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ORIGINAL PAPER

Toxicity and behavioral response of zebrafish exposed to combined microplastic and bisphenol analogues

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Abstract

Microplastics and bisphenol analogues are emerging environmental pollutants widely occurring in freshwaters. Harmful effects of microplastics and bisphenols have been studied individually, yet there is few knowledge on their combined effect. Here, we conducted acute toxicity tests using embryonic and larval zebrafish to assess the combined lethality after co-exposure of bisphenol A or F and 0.5- and 25-µm polystyrene microplastic particles. We monitored the accumulation of microplastics in zebrafish. We also studied the impact on the behavior of larval zebrafish. Results show that co-exposure of bisphenols and polystyrene microplastics increased lethality of larvae by 6.8–51% for bisphenol F and by 6.7–30.1% for bisphenol A. However, the bisphenol lethality toward embryos remains unchanged in the presence of microplastics. Fluorescence analysis shows that 0.5- and 25-µm microplastics accumulate in the larvae gastrointestinal area in a dose-dependent pattern, but did not concentrate in the embryo. Overall, co-exposure of polystyrene microplastics and bisphenol analogues displayed stronger behavioral effects, e.g. reduced moving distance and activity,) toward zebrafish larvae, compared with single pollutant exposure.

Keywords Bisphenol A · Bisphenol F · Microplastics · Joint toxicity · Behavioral effect · Zebrafish

Introduction

Microplastics is a very broad class of pollutant in the aquatic environment. Increasing evidences showed that microplastics can be detected in a variety of freshwater areas such as rivers, lakes and estuaries, and the microplastic pollution in freshwater is at the same level as the ocean (Razeghi et al. 2021). Based on recent studies, microplastics have been detected in drinking water and its freshwater sources (Koelmans et al. 2019; Chia et al. 2021). In China, microplastic pollution has been reported in a number of inland waters such as Taihu Lake, Yangtze River Basin, Pearl River, Yellow River Basin and Ulansuhai Lake (Su et al. 2016; Naqash et al. 2020; Yan et al. 2019; Wang et al. 2019). In addition to China, the existence of microplastics in freshwater was also reported in European, Africa, USA, Brazil and Australia (Naqash et al. 2020; Dahms et al. 2020; Lenaker et al. 2019; Bertoldi et al. 2021; Nan et al. 2020), indicating the universality of microplastic pollution in global freshwater environment.

Bisphenol analogues are widely used as important epoxy resin raw materials. Bisphenol A, which is the most broadly used bisphenol compound, has been shown to possess estrogen-like effect and is associated with many human diseases (Tijani et al. 2016; Colombo et al. 2011; Rezg et al. 2014). Thus bisphenol A has been restricted since 2010, and the use of its alternatives, such as bisphenol F, has increased sharply (Rochester and Bolden 2015). Water body is an important carrier of bisphenol pollutants in the environment, and studies have found that bisphenol

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analogues are detected frequently in surface waters of China (Yan et al. 2017; Wang et al. 2013; Jin and Zhu 2016; Yamazaki et al. 2015). Although bisphenol A is still the primary component of bisphenol compounds in water environment, the detection level of bisphenol F has a clear upward trend (Jin and Zhu 2016).

There is a large coexistence opportunity for microplastics and bisphenol analogues due to their universal pollution in inland surface waters. For example, the detection content of microplastics in Guangzhou section of the Pearl River and the Pearl River Estuary reached 19,860/m^3 and 8902/m^3 (Yan et al. 2019), and the concentration of microplastics in the Taihu Lake sample reached 3400–25,800/m^3 (Su et al. 2016), while these areas also possess high detection frequency of bisphenol pollutants with detection rates of 100% and 67% for bisphenol A and bisphenol F, respectively, in Taihu Lake and 100% for both in Pearl River (Zhao et al. 2019).

Current environmental risk assessment is mainly focused on the single substances rather than chemical mixtures (Bureš et al. 2021). Although the negative effects of microplastics and bisphenol analogues toward aquatic organisms have been well studied based on single-component exposure (Supplementary Section S1; Mu et al. 2018a; Yuan et al. 2019; Xu et al. 2020; Liu et al. 2021), few data have been reported on the combined toxic effects under their coexistence scenario. Additionally, previous works reported that the presence of microplastics would enhance the toxicity of pollutants including heavy metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, toward aquatic organisms (Batel et al. 2016; Jeong et al. 2018; Kim et al. 2017). Thus, it is urgent to clarify the composited harmful effect of microplastics and bisphenol compounds toward water animals. Here, the joint toxicity of polystyrene microplastics and bisphenol A/bisphenol F was assessed via zebrafish (*Danio rerio*), and the involved mechanism of composite effect was further analyzed.

Experimental

Animals

Adult zebrafish (strain AB) were obtained from China Zebrafish Resource Center. Adult fish maintenance and embryos collection were conducted as previously described (Mu et al. 2013). All animal experiments were in accordance with current Chinese legislation and approved by the independent animal ethics committee of Chinese Academy of Fishery Sciences.

Chemicals

Standard water was prepared with the formula of iso-7346-3 (ISO 1996). 99% bisphenol A (CAS: 80-05-7) and 99% bisphenol F (CAS: 620-92-8) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Polystyrene microplastics with particle size of 0.5 and 25 μm and green fluorescence-labeled polystyrene microplastics (with particle size of 0.5 and 25 μm) with excitation/emission wavelengths of 470 nm/526 nm were purchased from Baseline Chromtech Research Center (Tianjin, China). The particle size distribution (Supplementary Section S2 and Fig. S1) of the microplastics standards was measured by a laser nanoparticle size analyzer (Malvern PANalytical Ltd, UK). The stock solution of bisphenol compounds used for exposure was prepared using acetone.

Acute toxicity test of bisphenol A/bisphenol F combined with microplastics

Embryo. Test solutions of bisphenol A and bisphenol F were prepared using standard water with different series of geometric concentrations on the basis of pre-experiments (Fig. S2A). Thus, the 0.5- and 25-μm microplastic particles were added into the solutions and the final concentration was 2 (2.9×10^{10} particles/L for 0.5-μm microplastics; 2.3×10^{5} particles/L for 25-μm microplastics), 10 (1.4×10^{11} particles/L for 0.5-μm microplastics, 1.1×10^{6} particles/L for 25-μm microplastics) and 50 (7.2×10^{11} particles/L for 0.5-μm microplastics, 5.7×10^{5} particles/L for 25-μm microplastics) mg/L, respectively. Embryos at 2 h post-fertilization (hpf) were randomly transferred into test solutions in 24-well plates (20 wells were used in each plate), with 1 embryo and 2 mL exposure solution per well. The exposure lasted four days and the acute toxicity test was repeated three times.

Larvae. The exposure concentrations are provided in Fig. S2 for most cases (with exceptions in several co-exposure cases), which are designed on the basis of pre-experiments. Zebrafish larvae exposure was conducted in a 1-L tank, 20 zebrafish larvae at 6 days post-fertilization (dpf), and 500 mL solution was added in one tank. The exposure solution for larvae was prepared with standard water and concentrated paramecium solution (density of 100/mL) in order to provide exogenous food resource for larvae (Mu et al. 2018b). The exposure process and condition parameters are the same as described in embryonic exposure.

Detection of microplastics accumulation in zebrafish

During the acute toxicity test, green fluorescent polystyrene microplastic particles (0.5 and 25 μm) were used in

one replicate of all treatments (including both single- and double-component exposure) to assess the accumulation of microplastics in zebrafish embryo and larvae (Fig. S2B). Ten zebrafish embryos/larvae were taken from each treatment group, and the fluorescence signal in treated zebrafish was identified and recorded using a ZEISS Vert.A1 microscope (Jena, Germany) at 48, 72 and 96 h post-exposure (hpe), respectively.

Larvae behavior test

Healthy larvae at 6 dpf were exposed to a series of co-exposure scenarios of bisphenols and microplastics: 0.1, 1.0, 10 and 100 μg/L for bisphenol A and bisphenol F; 0.05 (7.2×10^8 particles/L for 0.5-μm microplastics; 5.7×10^3 particles/L for 25-μm microplastics), 2 and 10 mg/L for 0.5/25-μm microplastics. At the same time, bisphenol A, bisphenol F and microplastics solely exposure was also conducted with same concentration series. The behavior assessment was conducted as our previous study (Qian et al. 2020) and provided in detail in Supplementary Section S3.

Chemical confirmation of bisphenol analogues

Actual concentrations of bisphenol A and bisphenol F in exposure solutions of each replicate for all treatments were analyzed twice, respectively, at 0 hpe (the beginning of exposure) and 24 hpe (before the first replacement of exposure solution). The pretreatment of water samples and chromatographic analysis are provided in Supplementary Section S4, and the analytical results are provided in Table S1-S2.

Statistical analysis

All statistical analyses were undertaken using SPSS 16.0 software. The median lethal concentration (LC_{50}) values and 95% confidence limits were calculated by probit regression analysis. The normality (Kolmogorov – Smirnov and Shapiro–Wilk tests) and homogeneity of variance (Levene's test) of the data were determined during the analysis of variance. Differences were determined by one-way ANOVA and completed by Dunnett post hoc comparison. $P<0.05$ was considered significant.

Results and discussion

Combined toxicity of bisphenol A or F and polystyrene microplastics

No significant death was found in the groups exposed to 0.5- and 25-μm polystyrene microplastics at tested concentrations (0.1–100 mg/L), indicating a very low toxicity of polystyrene microplastic toward zebrafish embryo and larvae. Based on the 96-h LC_{50} values (Table S3–S4), the acute toxicity of bisphenol A and bisphenol F toward zebrafish larvae enhanced significantly with the existence of two sizes of microplastics (≥ 10 mg/L) (Fig. 1A, B), with increase rate of 6.8–51% and 6.7–30.1% (dependent on the size and concentration of microplastic particles) for bisphenol F and bisphenol A, respectively. This indicated the promotional effect of polystyrene microplastics toward the toxicity of bisphenol compounds. On the contrast, the presence of polystyrene microplastic particles failed to affect the toxicity of bisphenol analogues toward zebrafish embryos (Fig. 1C, D).

The enhanced bioaccumulation through microplastic particles is considered as one of the main approaches mediating the toxicity promotion of environmental pollutants with the presence of microplastics. Qu et al. (2019) found that the bioconcentration factor of the antidepressant venlafaxine in loach tissue could be increased by nearly 10 times in the presence of microplastics and caused stronger oxidative damage in loach tissue. For bisphenol analogues, a previous study reported that multiple microplastics (including polystyrene) exhibited sorption capacity for bisphenol A, which indicated a potential role of microplastic particles serving as transportation vectors for bisphenol compounds in water environment (Liu et al. 2019). Barboza et al. (2020) further reported that fish polluted by microplastics had significantly higher concentrations of bisphenol analogues than fish where no microplastics were found, suggesting a relation between microplastics and bisphenol contamination in fish. Therefore, we assumed that polystyrene microplastics could increase the accumulation of bisphenol analogues in zebrafish larvae through their adsorption and transported enrichment and thus induced higher acute toxicity.

Bioaccumulation of polystyrene microplastic particles in zebrafish larvae and embryo

After exposure to 0.5- and 25-μm polystyrene microplastics, zebrafish larvae showed obvious accumulation of particles, and the fluorescence signal enhanced apparently along with the increase of exposure concentration (Fig. 2). The fluorescence signal was mainly concentrated in the gastrointestinal area, indicating that the microplastics entered mainly through ingestion. A large number research evidences from both laboratory and field showed that microplastics can be absorbed by aquatic organisms, and one important approach is ingestion (Wright et al. 2013). Since the zebrafish larvae we used were at 6 dpf and had the ability to ingest exogenous food source, it is not surprising to detect the dose-dependent ingestion of microplastics. Previous study also reported that 5- and 50-μm sizes polystyrene microplastics could be ingested by zebrafish larvae and mainly enriched in

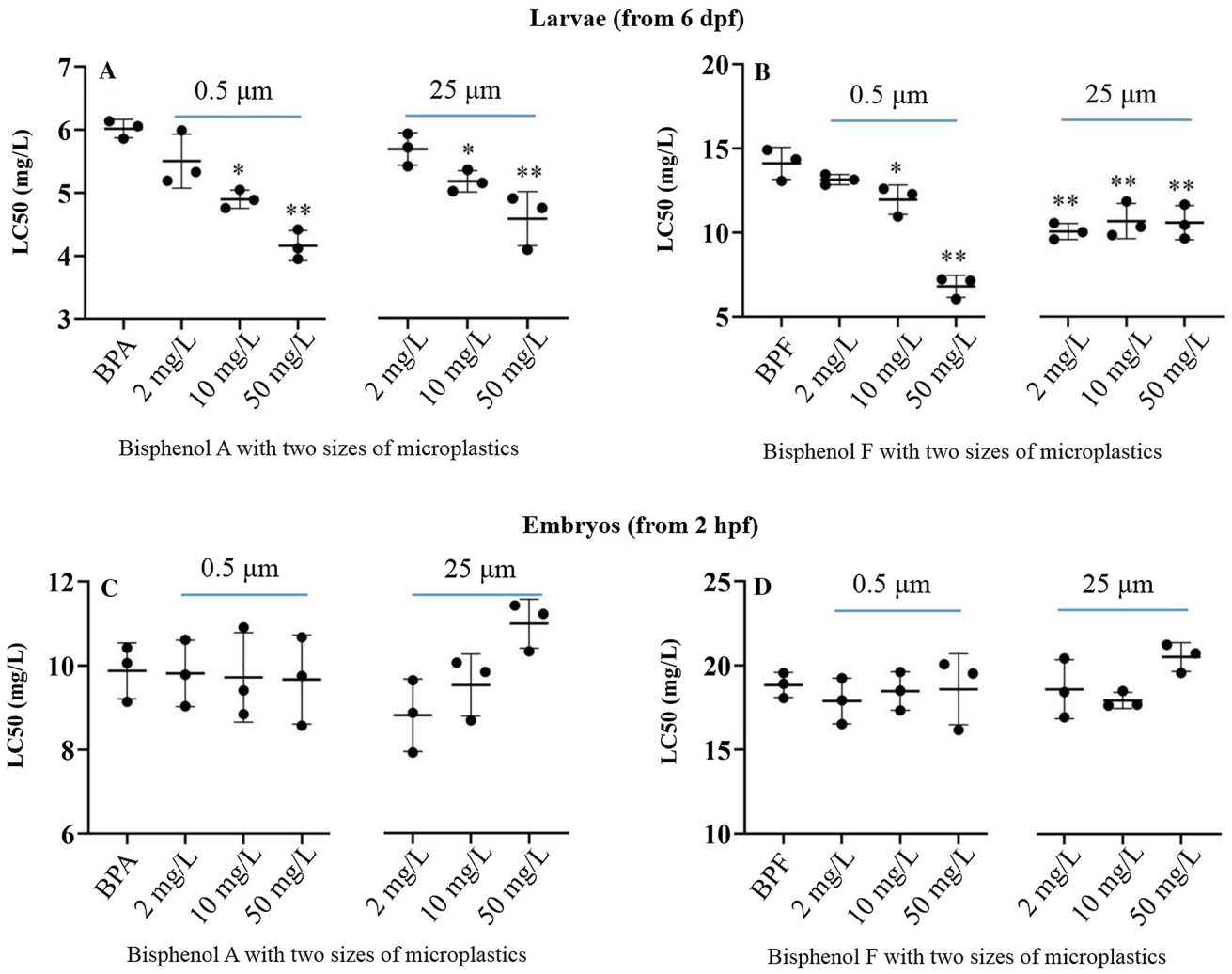

Fig. 1 Acute toxicity of bisphenol A and bisphenol F toward zebrafish with the presence of polystyrene microplastics. **A**, **B** The 96-h median lethal concentration (LC_{50}) post-co-exposure of bisphenol analogues and microplastics (0.5 and 25 μm) toward zebrafish larvae. **C**, **D** The 96-h LC_{50} post-co-exposure of bisphenol analogues and microplastics (0.5 and 25 μm) toward zebrafish embryo. *Significant difference between control and exposure treatments (*$p < 0.05$, **$p < 0.01$). *BPA* Bisphenol A; *BPF* bisphenol F; *dpf* days post-fertilization; *hpf* hours post-fertilization; *LC50* median lethal concentration

gut (Wan et al. 2019), which showed good agreement with the present work.

However, for zebrafish embryos, the microplastic particles are mainly distributed on the outer side of chorion and can hardly enter into the membrane (Fig. S3-S5). Even after the embryo hatches, the microplastic particles cannot accumulate in the larvae based on the graphs screened at 96 hpf (Fig. S6). A reasonable explanation for this result is that larvae post-hatch is still in the yolk sac stage and would not ingest external substances (like microplastics) through mouth until 96 hpf, which further illustrates that the early life stage of zebrafish absorbs microplastics particles (≥ 0.5 μm) mainly through ingestion. Thus, the presence of chorion during the embryonic stage may prevent the enrichment of polystyrene microplastics, transported bisphenols and the corresponding toxic effect (further discussed in Supplementary Section S5). This implicates that most microplastic particles would not directly contact nor enrich in the zebrafish embryos (including larvae post-hatch) during the 96-h exposure period, which is quite different with that of larvae exposure. The comparative analysis further supported that the varying ingestion of microplastics between embryo and larvae might be associated with their different sensitivity toward bisphenol-microplastics co-exposure. Another study also reported that the freshwater algae *Chlamydomonas reinhardtii* (with cell wall) could block the internalization of nanoparticles (NPs), while another algae *Ochromonas danica* without a cell wall showed significant adsorption of NPs (Huang et al. 2019).

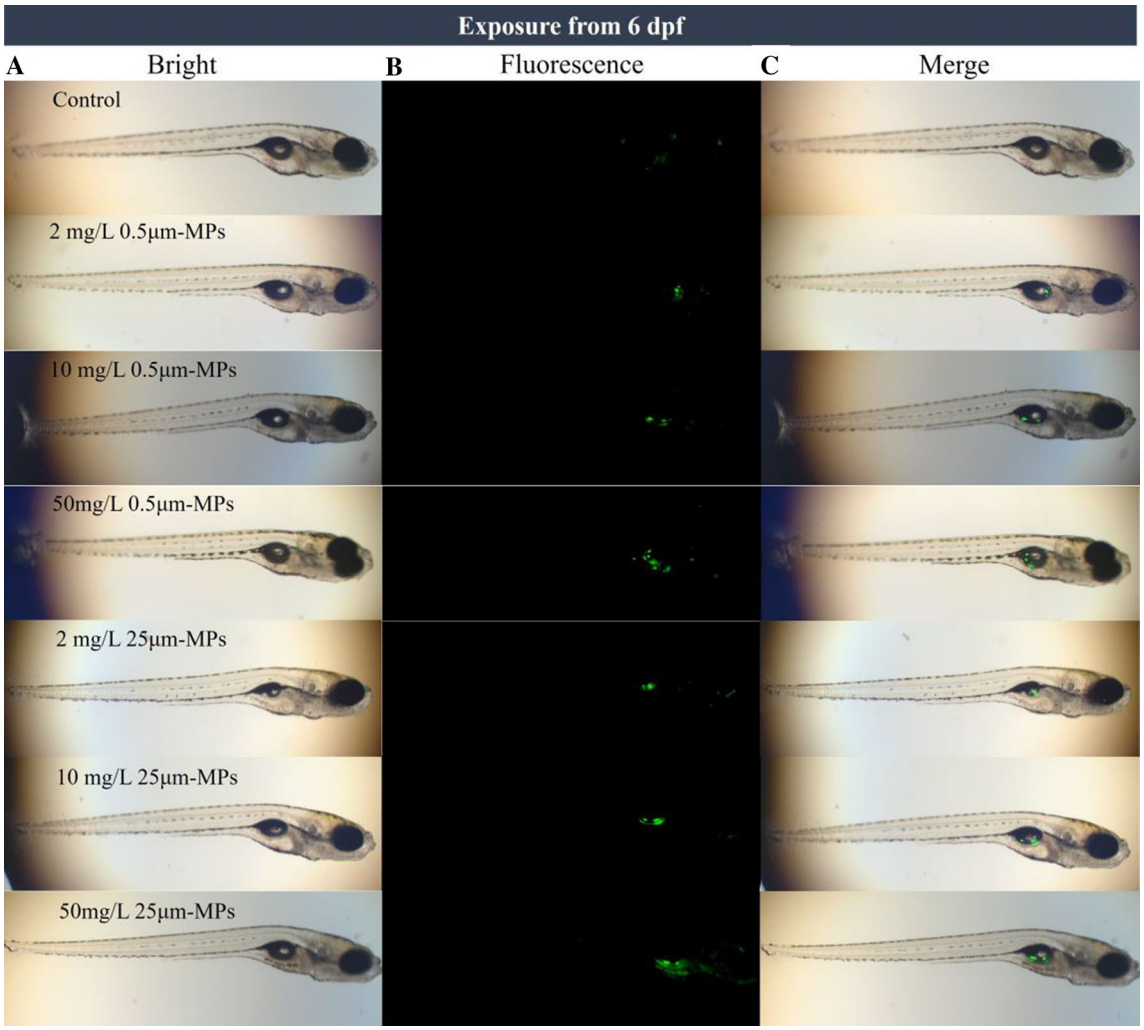

Fig. 2 Bioaccumulation of polystyrene microplastics in zebrafish larvae. **A**–**C** Typical graphs of zebrafish larvae in control and polystyrene microplastics treated groups (0.5 and 25 μm sizes, 2, 10 and 50 mg/L) at 9 days post-fertilization (3 days post-exposure) under bright (**A**), fluorescence (**B**) and merge (**C**) light conditions. *MPs* microplastics; *dpf* days post-fertilization

Combined behavioral effect of bisphenol A or F and polystyrene microplastics

Total moving distance. No significant change was observed in total moving distance post-single component exposure of bisphenol A/microplastics under tested concentrations. Surprisingly, significant reduced total moving distance was identified in zebrafish larvae that were co-exposed to bisphenol A (0.1, 1.0 and 100 μg/L) and 25-μm microplastics at 48 hpe (Fig. 3A) with 0.66, 0.64 and 0.56 fold of control, respectively, and the inhibition getting more serious at 96 hpe (Fig. 3B). Among them, the worst case was found in the co-exposure of 100 μg/L bisphenol A and 10 mg/L 25-μm microplastics, with average moving distance of 86.4 mm at 96 hpe (only 0.49 fold of control). 0.1 and 1.0 μg/L of bisphenol F could significantly inhibit larvae moving distance with the presence of 10 mg/L 25-μm microplastics at 96 hpe and performed an effective improvement in this change compared with the exposure of bisphenol F alone (Fig. 3D). However, less promotion effect was found toward behavioral toxicity post-bisphenols exposure with the presence of 0.5-μm microplastics, comparing with 25-μm microplastics (Figure S7C-D, S7G-H).

Moving activity Similar to the results of total moving distance, reduced moving activity was identified in most co-exposure cases of bisphenol A and 25-μm microplastics at 48 and 96 hpe (Figure S8A-B, Table S6). 10 and 100 μg/L bisphenol F significantly inhibited zebrafish activity, and the effect was strengthened by co-exposure with 10 mg/L 25-μm microplastics (Figure S8F). In addition, most co-exposure cases of bisphenol F and 0.5-μm microplastics significantly decreased zebrafish moving activity when bisphenol F concentration reached 10 μg/L at 96 hpe, but showed no significant change compared with that of solely bisphenol F

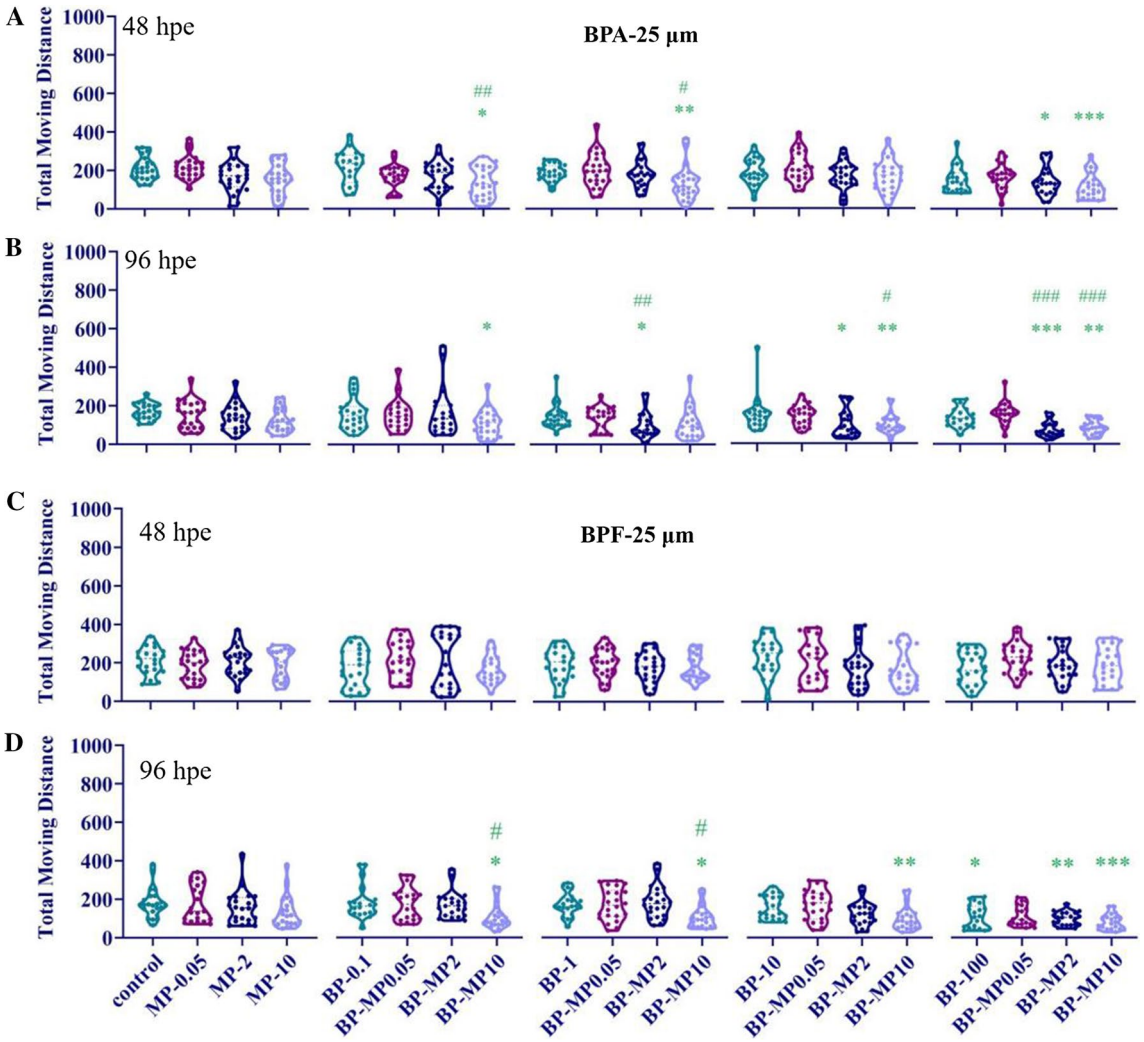

Fig. 3 Effects of single- and double-component treatments toward total moving distance (mm) of zebrafish larvae. **A**, **B** Total moving distance of zebrafish larvae in control, single microplastics (25 μm), single bisphenol A and co-exposure of bisphenol A (BPA) and microplastics at 48 (**A**) and 96 (**B**) hours post-exposure. **C**, **D** Total moving distance of zebrafish larvae in control, single microplastics (25 μm), single bisphenol F (BPF) and co-exposure of bisphenol F and microplastics at 48 (**C**) and 96 (**D**) hours post-exposure. *Significant difference between control and exposure treatments ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). # denotes the significant difference between co-exposure and single bisphenol analogue exposure ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$). *MP* microplastics; *BP* bisphenol analogue; *hpe* hours post-exposure

exposure (Figure S8H). This indicated that 0.5-μm microplastics possessed less contribution to the combined behavioral effect. Detailed results of all behavioral indicators are provided in Supplementary Material (Section S3, Fig. S7-10, Table S5-8).

Although there was no obvious evidence for the existence of synergy effect, co-exposure of polystyrene microplastics and bisphenol analogues possessed certain impact on zebrafish larvae behaviors and showed stronger behavioral toxicity than single pollutant exposure. Since both the tested concentrations of bisphenol analogues and microplastic particles are relevant with detected levels in samples from potential exposure sources (Koelmans et al. 2019; Yamazaki et al. 2015; 2019; Liu et al. 2017), the present study reveals the combined effect of their co-existence in water environment toward fish behavior. Taken together, when co-exposing with polystyrene microplastics, bisphenol analogues would contribute to severer risk toward aquatic organisms, which should be highly concerned (further discussed in Supplementary Section S6).

Conclusion

In this study, we assessed the toxicity and behavioral effects of bisphenol analogues with the presence of polystyrene microplastics using zebrafish as model. Both bisphenol A

and bisphenol F showed increased lethality toward larvae zebrafish when co-exposed with two sizes of microplastic particles. However, polystyrene microplastics failed to change the toxicity of bisphenol analogues toward embryos. The disparity in the composition effects between larvae and embryonic zebrafish may be due to the different accumulative capacity of microplastic particles toward fish during the two life stages. These findings suggest that microplastics would enhance the toxicity toward larvae zebrafish when co-existed with bisphenol analogues, while embryos may be spared from this effect due to the protection of the chorion.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10311-021-01320-w.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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