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Neurodevelopmental toxicity assessments of alkyl phenanthrene and Dechlorane Plus co-exposure in zebrafish

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ABSTRACT

Alkyl phenanthrene (A-Phen) and Dechlorane Plus (DP) are ubiquitous environmental pollutants that widely co-exist in the environment. It has been established that both A-Phen and DP elicit neurotoxicity, but the potential interactive toxicity of these contaminants is not well-known. To determine whether a mixture of A-Phen and DP would exhibit interactive effects on neurodevelopment, we co-exposed 3-methylphenanthrene (3-MP), a representative of A-Phen, with DP. Our results illustrated that exposure to 5 or 20 μg/L 3-MP alone or in combination with 60 μg/L DP caused neurobehavioral anomalies in zebrafish. In accordance with the behavioral deficits, 3-MP alone or co-exposed with DP significantly decreased axonal growth of secondary motoneurons, altered intracellular Ca^{2+} homeostasis and induced cell apoptosis in the muscle of zebrafish. Additionally, 3-MP alone or co-exposed with DP significantly increased reactive oxygen species (ROS) and the mRNA levels of apoptosis-related genes. These findings indicate that 3-MP alone or co-exposed with DP induces neurobehavioral deficits through the combined effects on neuronal connectivity and muscle function. Chemical analysis revealed significant increases in 3-MP and DP bioaccumulation in zebrafish co-exposed with 3-MP and DP. Elevated bioaccumulation resulting from mixture exposure may represent a significant contribution of the synergistic effects observed in combined chemical exposure.

1. Introduction

Alkyl polycyclic aromatic hydrocarbons (A-PAHs) are the dominant components of PAHs in crude oil, and they can be released into water because of various activities, including oil spills and offshore oil drilling (Sorensen et al., 2017). Dechlorane Plus (DP), an additive chlorinated flame retardant, has been extensively applied in electronic products (Wang et al., 2016). Thus, DP can be discharged into water during the processing of electronic waste. Although the primary sources of A-PAHs and DP differ, these compounds have similar properties to those of persistent organic pollutants (POPs) that widely coexist in the environment (Abdel-Shafy and Mansour, 2016; Wang et al., 2016). In particular, aquatic compartments such as water and sediment often constitute the ultimate reservoirs for A-PAHs and DP. Many studies have shown that A-PAHs and DP are widely detected in water and/or sediments of the same regions, including the North American Great Lakes (McDonough et al., 2014; Olukunle et al., 2018) and Atlantic Ocean (Lohmann et al., 2013; Möller et al., 2010). In our previous study, the concentrations of A-PAHs and DP were up to 96.1 ng/L and 3.5 ng/L, respectively, in the water of the Jiulong River Estuary in China (Chen et al., 2018). Additionally, A-PAHs and DP are detectable not only in the water and sediment of the Dalian coast in Northeast China but also in oysters with up to 91.1 ng/g wet weight (ww) and 19.1 ng/g ww (Hong et al., 2016; Jia et al., 2011), respectively. However, despite growing evidence that A-PAHs and DP are detectable in the same aquatic compartments, the ecological risks of A-PAH and DP

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co-exposure to aquatic creatures are not fully characterized.

Mixture toxicity occurs when different contaminants coexist in aquatic organisms, and the combined toxicities of pollutants, such as pesticides, heavy metals and brominated flame retardants (Kim et al., 2018; Li et al., 2019; Zhu et al., 2016), have been well understood, but limited information exists regarding A-PAHs and DP. For instance, the combined toxicities of endosulfan (ENDO) and phenanthrene (Phen) exhibited a synergistic effect on bile acid biosynthesis in zebrafish (Kim et al., 2018). Alkyl phenanthrene (A-Phen) is a Phen derivative with an alkyl substitution, and its acute toxicity has been shown to be higher than that of Phen in medaka embryos (Liu et al., 2016). Previous research has demonstrated that crude oil causes neurotoxicity in rats, and A-Phen is the major component in crude oil (Ebokaiwe et al., 2013). Currently, few reports are available on the neurotoxicity of A-Phen in aquatic organisms. DP has the same structure as cyclohexadiene as ENDO and can cause a thyroid interference effect (Kang et al., 2016) and neurotoxicity (Zhang et al., 2014). Moreover, our previous study (Chen et al., 2017) showed that DP exposure at a concentration of 60 μg/L caused neurobehavioral anomalies in zebrafish (*Danio rerio*) larvae, such as an increase in spontaneous movement at 24 h post fertilization (hpf) and decreased touch responsiveness and free swimming activity at 72 hpf and 120 hpf, respectively. Although the developmental toxicity of DP in zebrafish is well established, the neurodevelopmental effects of A-Phen and DP co-exposure in aquatic organisms are not well known.

Zebrafish (*Danio rerio*) have been widely used in development biology and toxicology due to the specific characteristics of the organisms, including small size, rapid development, transparency during embryonic development and high similarities at the physiological level to humans (Qian et al., 2019). Recent developments in the behavioral assessment methods available in zebrafish have resulted in an increase in studies employing zebrafish to evaluate the developmental neurotoxicity of various environmental contaminants, including organophosphate flame retardants (Glazer et al., 2018) and PAHs (Geier et al., 2018). Therefore, the zebrafish model was employed to assess the neurodevelopmental toxicity of A-Phen and DP. Based on the half maximal effective concentration (EC_{50}) of four A-Phens tested in our preliminary trials using the teratogenic endpoint of zebrafish, 3-methylphenanthrene (3-MP) had the lowest EC_{50} (Table S1), suggesting that 3-MP is the most toxic compound among the four A-Phens. Additionally, the concentrations of 3-MP were the highest among A-Phens in the water, sediment and oysters of the Dalian coast in Northeast China (Hong et al., 2016). Thus, 3-MP was selected as a representative A-Phen compound. In the present study, the acute neurotoxicity of 3-MP, DP and 3-MP/DP co-exposure was evaluated in zebrafish using static waterborne exposure. Three types of behavioral assays were performed following the treatments throughout development: spontaneous movements, touch response and free swimming activity. Furthermore, axonal growth of secondary motoneurons, Ca^{2+} homeostasis, reactive oxygen species (ROS) levels, and the mRNA expression levels of related genes were examined to illustrate the molecular mechanism of chemical-induced neurodevelopmental toxicity. The purposes of the present study were to determine whether a mixture of 3-MP and DP would exhibit interactive effects on neurodevelopment in zebrafish.

2. Materials and methods

2.1. Zebrafish husbandry

Wild-type AB line zebrafish were maintained at a set temperature (28 ± 0.5 °C) with a 14-h:10-h light-dark period in a recirculation system. Water was filtered by reverse osmosis before being entered into the system, and ocean salt was added to the system water to maintain the conductivity in the range of 750–1000 mS/cm. The adult zebrafish were raised twice a day with freshly hatched brine shrimps (Jiahong Feed Co., Tianjing, China). Embryos were collected from spawning adult zebrafish at a sex ratio of 1:1. Embryos with normal morphology were inspected under a Nikon stereomicroscope SMZ1500.

2.2. Embryo exposure and teratology screening

DP (purity > 97%) was obtained from Anpon Electrochemical Co., Ltd. (Jiangsu, China), and 3-MP (purity > 98%) was purchased from Sigma Aldrich Chemical Co. (Washington, USA). Stock solutions of 3-MP (5, 20 mg/L) and DP (60 mg/L) were made by directly dissolving these compounds in dimethyl sulfoxide (DMSO). Exposure solutions of 3-MP (5, 20 μg/L) and DP (60 μg/L) were prepared by diluting these stock solutions in fish water. The concentration of DMSO in the exposure groups and control group was 0.1% (v/v in fish water). The exposure concentration of DP was based on our previous research (Chen et al., 2017). The concentrations of 3-MP used in this study were selected based on our previous range-finding study, which showed that, after exposure to the concentration combination of 3-MP (20 μg/L) and DP (60 μg/L), the malformation of embryos/larvae showed a small increase (< 10%, Table S2). In this study, all embryo exposure procedures started at 6 hpf, and closure was observed at various developmental stages (Table S3). For teratology screenings, malformation, hatching and survival were observed from 8 to 120 hpf.

2.3. Behavioral assays

To assess whether motor behavior in zebrafish was influenced after co-exposure with 3-MP and DP, three types of behaviors were used: embryonic spontaneous movement, touch response and swimming activity (Chen et al., 2017). In total, 72 embryos (24 embryos per replicate, n = 3 replicates) from each exposure group were used for each behavioral assay. The behavioral assays were performed step by step at various developmental stages to evaluate the neurobehavioral toxicity of 3-MP and co-exposure with DP. At 24 hpf, spontaneous embryonic movement was recorded for 1 min using a Nikon CCD camera attached to a stereomicroscope. After 5 min of adaption, spontaneous movement was videotaped, and the recordings from the first to the last well ensured completion in 8 min. The touch response of the zebrafish larvae was assessed at 72 hpf. Embryos were first dechlorinated by protease E digestion (0.1 mg/mL; Roche) and then transferred into 24-well plates to adapt for 10 min (one embryo per well). The larval response was stimulated by a single gentle touch on the larval tail using an eyelash probe. The movement distance was monitored by camcorder software and calculated using Image-Pro Plus 6 (Yang et al., 2011). Normal larvae at 96 hpf were rinsed and transferred to a clean 24-well plate (one larval each well). At 120 hpf, the free swimming activity during a 10-min period in visible light was monitored after 5-min adaption using a Video-Track system (Zebralab ViewPoint 3.5, France).

2.4. Immunohistochemistry

Zebrafish at 48 hpf were anesthetized with 0.02% MS-222 and then fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. Whole-mount immunofluorescence staining was performed using a zn-5 antibody (1:200) to display a distinct image of neuron axons, as described previously (Chen et al., 2017). The secondary motoneurons were observed under a Nikon fluorescence microscope, and the average length of the axons was measured using Nikon analysis software (Nis-Elements-BR-4.0). Thirty embryos (10 embryos per replicate, n = 3 replicates) in each exposure and control group were used for data analysis.

2.5. Determination of the intracellular Ca^{2+} levels and Ca^{2+}-ATP activity

The quantification of the intracellular free Ca^{2+} content was conducted by flow cytometry. The cell preparation was performed following a previous method with minor modifications (Wu et al., 2018). Briefly, zebrafish larvae at 96 hpf from all treatment groups were rinsed

twice with water. The brain tissues for each treatment group were dissected from 60 larvae per replicate (n = 3 replicates) and were placed into a sterile tube containing 0.5 mL of lysis buffer (0.25% Trypsin-EDTA (Gibco) supplemented with 1 mg/mL of DNase I). The tissues were digested for 3 h at 28 °C in a 5% CO_2 incubator and were gently pipetted continuously for 1 min to ensure complete digestion. Dulbecco's modified Eagle medium (DMEM) mixed with 5% fetal bovine serum (FBS) was used to terminate the digestion. Next, the cell suspension was filtered through a 40-μm mesh and incubated with Fluo-4 AM dye (Beyotime, Jiangsu, China) for 30 min at 28 °C in a 5% CO_2 incubator. The mean fluorescent intensity was measured in 10,000 cells by flow cytometry (BD FACSCalibur, USA). Ca^{2+}-ATPase activities were tested using a commercially available Ca^{2+}-ATPase assay kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions.

2.6. Cellular death assays

Acridine orange (AO) staining was used to assess cell death in live larvae (Çomakli et al., 2018). Twelve zebrafish at 96 hpf from each treatment group were incubated with 5 μg/mL of AO for 1 h at 28 °C in the dark. The larvae were then washed three times with water for 5 min per wash. Live larvae were observed after being anesthetized under a Nikon fluorescence microscope (TE2000, Japan).

2.7. Measurement of ROS

Dichlorofluorescein-diacetate (DCFH-DA) staining was used to detect ROS generation (Zhu et al., 2016). At 96 hpf, 20 larval zebrafish per replicate (n = 3 replicates) from each treatment group were homogenized in cold phosphate buffer on ice, and then DCFH-DA was added and incubated at 37 °C for 30 min. The ROS content was assessed by green fluorescence intensity using an automatic microplate reader (SynergyH1, Biotek, USA).

2.8. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to analyze gene expression in zebrafish (Chen et al., 2017). Forty larvae or brain tissues at different developmental stages (48 or 96 hpf) were collected from each treatment group. mRNA extraction was performed using TRIzol reagent (Life Technology, CA, USA) according to the manufacturer's instructions. Quantification of total RNA was performed using the NanoDrop 2000 system (Thermo Fisher, USA), and cDNA was synthesized from total RNA using the PrimeScript® RT reagent kit (Takara, Japan). The specific primer sequences are listed in Table S4. For qRT-PCR, the 10-μL PCR system included 5 μL of Eva Green Master mix (Bio-Rad, USA), 0.3 μL of each primer, 2 μL of cDNA (20 ng/μL) and 2.4 μL of water. qRT-PCR was performed using the Eppendorf Mastercycler® Realplex 2 system. Gene expression was detected in three biological replicates for each exposure group. The relative expression of target genes in the different treatment groups was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.9. Quantification of DP and 3-MP in zebrafish

To measure the DP and 3-MP concentrations in water and larval zebrafish, the same methods were used as previously described (Chen et al., 2017). Briefly, at 120 hpf, larvae samples (n = 3 replicates) were spiked with $^{13}C_{12}$-PCB-208 and Phen-d_{10} used as a surrogate standard for DP and 3-MP, respectively. The concentrations of 3-MP and DP were detected using Agilent 7890A GC coupled to a 5975C MS equipped with an HP-5 MS capillary column (15 m × 0.25 mm × 0.1 μm; J&W Scientific). A procedural blank was performed for each batch of water and zebrafish samples, with the compounds of interest not being detected in any of the blanks.

2.10. Data analysis

Statistically significant differences among the exposure groups were determined by ANOVA followed by Tukey's test. All the data are reported as the means ± standard error of the mean (SEM). Statistical analyses were processed using SPSS 20.0 software (SPSS, Chicago, IL, USA), and the level of significance was identified by $P < 0.05$. Pearson's correlation analyses were carried out to determine the relationship between the content of 3-MP or DP and levels of Ca^{2+} in zebrafish.

3. Results

3.1. Teratogenic effects

To evaluate the teratogenic effects of 3-MP and DP co-exposure in zebrafish, several teratogenic phenotypes, including reduced pigmentation, pericardial edema, yolk-sac edema, curvature of the body axis, hatching rate and mortality, were scored from 8 to 120 hpf. All groups showed < 10% developmental anomalies (Table S2), and no significant differences were found in the hatch and mortality rates between the control and treated embryos/larvae during the exposure period of 6–120 hpf. However, only normal fertilized embryos/larvae were used for all subsequent experiments.

3.2. Effects on motor behavior

Zebrafish motor behavior appears sequentially during development and includes an early, transient period of spontaneous tail coiling, followed by an escape response to touch and free swimming (Wang et al., 2013). To determine whether a mixture of A-Phen and DP would exhibit interactive effects on neurodevelopment, three types of behavioral assays were performed—spontaneous movement, touch response and free swimming activity. At 24 hpf, the spontaneous movement frequency of larvae from all treatment groups, except the 60 μg/L DP group, was significantly decreased compared with the spontaneous movement frequency of larvae from the control group (Fig. 1A, $P < 0.05$). DP exposure alone increased the spontaneous movement frequency in zebrafish larvae, while co-exposure with 3-MP induced a concentration-dependent reduction in the spontaneous movement frequency compared with DP exposure alone (Fig. 1A, $P < 0.05$). The touch response is an instinctive movement of the escape reaction during the early development of zebrafish. At 72 hpf, all treated larvae exhibited reduced swimming distance in response to touch compared with the control larvae (Fig. 1B, $P < 0.05$). Relative to DP exposure alone, co-exposure with 3-MP decreased the swimming distance in a concentration-dependent manner. In particular, a synergistic effect was observed in DP co-exposed with 20 μg/L 3-MP. Free swimming activity was evaluated at 120 hpf and exhibited a similar pattern to that of the touch response between treated and control larvae (Fig. 1C).

3.3. Effects on axonal growth and related gene expression

At 48 hpf, the axonal lengths of secondary motoneurons in zebrafish larvae from all exposure groups were significantly shorter than those from the solvent control (Fig. 2A, $P < 0.05$). Similar to the pattern observed in the motor behavior tests, the axonal lengths in groups co-exposed to DP and 3-MP were shorter than those in groups exposed to DP or 3-MP exposure alone.

To further investigate the mechanism underlying 3-MP and DP co-exposure-induced axonal growth inhibition of secondary motoneurons, the axonal growth-related genes *gap*-43 and a1-tubulin in 48 hpf zebrafish were evaluated by qRT-PCR. The expression levels of *gap*-43 and *a1-tubulin* were significantly upregulated in the DP or 3-MP exposure group compared with that in the solvent control (Fig. 2B, C, $P < 0.05$). Larvae co-exposed to DP and 3-MP (5, 20 μg/L) showed remarkably

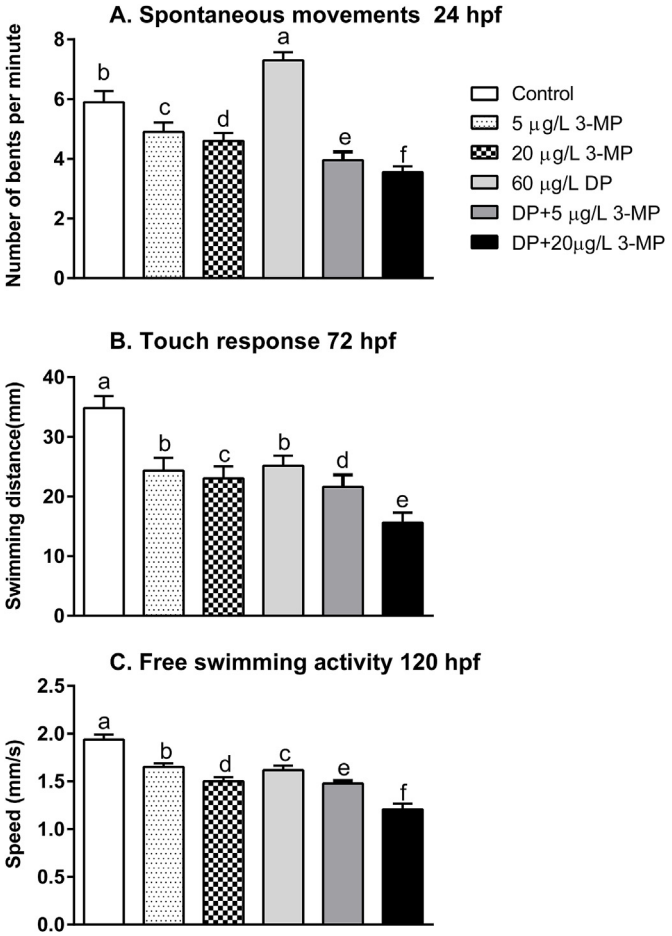

Fig. 1. 3-MP and DP treatment elicited neurobehavioral anomalies in zebrafish larvae. Zebrafish embryos were exposed to 3-MP (5 and 20 μg/L), DP (60 μg/L) or a co-exposure of 3-MP/DP at 5 or 20 μg/L of 3-MP in DP (60 μg/L) beginning from 6 hpf, and behaviors were tested at different stages. Spontaneous movement at 24 hpf was assessed by number of tail bends per minute (A) and touch response at 72 hpf was evaluated by swimming distance (B). Free swimming activity of larvae at 120 hpf was measured by swimming speed (C). The different letter above the bar indicates significant difference among groups ($P < 0.05$).

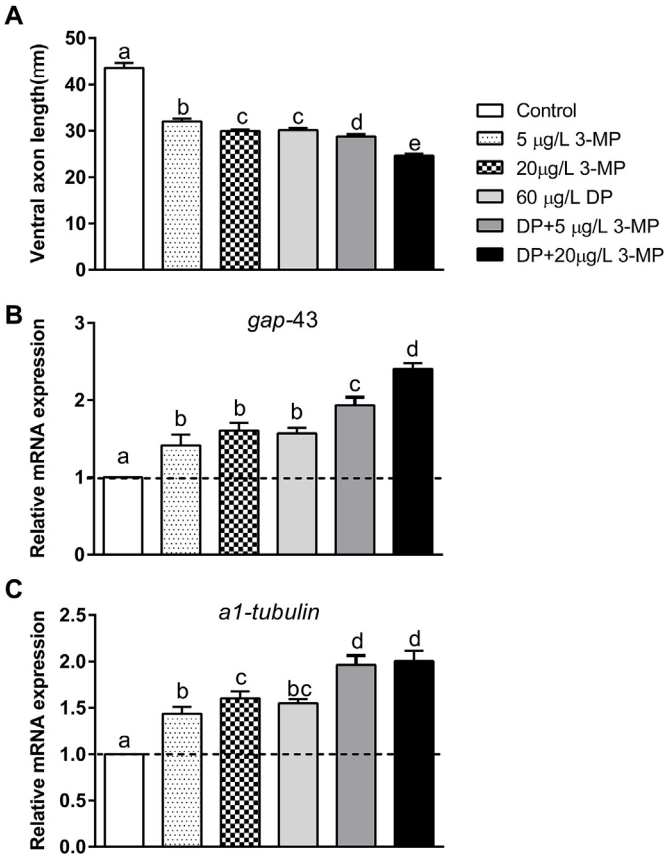

Fig. 2. 3-MP and DP treatment inhibited axonal growth and the expression of axon -related genes. Axonal length (A) and the gene expression of *gap*-43 (B) and *α1-tubulin* (C) were measured in 48 hpf zebrafish. The different letter above the bar indicates significant difference among groups ($P < 0.05$).

elevated expression levels of *gap*-43 and *α1-tubulin* compared with those in the single-compound-exposure groups (Fig. 2B, C, $P < 0.05$).

3.4. Effects on Ca^{2+} homeostasis

The intracellular Ca^{2+} levels were measured in the brain tissue of zebrafish larvae at 96 hpf. Significant increases in the intracellular Ca^{2+} levels were detected in the brain tissue from all treatment groups compared with those from the control group, and a synergistic increase was observed in the co-exposed groups compared with that in the groups with individual chemical exposure (Fig. 3A, $P < 0.05$). Measurement of Ca^{2+} ATPase activity in the brain tissue showed significant decreases in all exposure groups (Fig. 3B, $P < 0.05$).

To further explore the effect of 3-MP and/or DP exposure on Ca^{2+} homeostasis, we quantified the expression of the Ca^{2+} ATPase gene *atp2a2a* and skeletal muscle calcium release channel genes *ryr1a* and *ryr1b* in 96-hpf brain tissues from zebrafish. A significant reduction in *atp2a2a* expression was detected in all exposure groups, and a synergistic effect was exhibited with DP co-exposure with 20 μg/L of 3-MP (Fig. 3C, $P < 0.05$). The expression levels of *ryr1a* and *ryr1b* were downregulated in all treated groups compared with those of the controls (Fig. 3D and E). A significant reduction in *ryr1a* expression was observed in the 3-MP and DP co-exposed groups compared with that in the single-chemical-exposure groups. The gene expression findings are aligned with the observations of increased intracellular Ca^{2+} and reduced Ca^{2+} ATPase activity in the treatment groups.

3.5. Effects on cell apoptosis and oxidative stress

The amount of cell apoptosis in the tail region of 96-hpf larvae was significantly increased for all treatment groups compared with that in the solvent control. Significant increases were observed in the 3-MP and DP co-exposed groups compared with those in the groups with individual chemical exposure (Fig. 4, $P < 0.05$).

To determine whether ROS were generated as a possible mechanism underlying the increased cell apoptosis following 3-MP and DP exposure, we assessed the ROS levels and expression of cell apoptosis-related genes (*bcl-2*, *bax* and *caspase*-3). The ROS levels in all treatment groups were significantly elevated compared with those in the solvent control group (Fig. 5A). The expression level of *bcl*-2 was not significantly different among the 3-MP, DP, or co-exposure groups (Fig. 5C). However, the expression levels of *caspase*-3 and bax in all exposure groups were significantly elevated compared with those in the control group (Fig. 5B, D, $P < 0.05$). Co-exposure with 20 μg/L of 3-MP and 60 μg/L of DP elicited significant upregulation of *caspase*-3 and *bax* expression compared with DP or 3-MP exposure alone.

3.6. Bioaccumulation of 3-MP and DP in zebrafish larvae

The measured concentrations of DP and 3-MP in water were similar to the nominal concentrations of the exposure groups (Table S5). The

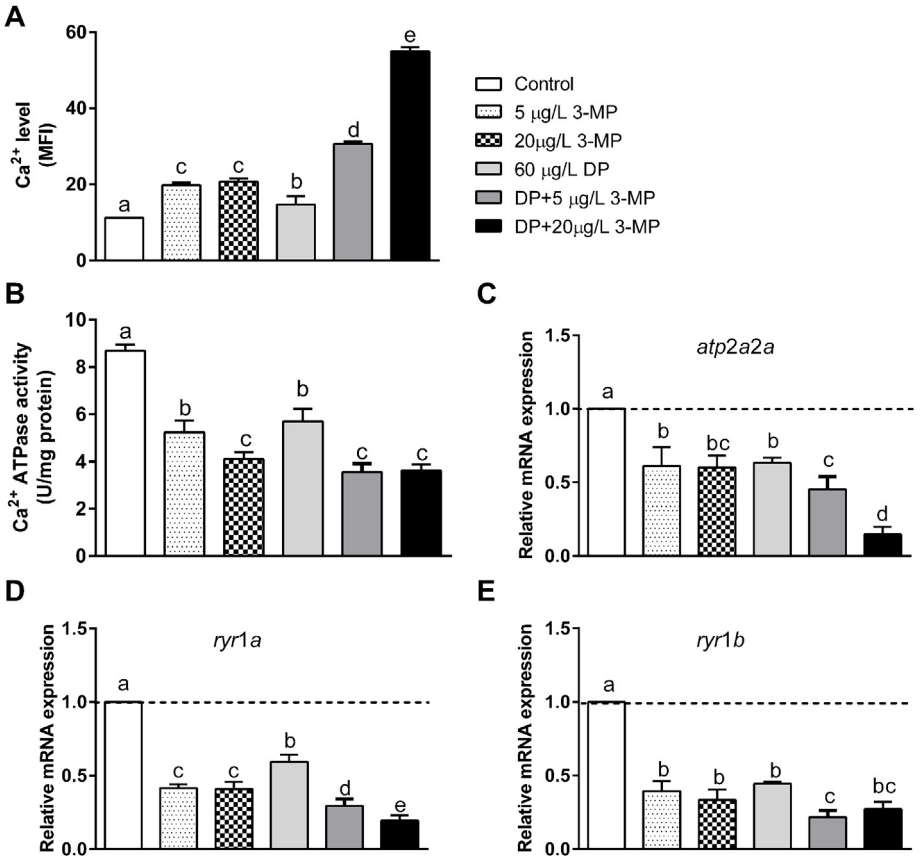

Fig. 3. 3-MP and DP exposure disturbed the intracellular Ca^{2+} homeostasis. Intracellular Ca^{2+} level (A), Ca^{2+}-ATPase activity (B) and mRNA expression of *atp2a2a* (C), *ryr1a* (D) and *ryr1b* (E) were detected in 96 hpf zebrafish larvae. The different letter above the bar indicates significant difference among groups ($P < 0.05$).

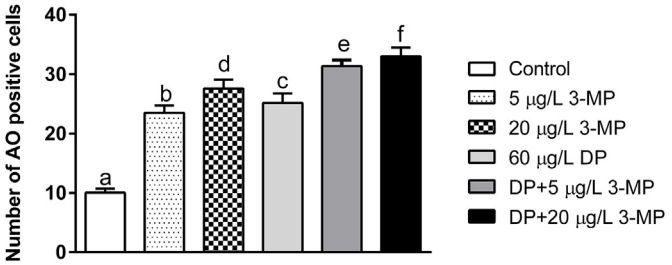

Fig. 4. 3-MP and DP exposure increased cell apoptosis in the tail region of zebrafish. The number of cells undergoing apoptosis were measured by acridine orange (AO) staining at 96 hpf. The different letter above the bar indicates significant difference among groups ($P < 0.05$).

larval body burden of DP was 583.2 ± 33.5 ng/g wet weight (ww) for the DP (60 μg/L)-alone group and 665.0 ± 33.5 and 1061 ± 85.7 ng/g ww for DP co-exposed with 5 μg/L and 20 μg/L of 3-MP, respectively (Fig. 6A). Relative to DP exposure alone, co-exposure with low and high doses of 3-MP increased the accumulation of DP in larvae by 14.0% (not statistically significant) and 81.9%, respectively. The larval body burden of 3-MP was 339.8 ± 23.2 and 594.4 ± 58.5 ng/g ww for 5 and 20 μg/L of 3-MP for the single-treatment groups, respectively, and 492.6 ± 24.8 and 872.8 ± 47.8 ng/g ww for the groups with co-exposure with DP and 5 and 20 μg/L 3-MP, respectively (Fig. 6B). Compared with the 3-MP-alone treatment, co-exposure with DP also increased the accumulation of 3-MP in larvae by 45.0% and 46.8% for the 5- and 20-μg/L 3-MP groups, respectively (Fig. 6B).

4. Discussion

This study showed that 3-MP alone or co-exposed with DP causes neurobehavioral anomalies in zebrafish. Furthermore, exposure to 3-MP alone or in combination with DP significantly inhibited axonal growth of secondary motoneurons, disrupted intracellular Ca^{2+} homeostasis in the brain tissues and increased cell apoptosis in the muscle of zebrafish, suggesting that 3-MP alone or with DP co-exposure induced neurobehavioral deficits through the combined effects on neuronal connectivity and muscle function.

We observed that 3-MP or DP exposure significantly induced neurobehavioral anomalies, including the alteration of spontaneous movement and reduction of touch response and free swimming activity, and co-exposure with 3-MP and DP showed additive or synergistic effects on neurobehavioral deficiencies. These findings indicate that 3-MP and DP are neurotoxic to developing zebrafish. This effect was similar to that exhibited following Phen exposure, where Phen exposure at 195.0 μg/L resulted in severe behavioral impairment, including reduction of swimming activity and ventilation in the early stage of the aquatic amphipod *Hyalella azteca* (Gauthier et al., 2016). However, co-exposure to the heavy metal Cu significantly attenuated the Phen-induced decrease in ventilation. DP exposure caused neurobehavioral deficits in zebrafish, findings that were consistent with those in our previous published study (Chen et al., 2017).

Neurobehavioral anomalies are closely associated with chemical-induced dysfunction of the nervous system, and the secondary motoneuron is a type of neural cell that governs motor behavior in the early development of zebrafish (Wang et al., 2013). In this study, significant decreases in the axonal growth of secondary motoneurons were observed in zebrafish treated with 3-MP and DP, indicating a potential causal relationship between 3-MP/DP-elicited neurobehavioral

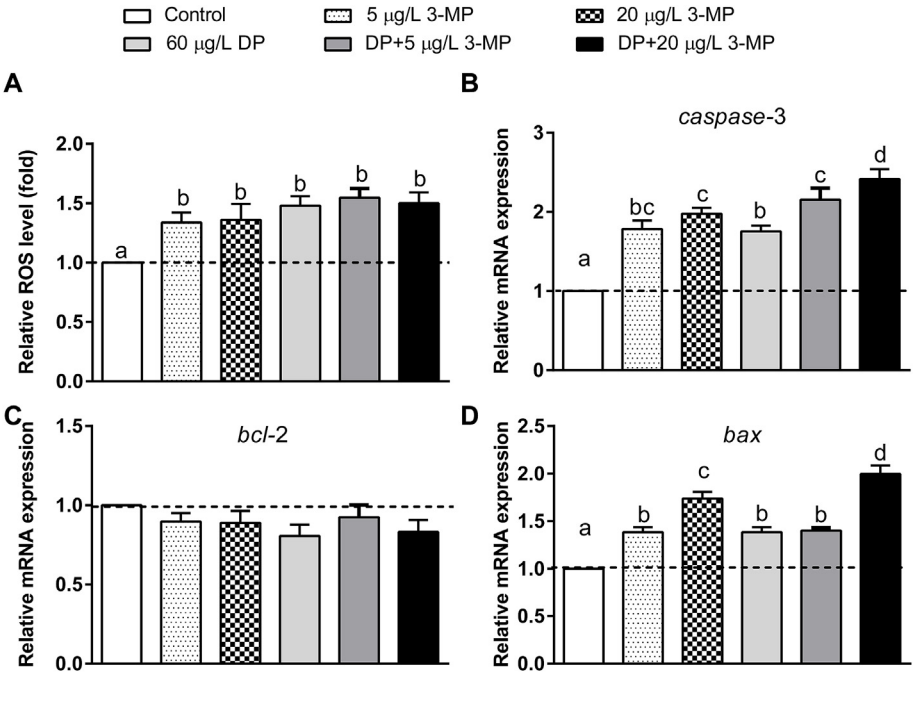

Fig. 5. 3-MP and DP treatment elicited oxidative stress and affected the expression of apoptosis-related genes. Following exposure to 3-MP, DP, or a combination, levels of ROS (A) and the gene expression of *capase*-3 (B), *bcl*-2 (C) and *bax* (D) were measured in zebrafish at 96 hpf. The different letter above the bar indicates significant difference among groups ($P < 0.05$).

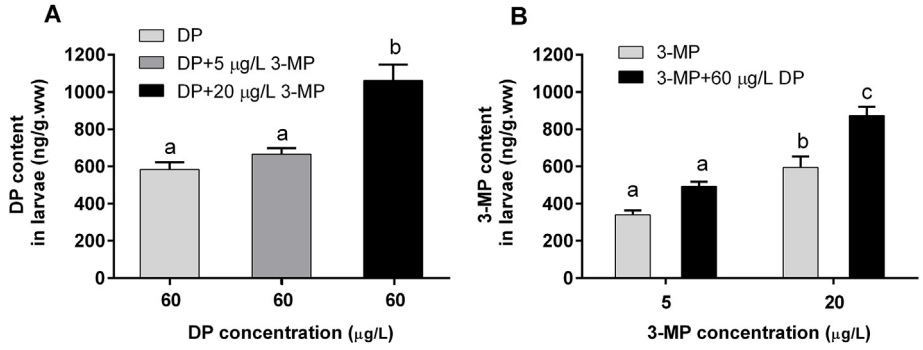

Fig. 6. The bioaccumulation of DP and 3-MP in 120 hpf zebrafish larvae. Levels of DP (A) and 3-MP (B) were detected in larvae after continuous exposure to 3-MP (5, 20 μg/L), DP (60 μg/L) and 3-MP/DP mixture from 6 to 120 hpf. The different letter above the bar indicates significant difference among groups ($P < 0.05$).

anomalies and disturbance of secondary motoneuron growth. *Gap*-43 is a typical glial protein with high expression in nerve growth cone cells and plays an important role in synaptic formation and axonal regeneration (Chen et al., 2012). *Tubulins*, including *a*1-*tubulin*, are highly expressed microtubule proteins in the differentiation of neurons (Chen et al., 2012). 3-MP alone or co-exposure with DP significantly increased the expression levels of *gap*-43 and *a*1-*tubulin*, which may be due to a compensatory feedback mechanism *in vivo* in response to 3-MP/DP-induced suppression of secondary motoneuron growth. Together, our results indicated that 3-MP alone or co-exposure with DP induced neurobehavioral deficiencies in zebrafish larvae, which may be a result of an alteration of neuronal connectivity.

The Ca^{2+} signaling pathway plays a key role in regulating neurotransmitter transmission and neuronal function (Williams and Smith, 2018). Exposure to 3-MP or DP alone significantly increased intracellular Ca^{2+} levels. Particularly, the synergistic effects on the intracellular Ca^{2+} levels were observed in DP co-exposed to 20 μg/L of 3-MP. The potential mechanisms underlying the elevated intracellular Ca^{2+} levels in fish due to DP and 3-MP are not known. It is well established that Ca^{2+} ATPase is an important transport enzyme for Ca^{2+}, and *atp2a2a* encodes endoplasmic reticulum Ca^{2+}-ATPase (Zhang et al., 2013). In the present study, exposure to 3-MP or DP alone caused decreases in Ca^{2+} ATPase activities and the mRNA expression of *atp2a2a* in brain tissue, and a synergistic effect on the reduction of *atp2a2a* expression was exhibited in DP co-exposure with 20 μg/L 3-MP, which may result in alteration of the intracellular Ca^{2+} levels in zebrafish. Meanwhile, 3-MP and/or DP exposure also significantly inhibited the expression of the skeletal muscle calcium release channel genes ryr1a and *ryr*1*b*. It is known that *ryr*1*a* and *ryr*1*b* encode the skeletal muscle calcium release channel (Agrawal et al., 2018). Down-regulation of *atp2a2a* and *ryr*1*a/b* was reported to have a close association with skeletal muscle dysfunction (Agrawal et al., 2018; Lamboley et al., 2014; Zhang et al., 2013). Therefore, 3-MP alone or co-exposure with DP may induce an adverse effect on muscle function in zebrafish. Our hypothesis was further supported by increased cell apoptosis as observed by an increase in AO staining and increased expression of apoptosis-related genes *caspase*-3 and *bax* in zebrafish treated with 3-MP and DP. Subsequent ROS analysis also showed increased production in larvae co-exposed to 3-MP and DP. Together, these findings indicate that 3-MP- and DP-induced behavioral anomalies may result from muscle function deficits that originated from altered intracellular Ca^{2+} homeostasis and increased ROS levels. Future pathological analysis is necessary to validate that the observed behavioral changes were due to muscle function-mediated motor deficits. More recently, DP exposure has been shown to interfere with the secretion of thyroid hormone (TH) in zebrafish (Kang et al., 2016). Changes in the *in vivo* TH levels can affect neurodevelopment and neural function (Kodavanti and Curras-Collazo, 2010). Thus, 3-MP- and

DP-induced behavioral anomalies could also be mediated through TH.

Importantly, we discovered increased accumulation of DP and 3-MP following co-exposure compared with that following single chemical exposure at the same concentrations. Although the exposure concentrations of DP and 3-MP in the present study were higher than those measured in surface water (< 100 ng/L, Salamova and Hites, 2011; Hong et al., 2016), relatively higher concentrations of toxicants are often used in laboratory short-term exposure procedures to elicit clearly distinguishable effects that allow the possible toxicity mechanisms to be determined (Zhu et al., 2014). However, the measured DP content in the exposed zebrafish larvae was within the range detected in aquatic species (19-9630 ng/g) near an electronic waste recycling workshop in South China (Wu et al., 2010), and the body burden of 3-MP in the present study was also similar to methyl PAH detected in fish (303-603 ng/g) from Kharg coral Island, Persian Gulf, Iran (Ranjbar Jafarabadi et al., 2019), suggesting that the DP and 3-MP doses used in the present study were relevant to environmental exposure. Significant elevation of the DP or 3-MP tissue body burden was found in DP co-exposure with 20 μg/L 3-MP. Thus, we speculate that the synergistic effects on motor deficits observed in zebrafish co-exposed with DP and 3-MP are most likely due to higher actual exposure concentrations of 3-MP and DP that resulted from elevated bioaccumulation in the co-exposure. Similar results were found in fish following pesticide exposure. For example, fish co-exposed to cypermethrin and chlorpyrifos showed that the accumulation of both pesticides was increased compared with single-chemical exposure (Bonansea et al., 2017). It was speculated that the higher bioaccumulation of chlorpyrifos and cypermethrin in fish was related to biotransformation system inhibition. Previous studies *in vivo* have shown that DP or A-Phen exposure could affect the activity of biotransformation enzymes in different tissues (Mu et al., 2016; Wu et al., 2012). Thus, DP and 3-MP co-exposure can enhance the bioaccumulation of both chemicals, a finding that may be attributed to their effect on inhibiting the biotransformation system. Together, our finding suggests that the additive or synergistic effects observed in exposure to chemical mixtures result from increased absorption or bioaccumulation of individual chemicals rather than different chemicals targeting an underlying common mechanism. Thus, this study, for the first time, provides insight into the neurotoxic effect and mechanisms of action of 3-MP and DP, and it would be useful for the risk assessment of these chemicals in aquatic ecosystems.

5. Conclusion

This study is the first to assess neurodevelopmental toxicity following co-exposure to 3-MP and DP in zebrafish. Exposure to 3-MP alone or in combination with DP significantly altered spontaneous movement, decreased touch response and reduced free swimming activity in zebrafish. In accordance with these neurobehavioral deficits, 3-MP single or co-exposure with DP significantly inhibited axonal growth of secondary motoneurons, disturbed intracellular Ca^{2+} homeostasis in brain tissues, induced ROS formation and increased cell apoptosis in the muscle of zebrafish. These findings suggest that 3-MP alone or co-exposure with DP causes neurobehavioral anomalies in zebrafish, a finding that may be due to joint effects on the alteration of neuronal connectivity and muscle function. Additionally, co-exposure to 3-MP and DP shows synergistic effects on neurobehavioral anomalies in zebrafish. These synergistic effects of 3-MP and DP on neurobehavioral deficits were due to higher 3-MP and DP tissue accumulation resulting from the co-exposure setting rather than any potential common underlying mechanism contributed by both chemicals. However, due to the complex interaction between neuronal function and endocrine disruptions, the effects of 3-MP and DP co-exposure on the disruption of thyroid hormone should be investigated further in the near future.

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Appendix A. Supplementary data

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