Trophic transfer of microplastics enhances tissue accumulation of chemical additives in fish

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Abstract

A variety of chemical additives are incorporated into plastics during their production process to give desirable properties. Although studies have suggested that microplastic ingestion can lead to accumulation of these chemicals in marine organisms, none provided the direct evidence in fish species. Here, we demonstrated the tissue accumulation of chemical additives in fish following microplastic ingestion. We exposed fish (*Myoxocephalus brandti*) to polyethylene microplastics compounded with brominated flame retardants (BFRs; BDE209, DBDPE) and ultraviolet stabilizers (UVs; UV-234, UV-327, BP-12) suspended in the water column, or to mysids (*Neomysis* spp.) pre-exposed to the same microplastics. Our results showed a maximum of 345-fold higher concentrations of additives in fish exposed to microplastics than the ambient sample. Also, fish fed plastic-exposed mysids accumulated significantly greater concentrations of BFRs in muscle than fish exposed to microplastics suspended in the water column ($p < 0.001$). This indicates that trophic transfer of microplastics has greater contribution to tissue accumulation of BFRs in fish than the waterborne ingestion. In contrast, no significant difference in the accumulation of UVs was found between the treatments, except for UV-327 in liver. These results suggests that the relative contribution between direct ingestion of microplastics from the water column and indirect ingestion via trophic transfer on tissue accumulation of additives in fish varies among compounds. Compounds with high molecular weight and high hydrophobicity like BDE209 and DBDPE were considered difficult to leach out from plastics, however, our study showed that trophic transfer of microplastics can facilitate the accumulation of such compounds from plastics to fish.

Keywords: Plastic ingestion, Brominated flame retardants, Ultraviolet stabilizers, *Neomysis* spp. *Myoxocephalus brandti*

41

42 **1. Introduction**

43 As plastic production has been increasing over the years, plastic debris have become
44 ubiquitous pollutants found throughout marine environments (Auta et al., 2017; Burns and
45 Boxall, 2018; Desforges et al., 2014; Duis and Coors, 2016). Especially, plastic particles
46 smaller than 5 mm called microplastics are concerned as an emerging pollutant in marine
47 ecosystems (NOAA, 2020). Their small size allows a variety of organisms to ingest such as
48 whales, seabirds, fish, and invertebrates (Auta et al., 2017; Cole et al., 2013; Rummel et al.,
49 2016; Zhang et al., 2019). Many studies have shown that ingesting microplastics can cause
50 negative physical impacts on organisms such as blockage and laceration of the digestive tract
51 (Derraik, 2002; Khosrovyan et al., 2012; Wright et al., 2013). In most cases, however, these
52 negative effects were observed with unrealistically high exposure concentration in laboratory
53 experiments (Cunningham and Sigwart, 2019; Lenz et al., 2016), and the current monitored
54 concentration in the ocean is considered to have negligible impacts on organisms because
55 microplastics can be egested from the body without long-term accumulation (Beiras and
56 Schönemann, 2020; A. Dawson et al., 2018).

57 Aside from their physical effects, microplastics also have chemical effects on
58 organisms. Plastics contain an array of chemicals that are either adsorbed to the polymer
59 surface from the surrounding water or incorporated during the manufacturing process, the latter
60 called plastic additives (Andrady, 2011; Cole et al., 2011; Ziccardi et al., 2016). These
61 chemicals have been shown to transfer to biological tissues following the ingestion of
62 microplastics and are concerned about their impacts on ecosystems because some of them have
63 persistent and toxic natures (Hermabessiere et al., 2017a; Wardrop et al., 2016; Ziccardi et al.,
64 2016). However, some model studies suggested that plastics are not important for the
65 accumulation of these pollutants in organisms because their concentrations in plastics would

be at equilibrium with those in organisms and the chemical diffusion from plastics to organisms would not occur (Gouin et al., 2011; Koelmans et al., 2016). In contrast, microplastics may play a more relevant role in the transfer of chemical additives as they are added to plastic polymers at high concentrations (~80% by weight (Gangi, 1999)), whereas the information about the tissue accumulation of additives "compounded" in plastics is still far limited compared to that of adsorbed pollutants. A recent study by Tanaka et al. (2020) demonstrated that the ingestion of plastics resulted in the tissue accumulation of chemical additives in seabird. While no study has so far provided the direct evidence that microplastic ingestion can lead to the tissue accumulation of chemicals additives in fish species, Coffin et al. (2019) demonstrated that *in vivo* fish gut simulated condition using the digestive enzyme enhanced the leaching of additives from plastics, which suggests that tissue accumulation in fish species is plausible. Studies using fish species are especially important considering the food web structure of marine ecosystems and the potential impact on human health through direct consumption (Campanale et al., 2020; Huang et al., 2020).

There are two possible pathways for predatory fish to ingest microplastics, either from the water column or their prey (Walkinshaw et al., 2020). Most microplastic exposure studies focus either on waterborne or dietary exposure, but none of them compared their relative contribution to the total ingestion. Our prior work compared the waterborne ingestion of microplastics and the trophic transfer using fish and mysids, and revealed that the trophic transfer is more important than the waterborne ingestion (Hasegawa and Nakaoka, 2021). If microplastic ingestion by fish leads to tissue transfer of plastic-derived chemicals, fish may receive more impacts on the exposure of those chemicals via trophic transfer of microplastics than the waterborne exposure.

The present study aims to examine the tissue accumulation of chemical additives in fish following microplastic exposure and the relative contribution of the waterborne and trophic transfer exposure on the accumulation. We used a crustacean mysid (*Neomysis* spp.) and a

benthic fish (*Myoxocephalus brandti*) as a model prey-predator system that was also used in our prior work (Hasegawa and Nakaoka, 2021). We conducted the exposure experiments using polyethylene microplastics compounded with two brominated flame retardants (BFRs; BDE209, DBDPE) and three ultraviolet stabilizers (UVs; UV-234, UV327, BP-12). To examine the relative importance of the two exposure pathways, we exposed the fish to polyethylene microplastics suspended in the water column or to mysids pre-exposed to polyethylene microplastics, and analyzed the concentration of each additive in fish muscle and liver. We hypothesized that the fish would accumulate the additives following microplastic ingestion and that trophic transfer of microplastics would yield the greater accumulation of the additives than the waterborne ingestion.

2. Materials & Methods

2.1 Animal collection and acclimation

We collected organisms for the experiment on September 7, 2020 at a seagrass bed in Akkeshi-ko estuary (43°02′ N, 144°52′ E), which is located in eastern Hokkaido, northern Japan. Mysids (*Neomysis* spp., body length: 7.70 ± 1.53 mm standard deviation, wet weight: 8.20 ± 3.59 mg standard deviation) and juvenile *M. brandti* (body length: 7.40 ± 0.52 cm standard deviation, wet weight: 4.35 ± 0.81 g standard deviation) were collected using an epibenthic sled. For the measurement of contaminant concentrations in the ambient environment, 90 mysids and three fish were immediately frozen and stored until the later chemical analysis, and the other fresh animals were brought to the laboratory for the experiments. Mysids were acclimated in 30-L aquaria overnight with flow-through seawater that were filtered by fine sand to allow them clear possible microplastics from their guts. Ten glass aquaria (245×165×160 mm) were prepared and one randomly selected fish was assigned to each aquarium, and then acclimated for 7 days prior to the start of the experiment. During

117 the acclimation, we daily fed the mysids microalgae (Shellfish diet 1800; Reed Mariculture)
118 and the fish fresh mysids. Each aquarium contained approximately 100 mysids or 20 fish.
119
120 **2.2 Preparation of plastics with additives**
121 We used cylindrical low-density polyethylene pellets (diameter 5 mm, length 5 mm;
122 DJK Corporation, Chiba, Japan) compounded with five plastic additives. Polyethylene is one
123 of the most produced plastic polymers and is commonly found in marine environments
124 (Andrady, 2011; Beiras et al., 2018; Burns and Boxall, 2018). Following five additives were
125 industrially compounded in the polyethylene pellets: 1,2,3,4,5-pentabromo-6-(2,3,4,5,6-
126 pentabromophenoxy)benzene (BDE209, CAS no. 1163-19-5), 1,2,3,4,5-pentabromo-6-[2-
127 (2,3,4,5,6-pentabromophenyl)ethyl]benzene (DBDPE, CAS no. 84852-53-9), 2-(benzotriazol-
128 2-yl)-4,6-bis(2-phenylpropan-2-yl)phenol (UV-234, CAS no.70321-86-7), 2,4-ditert-butyl-6-
129 (5-chlorobenzotriazol-2-yl)phenol (UV-327, CAS no. 3864-99-1), and (2-hydroxy-4-
130 octoxyphenyl)-phenylmethanone (BP-12, CAS no. 1843-05-6). BDE209 and DBDPE are
131 widely used in plastic products and electrical appliances as flame retardants and are suspected
132 to be non-readily degradable and have long-term toxicity (Hermabessiere et al., 2017b; NITE,
133 2017). UV-234, UV-324 and BP-12 are also commonly added to plastic products as UV-
134 stabilizers, which have the potential for bioaccumulation and toxicity on organisms (Kim et al.,
135 2011; Nakata et al., 2009). These compounds were selected as previous studies reported their
136 frequent occurrences in plastic debris collected in natural environments and marine organisms
137 (Rani et al., 2015; Rochman et al., 2014; Tanaka et al., 2019). The concentration of each
138 additive in the pellets was set below 0.04 % by weight, which is of the same order of magnitude
139 as those observed in these studies. Polyethylene powder (Flo-Thene, FG701N, Sumitomo Seika
140 Chemicals Co., Ltd., Osaka, Japan) and authentic standards of the additives in powder form
141 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were mixed well and molded into pellets

in a co-rotating twin-screw kneading extruder (HK-25D; Parker Corporation, Inc., Tokyo, Japan). The pellets were then melted and re-extruded twice to obtain a uniform distribution of the constituents. The pellets were further grinded into powders by a plastic cutting mill (PLC-2M, Osaka Chemical Co., Ltd., Osaka, Japan) and the powders were sieved over a 63 μm and 32 μm mesh. Particles left on the 32 μm sieve were collected and used for the experiments.

For measurement of the particle size, the quantification method with nile red was adapted from Erni-Cassola et al. (2017) using a nylon mesh filter with 8 μm pore size. Particle area was measured, and the square root was calculated as particle size. The targeted polyethylene microplastics had an average size of 29.90 ± 13.40 μm and their size distribution is shown in Fig.1. The concentrations of five additives in polyethylene microplastics were quantified (Table 1) and the composition of each additive is shown in Fig. 2.

To prepare the microplastic-suspended solution, a glass beaker was filled with 100 mL of distilled water and boiled in a microwave. Ten microliters of surfactant (Tween 80; polyethylene sorbitol ester, Cospheric LLC) were added and stirred with a glass rod for 30 s. Next, 5 mL of the solution was transferred to another grass beaker filled with 95 mL of boiled distilled water to make 0.004 % surfactant solution. The solution was left at room temperature for 1 h. Before starting the exposure experiment, 8 mg of polyethylene microplastics were added to a plastic tube containing 8 mL of 0.004 % surfactant solution. The tube was vortexed and left at room temperature for 1 h to immerse into the solution. The suspension solution was prepared each time for the experiments to minimize the leaching of the chemicals from polyethylene microplastics during the immersion process.

2.3 Leaching experiment

To determine the amount of chemicals leaching from microplastics to seawater, three beakers filled with 1-L filtered seawater (< 1 μm) were prepared. 250 μL of suspension solution

(corresponds to 500-µg microplastics) was added to each beaker and left for 24 h. We constantly circulated the seawater by aeration during the test. After 24 h, the seawater was transferred to a 1-L stainless container and stored in the freezer until the chemical analysis.

2.4 Ingestion experiment for mysids

Thirty 1-L glass beakers were prepared and divided into the following two groups; fifteen bottles filled with 1-L filtered seawater (< 1 µm) containing 500-µg/L microplastics, and 15 bottles filled with 1-L leachate seawater. The microplastic concentration was chosen as an environmentally relevant concentration as it is within the same order of magnitude of the microplastic concentration reported in the North Pacific Subtropical Gyre, which is among the most heavily contaminated area around the world (Goldstein et al., 2012). Leachate seawater was prepared by filtering 1-L filtered seawater (< 1 µm) containing 500-µg/L microplastics to determine the uptake of the chemicals leaching from microplastics during the experiment by mysids. We randomly selected mysids from the acclimation aquaria and placed three in each bottle. We then fed the mysids 2 mg (dry weight) of microalgae. We constantly circulated the seawater by aeration to ensure that the microplastics were evenly distributed in the bottles. After 24 h of microplastic exposure, the mysids were flushed gently with filtered seawater to remove beads from the exoskeleton and their body length and wet weight were measured. Then, the mysids were frozen at - 30℃ and stored until the later analysis. The same procedure was repeated 4 days to collect 150 mysids in total from each group except that only 15 mysids in each group were used at 4th day.

2.5 Trophic transfer experiment

After the acclimation for fish, ten fish aquaria were divided into the following two groups: Five aquaria contained 2-L filtered seawater (< 1 µm) with a 500-µg/L microplastic

192 suspension and nine plastic-free mysids each, so that the fish would ingest polyethylene
193 microplastics only from the water column. Five other aquaria each contained nine mysids that
194 had been pre-exposed to 500-µg/L microplastics. The five aquaria containing pre-exposed
195 mysids were filled with 2-L filtered seawater (1 µm) without a microplastic suspension so that
196 uptake of polyethylene microplastics would be solely from the food source. The number of
197 mysids was determined from a preliminary experiment to ensure that all mysids were consumed
198 by the fish within 24 h. Pre-exposure of the mysids followed the protocol for the first
199 experiment. We carried out the exposure for 10 days and exchanged the seawater once a day.
200 We constantly circulated the seawater by aeration throughout the experiment to ensure that
201 microplastics were evenly distributed in the aquaria. We did not observe any microplastics on
202 the bottom of the tanks throughout the experiment. After 10 days of exposure, the fish were
203 gently flushed with filtered seawater to remove beads from the body surface. Following
204 measurement of their body length and wet weight, the fish were frozen at - 30℃ and stored
205 until the later analysis.
206
207 **2.6 Sample analysis**
208 **2.6.1 Chemical and reagents**
209 Acetone, n-hexane, 2,2,4-trimethylpentane (iso-octane), methanol (MeOH), pyridine,
210 acetic anhydride, hydrochloric acid, anhydrous sodium sulfate, Wakogel Q-22 (75 µm, 200
211 mesh) and Wakogel Q-23 (75-150 µm, 100–200 mesh) were purchased from Wako Pure
212 Chemical Ltd. (Osaka, Japan). Dichloromethane (DCM) were purchased from Kanto Chemical
213 Co., Inc. (Tokyo, Japan). Analytical standards for BDE209 and DBDPE were purchased from
214 Wellington Laboratories Inc. (Guelph, Ontario, Canada). Hexa-nona BDE individual congeners
215 were obtained from Wellington Laboratories Inc. (BDE179, 183, 188 and 202), Cambridge
216 Isotope Laboratories Inc. (BDE207; Tewksbury, MA, US) and Accustandard Inc. (BDE155,
217 184, 196, 197, 203, 206 and 208; New Haven, CT, US) respectively. UV327 and BP12 were

also obtained from Accustandard Inc. Analytical surrogates were used the followings; f-BDE208 (4'-fluoro-2,2',3,3',4,5,5',6,6'-nonabromo-diphenylether) from Chiron AS (Trondheim, Norway) for BFRs, UV327-d20 were from Toronto Research Chemicals, Inc. (North York, ON Canada) for UV327 and UV234 and BP12-d17 were from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan) for BP12. As internal injection standard (IIS), chrysene-d12 was purchased from Sigma–Aldrich (St. Louis, MO, USA). All glassware was rinsed with methanol, acetone, and distilled n-hexane three times respectively or pre-baked at 550 ℃ for 4 hours.

2.6.2 Biological samples

For chemical analysis, the liver and whole-body (mainly muscle tissues, excluding the intestines, head and fins) were used. Each freeze-dried sample was extracted by accelerated solvent extractor (ASE200; Dionex, Sunnyvale, CA, USA) with acetone/DCM (1:3, v/v). The samples were extracted in 11 mL stainless steel cells under the following conditions: preheat time, 0 min; heat time, 5 min; static time, 5 min; flush%, 100%; purge, 60 s; 2 cycles; pressure, 1500 psi; temperature, 100 ℃.

To determine lipid contents of whole-body sample from the weight of the dried extracts, aliquot of the extract was taken into another weighed-4 mL vial. After injection the surrogates (f-BDE208, UV327-d20 and BP12-d17) into another aliquot, it was concentrated just to dryness in a rotary evaporator and transferred into a 10 mL centrifugation tube. After reducing the solvent less than 100 mL by gentle nitrogen stream, we added 50 mL each of pyridine and acetic anhydride into each tube. After holding at room temperature for >8 h, the reaction was stopped with the addition of 200 mL 4 M HCl. Acetylates were extracted with n-hexane 5 times. The n-hexane extract was passed through anhydrous sodium sulfate for dehydration and collected into pair-shaped flask.

After a solvent substitution from n-hexane to DCM, the acetylated sample was purified by gel permeation chromatography (GPC). About 2 mL of sample was subjected to GPC (2 cm i.d. ×30 cm, CLNpak EV-2000; Showa-denko, Tokyo, Japan) to separate target compounds from biolipids in DCM at an eluent at flow rate of 4 mL/min. The fraction with a retention time of 12 to 25 min was collected for further purification.

The GPC eluent was concentrated just to dryness in a rotary evaporator and placed in a 10% H2O-deactivated silica gel column (Wakogel Q-22, 1 cm i.d. × 9 cm). The first fraction including BFRs was eluted with 35 mL n-hexane/DCM (3:1, v/v). The second and third fraction including UVs were eluted with 15 mL MeOH/DCM (1:99, v/v) and 20 mL (1% MeOH/DCM)/Acetone (1:4, v/v) respectively. The second and third fraction were combined and evaporated the solvents and transferred into a 1 mL amber ampoule for instrumental analysis.

The first eluent containing BFRs was concentrated just to dryness in a rotary evaporator and placed in a fully activated silica gel column (Wakogel Q-23, 0.47 cm i.d. ×18 cm). The first fraction, containing aliphatic hydrocarbons, was eluted with 5 mL n-hexane. The second fraction, containing BFRs was eluted with 15 mL n-hexane/DCM (85:15, v/v) or 10 mL n-hexane/DCM (3:1, v/v). The BFR fraction was concentrated and transferred into a 1 mL amber ampoule for instrumental analyses.

2.6.3 Water samples

To analyze the water samples from the leaching experiment, the water was filtered through a glass fiber filter (GF/F), and f-BDE208, UV327-d20, and BP12-d17 dissolved in acetone were added to the filtrate as surrogates. Then, liquid-liquid extraction was carried out with dichloromethane (DCM) at 10% of the volume of the water sample, and the organic phase was collected while dehydrating with anhydrous sodium sulfate. This process was repeated

three times. The extracts were concentrated using a rotary evaporator and fractionated and purified using acetylation and 10 % water-inactivated silica gel column chromatography (Wakogel Q-22) as with the biological samples.

2.6.4 Instrumental analysis

For analysis of BFRs, the solvent was evaporated just to dryness under a nitrogen stream, and the residue was redissolved in 100 mL iso-octane.

UVs were analyzed on a 5977 quadrupole mass spectrometer fitted with a 7890 gas chromatograph (GC–MS; Agilent technologies, Inc., Santa Clara, CA, US). An Agilent HP-5MS 30-m fused silica capillary column (0.25 mm i.d., 0.25 mm film thickness) was used with helium as the carrier gas at 100 kPa. GC–MS operating conditions were set at 70 eV ionization potential with the source at 240 ℃ and the electron multiplier voltage at ~2000 eV. The injection port was maintained at 300 ℃, and the sample was injected in splitless mode, then purged 2 min after injection. The column temperature was held at 100 ℃ for 1 min, rose at 30 ℃/min to 160 ℃, at 10 ℃/min to 310 ℃ and was then held for 10 min. After a solvent delay of 4 min, selected ion monitoring mode was used. We monitored UV327 at m/z = 342 UV234 at 432, BP-12 at 213. Surrogate standards and IIS were monitored at m/z = 359 (UV327-d20), m/z = 343 (BP12-d17), at m/z = 240 (chrysene-d12) respectively. These were quantified by comparing the integrated peak area of the quantification ion with that of the IIS as derived from calibration lines drawn for individual UVs.

BFRs were determined by a micro electron capture detector fitted with a HP 7890 gas chromatograph (GC-mECD, Agilent Technologies Inc.). An Agilent DB-5 15-m fused silica capillary column (0.25 mm i.d., 0.25 mm film thickness) was used with helium as the carrier gas. To separate BDE208 and f-BDE208 clearly and gain higher peaks of BDE209 and DBDPE, we used ramped pressure mode for the analysis. The column pressure was held at 20.19 psi for

31 min, rose at 15 psi/min to 35 psi, and was then held for 6 min. The injection port was maintained at 250 ℃, and the sample was injected in splitless mode, then purged 0.75 min after injection. The column temperature was held at 80 ℃for 2 min, rose at 30 ℃/min to 240 ℃, at 3 ℃/min to 300 ℃ and was then held for 15 min. BFRs were identified and quantified against the standards.

2.6.5 Analytical quality control and quality assurance

A procedural blank was run in every set analyzed. Analytical values less than three times of the corresponding blank are expressed as ‘‘below the limit of quantification”, <LOQ. To certificate analytical quality control and quality assurance, quadruplicate mussel samples which were spiked the standard solution of target compounds were analyzed. The relative standard deviations of the concentrations of BDE209, DBDPE, UV327, UV234 and BP12 were 2%, 2%, 2%, 10% and 12% respectively. The averaged recoveries of BDE209, DBDPE, UV327, UV234, and BP12 were 90%, 114%, 92%, 100%, and 83% respectively.

2.7 Statistical Analysis

All analysis were conducted in R (R Core Team, 2020). To compare the differences in additive accumulation by mysids, fish muscle, and fish liver among treatments, we used generalized linear models (GLMs) with log link functions. A gamma distribution was assumed to account for the positive continuous values because the Shapiro-Wilk normality tests showed that the response variable had a non-normal distribution. We used log-transformed sample dry weight as an offset in the models. To test the effect of treatment, we performed a likelihood ratio test using the Anova function in the “car” package (Fox and Weisberg, 2019). For pairwise comparisons, we used Tukey’s HSD test as post-hoc analysis.

3. Results

3.1 Leaching experiment

Overall, 2.33% of the amount of additives were leached out from polyethylene microplastics for 24 h. The leaching rate varied among different additives (Table 2) with the mean of 3.16 %. BP-12 showed the higher leaching rate (8.79 %) than other additives (<3 %).

3.2 Ingestion experiment for mysids

Concentration of five additives accumulated in mysids significantly differed among treatments (Table 3). Mysids contained approximately 4-2450 times higher concentrations of additives by ingesting microplastics than those from the ambient environment (Fig. 3). Mysids treated with the leachate seawater also showed slightly elevated concentrations of additives than those from the ambient environment. For BP-12, the concentration in mysids treated with the leachate seawater was not significantly different from those exposed to microplastics.

3.3 Trophic transfer experiment

Concentration of the five additives in fish varied significantly among treatments (Table 5) although the patterns of variation greatly differed among additives and between body tissues (Fig. 4).

For BFRs, the treatment had significant effects on their concentrations both in muscle and liver. In muscle, fish fed plastic-exposed mysids showed the higher concentrations than those exposed to microplastics suspended in the water column. The mean effect was greater in BDE209 (71-fold on dry weight basis, 161-fold on lipid weight basis) than DBDPE (22-fold on dry weight basis, 52-fold on lipid weight basis). Although the lipid weight basis analysis showed the significantly higher concentration in the water treatment than the ambient control, no significant differences were observed between them on dry weight basis. Notably, the

343 within-treatment variation in the mysid treatment was also greater than the other two treatments.
344 In contrast, liver showed no significant difference in the concentration of BDE209 between the
345 mysid and water treatments although they were more than 3 fold greater than the ambient
346 control. The same patterns of significant variation was also found for DBDPE except that the
347 concentration in liver in the mysid treatment was not different from the ambient controls. Liver
348 showed different accumulation patterns of BFRs between the treatments. In the water treatment,
349 the mean concentrations were higher in liver (BDE209: 5.83 ng/g-dry, DBDPE: 9.79 ng/g-dry)
350 than muscle (BDE209: 0.47 ng/g-dry, DBDPE: 0.60 ng/g-dry) while the mysid treatment
351 showed the lower concentrations in liver (BDE209: 3.99 ng/g-dry, DBDPE: 2.52 ng/g-dry) than
352 muscle (BDE209: 4.30 ng/g-dry, DBDPE: 5.67 ng/g-dry).

353 For UVs, the treatment had significant effects on their concentration except for UV-
354 234 in muscle where no treatment effect was observed. In muscle, UV-327 and BP-12 showed
355 3-5 fold higher concentrations in the treatment groups than the ambient control (7-12 fold on
356 lipid weight basis). Unlike BDE209 and DBDPE, however, no significant differences were
357 found between the mysid and water treatments. In liver, UV-234 and UV-327 respectively
358 showed 9-13 fold and 35-89 fold higher concentration in the treatment groups than the ambient
359 controls, and also the concentration of UV-327 in the mysid treatment was significantly higher
360 than that in the water treatment. While BP-12 showed 3-fold higher concentration in the mysid
361 treatment than the ambient control, the concentration in the water treatment was not
362 significantly different from both the mysid treatment and the ambient control. In general, liver
363 showed the higher concentrations of UVs in liver than muscle in both treatments, whereas the
364 accumulation tendency between the tissues greatly differed among the chemicals. For example,
365 UV-234 showed the greatest increase in liver compared to muscle among UVs (Water: 29-fold,
366 Mysid: 53-fold), whereas BP-12 only showed 7-fold increase in the water treatment and 13-
367 fold increase in the mysid treatment.

368

4. Discussion

This study provides the first evidence that microplastic ingestion leads to the tissue accumulation of chemical additives in fish. We also revealed that trophic transfer of microplastics enhanced the accumulation of BFRs in fish muscle compared to the waterborne ingestion. However, as we observed the equal contribution to the accumulation of UVs between direct ingestion of microplastics from the water column and indirect ingestion via trophic transfer, their relative contribution varies among additives.

Previous studies showed that microplastics can act as a vector for the transfer of plastic-associated chemicals to marine organisms, but mostly targeted at the environmental contaminants adsorbed onto plastics, such as PCBs and PAHs (Besseling et al., 2013; Rochman et al., 2013). However, some model prediction studies pointed out that microplastics would not increase the body burden of these contaminants in organisms because other input sources like water and food would play a more important role in their accumulation (Bakir et al., 2016; Gouin et al., 2011; Koelmans et al., 2016). In contrast, plastic additives are different from pollutants adsorbed to plastics with respect that they are not attached to the polymer surface, but incorporated into plastics at high concentrations (Gangi, 1999). Several field observation studies found a positive relationship between plastic ingestion and chemical additives detected from marine fish and seabirds (Gassel et al., 2013; Tanaka et al., 2013), suggesting the potential occurrence of the tissue transfer of plastic-derived additives in natural environments. Our results support these evidence along with the previous finding by Tanaka et al. (2020) where they demonstrated that plastic ingestion led to the tissue accumulation of additives in seabird (*Calonectris leucomelas*) by the laboratory experiment. Gut fluid in both seabird and fish have been shown to play a key role in the leaching of chemicals from plastics (Coffin et al., 2019; Tanaka et al., 2015), which explains the tissue accumulation of chemical additives following plastic ingestion. Given the generality of plastic ingestion in fish and detection of additives in

plastics found in natural environments (Granek et al., 2020; Wang et al., 2020), our study raises a concern about the exposure of these additives to fish.

We found that trophic transfer of microplastics has greater effects on the accumulation of BDE209 and DBDPE in fish than waterborne ingestion. There are three possible explanations for this result which are not exclusive. First, fish was exposed to more chemical additives by ingesting microplastics via trophic transfer. Our previous study demonstrated that fish ingested greater amount of microplastics via trophic transfer than the waterborne ingestion (Hasegawa and Nakaoka, 2021). Higher ingestion rate of microplastics via trophic transfer should have resulted in the greater exposure intensity of chemical additives in fish stomach. Secondly, greater secretion of gut fluid may have elevated the leaching rate of additives from plastics in stomach. Gut fluid plays a key role in leaching hydrophobic chemicals from ingested plastics (Coffin et al., 2019; Tanaka et al., 2015). Digesting plastic-contaminated prey could facilitated the secretion of gut fluid in stomach, which may have enhanced the leaching of chemicals from plastics. Thirdly, mysids fragmented microplastics into smaller particles and helped the release of chemicals from plastics. Some crustacean species can fragment plastics into smaller particles, including mysids (A. L. Dawson et al., 2018; Hasegawa and Nakaoka, 2021; Mateos-Cárdenas et al., 2020). Generally, smaller plastics can release chemicals more easily due to their larger surface-to-volume ratio (Wright and Kelly, 2017). Ingesting microplastics from mysids, therefore, changed the size composition of plastics in fish stomach, and may have enhanced the leaching of chemicals. Compounds with high molecular weight and high hydrophobicity such as BDE209 and DBDPE (Table 1) were considered difficult to leach out from plastics and to transfer into biological tissues (Braekevelt et al., 2003; Cheng et al., 2020; Sun et al., 2019). However, we demonstrated the elevated accumulation of these compounds in the fish by ingesting microplastics from mysids. Thus, our study highlights the importance of indirect exposure via trophic transfer in chemical effects of microplastics on organisms.

Although we observed the greater accumulation of BDE209 and DBDPE via trophic transfer of microplastics, the waterborne exposure equally contributed the accumulation of UV-327, UV-234 and BP-12. This suggests that the trend in accumulation of chemical additives vary depending both on the properties of the chemicals and on exposure pathway. Given the ingestion of microplastics from the water column in fish are much lower than the trophic transfer (Hasegawa and Nakaoka, 2021), there could be other accumulation routes in the waterborne exposure of chemical additives. In marine fish, there are three possible routes for chemical uptake from the environment: gill uptake, dermal absorption and gut absorption by drinking water (Qiao et al., 2000). Although microplastic ingestion is assumed to only contribute to gut absorption, gill uptake could also be important because microplastics can also accumulate in gill. Water flux in the gill of marine benthic fish *Oligocottus maculosus*, which shares the same family of our target fish *M. brandti*, by ventilation is 300-fold higher than that in stomach by drinking water (Somo et al., 2020; Webb et al., 2001). Also, UV-234, UV-324 and BP-12 are likely to leach out from plastics more easily than BDE209 and DBDPE due to their lower molecular weight and less hydrophobic nature (Table 1). Considering the more frequent contact with microplastics and the lower hydrophobic nature, gill may have also played a role in the transfer of the UVs into fish body. Given that mysids accumulated additives leached out from plastics to the water column, fish was also likely to accumulate the additives from the water column in the same manner, especially for BP-12 that showed the higher leaching rate (10%) and the higher accumulation in mysids. Although we need further investigation on the mechanism that differentiates the behavior of additives depending on each exposure pathway, both waterborne exposure and trophic transfer of microplastics are important for the accumulation of plastic additives in fish.

Overall, we observed the preferential accumulation of the chemicals in liver over muscle, which is consistent with the previous findings targeted at the same class of compounds (Nakata et al. 2009; Tanaka et al. 2020). However, the effect size was much smaller in BFRs

(0.4-16 fold) than UVs (7-53 fold). Tanaka et al. (2020) observed the totally opposite trend in seabird that showed the considerably high level of accumulation for BDE209, but less for UV-327 and BP-12 in the liver. This indicates the species-specific accumulation patterns even in the same compounds. Tanaka et al. (2020) explained the lower accumulation of BP-12 by the degradation in liver due to the susceptible nature of BP-12 to the metabolization. In contrast, BDE209 is known to be resilient to biodegradation because of their high molecular weight. As several studies showed that BDE209 and DBDPE can be readily metabolized to lower brominated congeners in fish species (Chen et al., 2012; X. Wang et al., 2019), lower accumulation level for these two compounds could be explained by the metabolization. Still, we cannot fully exclude this possibility without conducting a depuration experiment to examine *in vivo* kinetics of these chemicals. Future studies should investigate the temporal variation of chemical concentrations in different tissues to understand the fate of plastic additives in organisms.

Studies have demonstrated that both BFRs and UVs have negative impacts on organisms (Kim et al., 2011; Morohoshi et al., 2005; X. Wang et al., 2019). Especially, extensive research have been conducted for BDE209 and have shown that the compound can act as an endocrine disrupter and cause neurotoxicity on fish (Sun et al., 2020; X. Wang et al., 2019; Y. Wang et al., 2019). BDE209 is also concerned for the accumulation in a human body because of the potential adverse health effects (Darnerud, 2008). As global seafood consumption has been increasing over the years, humans may be more frequently exposed to such additives through seafood consumption. A simple risk assessment of plastic additives on humans through consumptions of fish can be made as follows. First, BDE209 accumulation in fish, dairy intake of BDE209 through seafood consumption was estimated based on the result of BDE209 concentration in fish muscle. Assuming annual seafood consumption of 24.6 kg year^{-1} and the average body weight of 60 kg for adult Japanese (Ministry of Agriculture, 2016; Ministry of Health, 2018) and using our observed data on the mean BDE209 concentration of

4.70 ng/g.wet weight in fish muscle considering the both microplastic exposure pathways, dairy intake of BDE209 can be estimated as 0.46 ng/kg/day. According to the report from National Institute of Technology and Evaluation, lowest observed adverse effects levels (LOAEL) of BDE209 is estimated as 50 ng/kg/day to induce chronic toxicity for a human (NITE, 2017), which is approximately 110-fold higher than the estimated dairy intake value through seafood consumption. As our study used the environmentally relevant concentration of additives, accumulation of plastic additives in fish through microplastic exposure should be negligible for a human body in the current plastic pollution level of the world's ocean. However, current study only shows the marginal results with 10-day microplastic exposure to fish. Considering a long-term exposure of microplastics to fish throughout the life, fish would accumulate far more plastic-derived additives. A study predicted the abundance of plastics in the ocean would increase 50-fold by 2100 from the present condition (Everaert et al., 2018), suggesting that plastic exposure through seafood consumption would provide a relevant pathway for BDE209 to have adverse health effects on a human in the future scenario. We are also being exposed to these additives from the daily use of plastic products. The risk assessment study estimated a daily exposure amount of BDE209 to humans from different exposure routes such as inhalation of inside dusts or elution from plastic products, but did not include the exposure through seafood consumption (NITE, 2017). This study clearly showed the chemical accumulation in fish following microplastic exposure. Thus, these risk assessment studies should consider seafood consumption as a factor for the exposure of plastic-derived chemicals to a human body.

Conclusion

This study is the first to demonstrate that microplastic ingestion can lead to tissue accumulation of chemical additives in fish. We also revealed that indirect exposure of microplastics via trophic transfer can enhance the accumulation of BDE209 and DBDPE that were assumed difficult to accumulate in organisms because of their low leachability from

plastics. Some studies have suggested that microplastics themselves do not accumulate in the body and have negligible effects on organisms because they can be excreted from the body. Whereas, our findings indicate that microplastics play an important role in chemical bioaccumulation. As many chemical additives are potentially toxic, this study raises the concern about their negative impacts on marine ecosystems. To better understand the fates and the effects of chemical additives accumulated in organisms, further research should examine their *in vivo* kinetics and toxicity.

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720 **Tables**

721 **Table 1** Profile of additives compounded in the plastics used for the experiment. Octanol-water

722 partition coefficient (K_{ow}) is represented as a hydrophobicity parameter of each chemical.

Compounds	Concentration (μg/g-plastic)	Molecular weight (g/mol)	$logK_{ow}$
BDE209	357	959	12.1
DBDPE	74.2	971	13.6
UV-234	361	448	7.67
UV-327	397	358	6.91
BP-12	123	326	6.96

723

724 **Table 2** Result of the leaching experiment with 2 mg of microplastics exposed to 1-L seawater
725 for 24 h. SE represents standard error. Grey colored cells represent values under the limit of
726 quantifications (LOQ). All DBDPE values are technically lower than LOQ because the blank
727 value was very high.

Additive	Blank	Initial amount in plastics (ng)		Final amount in seawater (ng)		Leaching rate (%)	
		Mean	SE	Mean	SE	Mean	SE
BDE 209	0.127	713	60951	6.27	5.27	0.86%	0.72%
DBDPE	60.0	148	21930	4.13	3.53	2.61%	2.20%
UV 327	0.208	722	66262	5.12	1.34	0.70%	0.18%
UV 234	0.327	794	9662	23.2	5.94	2.83%	0.71%
BP 12	4.83	246	12082	23.8	3.14	8.79%	1.07%

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730 **Table 3** Results of likelihood ratio test on the generalized linear model for the concentrations
731 of additives in mysid as functions of treatment (ambient control, leachate treatment, and
732 microplastic treatment).

Additive	Fixed factor	LR Chisq	df	*p*-value
BDE209	Treatment	253	2	< 0.001
DBDPE	Treatment	186	2	< 0.001
UV234	Treatment	134	2	< 0.001
UV327	Treatment	280	2	< 0.001
BP12	Treatment	10.8	2	0.004

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735 **Table 4** Results of likelihood ratio test on the generalized linear model for the concentrations
736 of additives in fish muscle and liver as functions of treatment (ambient control, water treatment,
737 and mysid treatment).

Body tissue	Additive	Fixed factor	LR Chisq	df	*p*-value
Muscle (Dry weight)	BDE209	Treatment	27.9	2	< 0.001
	DBDPE	Treatment	27.2	2	< 0.001
	UV234	Treatment	0.441	2	0.802
	UV327	Treatment	9.72	2	0.00775
	BP12	Treatment	99.5	2	< 0.001
Muscle (Lipid weight)	BDE209	Treatment	38.2	2	< 0.001
	DBDPE	Treatment	36.4	2	< 0.001
	UV234	Treatment	5.15	2	0.0763
	UV327	Treatment	17.6	2	< 0.001
	BP12	Treatment	91.0	2	< 0.001
Liver (Dry weight)	BDE209	Treatment	18.5	2	< 0.001
	DBDPE	Treatment	11.9	2	0.00257
	UV234	Treatment	33.4	2	< 0.001
	UV327	Treatment	40.4	2	< 0.001
	BP12	Treatment	7.01	2	0.0301

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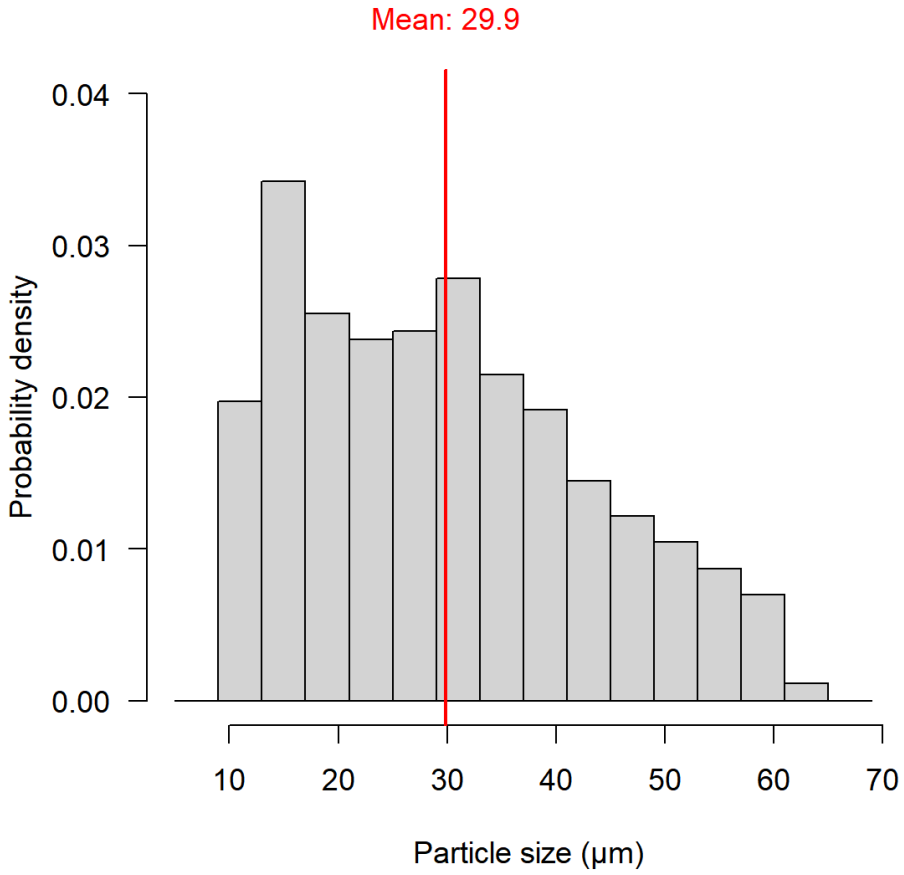
740 **Figures**



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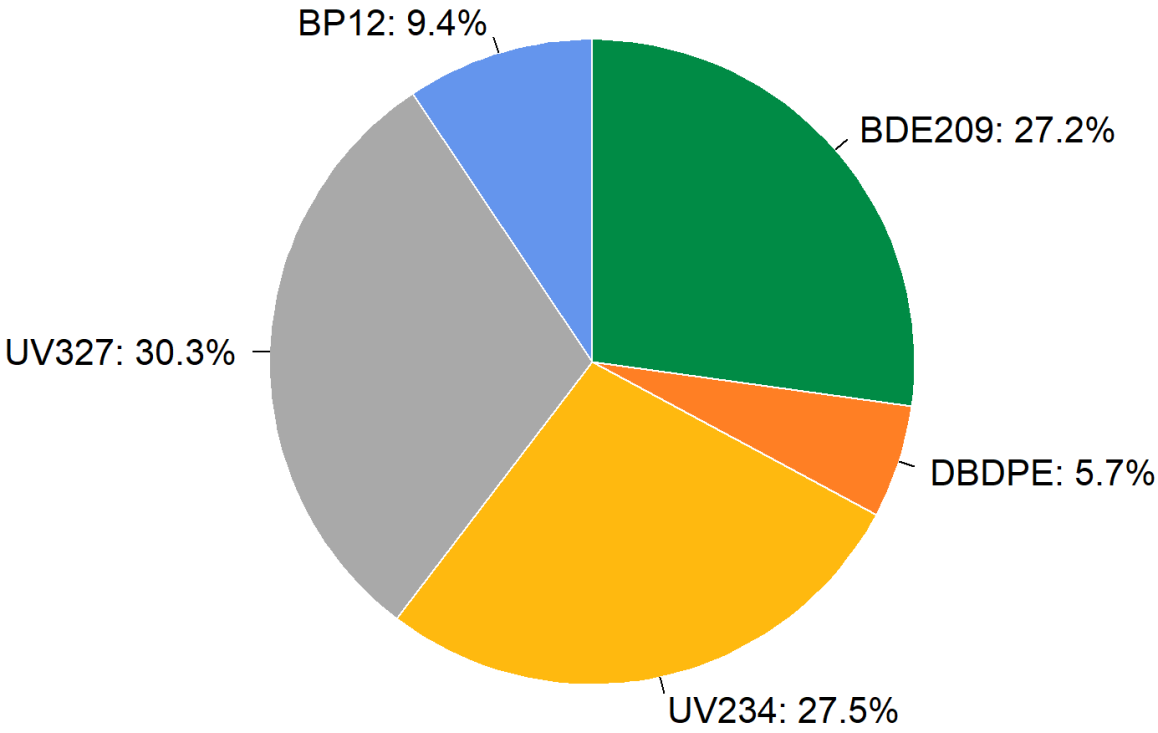
742 **Fig. 1.** Size distribution of polyethylene microplastics used for the experiment. Red line
743 denotes the mean particle size.



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745 **Fig. 2.** Composition of additives in polyethylene microplastics used for the experiment.

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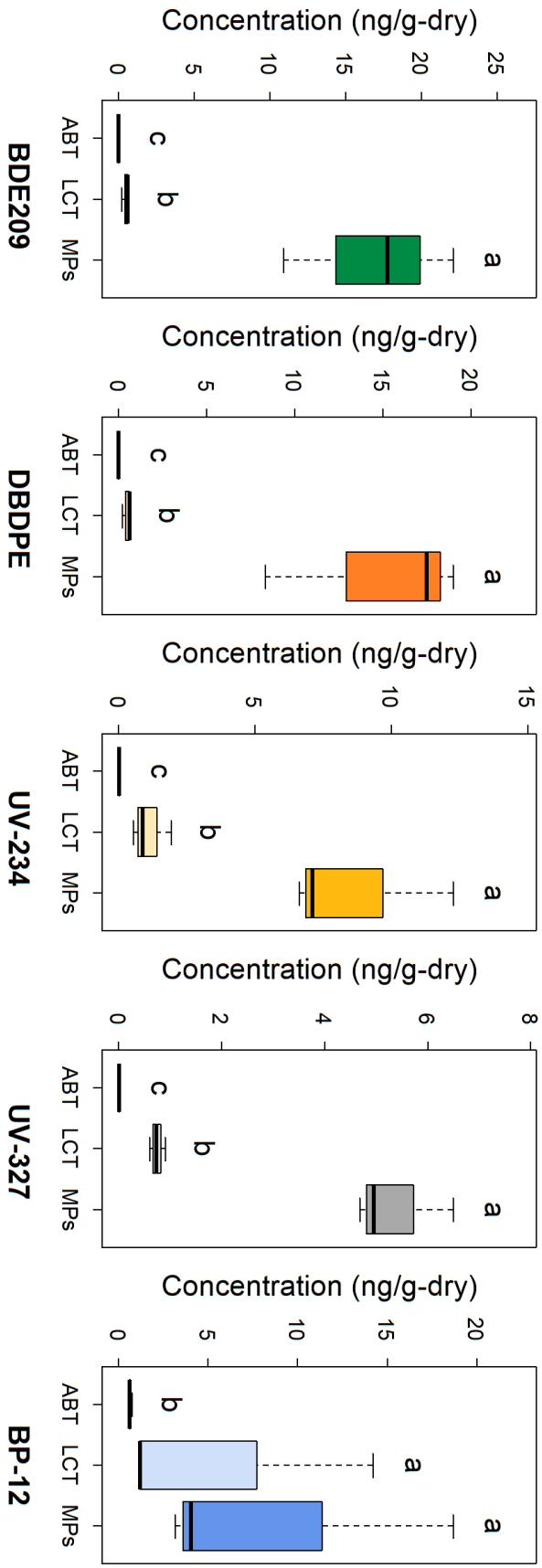

748 **Fig. 3** Concentration of the five additives in mysids per individual, collected from the ambient
749 environment (ABT), treated with the leachate seawater (LCT), and exposed to microplastics
750 (MPs). Results are shown as box and whisker plots with median (solid horizontal line),
751 interquartile range (25th and 75th percentiles; box), and the 10th and 90th percentiles
752 (whiskers). Different letters denote significant differences by post-hoc comparison ($p < 0.05$
753 GLM with post-hoc Tukey's HSD).

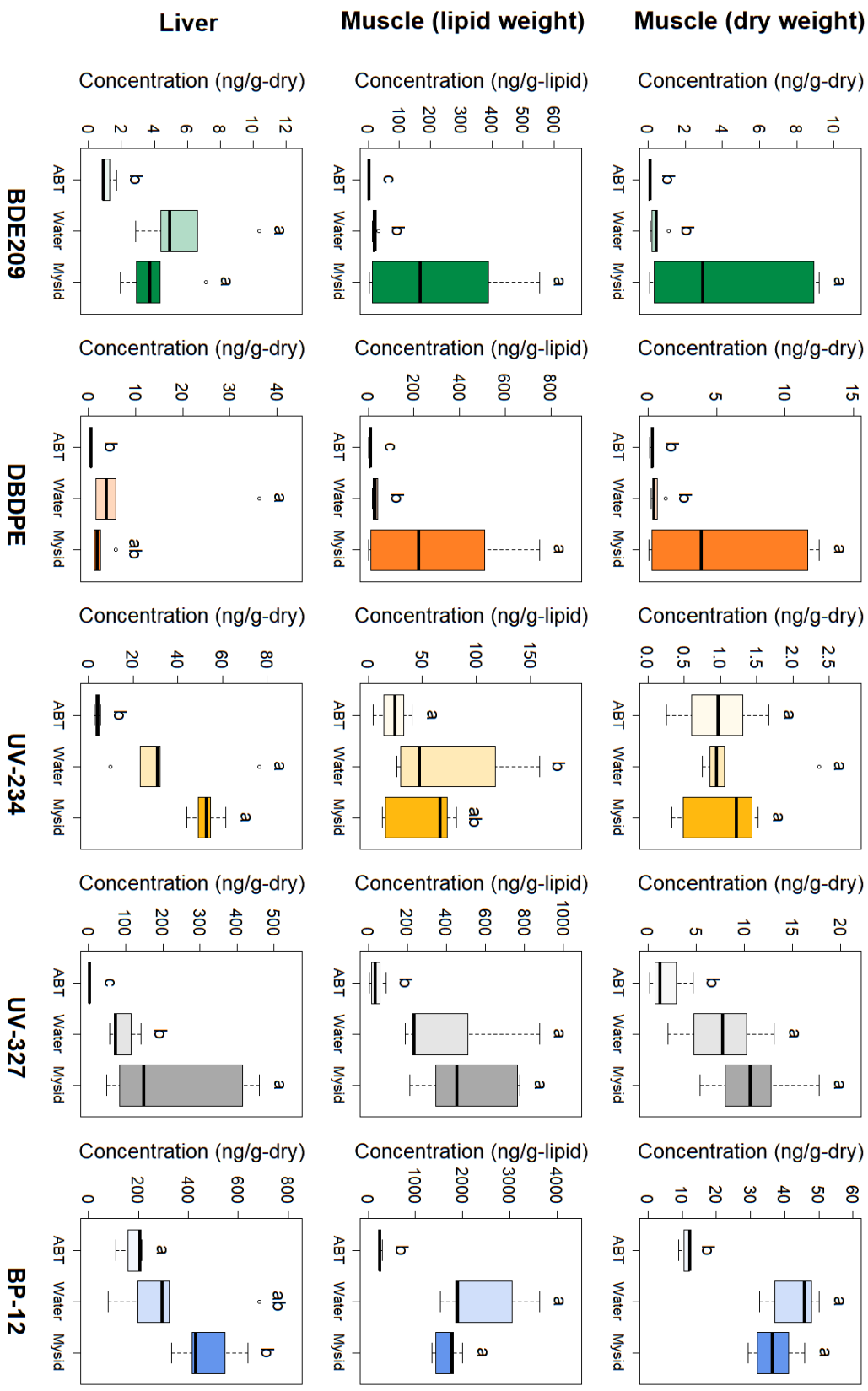

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Fig. 4. Concentration of the five additives in fish body and liver, collected from the ambient environment (ABT), exposed to microplastics suspended in the water column (Water), and fed plastic-exposed mysid (Mysid). Both dry weight and lipid weight base concentrations are shown for body, but only the dry weight base concentration is shown for liver because the available sample volume was limited. Results are shown as box and whisker plots with median (solid horizontal line), interquartile range (25th and 75th percentiles; box), and the 10th and 90th percentiles (whiskers). Different letters denote significant differences by post-hoc comparison ($p < 0.05$ GLM with post-hoc Tukey's HSD).