amount of BkF, 10 individuals of *Daphnia magna* and 40 *Chironomus riparius* larvae were washed and sampled. Excess water was removed, and duplicate samples were frozen immediately in liquid nitrogen for analysis with GC-MS.

IV.3.4.2 Experimental setup for food web experiments

The experimental groups and the tank setup were identical for the two different food web experiments (see Fig. 16). Static water renewal (50 % daily exchange) was carried out for the positive control groups with 400 µM BkF dissolved in water with a final DMSO concentration of 0.005 %. To this end, the first setup consisted of daphnids as primary consumers, which were fed to zebrafish, and the second setup

consisted of chironomid larvae as primary consumers. For animal welfare reasons, the two different food web experiments with *Daphnia magna* and *Chironomus riparius* as primary consumers and *Danio rerio* as secondary consumers were conducted in two independent replicates. Each experimental group consisted of three randomly allocated zebrafish, a total of 72 zebrafish.

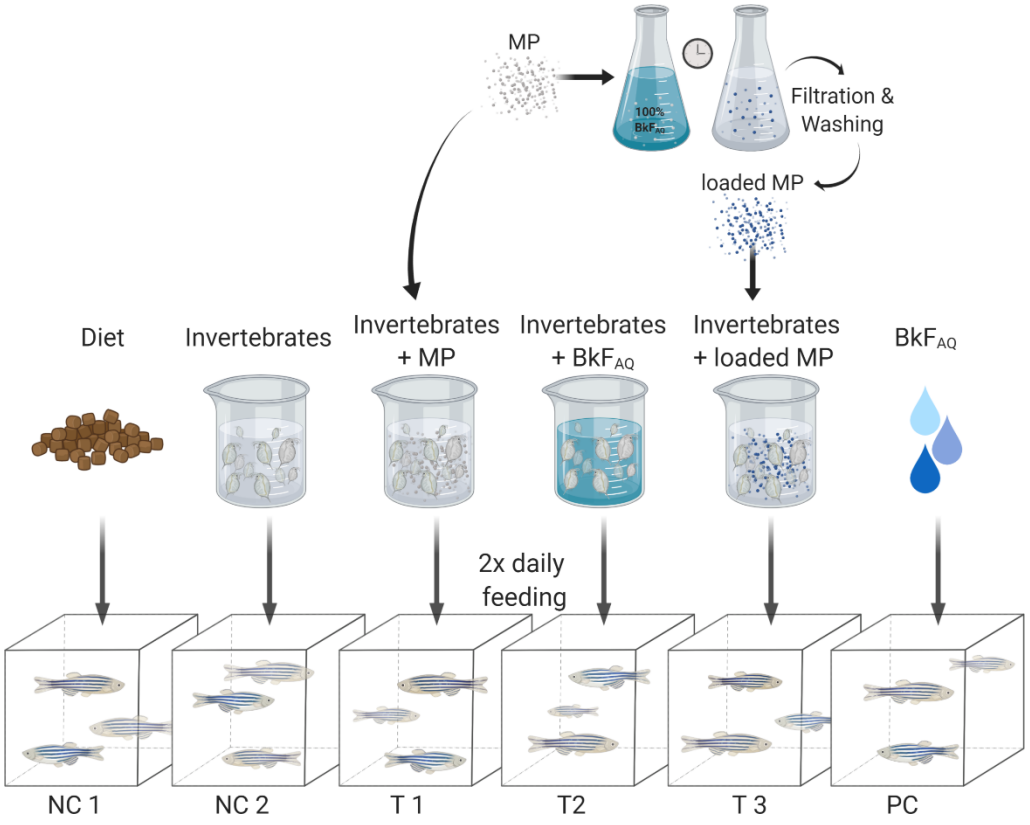

Fig. 16. Experimental setup for food web experiments with invertebrates (*Daphnia magna* and *Chironomus riparius*) and zebrafish to assess possible vector effects of MPs by transport of MP-sorbed benzo(k)fluoranthene.

After loading of PMMA particles with BkF and subsequent exposure of invertebrate organisms to BkF-sorbed MPs followed, and subsequently, trophic transfer experiments were conducted. Therefore, depending on the treatment group, zebrafish were fed with differently exposed invertebrates or untreated daphnids or chironomid larvae, or a commercial diet (1.00 ± 0.06 % wet weight per zebrafish per day). Thus, each experimental setup consisted of two negative controls in which zebrafish were either fed a commercial diet (negative control 1) or untreated daphnids/chironomid larvae (negative control 2), one positive control (400 µM waterborne BkF_{AQ}, PC) and three different exposure groups: Individual zebrafish were either fed with (**1**) daphnids/chironomid larvae pre-exposed to pristine PMMA (treatment 1), (**2**) daphnids/chironomid larvae incubated with waterborne 400 µM BkF (treatment 2) or (**3**) daphnids/chironomid larvae pre-exposed to MPs loaded with BkF (treatment 3). Particular care was taken, that each zebrafish was fed with the designated number of invertebrates, by adding the washed specimen with a pipette to the corresponding tanks.

To guarantee for comparable feed quantities in the different exposure scenarios, literature data for dry weights of *Daphnia magna* (Skjolding et al., 2014) and *Chironomus riparius* (Lee et al., 2009) were used. The total dry weight of daphnids fed to one zebrafish per day added up to approximately 1.5 % of wet weight, whereas the dry weight of 80 chironomid larvae added up to approximately 2.4 % of wet weight per zebrafish per day.

IV.3.4.3 Sampling of zebrafish for intestinal tract cryosectioning and hepatic EROD activity determination

According to Jönsson et al. (2009), CYP1A induction in zebrafish is maximal after 24 h of incubation to waterborne PAHs; thus, the exposure period was extended, since zebrafish were exposed to lower amounts BkF either sorbed to MPs or to daphnids/chironomid larvae. To prevent contamination by excess food, all fish were allowed to incubate in clean water without feeding for another 16 hours after two days of continuous feeding on BkF-loaded invertebrates. Fish were sacrificed for analysis of the fluorescence signal of BkF in the gastrointestinal tract (GI) and for the indirect measurement of CYP1A induction *via* hepatic EROD assay (modified from Batel et al. (2016). After euthanasia with 1.5 M tricaine (MS 222) and decapitation, the whole gastrointestinal (GI) tract was dissected, immersed in modified Davidson's fixative (Mulisch and Welsch, 2015) and stored at 4 °C until the specimen was subsided (3 - 5 days) prior to embedding for cryosectioning. Liver samples for ethoxyresorufin-*O*-deethylase (EROD) assay were rapidly dissected, frozen immediately in liquid nitrogen, and stored at -80 °C until further analysis.

IV.3.5 Cryosectioning and confocal laser scanning microscopy

Particle-associated transfer and possible accumulation of BkF within the intestine of zebrafish was investigated in unstained cryosections of the gastrointestinal tract, using a Nikon Eclipse 90i/C1 confocal laser scanning microscope (CLSM), since BkF emits fluorescence at 403 nm (Rivera-Figueroa et al., 2004). Cryosectioning was preferred over paraffin embedding to avoid extraction of BkF by a graded dehydration series of ethanol. After fixation, tissue samples were dehydrated (3 × 5 min in 1 × phosphate-buffered saline) and incubated in 10 % sucrose solution in PBS for 50 min at room temperature. After overnight incubation in 30 % sucrose solution in PBS at 4 °C, samples were transferred for 4 hours at room temperature to a 1:1 mixture of 30 % sucrose solution and Tissue Freezing Medium (Leica Biosystems, Germany) to prevent tearing of the intestinal cavity during the sectioning. Samples were embedded and frozen in Leica Tissue Freezing Medium and 20 µm sagittal sections were cut at -20 °C, using a Leica CM 3050 S cryostat (Leica Biosystems, Nussloch, Germany). Sections were transferred to Superfrost Plus™ Adhesion Microscope Slides (Thermo Fisher Scientific, Wiesbaden, Germany), left unstained and stored for a maximum of seven days at 4 °C until microscopic analysis.

According to Rivera-Figueroa et al. (2004), the fluorescence signal of BkF can be detected down to 0.16 nM. Fluorescence was recorded in optical sections in individual confocal images (λ_{EX} = 405 nm, λ_{EM} = 450 nm) together with a corresponding brightfield image. All cryosections from a given experiment were imaged with consistent settings at 10 × magnification and processed with FIJI (Preibisch et al., 2009; Schindelin et al., 2012). Fluorescence signals were quantified in stitched images within intestinal tissue as mean pixel intensities (sum of foreground intensities/number of foreground pixels) above the background pixel signal. The possible presence of liver tissue, chyme, or fluorescent particles within the chyme was excluded from the analysis (see Annex II.2 for details).

IV.3.6 GC-MS analysis of invertebrate organisms

In order to determine the uptake of (sorbed) BkF by invertebrate organisms and to track the possible trophic transfer to zebrafish, analytical GC-MS measurements were conducted. Therefore, duplicates of daphnids (10 individuals/sample) and chironomid larvae (40 individuals/sample) exposed for 24 h to either 400 µM waterborne BkF_{AQ}, pristine PMMA particles or particles loaded with BkF, were extracted as described by Kurth et al. (2017): Samples were homogenized in 600 µl acetonitrile with a TissueLyser II (30 Hz, 1 min, Qiagen, Hilden, Germany), and 7 mg NaCl and 28 mg of anhydrous $MgSO_4$ were added for an integrated liquid-liquid extraction to facilitate the extraction of BkF into the hydrophobic acetonitrile phase. Mixing and phase separation were enhanced by vortexing for 1 min and centrifuging the samples for 10 min at 4000 rpm using a Heraeus Multifuge 1 S-R (Thermo Scientific, Schwerte, Germany). Eventually, 400 µl of the acetonitrile phase was transferred into a new glass vial. As final internal BkF concentrations were unknown, an external five-point calibration for GC-MS measurements (10 - 100 ng/ml) was carried out separately for *Daphnia magna* and *Chironomus riparius* in BkF-free samples (for calibration data and detailed GC-MS settings, please refer to Annex II.4).

Only minute amounts of BkF might have derived from leftover PMMA particles in the intestine of chironomid larvae and daphnids since visual fluorescence detection documented that BkF was dispersed throughout invertebrate tissue (see results for details). GC-MS measurements were conducted with 10 µl injection volume (QP2010 SE, Shimadzu Scientific Instruments, Duisburg, Germany, column HP5 MS, Agilent technologies, carrier gas: helium), and results were evaluated with GCMSsolution software (Shimadzu).

IV.3.7 Ethoxy-resorufin-*O*-deethylase (EROD) assay

Frozen liver samples of all treatments were homogenized for 30 s at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany) in 800 µl ice-cold extraction buffer (15 mM $NaH_2PO_4 \times H_2O$, 68 mM $Na_2HPO_4 \times 2\ H_2O$, 1.2 mM $MgCl_2^- \times 6\ H_2O$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol, pH 7.4) based on protocols by Örn et al. (1998) and Batel et al. (2016). Samples were measured in

triplicates and emitted resorufin signals were compared to a resorufin standard curve (0.156 - 5 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). Phosphate buffer as blank (250 µl), duplicate wells of resorufin, and homogenized samples in triplicates (50 µl) were added to black 96-well microtiter plates (Brand, Wertheim, Germany). The enzymatic reaction was started by adding 100 µl of a mix of 6 µM 7-ethoxyresorufin and 10 µM dicoumarol (2.5 mM stock in DMSO), followed by 100 µl of 0.8 mM NADPH (in phosphate buffer). EROD activity was measured in crude lysates over 20 min 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, using 0.125 - 2 mg/ml bovine serum albumin in phosphate buffer as standard. Fluorescence was measured at 690 nm (GENios plate reader) after 20 min incubation at room temperature. 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).

IV.3.8 Data analysis

For statistical evaluation, data were tested for normality and equal variances. To identify statistically significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), Mann-Whitney U test was used for pairwise comparison. Comparing different treatment groups versus a control group, either parametric ANOVA followed by Holm-Sidak *post-hoc* test or nonparametric Kruskal-Wallis analysis followed by Dunn's *post-hoc* test were performed using SigmaPlot 13 (Systat Software, Erkrath, Germany). Results were expressed as mean values ± standard deviation.

IV.4 Results

IV.4.1 PMMA loading with benzo(k)fluoranthene and particle recovery

The quantification of the final amount of BkF sorbed to MP particles is crucial to assess possible effects in all organisms of the trophic food web experiments. Measurements with GC-MS documented that up to 80 % of BkF was sorbed to PMMA particles, and less than 10 % remained in the aqueous phase after 24 h of incubation. Extraction of the sample containers showed that approximately 10 % of BkF was sorbed to glass surfaces and was, therefore, not available for sorption to MP. Up to 20 % of BkF has leached from BkF-loaded particles over a 24 h incubation period in clean water and might thus have been available for direct uptake without particle-mediated transfer (Tab. 7).

Tab. 7. Sorption of benzo(k)fluoranthene to PMMA particles determined by GC-MS. The amount of BkF sorbed to MP was measured in *n*-hexane extracted PMMA particles. Residues of BkF and desorption of BkF from loaded particles were measured directly in the aqueous phase after filtration. Water from washing steps was measured in the aqueous phase. Sample containers were extracted after incubation of PMMA suspensions. Data are given as means ± SD (n = 3). *Copyright © Elsevier 2020. Data were produced jointly with Sven Huppertsberg.*

	Nominal concentration	PMMA	Aqueous phase	Desorption (24h)	Washing water	Sample container
[µM]	200	127.2 ± 32.6	16.9 ± 0.8	29.8 ± 13.4	9.6 ± 1.3	22.8 ± 1.1
[%]	100	63.6 ± 16.3	8.5 ± 0.2	14.9 ± 6.7	4.8 ± 0.6	11.4 ± 0.6

In addition to the analytical quantification of BkF, a possible particle loss during necessary filtration when preparing BkF-loaded microplastic suspensions was investigated using a particle counting system (SVSS, PAMAS) in four replicate measurements. Background particle numbers were determined from blanks for both initial PMMA suspensions (< 1 %) and resuspensions (< 10 % in total) and subtracted from each measurement. With respect to particles counted over the entire measuring range of 5 to 200 µm, 38.7 ± 4.3 % of the PMMA particles were recovered in the resuspension (Fig. A3 A). Since all treatments, including the control with pristine PMMA, were treated alike, particle numbers were similarly reduced in all MP exposure scenarios. Deviations occurred for different size fractions. Especially the recovery of larger particles (< 30 µm) showed larger variation due to a lower number of particles detected over a wide size range (Fig. A3 B). Nonetheless, it could be demonstrated that the method used for loading PMMA particles with dissolved BkF and filtering the solution to obtain only BkF-loaded particles was successful and reproducible.

IV.4.2 Particle uptake and benzo(k)fluoranthene transfer in invertebrate organisms

During the 24 h incubation period, *Daphnia magna* and *Chironomus riparius* readily ingested PMMA particles, which could immediately be detected under the microscope at 10 × magnification (Fig. 17). Preliminary tests detected only faint fluorescence within the invertebrates after 2 - 12 h exposure (see Fig. A2). A clear fluorescence signal of BkF at 403 nm was visible after 24 h of exposure, which was most likely due re-uptake of egested particles. Overall, more than 80 % of daphnids showed ingested PMMA particles in the gut, whereas in chironomid larvae over 60 % ingested MP. Even though the spiking process of PMMA particles led to a decrease in the initial number of particles, enough PMMA spheres were thus readily accessible for the invertebrates. During microscopy of the individuals, no egestion of particles could be detected. Nevertheless, sampling, handling and washing of the organisms might have led to the excretion of particles. Thus, the particle numbers determined might be a snapshot over an extended period of ingestion and egestion.

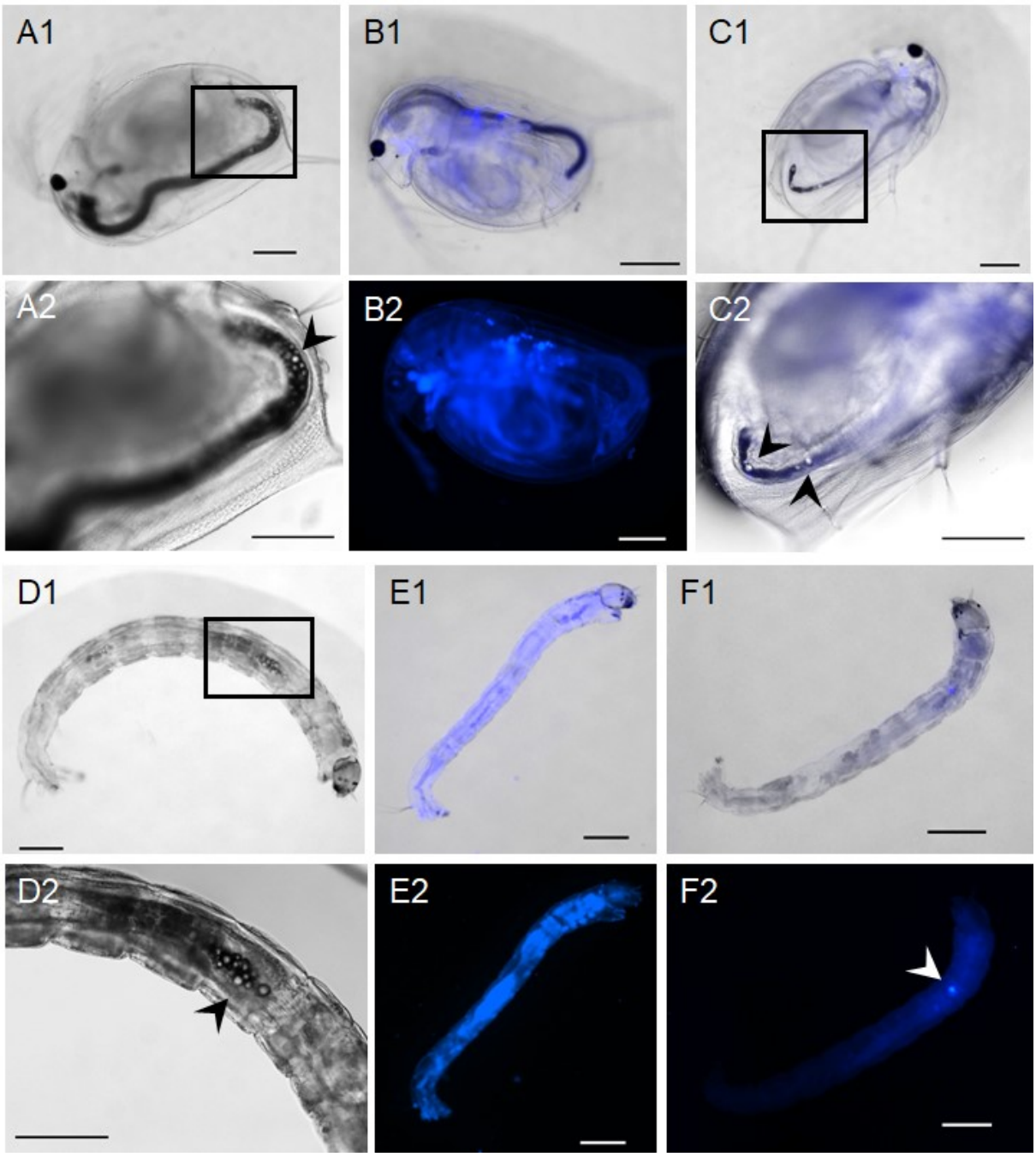

Fig. 17. Particle uptake and transfer of BkF in *Daphnia magna* (**A - C**) and *Chironomus riparius* (**D - F**). (**A1**/**A2**) PMMA-particles (arrowheads) in the gut of *Daphnia magna*. (**B1**/**B2**) Fluorescence signal after exposure to 400 µM BkF_{AQ} (B2: exposure time 200 ms). (**C1**/**C2**) Fluorescence signal after exposure to BkF-loaded PMMA. (**D1**/**D2**) PMMA-particles (arrowheads) in the gut of *Chironomus riparius*. (**E1**/**E2**) Fluorescence signal after exposure to 400 µM BkF_{AQ} (E2: exposure time 2 s). (**F1**/**F2**) Fluorescence signal after exposure to BkF-loaded PMMA (F2: exposure time 2 s). Brightfield and fluorescence image (excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm, Nikon Eclipse 90i), Scale bars 250 µm.. *Copyright © Elsevier 2020. Data were produced jointly with Carmen Sommer.*

Fluorescence measurements in *Daphnia magna* showed a similar pattern of BkF accumulation, as no significant differences could be detected between the control group and BkF sorbed to PMMA (Fig. 18). Uptake of BkF from the ambient medium was maximal ($p < 0.001$) upon direct exposure to waterborne BkF_{AQ}. In contrast, *Chironomus riparius* larvae accumulated significant amounts of BkF ($p < 0.05$) from

BkF-loaded PMMA in comparison to *Daphnia magna*, where no uptake of BkF sorbed to PMMA could be detected (Fig. 18). Only minute amounts of BkF might have derived from leftover PMMA particles in the intestine of chironomid larvae and daphnids since visual fluorescence detection documented that BkF was dispersed throughout invertebrate tissue. Control daphnids and chironomids only expressed a slight background fluorescence, which was not altered by the ingestion of pristine PMMA particles. Findings were corroborated by GC-MS measurements; no background contamination with BkF was present in both invertebrate species.

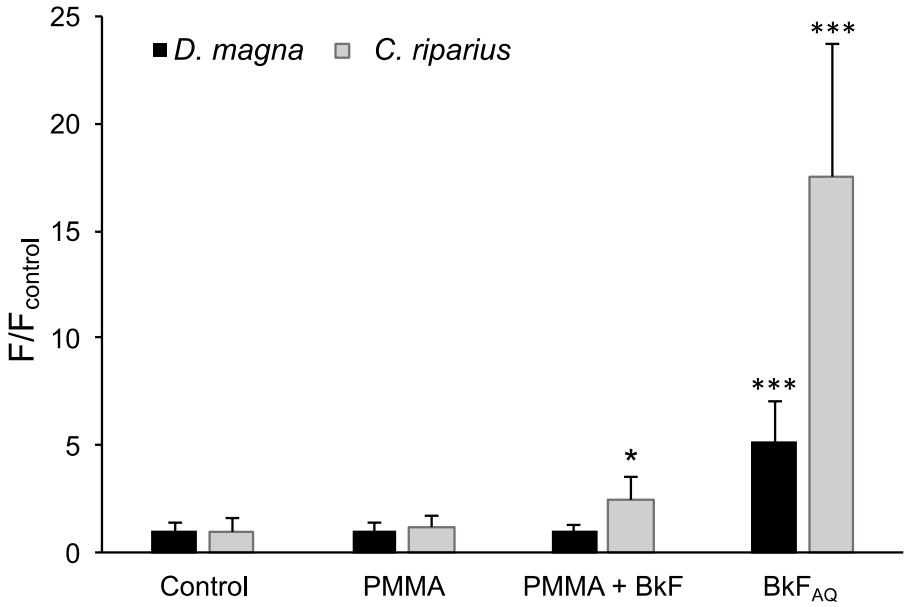

Fig. 18. Fluorescence signal of BkF in invertebrate organisms after 24 h of exposure to either Elendt M4 medium (Control), Elendt M4 medium with pristine PMMA (PMMA), BkF-loaded PMMA (PMMA + BkF) or dissolved BkF (BkF_{AQ} in Elendt M4 medium. Asterisks indicate groups with significantly different fluorescence intensities compared to control (ANOVA, Holm-Sidak *post-hoc*, * $p < 0.05$, *** $p < 0.001$, $n = 14$, $N = 2$, Data are given as means ± SD). *Copyright © Elsevier 2020. Data were produced jointly with Carmen Sommer.*

Daphnids and chironomid larvae exposed to waterborne BkF_{AQ} and PMMA loaded with BkF were measured in comparison to negative controls (for chromatograms see Annex II.4., Fig. A5, Fig. A6). Exposure to waterborne BkF_{AQ} (2 µg BkF in 20 ml Elendt M4 medium) induced accumulation of BkF in daphnids at 13.8 ± 2.1 ng/ml BkF per 10 individuals and 10.56 ± 0.55 ng/ml BkF in chironomid larvae per 40 individuals (Tab. 8). A single zebrafish was fed either with 40 daphnids per day or 80 individuals of *Chironomus riparius* per day. Over two days, an estimated amount of 110 ng of BkF_{AQ} was transferred to *Danio rerio* by daphnids (T 2), whereas *Chironomus riparius* only transferred 42 ng of BkF_{AQ} (T 2). Chironomid larvae transferred 63.4 ± 0.8 ng BkF sorbed to PMMA (T 3) to one zebrafish per day (126 ng BkF over two days). In contrast, daphnids pre-exposed to PMMA loaded with BkF (T 3) failed to accu-mulate any BkF (Annex II.4., Fig. A5).

Tab. 8. Determination of benzo(k)fluoranthene in *Daphnia magna* and *Chironomus riparius* either taken up as dissolved BkF (BkF_{AQ}) or sorbed to PMMA (PMMA + BkF) as determined by GC-MS in acetonitrile. The estimated amount of daily BkF uptake by zebrafish (in ng), feeding on pre-exposed daphnids and chironomid larvae. Limit of Detection for BkF (LoD): ≤ 3 ng/ml. Data are given as means ± SD (n = 2). [a] 10 daphnids/measurement, [b] 40 chironomid larvae/measurement. *Copyright © Elsevier 2020.*

	Exposure	BkF [ng/ml]	Amount BkF/day/fish
Daphnia magna [a]	BkF_{AQ}	13.8 ± 2.1	55.2 ng ± 8.4
	PMMA + BkF	< 3	-
Chironomus riparius [b]	BkF_{AQ}	10.6 ± 0.6	21.1 ng ± 1.1
	PMMA + BkF	31.7 ± 0.4	63.4 ng ± 0.8

IV.4.3 Trophic transfer of benzo(k)fluoranthene in food web experiments

IV.4.3.1 Fluorescence tracking of benzo(k)fluoranthene in zebrafish intestine

Analysis of the fluorescence signals in unstained cryosections revealed that no significant transfer of BkF had taken place in both trophic transfer experiments. In contrast, exposure of zebrafish to waterborne BkF_{AQ} (PC) led to a significant increase in fluorescence ($p < 0.001$) in the gastrointestinal tracts in both experiments (Fig. 20A). Transfer of PMMA particles could be confirmed microscopically since individual MPs could be detected in intestinal sections of MP treatment groups only (arrowheads in Fig. 19A - C). Fluorescence intensity was not altered by feeding with BkF-loaded daphnids or chironomid larvae (T 2). PMMA particles were exclusively found in the intestinal lumen and the chyme, i.e., there was no sign of translocation to organs beyond the intestinal lining. Furthermore, there was no evidence for adverse effects of MPs on tissue integrity.

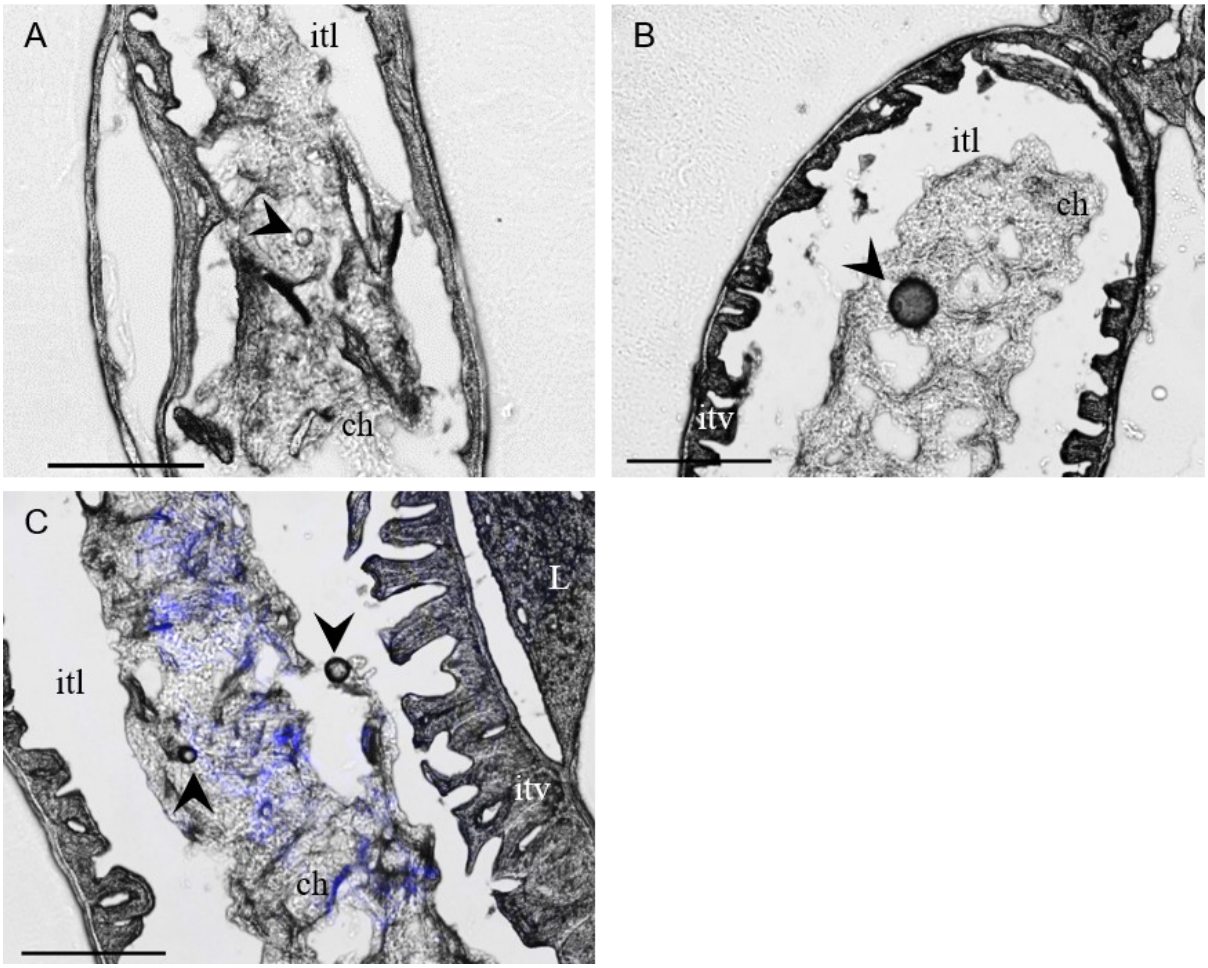

Fig. 19. Different sections of gastrointestinal tracts showing PMMA particles (black arrowheads) in the intestinal lumen and chyme of *Danio rerio* and associated BkF fluorescence signal (blue fluorescence signal). (**A**) Exposure to daphnids fed pristine PMMA. (**B**) Exposure to chironomid larvae fed pristine PMMA. (**C**) Exposure to chironomid larvae fed with BkF-loaded PMMA. L = liver, itl = intestinal lumen, itv = intestinal villi, ch = chyme. Brightfield and blue fluorescence image (excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm, Nikon Eclipse 90i), scale bars 250 µm. *Copyright © Elsevier 2020. Data were produced jointly with Carmen Sommer.*

IV.4.3.2 EROD activity in zebrafish

Frozen liver samples from the independent experimental runs were analyzed using the *in vitro* EROD assay according to Batel et al. (2016) and Örn et al. (1998). EROD activity in zebrafish liver samples could not be altered by feeding with either invertebrate species within two days of trophic transfer experiments, i.e., zebrafish fed with invertebrates pre-incubated with BkF-loaded MPs did not show an increase in CYP450 activity (Fig. 20B). Even uptake of Chironomus riparius that previously accumulated BkF sorbed to PMMA (Fig. 18), failed to alter the CYP 450 in zebrafish (Fig. 20B, T3). Only the daily uptake of 21.1 ± 1.1 ng waterborne BkF_{AQ} by chironomid larvae and direct exposure of zebrafish to BkFAQ (positive control) significantly induced EROD activity in zebrafish ($p < 0.05$, $p < 0.001$, respectively, Fig. 20B). Since daphnids did not accumulate BkF sorbed to PMMA over 24 h, they could not transfer BkF to zebrafish (Tab. 8). These findings are in line with the internal BkF concentrations of both primary consumers quantified by GC-MS.

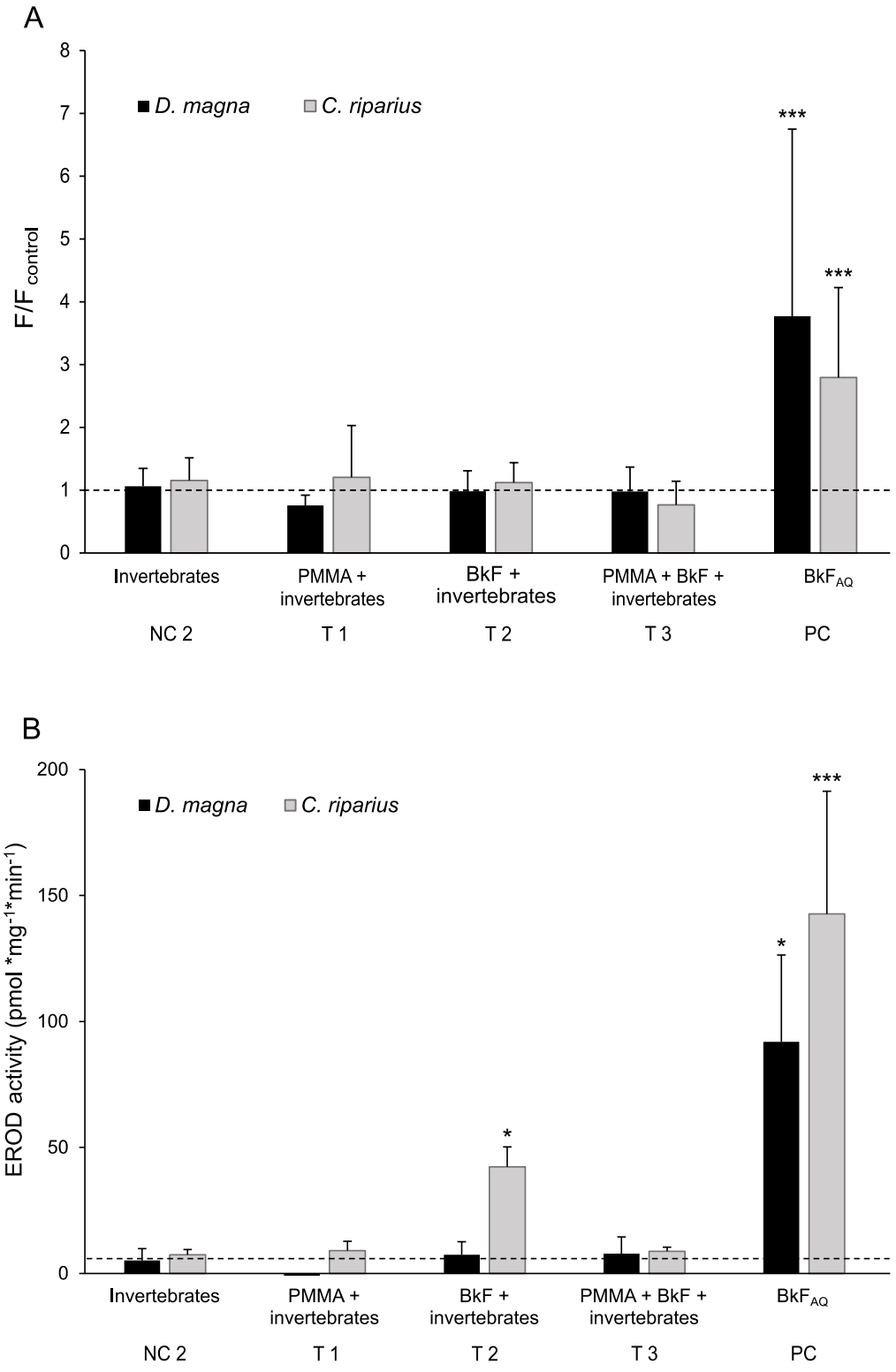

Fig. 20. Effects on (**A**) Fluorescence signal in gastrointestinal tracts and (**B**) *In vitro* EROD activity in liver of *Danio rerio* after two days of consecutive feeding with either untreated invertebrates (Invertebrates = NC 2) or different pre-exposed invertebrate organisms (*Daphnia magna*, *Chironomus riparius*). Treatment groups comprised organisms with ingested, pristine PMMA (PMMA + invertebrates = T 1), invertebrates pre-exposed to BkF_{AQ} (BkF + invertebrates = T 2) or invertebrates with ingested PMMA loaded with BkF (PMMA + BkF + invertebrates = T 3). Zebrafish exposed solely to waterborne BkF_{AQ} served as a positive control (BkF_{AQ} = PC). Control is indicated as dashed line (NC 1). Asterisks indicate groups with significantly different fluorescence intensities and EROD activity (Kruskal-Wallis analysis, Dunn's *post-hoc,* * $p < 0.05$, *** $p < 0.001$, n = 6, N = 2, MV ± SD). *Copyright © Elsevier 2020. Data (A) were produced jointly with Carmen Sommer.*

IV.5 Discussion

There is only limited number of studies on (semi-)natural trophic transfer of MPs and MP-associated contaminants with vertebrates as high-level consumers. Previous studies covered marine environments (Avio et al., 2015; Santana et al., 2017; Diepens and Koelmans, 2018; Nelms et al., 2018) as well as intertidal (Tosetto et al., 2017) and freshwater habitats (Rochman et al., 2017). The interpretation of the effects by MP-associated contaminants after transfer across different trophic levels in aquatic food webs are inconclusive (de Sá et al., 2018; Franzellitti et al., 2019). In fact, there is no clear evidence for bioaccumulation or biomagnification of substances transported by MPs (Triebskorn et al., 2019).

Given the increasing production volume and the broad application spectrum of PMMA in automotive and computer industries (PlasticsEurope, 2018), PMMA is becoming a potential environmental threat. So far, however, PMMA is clearly underrepresented in environmental studies (de Sá et al., 2018). Although the amount of PMMA particles applied in the laboratory experiments exceeded reported environmental concentrations in freshwaters ranging from 0.027 to 18,700 particles/m^3 (Adam et al., 2019; Triebskorn et al., 2019), the spatial distribution of MPs varies considerably (Duis and Coors, 2016; Anbumani and Kakkar, 2018). Therefore, the present study aimed to provide a more comprehensive insight into the potential transfer of a persistent organic pollutant sorbed to PMMA, from invertebrates to a model vertebrate organism in a simplified freshwater food web.

IV.5.1 Loading of PMMA particles with benzo(k)fluoranthene and particle recovery

GC-MS measurements documented up to 80 % sorption of BkF to PMMA particles. The ability to bind and transfer hazardous organic compounds such as polychlorinated biphenyls (PCBs) or PAHs strongly depends on polymer type, surface area and surrounding medium (Rochman et al., 2013b; Koelmans et al., 2016; Beckingham and Ghosh, 2017; Diepens and Koelmans, 2018; Müller et al., 2018). In this study, 0.6 - 0.7 mg BkF were sorbed to 1 g PMMA particles after the incubation and washing procedure. Rochman et al. (2013b;a) showed that, BkF can sorb to MPs from marine environments and may be transferred within food webs. Environmental concentrations of PAHs sorbed to mixtures of marine plastic samples from the North Pacific Subtropical Gyre or MPs deposited on beaches, accumulated PAHs up to 24 µg/g plastics (Yeo et al., 2017; Chen et al., 2019; Pannetier et al., 2019a). However, sorption of PAHs to MPs in freshwater ecosystems might differ from marine ecosystems (e.g., due to salinity, concentration gradients). Most importantly, in limnic ecosystems, point sources are likely to play a more significant role than in marine systems (Klein et al., 2015; Duis and Coors, 2016; Besseling et al., 2017), especially in close vicinity to pollutant hot spots (Li et al., 2018a; Triebskorn et al., 2019).

IV.5.2 Trophic transfer of microplastics and MP-associated benzo(k)fluoranthene

IV.5.2.1 Uptake into invertebrate organisms

The majority of recent studies detected minor effects by various types of MPs and almost negligible MP-associated transfer of hazardous organic compounds (HOCs) like PCBs, PAHs and PBDEs (Frydkjær et al., 2017; Rochman et al., 2017; Tosetto et al., 2017; Rehse et al., 2018). Particle uptake and accumulation of BkF strongly depends on feeding strategies of invertebrate organisms. Both *Daphnia magna,* as a pelagic filter feeder, and *Chironomus riparius* larvae, as a benthic collector-gatherer, feed non-selectively on particles (Rehse et al., 2016; Scherer et al., 2017; Silva et al., 2019). In our study, both invertebrate species ingested PMMA particles over 24 h at comparable amounts, as already described by Scherer et al. (2017) and Nel et al. (2018).

Trophic transfer of PCBs to a vertebrate as a high-level consumer was investigated by Rochman et al. (2017). Feeding white sturgeon (*Acipenser transmontanus*) over 28 days with a diet consisting of 20 % homogenized Asian clams (*Corbicula fluminea*) chronically pre-exposed to PCB-loaded MPs did not result in a quantifiable transfer of PCBs (Rochman et al., 2017). In the present study, only *Chironomus riparius* was able to transfer significant amounts of BkF *via* MPs to zebrafish (63.4 ± 0.8 ng). In contrast, in a previous study from the same laboratory, Batel et al. (2016) reported the transfer of much higher amounts of benzo(*a*)pyrene (BaP) from saltwater brine shrimp (Artemia spec.) Nauplii to zebrafish *via* 10 - 20 µm polyethylene particles of 60 µg BaP. Nevertheless, seemingly the amount of transferred BaP was too low to induce significant changes in hepatic CYP450 levels in zebrafish.

In the present study, exposure to BkF sorbed to PMMA particles led to a three-fold increase of BkF taken up by chironomid larvae, whereas daphnids did not accumulate BkF above analytical detection limits (LoD: 3 ng/ml, see Tab. 8). This might be due to a longer retention time of particles in the intestine of chironomid larvae (3 h) if compared to daphnids (2 min; Scherer et al., 2017), allowing more BkF to desorb within *Chironomus riparius* despite a lower MP uptake rate. Nevertheless, BkF concentrations within invertebrates were one order of magnitude lower than the initial BkF concentration in the aqueous phase, providing only minor amounts of BkF to be subsequently transferred to zebrafish. Most likely, the increased feeding rate of 0.9 % in experiments with *Chironomus riparius* (2.4 % of zebrafish body wet weight) compared to daphnid experiments (1.5 % of zebrafish body wet weight) was already sufficient to induce alterations in EROD activity.

The amount of BkF taken up by chironomid larvae *via* loaded MPs (31.7 ng/ml) was three times higher than the amount taken up by direct exposure to waterborne BkF_{AQ} (10.6 ng/ml), but apparently not bioavailable for zebrafish. Bioavailability to zebrafish might have been compromised by the metabolic capacities of *Chironomus riparius* and *Daphnia magna* to transform BkF into water-soluble products. Both species have been documented to effectively metabolize PAHs such as pyrene, fluoranthene or benzo(*a*)pyrene within 24 h (Gourlay et al., 2002, 2003; Verrengia Guerrero et al., 2002; Ikenaka et al.,

2006; Berrojalbiz et al., 2009; Carrasco Navarro et al., 2013). Trophic transfer of resulting pyrene metabolites has been demonstrated for larvae of *Chironomus riparius* as prey and *Gammarus setosus* as a predator (Carrasco Navarro et al., 2013), and further, metabolites produced by prey proved less bioavailable to predator organisms than metabolites artificially spiked to food. Thus, in the present experiment, daphnids and chironomid larvae may be assumed to have metabolized BkF (waterborne and MP-associated), resulting in low levels of accumulated BkF and BkF metabolites which could most likely not be detected by both fluorescence tracking and GC-MS measurements. Presumably, upon exposure to waterborne BkF_{AQ}, *Chironomus riparius* produced metabolites to a larger extent, which significantly increased EROD activity ($p < 0.05$) in zebrafish liver, similar to a trophic transfer study on significantly different amounts of fluoranthene biotransformation products in two closely related polychaete species (Palmqvist et al., 2006). In fact, some BkF metabolites have been proven to be more toxic than the parent compound (Spink et al., 2008). In contrast, rapid metabolization of BkF in zebrafish larvae was also shown by Barranco et al. (2017), where BkF was the major inducer of the *cyp1a1* gene; yet, significant bioaccumulation could not be measured over 48 h. Hence, metabolic degradation of BkF and a diminished fluorescence signal as well as no alterations in EROD activity were likely. Eventually, it is important to note the negative impact of dissolved organic matter (DOM) on the bioavailability and bioaccumulation of PAHs in daphnids (Gourlay et al., 2003). Naturally occurring DOM may reduce the bioavailability and potential transfer of PAHs along the food web in freshwater ecosystems, in addition to the high amounts of natural particles affecting the sorption of pollutants (Koelmans et al., 2016; Diepens and Koelmans, 2018)

IV.5.2.2 Transfer of PMMA and MP-associated benzo(k)fluoranthene from invertebrates to zebrafish (*Danio rerio*)

Transfer of PMMA particles by invertebrates to *Danio rerio* was successful in all microplastic treatments (see Fig. 19). Localization of MPs, however, was exclusively restricted to the gastrointestinal lumen and chyme; there was no evidence found for MPs being retained by the intestinal lining or penetrating gastrointestinal epithelial cells. As a consequence, this short-term feeding did not cause any macroscopically detectable tissue damage in fish. Likewise, Batel et al. (2016) demonstrated the MP transfer to zebrafish by feeding *Artemia* spec. nauplii at relatively high concentrations (1.2×10^6 polyethylene particles/L) without any damage to the fish. In contrast, other studies applying numbers of MPs closely to environmentally relevant concentrations even failed to detect MPs in vertebrate consumers (Grigorakis et al., 2017; Santana et al., 2017).

In the present food web experiments, EROD activity in zebrafish liver tissue only showed a significant increase, when fish were fed with BkF_{AQ}-exposed larvae of *Chironomus riparius* or upon direct exposure to waterborne BkF_{AQ}. However, the ingestion of organisms pre-treated with BkF-sorbed PMMA particles modulated hepatic EROD activities only to a minor extent. As demonstrated by Batel et al.

(2016, 2018), the lack of external pathological alterations in fish exposed to MPs and MP-associated contaminants does not necessarily exclude alterations in more sensitive biological endpoints such as the induction of CYP450-driven biotransformation processes. Given considerable interspecific variations of adverse effects by MPs and contaminants associated with MPs, MP-mediated transfer of PAHs has been discussed highly controversially for both invertebrates and vertebrates (Avio et al., 2015; Batel et al., 2016; Tosetto et al., 2016, 2017; Rochman et al., 2017). Avio et al. (2015) detected the accumulation of MPs and pyrene transferred by MPs in filter-feeding Mediterranean mussels (*Mytilus galloprovincialis*), and concluded that bioavailability of microplastic-associated PAHs is increased through ingestion by filter feeders. Whereas, other studies detected MP uptake by aquatic and terrestrial invertebrates (*Corbicula fluminea, Platorchestia smithi*), but failed to detect any effects through contaminant transfer or even bioaccumulation at both first and second trophic levels (Tosetto et al., 2016, 2017; Rochman et al., 2017). Exposure of medaka prolarvae (*Oryzias latipes*) to DMSO extracts from environmental MP samples loaded with PAH concentrations ranging from 2 ng/g to 18 ng/g over 48 h did not induce significant alterations in hepatic EROD activities (Pannetier et al., 2019b). Likewise, Rochman et al. (2013b) observed no change in CYP1A enzyme activities in Japanese medaka (*Oryzias latipes*) after two months of chronic exposure to MPs loaded with up to 129 ng/g PAHs. However, it should be considered that EROD activity may vary considerably between fish species (*Oryzias latipes* and *Danio rerio*) and life stages (prolarvae and adult; Whyte et al., 2000; Vehniäinen et al., 2012). Results from the present study, which used absolute amounts of BkF in *Chironomus riparius* and *Daphnia magna* in a similar nanogram range to PAHs extracted from microplastic beach samples (Pannetier et al., 2019a), corroborates the conclusion that the amounts of PAHs transferred *via* MPs are most likely too small to induce enzymatic CYP1A activities.

So far, only a few studies investigated the effects of PMMA micro- and nanoplastics on freshwater invertebrates with only minor adverse impact on weight gain and metabolic activities (Straub et al., 2017; Kratina et al., 2019). In case of accidental discharge of MPs in freshwater ecosystems, PAH concentrations on MPs similar to those used in the present study might well be reached. However, in line with previous results (Tosetto et al., 2016, 2017; Rochman et al., 2017), BkF uptake from particles and bioaccumulation only occurred in chironomid larvae, and trophic transfer of microplastic-sorbed BkF could not be confirmed. It seems more likely, that in aquatic ecosystems the pollutant transfer by particle ingestion will be dominated by natural particles such as clay minerals, algae, biochar, black carbon, but not by MPs (Gouin et al., 2011; Koelmans et al., 2016; Beckingham and Ghosh, 2017; Diepens and Koelmans, 2018; Lee et al., 2019).

IV.6 Conclusions

In an attempt to further improve our understanding of the potential of MPs to transfer sorbed pollutants in trophic food webs, the present study developed a reproducible approach to load MPs with model substances. Despite, trophic transfer of BkF *via* MPs could not be confirmed using a combination of highly sensitive fluorescence tracking *via* CLSM and advanced chemical-analytical methods. In contrast, hepatic EROD induction proved superior in sensitivity compared to fluorescence measurements, since, at least for direct exposure to waterborne BkF_{AQ} (positive control) and BkF-transfer *via* chironomid larvae (treatment 2), an induction of CYP450 could be documented. Although the potential of PMMA to transfer hydrophobic chemicals to invertebrate organisms could principally be demonstrated, results indicate that – relative to the likely transfer of organic trace contaminants by natural particles (Koelmans et al., 2016; Wagner and Lambert, 2018) – the contribution of PMMA particles to act as a carriers for hydrophobic chemicals may be limited.

IV.7 Annex II

II.1. GC-MS settings for benzo(k)fluoranthene (BkF) determination and particle sorption measurements

Instrument settings

Injection Volume: 1 µl

Solvent washes (acetone) 3 × 3 µl preinjection, 3 × 3 µl postinjection

Injection Type: Standard

Split-Splitless Inlet

Heater 250 °C, Pressure 64 kPa, Septum purge flow 3 ml/min (He)

Temperature MSD Transfer Line 280 °C

Tab. A3. Settings for GC-MS oven program for BkF determination. *Copyright © Elsevier 2020.*

	Heating rate [°C/min]	Temperature [°C]	Hold time [min]
Initial		60	2
Ramp 1	40	325	0
Ramp 2	20	325	2
Post run	0	300	10

Detection parameter

Ion Source Electron ionization (EI), MS Source 230 °C, MS Quadrupole 150 °C,

SIM Phenanthrene (ISTD) m/z 176 (qualifier), m/z 178 (quantifier)

Benzo(k)fluoranthene m/z 250 (qualifier), m/z 252 (quantifier)

Calibration for BkF determination

12-point calibration was performed between 2.5 - 350 ng/ml, and evaluation was achieved by equal weighting of standards. Limit of Detection (LoD) ≤ 1 ng/ml and Limit of Quantification (LoQ) ≤ 2.5 ng/ml. Quality Control (QC, 100 ng/ml standard) was determined before and after the measurement of the samples and the deviation was smaller than 10 %.

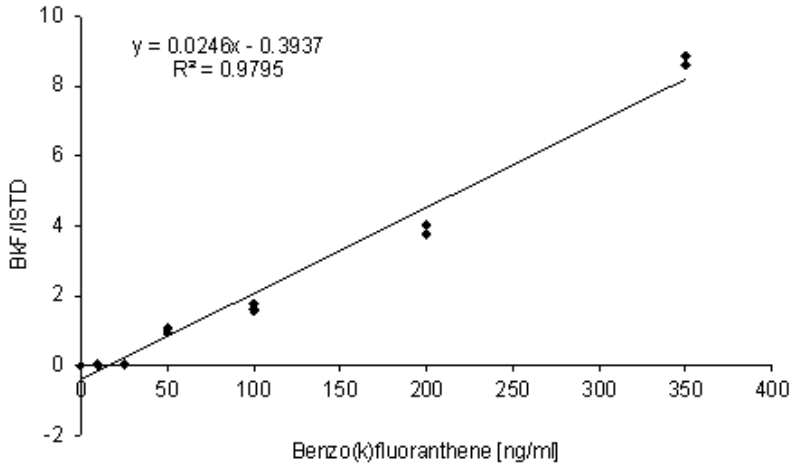

Fig. A3. Calibration curve for BkF measurements *via* GC-MS (Agilent Technologies, GC 7890B, column 19091S-433 I HP, MSD 5977B). *Copyright © Elsevier 2020. Data were produced jointly with Sven Huppertsberg*.

II.2. Fluorescence tracking in gastrointestinal cryosections of zebrafish and invertebrate organisms with image data analysis using FIJI

Preliminary tests. Fluorescence signal in *Daphnia magna.*

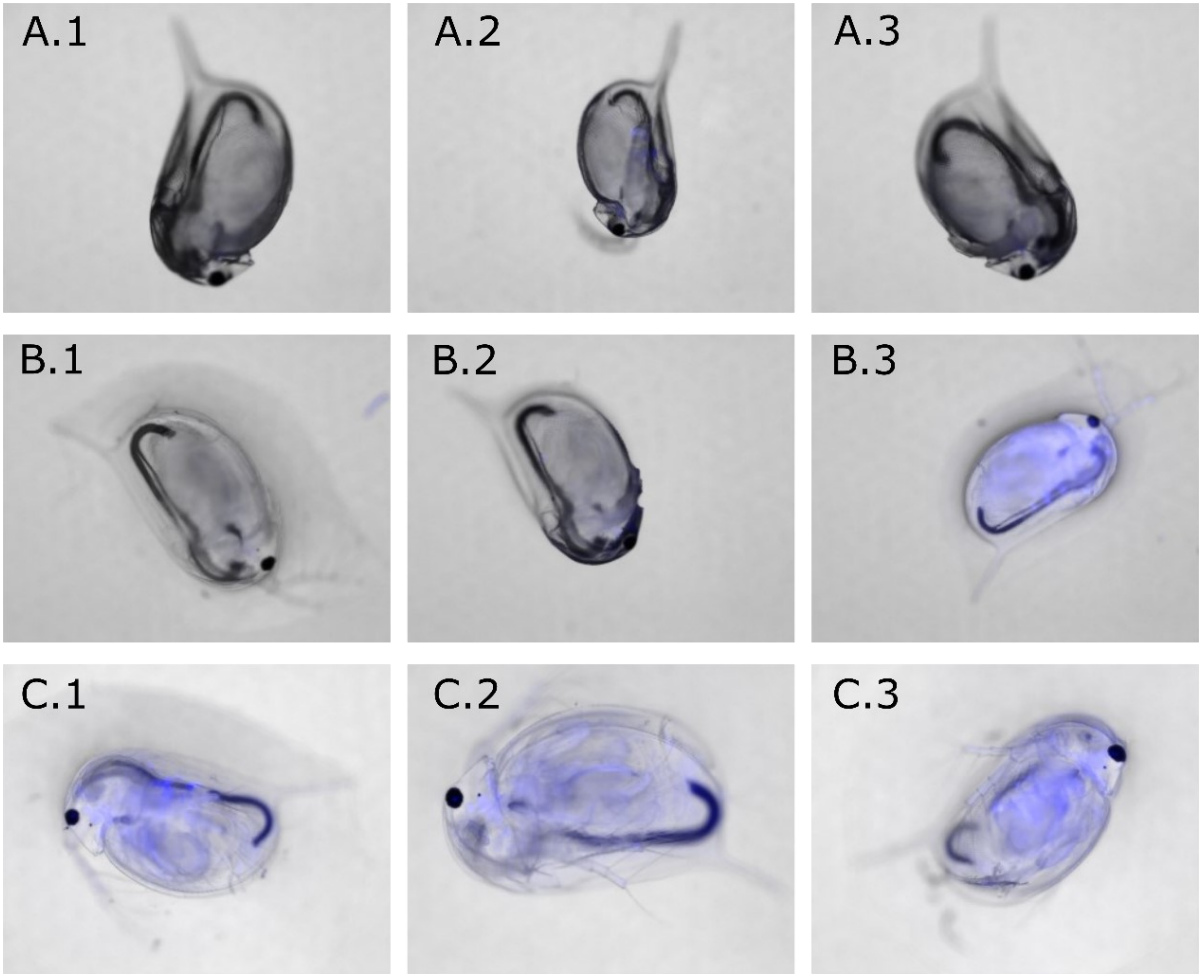

Fig. A4. Development of BkF fluorescence signal intensity after exposure to 400 µM BkF_{AQ} in different individuals of *Daphnia magna* over 2 - 24 h. (**A**) 2 h of exposure to BkF_{AQ}. (**B**) 12 h of exposure. (**C**) 24 h of exposure. Overlay of brightfield and fluorescence image, exposure time: 200 ms (excitation filter: 340 - 380 nm, emission filter: 435 - 485 nm, Nikon Eclipse 90i). *Copyright © Elsevier 2020. Data were produced jointly with Carmen Sommer.*

Image Acquisition details for gastrointestinal tract sections

HV: 38, Offset: 0, Laser power: 45, Pinhole: 3.1 AU, Scan speed: ¼, Scan size: 1024

FIJI Plugins used and parameters

Grid/Collection stitching for stitching of single 10 × images to whole image of the intestinal tract with Overlap: 5 %, Fusion method: linear blending, Default parameters (Preibisch et al., 2009).

II.3. Particle recovery of PMMA particles after incubation with BkF

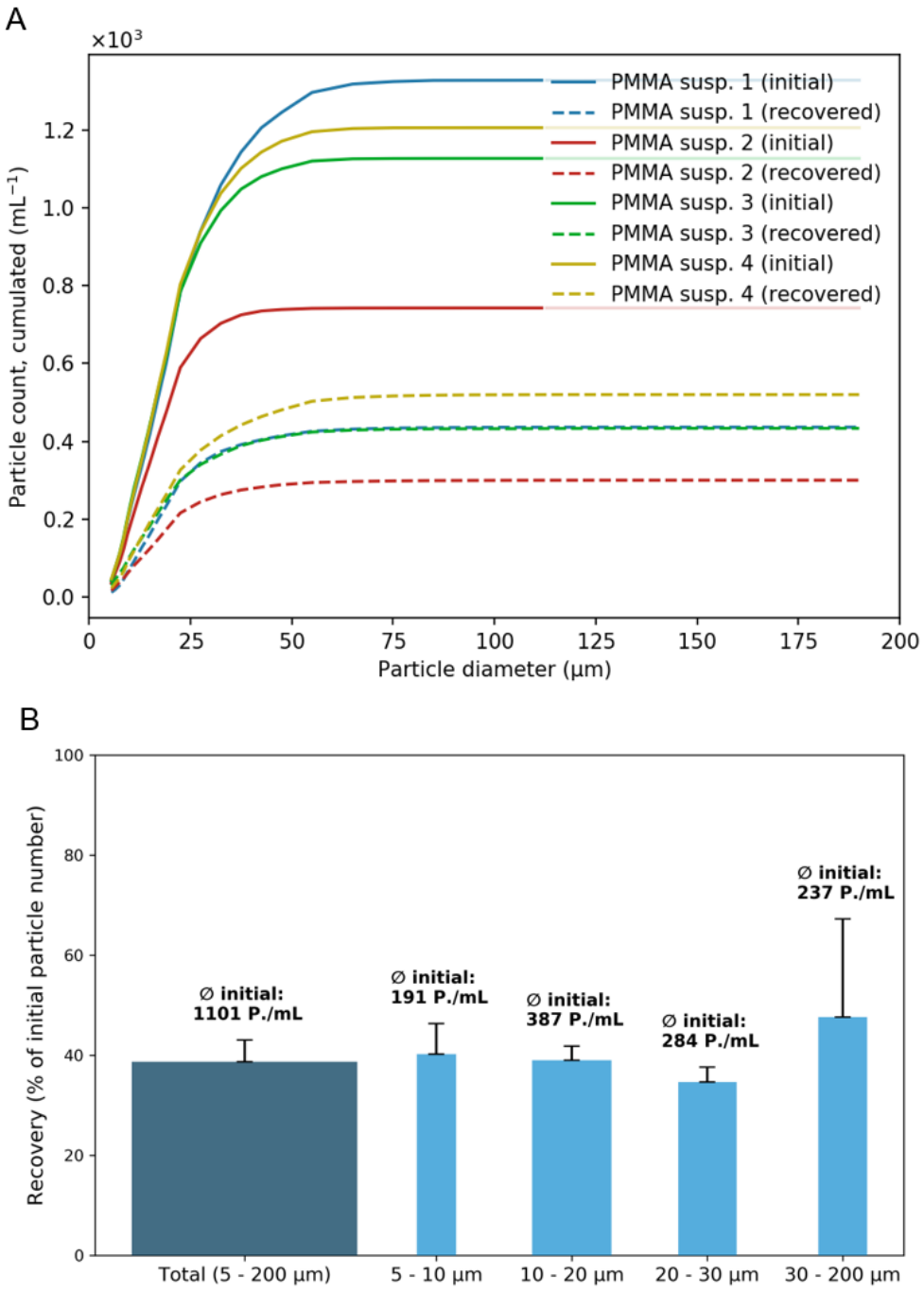

Fig. A5. Particle recovery of PMMA suspension after filtration, washing steps and resuspension in M4 medium. (**A**) Cumulated particle count of four replicate measurements. Solid line = initial particle suspension, dashed line = resuspension. (**B**) Composition of recovered PMMA particles after filtration as percentage of particle number of initial PMMA suspension. n = 4, mean initial particle numbers per ml indicated above the bars. *Copyright © Elsevier 2020. Data were produced jointly with Stefan Dittmar.*

II.4. GC-MS measurements of benzo(k)fluoranthene in invertebrate organisms

Instrument settings

Injection Volume: 10 µl

Injection Type: Standard

Injection mode: Splitless

Injection temperature: 290 °C, Pressure 51.4 kPa, Purge flow 5.0 ml/min (He)

Tab. A4. Settings for GC-MS oven program for BkF determination in *Daphnia magna* and *Chironomus riparius*. *Copyright © Elsevier 2020.*

	Heating rate [°C/min]	Temperature [°C]	Hold time [min]
Initial	-	50	1
Ramp 1	60	150	0
Ramp 2	5	300	3.4

Detection parameter

Flame thermionic detector (FTD), MS Quadrupole

Ion source Temp. 200 °C

SIM Benzo(k)fluoranthene m/z 250 (qualifier), m/z 252 (quantifier)

Retention time BkF: 27.854 min

Calibration for BkF in invertebrate organisms

5-point calibration was performed between 10 - 100 ng/ml, Limit of Detection (LoD) ≤ 3 ng/ml, recovery rate 85 ± 13 % for *Daphnia magna* and 103 ± 18 % for *Chironomus riparius.*

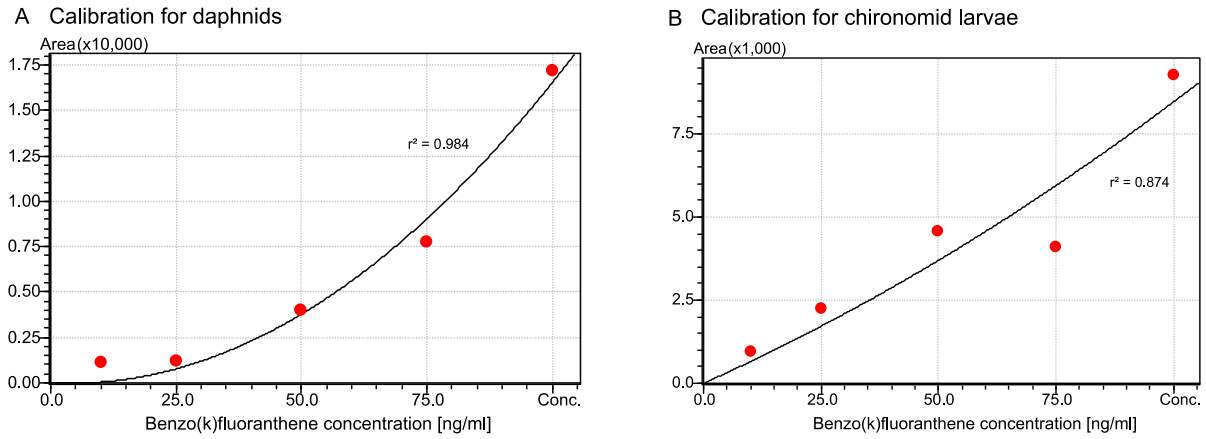

Fig. A6. Calibration curves (quadratic curve fit) for benzo(k)fluoranthene detection in daphnids **(A)** and chironomid larvae **(B)** using GC-MS (QP2010 SE, Shimadzu, column HP5 MS, Agilent technologies). *Copyright © Elsevier 2020.*

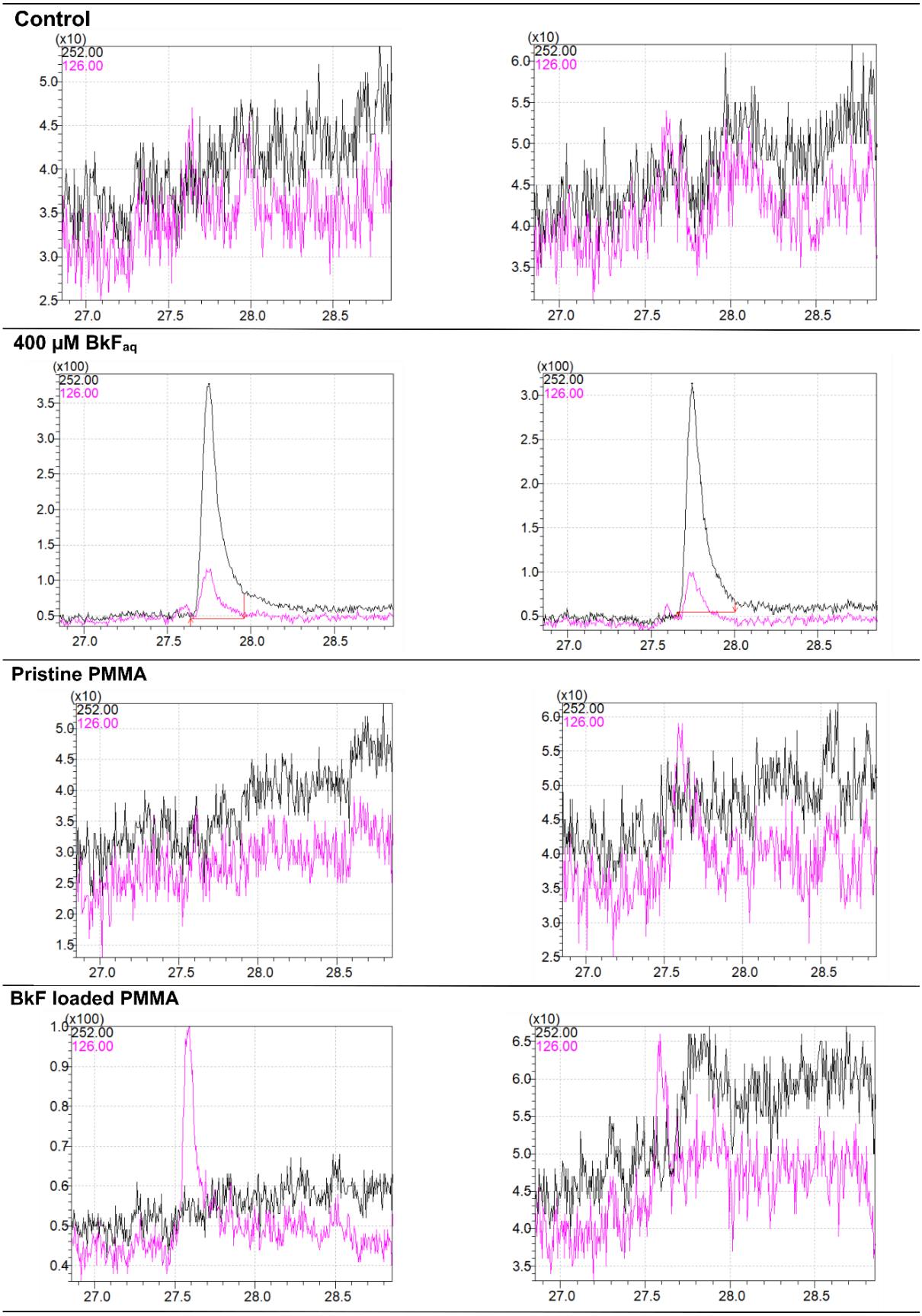

Fig. A7. GC-MS chromatograms of daphnid (*Daphnia magna*) samples from GC-MS measurements, control samples and samples exposed either to dissolved 400 µM BkF, pristine PMMA particles or BkF-sorbed PMMA particles over 24 h. BkF signal = black line, n = 2 (GCMSsolution software, Shimadzu Scientific Instruments, Germany). *Copyright © Elsevier 2020.*

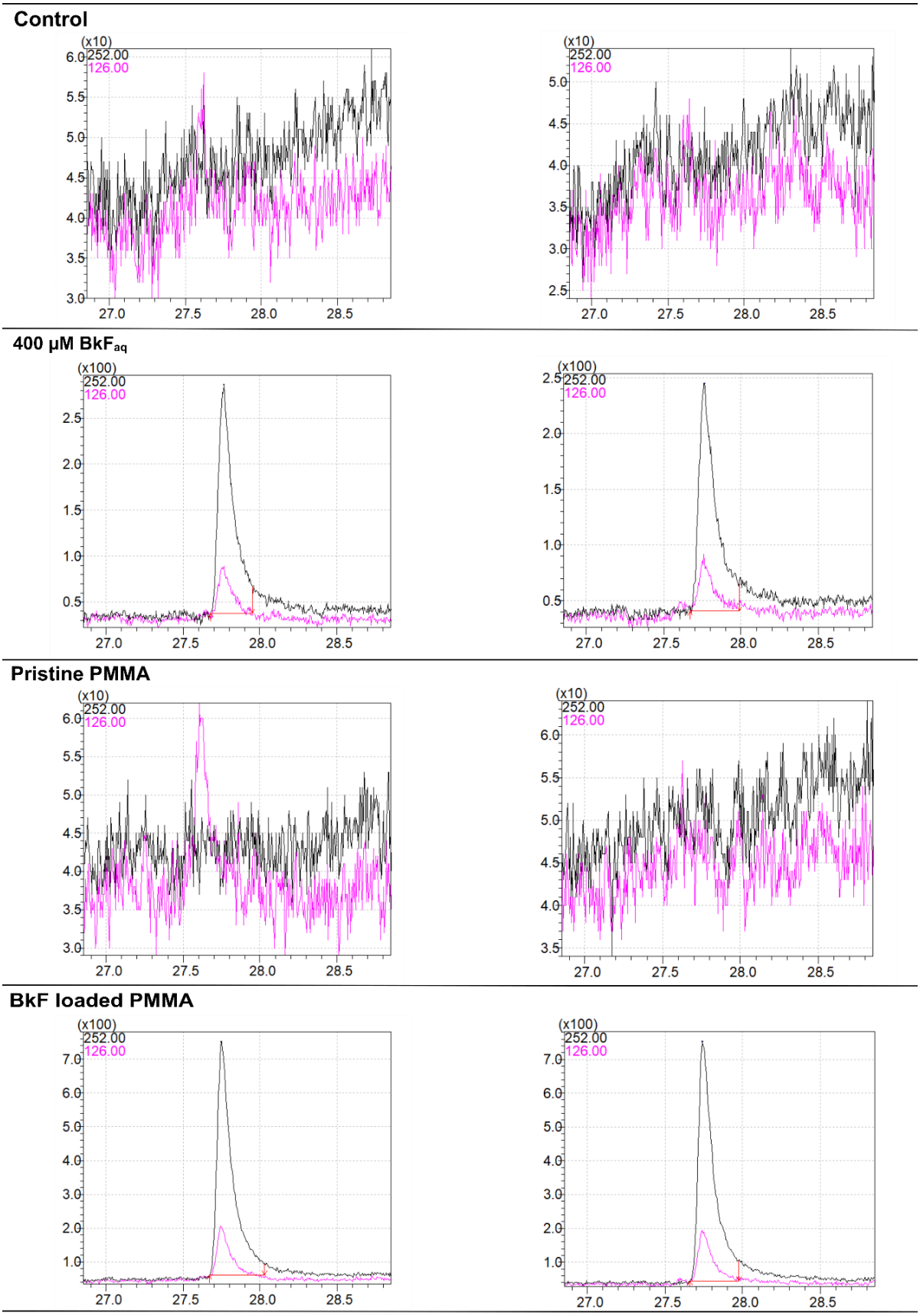

Fig. A8. GC-MS chromatograms of chironomid larvae (*Chironomus riparius*) samples from GC-MS measurements, control samples and samples exposed either to dissolved 400 µM BkF, pristine PMMA particles or BkF-sorbed PMMA particles over 24 h. BkF signal = black line, n = 2 (GCMSsolution software, Shimadzu Scientific Instruments, Germany). *Copyright © Elsevier 2020.*

V. Environmental exposure of microplastics in a highly polluted stream in Germany. Analytical quantification and toxicological effects in zebrafish (*Danio rerio*)

This chapter is based on a manuscript written entirely by me and currently prepared for submission in Environmental Pollution and includes content that was created jointly with Nadine Kämmer and Sven Huppertsberg. Nadine Kämmer conducted visual motor response tests and Sven Huppertsberg conducted the analytical quantification of water and matrix samples and the corresponding figures and tables are marked. Conceptualization, sample preparation, fieldwork, toxicological tests and data analysis were conducted by myself.

V.1 Abstract

Given the increasing amounts of plastic debris entering marine and freshwater ecosystems, there is a growing demand for environmentally relevant exposure scenarios to improve the risk assessment of microplastic particles (MPs) in aquatic environments. So far, data on adverse effects in aquatic organisms induced by naturally exposed MPs are scarce and controversially discussed. Consequently, we investigated the potential role of MPs regarding the sorption and transfer of environmental contaminants under natural conditions. Therefore, a mixture of four common polymer types (polyethylene, polypropylene, polystyrene, polyvinyl chloride) was exposed for three weeks in polluted surface water. The water, MP mixture, sediment and suspended matter were target screened for present pollutants using GC/LC-MS, resulting in up to 94 different compounds. Possible adverse effects were investigated using several biomarkers in early developmental stages of zebrafish (*Danio rerio*). Exposure to water samples significantly inhibited acetylcholine esterase activity, altered CYP450 induction as well as behavioral patterns of zebrafish. Particle samples and exposed MPs induced effects in zebrafish to a lesser extent compared to water samples. The analytical target screening documented only a few compounds sorbed to natural particles and MPs. Regarding acute toxic effects, no clear differentiation between different particle types could be made, concluding that MPs - upon exposure in natural water bodies - seem to approximate the sorption behavior of natural particles, presumably due to biofilm formation. Thus, MPs most likely do not transfer elevated amounts of environmental pollutants compared to natural inorganic particles and therefore do not pose an additional threat to aquatic biota.

V.2 Introduction

The continuously increasing plastic production and deficient waste management lead to an estimated 5 to 12 million tons of plastic debris being disposed of on land, potentially ending up in the oceans within one year (Jambeck et al., 2015). Recent estimates also predict that rivers can transport between 1.2 and 2.4 million tons of plastic debris into the seas and oceans (Lebreton et al., 2017). Besides, land use and population density are correlated with aquatic plastic pollution, suggesting that rivers are the crucial link between global land surface and oceans, contributing up to 94 % of the total aquatic plastic contamination (Schmidt et al., 2017). Due to the ubiquitous occurrence and the constant increase of microplastic particles (MPs) in aquatic environments, MPs are considered a global threat to marine and freshwater ecosystems (Avio et al., 2017; Jahnke et al., 2017).

At present, synthetic polymer fragments are being detected in planktonic samples (Thompson et al., 2004; Beer et al., 2018), on marine and freshwater shorelines (Browne et al., 2011; Imhof et al., 2013), in deep-sea sediments (Van Cauwenberghe et al., 2013) and several European rivers (Lechner et al., 2014; Dris et al., 2015; Mani et al., 2015; Scherer et al., 2020a). The particle numbers detected in surface waters ranged from 0.027 particles/m^3 in the Laurentian Great Lakes (Eriksen et al., 2013) to 18700 particles/m^3 in Amsterdam canal water (Leslie et al., 2017) and the river Rhine discharges approximately 191.6 million of MPs into the North Sea every day (Mani et al., 2015). As shown by Scherer et al. (2020a), the composition of plastic debris in rivers (water column and sediment) reflects the most commonly produced and used polymers (polyethylene, polypropylene, polyvinyl chloride and polystyrene). It has been demonstrated that MP pollution markedly increased in the vicinity of urbanized or industrialized areas (Alimi et al., 2017; Kooi et al., 2018; Bikker et al., 2020).

Compared to natural particles (e.g., algae, clay minerals, detritus), MPs persist and accumulate in the environment over much longer timescales. Therefore, MPs are prone to further transport and are available to a wide range of organisms in all compartments of marine and limnic ecosystems (Kooi et al., 2018). The sorption and desorption of complex contaminant mixtures to MPs from the aquatic environment has already been investigated in several laboratory studies (Bakir et al., 2012; Allen et al., 2018; Hüffer et al., 2018; Liu et al., 2019), indicating a possible transport of sorbed contaminants to organisms through ingestion (Rochman et al., 2013a; Batel et al., 2016; Chen et al., 2019; Pannetier et al., 2019b; Hanslik et al., 2020). The successful accumulation of particularly lipophilic organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, polybrominated biphenyl ethers (PBDEs) or metals has already been documented for MPs collected from surface waters, shorelines and beaches or after exposure to wastewater (Rochman et al., 2013a; Ašmonaite et al., 2018; Vedolin et al., 2018; Le Bihanic et al., 2020). Amounts of PCBs and PAHs extracted from collected MPs ranged from 1 ng/g up to 850 ng/g plastic (Rochman et al., 2013a; Rios Mendoza and Jones, 2015; Pannetier et al., 2019a). These findings illustrate that MPs, due to their

hydrophobic surface, can accumulate hazardous organic compounds in concentrations that are several orders of magnitudes higher compared to the surrounding medium (Hüffer and Hofmann, 2016).

So far, hardly any studies have been conducted on biofouling, weathering and potential alterations of sorption capacities of MPs under natural conditions and their potential effects for biota (Jahnke et al., 2017). As a consequence, there is an increasing demand for more environmentally relevant exposure scenarios to improve the risk assessment of MPs (Wagner and Lambert, 2018; Triebskorn et al., 2019).

Early life stages of zebrafish (*Danio rerio*) have already proven sensitive for the identification of potential environmental hazards and as a basis for risk assessment of xenobiotics such as pesticides, pharmaceuticals, personal care products or environmental pollutants (Lammer et al., 2009; Braunbeck et al., 2015). Therefore, the testing of microplastics and associated contaminants are considered indispensable.

In an attempt to further close this gap, we exposed a mixture of common polymer types (polyethylene, polypropylene, polystyrene, polyvinyl chloride) for 21 days in well-monitored surface water in a tributary of the river Rhine (south of Frankfurt/Main, Germany). The analytical target screening for trace pollutants in water, sediment, suspended matter and exposed MPs was performed either by liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). In addition, adverse effects of water and particle samples were assessed in terms of acute toxicity for zebrafish using the fish embryo toxicity test according to OECD TG 236 (2013), potential neurotoxic effects by means of acetylcholine esterase (AChE) activity and larval visual motor response test (VMR), as well as effects of dioxin-like substances *via* the ethoxyresorufin-*O*-deethylase (EROD) assay. By coupling analytical target screening for anthropogenic contaminants with various sensitive biomarkers and endpoints in early developmental stages of zebrafish, we provide further evidence for the low capacity of MPs to act as a vector for environmental pollutants.

V.3 Material and methods

V.3.1 Material and chemicals

A mixture of irregular particles of four different MPs was used for environmental exposure, which comprised 50 % polyethylene (PE), 25 % polypropylene (PP), 15 % polystyrene (PS) and 10 % polyvinyl chloride (PVC). Polymers were purchased from Basell Polyolefine (Wesseling, Germany) and BASF (Ludwigshafen, Germany). A final concentration of 100 mg/L of particle samples (sediment, suspended matter, MP mixture) was used in the experiments, except for larval VMR tests where only 5 mg/L was used to avoid masking of behavioral effects due to morphological malformations or incorrect video tracking of particles instead of larval zebrafish. All chemical reagents were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany). Measurement of protein contents was carried out with the DC™ Protein Assay kit from Bio-Rad (Feldkirchen, Germany), using bovine serum albumin (0.125 - 2 mg/ml, in phosphate buffer) as standard.

V.3.2 Exposure of microplastics in Schwarzbach and sampling

The Schwarzbach is a tributary of the river Rhine (south of Frankfurt/Main, Germany, Fig. 21A) and was chosen as the site for MP exposure. Due to continuous monthly monitoring by the Hessian Agency for Nature Conservation, Environment and Geology (HLNUG, Wiesbaden, Germany), the presence of different organic pollutants, pharmaceuticals and pesticides has been well documented in Schwarzbach water over the past years (HLNUG, 2019, 2020). Exposure started at the end of June 2019 and lasted 21 days. For exposure and recovery, the MP mixture was placed in cellulose filters and tightly sealed in stainless steel tea infusers (Fig. 21B/C). A total of four infusers were freely hanging in a metal basket that was placed in a water depth of approximately one meter in the Schwarzbach. Suspended matter samplers were deployed in close vicinity to the MP exposure site (Fig. 21D). A chemically well-characterized natural spring 30 km north of Schwarzbach (Niedernhausen, Hesse, Germany, Fig. 21A) was selected as reference site, and water samples were taken at identical time points. Water parameters such as oxygen, temperature, pH, conductivity and salinity were measured on-site using a WTW Multi 350i and WTW Oxi 3310 (Xylem Analytics, Weilheim, Germany). Further parameters (nitrite, nitrate, ammonia, phosphate, hardness) were subsequently determined in the lab using sera® aqua-tests (sera, Heinsberg, Germany). Microplastic samples were exposed in the Schwarzbach for 21 days and water samples were taken at the start and end of exposure (two samplings). Sediment and suspended matter samples from Schwarzbach were collected after 21 days (Fig. 21E).

Fig. 21. (**A**) Sampling points (Schwarzbach and Niedernhausen) and MP exposure site at the Schwarzbach. (**B/C**) The MP mixture was exposed using tea infusers with cellulose lining to minimize particle loss, hanging freely in a metal basket in the stream. (**D/E**) Suspended particle samplers with a total volume of 2 L were deployed in the Schwarzbach (black arrow, **D**). (**E**) Accumulation of suspended particles after 21 days of exposure in the Schwarzbach.

Particle samples were subsequently sieved (< 500 µm) and dried at 40 °C for 120 h and stored at 4 °C. Water samples for toxicological tests from the Schwarzbach and the natural spring were filtered using an 8 µm cellulose filter MN 640 w (Machery Nagel, Düren, Germany) before use and stored at 4 °C (storage did not exceed one week). Further aliquots for behavioral tests were frozen and stored at -20 °C. The particle samples (MPs clean, MPs exposed, suspended matter and sediment) were prepared by adding 100 mg/L of the dried matrix to artificial water (according to OECD TG 236). Uniform mixing was achieved by aeration with glass Pasteur pipettes (Brand, Wertheim, Germany). Water samples were applied without further dilution. Before use, samples were thawed if necessary, brought to temperature, aerated and pH was adjusted (pH 7.75 ± 0.02) according to OECD TG 236.

All toxicological tests were performed independently in triplicates. For this purpose, zebrafish embryos were exposed to either water samples (Schwarzbach or spring water) or particle samples consisting of clean MP mixture (pre-exposure), loaded MPs (post-exposure), sediment and suspended matter. Effects were compared with zebrafish exposed to negative control (artificial water) and respective positive controls. The tests were performed with water samples from the first and last days of the exposure period. Effects on larval swimming activity (VMR tests) were investigated by exposure to water samples from the end of the exposure period. Particle samples (MP mix, sediment, suspended matter) were collected and analyzed after 21 days.

V.3.3 Analytical quantification of water, particle and microplastic samples

Particle size distribution measurements and particle counting were jointly conducted with Stefan Dittmar.

For target analysis of water and particle samples, either gas chromatography with electron ionization mode coupled to a mass spectrometer (GC-EI-MS, carrier gas helium, 7890B, 5977B, Agilent Technologies, Santa Clara, USA) with a HP5ms capillary column (30 m × 250 µm × 0.25 µm, Agilent, Waldbronn, Germany) or an ultra high performance liquid chromatography (LC-MS, Nexera Shimadzu, Duisburg, Germany) with an ACQUITY UPLC HSS T3 column (2.1 × 50 mm, 1.8 µm, flow rate 0.5 ml/min, Waters, Eschborn, Germany) coupled with a Sciex 5500 spectrometer (Darmstadt, Germany) were used.

With regard to the variety of contaminants and different quantification methods, water samples were either measured directly or had to be pretreated prior to measurements. Depending on the targeted compounds, samples had to be enriched by solid-phase extraction according to DIN EN 12393-1:2014-01 (DIN e.V., 2014) or according to DIN 38407-42:2011-03 (DIN e.V., 2011), the corresponding methods for pretreatment are indicated in Tab. 10. Dry particle samples were extracted for 60 min in acetone using ultrasound. After filtration and before measurement, extracts were concentrated down to 1 ml for GC-EI-MS measurements or dried and resuspended in methanol and water (1:1) for quantification using

LC-MS. Injection volume was set to 1 µl, and atrazine and N,N'-diphenyl guanidine-d10 were used as internal standards.

After exposure in the Schwarzbach, the recovered MP mixture, as well as sediment and suspended matter samples, were analyzed according to Eitzen et al. (2019) to determine particle size distributions and quantify particle amounts using a small volume syringe system particle counter (SVSS, PAMAS, Rutesheim, Germany). The laboratory glassware was cleaned with distilled water before use, rinsed twice using ultra-pure water with an intermediate ultrasound treatment step (10 min) to prevent cross-contamination. Suspensions of 0.01 g/L were prepared with ultra-pure water (resistivity > 17 MΩ·cm, ELGA Berkefeld LabWater, Veolia Water Technologies, Celle, Germany) and 0.01 % of the surfactant SF100 (NovaChem) for homogenous dispersion of particles. All samples were treated with ultrasound for 10 min before measurements, to break up particle agglomerations and to improve single particle detection. Four replicate measurements were performed for each particle type with a laser-diode sensor in a size range of 5 µm to 200 µm.

V.3.4 Zebrafish (*Danio rerio*) husbandry and egg collection

Adult zebrafish (*Danio rerio*) from the "Westaquarium" strain maintained at the animal rearing and maintenance facility at the Aquatic Ecology and Toxicology Group at University of Heidelberg (licensed by regional animal welfare authorities under no. 35–9185.64/BH) were used for breeding. Rearing, breeding conditions and egg collection were conducted as described by Lammer et al. (2009).

V.3.5 Toxicity testing of microplastics and environmental samples with zebrafish

To prevent sorption of contaminants from particle samples on plastic surfaces and thus alter concentrations unpredictably, all tests with particle samples were carried out in HPLC flat-bottom glass vials (neoLab Migge, Heidelberg, Germany), inserted into 24-well plates (TPP, Trasadingen, Switzerland). Furthermore, the daily exchange of test solutions was performed with glass Pasteur pipettes. In general, all samples (water and particles) have been applied in the selected bioassays. An exception had to be made for sediment samples from Schwarzbach, which have not been tested in VMR tests due to the increased particle size that impaired detection of zebrafish using the EthoVision XT software (refer to chapter V.3.5.4).

V.3.5.1 Zebrafish embryo toxicity test

The acute toxicity was assessed according to OECD TG 236 (2013) by exposure of freshly fertilized eggs of *Danio rerio* (< 1 hpf) to water (Schwarzbach and spring) and particle samples (clean MP mix,

exposed MP mix, sediment, suspended matter), always in comparison to negative control (artificial water according to OECD TG 236) and positive control (4 mg/L 3,4-dichloroaniline). After fertilization was confirmed, the eggs were either transferred to HPLC flat-bottom glass vials or directly into 24-well plates with 2 ml of the test solution. All test vessels were pre-incubated with the according solution and sealed with self-adhesive foil (SealPlate®, EXCEL Scientific, Asbach, Germany) to prevent evaporation and cross-contamination. Semi-static exposure with daily renewal of solutions was conducted at 26 ± 1 °C at 16:8 light:dark regime with 20 embryos per treatment and per replicate (n = 3 × 20). Lethal and sublethal effects were documented at 24, 48, 72, 96 hpf, according to OECD TG 236 and Nagel (2002), using a Zeiss Olympus CKX41 microscope with Zeiss Axio Cam ICc1 camera (software Zen lite 2011, Zeiss, Oberkochen, Germany). Tests were considered valid with an effect rate below 10 % in negative controls and > 30 % mortality in positive controls, and results were expressed as cumulated effects in percent after 96 hpf.

V.3.5.2 Acetylcholine esterase activity in zebrafish

The acetylcholine esterase activity was investigated to assess possible neurotoxic effects of present contaminants in water and particle samples (e.g., organophosphate pesticides, PAHs, dioxins, plasticizers, etc.) according to a protocol established by Kais et al. (2015). In short, 15 zebrafish embryos per treatment were exposed as described for the zebrafish embryo toxicity test. A negative and positive control (artificial water and 2 mg/L of paraoxon-methyl) were included as well. After 96 hpf, embryos were washed three times in artificial water for 10 min and excess water was removed before shock freezing in liquid nitrogen. For analysis, pseudoreplicates of 3 × 5 pooled zebrafish per treatment (15 embryos in total per treatment and replicate, n = 3 × 15) were thawed on ice and homogenized for 60s at 30 Hz in 400 µl ice-cooled phosphate buffer with 0.1 % Triton X-100 using a TissueLyser II (Qiagen, Hilden, Germany). After centrifugation with a Heraeus Multifuge 1 S-R, for 15 min at 4 °C and 10.000 × G (Thermo Scientific, Schwerte, Germany), the supernatant was transferred and stored on ice for further enzyme kinetic and protein measurements. Enzyme kinetics were determined in a 96-well plate (TPP, Trasadingen, Switzerland) at 415 nm for 10 min using a GENios plate reader (Tecan, Crailsheim, Germany). Phosphate buffer served as a blank and samples were applied in quadruplicates (50 µl), before 50 µl phosphate buffer and 100 µl of 0.89 mM 5,5′-dithiobis-2-nitrobenzoic acid were added. The kinetic reaction was initiated by the addition of 100 µl of 0.9 mM acetylthiocholine iodide and changes in optical density and protein content (ΔOD/min/mg of protein) were recorded over time. AChE activity values were expressed as a percentage of the negative control and the enzyme activities were standardized to the measured protein content.

V.3.5.3 *In vivo* 7- Ethoxy-resorufin-*O*-deethylase (EROD) assay in zebrafish

To evaluate the potential effects of dioxin-like substances in the samples, the *in vivo* ethoxyresorufin-*O*-deethylase (EROD) induction was investigated according to Kais et al. (2018). In brief, 10 zebrafish embryos per treatment/replicate (n = 3 × 10) were raised in artificial water until 96 hpf and were subsequently exposed to the different samples for 3 h. Exposure to 75 µg/L β-naphthoflavone served as a positive control. The embryos were then rinsed twice in artificial water for 5 min and incubated in 0.6 mg/L 7-ethoxyresorufin for 20 min. After immobilization with 0.016 % tricaine (MS 222, methane sulphonate), embryos were embedded in 1 % low-melting agarose (SeaKem HGT Agarose, Cambrex BioScience, Rockland, USA) in lateral orientation (left side down) and covered with 0.016 % tricaine solution to maintain viability. The resorufin fluorescence signal was measured using an inverted Nikon Eclipse 90i epifluorescence microscope (λ_{EX} = 560 ± 20 nm, λ_{EM} = 630 ± 30 nm, Nikon, Duesseldorf, Germany) at 10 × magnification (Nikon Plan Flour water immersion objective NA 0.17, WD 16.0 mm) with a Nikon DS-Ri-1 camera. The images were acquired and processed with NIS-Elements 4.0 imaging software (Nikon, Duesseldorf, Germany), and signals were normalized to basal background fluorescence signals of the negative controls.

V.3.5.4 Visual motor response test with zebrafish

Locomotor behavior of larval zebrafish was investigated *via* the light-dark transition test (visual motor response, VMR) to determine potential adverse effects of neuroactive substances in water and particle samples (Kokel et al., 2010; Marcato et al., 2015). Therefore, zebrafish embryos were continuously exposed in a semi-static exposure scenario for 96 h to either water or particle samples (without sediment samples), artificial water (negative control) and positive control (0.05 mg/L chlorpyrifos) in 25 ml sample volume. Behavioral effects of chlorpyrifos, such as reduced larval locomotion, have already been described in detail (Kienle et al., 2009; Dishaw et al., 2014). At 96 hpf, 12 zebrafish without morphological alterations per treatment and replicate (n = 3 × 12) were chosen and transferred to HPLC flat-bottom glass vials with 400 µl sample volume and subsequently inserted into a custom-made plate to analyze 6 × 6 zebrafish embryos simultaneously. For video analysis, the plate was then placed in a water bath (26.4 °C) inside the DanioVision™ box (Noldus, Wageningen, Netherlands), as described by Heinrich et al. (2020). During the acclimatization phase of 4 h, the lights remained on. Recording started with an initial light phase of 10 min to track the basal activity of zebrafish, followed by a dark phase of 10 min. In total, seven light and six dark phases were recorded. If the movement tracking was erroneous (threshold of "missing samples" was set to ≥ 0.5 % per fish/per phase), individual zebrafish were excluded from the analysis, resulting in a total of 31 - 36 zebrafish per treatment. The activity (total distance moved during light/dark phase in cm/min) was tracked and recorded using EthoVision XT software (Version 11.5, Noldus, Wageningen, Netherlands).

V.3.6 Data analysis

For statistical evaluation, data were tested for normality and equal variances. To identify statistically significant differences (p-value = 0.05) of treatments compared to control groups, either parametric ANOVA followed by Holm-Sidak *post-hoc* test or nonparametric Kruskal-Wallis analysis followed by Dunnett's *post-hoc* test was performed using SigmaPlot 13 (Jandel, Systat, Erkrath, Germany). Significant differences between samplings were analyzed using a t-test. VMR data were analyzed using GraphPad Prism (v 6.07 GraphPad, La Jolla, California, USA) with two-way ANOVA for repeated measurements followed by Holm-Sidak *post-hoc* test to distinguish effects over time and between light/dark phases. Results were expressed as mean values (MV) ± standard deviation (SD).

V.4 Results

V.4.1 Analytical quantification of water and particle samples from Schwarzbach

Water samples from Schwarzbach and spring did not differ significantly regarding physicochemical properties, except for nitrate (Tab. 9). However, all sample parameters were within the range of optimal rearing conditions for zebrafish and were therefore suitable for immediate application (Lawrence, 2007; Varga, 2016). Prior to the experiments, the pH was always adjusted according to OECD TG 236, and all samples (water and particle samples) were aerated to facilitate uniform mixing.

Tab. 9. Water parameters from Schwarzbach and spring water samples from both sampling time points. Oxygen, temperature, conductivity, pH and salinity measured on-site with WTW Multi 350i and WTW Oxi 3310 (Xylem Analytics, Weilheim, Germany). Nitrite, nitrate, ammonia, phosphate and hardness determined in the laboratory using sera® aqua-tests (sera, Heinsberg, Germany).

	Schwarzbach			**Schwarzbach**		**Spring**	
On-site measurements	**1 day**	**21 days**	**Lab measurements**	**1 day**	**21 days**	**1 day**	**21 days**
Oxygen [%]	63.4	69.0	Nitrate [mg/L]	10.0	10.0	0.0	0.0
Temperature [°C]	27.6	21.0	Nitrite [mg/L]	0.0	0.0	0.0	0.0
Conductivity [µS/cm]	911	720	Ammonia [mg/L]	0.2	0.2	0.0	0.0
pH	7.7	7.3	Carbonate hardness [°dH]	9	7	2	2
Salinity [pSU]	0.4	0.3	Total hardness [°dH]	11	11	2	2
			Phosphate [mg/L]	0.5	0.25	0.0	0.0

No contaminants were detected in spring water from Niedernhausen and therefore the samples were classified as pollutant-free (Annex III.1, Tab. A5). The analytical target screening for a total of 378 different substances and metabolites in Schwarzbach samples revealed 70 compounds at the beginning of the exposure period (June) and 94 compounds at the end of the exposure period in (July) as shown in the Annex III, Tab A6. These included pharmaceuticals (beta blocker, antibiotics, antidepressants, etc.) plant protection products (herbicides, insecticides, plant growth regulator, etc.), additives (per- and polyfluoroalkyl substances, plasticizers, flame retardants, etc.), sweeteners (sucralose, acesulfame, sodium cyclamate, etc.) and caffeine. For all substances detected, literature data were screened for potential adverse effects in zebrafish. Of 94 compounds, 41 substances (44 %) have not been investigated with regard to (eco-)toxicological effects in zebrafish. And for additional 21 compounds, no adverse effects have been detected in zebrafish at environmentally relevant concentrations. For another five compounds, the described effects in the literature were contradictory, meaning that these compounds could not be excluded for the evaluation of acute toxic effects in zebrafish presented in this study. When comparing the measured amounts of pollutants with the annual mean values, a distinct exceedance of the annual mean concentrations by a factor of 1.3 - 6 was observed for several pharmaceuticals (cetirizine, citalopram, diclofenac) and additives (benzotriazole, PFBS, PFOA, TPP, TCEP, TCPP) in water samples from Schwarzbach (Tab. 10). An overview of the compounds that gave rise to potential effects in zebrafish (32 substances) is given in Tab. 10, including measured concentrations [µg/L] and annual mean concentrations from 2019.

Tab. 10. List of detected compounds [µg/L] in water samples from Schwarzbach, causing adverse effects in zebrafish. Detection method: Liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS), superscripts indicate the different pretreatment protocols ([1] DIN 12393-1:2014-01, [2] DIN 38407-42:2011-03). Concentrations below the limit of quantification are indicated (<). Compounds: Per- and polyfluoroalkyl substances (PFAS). Possible modulation of effects (↑↓ up/down regulation). Endpoints: Endocrine disruptor (EDC), cytochrome P450 subfamily (CYP), acetylcholine esterase (AChE), Aryl hydrocarbon receptor (AhR). Literature data with contradictory results are marked (*). *Data were produced jointly with Sven Huppertsberg*.

			2019					
		Compound	**June**	**July**	**Annual mean**	**Detection**	**Adverse effects in zebrafish**	**Reference**
Pharmaceuticals	lipid regulator	Bezafibrate	0.00	0.03	0.05	LC-MS	↑ Genotoxic effects (*in vitro*), EDC	Rocco et al., 2010; Velasco-Santamaría et al., 2011
	anticonvulsant drug	Carbamazepine	0.26	0.54	0.46	LC-MS	↑ Mortality, feeding behavior, ↑ AChE, EDC	van den Brandhof and Montforts, 2010; da Silva Santos et al., 2018; Fraz et al., 2018; Pohl et al., 2019
	metabolite of Carbamazepine	Dihydroxycarbazepine	0.68	0.88	0.89	LC-MS	↑ Acute toxicity	Pohl et al., 2019
	metabolite of Carbamazepine	Carbamazepine-10,11 epoxide	<	0.05	0.06	LC-MS [1]	↑ Acute toxicity	Pohl et al., 2019
	antihistamine	Cetirizine	0.34	0.83	0.50	LC-MS	↑ Mortality, ↓ Activity	Bittner et al., 2019
	antidepressant, SSRI	Citalopram	0.00	0.05	0.01	LC-MS	↑ Neurotoxic effects, ↓ Activity	Bachour et al., 2020; Steele et al., 2018; Zindler et al., 2019
	analgesic, anti-inflammatory drug	Diclofenac	0.33	5.30	1.35	LC-MS [1]	No toxicity, ↓ Embryonic motion, ↑↓ Behavior/neurotoxicity, No effects	Hallare et al., 2004; Memmert et al., 2012; Xia et al., 2017 *
	antibiotic	Erythromycin	0.04	0.09	0.08	LC-MS	↓ CYP1 inhibition (*in vitro*)	Smith et al., 2012
	local anesthetic, antiarrhythmic agent	Lidocaine	0.06	0.12	0.10	LC-MS [1]	↓ AChE, ↓ Activity	de Abreu et al., 2019

Tab. 10. continued

Pharmaceuticals	antihypertensive drug	Metoprolol	0.23	0.38	0.38	LC-MS	Cardiac dysfunction	Meng et al., 2020; Sun et al., 2014	
	tranquilizer, metabolite of Diazepam	Oxazepam	0.00	0.03	0.03	LC-MS	No toxicity, ↓ Activity	Pohl et al., 2019; Vossen et al., 2020	*
	antibiotic	Sulfamethoxazole	0.12	0.23	0.18	LC-MS	Thyroid disruption	Raldúa et al., 2011	
	opioid, analgesic	Tramadol	0.17	0.56	0.45	LC-MS [1]	↓ Activity, No effects	Sehonova et al., 2016; Bachour et al., 2020	*
	antidepressant	Venlafaxine	0.08	0.19	0.19	LC-MS [1]	↓ Reproductive output, ↑ Acute toxicity	Galus et al., 2013	
Plant protection products	fungicide	Azoxystrobin	0.00	0.05	0.01	LC-MS	↑ Hepatotoxic effects, ↑ Oxidative stress, genotoxic effects	Cao et al., 2016; Han et al., 2016; Jia et al., 2018	
	herbicide	Metribuzin	0.00	0.05	0.03	LC-MS	↑ Mortality	Plhalova et al., 2012	
	herbicide	Terbutryn	<	0.036	0.03	LC-MS [1]	↑ Acute toxicity	Plhalova et al., 2009	
Additives	anticorrosive	Benzotriazole	3.8	7.8	5.29	LC-MS [1]	Induction of AhR pathway, ↑↓ *cyp1a1*, ↑ Oxidative stress, liver damage	Fent et al., 2014; Hemalatha et al., 2020; Tangtian et al., 2012	
	plasticizer	Bisphenol A	0.04	0.01	0.04	LC-MS [1]	↑ Activity, EDC, ↑ Mutagenic effects	Saili et al., 2012; Staples et al., 2011	
	PFAS	PFBS	0.013	0.005	0.006	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Sant et al., 2019; Ulhaq et al., 2013	
	PFAS	PFHpA	0.010	0.007	0.004	LC-MS [2]	↑ Activity	Menger et al., 2020	
	PFAS	PFHxS	0.009	0.004	0.006	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020	
	PFAS	PFNA	<	0.001	0.001	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020	
	PFAS	PFOA	0.008	0.007	0.006	LC-MS [2]	↑ Activity	Menger et al., 2020	

Tab. 10. continued

Additives	PFAS	PFOS	0.009	0.010	0.009	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020	
	plasticizer, flame retardant	Triphenyl phosphate (TPP)	0.00	0.02	0.00	GC-MS [1]	↓ Activity, Thyroid disruption	Jarema et al., 2015	
	flame retardant	Tris(1,3-dichloro-2-propyl) phosphate (TDCP)	0.05	0.05	0.06	GC-MS [1]	Neurotoxic effects, EDC	McGee et al., 2012; Wang et al., 2015a;b	
	reducing agent	Tris(2-carboxyethyl) phosphine (TCEP)	0.10	1.50	0.25	GC-MS [1]	↑↓ Behavior/neurotoxic effects, No toxicity	Dishaw et al., 2014; Behl et al., 2015; Jarema et al., 2015	*
	plasticizer, flame retardant	Tris(2-chloroethyl)-phosphate (TCPP)	0.44	1.04	0.60	GC-MS [1]	↓ Activity	Dishaw et al., 2014	
	PFAS	6:2 FTS	0.003	0.004	0.006	LC-MS [2]	↑ Activity	Menger et al., 2020	
Sweeteners	sugar substitute	Acesulfame K	0.92	1.20	1.36	LC-MS	↑↓ Activity, No toxicity	Dong et al., 2020; Stolte et al., 2013	*
Others		Caffeine	0.61	0.11	0.22	LC-MS	↑ Activity, neuromuscular effects, ↑ Acute toxicity	Rah et al., 2017; Rodriguez et al., 2014	

Tab. 11. List of detected compounds [ng/10 mg dry mass] in particle samples from Schwarzbach and possible adverse effects in zebrafish. Detection method: Liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). Octanol-water partitioning coefficient (log K_{OW}). Possible modulation of effects (↑↓ up/down regulation). Endpoints: Acetylcholine esterase (AChE). Data are given as mean values (n = 2 - 3). *Data were produced jointly with Sven Huppertsberg.*

Class		Compound	Concentration [ng/10 mg dry mass] MP mix	Sediment	Suspended matter	Method	log K_{OW}	Adverse effects in zebrafish	Reference
Additives	vulcanization accelerator	1,3-diphenyl guanidine	576.5	120.3	105.7	LC-MS	1.7 - 2.9	Not validated	-
	plasticizer, flame retardant	Tris(2-chloroethyl) phosphate (TCPP)	-	1.7	1.9	GC-MS	1.4 - 1.8	↓ Activity	Dishaw et al., 2014
Pharmaceuticals	antiemetic, neuroleptic drug	Amisulpride	-	2.2	2.8	LC-MS	1.5	Not validated	-
	local anesthetic, antiarrhythmic agent	Lidocaine	7.7	6.8	5.6	LC-MS	2.3	↓ AChE, ↓ Activity	de Abreu et al., 2019
	antihypertensive drug	Telmisartan	-	22.4	29.4	LC-MS	6.9 - 8.4	Not validated	-

During the exposure period, the number of substances detected in water samples from the Schwarzbach increased by 34 % within the experimental period. Moreover, the concentration of the majority of detected substances increased over time, for example, the anticorrosive agent benzotriazole increased from 3.80 µg/L to 7.80 µg/L and the anti-inflammatory drug diclofenac from 0.33 µg/L to 5.30 µg/L (Tab. 10).

The particle samples from the Schwarzbach (MP mixture, sediment and suspended matter) were screened for 124 different substances (Annex III.1, Tab. A7) similar to the water samples. However, the analysis could only detect five compounds belonging either to the class of pharmaceuticals or additives (Tab. 11). Due to the extraction of particles with acetone, unknown additives and organic substances were most likely extracted as well, and thus suppressed the measurement signals during GC/LC-MS screening. Therefore, only the presence of substances could be successfully documented, but the quantification was impaired. Of five detected compounds, only two substances (lidocaine and 1,3-diphenyl guanidine) were also detected in the exposed MP samples.

V.4.2 Characterization of microplastic mixture and particle samples

This section includes content that was created jointly with Stefan Dittmar.

The SVSS measurements were performed to determine the potential particle loss during exposure of the MP mixture and the particle size distribution of all particle samples. Before exposure in the Schwarzbach, the MP mixture consisted of 5265 ± 128 particles/ml (Fig. 22A, red line), and after the exposure 3600 ± 94 particles/ml could be recovered from the infusers (Fig. 22A, yellow line), which corresponds to a loss of approximately 32 %. The particle fraction < 50 µm was affected the most (Fig. 22B), as the small particles were probably washed out during the exposure. The natural particle samples were analyzed similarly to the MP samples. The suspended particle samplers were characterized to be most successful in collecting particles ≤ 60 µm (Liess et al., 1996), which has been reflected in the measured particle size distribution of the suspended matter in our study (Fig. 22B, blue line). The cumulated particle number of suspended matter amounted to 7734 ± 573 particles/ml, with the fraction of small particles being highest compared to the other samples. The sediment samples were collected in the embankment close to the water surface and were composed of larger particles (≥ 50 µm) compared to the suspended matter (Fig. 22B, green line). Nevertheless, the cumulated particle number was similar to the exposed MP mixture with 4533 ± 125 particles/ml. Besides the increased fraction of small particles in the suspended matter samples and thus an overall increased particle number, both the MP mixture (pre-exposure) and the sediment samples were comparable in their particle size distribution and overall quantity.

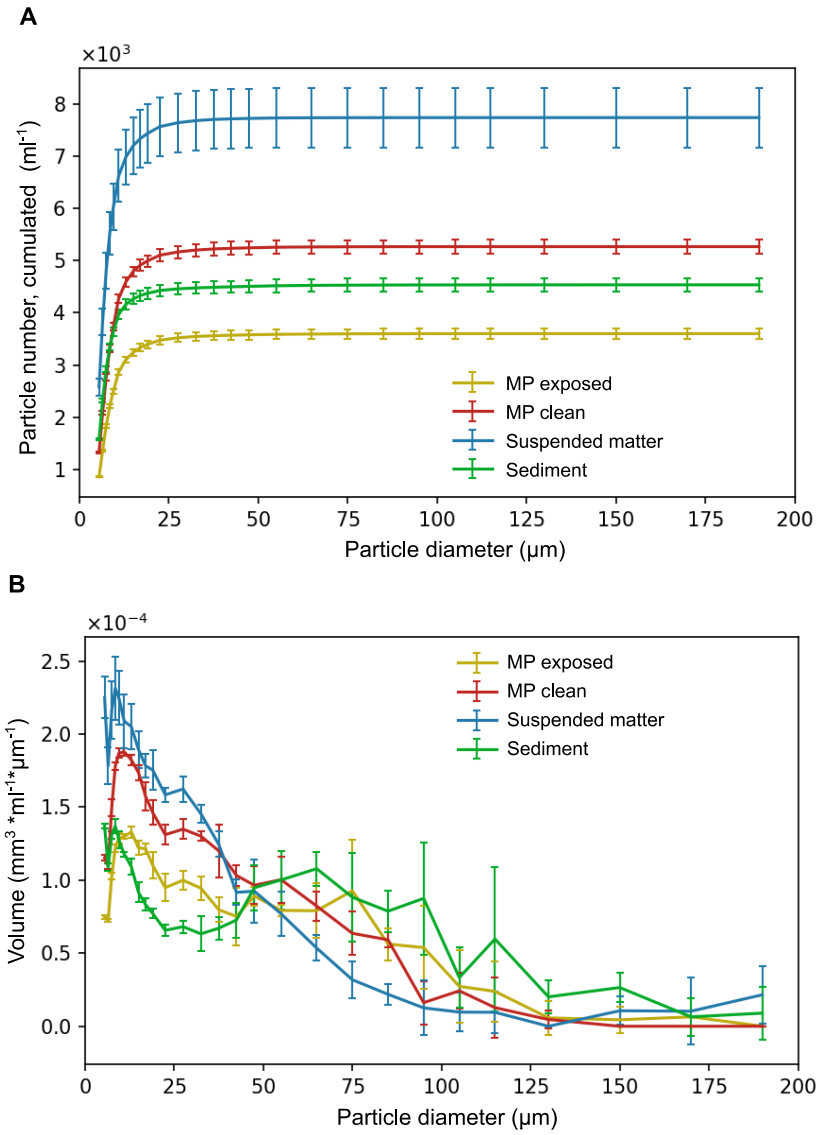

Fig. 22. Particle numbers and particle size distribution of particle samples measured using a SVSS (PAMAS, Rutesheim, Germany). **(A)** Cumulated particle numbers of particle samples, indicating slight particle loss of MP after exposure. **(B)** The particle size distribution of particle samples, indicating larger fractions of small particles (< 50 µm) in suspended matter samples and clean MP mix compared to sediment samples. Data are given as mean values ± SD (n = 4). *Data were produced jointly with Stefan Dittmar.*

V.4.3 Acute toxicity test with zebrafish

The continuous exposure of zebrafish embryos to water samples induced only sublethal effects over 96 h. In general, pericardial and yolk sac edema, as well as cardiovascular effects (reduced blood circulation), were displayed as early as 48 hpf. Exposure to spring water samples induced effects in 4 - 9 % of individuals, while Schwarzbach samples induced effects in 6 - 10 % of zebrafish embryos at 96 hpf (Fig. 23A). No significant differences were detected, although an increased number of sublethal effects was detected in Schwarzbach samples compared to the control (Schwarzbach > spring water > control). Exposure to particle samples induced an increase in sublethal effects (Fig. 25A). Suspended matter and sediment samples induced significantly more sublethal effects after 96 h (34 - 36 % of individuals)

compared to the control ($p < 0.05$). Morphological alterations included pericardial/yolk sac edema, cardiovascular effects (reduced blood circulation, reduced heartbeat) starting at 48 hpf and spinal deformities (lordosis, scoliosis) starting at 72 hpf. Especially for suspended matter and sediment samples these effects resulted in impaired swimming behavior of zebrafish at 96 hpf. Exemplary acute toxicity malformations are shown in Annex III (Fig. 9A). However, clean MPs and exposed MPs induced a similar number of effects (24 % and 28 % respectively) in zebrafish as wells as suspended matter and sediment samples with 34 - 36 % (sediment ≈ suspended matter > MP exposed ≈ MP clean > control).

V.4.4 AChE activity in zebrafish

The acetylcholine esterase activity in whole body homogenates was significantly reduced by exposure to both types of water samples ($p < 0.05$, $p < 0.01$). Both spring water samples significantly reduced AChE activity by 7 - 8 % (1 day, 21 days, $p < 0.05$). Schwarzbach water samples led to a 6 - 11 % reduction in AChE activity compared to control samples, whereby the reduction in zebrafish was significant only upon exposure to water from the last sampling ($p < 0.01$, see Fig. 24A). The AChE activity was altered by water samples accordingly: Control > spring water > Schwarzbach. Nevertheless, no significant differences were detected between the two samplings (t-test). In comparison, particle samples did not induce significant inhibition of AChE activity, although a maximum reduction of 5 % was induced by clean MPs (Fig. 26A).

V.4.5 EROD activity in zebrafish

A significant increase in CYP450 activity ($p < 0.01$, $p < 0.001$) was induced in zebrafish embryos by exposure to Schwarzbach water from both samplings (Fig. 23B, white/grey bars). Metabolic activity was up to 2.5-fold higher in zebrafish compared to controls and spring water samples. Samples from different time points did not induce significant changes for either spring water or Schwarzbach. Thus, EROD activity was altered by Schwarzbach > spring water ≈ control. In contrast, the EROD expression pattern for particle samples differed considerably from water samples, as the EROD induction rates were slightly reduced (Fig. 25B). The exposed MPs induced a significant inhibition of 50 % ($p < 0.05$) compared to the control and clean MP mixture. The suspended matter did not induce any changes deviating from the control. When comparing the exposed particle samples (MP mix exposed, suspended matter, sediment), a significant reduction in CYP450 activity by 50 % ($p < 0.001$) was observed for sediment and exposed MP mixture compared to suspended matter. However, clean MPs also slightly reduced EROD activity, but changes were not significant compared to the control. CYP450 induction pattern was altered by particle samples accordingly: Control ≈ suspended matter ≈ MP clean > MP exposed ≈ sediment.

V.4.6 Zebrafish visual motor response

Based on the results of embryo toxicity tests, the amount of matrix for VMR tests was reduced to 5 mg/L, as previous studies have shown that gross morphological perturbations in larval development could mask behavioral patterns and diminish the sensitivity of these tests (Ali et al., 2012; Legradi et al., 2014; Le Bihanic et al., 2015). The water samples induced fewer sublethal effects in the FET and were therefore not diluted. The VMR tests were conducted to analyze the adaptive behavior to photic stimulation (light-dark transition). The stimulation causes an increase in larval swimming activity in sudden dark phases compared to low activity patterns in light phases (basal level). Adaption proceeds over a certain time, resulting in reduced activity (monotonic suppression) in later phases (Burgess and Granato, 2007; Rihel and Schier, 2012; Nüßer et al., 2016). This native behavior was evident in the control samples, as shown in Fig. 24B (solid black line).

Exposure to water samples from Schwarzbach (Fig. 24B, green line) tended to increase the activity of *Danio rerio* embryos compared to the control, while exposure to spring water samples (red line) caused a slightly reduced activity, which approached the control level in later dark phases. Sustained hypoactivity ($p < 0.05$) was induced by exposure to chlorpyrifos (positive control), whereas Schwarzbach water significantly increased activity ($p < 0.05$) in the final dark phase. Thus, zebrafish swimming activity was altered by Schwarzbach > control ≈ spring water > CPF, indicating slight hyperactivity in light phases and a reduced adaptation to light-dark transition when fish were exposed to Schwarzbach water samples. Exposure of zebrafish to particle samples (Fig. 26B) did not induce behavioral alterations and no clear differences were detected between clean MPs (red line), exposed MPs (green line) or suspended matter (magenta line) compared to the control (black line): Control ≈ MP clean ≈ MP exposed ≈ suspended matter.

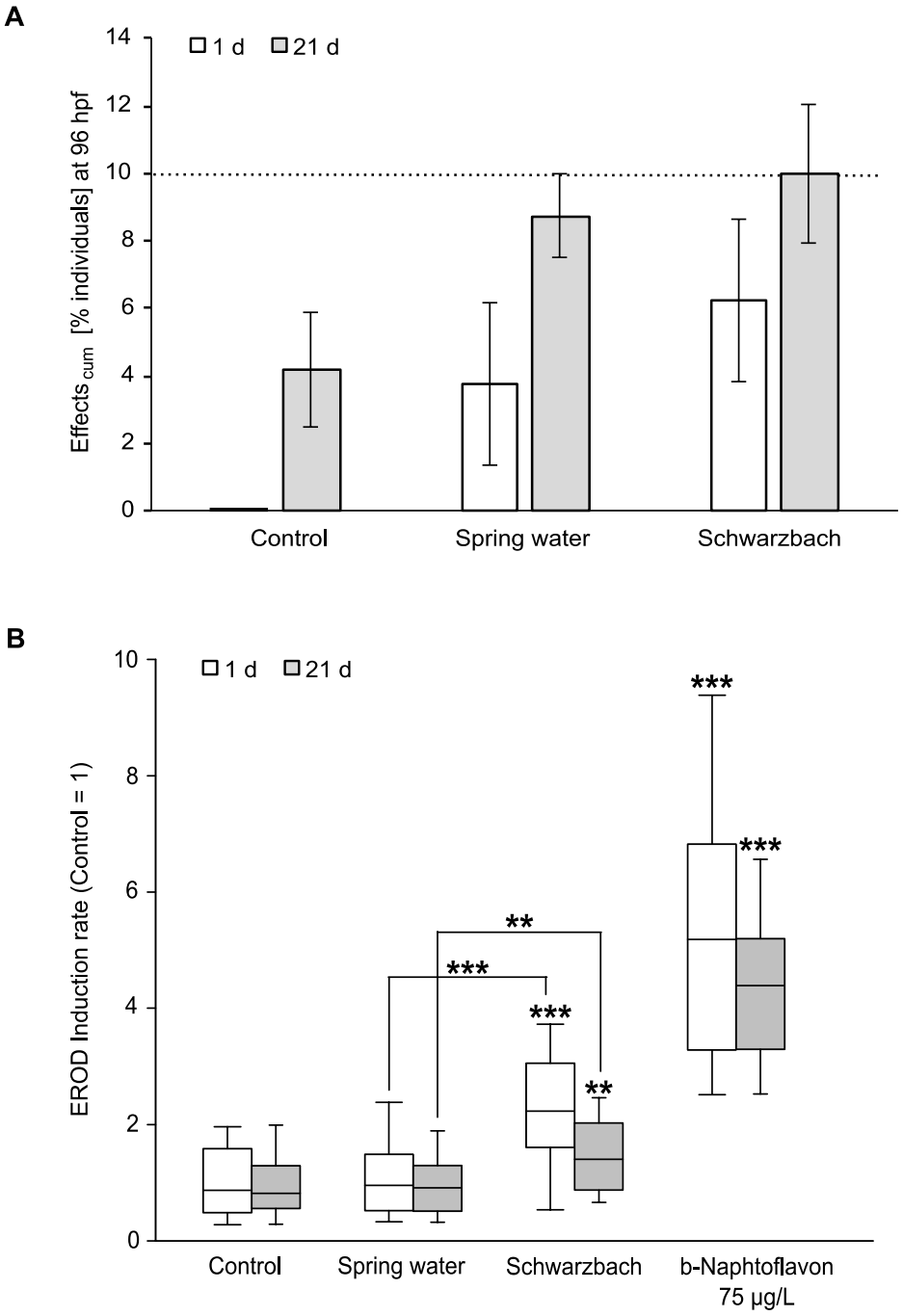

Fig. 23. Results of acute toxicity tests of Schwarzbach and spring water samples with zebrafish (*Danio rerio*). Water samples were investigated at the start (1 d, white bars) and end of the exposure period (21 d, grey bars) except for VMR tests. **(A)** Cumulated sublethal effects of zebrafish embryo toxicity test according to OECD TG 236 at 96 hpf, the dotted line indicates the 10 % validity threshold (nonparametric Kruskal-Wallis analysis and Dunnett's *post-hoc*, N = 3, n = 60). **(B)** EROD induction rate upon 3 h of acute exposure of 96 hpf *Danio rerio* embryos, normalized to background fluorescence of control (nonparametric Kruskal-Wallis analysis and Dunnett's *post-hoc*, data are presented as median, whiskers represent 95 % confidence intervals, N = 3, n = 30). ** $p < 0.01$, *** $p < 0.001$, MV ± SD.

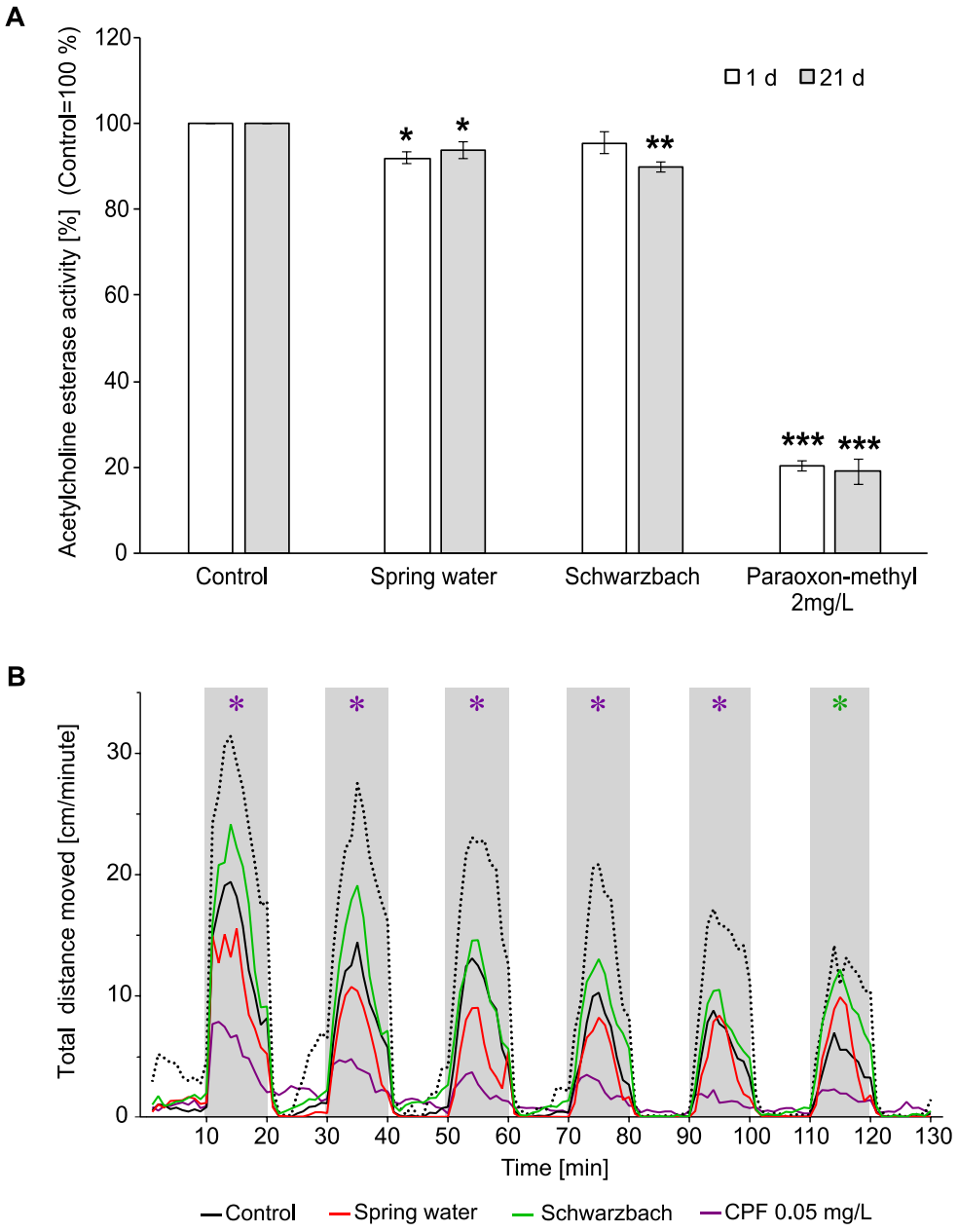

Fig. 24. Results of acute toxicity tests of Schwarzbach and spring water samples with zebrafish (*Danio rerio*). Water samples were investigated at the start (1 d, white bars) and end of the exposure period (21 d, grey bars) except for VMR tests. **(A)** Acetylcholine esterase activity after continuous exposure over 96 h, expressed as % of the control (ANOVA and Holm-Sidak *post-hoc*, N = 3, n = 45). **(B)** Larval visual motor response upon continuous exposure over 96 hpf and light-dark transition test, expressed as total distance moved (cm/per minute). Dotted lines indicate 95 % confidence intervals. Grey columns indicate the dark phases, colors of asterisks indicate the assignment to corresponding exposure scenario (two-way ANOVA and Holm-Sidak *post-hoc*, N = 3, n = 31 - 36, *data were produced jointly with Nadine Kämmer*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, MV ± SD.

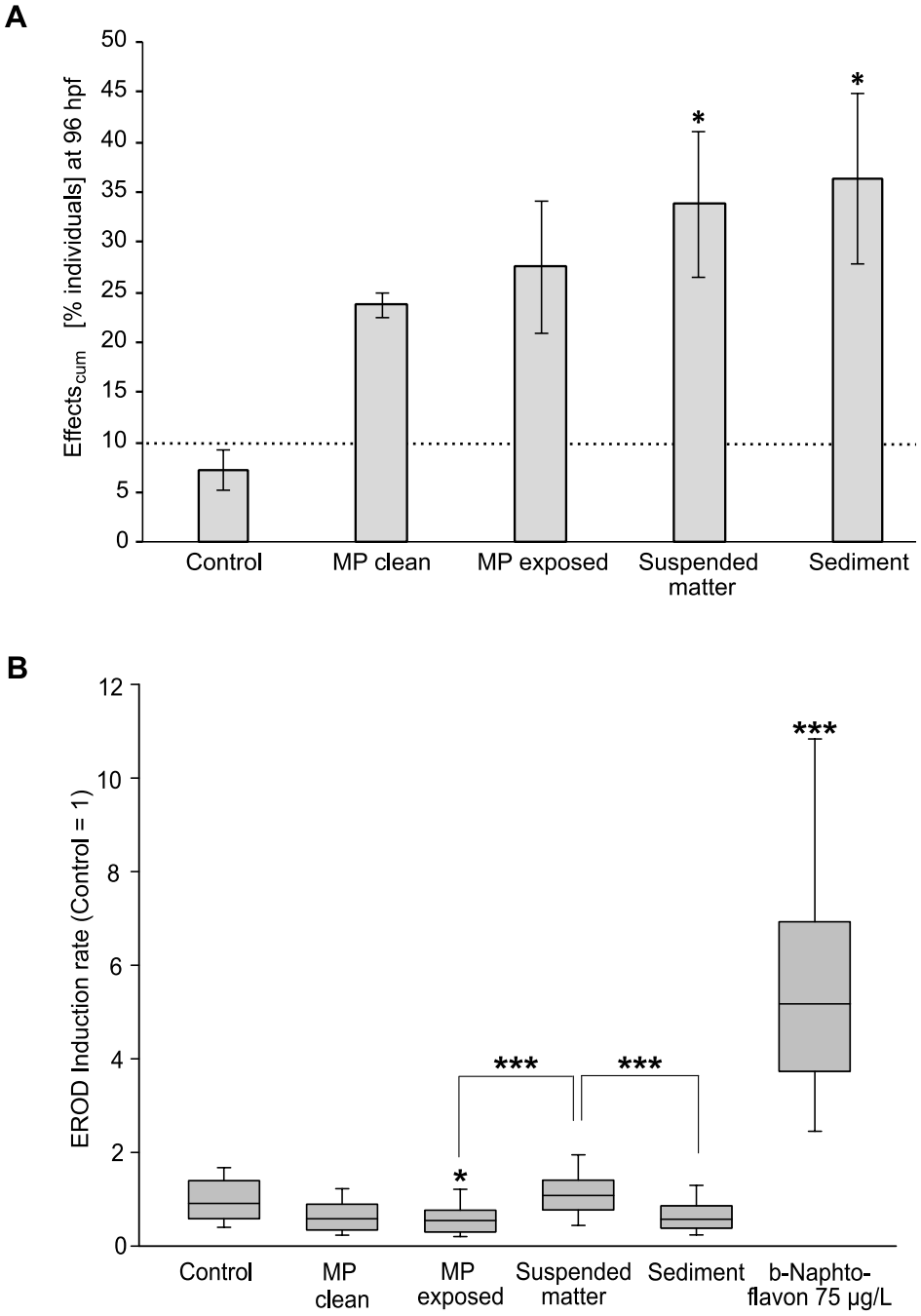

Fig. 25. Results of acute toxicity tests of particle samples (MP, sediment, suspended matter) with zebrafish (*Danio rerio*). Particle samples were investigated only after 21 days of exposure. **(A)** Cumulated sublethal effects of zebrafish embryo toxicity test according to OECD TG 236 at 96 hpf, the dotted line indicates the 10 % validity threshold (nonparametric Kruskal-Wallis analysis and Dunnett's *post-hoc*, N = 3, n = 60). **(B)** EROD induction rate upon 3 h of acute exposure of 96 hpf *Danio rerio* embryos, normalized to background fluorescence of control (nonparametric Kruskal-Wallis analysis and Dunnett's *post-hoc*, data are presented as median, whiskers represent 95 % confidence intervals, N = 3, n = 30). * $p < 0.05$, *** $p < 0.001$, MV ± SD.

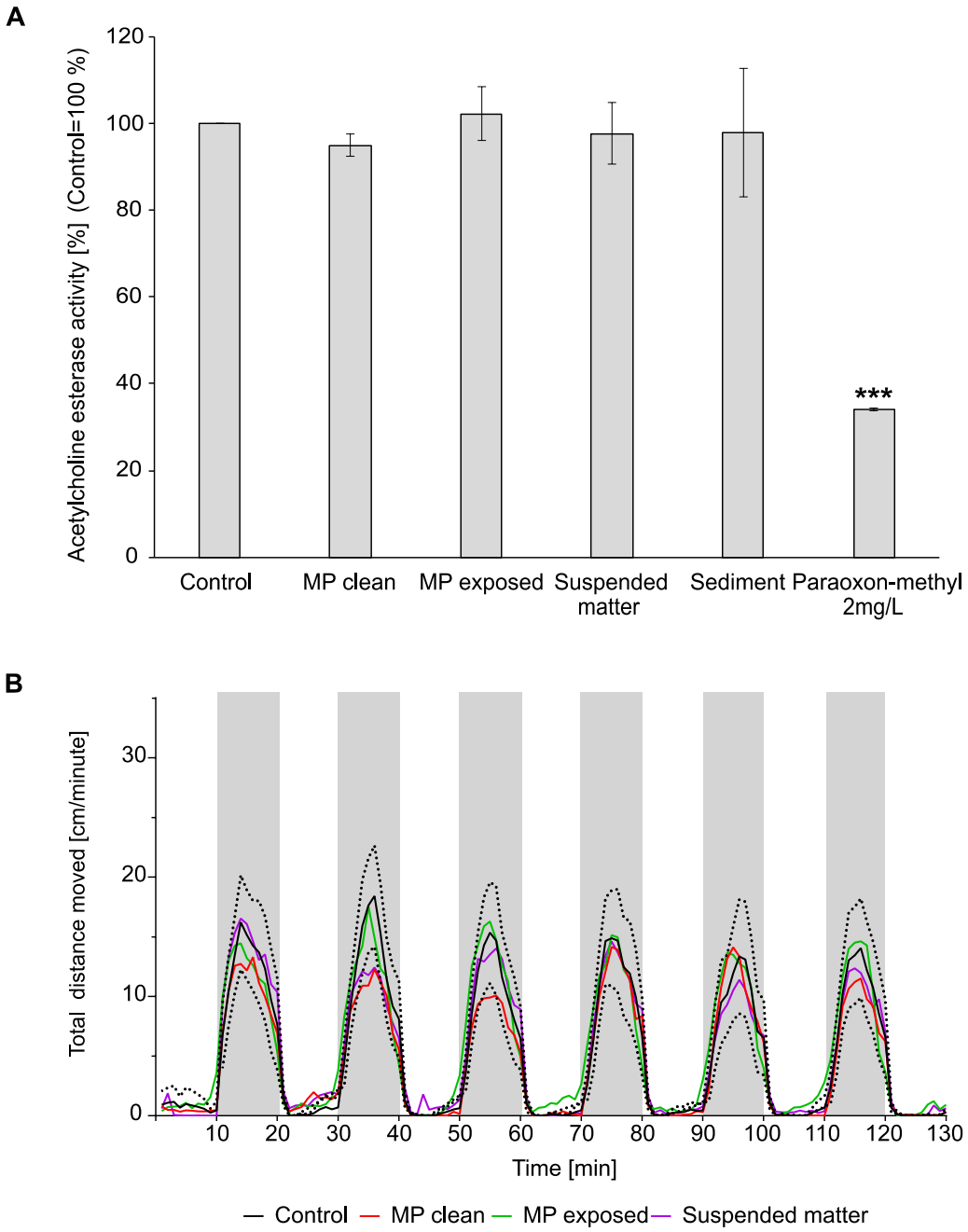

Fig. 26. Results of acute toxicity tests of particle samples (MP, sediment, suspended matter) with zebrafish (*Danio rerio*). Particle samples were investigated only after 21 days of exposure. **(A)** Acetylcholine esterase activity after continuous exposure over 96 h, expressed as % of the control (ANOVA and Holm-Sidak *post-hoc*, N = 3, n = 45). **(B)** Larval visual motor response upon continuous exposure over 96 hpf and light-dark transition test, expressed as total distance moved (cm/per minute). Dotted lines indicate 95 % confidence intervals. Grey columns indicate dark phases (two-way ANOVA and Holm-Sidak *post-hoc*, N = 3, n = 31 - 36, *data were produced jointly with Nadine Kämmer*). *** $p < 0.001$, MV ± SD.

V.5 Discussion

V.5.1 Effects in zebrafish induced by exposure to water samples

Given the increasing amounts of plastic debris in the environment and the proven capacity of MPs to transfer hazardous organic pollutants, studies assessing the potential adverse effects of MPs in aquatic environments under realistic conditions are still scarce but highly demanded (Koelmans et al., 2016; Wagner and Lambert, 2018; Triebskorn et al., 2019). To further close this gap, we exemplarily exposed a mixture of MPs in a polluted stream, determined the occurrence of various anthropogenic contaminants by analytical target screening and investigated acute adverse effects induced by environmental samples and exposed MPs in different biological endpoints using zebrafish embryos.

Throughout the exposure period, the physicochemical water parameters, including ammonia, nitrogenous compounds, phosphate and hardness, were at non-toxic levels (see Tab. 9) and most likely did not induce adverse effects in *Danio rerio*. Regarding the effects of Schwarzbach samples, it became evident that the increase in the number and concentration of compounds over the experimental period was reflected in the increase of acute toxic effects in FET tests (Fig. 23A) and inhibition of AChE activity (Fig. 24A) when zebrafish were exposed to samples from the end of the test period (grey bars). Moreover, spring water slightly inhibited AChE activity ($p < 0.05$) and exposed individuals showed a tendency of hypoactivity in the early light phases of VMR tests (Fig. 24B).

An increase in acute toxic effects and mortality in zebrafish embryos has already been described for 12 of the listed compounds (Tab. 10); pharmaceuticals such as carbamazepine and related metabolites (Pohl et al., 2019), cetirizine (Bittner et al., 2019) and venlafaxine (Galus et al., 2013), plant protection products like metribuzin and terbutryn (Plhalova et al., 2009, 2012) or per- and polyfluoroalkyl substances (Sant et al., 2019; Menger et al., 2020) and caffeine (Rodriguez et al., 2014). All compounds associated with acute toxic effects have been detected in Schwarzbach samples at concentrations between 1 ng/L and 1 µg/L and were thus below the concentrations tested in the literature, explaining the minor increase of cumulated sublethal effects for Schwarzbach water samples over the test period (Fig. 23A).

Exposure to samples from Schwarzbach induced contradictory neurotoxic effects in zebrafish by increasing the swimming activity of embryos during light phases and inhibiting AChE activity. Known AChE inhibitors (e.g., organophosphate pesticides, flame retardants, pharmaceuticals, metals, etc.) can lead to reduced swimming activity in behavioral tests (Tilton et al., 2011; Dishaw et al., 2014; Cao et al., 2018; Massei et al., 2019) and thus impair the adaption to light-dark transition, as observed for 0.05 mg/L chlorpyrifos (positive control) and Schwarzbach samples in the last light phase. Furthermore, Scott and Sloman (2004) assumed that behavioral alterations in fish are coupled to the physiological impact of environmental pollutants, which, compared to the development of morphological deformities, might indicate adverse effects considerably earlier and are therefore a useful measure for assessing possible downstream effects. The increased amounts (> 1 µg/L) of diclofenac, TCEP, TCPP and acesulfame

(Tab. 10) may have altered AChE activity and larval behavior as described previously in several studies (Dishaw et al., 2014; Jarema et al., 2015; Xia et al., 2017; Dong et al., 2020). Besides, several other pollutants have been detected in Schwarzbach water samples at much lower concentrations (< 1 µg/L). Pharmaceuticals such as carbamazepine (Zhou et al., 2019), cetirizine (Bittner et al., 2019), citalopram (Zindler et al., 2019), oxazepam (Vossen et al., 2020) or tramadol (Bachour et al., 2020) have already proven to influence larval behavior, and induce neurotoxic effects in zebrafish. Similar effects have been documented for plasticizers and flame retardants such as PFOA, PFOS, PFNA, PFBS (Menger et al., 2020), TPP (Jarema et al., 2015) and TDCP (McGee et al., 2012), which have also been detected in Schwarzbach samples (0.001 - 0.05 µg/L). Whereas, the increased activity pattern displayed by zebrafish in VMR tests might have been related to the presence of diclofenac (Xia et al., 2017), additives such as bisphenol A (Saili et al., 2012), PFAS (e.g., PFHpA, PFOA, TCEP 6:2 FTS), acesulfame (Dong et al., 2020) or caffeine (Rodriguez et al., 2014).

Since the analytical quantification failed to detect any harmful substances in spring water, effects might be caused by naturally occurring compounds such as dissolved organic carbon (DOC) or humic substances (HS) as described by Steinberg et al. (2008). Successful inhibition of acetylcholine esterase enzymes by humic acids has already been demonstrated (Akincioglu et al., 2017), and the variety of humic compounds is known to affect cytochrome P450 expression and the aryl hydrocarbon receptor (AhR) pathway (Bittner et al., 2006; Matsuo et al., 2006; Janošek et al., 2007). As a consequence, HS could increase the toxicity and bioavailability of anthropogenic pollutants for biota (Chen et al., 2008; Almeida et al., 2019). In addition, HS have been shown to alter locomotion patterns in zebrafish embryos already at sublethal levels by reducing the swimming activity in dark periods, as shown by Almeida et al. (2019), which is consistent with the observations from our VMR tests. However, it should be considered that the overall amount of HS in surface water only contributes a small fraction to the total amount of DOC (Ma et al., 2001; De Schamphelaere and Janssen, 2004) and therefore, the observed effects may not be associated solely with HS.

Further, the EROD activity in zebrafish was not altered by spring water compared to the control. Whereas, Schwarzbach samples significantly increased EROD induction ($p < 0.01$, $p < 0.001$, Fig. 23B) in zebrafish embryos (96 hpf) compared to controls and spring water samples. These effects may have been caused by high concentrations of the anticorrosive benzotriazole (3.8 - 7.8 µg/L), which affects the AhR pathway stimulation according to Fent et al. (2014) as well as *cyp1a* gene induction and caused histopathological lesions in liver tissue of zebrafish (Hemalatha et al., 2020). Further hepatotoxic effects were described for the detected fungicide azoxystrobin (0.05 µg/L) by Cao et al. (2016), and inhibition of CYP P450 in zebrafish has been described for the antibiotic compound erythromycin (Smith et al., 2012), which has been detected at 0.04 - 0.09 µg/L in Schwarzbach samples. Most likely, the presence of HS and DOC might have interfered with present EROD inducers, as HS themselves are considered a chemical stressor and may lead to additive effects and interactions with environmental pollutants (Almeida et al., 2019). Furthermore, the metabolic activity might also influence behavioral patterns

(Scott and Sloman, 2004) and might have led to a reduced adaptation pattern of zebrafish in the VMR tests upon exposure to Schwarzbach water samples.

However, all laboratory studies have documented adverse effects of these pollutants at elevated, non-environmentally relevant concentrations in single application scenarios or as binary mixtures, rendering a direct comparison of laboratory and literature data difficult. As already argued by Menger et al. (2020), mixture toxicity cannot be predicted reliably by additive effects only, even when investigating similar compounds such as per- and poly-fluoroalkyl substances. Synergistic toxic effects have been documentted for certain pesticides (organophosphates and carbamates) and azole fungicides in a systematic review by Cedergreen (2014). The author also stated that additive effects of co-occurring pollutants were more likely and therefore can pose a higher risk to aquatic organisms than synergistically acting substances, which require elevated concentrations to elicit synergistic effects (Cedergreen, 2014). However, several compounds detected in Schwarzbach water samples have not been investigated regarding their potential adverse effects on aquatic biota and zebrafish. Several pharmaceuticals and metabolites (e.g., metformin, gabapentin, diatrizoate, candesartan, etc.), plant protection products (dikegulac) and artificial sweeteners (sucralose) have been detected in concentrations well above 1 µg/L (Annex III.1, Tab. A6). Hence, unintended harmful interactions with other compounds should not be excluded. Furthermore, several substances have been concentrated during the exposure period compared to annual mean values. Nonetheless, it seems likely that the complex mixture of 21 contaminants that have been associated with predominantly neurotoxic effects and behavioral alterations, may have caused the illustrated effects in the present study.

V.5.2 Effects in zebrafish induced by exposure to particle samples

The acute toxic effects of the particle samples were less pronounced compared to water samples. Significantly more sublethal effects were documented for the natural particles, possibly due to the variety of sorbed compounds (see Tab. 11). In contrast, only two substances were extracted from the exposed MP mixture. Sediment and suspended matter can be considered to act as passive samplers for hydrophobic environmental contaminants (Pohlert et al., 2011). Despite the relatively short exposure time of the MP mixture compared to the natural particles, the MPs accumulated compounds similar to natural particles. As shown by Jemec Kokalj et al. (2019), MPs exposed to wastewater and landfill leachate for three weeks induced only minor effects in zebrafish embryos, which is in line with the results of our study. However, it should be noted that the number of cumulated sublethal effects was elevated for both MP mixtures (clean and exposed). These effects might be attributed to the fraction of 10 % polyvinyl chloride (PVC) in the MP mixture, as PVC contains toxic plasticizers such as di-2-ethylhexyl phthalate. Phthalates are known to be highly mobile, as they are not chemically bound to the vinyl chloride but embedded in chains of polyvinyl molecules (Akovali, 2012). Furthermore, it has been shown that

di-2-ethylhexyl phthalate induces AhR activity in zebrafish already at low microgram concentrations (Junaid et al., 2018), and therefore might have affected the EROD activity of zebrafish embryos.

The presence of DPG was noticeable among the extracted substances (Tab. 11). DPG originates from tire wear particles and is used as a vulcanization accelerator in rubber processing and polymer production (Evans, 1997). The toxic potential for aquatic organisms has already been investigated, as tire wear particles are omnipresent in surface waters due to road runoff (Marwood et al., 2011; Wagner et al., 2018). So far, no acute toxic potential (≤ 100 mg/L) for aquatic organisms has been documented for tire wear particles or leachates (Gualtieri et al., 2005; Wik and Dave, 2006; Marwood et al., 2011). It is therefore unlikely that DPG extracted from suspended matter, sediment, and exposed MPs has induced adverse effects in *Danio rerio* embryos. However, based on quantitative structure-activity relationship models (QSAR), it was assumed that especially halogenated transformation products of DPG forming under natural conditions might pose an increased toxicological hazard and could therefore pose a risk to aquatic organisms (Sieira et al., 2020).

Another detected compound was the local anesthetic lidocaine, which has been widely accepted as an alternative sedative for the experimental handling of fish (Collymore et al., 2016). Yet, no lethal toxic effects have been documented at concentrations ≤ 100 mg/L (Collymore et al., 2014). The mode of action of lidocaine is based on the blocking of voltage-gated Na^+-channels, leading to altered movement patterns and reduced activity in zebrafish (Collymore et al., 2016; de Abreu et al., 2019). Although lidocaine was extracted from sediment, suspended matter and exposed MPs, behavioral changes or reduction of AChE activity could not be detected for either particle sample, probably due to only a low amount of lidocaine.

Furthermore, only three other substances have been extracted from sediment and suspended matter samples: The antihypertensive drug telmisartan, the plasticizer and flame retardant TCPP and the neuroleptic agent amisulpride. So far, the adverse effects of amisulpride and telmisartan on aquatic organisms have not been studied in detail. Still, it was suggested that transformation products of amisulpride might be more toxic than the parent compound, based on Microtox® tests with *Vibrio fischeri* (Gros et al., 2015). As demonstrated by Dishaw et al. (2014), TCCP did not induce acute toxic effects in zebrafish embryos, but swimming behavior was altered significantly at 100 µM TCPP. However, no behavioral alterations or neurotoxic effects were observed in zebrafish embryos being exposed to natural particles from Schwarzbach. Neither substance had sorbed to the exposed MP mixture, possibly due to the hydrophilic character of TCPP and amisulpride (log K_{OW} 1.4 - 1.8). Nevertheless, the high lipophilicity of telmisartan (log K_{OW} 6.9 - 8.4) might have led to increased sorption and accumulation in natural particle samples but failed to sorb to MPs during 21 days of exposure.

It should be noted that the VMR tests had to be limited in terms of particle load. A high particle load (100 mg/L) interfered with video tracking (pre-test not shown) and induced sublethal morphological deformities in zebrafish embryos (FET), resulting in falsified tracking of zebrafish and impaired

swimming behavior. Therefore, the particle load was reduced to 5 mg/L for all particle samples in the VMR tests. This limitation might be responsible for the reduced effect size in VMR tests and consequently prevented the detection of significant changes in larval swimming behavior, thereby underestimating the effects of particle samples. In future studies, this issue could be addressed by using solvent extracts of particle samples instead of resuspending the particles, as demonstrated by Pannetier et al. (2019b). However, solvent extracts might not constitute environmental conditions of native particles, as particle-associated contaminants are artificially re-dissolved, thus changing the water solubility of contaminants (Kais et al., 2015), apart from the known adverse effects of different solvents on fish larvae (Hallare et al., 2006; Chen et al., 2011; Maes et al., 2012). Therefore, the dispersion of particles in an aqueous phase – as performed in the present study – seems to be a better approach to mimic natural conditions in surface waters. Furthermore, by excluding the masking of behavioral patterns by morphological deformities in zebrafish embryos, potential neurotoxicity can be detected at much lower concentrations than in conventional acute toxicity studies, thereby increasing the sensitivity of this endpoint (Ali et al., 2012).

As argued by Wincent et al. (2015), other environmental pollutants and their metabolites might have been overlooked by the analytical target screening but may have contributed to the acute toxic effects. The suppressed EROD activity by exposure to the exposed MP mixture ($p < 0.05$) and sediment samples may be attributed to the undetected presence of estrogenic compounds, as shown in flounder (Kirby et al., 2007), or due to the co-exposure of polycyclic aromatic hydrocarbons and metals which significantly decreased CYP1A activity in crucian carp (Ding et al., 2014). Other known EROD inhibitors such as carbamazepine, diclofenac and erythromycin (Smith et al., 2012) have also been detected in Schwarzbach water samples. Yet, literature research also revealed that approximately 44 % of the substances detected in water samples have not yet been tested for potential (eco-)toxicological effects in aquatic organisms and therefore require future investigation.

Even though the particle numbers varied among all types of particle samples, no distinct differences in the investigated endpoints based on the particle load could be found. Also, the particle size distribution did not seem to influence the sorption behavior of pollutants or toxic effects in zebrafish. Overall, sediment, suspended matter and exposed MPs caused similar toxic effects in zebrafish. As a result, after 21 days of exposure in the Schwarzbach, MPs seemed to express a similar sorption behavior compared to the natural particles. A possible explanation could be the formation of a biofilm on MPs similar to the natural biofilm on sediment and suspended matter. Biofilm formation has been shown to influence the sorption behavior of MPs by reducing the sorption capacity due to the incorporation of HS and the metabolic capacities of biofilm-associated microbes (Rummel et al., 2017). It seems probable that phthalates derived from the PVC fraction in the MP mixture (pre- and post-exposure) could have induced adverse effects in zebrafish even when exposed to the clean MP mixture. Most likely, the few documented effects could be attributed to the presence of a natural biofilm and associated humic

substances, as well as undetected pollutants or metabolites that were not among the five detected compounds, leading to more complex chemical interactions, as already stated by Cedergreen (2014).

Overall, it should be noted that upon entering aquatic ecosystems, biofilm formation and microbial colonization on MPs significantly alter polymer-specific properties (e.g., surface modification, weight) and consequently induce sedimentation processes (Wright et al., 2013). Over time, MPs may tend to settle in the sediment, to be finally buried and are thus no longer available for biota (Kooi et al., 2018; Scherer et al., 2020a).

V.6 Conclusions

Considering the increasing demand for environmental exposure scenarios to assess the potential of MPs to sorb and transfer hazardous pollutants, a mixture of common MPs was exposed in well-monitored surface water. The detection of several dissolved anthropogenic pollutants has corroborated documented acute toxic effects in zebrafish elicited by water samples. In contrast, exposed MPs caused similar effects compared to natural particles, suggesting an approximation of sorption properties and the formation of a biofilm. Consequently, the function of MPs to act as a vector for anthropogenic pollutants seems to be negligible compared to the vast amount of natural particles and thus, MPs pose hardly any risk to aquatic organisms. However, biofilm formation and behavior of MPs in natural surface water bodies is poorly studied and requires further investigation. It can be assumed that MPs behave similarly to natural inorganic particles when entering aquatic ecosystems and only marginally change the proportion of pollutants available for biota.

V.7 Annex III

III.1. Compounds detected in water samples (Spring and Schwarzbach) and compounds extracted from particle samples

Tab. A5. Complete list of screened compounds [µg/L] in spring water samples (Niedernhausen). Detection method: Liquid chromatography-mass spectrometry (LC-MS). Limit of detection (LoD) and limit of quantification (LoQ) are given as a reference. *Data were produced jointly with Sven Huppertsberg.*

Class		Compound	Concentration [µg/L]	Method	LoD	LoQ
Pharmaceuticals	antihypertensive drug, diuretic	Furosemide	0.00	LC-MS	0.01	0.02
	analgesic, anti-inflammatory drug	Ibuprofen	0.00	LC-MS	0.01	0.02
	antihypertensive drug	Valsartan	0.00	LC-MS	0.01	0.02
	metabolite of Valsartan	Valsartan acid	0.00	LC-MS	0.01	0.02
Plant protection products	herbicide	2,4,5-Trichloro-phenox-yacetic acid	0.00	LC-MS	0.02	0.04
	herbicide	2,4-Dichloro-phenoxya-cetic acid	0.00	LC-MS	0.01	0.02
	herbicide	4-(2,4-dichloro-phe-noxy)butyric acid	0.00	LC-MS	0.01	0.02
	herbicide	Bentazon	0.00	LC-MS	0.01	0.02
	herbicide	Clodinafop	0.00	LC-MS	0.01	0.02
	herbicide	Dicamba	0.00	LC-MS	0.01	0.02
	herbicide	Dichlorprop	0.00	LC-MS	0.01	0.02
	herbicide	Diclofop-methyl	0.00	LC-MS	0.01	0.02
	plant growth regulator	Dikegulac	0.00	LC-MS	0.01	0.02
	herbicide	Fenoxaprop-P-ethyl	0.00	LC-MS	0.005	0.01
	herbicide	Fluazifop-P	0.00	LC-MS	0.01	0.02
	herbicide	Fluroxypyr	0.00	LC-MS	0.01	0.02
	herbicide	Haloxyfop	0.00	LC-MS	0.01	0.02
	metabolite of Icaridin	Icaridin acid	0.00	LC-MS	0.01	0.02
	herbicide	Isoxaflutole	0.00	LC-MS	0.01	0.02
	herbicide	MCPA	0.00	LC-MS	0.01	0.02
	herbicide	Mecoprop	0.00	LC-MS	0.01	0.02
	herbicide, fungicide	Triclopyr	0.00	LC-MS	0.01	0.02

Tab. A6. List of all 94 detected compounds [µg/L] in Schwarzbach water samples and their potential adverse effects in zebrafish. Detection method liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS), superscripts indicate the pretreatment protocols ([1] DIN 12393-1:2014-01, [2] DIN 38407-42:2011-03). Concentrations below limit of quantification are indicated (<). Compounds: Per- and poly-fluoroalkyl substances (PFAS). Possible modulation of effects (↑↓ up/down regulation). Endpoints: Endocrine disruptor (EDC), cytochrome P450 subfamily (CYP), ethoxy-resorufin-*O*-deethylase (EROD), acetylcholine esterase (AChE), Aryl hydrocarbon receptor (AhR). Contradictory results in literature data (*) and no available literature data (not validated) are indicated. *Data were produced jointly with Sven Huppertsberg*.

		Compound	June	July	2019 Annual mean	Min	Max	Detection	Adverse effects in zebrafish	Reference
Pharmaceuticals	antiemetic, neuroleptic drug	Amisulpride	0.06	0.46	0.24	0.00	0.50	LC-MS [1]	Not validated	-
	metabolite of Metoprolol	Atenolol acid	0.21	0.20	0.32	0.20	0.56	LC-MS	Not validated	-
	lipid regulator	Bezafibrate	0.00	0.03	0.05	0.00	0.12	LC-MS	Genotoxic effects (*in vitro*), EDC	Rocco et al., 2010; Velasco-Santamaría et al., 2011
	antihypertensive drug, beta blocker	Bisoprolol	0.10	0.13	0.22	0.10	0.36	LC-MS	No toxicity	Godoy et al., 2020
	antihypertensive drug	Candesartan	0.95	1.80	1.40	0.60	2.30	LC-MS	Not validated	-
	anticonvulsant drug	Carbamazepine	0.26	0.54	0.46	0.26	0.65	LC-MS	↑ Mortality, feeding behavior, ↑ AChE, EDC	van den Brandhof and Montforts, 2010; da Silva Santos et al., 2018; Fraz et al., 2018; Pohl et al., 2019
	metabolite of Carbamazepine	Dihydroxycarbazepine	0.68	0.88	0.89	0.59	1.20	LC-MS	↑ Acute toxicity	Pohl et al., 2019
	metabolite of Carbamazepine	Carbamazepine-10,11 epoxide	<	0.05	0.06	0.04	0.07	LC-MS [1]	↑ Acute toxicity	Pohl et al., 2019
	antihistamine	Cetirizine	0.34	0.83	0.50	0.21	0.83	LC-MS	↓ Activity, ↑ Mortality	Bittner et al., 2019
	antidepressant, SSRI	Citalopram	0.00	0.05	0.01	0.00	0.06	LC-MS [1]	↑ Neurotoxic effects, ↓ Activity	Steele et al., 2018; Zindler et al., 2019; Bachour et al., 2020
	antibiotic	Clarithromycin	0.00	0.09	0.13	0.00	0.27	LC-MS	No toxicity	Baumann et al., 2015

Tab. A6. continued

Pharmaceuticals	antibiotic	Clindamycin	0.05	0.13	0.11	0.05	0.18	LC-MS	Not validated	-	
	platelet aggregation inhibitor	Clopidogrel acid	0.00	0.02	0.01	0.00	0.03	LC-MS	Not validated	-	
	contrast agent	Diatrizoate	2.20	3.10	2.86	1.90	3.90	LC-MS	Not validated	-	
	analgesic, anti-inflammatory drug	Diclofenac	0.33	5.30	1.35	0.33	5.30	LC-MS	No toxicity, ↓ Embryonic motion,↑↓ Behavior/ neurotoxicity, No effects	Hallare et al., 2004; Memmert et al., 2012; Xia et al., 2017	*
	antibiotic	Erythromycin	0.04	0.09	0.08	0.02	0.18	LC-MS	↓ CYP1 inhibition (*in vitro*)	Smith et al., 2012	
	antimycotic	Fluconazole	0.00	0.05	0.04	0.00	0.07	LC-MS [1]	Not validated	-	
	anticonvulsant drug	Gabapentin	0.63	1.0	0.95	0.63	2.00	LC-MS	No toxicity	Li et al., 2018 b	
	antihypertensive drug, diuretic	Hydrochlorothiazide	0.62	0.22	1.89	0.22	8.80	LC-MS	Not validated	-	
	metabolite of Bupropion	Hydroxybupropion	0.00	0.04	0.02	0.00	0.04	LC-MS [1]	Not validated	-	
	contrast agent	Iomeprol	1.60	0.7	1.63	0.70	2.50	LC-MS	Not validated	-	
	contrast agent	Iopromide	1.50	0.9	1.63	0.79	3.30	LC-MS	No toxicity	Steger-Hartmann et al., 2002	
	antihypertensive drug	Irbesartan	0.30	0.29	0.40	0.24	0.65	LC-MS [1]	Not validated	-	
	anticonvulsant drug	Lamotrigine	0.39	0.63	0.69	0.63	1.10	LC-MS	Not validated	-	
	anticonvulsant drug	Levetiracetam	0.00	0.04	0.02	0.00	0.14	LC-MS	No toxicity	Beker van Woudenberg et al., 2014; Martinez et al., 2018	
	local anesthetic, antiarrhythmic agent	Lidocaine	0.06	0.12	0.10	0.04	0.17	LC-MS [1]	↓ AChE, ↓ Activity	de Abreu et al., 2019	

Tab. A6. continued

Pharmaceuticals	insulin regulator	Metformin	1.40	200.00	21.14	0.86	200.00	LC-MS	No toxicity	Godoy et al., 2018	
	antihypertensive drug	Metoprolol	0.23	0.38	0.38	0.23	0.48	LC-MS [1]	Cardiac dysfunction	Meng et al., 2020; Sun et al., 2014	
	antihypertensive drug	Olmesartan	0.34	0.53	0.54	0.34	0.86	LC-MS	No toxicity	Bayer et al., 2014	
	tranquilizer, metabolite of Diazepam	Oxazepam	0.00	0.03	0.03	0.00	0.05	LC-MS	No toxicity, ↓ Activity	Pohl et al., 2019; Vossen et al., 2020	*
	anticonvulsant drug	Oxcarbazepine	0.00	0.06	0.08	0.00	0.20	LC-MS [1]	Not validated	-	
	metabolite of Allopurinol	Oxipurinol	7.20	12.00	9.08	1.00	13.00	LC-MS	Not validated	-	
	analgesic, anti-inflammatory drug	Phenazone	0.03	0.05	0.05	0.03	0.06	LC-MS	No toxicity (*in vitro*)	Caminada et al., 2006	
	anticonvulsant drug	Primidone	0.08	0.15	0.14	0.08	0.18	LC-MS	No toxicity, No biotrans-formation	Berghmans et al., 2007	
	antibiotic	Roxithromycin	0.00	0.09	0.04	0.00	0.17	LC-MS	↓ AChE, ↑ EROD (*Carassius auratus*)	Liu et al., 2014	
	diabetes drug	Sitagliptin	0.71	0.92	1.05	0.71	1.50	LC-MS	No toxicity	Vestel et al., 2016	
	antiarrhythmic agent, beta blocker	Sotalol	0.08	0.09	0.10	0.06	0.12	LC-MS	No toxicity	Diekmann and Hill, 2013; Gauthier and Vijayan, 2019	
	antibiotic	Sulfamethoxazole	0.12	0.23	0.18	0.08	0.32	LC-MS	Thyroid disruption	Raldúa et al., 2011	
	metabolite of Sulfamethoxazole	N4-Acetyl-sulfamethoxazole	0.02	0.02	0.04	0.00	0.09	LC-MS	Not validated	-	
	antibiotic	Sulfapyridine	0.07	0.19	0.14	0.07	0.22	LC-MS	Not validated	-	
	antihypertensive drug	Telmisartan	0.39	0.88	0.68	0.25	1.29	LC-MS [1]	Not validated	Wienen et al., 2000	

Tab. A6. continued

Pharmaceuticals	opioid, analgesic	Tramadol	0.17	0.56	0.45	0.06	0.73	LC-MS [1]	No toxicity, ↓ Activity	Sehonova et al., 2016; Bachour et al., 2020	*
	antibiotic	Trimethoprim	0.03	0.03	0.04	0.00	0.07	LC-MS	No toxicity	Halling-Sørensen et al., 2000	
	antihypertensive drug	Valsartan	0.22	0.03	0.08	0.00	0.24	LC-MS	Not validated	-	
	metabolite of Valsartan	Valsartan acid	1.41	2.15	2.07	1.14	3.47	LC-MS	No data available	Godoy et al., 2015	
	antidepressant	Venlafaxine	0.08	0.19	0.19	0.02	0.35	LC-MS [1]	↓ Reproductive output, ↑ Acute toxicity	Galus et al., 2013	
	antidepressant, metabolite of Venlafaxine	O-desmethyl-venlafaxine	0.36	1.06	0.93	0.36	1.58	LC-MS [1]	Not validated	-	
	metabolite of Venlafaxine	N-desvenlafaxine	0.05	0.07	0.09	0.05	0.11	LC-MS	Not validated	-	
	metabolite of Venlafaxine	N,O-didesvenlafaxine	0.13	0.23	0.22	0.12	0.34	LC-MS	Not validated	-	
	metabolite of Metamizole	4-acetamidoantipyrine	0.95	1.20	0.87	0.61	1.20	LC-MS	No toxicity	Baumann et al., 2016	
	metabolite of Aminophenazone	4-formyl-aminoantipyrine	0.79	1.10	1.05	0.71	1.40	LC-MS	Not validated	-	
	metabolite of Verapamil	D617	0.04	0.05	0.06	0.04	0.08	LC-MS	Not validated	-	
	metabolite	Acridine-9-carboxylic acid	0.04	0.09	0.07	0.04	0.10	LC-MS	Not validated	-	
PPP	fungicide	Azoxystrobin	0.00	0.05	0.01	0.00	0.05	LC-MS	↑ Hepatotoxic effects, ↑ Oxidative stress, genotoxic effects	Cao et al., 2016; Han et al., 2016; Jia et al., 2018	
	insecticide	DEET	0.04	0.215	0.07	0.01	0.22	GC-MS [1]	No toxicity	Costanzo et al., 2007	

Tab. A6. continued

Plant protection products (PPP)	metabolite of Terbuthylazine	Desethyl-terbuthylazine	0.00	0.03	0.01	0.00	0.04	LC-MS	Not validated	-
	metabolite of Chloridazon	Desphenylchloridazon	0.12	0.13	0.12	0.07	0.15	LC-MS	Not validated	-
	plant growth regulator	Dikegulac	1.52	1.69	1.44	0.87	2.45	LC-MS	Not validated	-
	herbicide	Dimethenamid	0.00	0.02	0.01	0.00	0.07	LC-MS	Not validated	-
	herbicide	Dimethenamid-P	0.00	0.02	0.01	0.00	0.07	LC-MS	Not validated	-
	metabolite of Icaridin	Icaridin acid	0.13	0.66	0.21	0.11	0.66	LC-MS	Not validated	-
	herbicide	MCPA	0.02	0.04	0.02	0.00	0.06	LC-MS	Not validated	-
	herbicide	Mecoprop	0.00	0.12	0.04	0.00	0.12	LC-MS	Not validated	-
	herbicide	Metribuzin	0.00	0.05	0.03	0.00	0.26	LC-MS	↑ Mortality	Plhalova et al., 2012
	herbicide	Terbutryn	<	0.036	0.03	0.01	0.05	LC-MS [1]	↑ Acute toxicity	Plhalova et al., 2009
	insecticide	Thiacloprid	0.00	0.03	0.00	0.00	0.03	LC-MS	No toxicity	Osterauer and Köhler, 2008
	metabolite of Terbuthylazine	Terbuthylazine-desethyl-2-hydroxy	0.00	0.07	0.02	0.00	0.09	LC-MS	Not validated	-
Additives	anticorrosive	Benzotriazole	3.80	7.80	5.29	3.80	7.80	LC-MS [1]	Induction of AhR pathway, ↑↓*cyp1a1*, ↑ Oxidative stress, liver damage	Tangtian et al., 2012; Fent et al., 2014; Hemalatha et al., 2020
	plasticizer	Bisphenol A	0.04	0.01	0.04	0.00	0.08	LC-MS [1]	↑ Activity, EDC, ↑ Mutagenic effects	Saili et al., 2012; Staples et al., 2011
	chromatographic agent	PFBA	0.072	0.118	0.068	0.010	0.262	LC-MS [2]	No toxicity	Godfrey et al., 2017; Hagenaars et al., 2011

Tab. A6. continued

Additives	PFAS	PFBS	0.013	0.005	0.006	0.003	0.012	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Sant et al., 2019; Ulhaq et al., 2013
	PFAS	PFHpA	0.010	0.007	0.004	0.002	0.010	LC-MS [2]	↑ Activity	Menger et al., 2020
	PFAS	PFHxA	0.02	0.021	0.015	0.003	0.021	LC-MS [2]	No toxicity	Menger et al., 2020
	PFAS	PFHxS	0.009	0.004	0.006	0.003	0.009	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020
	PFAS	PFNA	<	0.001	0.001	0.000	0.002	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020
	PFAS	PFOA	0.008	0.007	0.006	0.004	0.009	LC-MS [2]	↑ Activity	Menger et al., 2020
	PFAS	PFOS	0.009	0.010	0.009	0.004	0.015	LC-MS [2]	↑ Acute toxicity, ↓ Activity	Menger et al., 2020
	PFAS	PFPeA	0.014	0.012	0.009	0.005	0.014	LC-MS [2]	No toxicity	Menger et al., 2020
	plasticizer, catalyst, solvent	Triethyl phosphate	0.09	0.02	0.01	0.00	0.09	LC-MS [1]	No toxicity	Du et al., 2015
	defoamer	Triisobutyl phosphate (TiBP)	0.06	0.02	0.04	0.00	0.08	GC-MS [1]	Not validated	-
	plasticizer, flame retardant	Triphenyl phosphate (TPP)	0.00	0.02	0.00	0.00	0.02	GC-MS [1]	↓ Activity, Thyroid disruption	Jarema et al., 2015
	crystallization agent	Triphenylphosphine oxide (TPPO)	0.00	0.03	0.05	0.00	0.18	GC-MS [1]	Not validated	-
	flame retardant	Tris(1,3-dichloro-2-propyl)phosphate (TDCP)	0.05	0.05	0.06	0.03	0.11	GC-MS [1]	Neurotoxic effects, EDC	McGee et al., 2012; Wang et al., 2015a;b
	reducing agent	Tris(2-carboxyethyl)-phosphine (TCEP)	0.10	1.50	0.25	0.02	1.50	GC-MS [1]	↑↓ Behavior/neurotoxic effects, No toxicity	Dishaw et al., 2014; Behl et al., 2015; Jarema et al., 2015 *

Tab. A6. continued

Additives	plasticizer, flame retardant	Tris(2-chloroethyl)-phosphate (TCPP)	0.44	1.04	0.60	0.41	1.04	GC-MS [1]	↓ Activity	Dishaw et al., 2014	*
	vulcanization accelerator	1,3-Diphenyl guanidine	<	0.235	0.12	0.03	0.25	LC-MS [1]	Not validated	-	
	anticorrosive	4/5-Methyl-benzotria-zole	0.32	0.76	0.64	0.30	0.95	LC-MS [1]	Not validated	-	
	PFAS	4:2 FTS	0.007	0.006	0.006	0.000	0.011	LC-MS [2]	Not validated	-	
	PFAS	6:2 FTS	0.003	0.004	0.006	0.003	0.010	LC-MS [2]	↑ Activity	Menger et al., 2020	
Sweeteners	sugar substitute	Acesulfame K	0.92	1.20	1.36	0.55	2.40	LC-MS	↑↓ Behavioral effects, No toxicity	Dong et al., 2020; Stolte et al., 2013	*
	sugar substitute	Sodium cyclamate	0.59	0.12	0.15	0.00	0.59	LC-MS	No toxicity	Stolte et al., 2013	
	sweetener	Sodium saccharin	0.25	0.12	0.19	0.00	0.83	LC-MS	Not validated	-	
	sugar substitute	Sucralose	10.0	18.0	15.67	10.00	20.00	LC-MS	Not validated	-	
Others		Caffeine	0.61	0.11	0.22	0.00	1.10	LC-MS	↑ Activity, neuromuscular effects,↑ Acute toxicity	Rodriguez et al., 2014; Rah et al., 2017	

Tab. A7. Complete list of 124 screened compounds [ng/10 mg dry mass] in particle samples from Schwarzbach. Detection method: Liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). *Data were produced jointly with Sven Huppertsberg*.

	Concentration [ng/10 mg dry mass]			
Compound	**MP mix**	**Sediment**	**Suspended matter**	**Method**
1,3-diphenyl guanidine	576.5	120.3	105.7	LC-MS
2,4,5-Trichlorophenoxyacetic acid	0.0	0.0	0.0	LC-MS
2,4-Dichlorophenoxyacetic acid	0.0	0.0	0.0	LC-MS
2,6-Dichlorobenzonitrile	0.0	0.0	0.0	GC-MS
2-Aminobenzimidazole	0.0	0.0	0.0	LC-MS
4-(2,4-dichlorophenoxy)butyric acid	0.0	0.0	0.0	LC-MS
4,4'-DDT 235	0.0	0.0	0.0	GC-MS
4/5-Methyl-Benzotriazole	0.0	0.0	0.0	LC-MS
Alachlor	0.0	0.0	0.0	GC-MS
Ametryn	0.0	0.0	0.0	GC-MS
Amisulpride	-	2.2	2.8	LC-MS
Atrazine	0.0	0.0	0.0	GC-MS
Azinphos-ethyl	0.0	0.0	0.0	GC-MS
Azinphos-methyl	0.0	0.0	0.0	GC-MS
Bentazon	0.0	0.0	0.0	LC-MS
Benzotriazole	0.0	0.0	0.0	LC-MS
Bicalutamide	0.0	0.0	0.0	LC-MS
Bifenox	0.0	0.0	0.0	GC-MS
Bixafen	0.0	0.0	0.0	LC-MS
Bupropion	0.0	0.0	0.0	LC-MS
Carbamazepine-10,11 epoxide	0.0	0.0	0.0	LC-MS
Chlorfenvinphos	0.0	0.0	0.0	GC-MS
Chlorpyrifos-ethyl	0.0	0.0	0.0	GC-MS
cis/trans Heptachlor epoxide	0.0	0.0	0.0	GC-MS
cis-Chlordane	0.0	0.0	0.0	GC-MS
Citalopram	0.0	0.0	0.0	LC-MS
Climbazole	0.0	0.0	0.0	LC-MS
Clodinafop	0.0	0.0	0.0	LC-MS
Coumaphos	0.0	0.0	0.0	GC-MS
Coumarin	0.0	0.0	0.0	LC-MS
Cycloxydim	0.0	0.0	0.0	LC-MS
Cypermethrin	0.0	0.0	0.0	GC-MS
Cyprodinil	0.0	0.0	0.0	GC-MS
DEET	0.0	0.0	0.0	GC-MS
Demeton-MS	0.0	0.0	0.0	LC-MS

Tab. A7. continued

Demeton-O	0.0	0.0	0.0	GC-MS
Demeton-S	0.0	0.0	0.0	GC-MS
Demeton-S-methyl	0.0	0.0	0.0	GC-MS
Desmetryn	0.0	0.0	0.0	GC-MS
Dicamba	0.0	0.0	0.0	LC-MS
Dichlorprop	0.0	0.0	0.0	LC-MS
Dichlorvos	0.0	0.0	0.0	GC-MS
Diclofop-methyl	0.0	0.0	0.0	LC-MS
Dicofol	0.0	0.0	0.0	GC-MS
Diflufenican	0.0	0.0	0.0	LC-MS
Dikegulac	0.0	0.0	0.0	LC-MS
Dimethoate	0.0	0.0	0.0	GC-MS
Disulfoton	0.0	0.0	0.0	GC-MS
Dithianon	0.0	0.0	0.0	LC-MS
Endosulfan	0.0	0.0	0.0	GC-MS
Endosulfan A	0.0	0.0	0.0	GC-MS
Endosulfan sulfate	0.0	0.0	0.0	GC-MS
Etrimfos	0.0	0.0	0.0	GC-MS
Fenhexamid	0.0	0.0	0.0	LC-MS
Fenitrothion	0.0	0.0	0.0	GC-MS
Fenoxaprop-P	0.0	0.0	0.0	LC-MS
Fenoxaprop-P-ethyl	0.0	0.0	0.0	GC-MS
Fenpropimorph	0.0	0.0	0.0	GC-MS
Fenthion	0.0	0.0	0.0	GC-MS
Fluazifop-P	0.0	0.0	0.0	LC-MS
Fluchloralin	0.0	0.0	0.0	GC-MS
Fluconazole	0.0	0.0	0.0	LC-MS
Fluoxetine	0.0	0.0	0.0	LC-MS
Fluroxypyr	0.0	0.0	0.0	LC-MS
Flurtamone	0.0	0.0	0.0	LC-MS
Furalaxyl	0.0	0.0	0.0	GC-MS
Furmecyclox	0.0	0.0	0.0	LC-MS
Furosemide	0.0	0.0	0.0	LC-MS
Haloxyfop	0.0	0.0	0.0	LC-MS
Hexabromocyclododecane	0.0	0.0	0.0	GC-MS
Hexachlorocyclohexane	0.0	0.0	0.0	GC-MS
Hydroxybupropion	0.0	0.0	0.0	LC-MS
Ibuprofen	0.0	0.0	0.0	LC-MS
Icaridin acid	0.0	0.0	0.0	LC-MS
Iprodione	0.0	0.0	0.0	GC-MS

Tab. A7. continued

Irbesartan	0.0	0.0	0.0	LC-MS
Isoxaflutole	0.0	0.0	0.0	LC-MS
Ketoconazole	0.0	0.0	0.0	LC-MS
Kresoxim-methyl	0.0	0.0	0.0	GC-MS
Lidocaine	7.7	6.8	5.6	LC-MS
Losartan	0.0	0.0	0.0	LC-MS
Malathion	0.0	0.0	0.0	GC-MS
MCPA	0.0	0.0	0.0	LC-MS
Mecoprop	0.0	0.0	0.0	LC-MS
Mesotrione	0.0	0.0	0.0	LC-MS
Methamidophos	0.0	0.0	0.0	LC-MS
Methidathion	0.0	0.0	0.0	GC-MS
Metribuzin-desamino	0.0	0.0	0.0	LC-MS
Mevinphos	0.0	0.0	0.0	GC-MS
Moclobemide	0.0	0.0	0.0	LC-MS
N-Acetyl Sulfadiazine	0.0	0.0	0.0	LC-MS
Napropamide	0.0	0.0	0.0	LC-MS
O-desmethylvenlafaxine	0.0	0.0	0.0	LC-MS
Oxcarbazepine	0.0	0.0	0.0	LC-MS
Parathion-ethyl	0.0	0.0	0.0	GC-MS
Parathion-methyl	0.0	0.0	0.0	GC-MS
Pendimethalin	0.0	0.0	0.0	GC-MS
Pentobarbital	0.0	0.0	0.0	GC-MS
Phenobarbital	0.0	0.0	0.0	GC-MS
Picolinafen	0.0	0.0	0.0	GC-MS
Propham	0.0	0.0	0.0	GC-MS
Quinoxyfen	0.0	0.0	0.0	GC-MS
Ranitidin Hydrochloride	0.0	0.0	0.0	LC-MS
Sulfadiazine	0.0	0.0	0.0	LC-MS
Telmisartan	-	22.4	29.4	LC-MS
Terbutryn	0.0	0.0	0.0	LC-MS
Tramadol	0.0	0.0	0.0	LC-MS
trans-Chlordane	0.0	0.0	0.0	GC-MS
Triazophos	0.0	0.0	0.0	GC-MS
Tributyl phosphate (TBP)	0.0	0.0	0.0	GC-MS
Triclopyr	0.0	0.0	0.0	LC-MS
Triclosan	0.0	0.0	0.0	GC-MS
Trifluralin	0.0	0.0	0.0	GC-MS
Triisobutyl phosphate (TiBP)	0.0	0.0	0.0	GC-MS
Triphenyl phosphate (TPP)	0.0	0.0	0.0	GC-MS

Tab. A7. continued

Triphenylphosphine oxid (TPPO)	0.0	0.0	0.0	GC-MS
Tris(1,3-dichloro-2-propyl)phosphate (TDCP)	0.0	0.0	0.0	GC-MS
Tris(2-carboxyethyl)phosphine (TCEP)	0.0	0.0	0.0	GC-MS
Tris(2-chloroethyl)phosphate (TCPP)	-	1.7	1.9	GC-MS
Tritosulfuron	0.0	0.0	0.0	LC-MS
Valsartan	0.0	0.0	0.0	LC-MS
Valsartan acid	0.0	0.0	0.0	LC-MS
Venlafaxine	0.0	0.0	0.0	LC-MS
Vinclozolin	0.0	0.0	0.0	GC-MS

III.2. Exemplary sublethal effects of water and particle samples in zebrafish embryos

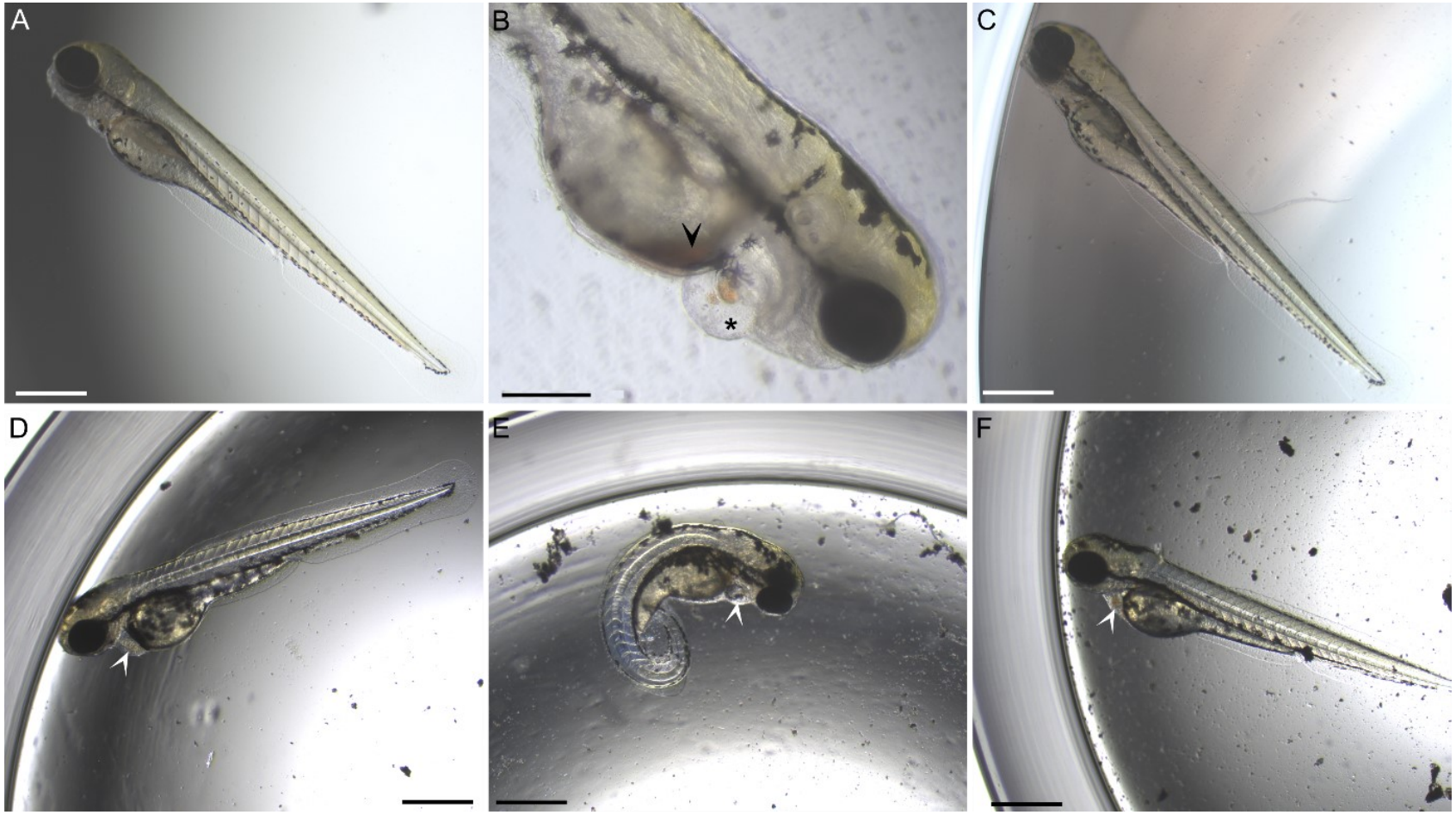

Fig. A9. Examples of sublethal effects in zebrafish from embryo toxicity test after 72 - 96 hpf exposure to control, water and particle samples. **(A)** Negative control at 96 hpf. **(B)** Exposure to Schwarzbach water at 96 hpf, causing pericardial edema and heart deformities (*), yolk sac edema (arrowhead), scale bar 250 µm. **(C)** Zebrafish embryo after spring water exposure at 96 hpf. **(D)** Pre-exposed MP at 72 hpf, causing pericardial edema (arrowhead) in zebrafish. **(E)** Suspended matter at 96 hpf, inducing pericardial/yolk sac edema (arrowhead) and spinal deformities. **(F)** Exposure to sediment, caused pericardial edema (arrowhead) after 72 h. Scale bars 500 µm. Zeiss Olympus CKX41 (Oberkochen, Germany).

VI. Overall conclusions

In the present thesis, the fate and effects of two different types of polymer particles (spherical particles of PMMA, irregular fragments of PS) in combination with two environmentally relevant contaminants (benzo(k)fluoranthene, chlorpyrifos) on different life stages of the freshwater fish species (*Danio rerio*) were considered. To this end, alterations in different biological endpoints in zebrafish were assessed: Following acute and chronic exposure, in different compartments of a simplified food web and after exposure of MPs in surface water. This multidisciplinary approach of coupling highly sensitive analytical quantification methods with different biological endpoints and biomarkers conclusively illustrated only minor adverse effects and impacts of both model pollutants and MPs in different aquatic species. Therefore, this approach has proven to be advantageous for a comprehensive assessment of the potential adverse effects of MPs in limnic ecosystems.

Overall, the necessary pre-incubation of PMMA and PS particles with the model pollutants resulted in sorption of 60 - 80 % of the substances to both types of MPs, thereby enabling the development of a reproducible incubation method. In various exposure scenarios, MPs were readily ingested by all aquatic organisms and it has been demonstrated that pristine MPs did not cause adverse effects or physical harm to zebrafish, which was in clear contradiction to the majority of recent research studies.

Acute exposure of zebrafish embryos to benzo(k)fluoranthene and chlorpyrifos induced sublethal effects in the fish embryo toxicity test, EROD assay and AChE activity. These effects were significantly reduced when both substances were applied sorbed to MPs. The acute toxic effects were reduced by up to 70 % compared to waterborne exposure scenarios, due to the strong sorption affinity of BkF and CPF to MPs and only minor leaching into the aqueous phase. The availability of BkF and CPF was thus reduced for zebrafish embryos. Furthermore, no synergistic or additive effects of MPs and sorbed contaminants were observed. These results are in clear contrast to recent study results on the adverse effects of MPs on aquatic organisms, possibly due to the variability of exposure scenarios and varying amounts of MPs and model contaminants. Considering the variety of polymer types produced, other MPs than polystyrene and polyethylene require further research. Furthermore, a detailed investigation of delayed toxic effects is necessary as later developmental stages of zebrafish could also be adversely affected.

No adverse effects have been induced in adult zebrafish by chronic exposure to MPs and environmentally relevant concentrations of MP-associated BkF and CPF. The gastrointestinal tract is considered the main site for chemical desorption and is relevant for metabolic activities. Therefore, the novel approach to quantify the intestinal uptake of BkF in cryosections of the gastrointestinal tract of zebrafish using CLSM proved to be useful to complement the results of the hepatic EROD assay. Only exposure to waterborne BkF altered the biomarker responses in terms of fluorescence intensity and hepatic EROD activity over the entire experimental time course. In contrast, MP-associated CPF and BkF induced only transient effects. After 21 days of continuous exposure, the biomarker responses did not differ considerably from negative controls. Even the highly sensitive fluorescence UPLC detection could only detect

minute amounts of waterborne BkF in zebrafish liver samples. It has been argued that bioaccumulation and biotransformation processes in zebrafish seem to be rather insignificant at environmentally relevant concentrations of both substances and that the overall relevance of MPs as vectors for environmental pollutants can be considered negligible. Data on chronic MP exposure, with or without sorbed contaminants, are nevertheless scarce and therefore require further investigation.

The previous findings were substantiated by limnic food web experiments, where the trophic transfer of MP-associated BkF from invertebrates to zebrafish could not be confirmed. However, it became evident that daphnids and chironomid larvae differed in their metabolic capacities, as only waterborne BkF was transferred from chironomid larvae to zebrafish and caused a significant increase in hepatic EROD activity. Evidence has also been provided that the ingestion of MPs was an additional route of exposure to lipophilic anthropogenic pollutants. Other studies have demonstrated that aquatic invertebrates were able to metabolize PAHs and thus reduce the risk of transferring HOCs to higher trophic levels. To this end, further research on aquatic species is still necessary to improve the understanding of biotransformation processes of anthropogenic pollutants such as insecticides and PAHs. The indicated vector potential of MPs appeared to be insignificant compared to the considerable amount of naturally occurring particles in aquatic environments and their well-documented sorption capacities. Concerning the demand for experiments with natural reference particles, future food web experiments might be extended with regard to the effects of chronic feeding and supplemented with particulate inorganic matter (e.g., kaolin, clay minerals, quartz sand) to distinguish possible effects between anthropogenic and natural inorganic particles.

The exposure of a MP mixture in a natural surface water did not induce significantly different acute toxic effects in zebrafish embryos compared to natural particles from sediment and suspended matter samples. However, the toxic potential of water samples from the Schwarzbach differed significantly from spring water samples with regard to neurotoxic effects (AChE activity, larval visual motor response test) and effects of dioxin-like substances (EROD assay). The induced effects of Schwarzbach water samples were related to quantifiable amounts of pharmaceuticals, flame retardants and pesticides based on chemical target analyses. Nevertheless, the toxic potential of the MP mixture had to be evaluated carefully. Presumably, the percentage of polyvinyl chloride in the MP mixture as well as the presence of a natural biofilm, associated with humic substances or undetected pollutants and metabolites, could have been responsible for adverse effects documented by FET, EROD assay, AChE activity and VMR tests due to more complex chemical interactions. Similar to previous studies, prolonged exposure of MPs in natural aquatic environments can lead to biofilm formation and the attachment of microbes or humic substances on MP surfaces, resulting in the assimilation of anthropogenic MPs to natural particles. As a consequence, these processes might alter the sorption behavior of MPs, but data still remains scarce. Hence, further investigations on surface modification and sorption behavior under realistic environmental conditions are necessary.

With regard to the results of the present thesis, the contribution of MPs to the sorption and transfer of hazardous organic pollutants appeared to be of minor importance compared to the abundance of abiotic particles and overall contaminant fluxes in limnic ecosystems. Given that the amount of MP applied in the present thesis has exceeded reported environmental concentrations, adverse effects of MPs in limnic ecosystems seem even less likely. Still, the ever-increasing polymer production and waste generation will inevitably increase the abundance of plastic fragments in the environment. Due to their persistence and slow and only partial degradation, plastic fragments have already been classified as a novel environmental pollutant of concern and the (eco-)toxicological hazard remains uncertain. Laboratory studies have so far focused on the effects of high amounts of only a few types of pristine, spherical and monodisperse polymers within a narrow size range. Important mechanisms that might considerably influence the properties of MPs, such as weathering, aging, biofilm formation, or fragmentation, have been rarely considered.

The development of suitable methods to obtain reliable data on the abundance of MPs in the environment has not yet been completed. For this reason, the discrepancy between laboratory studies and exposure results from field studies on the hazard potential of MPs will hinder an appropriate risk assessment of MPs. Although the field of microplastic research is still evolving, knowledge from the established area of nanomaterial research might be transferred to consider the effects of the ongoing fragmentation process of larger plastic debris. Complex contaminant mixtures and the occurrence of natural particles have to be considered, as these processes are known to alter the sorption equilibrium for MPs. Consequently, the impact of MPs on different life stages of aquatic freshwater species should be assessed in future studies in accordance with the demand for more environmentally relevant exposure scenarios, including natural particles, biofilm formation, complex contaminant mixtures and secondary and weathered MPs from various polymer types.

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