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1 Microplastics and sorbed contaminants – Trophic exposure in fish sensitive early life stages

2

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18

19 **Abstract**

20 The present study evaluated very small microplastic particle (MPs) transfer to zebrafish and
21 marine medaka larvae *via* prey experimentally exposed to MPs from the onset of feeding.
22 Larvae were fed *Paramecium* or *Artemia* nauplii loaded with fluorescent 1-5 or 10-20 μm
23 MPs. Pollutant accumulation was analyzed by optically tracking of benzo[a]pyrene (BaP) and
24 recording *cyp1a* transcription. *Paramecium* transferred 1-5 μm particles only, whereas *Arte-*
25 *mia* efficiently transferred both MPs. Although zebrafish and medaka larvae fed from the on-
26 set of active food intake (2-3 dph, respectively) on *Paramecium* and from days 6-7 post-hatch
27 on *Artemia* nauplii, neither MP accumulation nor translocation to tissues was detected. MP
28 egestion started within few hours after ingestion. *Cyp1a* induction and fluorescent analyses
29 proved BaP bioavailability after transfer *via* *Paramecium* and *Artemia*. Unicellular or plank-
30 ton organisms ingest contaminants *via* MPS and transfer effectively these to sensitive early
31 life-stages of vertebrates, giving rise to whole-life exposure.

32

33 Keywords: Trophic transfer, microplastics, fish, larvae, benzo[a]pyrene, *Paramecium*, *Arte-*
34 *mia*, zebrafish, marine medaka

35

36

37 Introduction

38 Today, microplastic particles (MPs) are found ubiquitously in any water body (Browne et al.,
39 2011; Eerkes-Medrano et al., 2015; Eriksen et al., 2014; Van Cauwenberghe et al., 2015).
40 Along with increasing occurrence in the environment, detection rates of MPs in marine biota
41 have been well documented especially in zooplankton and fish (Cole et al., 2013; Desforges et
42 al., 2015; Hermsen et al., 2017; Rummel et al., 2016; Steer et al., 2017; Watts et al., 2015).
43 However, eventual effects by MPs in marine and freshwater organisms and their potential
44 impact on ecosystems have remained a matter of debate for more than a decade (Heinrich et
45 al., 2020; Koelmans et al., 2016; Lohmann, 2017; Thompson et al., 2004; Wright et al., 2013).

46 Ingestion of MPs has been demonstrated across a wide range of phyla (*e.g.* zooplankton, mol-
47 lusks, marine worms, fish), and their adverse effects might be attributed to different processes
48 including the lack of egestion (occlusion effects) or the induction of false satiation after inges-
49 tion of MPs and the devoid of nutritional value as reviewed in Eerkes-Medrano et al. (Eerkes-
50 Medrano et al., 2015). In some cases ingestion of virgin MPs induced no or limited toxicity
51 (Beiras et al., 2018; Cormier et al., 2019; Le Bihanic et al., 2020; Mazurais et al., 2015) while
52 in other cases they have been shown to induce inflammatory processes and physiological
53 stress responses (Jovanovic, 2017; Karami et al., 2017; von Moos et al., 2012; Wright et al.,
54 2013), which may be the consequence of physical or chemical insults (due to additives for the
55 latter). In a review, (Hermabessiere et al., 2017) summarize reports on effective transfer of
56 plastic additives to marine organisms including phthalates, nonylphenol, bisphenol A and
57 brominated flame retardants.

58 Beyond such intrinsic toxicity, another potential threat attributed to MPs is due to their physi-
59 cochemical properties, which favor the adsorption of hydrophobic chemicals on their surface.
60 As a consequence, hydrophobic compounds present in aquatic environments accumulate on
61 MPs, as shown for a wide variety of substances including polychlorinated biphenyls (PCBs),
62 polycyclic aromatic hydrocarbons (PAHs) and metals (Alimba and Faggio, 2019; Ashton et
63 al., 2010; Bakir et al., 2012; Endo et al., 2005; Fisner et al., 2013; Mato et al., 2001; Ogata et
64 al., 2009; Rios et al., 2007; Strungaru et al., 2019; Velzeboer et al., 2014). Yet, the importance
65 of MPs as vectors for adsorbed contaminants has been under discussion and – according to
66 modeling approaches – has been assumed not to be major (Bakir et al., 2016; Heinrich et al.,
67 2020; Koelmans et al., 2016; Lohmann, 2017). In part, experiments agree with such modeling
68 by documenting the absence or a minor role for MPs as vectors for pollutants (Besseling et
69 al., 2017; Devriese et al., 2017). In contrast, other experimental studies demonstrated that con-
70 taminants adsorbed on MPs can effectively be transferred to organisms (Batel et al., 2016;
71 Gassel and Rochman, 2019; Rochman et al., 2013; Scopetani et al., 2018; Teuten et al., 2009;
72 van der Hal et al., 2020; Wardrop et al., 2016) and can elicit molecular responses (Avio et al.,
73 2015; Rochman et al., 2013; Sleight et al., 2017). Most likely, the potential role of MPs as
74 vectors for pollutants depends on the physicochemical properties of the pollutants as revealed
75 by both experimental approaches and field sampling (Diepens and Koelmans, 2018; Gassel
76 and Rochman, 2019).

77 In the marine environment, coastal areas are considered of major importance, as they repre-
78 sent nurseries for many fish species (Beck et al., 2001). Simultaneously, these areas are close
79 litter sources and, thus, characterized by relatively high concentrations of MPs and chemical
80 pollutants (Browne et al., 2011; Guzzetti et al., 2018; Hermsen et al., 2017). The increase in
81 MPs and MPs ingested in larvae observed in coastal slick supports this hypothesis (Gove et
82 al., 2019). As a consequence, fish early development and larval stages may be speculated to
83 be affected in different or stronger manners by MPs and associated hazardous chemicals than
84 adult organisms. Fish larvae mainly feed on diverse zooplankton species, which have been
85 shown to ingest MPs (Cole et al., 2013; Desforges et al., 2015). Zooplankton may then act as
86 a funnel delivering high amounts of MPs to fish larvae; relative to body weight, amounts of
87 pollutants may be larger than in adult fish. The contribution of zooplankton to the transfer of
88 MPs to fish larvae might, therefore, be a major issue for fish stock sustainability, but is largely
89 unknown so far (Au et al., 2017). Thus, even if exposure to contaminants through MP inges-
90 tion may theoretically not be a major source for the uptake of contaminants, trophic transfer
91 *via* food webs might still represent an important route for the transfer of MP and associated
92 contaminants to ecologically sensitive life-stages of aquatic organisms. Especially early expo-
93 sure to elevated amounts of MPs might potentially have larger impact on fish early life-stages
94 than on adult fish.

95 For these reasons, the present study was designed to evaluate how very small MPs and associ-
96 ated contaminants transfer *via* prey exposed to MPs and how they affect early life-stages of
97 fish. In order to avoid possible species-specific findings, we used two evolutionary distant
98 model fish species, the freshwater zebrafish (*Danio rerio*) and the marine medaka (*Oryzias*
99 *melastigma*) both being frequently used for ecotoxicology studies. Similarly, as prey, we used
100 two unrelated organisms, *Paramecium* and *Artemia*, as two plankton-like models of con-
101 trasting sizes. Following the approach by Batel et al. (2016) in adult fish, we analyzed (1) the
102 trophic transfer of MPs to larvae of zebrafish and of the marine medaka *via Paramecium* and
103 *Artemia*, (2) the quantitative and qualitative transfer of MPs and associated chemicals, and
104 finally (3) the bioavailability of chemicals transferred from MPs to fish larvae.

105

106 **Materials and Methods**

107 **Fish husbandry**

108 Adult zebrafish (*Danio rerio*) aged 12 months were obtained from the breeding and mainte-
109 nance facilities of the Aquatic Ecology and Toxicology Group at the Center for Organismal
110 Studies Heidelberg (licensed by regional animal welfare authorities under 35-9185.64/BH
111 Braunbeck). Temperature was maintained at 25.0 ± 1.0 °C, and fish were kept under a con-
112 stant artificial dark/light cycle of 8/16 h. Constant filtering plus permanent flow-through con-
113 ditions guaranteed that ammonia, nitrite, and nitrate concentrations were kept below detection
114 limits (5, 1 and 140 mg/L, respectively). Fish were fed commercially available artificial diets
115 (TetraMin™ flakes; Tetra, Melle, Germany) twice daily *ad libitum*, supplemented with *Arte-*
116 *mia* nauplii of appropriate size (Instar II, 2 pipettes per 10 fish; (Lim et al., 2003) obtained

117 from uncontaminated sources. Zebrafish eggs were obtained according to Lammer et al.
118 (2009).

119 Marine medaka (*Oryzias melastigma*) brood stocks are routinely maintained in the La-
120 boratoire Ressources Halieutiques, Ifremer (facility authorization A171901; project authoriza-
121 tion APAFIS#10883) from a stock obtained as embryos in 2007 from the laboratory of Dr.
122 Doris Au (State Key Laboratory in Marine Pollution, City University of Hong Kong, China).
123 Juveniles and adult fish were reared in recirculating systems in water at 25 ‰ salinity in an
124 isothermal room at 26 ± 1 ° C with a 14/10 h light/dark photoperiod. Fish were fed twice a
125 day with food pellets of various size depending on their age (Special Diets Services, Dietex
126 France, Argenteuil, France) with an additional feeding at noon with freshly hatched brine
127 shrimp (*Artemia*) nauplii (Ocean Nutrition Europe, Essen, Belgium).

128 Eggs were collected by siphoning tanks bottom within 4 hours after light onset and quickly
129 cleaned. In order to remove chorionic projections, eggs were rolled on sandpaper (p.2000) as
130 described for Japanese medaka (Porazinski et al., 2010), extensively rinsed in order to remove
131 debris and finally transferred to Petri dishes filled with artificial 20 µm-filtered synthetic arti-
132 ficial seawater (Instant Ocean; salinity 25 ‰, pH = 8.0) containing methylene blue.

133

134 **Chemicals**

135 Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Deisenhofen,
136 Germany, or St. Quentin Fallavier, France) at the highest purity available.

137

138 **Microplastics (MPs) and benzo[a]pyrene (BaP) spiking**

139 Green fluorescent microspheres of two sizes were purchased from Cospheric (Santa Barbara,
140 CA, USA): (1) proprietary plastic microspheres with a size range of 1 - 5 µm range and a den-
141 sity of 1.3 g/cc (catalogue code: FMG-1.3 1-5 µm; later referred to as MP5), and (2) polyeth-
142 ylene plastic microspheres with a size range of 10 - 20 µm and a density of 1.00 g/cc (cata-
143 logue code: UVPMS-BG-1.00 10-20 µm; later referred to as MP20). Characterization of MPs
144 polymers was performed as described in Supporting information (S1).

145 MP spiking was performed according to previously published protocols (Batel et al., 2018;
146 Batel et al., 2016). BaP from stock solution in acetone (maximum amount used 100 µl) was
147 added to double-distilled water to a total volume of 10 ml in a 50 ml glass bottle. Approxi-
148 mately 1.2×10^6 particles, corresponding to 0.5 mg of MP5 or 2.5 mg of MP20 were added
149 and incubated overnight on a shaker (Rocky 1010; Fröbel, Lindau, Germany). After spiking,
150 solutions were filtered over sterile 0.45 µm nitrocellulose filters (TOP CA Sterile Syringe
151 Filter, Berrytec, Grünwald, Germany), washed three times with double-distilled water and
152 finally recovered in 10 ml of double-distilled water. Nominal BaP concentrations used for
153 spiking were 10, 100 and 300 µM; spiked MPs will later be called MP-BaP10, MP-BaP100 or
154 MP-BaP300. In previous studies, chemical analyses had shown that over 90 % of BaP bound

155 to MPs and that there were no solubility problems (Batel et al., 2016). An additional desorp-
 156 tion control was used to analyze the potential desorption of BaP from spiked MPs after 24 h
 157 incubation in clean water and to simulate the potential direct transfer of desorbed BaP *via* the
 158 water column. The desorption control (DC) was prepared by incubating the spiked, washed
 159 and filtered MPs for 24 h in double-distilled water under continuous agitation. MPs were then
 160 filtered out, and larvae were exposed to the residual water and BaP potentially re-dissolved.

161 For definition of exposure groups, see Table 1.

162 **Table 1:** Definition of exposure group codes used in the present study

Exposure group	Live prey	MPs	Benzo[a]pyrene (BaP)
MP5	-	MP 1-5 μm	-
MP20	-	MP 10- 20 μm	-
Par-MP5	<i>Paramecium</i>	MP5	-
Par-MP20	<i>Paramecium</i>	MP20	-
Par-MP5-BaP10	<i>Paramecium</i>	MP5	10 μM
Par-MP20-BaP10	<i>Paramecium</i>	MP20	10 μM
Art-MP5	<i>Artemia</i>	MP5	-
Art-MP20	<i>Artemia</i>	MP20	-
Art-MP5-BaP100	<i>Artemia</i>	MP5	100 μM
Art-MP5-BaP300	<i>Artemia</i>	MP5	300 μM
NC (negative control)	-	-	-
PC (positive control: waterborne BaP)	-	-	100 nM
DC (desorption control)	-	-	BaP re-solution control (see text)

163

164

165 **Trophic transfer from *Paramecium* to fish larvae**

166 **MP uptake by *Paramecium***

167 *Paramecia* (*Paramecium spec.*) were obtained from a permanent culture of the Aquatic Ecolo-
 168 gy and Toxicology Group at the Center for Organismal Studies (University of Heidelberg,
 169 Germany). The culture was kept in glass bottles darkened with aluminum foil and filled with
 170 artificial seawater (1.5 g *Artemia* salt [9.52 g/L Na⁺, 0.71 mg/L Mg²⁺, 0.24 g/L Ca²⁺, 0.22 K⁺,
 171 11.0 g/L Cl⁻, 1.54 g/L SO₄²⁻ per 5 L distilled water]; Preis Aquaristik, Bayerfeld-Steckweiler,
 172 Germany). *Paramecium* was fed with 5 cooked wheat grains per L (50 grains cooked in 50
 173 ml distilled water for 10 min at 300 °C). *Paramecium* cultures were split every two weeks in
 174 order to prevent a decrease in population density.

175 For the monitoring of MP ingestion, approx. 1.2×10^6 MP particles (about 0.5 or 2.5 mg of
176 MP5 or MP20, respectively) were mixed in 9 ml of *Paramecium* rearing medium in glass Pe-
177 tri dishes. For better mixing of *Paramecium* and MPs, Petri dishes were incubated on a REAX
178 3 hinged plate (Heidolph, Schwabach, Germany). After 1, 3 and 5 h incubation, 1.5 ml of the
179 solution were transferred to a 2 ml tube. *Paramecium* rearing media were filtered through a
180 100 μm filter (EASY strainer, Greiner, Frickenhausen, Germany) into new tubes and washed
181 with 1 ml artificial sea water. *Paramecium* passed the filter, whereas MPs attached to the filter
182 or to the plastic walls of the tubes due to hydrophobic interactions. The tubes were then spun
183 in a table centrifuge (Mini centrifuge, National Labnet, Woodbridge, USA) for 2×10 sec.
184 Remaining MP5 with higher density than water sedimented at the bottom or along the wall of
185 the vessel, whereas MP20 particles with lower density than water floated at the water surface.
186 Separated *Paramecium* were sampled from the layers in-between and later called Par-MP.

187 *Paramecium* were transferred to confocal dishes, and MP uptake was tracked with a Nikon
188 Eclipse 90i epifluorescence microscope (Nikon, Düsseldorf, Germany). For photography,
189 *Paramecium* were immobilized with a saturated EDTA (5 g/15 ml) solution.

190

191 **MPs transfer from *Paramecium* to fish larvae**

192 The transfer of MPs from *Paramecium* to zebrafish larvae was tested at different ages of the
193 larvae (96 - 192 hours post-fertilization, hpf) and for different feeding durations (1 - 6 h, Ta-
194 ble 2). One zebrafish larva was transferred to each well of a 24-well plate (TPB, Trasadingen,
195 Switzerland) in 1.5 ml ISO 7346/3 water (64.7 mg/L NaHCO_3 , 5.7 mg/L KCl , 294.0 mg/L
196 $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 123.3 mg/L MgSO_4 ; (ISO, 1996)). *Paramecium* were fed MPs for 1 h and
197 separated from particles in solution as described above. 500 μl of Par-MP solution were added
198 to each well to reach approximately 30 *Paramecium* per fish larva. The 24-well plates were
199 sealed (Excel scientific, SealPlate, USA), and zebrafish larvae were incubated at 26 °C for 1 -
200 6 h with Par-MP. After incubation, the medium was carefully removed from the larvae, which
201 were then euthanized by adding 1 ml tricaine (400 mg/L ethyl-4-aminobenzoate). After trans-
202 fer to confocal dishes, the uptake of MPs *via Paramecium* was analyzed with a Nikon Eclipse
203 90i epifluorescence microscope.

204 Marine medaka exposure to Par-MP followed the same protocol with the exception that expo-
205 sure started 1-2 days post-hatch corresponding to 11-12 days post-fertilization (dpf).

206

207 **MP transfer from *Artemia* to fish larvae**

208 **MP uptake by *Artemia* nauplii**

209 *Artemia* nauplii were exposed to MPs as previously described (Batel et al., 2016). In brief,
210 *Artemia* cysts were incubated in seawater at 26 °C under constant aeration. After 48 h incuba-
211 tion, Instar II stage nauplii (Lim et al., 2003) were collected and counted after dilution under a
212 dissecting microscope. This nauplii stage has been chosen as it is the first *Artemia* stage in-

213 gesting particles being still small enough to match larvae mouth size. Approx. 10.000 nauplii
214 were incubated further in 30 ml of seawater with approx. 1.2×10^6 MP5 or MP20 particles
215 and kept under constant aeration for oxygen supply and agitation of *Artemia* nauplii and MPs.
216 *Artemia* nauplii were then filtered using a 100 μm mesh and rinsed thoroughly. In the further
217 text, *Artemia* nauplii incubated with MPs will be referred to as Art-MP.

218 For 2 days of exposure, freshly harvested *Artemia* nauplii were distributed to zebrafish larvae
219 twice daily. For monitoring of MPs elimination kinetics, larvae were fed another 2 days with
220 non-loaded *Artemia* nauplii prepared as described above. Every evening, 1h after the last
221 feeding of the day, some larvae were collected and euthanized using an overdose of benzo-
222 caine solution (500 mg/L) and observed under an Olympus BX41 epifluorescence microscope
223 (Olympus, Rungis, France; magnification 40 \times , light source: X-Cite EXFO with GFP filter).
224 Fluorescent larvae were counted to calculate the proportion of larvae having ingested MP-
225 loaded *Artemia* and imaged using DMK 31AU03 camera and IC Capture software (The Imag-
226 ing Sources, Elvitec, Pertuis, France). Fluorescence was then quantified using ImageJ
227 (Schneider et al., 2012) with correction for background fluorescence. A total of 20 larvae for
228 each exposure group were monitored in triplicate.

229

230 **Trophic transfer of MP-associated benzo[a]pyrene (BaP) and *cyp1a* induction in fish** 231 **larvae**

232 **Fish larvae exposure**

233 The transfer of BaP from microplastics through planktonic organisms to fish larvae was eval-
234 uated in zebrafish *via Paramecium* and in marine medaka *via Artemia* nauplii to show the
235 potential of MP trophic transfer from the very first feeding of the fish larvae. In the case of
236 zebrafish, 10 individuals of 7 d old larvae per exposure group were analyzed in three inde-
237 pendent replicates. Larvae of the negative control (NC) were incubated in artificial water;
238 positive controls (PC) contained 100 nM waterborne BaP, with the wells pre-incubated over-
239 night with the BaP solution for of the plastic well walls. MPs were spiked, and *Paramecium*
240 were incubated with spiked MPs as described above. Six ml *Paramecium* rearing medium
241 were mixed with 2 ml of the MPs loaded with BaP; except for negative and positive controls,
242 the other test groups were also diluted 1:3 with ISO water. For this end, 500 μl of the test so-
243 lutions were added to the wells (final volume 2 ml), and larvae were incubated for 3 hours at
244 26 ± 1 $^{\circ}\text{C}$. After incubation, the medium was discarded, and the larvae were euthanized as
245 described above and used for either fluorescence tracking or *cyp1a* analysis.

246 In the case of marine medaka, 17 d old larvae were used. MP5-BaP and Art-MP5-BaP were
247 prepared as described above. Likewise, larval exposure was performed as described above for
248 2 days (4 meals), and larvae were collected 3 hours after the last meal and euthanized using an
249 overdose of benzocaine solution (500 mg/L), rinsed thoroughly in water and stored for *cyp1a*
250 transcription analyses. A total of 6 pools of 10 larvae were collected from three exposure rep-
251 licates.

252 The subsequent evaluation of the fluorescence signal of the BaP required a positioning of the
253 larvae as identical as possible. Within a drop of 0.1 % low melting agarose (26 ± 2 °C;
254 MatTek, Ashland, USA), larvae were positioned on their left sides and fixed by gelling of the
255 low melting agarose (100 mg/10 ml 0.016 % tricaine). Micrographs were taken in the DAPI
256 fluorescence channel (340 - 380 nm excitation, 435 - 485 nm emission) of a Nikon Eclipse 90i
257 epifluorescence microscope (Nikon, Tokyo, Japan) with an exposure time of 2 seconds.

258

259 ***Cyp1a* gene transcription analysis**

260 To evaluate bioavailability of BaP, we monitored transcription of *cyp1a* which is known to be
261 induced in vertebrates, by a wide range of chemicals including BaP (Ma and Lu, 2007). For
262 *cyp1a* gene transcription analyses, larvae were washed three times in RNase-free water im-
263 mediately after sampling and stored in RNALater at -20°C until further processing. Prior to
264 RNA extraction, RNALater was removed, and embryos were immersed in lysis buffer
265 (Quiagen, Les Ulis, France) before disruption in a Beadblaster (Benchmark Scientific,
266 Dutscher, France). The total RNA extraction followed the protocols of the RNeasy Plus Uni-
267 versal Mini Kit (Quiagen). Quality and quantity of the extracts were checked using electro-
268 phoresis migration and spectrophotometric dosing. Subsequently, cDNA was synthesized
269 from 2 µg of the total RNA, using 2 µl of Superscript III Reverse Transcriptase (Invitrogen,
270 Fisher Scientific, Illkirch, France) according to the manufacturer's protocol in a final volume
271 of 21 µl. Prior to analysis, all cDNA was diluted 5 times in Milli-Q water. The qPCR experi-
272 ments were run in a final volume of 20 µl, including an optimized primer concentration range
273 between 300 and 600 nM (Eurofins Genomics, Ebersberg, Germany) and 2X Fast SYBR
274 Green Master Mix (Applied Biosystems, Fisher Scientific, Illkirch, France). Technical tripli-
275 cates were run for each biological replicate. The analysis software Relative Expression Soft-
276 ware Tool (REST; (Pfaffl, 2001; Pfaffl et al., 2002)) automatically calculated fold changes in
277 transcription relative to negative controls (NC; cf. Table 1) using the 3 most stable references
278 genes between all groups, and combined into an index *via* the free access BestKeeper soft-
279 ware (Pfaffl et al., 2004). Reference genes were *g6pd*, *actb1* and *b2m* for zebrafish and *g6pd*,
280 *actb1* and *18s* for medaka.

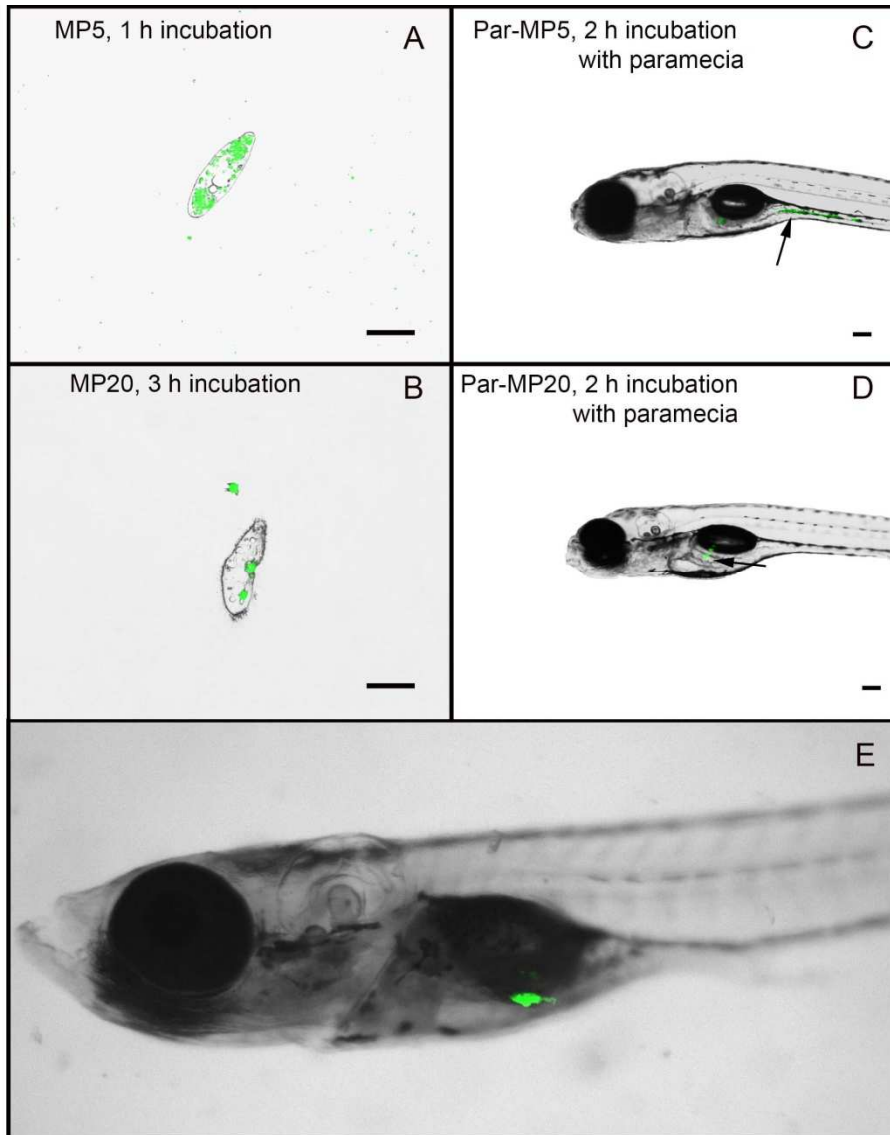
281

282 **Results**

283 **Trophic transfer of microplastic particles from *Paramecium* to zebrafish and medaka** 284 **larvae**

285 After 1, 3 and 5 h incubation to microplastics, *Paramecium* was analyzed for MP uptake. In
286 general, MP5 particles were taken up more efficiently than MP20: After 1 h incubation, all
287 *Paramecium* individuals contained high numbers of MP5 particles within nutritive vacuoles
288 (Fig. 1A), which remained constant after 3 and 5 h of incubation. In contrast, only a small
289 amount of MP20 particles were visible inside about 10 % of the *Paramecium* individuals (Fig.
290 1B). Although with longer incubation time the number of *Paramecium* with ingested MP20

291 increased, the overall number of microplastic particles absorbed remained low, if compared to
292 MP5. Because of the small number of MP20 particles within *Paramecium*, only very few of
293 the bigger MP20 particles were observed in the intestinal tract of zebrafish larvae after feed-
294 ing Par-MP20, whereas the signal was far stronger when feeding Par-MP5 (Figs. 1C-D). A
295 similar experiment was performed with marine medaka confirming efficient transfer of MP5
296 through *Paramecium* (Fig. 1E).



297

298 **Fig. 1:** Internalization of fluorescent MPs by *Paramecium* and transfer to zebrafish (*Danio*
299 *rerio*) larvae. Detection of fluorescent MPs inside *Paramecium* after 1 h incubation with MP5
300 (A) and 3 h incubation with MP20 (B). Ingestion of fluorescent MPs by fish larvae fed *Para-*
301 *mecium* previously incubated with MPs for 2 hours: 168 hpf zebrafish larvae fed Par-MP5 (C)
302 or Par-MP20 (D); 264 hpf marine medaka larvae fed Par-MP5 (E; green fluorescence overlaid
303 with bright field image). Scale bars: 100 μm.

304

305 To further characterize the trophic transfer, ingestion of particles by zebrafish larvae was
 306 monitored for different feeding durations (1 - 6 h) and starting at different ages (96 - 192 hpf;
 307 Table 2). Independent of the feeding duration, transfer could only be documented from \geq 120
 308 hpf. Starting at this age, fluorescence was readily detected in larvae. Furthermore, after 5 h
 309 feeding, one larva was observed egesting microplastic-containing feces. There were no MPs
 310 visible in intestinal tracts of larvae incubated with waterborne MPs, thus, larvae did not feed
 311 directly on MPs, only *via* intermediate prey at this age.

312 **Table 2:** Transfer of *Paramecium* loaded with small microplastic particles (Par-MP1) to
 313 zebrafish (*Danio rerio*) larvae after variable feeding duration and feeding starting at different
 314 ages. Numbers indicate fluorescent larvae *versus* total number of larvae analyzed (in brack-
 315 ets).

316

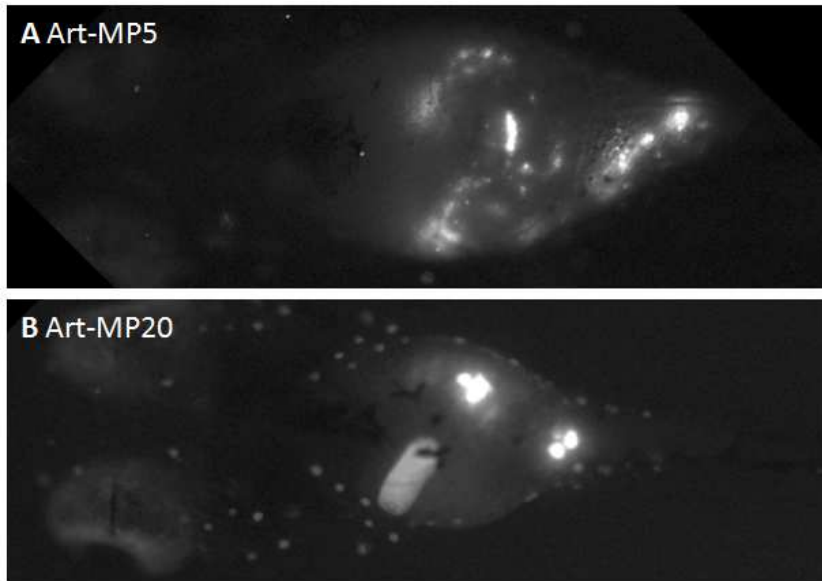
Feeding start time	96 hpf	120 hpf	144 hpf	168 hpf	192 hpf
1 h	-	4 (12)	-	-	-
2 h	0 (11)	12 (12)	7 (12)	11 (12)	8 (8)
3 h	-	9 (12)	5 (12)	10 (11)	3 (12)
	-	11 (11)	8 (12)	11 (12)	10 (12)
4 h	0 (11)	12 (12)	8 (12)	12 (12)	9 (11)
5 h	-	11 (12)	8 (11)	12 (12)	7 (7)
	0 (11)	10 (12)	12 (12)	12 (12)	12 (12)
6 h	-	-	7 (12)	11 (12)	-
	0 (11)	11 (11)	10 (11)	12 (12)	-

317

318

319 **Trophic transfer of microplastic particles from *Artemia* nauplii to fish larvae**

320 As described previously (Batel et al., 2016), *Artemia* Instar II successfully ingested MPs both
 321 MP5 and MP20. Since the uptake of particles by *Artemia* nauplii only starts at Instar II stage
 322 (body size \sim 200 μ m), feeding of fish larvae could only be initiated when the mouth opening
 323 of the fish larvae was wide enough, i.e. at about 6 d post-hatch (\sim 8 dpf = 192 hpf for zebrafish
 324 and \sim 17 dpf for medaka. At this age, larvae readily ingest *Artemia* nauplii and MPs transfer
 325 was easily visible (Fig. 2).

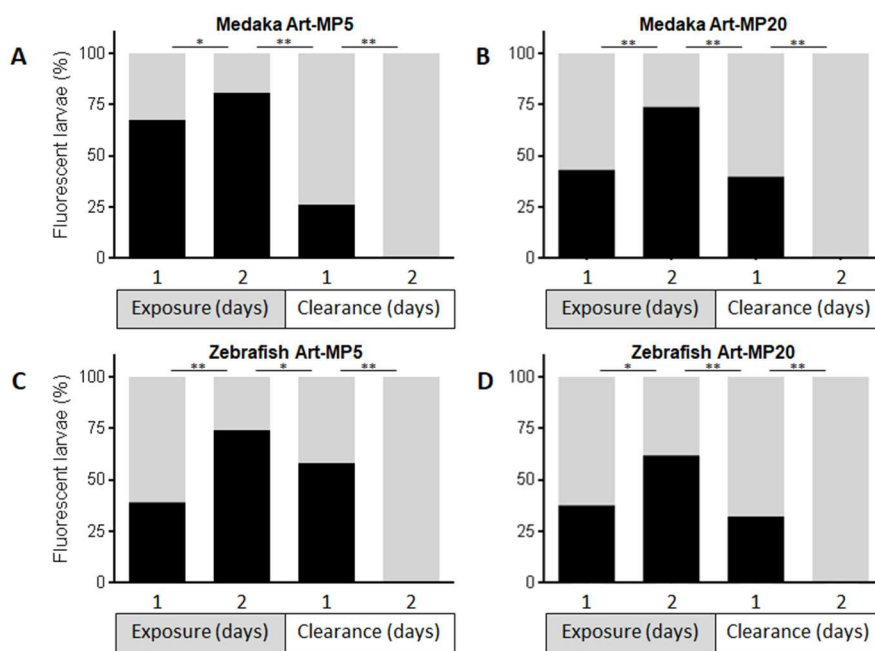


326

327 **Fig. 2:** Transfer of fluorescent MPs to marine medaka (*Oryzias melastigma*) larvae, dorsal
 328 view. Ingestion of fluorescent MPs by 17 dpf medaka larvae fed Art-MP5 (A) Art-MP20 (B);
 329 ventral views. Note some autofluorescent pigment cells in B.

330

331 Independent from species and MPs size, the evolution of the proportion of fish larvae showing
 332 fluorescence followed the same pattern (Fig. 3) with an increase between days 1 and 2 and a
 333 significant decrease of the proportion of larvae showing fluorescence at day 3 (i.e. day 1 of
 334 clearance after the end of exposure to MP). At day 4 (day 2 of clearance), there were no lar-
 335 vae with fluorescence left anymore, independent of species and MP size.



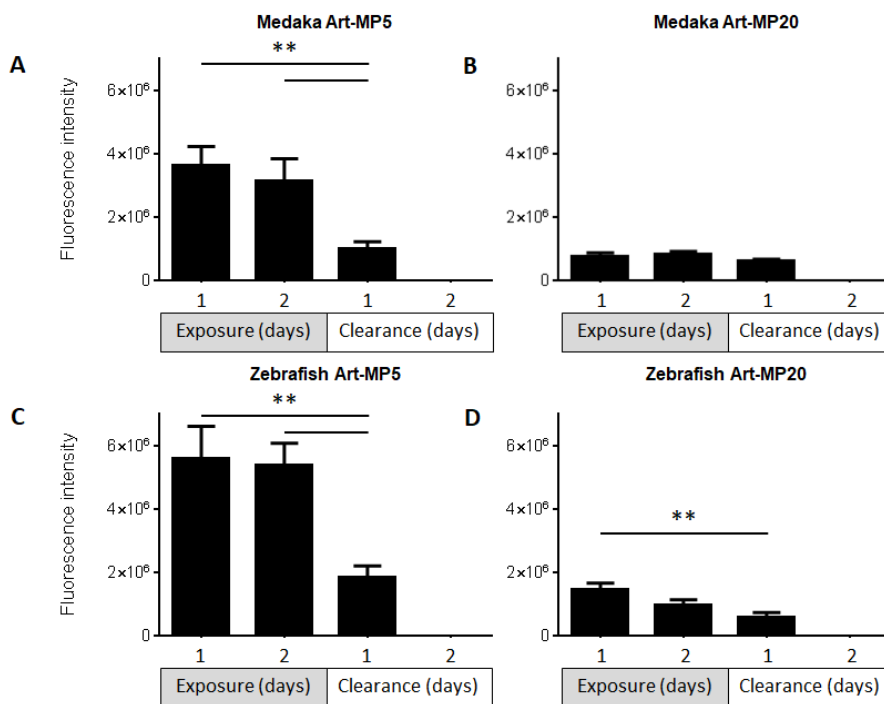
336

337 **Fig. 3:** Proportion of marine medaka (*Oryzias melastigma*) and zebrafish (*Danio rerio*) larvae
 338 showing fluorescence (MP uptake) over time after feeding with *Artemia* loaded with fluores-

339 cent MPs. Fish larvae were fed on days 1 and 2 with *Artemia* that had been exposed to fluo-
 340 rescent MPs, followed by two additional days of clearance with non-exposed *Artemia*. Marine
 341 medaka larvae fed Art-MP5 (A) and Art-MP20 (B). Zebrafish larvae fed Art-MP5 (C) and
 342 Art-MP20 (D). Triplicates of 20 individuals were monitored; Differences between successive
 343 days have been tested using Fisher's exact test: * = $p < 0.05$; ** = $p < 0.01$.

344

345 In order to describe ingestion in both fish species in more detail, fluorescence intensity was
 346 analyzed within the gut lumen of fluorescent larvae (Fig. 4). The temporal pattern was very
 347 similar between species, but differed with MP size: In the case of Art-MP5, fluorescence was
 348 higher during feeding days without difference between days 1 and 2, if compared to day 1 of
 349 clearance. In contrast, in medaka fed Art-MP20, the fluorescence remained constant during
 350 the first 3 days, while in the case of zebrafish fluorescence was slightly lower on day 1 of
 351 clearance when compared to day 1 of exposure. In all cases, fluorescence had completely dis-
 352 appeared on day 2 of clearance. It is noteworthy that, independent of the species, the fluo-
 353 rescence intensity obtained with MP20 was significantly lower than with MP5.



354

355 **Fig. 4:** Fluorescence intensity (MP uptake) in the gut lumen of zebrafish (*Danio rerio*) and
 356 marine medaka (*Oryzias melastigma*) measured in fluorescent larvae over time. Fish larvae
 357 were fed using the same schedule as described in Fig. 3. Marine medaka larvae were fed Art-
 358 MP5 (A) and Art-MP20 (B); zebrafish larvae were fed Art-MP5 (C) and Art-MP20 (D). Trip-
 359 licates of 20 individuals were monitored; data are given as means \pm SEM; one-way-ANOVA:
 360 ** = $p < 0.01$.

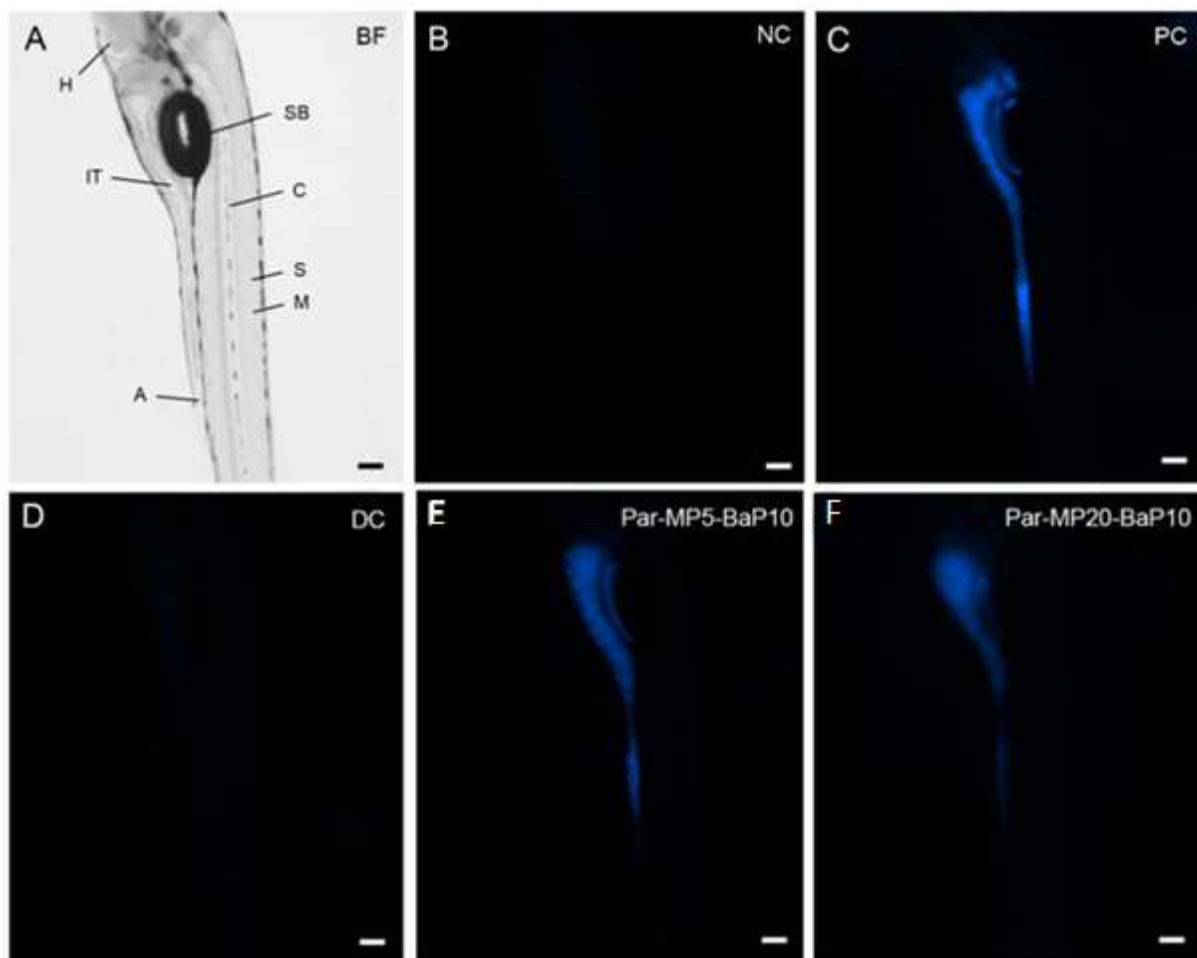
361

362

363 **Transfer of benzo[a]pyrene (BaP) to zebrafish and medaka larvae via MPs given to prey**

364 Due to its strong autofluorescence, BaP can easily be tracked within organisms. The BaP-
365 specific fluorescence in fish larvae that had fed on *Paramecium* or *Artemia* nauplii previously
366 loaded with BaP adsorbed to MPs thus confirmed successful transfer from MPs to fish larvae.
367 Independent of particle size, no BaP-specific fluorescence was detected in larvae from either
368 negative or desorption controls, or in larvae fed clean “virgin” MPs (Fig. 5).

369 The negative result for the desorption control specifically illustrates that in clean water BaP
370 did not re-dissolve off the MPs into the water column. Therefore, BaP-specific fluorescence
371 signals under other experimental conditions could not derive from previously desorbed BaP to
372 water. Direct exposure to waterborne BaP (PC) also resulted in a distinct accumulation of BaP
373 within fish larvae.

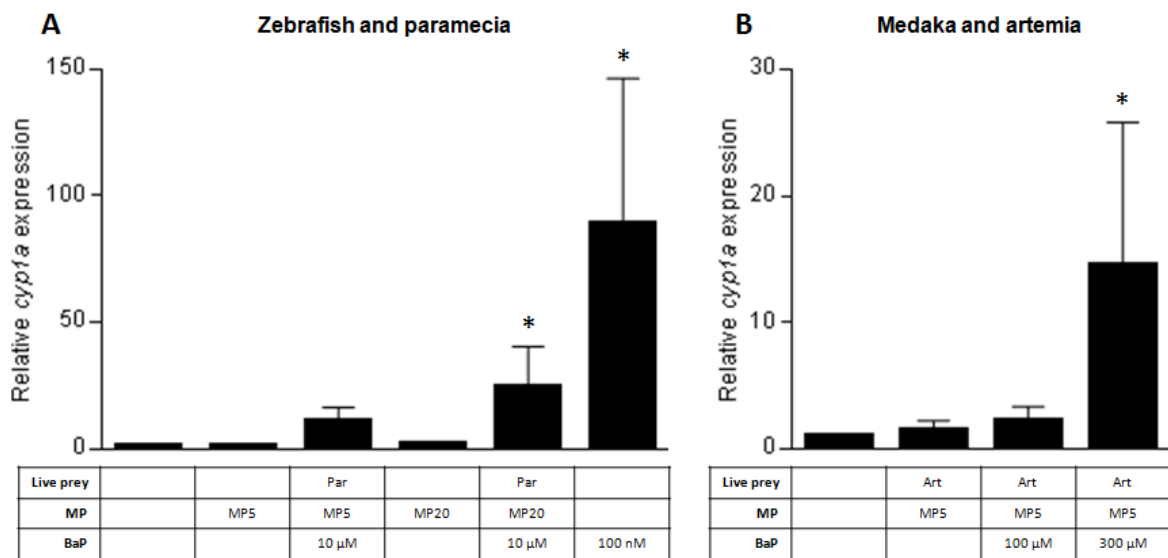


374
375 **Fig. 5:** Uptake of benzo[a]pyrene (BaP) into 168 h old zebrafish (*Danio rerio*) larvae after
376 exposure to MP-BaP or feeding with Par-MP-BaP. (A) Bright field micrograph of the larvae
377 (H – heart, IT – intestinal tract, A – anus, SB – swim bladder, C – chorda, S – somites, M –
378 muscles). (B) Negative control without any fluorescence signal. (C) Positive control (100 nM
379 waterborne BaP) with a strong signal along the entire intestinal tract. (D) Desorption control
380 (DC) without any fluorescence signal. (E) Larvae feeding on Par-MP5-BaP10 revealed a
381 strong signal. (F) Larvae fed Par-MP20-BaP10.

382 **Bioavailability of BaP transferred to fish larvae through MPs given *via* prey**

383 In order to biologically quantify the bioavailability of BaP transferred by MPs, the induction
 384 of *cyp1a* was monitored using qPCR in both species. In zebrafish, consistently with the ab-
 385 sence of BaP fluorescence, no *cyp1a* induction was visible in negative controls and in fish fed
 386 on virgin MPs (independent of size), whereas a 90 fold induction could be documented for the
 387 positive control (waterborne BaP; Fig. 6A; $p = 0.035$). In the case of Par-MP5-BaP10, there
 388 was no significant induction of *cyp1a*, when compared to the negative control. In contrast,
 389 exposure to MP20-BaP10 significantly induced *cyp1a* transcription after exposure *via Para-*
 390 *mecium* (Par-MP20-BaP10; fold change: 16.5, $p = 0.022$).

391 The same experiment was carried out using marine medaka and *Artemia* as live prey interme-
 392 diate. After 2 d of exposure (i.e. 4 meals), no change of *cyp1a* transcription was observed in
 393 marine medaka larvae fed Art-MP5, if compared to the control (*Artemia* only; fold change:
 394 1.455; $p = 0.22$; Fig. 6B). Similarly, the use of Art-MP5-BaP100 produced no change of
 395 *cyp1a* transcription (fold change: 2.18; $p = 0.17$). In contrast, when marine medaka were fed
 396 Art-MP5-BaP300, a significant induction of *cyp1a* transcription was observed, if compared to
 397 *cyp1a* transcription in Control larvae (fold change: 14.57; $p = 0.03$). These results demon-
 398 strated the efficiency of *Artemia* as an intermediate host to deliver MPs to fish larvae and the
 399 bioavailability of BaP after exposure to Art-MP5-BaP300.



400

401

402 **Fig. 6:** Transcription of *cyp1a* relative to the negative control (NC; first bar of each graph) in
 403 zebrafish (*Danio rerio*; A) and marine medaka (*Oryzias melastigma*; B). Tables below each
 404 graph describe exposure conditions. Note that scales are different between graphs. Three
 405 (zebrafish) and six (medaka) pools of ten larvae were monitored; data are given as means \pm
 406 SD; statistical analyses based on ANOVA-on-ranks against NC: * = $p < 0.05$.

407

408 Discussion

409 The results of this study reveal the efficient transfer of very small MPs with dimensions in the
410 lower μm size range *via* trophic transfer through exposed prey in the micro- or millimeter
411 range organisms to vertebrate larvae, highlighting the importance of considering trophic food
412 webs when analyzing microplastic exposure. Using two types of MPs different in both struc-
413 ture and size illustrates the variability of type- and size-dependent effects on organisms. In the
414 present experiment, we used well-defined 10 - 20 μm polyethylene particles as well as 1 -
415 5 μm particles made up of a CosphericTM proprietary plastic; both sizes are likely to regularly
416 occur in the environment.

417 As a common model organism for freshwater protozoans, *Paramecium* had readily ingested
418 the very small 1 - 5 μm MPs of as early as 1 h after initiation of the incubation. In contrast,
419 larger particles (10 - 20 μm) were apparently too large to be ingested efficiently. A similar
420 size range for plastic ingestion by *Paramecium* was mentioned by Holm et al. (2013), who fed
421 *Paramecium* with 1 μm fluorescent polystyrene particles (Holm et al., 2013). As representa-
422 tives of zooplankton filter feeders, *Artemia* nauplii also ingested both 1 - 5 and 10 - 20 μm
423 MPs at high amounts, which had already been demonstrated by (Batel et al., 2016).

424 In both medaka and zebrafish larvae feeding on *Paramecium* and *Artemia* nauplii containing
425 MPs could be documented inside the gastrointestinal tract within few hours of ingestion.
426 Some variability was observed in MPs uptake, especially in the case of exposure of zebrafish
427 through *Paramecium* (*e.g.* at 144 hpf in Table 2). This may be related to variation in larval
428 activity level through development (Vignet et al., 2013). In addition, in zebrafish, feeding
429 starts at 5 dpf and is associated with an increase in gut function at this age (Sadler et al.,
430 2013). It is thus likely that what is observed at 144 hpf is due to inter-individual variability in
431 developmental rate, potentially affecting these traits. Such transfer of MPs through prey has
432 already been shown from mesozooplankton to macrozooplankton (Setälä et al., 2014) as well
433 as from mussels to crabs (Farrell and Nelson, 2013), but – to the best of our knowledge – not
434 in fish larvae. Conversely, after direct exposure *via* water, no MPs were visible in the intesti-
435 nal tracts of the larvae. Thus, fish larvae primarily ingested the small MPs *via* the prey, but
436 not directly *via* the water column.

437 Trophic transfer of microplastics is also receiving increasing attention in microplastic field
438 studies. Nelms et al. (2018) analyzed sub-samples of scat from captive seals (*Halichoerus*
439 *grypus*) and whole guts of wild caught Atlantic mackerel (*Scomber scombrus*) the seals were
440 fed on. Results indicate that ‘trophic transfer represents an indirect, yet potentially major
441 pathway of microplastic ingestion for any species whose feeding ecology involves the con-
442 sumption of whole prey, including humans’ (Nelms et al., 2018). Trophic transfer of MPs has
443 also been documented in the field: Welden et al. (2018) analyzed microplastics contents in
444 plaice (*Pleuronectes platessa*), spider crab (*Maja squinado*) and ingested sand eels (*Ammo-*
445 *dytes tobianus*) and found microplastics in ingested sand eels, directly proving microplastic
446 trophic transfer in the wild (Welden et al., 2018). This has also been suggested for Mediterra-
447 nean fish species (Savoca et al., 2019a; Savoca et al., 2019b).

448 In the present laboratory study, focus was laid on the mode-of-action of microplastic trophic
449 transfer in early life-stages of fish, analyzing time scales and kinetics of first MPs uptake and
450 potential transfer of hazardous chemicals by MPs using benzo[a]pyrene (BaP) as a model
451 PAH. Zebrafish were feeding on *Paramecium* on MPs from 120 hpf, whereas marine medaka
452 started feeding on *Paramecium* immediately from hatching. In the case of the fish species
453 studied here, approx. 6 - 7 d after hatching (i.e. 8 dpf for zebrafish and 17 dpf for marine
454 medaka), larvae were able to switch to larger prey and started feeding on *Artemia* nauplii. As
455 a consequence, as shown above, exposure of fish larvae to MPs *via* live prey was able to reach
456 high levels of exposure. This is in agreement with field studies, which revealed MP uptake in
457 larvae of various fish species (Steer et al., 2017). Previous work showed that, in the case of
458 zebrafish, uptake of MPs through *Artemia* nauplii also occurred in juveniles or adults (Batel et
459 al., 2016); thus, trophic food chains can deliver significant amounts of very small MPs from
460 the first food uptake by early life-stages of fish until adulthood, meaning a whole life expo-
461 sure to very small MPs through uptake *via* prey.

462 Despite massive uptake of MPs, adverse effects never became obvious in neither *Paramecium*
463 and *Artemia* nauplii, nor fish larvae. A longer exposure may have resulted in biological ef-
464 fects. Likewise, neither accumulation nor translocation to tissues other than the gut could be
465 observed over time. This is in line with previous experiments performed in juvenile seabass
466 (*Dicentrarchus labrax*) fed with the same MPs as in the present study, however, contained in
467 regular food pellets (Mazurais et al., 2015). This lack of effects is likely due to the fast eges-
468 tion rate of fish larvae: Effective egestion started 5 h after the onset of exposure, and disap-
469 pearance of fluorescence in the gastrointestinal tracts of the larvae was complete after less
470 than two days after the last exposure. This corroborates observations in seabass exposed to
471 pellets spiked with MPs (Mazurais et al., 2015), where translocation to tissues could also not
472 be observed. Likewise, previous studies using similar trophic exposure of adult zebrafish,
473 Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) revealed no
474 translocation to tissues (Batel et al., 2020; Batel et al., 2016; Elizalde-Velazquez et al., 2020;
475 Zhu et al., 2020). However, there are also reports on translocation in fish, particularly to the
476 liver, after experimental exposure (Bakir et al., 2016; Ding et al., 2018) and in fish collected
477 in the field (Avio et al., 2015; Collard et al., 2017). Such discrepancies cannot be resolved
478 easily, since there is no correlation of translocation with MP size, shape, material or duration
479 in the case of experimental exposures (Avio et al., 2015; Bakir et al., 2016). In freshwater
480 studies, however, a recent review on MP uptake and translocation in a multitude of species
481 revealed that, at least partly, studies reporting translocation should be re-evaluated
482 (Triebkorn et al., 2019).

483 Another most important aspect in MP research is the much-discussed issue of a potential
484 transfer of hazardous organic chemicals to aquatic organisms upon MP uptake (Bakir et al.,
485 2016; Koelmans et al., 2016; Lohmann, 2017). In the present study, the transfer of ben-
486 zo[a]pyrene (BaP), a model PAH, was analyzed: Using BaP-specific autofluorescence and
487 *cyp1a* induction, absorption of BaP by larvae and, thus, bioavailability of BaP could clearly
488 be documented. However, concentrations of both microplastics and BaP were beyond envi-
489 ronmentally relevant concentrations and thus only document potential modes-of-action. Com-

490 parison of *cyp1a* transcription induction by positive control and prey is quite impossible since
491 actual exposure of larvae depends on the number of MPs ingested by the prey and the number
492 of preys ingested by larvae.

493 MP ingestion by *Paramecium* and *Artemia* nauplii, and consequently by fish larvae, was
494 higher with 5 µm MPs than with the larger 20 µm MPs. In contrast, this difference did not
495 become apparent when BaP transfer and subsequent BaP-specific fluorescence in larvae was
496 visualized by optical means. The induction of *cyp1a* was even higher with the larger 20 µm
497 MPs than with 5 µm MPs, which was most likely related to a difference in chemical composi-
498 tion of MPs. According to the suppliers, the 20 µm MP particles were made of polyethylene
499 (PE), whereas the smaller 5 µm particles were made of a non-disclosed polymer other than
500 PE, at least according to their melting temperatures. Interestingly, the desorption control,
501 which analyzed the desorption of BaP from MPs in clean water, produced no fluorescence in
502 larvae. This points out that not only trophic transfer, but also the type of plastic used as a ve-
503 hicle and chemical equilibria need to be taken into consideration when assessing the risk of
504 MPs in the aquatic environment.

505 Although there has been a critical discussion about testing “clean” laboratory animals and
506 MPs spiked with hazardous organic chemicals with regard to chemical equilibrium
507 (Lohmann, 2017), the present study illustrates that, in simple mode-of-action studies, the
508 feeding of MPs *via* prey may increase (1) the amount of MPs taken up into intestinal tracts of
509 fish larvae and (2) at least the uptake of hazardous organic chemicals. Thus, transfer of haz-
510 ardous organic chemicals bound to MP along trophic food chains might play an important role
511 in aquatic ecosystems with respect to MP toxicity and the transfer of hazardous organic chem-
512 icals. Furthermore, it is important to note that hazardous organic chemicals on MPs may reach
513 chemical equilibrium in one area, but – following drift of MPs – may reach areas that had not
514 been in touch with high concentrations of hazardous organic chemicals before, e.g. the deep
515 sea or remote areas (Van Cauwenberghe et al., 2013). Thus, the chemical equilibrium to
516 aquatic organisms in such remote areas is most likely different from that in the regions of MP
517 origin and might well account for the transfer of hazardous organic chemicals to biota.

518 Fish larvae can be exposed to MPs by prey from the very first day of feeding until adulthood,
519 making exposure potentially lasting a whole life. Already at small scale, the transfer along
520 food webs is most important in aquatic ecosystems. If MP transfer already starts at such
521 small-scale events and continues to higher levels within trophic food webs, the overall live-
522 long MP exposure in natural environments might well be higher than expected (Pannetier et
523 al., 2020). This seems especially true regarding nanoplastic particles, for which the actual
524 number is potentially by orders of magnitude higher than that of microplastics, and the biolog-
525 ical importance is likely to be much higher than that of microplastics (Ivleva et al., 2017).

526

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542

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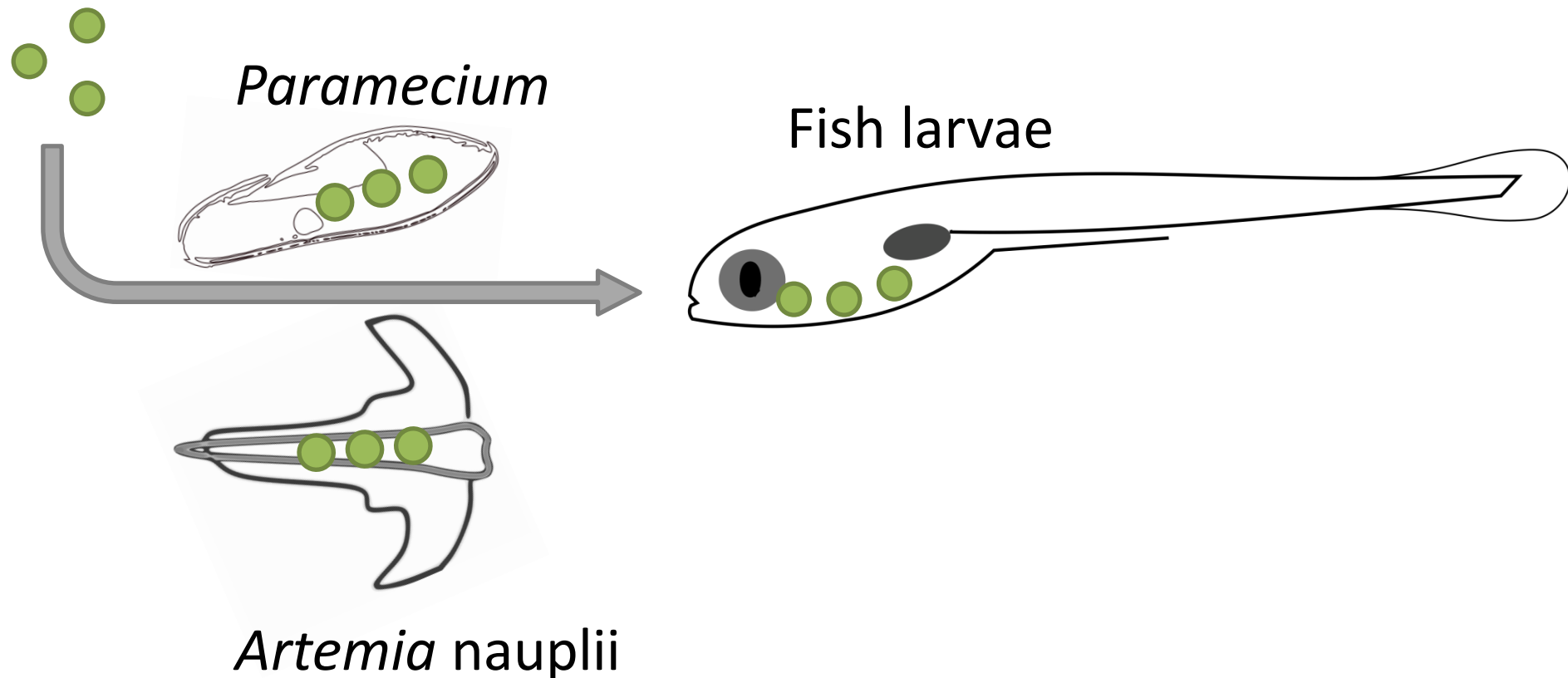
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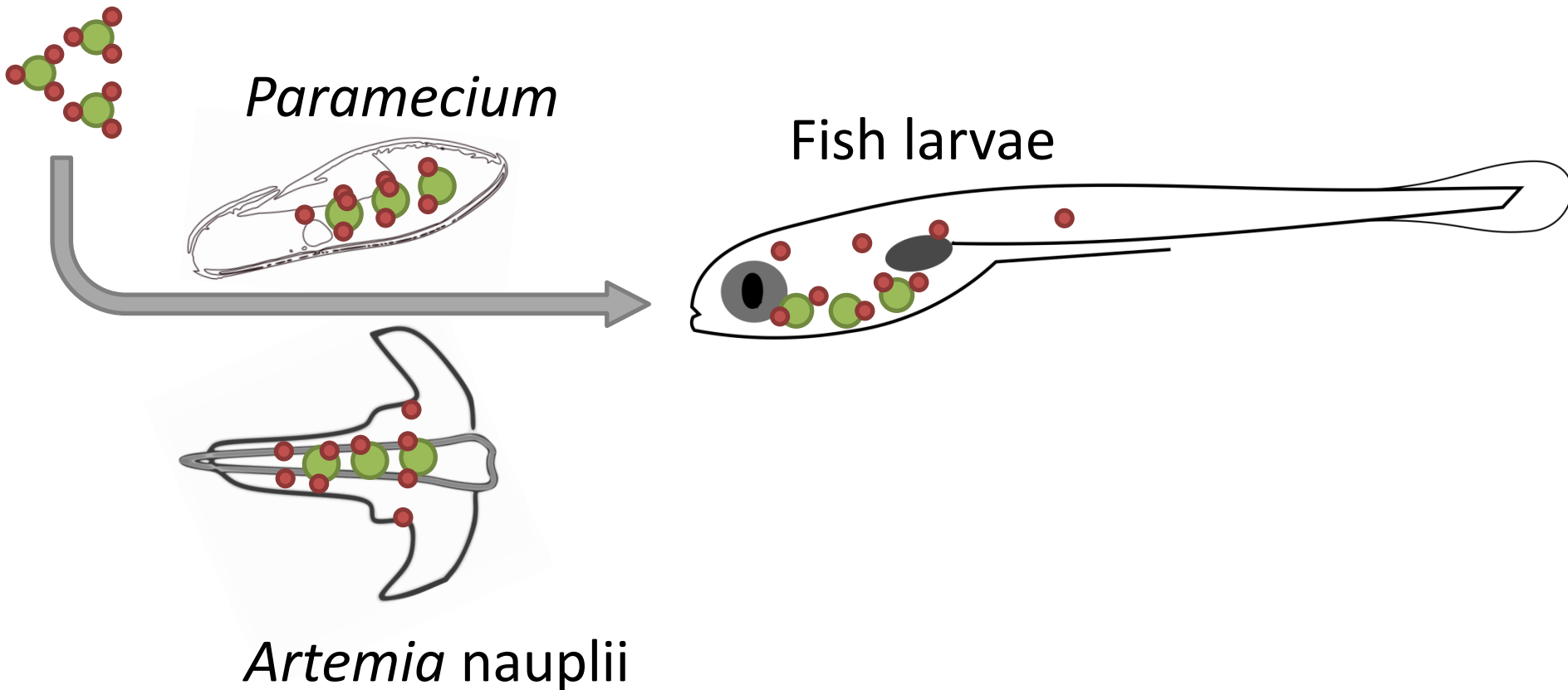
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Microplastics



Microplastics + Benzo[a]pyrene



Prey as *Paramecium* and *Artemia* nauplii efficiently transfer very small microplastics and associated benzo[a]pyrene to zebrafish and marine medaka larvae exposing sensitive early life stages of fish