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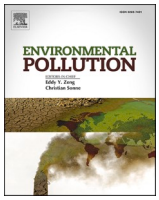

Neurobehavioral toxicity induced by combined exposure of micro/nanoplastics and triphenyltin in marine medaka (*Oryzias melastigma*)☆

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ABSTRACT

Microplastics/nanoplastics (MNPs) inevitably coexist with other pollutants in the natural environment, making it crucial to study the interactions between MNPs and other pollutants as well as their combined toxic effects. In this study, we investigated neurotoxicity in marine medaka (*Oryzias melastigma*) exposed to polystyrene micro/nanoplastics (PS-MNPs), triphenyltin (TPT), and PS-MNPs + TPT from physiological, behavioral, biochemical, and genetic perspectives. The results showed that marine medaka exposed to 200 ng/L TPT or 200 μg/L PS-NPs alone exhibited some degree of neurodevelopmental deficit, albeit with no significant behavioral abnormalities observed. However, in the PS-MP single exposure group, the average acceleration of short-term behavioral indices was significantly increased by 78.81%, indicating a highly stress-responsive locomotor pattern exhibited by marine medaka. After exposure to PS-MNPs + TPT, the swimming ability of marine medaka significantly decreased. In addition, PS-MNPs + TPT exposure disrupted normal neural excitability as well as activated detoxification processes in marine medaka larvae. Notably, changes in neural-related genes suggested that combined exposure to PS-MNPs and TPT significantly increased the neurotoxic effects observed with exposure to PS-MNPs or TPT alone. Furthermore, compared to the PS-MPs + TPT group, PS-NPs + TPT significantly inhibited swimming behavior and thus exacerbated the neurotoxicity. Interestingly, the neurotoxicity of PS-MPs was more pronounced than that of PS-NPs in the exposure group alone. However, the addition of TPT significantly enhanced the neurotoxicity of PS-NPs compared to PS-MPs + TPT. Overall, the study underscores the combined neurotoxic effects of MNPs and TPT, providing in-depth insights into the ecotoxicological implications of MNPs coexisting with pollutants and furnishing comprehensive data.

1. Introduction

Micro/nanoplastics (MNPs), pollutants resulting from industrial activities, have been a subject of growing environmental concern since their initial documentation in the 1970s (Law, 2017). The presence of MNPs in aquatic environments has been quantitatively assessed, with findings indicating weight concentrations ranging from 0.4 to 640 μg per liter (Lasee et al., 2017; Liang et al., 2021). The size of particles plays a crucial role in determining the relative surface area of MNPs (Peng et al., 2020), an influential factor influencing MNPs' behavior and consequent ecological ramifications. Notably, MNPs demonstrate propensities for dispersion and accumulation within biotic entities, with emerging evidence suggesting that particle size is a paramount determinant of MNPs' biodistribution (Liu et al., 2024b; Zhang et al., 2022). Comparative analyses reveal that smaller nanoplastics (NPs) more readily penetrate and accumulate in tissues, while larger microplastics (MPs) are unable to enter. MPs are mainly distributed in the intestines and gills of aquatic animals (Lu et al., 2016), while NPs have been found within the vascular systems (Ma et al., 2021). Interestingly, research indicated that 50 nm NPs have the capability to penetrate the brain barriers of fish larvae, intestinal barriers, and can also lead to abnormal embryo development (Liang et al., 2021; Liu et al., 2024a; Xian et al., 2024; Ye et al., 2024). It further underscores the intricate relationship between the size of MNPs and their toxicological effects. This complexity was

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further highlighted in studies on the synergistic toxicity of MNPs and contaminants on zebrafish (Chen et al., 2017; He et al., 2021a; Sim et al., 2023). These studies have showed that while the coexistence of larger MPs and pollutants may not change or reduce toxicological impacts, the presence of NPs consistently intensifies harmful effects. These findings collectively underscore the imperative need for a nuanced understanding of MNPs' environmental and biological interactions, an area that remains underexplored and contentious to date.

Organotins (OTs) are environmental pollutants widely distributed in coastal areas. OT compounds have been detected in coastal regions and river basins in China, with concentrations ranging from 33.9 to 203 ng/L in areas such as the Yangtze River (Chen et al., 2022; Li and Li, 2020). Previous studies have shown that triphenyltin (TPT), as a typical OT compound, can quickly adsorb to and interact with the surfaces of MNPs (Guo and Wang, 2019; Zhang et al., 2023). Thus far, the individual environmental toxicological effects of MNPs (Zhang et al., 2020) and TPT (He et al., 2021a; He et al., 2022; Li et al., 2023) have been extensively studied. For instance, MNPs may accumulate through the food chain, posing potential damages to associated organisms, including but not limited to brain injury and behavioral disorders in fish (Mattsson et al., 2015). TPT can disrupt the endocrine system (He et al., 2021b; Li et al., 2024) and penetrate the blood-brain barrier, leading to neurotoxicity (da Silva et al., 2018). Additionally, transcriptomic data of marine medaka suggested that TPT can significantly affect the behavioral functions and neuroendocrine system of marine medaka through transcriptome sequencing analysis, which further confirms the neurotoxicity of TPT on marine medaka (He et al., 2022). Although there have been studies on the combined toxicity of MNPs and TPT, studies on the neurotoxic effects of combined exposure are still lacking. Therefore, it is necessary to investigate the combined neurotoxicity of MNPs and TPT on marine organisms to better understand the ecological toxicological significance of MNPs.

A series of typical behavioral changes in fish, such as changes in swimming frequency and intensity, are often related to physiological changes in the organism (Feng et al., 2024; Li et al., 2019). Swimming behavior has long been utilized as a sensitive indicator of overall development defects and survival capabilities in fish (MacPhail et al., 2009; Weis, 2014). Since behavioral changes directly correlate with an organism's adaptability within its environment, even if pollutants do not directly induce mortality, alterations in behavior can impact feeding, predator avoidance, or reproductive potential, consequently reducing the organism's adaptability within the ecosystem (Takai et al., 2022). Empirical studies have indicated that concentrations at which organisms exhibit behavioral changes in pollution gradient exposure experiments are often lower than those causing deformities or mortality, underscoring the utility of behavioral effect detection in indicating relatively mild environmental pollution (Yuan et al., 2021). Some studies emphasize that the average duration of behavioral research is comparable to that of acute lethality, developmental, and reproductive experiments. Furthermore, behavioral experiments are usually more sensitive and statistically more efficient than developmental or reproductive experiments (Kiran et al., 2022; Melvin and Wilson, 2013). Furthermore, locomotor behaviors are associated with neurotoxicity, with research suggesting that permeability of neuronal membranes, ion channels, and mitochondria may induce neurotoxic effects in brain regions such as the cortex, hippocampus, and hypothalamus (Zhang et al., 2018; Zheng et al., 2017). A more comprehensive examination indicated that behavioral changes reflect the integrated expression of animals at molecular, biochemical, and physiological levels under conditions of environmental pollution (López-Rodríguez et al., 2021; Tao et al., 2022).

Marine medaka (*Oryzias melastigma*), originally indigenous to India and the western regions of Southeast Asia (Naruse, 1996), has emerged as a pivotal species in marine ecotoxicological investigations, owing to its experimental advantages such as diminutive size and rapid reproductive cycle (Dong et al., 2014). Marine medaka had been used to study the toxicity manifestations and mechanisms of various anthropogenic pollutants. In this study, we conducted an exposure study on marine medaka using two typical marine pollutants, TPT and MNPs. Polystyrene (PS) is one of the most widely used plastics in the world, and polystyrene micro/nanoplastics (PS-MNPs) are one of the most frequently detected MPs in the environment (Zhang et al., 2022). Therefore, PS particles usually served as the model to investigate the fate and risks of MPs and NPs in the environment. Domestic sewage is the main source of MNPs and TPT. Current research indicates that TPT and MNPs do coexist in the aquatic environment (Liu et al., 2024a; Mao et al., 2022). Moreover, MNPs have hydrophobic surfaces that can adsorb TPT, leading to their aggregation and the formation of composite pollutants, further exacerbating the level of marine pollution. This study aimed to investigate the potential impairment of neuroactivity and locomotor abilities in marine fish exposed to environmental concentrations of MNPs and organic compounds, from the perspectives of fish behavior, tissue, neurochemical indicators, and molecular analysis. The primary objective of this investigation is to elucidate the neurotoxic effects and mechanisms arising from the simultaneous exposure of marine medaka to PS-MNPs and TPT, and to analyze the differences in the toxic effects of polystyrene microplastics (PS-MPs) and polystyrene microplastics (PS-NPs) on TPT. We hypothesize that the addition of PS-MNPs would affect the TPT-induced neurotoxicity and that particle size differences would lead to different neurotoxic effect. The findings obtained in this study provide an important reference for subsequent research on the simultaneous exposure of MNPs and OTs, as well as their neurotoxicity in marine organisms.

2. Material and methods

2.1. Chemicals and test fish

The adult marine medaka were cultured under aquaculture conditions by Qingdao Favourite Biotechnology Co., Ltd (Qingdao, China). In short, we utilized artificial seawater with a salinity of 30‰, a light-dark cycle of 14 h of light and 10 h of darkness, maintained a temperature of 24 °C ± 0.5 °C, and aerated the culture. They were fed twice daily with newly hatched brine shrimp. Eggs were collected on the morning of the second day after pairing male and female fish in the tank. To ensure synchronous development of fertilized eggs, the status and quality of these eggs were checked under a stereomicroscope (Cnoptec, SZ650, China) before being transferred to culture (Anderson et al., 2020; Choe et al., 2021; Ng and Gong, 2013). Dimethyl sulfoxide (DMSO) with a purity of 99.5% was used to prepare the TPT stock solution from TPTCl powder (purity >96%). Wuxi Rigor Biotechnology Co., Ltd (Wuxi, China) provided suspensions of 50 nm PS-NPs and 5 μm PS-MPs in this study. Before exposure, the solution containing the mixture of TPT and PS-MNPs underwent 24 h of oscillation at 25 °C and 150 rpm to ensure complete homogenization.

The ATPase assay kit (A070-1-2, Colorimetric method), the acetylcholinesterase (AChE) test kit (A024-1-1, Colorimetric method), and the total protein (TP) test kit (A045-2-2, Coomassie brilliant blue method) were procured from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Additionally, the fish pigment P450 subenzyme CYP1A1 (EROD) assay kit and the fish thyroid hormone (T4) test kit (ELISA) were supplied by Pure Biotech (Wuhan, China).

2.2. The characteristics of PS-MNPs

The morphology of PS-MNPs was analyzed using scanning electron microscopy (SEM). To examine the surface morphology and particle size of PS-MNPs and explore the interactions between TPT and PS-MNPs, the PS-MNPs solution and PS-MNPs + TPT solution were centrifuged in a glass centrifuge tube and then the supernatant was poured off. The tube containing the precipitate was then covered with gauze and freeze-dried using a freeze dryer (Shunzhi, FD-1A-50, China) for 48 h. Subsequently,

the centrifuged and air-dried samples were imaged using SEM at an accelerating voltage of 5 kV. The particle size of PS-MNPs was assessed by analyzing images with ImageJ software (http://imagej.nih.gov/ij/), referencing a study published previously (Malafaia et al., 2020). In brief, the diameters of PS-MNPs and PS-MNPs + TPT in SEM images were measured using the straight-line tool in ImageJ, followed by calibration from pixels to actual dimensions using the image scale to obtain size. Additionally, fourier transform infrared spectroscopy (FTIR) was employed to analyze the surface functional group variations of PS-MNPs and PS-MNPs + TPT. The hydrodynamic diameter and zeta potential of PS-MNPs and PS-MNPs + TPT solutions were determined using dynamic light scattering (Malvern zetasizer, model ZEN3600, UK).

2.3. Experimental design and sampling

In the study, based on previous research, we selected 50 healthy blastula stage embryos [6 h post-fertilization (hpf)] and placed them in culture dishes filled with 50 mL of exposure solution (Liu et al., 2024a). Adopting a semi-static exposure approach, we ensured that the exposure solution was fully replaced each day, maintaining a continuous exposure period of 21 days. The experiment was divided into one control group and five treatment groups, each consisting of 5 culture dishes, totaling 250 embryos per group. Solvent control group (DMSO control group), TPT group, PS-NP group, PS-MP group, and two combined exposure group (PS-NPs + TPT group and PS-MPs + TPT group). The concentrations of 200 ng/L TPT and 200 μg/L PS-MNPs were determined based on environmentally relevant concentrations and extensive toxicity testing results in the literature (Ding et al., 2018; Qiao et al., 2023; Sham et al., 2020). In addition, we detected the actual concentration of TPT in the TPT exposure group as 200.54 ± 4.51 ng/L using gas chromatography-mass spectrometry (GC-MS, Agilent Technologies Ltd., USA). Specific TPT concentrations were provided in the Supplementary Material (Table S2).

2.4. Detection of developmental indicators in embryos

The number of deaths and hatches was recorded daily, and microscopic examination was conducted to check for any abnormalities. Based on the recorded data, the mortality rate (%) and hatching rate (%) were calculated. On the 5th, 8th, and 11th days, heart rate measurements were taken by counting the number of heartbeats for 15 s in 15 embryos from each group.

2.5. Behavior test

The marine medaka larvae from each group were placed in a 6-well plastic plate (1 fish per well) after 21 days of exposure. After a 10-min acclimatization period, swimming behavior was recorded using video recording for a duration of 10 min. This process was repeated three times, with each session providing 10 min of video footage for tracking and analyzing swimming trajectories. This process was repeated three times to obtain parallel tracking records for each group. The recorded videos were analyzed using the LoliTrack behavioral analysis system (Beijing EcoTech Science and Technology Ltd.) for movement data and swimming trajectories. Each video generated an analysis file and a tracking video. Using multi-region single target tracking mode as the main parameter, randomly selected larvae are tracked once.

2.6. Brain tissue histopathological examination

After 21 days of exposure, six marine medaka larvae from each group were rinsed with PBS and then fixed in 4% paraformaldehyde (PFA) for at least 24 h. After dehydration and embedding in paraffin wax, the specimens were sectioned into 5 $^{\mu m}$ thick slices and subjected to hematoxylin and eosin staining following established protocols cited in previous literature (Liu et al., 2024a).

2.7. Biochemical analyses

After the exposure, 30 larvae were selected per tube, with a total of 540 larvae chosen for subsequent experimental analysis. The medaka were fasted for 24 h before sampling. Tissues were washed with pre-chilled PBS solution (0.01 M, pH = 7.4), then homogenized with a tissue-to-PBS ratio of 1:9 on ice to create a uniform slurry. The supernatant obtained after centrifugation was used for subsequent testing. Na^+-K^+ ATPase, AChE, TP, and EROD levels were assessed using assay kits. Enzyme activities were determined following the provided instructions. All samples and standards were processed in triplicate.

2.8. Analysis of gene expression levels

After exposure, thirty marine medaka larvae from one replicate were pooled as one sample. According to our previous study, total RNA was extracted from marine medaka larvae and then amplified by quantitative real-time PCR (qRT-PCR) (Liu et al., 2024a). For specific procedures, refer to the Supplementary Material Text S1. The expression levels of thyroid-related genes, neurodevelopment-related genes, and energy metabolism-related genes were detected. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were provided in Table S1.

2.9. Data analysis

All graphs were generated using GraphPad Prism software, and statistical analyses were conducted using SPSS 19.0 software. After normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene) tests, Duncan's test was employed to compare the significant differences between sample groups. All results were presented as mean ± standard error of the mean (SEM).

3. Result

3.1. The SEM images and $^{particle\ sizes}$ of PS-MNPs

The collected data confirmed the consistency of the employed PS-MNPs, consistent with the manufacturer's description that they were composed of uniform plastic particles (Fig. 1). The measurement results indicated that the particle sizes of PS-MPs and PS-NPs were 51.7 ± 4.6 nm and 4.8 ± 0.2 μm, which were appropriate for this experiment. SEM images showed that both types of PS-MNPs were smooth, spherical, and had uniform morphology, and TPT formed irregular crystals that tightly combined with PS-MNPs. The infrared spectra of PS-NPs and PS-MPs both conformed to the standard structure of polystyrene [Fig. 1 (C)]. The absorption peaks were located in region (I) for the C–H bond stretching vibration on the benzene ring, region (II) for the C–H bond stretching vibration of $–CH_2$-, region (III) for the backbone vibration of the benzene ring, and region (IV) for the exoskeleton vibration of the benzene ring. However, the addition of TPT may introduce interference and masking effects on the above characteristic peaks of PS-NPs and PS-MPs, but it did not introduce new functional groups. As shown in Table 1, the hydrodynamic diameter of PS-NPs after adsorbing TPT increased from 55.890 ± 4.940 nm to 74.300 ± 10.420 nm; for PS-MPs, it increased from 5530.000 ± 335.730 nm to 5742.333 ± 433.400 nm. Additionally, the potential of PS-NPs after adsorbing TPT changed from −37.1 ± 9.45 mV to −27.333 ± 0.27 mV. However, compared to PS-MPs, there was no significant change in the zeta potential of PS-MPs + TPT.

3.2. TPT and PS-MNPs affected the mortality rate, hatching rate, and heart rate of medaka

Fig. 2 depicted the mortality, hatching, and heart rate for each group. In comparison to the control group, exposure to TPT, PS-NPs, PS-NPs +

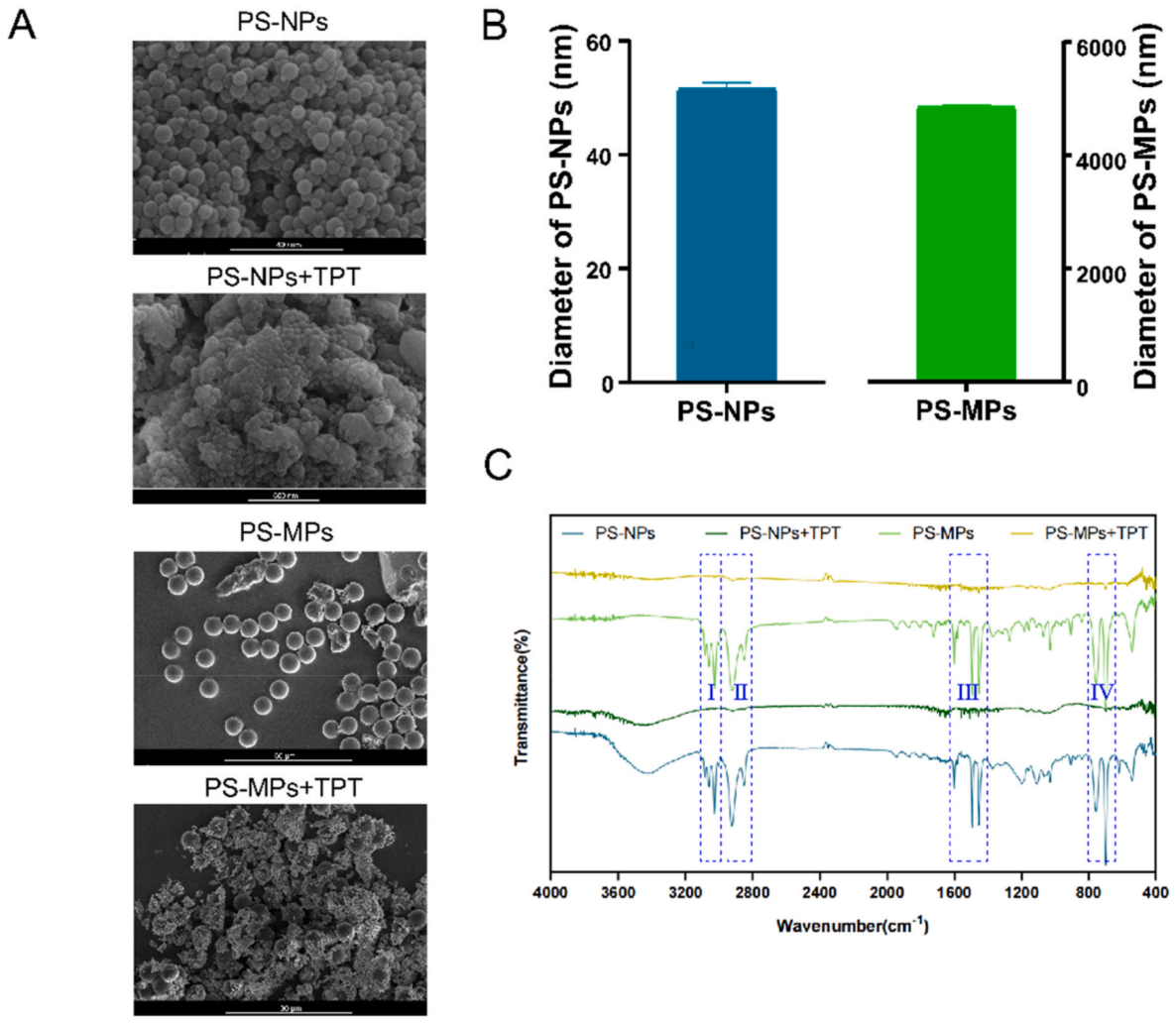

Fig. 1. (A) SEM images of dried PS-MNPs and PS-MNPs + TPT and (B) particle diameter in SEM images (C) The FTIR spectra of PS-MNPs and PS-MNPs + TPT.

Table 1
The hydrodynamic diameter and zeta potential of PS-MNPs and PS-MNPs + TPT. Data are presented as mean ± SEM.

Groups	hydrodynamic diameter (nm)	zeta potential (mV)
PS-NPs	55.890 ± 4.940	−37.100 ± 9.450
PS-NPs + TPT	74.300 ± 10.420	−27.333 ± 0.270
PS-MPs	5530.000 ± 335.730	−16.633 ± 2.830
PS-MPs + TPT	5742.333 ± 433.400	−16.633 ± 2.250

TPT, PS-MPs, and PS-MPs + TPT resulted in respective increases in mortality of 13.22%, 7.22%, 14.04%, 10.55%, and 12.55%, while hatching rates decreased by 16.51%, 9.84%, 20.07%, 15.84%, and 19.84%, respectively. There were no significant differences observed in the heart rate of embryos at 5 days post-fertilization (dpf) exposed to TPT or PS-MNPs compared to the control group. However, at 8 dpf, varying degrees of changes in heart rate were observed in each exposure group. TPT and PS-NPs induced an increase in heart rate, while PS-MPs resulted in a decrease. Combined exposure to PS-MNPs + TPT led to a

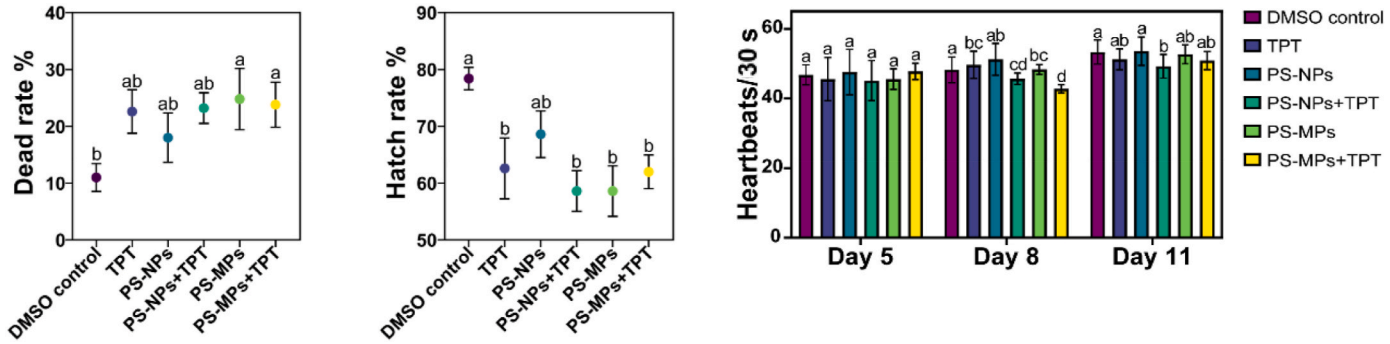

Fig. 2. The dead rate (left), hatch rate (middle) and the heart rate at day 5, day 8 and day 11 (right) of marine medaka embryos under the influence of TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT. Data are presented as mean ± standard error of the mean (SEM), and different letters denote statistical differences across treatments ($P < 0.05$).

significant reduction in heart rate. By 11 dpf, the heart rate in the PS-NPs + TPT group remained significantly depressed, while the heart rate in the PS-MPs + TPT group gradually returned to normal.

3.3. TPT and PS-MNPs affect the swimming behavior of medaka larvae

The locomotor behavior data analysis is shown in Fig. 3. The PS-MNPs + TPT exposure group showed a significant decrease in locomotor activity, possibly increasing anxiety. Compared to the control group, TPT showed no significant effects on marine medaka across four parameters of swimming behavior. Similarly, PS-MPs and PS-NPs did not exhibit significant effects on average velocity, active time, or distance moved. However, the results for average acceleration revealed a significant increase in marine medaka's average acceleration with PS-MPs compared to the control group, showing an increase of up to 78.81% [Fig. 2(B)]. Additionally, there was no significant difference in average acceleration between the PS-NPs + TPT group and the PS-NPs group; only a decreasing trend was observed. Furthermore, PS-MNPs + TPT exposure groups exhibited a significant decrease in data compared to PS-MNPs alone. For instance, in terms of active time, the PS-MPs + TPT group showed a decrease of 28.48% compared to the PS-MPs group, while a decrease of 34.15% was observed for the PS-NPs + TPT group compared to the PS-NPs group.

3.4. Transcriptional response to TPT and PS-MNPs on brain development of medaka

No obvious pathological damage was observed in brain tissue

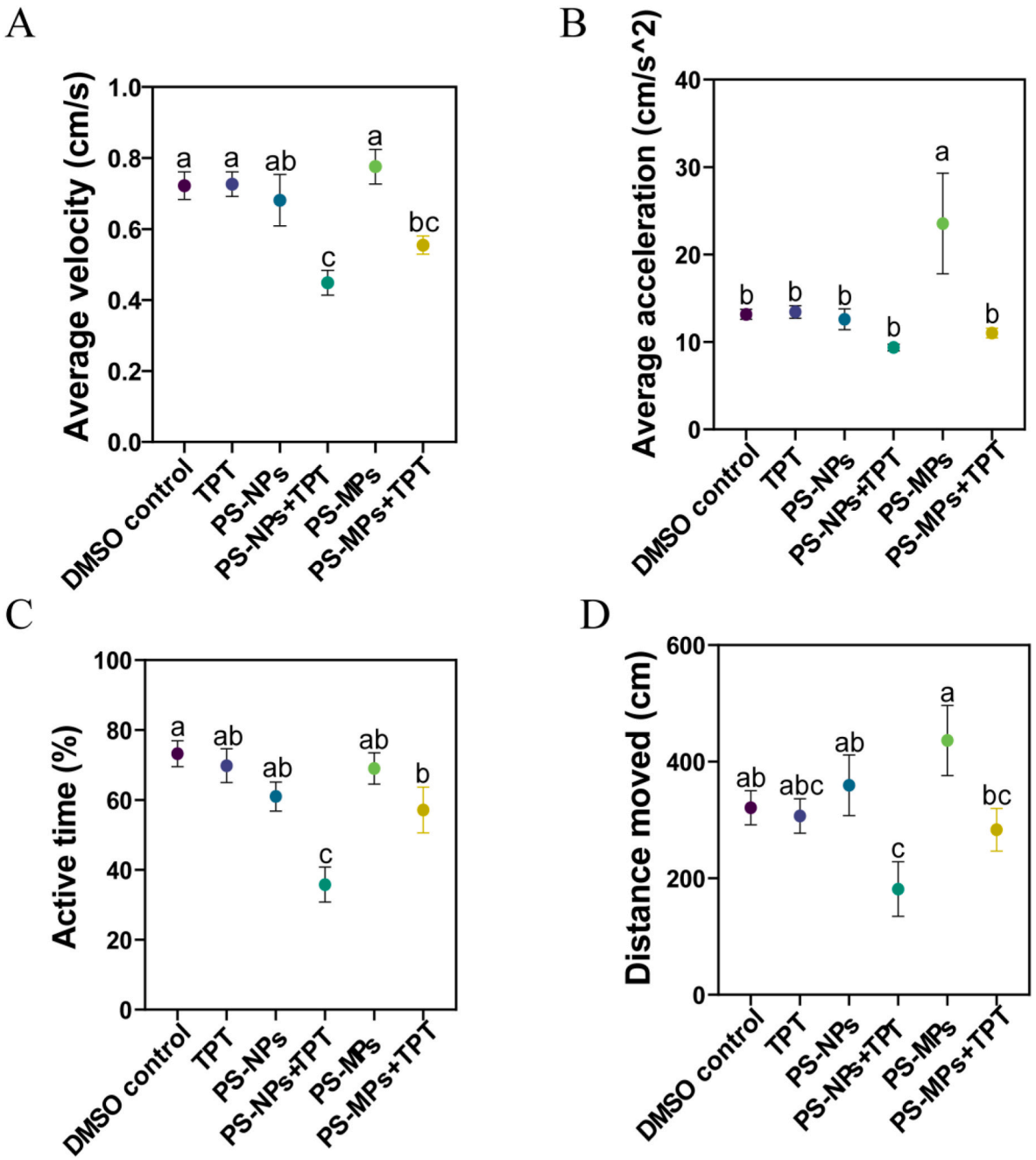

Fig. 3. Effects of TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT on the locomotor behavior of medaka larvae. Data are presented as mean ± standard error of the mean (SEM), and different letters denote statistical differences across treatments ($P < 0.05$).

sections of medaka larvae from the PS-MNPs and PS-MNPs + TPT groups (Fig. 4). Similarly, the levels of T4 showed no significant differences in groups, despite its close association with brain development. However, expression levels of the related genes *thr-α* and *thr-β* exhibited variations. Compared to the control group, the expression levels of *thr-α* were upregulated by 106.85% in the PS-NPs group and by 31.7% in the PS-MPs group. Additionally, the *thr-α* expression level was higher in the PS-NPs group compared to the PS-MPs group. Notably, TPT did not affect the expression level of *thr-α*, but the addition of PS-MNPs significantly enhanced its expression level. Furthermore, compared to the control group, TPT, PS-NPs, and PS-MPs upregulated the expression levels of *thr-β* by 95.05%, 177.13%, and 79.3%, respectively, with the expression level in the PS-NPs group being higher than that in the PS-MPs group. In the PS-MNPs + TPT group, there was a more pronounced increase in the expression level of the *thr-β* gene.

3.5. TPT and PS-MNPs induce the neurotoxicity in medaka

Fig. 5 A showed that the AChE activity in the control group was 0.5–0.6 mg prot/mL. TPT and PS-MPs had no significant effect on AChE activity, while PS-NPs led to a decrease in activity. Addition of PS-MNPs resulted in a significant reduction in AChE activity in the PS-MNPs + TPT co-exposure group, ranging from 0.2 to 0.3 mg prot/mL. Regarding EROD enzyme activity, TPT, PS-MNPs, and PS-MNPs + TPT significantly elevated its levels compared to the control group. Among them, the PS-NPs + TPT group exhibited higher enzyme activity than the PS-NPs group, and the PS-MPs + TPT group exhibited higher enzyme activity than the PS-MPs group.

Expression levels of six neurodevelopment-related genes (*gap43*, *elavl3*, *α-tubulin*, *gfap*, *mbpa*, *gst*) were quantified. The results showed that *gap43*, *α-tubulin*, *gfap*, and *mbpa* expression levels generally decreased with exposure to TPT and PS-MNPs. In terms of *gst*, TPT had

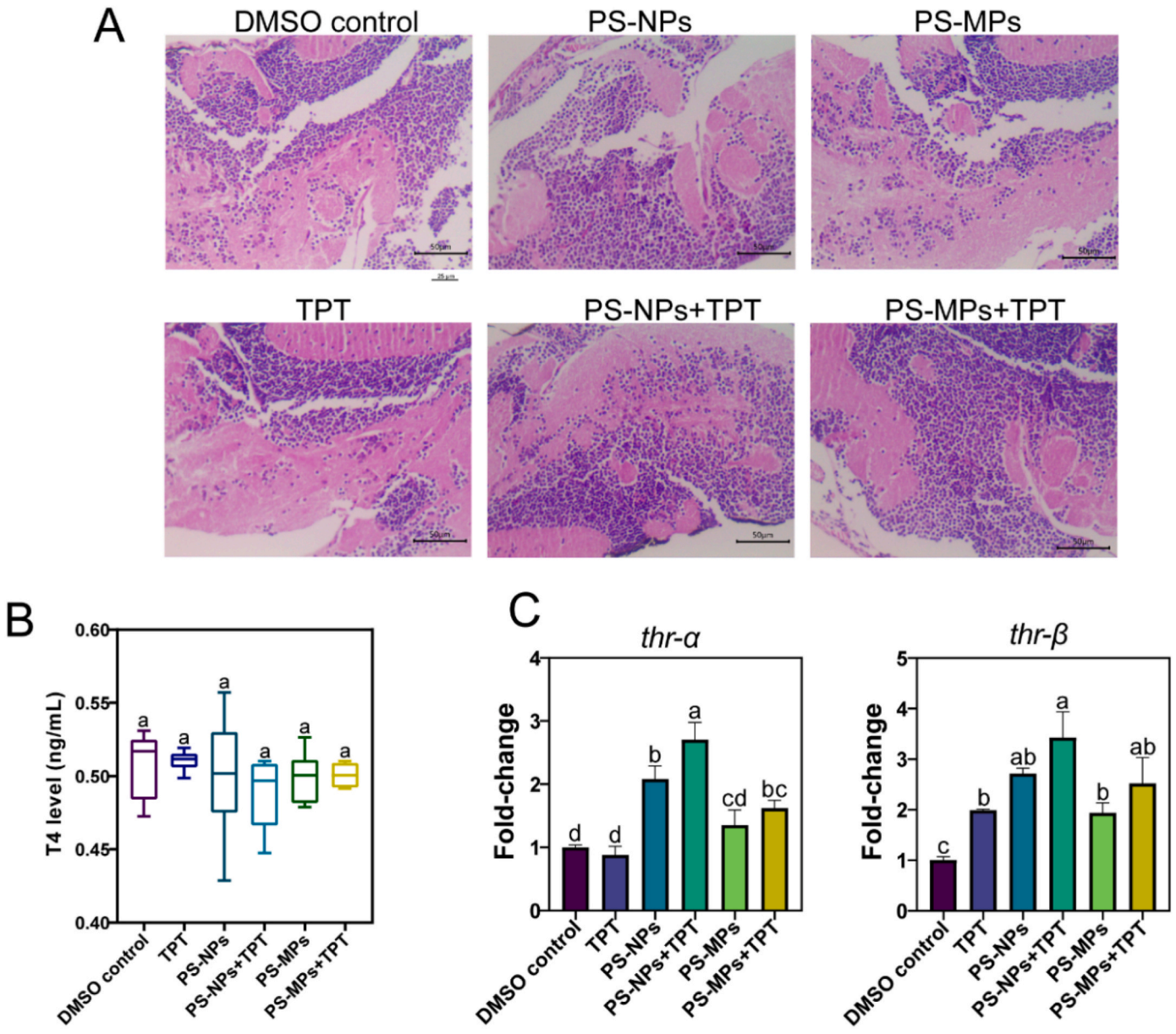

Fig. 4. (A) 400 × histological images of brain of medaka larvae exposed to TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT. (B) Effects of TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT on T4 content in medaka larvae. (C) Relative gene expression of thyroid development genes in 21 dpf (days post-fertilization) larvae exposed to TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT. Data are presented as mean ± standard error of the mean (SEM), and different letters denote statistical differences across treatments ($P < 0.05$).

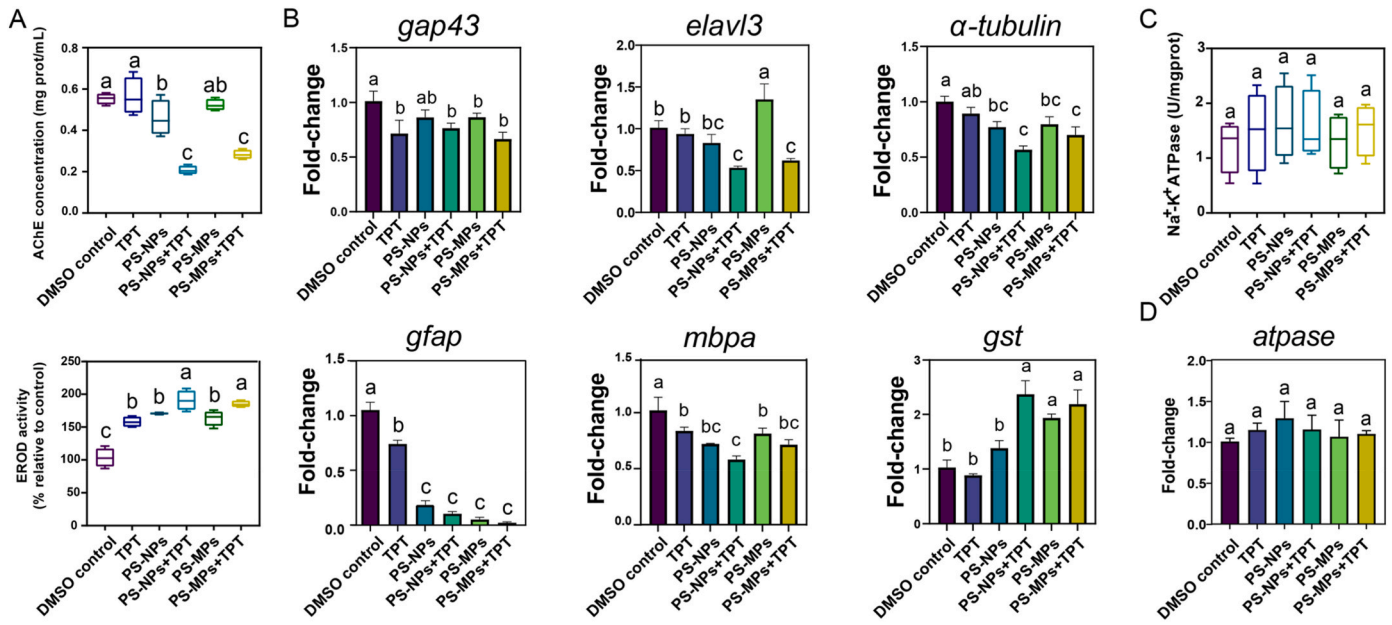

Fig. 5. (A) Effects of TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT on AChE and EROD activity in medaka larvae. (B) Relative gene expression of neurodevelopment genes in 21 dpf larvae exposed to TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT. (C) Effects of TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT on ATPase in medaka larvae. (D) Expression of *atpase* gene in 21 dpf larvae exposed to TPT, PS-NPs, PS-NPs + TPT, PS-MPs and PS-MPs + TPT. Data are presented as mean ± standard error of the mean (SEM), and different letters denote statistical differences across treatments ($P < 0.05$).

no significant effect on gene expression, while PS-MNPs upregulated gene expression. However, the expression levels of PS-NPs + TPT and PS-MPs + TPT exposed groups were increased by 135.23% and 128.78%, respectively. This indicated that the *gst* expression level was significantly increased after the addition of TPT. Notably, the PS-MPs exposure group upregulated *elavl3* expression by 36.92% compared to the control group. However, TPT, PS-NPs, PS-NPs + TPT and PS-MPs + TPT exposure groups decreased *elavl3* expression levels by 8.2%, 20.53%, 50.65%, and 47.55%, respectively. The determination of Na + -K + ATPase activity and the corresponding control gene *atpase* showed no significant differences in these two markers representing energy metabolism among the six groups.

4. Discussion

In the study, SEM images revealed that the addition of TPT induced significant aggregation effects in PS-MNPs [Fig. 1 (A)]. The particle sizes of 50 nm NPs and 5 μm MP met the study criteria [Fig. 1 (B)]. Furthermore, FTIR results indicated that TPT interfered with the vibrational modes of characteristic functional groups in PS-MNPs. Additionally, the presence of TPT increased the hydrodynamic diameter of PS-MNPs. In comparison to PS-NPs, the decrease in absolute Zeta potential values of PS-NPs + TPT implied reduced electrostatic repulsion, hence decreased stability [Fig. 1 (C)](Dong et al., 2018). Previous studies suggested that interactions between TPT and PS-MNPs promote aggregation and sedimentation behavior (Liu et al., 2024a). Consequently, these altered properties may affect the toxicity of PS-MNPs + TPT.

The results of early developmental indicators showed increased mortality [Fig. 2 (left)] and decreased hatching rates [Fig. 2 (middle)] in the PS-MNPs exposure groups, consistent with findings indicating that 100 nm NPs and 2 μm MP reduced zebrafish embryo survival and hatching rates (Feng et al., 2022; Liu et al., 2022; Malafaia et al., 2020). PS-MPs were particularly detrimental as PS-MPs readily adhered to the embryonic chorion, forming a dense PS layer on the embryo's surface and generating hypoxic microenvironments. The pronounced impact of PS-MPs on embryo mortality and hatching rates, compared to PS-NPs, can be attributed to the ability of PS-MPs to obstruct the chorionic pore, resulting in more severe hypoxia (Li et al., 2020). Previous studies have demonstrated the lethal effects of TPT on zebrafish embryos (Qiao et al., 2023). Our findings indicate that TPT can significantly increase the mortality rate in medaka. Furthermore, the fish heart is the initial organ to develop and is particularly vulnerable to environmental stress (Huang et al., 2011). Cardiac development and neural development are interdependent and mutually regulated processes. They both contribute to the overall embryonic development (Morton et al., 2017). A previous study had shown that 100 ng/L TPT did not significantly alter heart rate (Yi and Leung, 2017). In this study, 200 ng/L TPT did not change the embryo heart rate significantly [Fig. 2 (right)]. In this study, we observed that the combined exposure of PS-MPs and TPT did not significantly alter the embryo's heart rate. This may be attributed to the primary distribution of PS-MPs in the intestines and gills of aquatic organisms, which are not directly involved in heart rate regulation (Lu et al., 2016). In contrast, PS-NPs, due to their smaller size, can more easily penetrate biological barriers, including cardiac tissues, which may result in a significant decrease in heart rate (Liu et al., 2024a). Furthermore, the co-exposure of PS-NPs and TPT exacerbated the toxicity of TPT, as NPs are more prone to adsorb additional TPT compared to MPs (Liu et al., 2024a). Another study suggested that NPs, owing to their larger surface area and potential for pollutant adsorption, may play a more critical role in the bioaccumulation and toxicity of pollutants (Peng et al., 2020). This implies that PS-NPs worsen the hazard posed by TPT compared to PS-MPs. In summary, TPT and PS-MNPs exposure groups affect embryo mortality and hatchability. Nonetheless, combined exposure to PS-MNPs + TPT led to greater toxicity compared to TPT alone, resulting in a decrease in heart rate. This could be attributed to the PS-MNPs adhering to the surface of the embryo, causing hypoxia and leading to changes in heart rate. In particular, small particle size PS-NPs may be translocated to cardiac tissues and exacerbate the effects on the heart, thus the PS-NPs + TPT exposure group resulted in a significant decrease in heart rate.

The early swimming behavior and patterns of medaka larvae reflect their motor ability and the neurodevelopmental processes associated with movement during early development. Previous studies have shown that high concentrations of TPT lead to significant behavioral abnormalities, while low concentrations do not. The exposure to TPT did not

induce significant behavioral abnormalities, which could be attributed to the low concentration levels employed in our study. In our study, the larger particle size of PS-MPs resulted in a significant increase in swimming acceleration and displacement in medaka larvae, similarly, 1 μm and 10 μm MP particles significantly enhanced the movement distance and swimming speed of cladocerans (*Daphnia magna*). The increase in swimming activities could be explained by the organisms' attempts to escape the environment contaminated by MPs, which thereby increasing the frequency of swimming (Lopes et al., 2004). However, owing to their diminutive particle size, PS-NPs might be less conspicuous, thus single exposure to PS-NPs did not markedly amplify larval behavior. Conversely, the combined exposure to PS-MNPs and TPT substantially reduced larval swimming behavior and demonstrated a more potent inhibitory effect compared to separate exposures to PS-MNPs and TPT. This finding is consistent with previous studies that demonstrated enhanced toxic effects from co-exposure to MPs and 17α-ethinylestradiol (EE2) (Chen et al., 2017). Interestingly, independent exposure to PS-NPs or TPT did not induce behavioral abnormalities in marine medaka. However, co-exposure to PS-NPs and TPT resulted in aberrant locomotor behavior, exhibiting a significantly greater effect compared to co-exposure with PS-MPs and TPT. This outcome may be attributed to the agglomeration of PS-NPs into larger entities upon binding with TPT, thereby increasing the probability of larval contact.

T4 plays a crucial role in the early brain development of fish embryos. During the embryonic and larval stages, T4 is essential for the proliferation, migration, and differentiation of neurons, thus being highly associated with the formation of the complex structure of the brain and neural networks (Tang et al., 2020). Furthermore, abnormal levels of T4 are closely associated with the occurrence and development of neurological disorders (Tang et al., 2020). For instance, hypothyroidism (an underactive thyroid) may lead to cognitive decline, depression, and other neurological disorders. Thyroid hormones are also associated with the formation and function of the heart in early fish development; many cardiac genes are downstream targets of Thyroid Releasing Hormone (TR) hormones, and cardiac function is highly related to the swimming ability of fish (Han et al., 2021; Jabbar et al., 2017). In the study, no significant pathological damage was observed in brain tissue sections of exposed marine medaka larva, but there was a decreasing trend in T4 levels (Fig. 4 [A]). This may be because the concentrations of TPT used in the experiment were not sufficient to cause tissue-level changes. Additionally, T4 might be maintained at a stable level through internal physiological regulatory mechanisms. However, compared to the control group, the expression levels of *thr-α* and *thr-β* were upregulated in marine medaka exposed to PS-MNPs and TPT, indicating that PS-MNPs and TPT may have disrupted the homeostasis of the hypothalamic-pituitary-thyroid (HPT) axis (Lee et al., 2014). Additionally, the study found that TPT might have interfered with the thyroid regulation mechanism in fish, and the presence of PS-MNPs enhanced the disruption of the thyroid regulation axis by TPT, especially in the PS-NPs + TPT exposure group, which showed a more significant upregulation of gene expression levels, possibly exacerbating the disruptive effect of TPT.

Neuroendocrine factors play an important role in fish behavior. As a cholinergic neurotransmitter, acetylcholine (ACh) is an important driving factor for animal behavior and neurodevelopment (den Hartog et al., 2002). In addition, AChE is one of the key enzymes involved in regulating the uptake of neurotransmitters in organisms and is widely used as a biomarker for neurotoxicity (Lionetto et al., 2013). The significant decrease in AChE concentration in the PS-MNPs + TPT exposure groups indicates that the combined exposure severely interferes with the synthesis and degradation processes of ACh [Fig. 5 (A)], thereby disrupting normal neural excitability. The combined exposure groups with the addition of PS-MNPs (PS-MNPs + TPT) showed a significantly lower AChE concentration compared to the TPT single exposure group [Fig. 5 (A)], indicating that PS-MNPs exacerbate the interference of TPT on the synthesis and degradation processes of AChE. EROD is an enzyme in fish cytochrome P450 monooxygenase that plays an important role in detoxification processes within organisms (Goksoyr and Forlin, 1992). EROD is a specific biomarker, and the increase in EROD concentration in the TPT single exposure group indicates that neural activity in medaka may be impaired, which in turn activates detoxification processes in vivo (Fig. 5 (A)) (Volz et al., 2008). The combined exposure groups with the addition of PS-MNPs (PS-MNPs + TPT) showed a significantly higher EROD concentration compared to the TPT single exposure group [Fig. 5 (A)], indicating that PS-MNPs exacerbate the impairment of normal physiological functions of marine medaka by TPT and stimulate detoxification.

The expression levels of neuro-related genes were detected using qRT-PCR [Fig. 5 (B)]. *gap43* is an expression marker gene for axon growth during neurodevelopment, and *elavl3* is an expression gene for a neural-specific RNA-binding protein, both of which exhibited high levels of expression in fish development and neural axon regeneration processes (Chen et al., 2012a). In this study, the increased expression of *elavl3* caused by PS-MPs suggests that PS-MPs promoted neuronal axon regeneration, which might be associated with their ability to sense PS-MPs and rapid locomotion in the environment. Thus, the up-regulation of elavl3 expression level might be an important reason for the significant increase in average acceleration and consequently the high stress response exhibited in the PS-MPs exposure group. The decreased expression of *elavl3* caused by the combined exposure of TPT and PS-MNPs indicates that the combined effect inhibits the development of nerve axons and damages the nerve regeneration ability of medaka, which may have an irreversible impact on nerve excitability. *α-tubulin* is an intermediate filament protein that played a crucial role in maintaining the structural integrity of nerve cells (Fan et al., 2010). The notable reduction in α-tubulin expression levels observed in the PS-MNP single exposure groups indicates the high sensitivity of the cytoskeletal system to PS-MNPs. In the combined exposure group where PS-MNPs were added (PS-MNPs + TPT), *α-tubulin* gene expression levels were significantly lower compared to the TPT single exposure group, implying that PS-MNPs exacerbate TPT's interference with cytoskeleton formation. The cytoskeleton played a role in processes such as axonal branching, dendritic spines, and axon formation. Therefore, changes in the cytoskeleton could have profound effects on the brain function of medaka (Fan et al., 2010). *gfap* is an intermediate filament protein considered as a marker for astrocytes and is highly expressed in radial glia and astrocytes of the central nervous system (Fan et al., 2010). TPT and PS-MNPs decreased the gene expression levels of the *gfap* gene, indicating the damage to astrocytes. Meanwhile, PS-MNPs enhanced the damage effect of TPT on astrocytes. *mbpa* is a biomarker for myelination and played an important role in axon myelination during central nervous system development in fish (Fan et al., 2010). In this study, the significant decrease in *mbpa* gene expression levels in medaka indicated that TPT caused damage to axonal myelination. In the PS-MNPs + TPT co-exposure groups, the addition of PS-MNPs further enhanced the damage to axonal myelination (Chen et al., 2012b), which affected information communication between neural cells (Lai et al., 2015) and subsequently influenced behavior. The *gst* gene is associated with pollutant-mediated neurotoxicity (Gasmi et al., 2022). The upregulation of *gst* expression levels led to an increase in neurotoxicity. In summary, molecular evidence suggested that the combined exposure to PS-MNPs and TPT markedly amplified the neurotoxic effects observed with individual exposures to either PS-MNPs or TPT.

ATP is the energy currency of organisms, and the activity of ATPase and the expression level of related genes can reflect the metabolic capacity and energy supply intensity of organisms. Generally speaking, the energy metabolism of organisms is closely connected to various life activities (Goksoyr and Forlin, 1992). For example, research has shown a correlation between energy metabolism and brain activity in fruit flies (Mattila and Hietakangas, 2017). However, no significant differences were found in energy metabolism activities in this study [Fig. 5 (C) (D)]. This might suggest that TPT and PS-MNPs do not affect the brain activity

of medaka through energy metabolism activities.

Behavioral changes are external manifestations of intrinsic damage to the animals' nervous system. In a study on the neurotoxicity of moxifloxacin on zebrafish larvae, zebrafish swimming behavior was used as a biological indicator of neurotoxicity (Cheng et al., 2020). This study investigated the swimming behavior of medaka and demonstrated that TPT and PS-MNPs had neurotoxic effects on medaka, affecting the swimming behavior of larvae. Specifically, PS-MPs exert a significant impact on the swimming behavior of larvae. The combined exposure to PS-MNPs and TPT intensified neurotoxic effects. The PS-NPs + TPT group demonstrated greater toxicity in long-term physiological parameters (such as duration of activity, total distance traveled, gene expression levels, enzyme activity, etc.) compared to other groups. This may be attributed to the smaller nanoparticles causing less significant damage to organisms in vitro, but ultimately causing greater harm when entering the body. Behavioral effects could be used to indicate relatively mild environmental pollution and served as sensitive indicators for detecting toxic and harmful environmental chemical contaminants (Zhou et al., 2021). In addition, TPT increased the expression of the control genes *thr-α* and *thr-β*, which regulated the release of thyroid-stimulating hormones, but the concentration of thyroid hormone T4 did not show significant changes. This might be due to the organism's internal regulatory mechanisms maintaining normal T4 concentrations. While this study provides data and insights on the toxicity of PS-MNPs and TPT on marine medaka in terms of behavior, neurotransmitters, hormones, and genes, there are still certain limitations. The study mainly considered the stress conditions during the early developmental stages of marine medaka without covering adult marine medaka. The lack of coverage may introduce uncertainty regarding the extrapolation of results and potentially restrict the value of the study. However, conducting research on the full life cycle exposure and intergenerational effects on marine medaka is an important part of our future work.

5. Conclusion

This study comprehensively explored the combined neurotoxic effects of TPT and PS-MNPs on the initial stages of marine medaka development from the aspects of comprehensive exposure, behavioral analysis, biochemical experiments, and gene expression analysis. The findings revealed the distinct effects of TPT and PS-MNPs on the swimming behavior of medaka. Notably, TPT and PS-NPs did not cause obvious behavioral anomalies. However, in the PS-MPs exposure group, acute stress responses were observed with a significant increase in acceleration, indicating heightened short-term neural excitability in marine medaka exposed individually to PS-MPs. Co-exposure to PS-MNPs + TPT resulted in neurotoxic damage, characterized by pronounced deviations from normal levels in biochemical markers and gene expression, ultimately affecting the locomotor capabilities of marine medaka. Additionally, the study demonstrated that the damage caused by PS-NPs in the presence of TPT was greater than that caused by PS-MPs, possibly related to the particle size of the PS-MNPs. These discoveries lay a robust theoretical groundwork for further examining the cumulative toxicity and mechanisms of PS-MNPs and OTs in aquatic organisms within their native environments.

CRediT authorship contribution statement

Peiran Lin: Writing – original draft, Investigation. **Ling Liu:** Supervision, Funding acquisition. **Yuqing Ma:** Validation, Data curation. **Renyan Du:** Writing – original draft, Investigation. **Chuansen Yi:** Methodology. **Ping Li:** Supervision. **Yanan Xu:** Software, Investigation. **Haiyang Yin:** Validation, Software. **Le Sun:** Validation, Software. **Zhi-Hua Li:** Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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