

Article

The Effects of Single and Combined Exposure to Polystyrene Nanoplastics and Copper on the Behavior of Adult Zebrafish

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Abstract: Different pollutants often coexist in natural environments, making it crucial to monitor and study the ecotoxicological effects of composite pollutants in aquatic environments. Nanoplastics and heavy metals are emerging environmental pollutants that can affect the health of aquatic organisms and threaten human health via the food chain. In this study, zebrafish was employed as a model organism to explore the effects of short-term exposure to polystyrene nanoplastics (PS-NPs) and heavy metal copper ions (Cu^{2+}) either individually or in combination on fish behavior. First, the single and combined toxicity of Cu^{2+} and PS-NPs to adult zebrafish was investigated to obtain the LC_{50} values of the two pollutants at 24, 48, 72, and 96 h. Then, the effects of sub-lethal concentrations of Cu^{2+} (0.06, 0.15, and 0.3 mg/L), PS-NPs (5, 10, and 15 mg/L) and binary mixtures containing Cu^{2+} and PS-NPs (0.06 mg/L + 10 mg/L, 0.15 mg/L + 10 mg/L, and 0.3 mg/L + 10 mg/L) on the swim speed and individual distance of zebrafish within 4 h were studied. The results show that the LC_{50} value for single exposure of zebrafish to Cu^{2+} decreased with the increase in the exposure time, while PS-NPs showed no significant acute toxicity to zebrafish when the concentration was less than 20 mg/L and the exposure time was less than 96 h. The combined exposure of zebrafish to Cu^{2+} and PS-NPs resulted in a 3.1–32.2% reduction in the LC_{50} value at different time points compared with Cu^{2+} alone. In the behavioral study, both single and combined exposure to Cu^{2+} and PS-NPs induced hyperactivity and aggregation phenomena in the zebrafish at different levels; the duration of these two phenomena was correlated with the concentration of the pollutants. The combined exposure to Cu^{2+} and PS-NPs exacerbated the behavioral changes in zebrafish compared with exposure to Cu^{2+} alone, reducing their hyperactivity time, average swim speed and aggregation time by 30.7–41.0%, 13.6–15.4%, and 28.3–28.8%, respectively. Therefore, this study indicates that the combined short-term exposure to PS-NPs and Cu^{2+} can exacerbate the toxicity of pollutants, and also proves the feasibility for early warning of combined NPs and heavy metals pollution based on adult zebrafish behavioral indicators.

Keywords: zebrafish; heavy metals; nanoplastics; combined toxicity; swimming behavior



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1. Introduction

Pollutants in water usually appear in the form of complex mixtures. The pollutants interact with each other, resulting in different toxic effects, such as synergistic or antagonistic effects [1]. Copper is an essential trace micronutrient that can promote the metabolism and improve the immunity of organisms. However, an overdose of copper has several negative impacts on the health of humans and animals. There are numerous reports worldwide regarding Cu^{2+} pollution in aquatic environments, with Cu^{2+} concentrations ranging from

0.002 to 133 mg/L [2–4], potentially originating from copper mining, agriculture, and manufacturing activities [5]. Recent studies indicated that aquatic organisms exhibit a sensitivity to Cu that is 10 to 100 times greater than that of mammals. This could be because the liver and kidneys of mammals possess more sophisticated detoxification systems compared with aquatic organisms [2]. For example, in Nile tilapia, after 21 days of exposure to 0.5–2.5 mg/L of water-soluble copper, histopathological examination revealed that the gills of fish presented edema, the growth of the lamellar epithelium and the strong relaxation of the lamellar vascular axis, in addition to vacuoles and necrosis in the liver [6]. The locomotor activity and angular orientation of movements of *Arius felis* showed different changes when exposed to 0–0.2 mg/L Cu^{2+} for 72 h [7]. After the chemical perception of silver salmon was destroyed under Cu^{2+} exposure (5–20 $\mu\text{g/L}$), the probability of predation increased [8]. Ma et al. observed a decrease in the swimming and respiration activity of zebrafish exposed to CuSO_4 concentrations of 0.1 mg/L and 1.58 mg/L for 10 days. Therefore, swimming, consistency, and respiratory rate were regarded as effective non-invasive toxicity biomarkers for water quality monitoring [9].

With the widespread use of plastic products, it is estimated that the total global plastic production will reach 34 billion tons by 2050 [10]. Nano- and microplastics (NMPs) pollution has attracted extensive attention from scientists and the public [11]. NMPs refer to plastics with a size of less than 5 mm, which originate from commercial plastics at the microscale size, or are formed via the physical abrasion, weathering, and UV radiation of larger plastics to small pieces in the marine environment [12]. Common sources of NMPs include polystyrene (PS), polyethylene, polypropylene, polyvinyl chloride, polyurethane and polyethylene terephthalate, among which PS is one of the most widespread forms of plastic in aquatic environments [13,14], and has adverse effects on the growth, development and behavior of aquatic organisms [15–17]. According to previous reports, the detected PS concentrations in aquatic environments worldwide ranged from 0.02 to 10^6 items/ m^3 [18,19]. Currently, studies on the toxicity of NMPs to aquatic organisms have primarily focused on intestinal damage, liver inflammation and lipid accumulation, and reduced swimming ability induced by long-term exposure [19–21]. No biochemical or behavioral toxicity has been observed in aquatic organisms under short-term exposure. In addition, due to their large surface area and hydrophobicity, NMPs can act as carriers of other pollutants, including heavy metals and organic pollutants [22], thus influencing the bioavailability of pollutants and aggravating their toxicity to organisms [23]. Chen et al. reported that a 19-day combined exposure to nanoplastics (NPs) and Cd led to poor growth, liver oxidative stress, and gut microbiota imbalance in largemouth bass. Furthermore, the toxicity of combined NPs and Cd exposure was higher than that of exposure to either NPs or Cd alone [24]. Lu et al. reported that polystyrene microplastics increased the accumulation of cadmium in zebrafish livers, guts and gills, and thus caused oxidative damage and inflammation in adult zebrafish [25]. Based on biochemical and genetic analyses, Santos et al. found that the combination of microplastics and copper could promote neurotoxicity and oxidative stress damage in zebrafish larvae and embryos [26].

Sensory function and behavior are crucial for fish to detect water movement while swimming, avoiding predators, locating prey and spawning grounds [27,28]. They serve as the most sensitive indicators of pollutant toxicity to aquatic organisms, compared with the endpoints of reactive oxygen species formation, endocrine disruption and acute toxicity [29,30]. Water quality monitoring studies based on behavioral responses in zebrafish (*Danio rerio*) have attracted extensive attention due to the good dose-effect relationship between exposure level and abnormal behavioral characteristics and low operating cost [9,31]. However, the current studies primarily focus on the impact of combined exposure to NMPs

and heavy metals on zebrafish biochemical indicators, with few studies researching the effects of combined exposure on zebrafish behavior.

Therefore, in this study, adult zebrafish were employed to explore the effects of single and combined toxicity of a heavy metal (Cu^{2+}) and polystyrene nanoplastics (PS-NPs) on their behavior. First, the acute toxicity of the two pollutants was evaluated by determining the median lethal concentration (LC_{50}) values of Cu^{2+} and PS-NPs under single and combined exposure at 24, 48, 72 and 96 h. Then, under sub-lethal concentrations of the two pollutants, the swimming activity and social behavior of the zebrafish were characterized according to the swim speed and the individual distance, respectively, to evaluate the effects of Cu^{2+} and PS-NPs on the behaviors of the zebrafish under single and combined exposure.

2. Materials and Methods

2.1. Zebrafish Maintenance and Reagents

Adult AB wild-type zebrafish (*Danio rerio*), weighing approximately 0.3 g and 3–4 cm in length, were purchased from the zebrafish breeding center of Wuhan Institute of Aquatic Sciences, the Chinese Academy of Sciences, and adapted to the environmental conditions for two weeks. The fish culture water consisted of deionized water and 0.2% sea salt to keep the conductivity at approximately 400 $\mu\text{S}/\text{cm}$, under the following conditions: 26 ± 1 °C, pH 6.8–7.5, and a 14 h light/10 h dark photoperiod. The zebrafish were fed twice a day with commercial freeze-dried artemia. The feeding was stopped 24 h before the experiment. The Animal Care and Use Committee of Zhejiang University of Science and Technology approved all the experimental procedures in this study (approval ID: ZKJ2021311).

PS-NPs microspheres were purchased from Wuxi Ruige Biotechnology Co., Ltd., Wuxi, China. The particle size of the microspheres was 50 nm, and the coefficient of variation was <6%. The dispersant was deionized water, and the concentration was 25 g/L. Anhydrous copper sulfate (analytically pure) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China. A stock solution containing 1 g/L of Cu^{2+} was prepared using deionized water.

2.2. Experimental Method

2.2.1. Acute Toxicity Test of Single and Combined Treatments with PS-NPs and Cu^{2+}

Several 5 L glass beakers were prepared, and 4 L of the test solution was added to each beaker. The zebrafish culture water (deionized water with 0.2% sea salt) was studied as the control group, and the solutions containing different concentrations of PS-NPs and Cu^{2+} were studied as the treatment groups. The single exposure concentrations of Cu^{2+} were 0.1, 0.25, 0.35, 0.5, and 1 mg/L, and those of PS-NPs were 1, 5, 10, 15, and 20 mg/L. The combined exposure concentrations of Cu^{2+} and PS-NPs were 0.1 mg/L + 10 mg/L, 0.25 mg/L + 10 mg/L, 0.35 mg/L + 10 mg/L, 0.5 mg/L + 10 mg/L, and 1 mg/L + 10 mg/L. The pH of all experimental water was maintained at 7.2–7.3. In total, 10 zebrafish (male to female ratio of 1:1) were randomly placed in each beaker and set in three parallel rows of beakers for each treatment (control, single, and combined exposure). The exposure solutions were exchanged for fresh solutions daily. There were 16 beakers in total: 5 concentrations for the single and combined treatments each, and the control, replicated 3 times. All the experimental beakers were placed into an incubator under the conditions of 26 ± 1 °C and a 14 h light/10 h dark photoperiod. The number of dead fish was counted every day, and they were removed immediately from the beakers. The LC_{50} values of Cu^{2+} and PS-NPs in the single and combined exposure treatments were calculated by observing and recording the number of zebrafish deaths at 24, 48, 72, and 96 h.

2.2.2. Behavioral Analysis of Zebrafish Exposed to Single and Combined Treatments with PS-NPs and Cu^{2+}

The behavioral monitoring of the zebrafish was carried out in a round glass tank with a diameter of 30 cm and a height of 20 cm. In total, 6 adult zebrafish were randomly placed in each tank at a male-to-female ratio of 1:1 (three replicates were established for each group). The water conditions were maintained at 26 ± 1 °C, pH 6.8–7.5, and a water flow rate of 1 L/min. LED lights with a light intensity of 200–300 lux were equipped at the bottom of the fish tank. A camera (HUIBOS 2K X20, Huibos Technology Co., Ltd, Shenzhen, China) was installed directly above the fish tank to record the behavior of the zebrafish throughout the experiment, with a resolution of 1080×720 and 25 fps. The whole instrument was covered by a mini photo studio box, which not only reduces the interference of external light and noise on the behavior of zebrafish, but also maximizes the diffusive light and minimizes the reflections to ensure that the camera can capture clear images of the fish. The recorded videos were then analyzed using a behavior tracking software, idtracker.ai (Version 5.2.12) [32], which calculated the behavior parameters of zebrafish through recording the two-dimensional position coordinates of zebrafish with respect to each minute. The video recording device for zebrafish locomotion tracking is shown in Figure 1a.

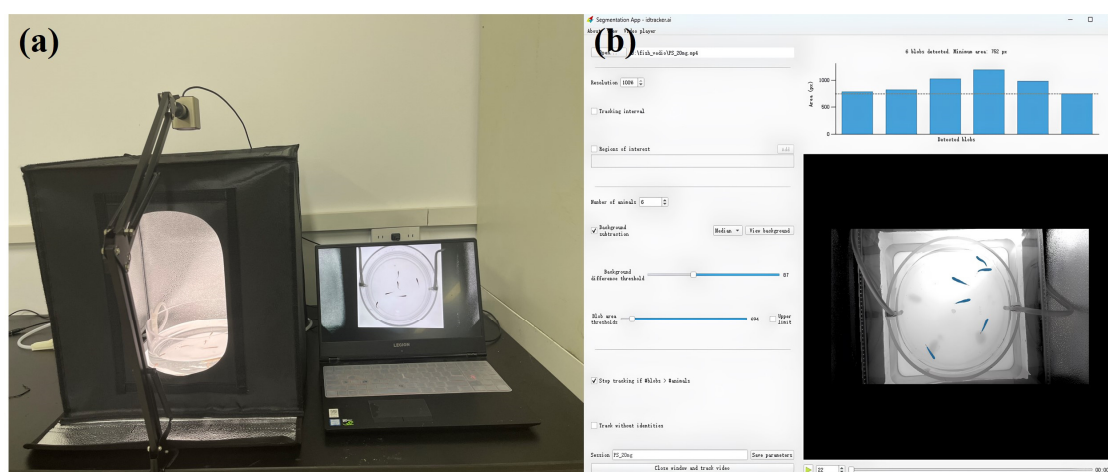


Figure 1. (a) Video recording device for fish locomotion tracking; (b) the user interface of idTracker when analyzing the movement of six fish in a fish tank.

The total duration of zebrafish movement behavior monitoring was 240 min. The first 60 min was under normal water quality conditions. At the 60th min, the pollutants were injected into the water pump pipe and allowed to flow into the fish tank with water, so the behavioral changes in zebrafish induced by pollutants could be compared before and after 60 min. In this experiment, the swim speed was used to characterize the individual swim activity of fish in each group, and the average individual distance was used to characterize the social behavior of fish in each group. The swim speed and individual distance results were calculated based on the average body length (bl) of six zebrafish in the corresponding fish tank, estimated using the monitoring software. The time taken from the onset of exposure until the fish's swimming speed recovered to its pre-exposure level was calculated as the hyperactivity time. The percentage change in the average swim speed of zebrafish during the last 30 min of experimental monitoring (from 210 to 240 min) compared with the average swim speed during the pre-exposure period (from 0 to 60 min) was calculated as the change in average swim speed. The time taken from the onset of exposure until the fish's individual distance recovered to its pre-exposure level was

calculated as the aggregation time. The user interface of idTracker showing the tracking of six fish is shown in Figure 1b.

2.3. Statistical Analysis

The experimental data were calculated and plotted using origin2021. The LC_{50} at different time points was calculated with the SPSS version 27 software. The differences between each group were evaluated via one-way analysis of variance (ANOVA) followed by Dunnett's t -test, with $p < 0.05$ indicating significant differences.

3. Results and Discussion

3.1. Acute Toxicity Effects of Cu^{2+} and PS-NPs on Zebrafish Under Single and Combined Exposure

As shown in Figure 2, the LC_{50} values of Cu^{2+} at 24, 48, 72 and 96 h were 0.601, 0.421, 0.305 and 0.293 mg/L, respectively. However, even when the concentration of PS-NPs reached 20 mg/L, no significant toxicity to zebrafish was detected at 96 h. Therefore, the experimental data are not presented here. Under the combined exposure of Cu^{2+} and PS-NPs, the LC_{50} values of zebrafish at 24, 48, 72 and 96 h were 0.407, 0.352, 0.292 and 0.284 mg/L, respectively. Both the LC_{50} values of Cu^{2+} alone and Cu^{2+} combined with PS-NPs decreased with the increase in the exposure time. In addition, compared with the single exposure to Cu^{2+} , the LC_{50} values of Cu^{2+} combined with PS-NPs at each time point were reduced by 32.2%, 16.4%, 4.3% and 3.1%, respectively, and there were significant differences at 24 h and 48 h.

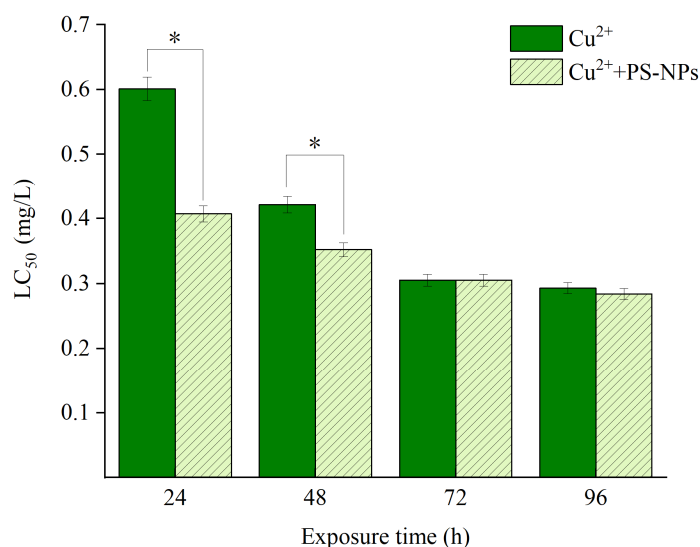


Figure 2. Acute toxicity of Cu^{2+} and PS-NPs, alone or combined toward zebrafish at 24, 48, 72 and 96 h. Data are expressed as mean $\pm$ S.D. * represents significant differences between two groups.

The results revealed that the toxicity of Cu^{2+} may have cumulative effects on zebrafish; thus, the LC_{50} value decreased with an increase in the exposure time. The phenomena of cheek hyperemia and redness were observed in all dead fish, suggesting that fish gills may be a target attacked by Cu, as Cu may cause sodium ion loss by inhibiting Na^+/K^+ -ATPase, leading to cardiovascular failure and respiratory dysfunction [27,33]. PS-NPs did not show significant acute toxicity at concentrations lower than 20 mg/L and exposure time of less than 96 h, which was consistent with previous reports indicating that NMPs usually do not show direct lethal effects on organisms in a short time of exposure [20]. Meanwhile, the results of the acute toxicity test under combined exposure showed that the addition of NPs significantly reduced the LC_{50} values at 24 h and 48 h, compared with the LC_{50} of Cu^{2+} exposure alone. This phenomenon may be due to the adsorption of more Cu^{2+} onto the

surface of the PS-NPs particles, thus increasing the bioavailability of Cu^{2+} to the zebrafish and aggravating the toxicity of Cu^{2+} [1,34].

3.2. Behavioral Effects of Cu^{2+} and PS-NPs on Zebrafish Under Single and Combined Exposure

3.2.1. Effects of Cu^{2+} and PS-NPs Exposure on Swim Activity of Zebrafish

Swimming activity is one of the most commonly measured behavioral responses in toxicological studies [35], since active swimming ability plays a crucial role in predation, mating and defense throughout the life of fish and reflects the status of internal biochemical homeostasis [36,37].

Based on the results of the acute toxicity test, the 72 h LC_{50} of Cu^{2+} was chosen as a toxic unit (1 TU); thus, concentrations of 0.2 TU, 0.5 TU and 1 TU (0.06, 0.15, and 0.3 mg/L) were employed to study the behavioral responses of zebrafish under single exposure to Cu^{2+} . The concentrations of 5, 10, and 15 mg/L were employed to study the behavioral responses of zebrafish under single exposure to PS-NPs. In the combined exposure experiment, a 10 mg/L PS-NPs solution was mixed with 0.06, 0.15, or 0.3 of Cu^{2+} to study the effect of the combined exposure on the behavioral responses of zebrafish.

The temperature and pH value were maintained at 26 ± 1 °C and 7.2 ± 1 throughout the entire experiment, respectively, both before and after exposure to the pollutants. As shown in Figure 3, the swim speed of zebrafish changed before and after exposure to different concentrations of Cu^{2+} . In the first 60 min, when the zebrafish were in the culture water without pollutant, their swim speed fluctuated within a small range, which was relatively stable. From 60 min, zebrafish exposed to different concentrations of Cu^{2+} quickly perceived the changes in the water environment in a short time and showed a hyperactive state; that is, a sharp increase in their swim speed. However, the change trend for the swim speed varied among different groups. Under exposure to a concentration of 0.06 mg/L of Cu^{2+} , the zebrafish exhibited hyperactive behavior from 60 min to 200 min, lasting for approximately 140 min. At the late stage of exposure, the swim speed of zebrafish returned to its original level. Under exposure to a concentration of 0.15 mg/L of Cu^{2+} , the hyperactive time of zebrafish lasted for 75 min, and the swim speed gradually decreased from 135 min to 240 min. Under exposure to a concentration of 0.3 mg/L of Cu^{2+} , the duration of behavioral hyperactivity of zebrafish was 61 min, from 122 min to 240 min, and the swim speed of zebrafish decreased at the late stage of exposure, finally reaching a level lower than the original before exposure. According to a previous report, metal ions might affect the swimming behavior of fish by increasing the plasma ammonia concentration and reducing the levels of Na^+ , K^+ , and Ca^{2+} in plasma. These ions are involved in metabolism and physiological activities, such as the central and peripheral nervous system, muscle contraction, and neuromuscular transmission connections [30].

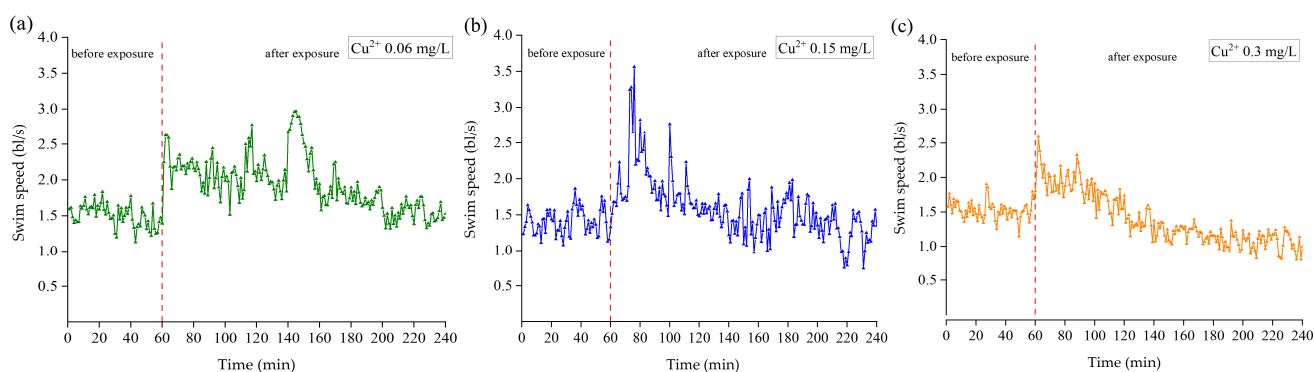


Figure 3. Swim speed of zebrafish during 240 min locomotion tracking before and after exposure to (a) 0.06 mg/L Cu^{2+} ; (b) 0.15 mg/L Cu^{2+} ; (c) 0.3 mg/L Cu^{2+} .

As shown in Figure 4, the swim speed of zebrafish changed before and after exposure to different concentrations of PS-NPs. After exposure to different concentrations of PS-NPs from 60 min, fish in all groups presented the process of hyperactivity first, and then returned to the original level. The hyperactive duration of zebrafish exposed to 5, 10, and 15 mg/L of PS-NPs was 10, 20, and 40 min, respectively, all lower than that of zebrafish exposed to 0.06 mg/L of Cu^{2+} (the lowest concentration). Additionally, the hyperactive time of zebrafish was prolonged with an increase in PS-NPs exposure concentration, which indicates that zebrafish may undergo adaptive changes in behavior in response to environmental pollutants. Under low concentrations of exposure, a small amount of PS-NPs is ingested through the mouth and gills and enters the fish, such that the zebrafish can quickly adjust and return to a normal state in a short time; meanwhile, under high concentrations of exposure, the zebrafish take a longer time to adapt to this change in water environment. Chen et al. found that, the stimulation of particles and the increase in estrogen content, rather than oxidative stress, after exposure to PS microplastics (5 μm) for 7 d led to a prolonged period of hyperactivity in zebrafish [38]. Limonta et al. reported that exposure to polyethylene and polystyrene microplastics for 20 days at 100 and 1000 $\mu\text{g/L}$ increased the nocturnal activity of zebrafish in a concentration-dependent manner [39]. These results were consistent with the behavior of zebrafish exposed to PS-NPs in this study. However, under the experimental conditions, PS-NPs did not cause significant physiological damage to zebrafish, such that the swim speed of zebrafish in each experimental group returned to its original level.

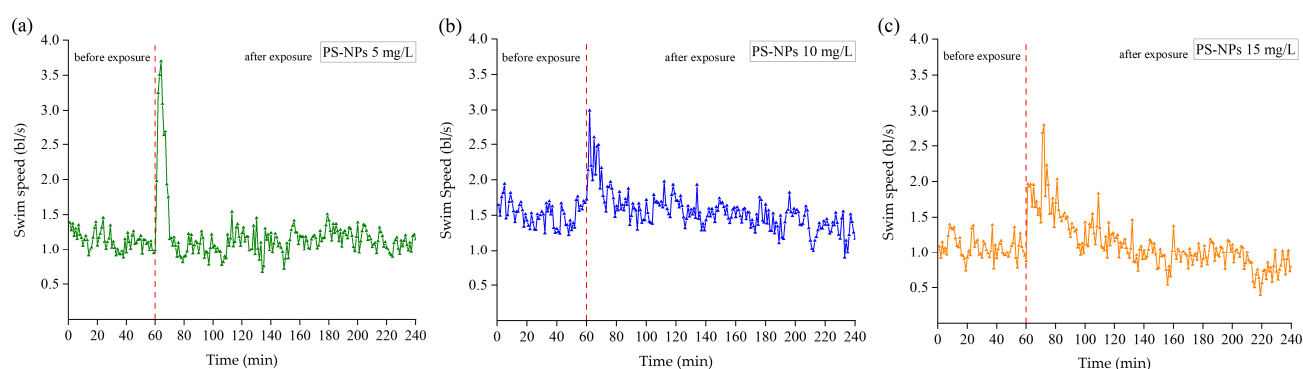


Figure 4. Swim speed of zebrafish during 240 min locomotion tracking before and after exposure to (a) 5 mg/L PS-NPs; (b) 10 mg/L PS-NPs; (c) 15 mg/L PS-NPs.

As shown in Figure 5, the swim speed of zebrafish changed before and after exposure to different Cu^{2+} and PS-NPs mixture concentrations. The change trend in the swim speed under the combined exposure was similar to that with the single exposure of Cu^{2+} . The hyperactive time of zebrafish under the combined exposure at low, medium and high concentrations of the pollutants were 143, 52, and 36 min, respectively, slightly shorter than those under the exposure to Cu^{2+} alone. Additionally, the swim speed in the combined exposure group with medium and high concentrations of Cu^{2+} and PS-NPs did not return to its original level at the end of the exposure.

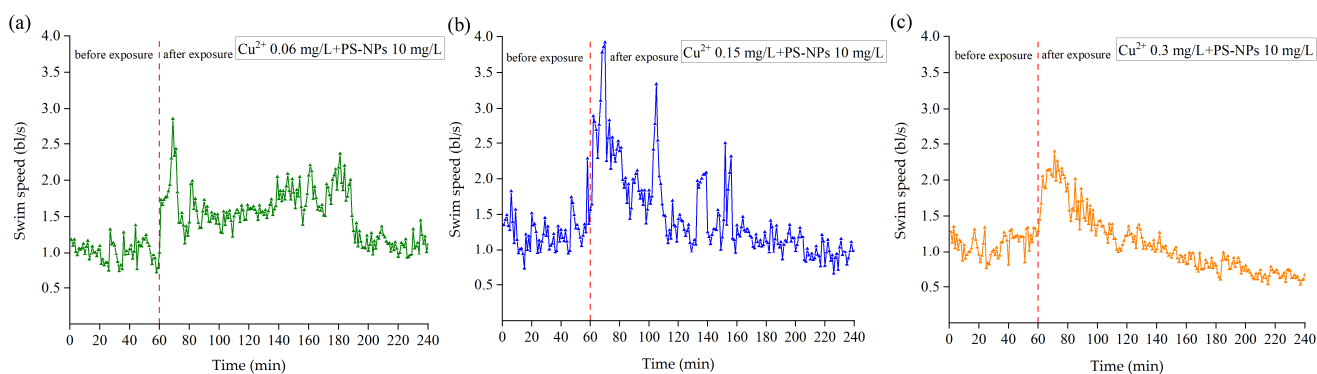


Figure 5. Swim speed of zebrafish during 240 min locomotion tracking before and after exposure to the combination of (a) 0.06 mg/L Cu^{2+} + 10 mg/L PS-NPs; (b) 0.15 mg/L Cu^{2+} + 10 mg/L PS-NPs; (c) 0.3 mg/L Cu^{2+} + 10 mg/L PS-NPs.

Figure 6a compares the hyperactive time among the experimental groups. Under the single exposure to different concentrations of Cu^{2+} , the hyperactive time gradually decreased with increasing concentration. Under the single exposure to different concentrations of PS-NPs, the hyperactive time gradually increased with increasing concentration. Under the combined exposure of Cu^{2+} and PS-NPs, the hyperactive time gradually decreased with increasing concentration. In addition, compared with exposure to Cu^{2+} alone, the combined exposure of Cu^{2+} and PS-NPs at medium-high concentrations reduced the hyperactive time of zebrafish by 30.7% and 41.0%, respectively. The changes in average swim speed of zebrafish before and after exposure in different exposure groups are shown in Figure 6b. The average swim speed of zebrafish in both groups exposed to Cu^{2+} alone (0.15 mg/L and 0.3 mg/L) and a combination of Cu^{2+} and PS-NPs (0.15 mg/L + 10 mg/L and 0.3 mg/L + 10 mg/L) decreased after exposure. The average swim speed in the combination exposure group decreased by 13.6% and 15.4% compared with the Cu^{2+} alone group, respectively. These results indicate that the addition of PS-NPs increased the toxicity of Cu^{2+} at medium and high concentrations of exposure, resulting in a synergistic effect of toxicity, similar to the results of acute toxicity. In this experiment, the behavioral changes in zebrafish exposed to different concentrations of pollutants conformed to the stepwise response model, including the no effect, stimulation, regulatory recovery and toxic effect stages [40]. Zebrafish can gradually adapt to changes in the water environment via self-regulation within a certain period of time in a low toxicity environment, returning to normal behavior. With an increase in environmental toxicity, zebrafish undergo significant injury and gradually lose their ability to recover to normal levels of movement. It is speculated that the toxicity of Cu^{2+} to zebrafish causes damage to its cognitive and behavioral functions with increasing exposure time. The higher the concentration of Cu^{2+} , the more serious the damage to fish, leading to a shorter hyperactive time and lower swim speed. The toxicity to the central nervous system may be the cause of abnormal fish behavior (swimming) [41]. Metals can accumulate in brain tissue, leading to abnormal behavior (reduced swimming activity) and cell damage. Unbalanced copper homeostasis exerts neurotoxic effects on organisms [42]. Therefore, the decrease in swimming intensity of zebrafish may be attributed to the neurotoxic effect of CuSO_4 (failure of copper homeostasis) [9]. NMPs were reported to act as carriers of heavy metals [22], thus influencing the bioavailability of pollutants and aggravating their toxicity to organisms [23]. Fu et al. used a pseudo second-order kinetic model and the Elovich model to describe the adsorption kinetics of PS-NPs for Cu^{2+} , and reported that the adsorption rate was the fastest in the first 180 min [43]. Therefore, during the observation time of this study, PS-NPs could complete the saturated adsorption of trace Cu^{2+} and affect the behavior of zebrafish.

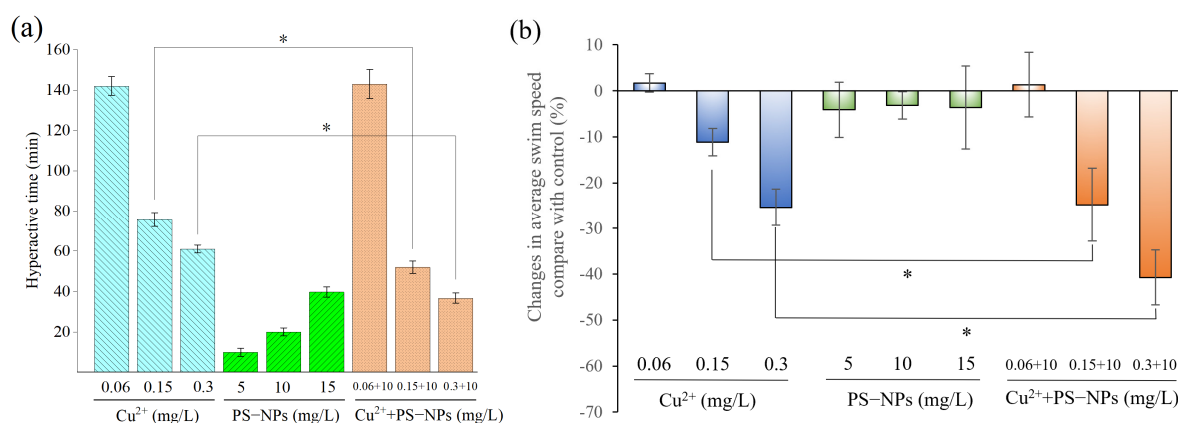


Figure 6. Comparison of (a) hyperactive time and (b) changes in average swim speed of zebrafish in different exposure groups. Data are expressed as mean $\pm$ S.D. * represents significant differences between two groups.

3.2.2. Effects of Cu^{2+} and PS-NPs Exposure on Social Behavior of Zebrafish

Figures 7–9 show the changes in the average individual distance of zebrafish before and after exposure to different concentrations of pollutants. In each group, the individual distance of fish in the first 60 min remained within a stable range under culture water conditions. After the addition of Cu^{2+} and PS-NPs at 60 min, the individual distance of fish showed a significant decrease within a short time; that is, the fish school tended to gather together, similar to Lu et al.'s results [44]. However, the change trend of individual distance in zebrafish differed from that in the swim speed in this experiment. With increasing exposure time, the individual distance of zebrafish in each group gradually increased and then returned to the level before exposure. This may be due to the long-term aggregation of fish leading to increased pressure on limited resources such as oxygen; therefore, the fish will disperse again even if their physiological state is damaged [45].

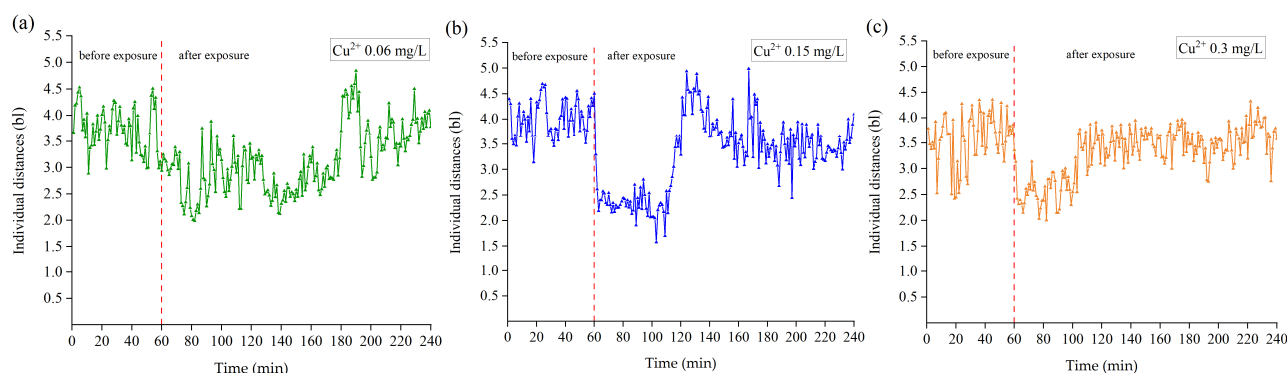


Figure 7. Individual distance of zebrafish during 240 min locomotion tracking before and after exposure to (a) 0.06 mg/L Cu^{2+} ; (b) 0.15 mg/L Cu^{2+} ; (c) 0.3 mg/L Cu^{2+} .

Figure 10 presents the aggregation time of zebrafish under different concentrations of Cu^{2+} and PS-NPs, single or combined. When exposed to 0.06, 0.15 and 0.3 mg/L Cu^{2+} alone, the aggregation times of zebrafish were 130, 59, and 46 min, respectively. When exposed to 5, 10, and 15 mg/L of PS-NPs alone, the aggregation times of zebrafish were 28, 41, and 62 min, respectively. Under the combined exposure to different concentrations of Cu^{2+} and PS-NPs, the aggregation times of zebrafish were 127, 42, and 33 min, respectively. The change trends in the aggregation time for zebrafish in each group were similar to those for the hyperactive time. The aggregation time gradually decreased with increasing concentration under the single exposure of Cu^{2+} . The aggregation time increased with increasing concentration

under single exposure to PS-NPs. The aggregation time gradually decreased with increasing concentration under combined exposure. Compared with exposure to Cu^{2+} alone, the combined exposure of Cu^{2+} and PS-NPs at medium-high concentrations reduced the aggregation time of zebrafish by 28.8% and 28.3%, respectively. These results further verify that PS-NPs aggravated the toxicity of copper ions at medium–high concentrations, consistent with the conclusion of the zebrafish swim activity experiment.

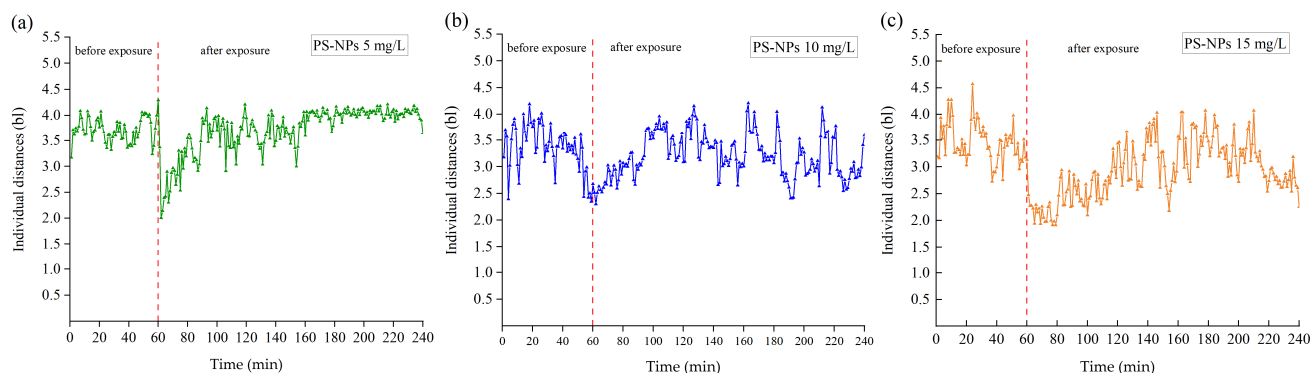


Figure 8. Individual distance of zebrafish during 240 min locomotion tracking before and after exposure to (a) 5 mg/L PS-NPs; (b) 10 mg/L PS-NPs; (c) 15 mg/L PS-NPs.

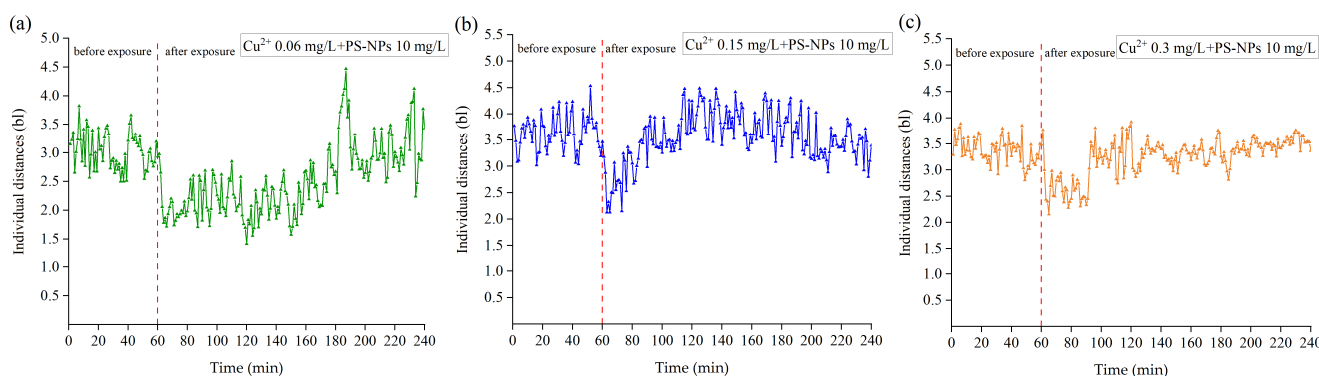


Figure 9. Individual distance of zebrafish during 240 min locomotion tracking before and after exposure to the combination of (a) 0.06 mg/L Cu^{2+} + 10 mg/L PS-NPs; (b) 0.15 mg/L Cu^{2+} + 10 mg/L PS-NPs; (c) 0.3 mg/L Cu^{2+} + 10 mg/L PS-NPs.

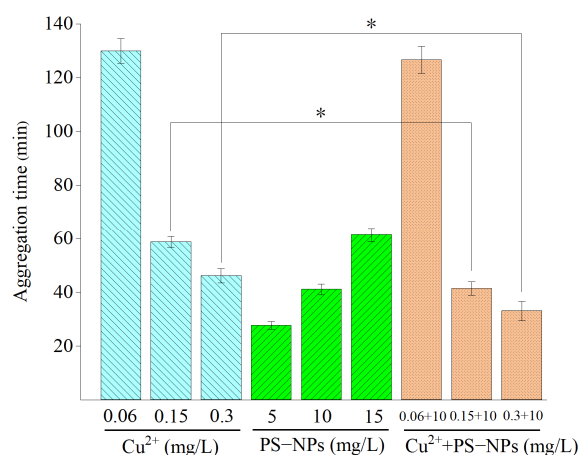


Figure 10. Comparison of aggregation duration of zebrafish in different exposure groups. Data are expressed as mean $\pm$ S.D. * represents significant differences between two groups.

The aggregation phenomenon may be because fish schools can reduce the energy consumed by individuals swimming during aggregation, thereby allocating more energy to resisting the invasion of pollutants in their bodies. Marras proved that the energy cost of swimming was reduced when schools of fish gathered and swam together in the water flow, compared with individual swimming alone [46]. However, in this experiment, although the zebrafish exhibited a temporary aggregation response to pollutants exposure, the individual distance among zebrafish in each group gradually reverted to its pre-exposure state. This indicates that under these experimental conditions (exposure concentration and time), the impact of the composite pollutants on social behavior of zebra fish is reversible. In future research, further studies are needed to explore how composite pollutants affect zebrafish's feeding, circadian rhythm, mating, and avoidance behavior through their impact on the swim speed and individual distance of fish. On the other hand, the adsorption of Cu^{2+} by PS-NPs and the distribution of complex compounds within fish will be further clarified, along with studying the correlation with changes in fish biochemical indicators, to investigate the mechanism of toxicity of these composite pollutants in fish.

4. Conclusions

This study indicated that Cu^{2+} and PS-NPs exhibited synergistic toxicity in zebrafish. The addition of PS-NPs increased the acute toxicity of Cu^{2+} and reduced the hyperactivity and aggregation time of zebrafish. Especially at medium-high concentrations, the swim speed of zebrafish was significantly affected. This could not be recovered via self-adjustment to the original level after exposure to the combined pollutants. Meanwhile, this study also revealed that behavioral indicators of zebrafish are sensitive to the toxicity of single and combined pollutants in water environments, presenting the characteristics of a rapid response to the toxicity of pollutants. These findings prove the feasibility of using zebrafish behavioral indicators for the detection and early warning of mixed pollutants in water and provide basic data for further studies on the effects of the combined toxicity of NPs and heavy metal ions on the behavior of zebrafish.

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