RESEARCH ARTICLE

The investigation of the behavioral toxicity of biodegradable microplastic polybutyleneadipate-co-terephthalate (PBAT) on Zebrafish (*Danio rerio*)

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Polybutyleneadipate-co-terephthalate (PBAT) has been extensively applied as a biodegradable plastic. However, its environmental safety is yet to be fully understood. This study explored the effects of subchronic exposure to PBAT on different behaviors of zebrafish by exposing 3-month-old zebrafish to PBAT microplastics for 21 days and evaluating their swimming, shoaling, anxiety-like, and avoidance behaviors. The results showed that subchronic exposure to PBAT impaired motor performance, reduced sociability, and diminished avoidance capability in zebrafish, while also exacerbating anxiety-like behaviors. Specifically, a decline in motor function was observed, evidenced by significantly reduced swimming speed ($P < 0.01$) and restricted swimming range ($P < 0.01$) in the 20 mg/day dose groups compared to the control. The reduction in sociability was reflected in the significantly increased duration of time spent alone ($P < 0.05$), decreased duration of aggregation ($P < 0.05$), and higher shuttling frequency across the midline ($P < 0.05$) in the 20 mg/day dose group. Impaired avoidance ability was demonstrated by a significantly lower number of zebrafish exhibiting high-sensitivity responses ($P < 0.05$) in the 20 mg/day dose group relative to controls. Furthermore, anxiety-like behaviors were aggravated as shown by a significantly longer latency to first cross the midline and a significantly increased total duration of time spent at the bottom of the tank ($P < 0.05$) in the 20 mg/day dose group. The results indicated that the 20 mg/day dose expose disrupted all four behavioral categories of zebrafish, suggesting that PBAT might exert toxic influences on zebrafish and further investigation on its toxicological mechanisms was required.

Keywords: polybutyleneadipate-co-terephthalate (PBAT); biodegradable microplastics; zebrafish (*Danio rerio*); behavior; toxicity.

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Introduction

Traditional plastics are mainly derived from petrochemical sources and are difficult to degrade in the natural environment. They usually take centuries to decompose and produce microplastics (MPs), which pollute the ecosystem. Therefore, biodegradable plastics emerged as a sustainable alternative. These eco-friendly materials are commonly applied as agricultural mulch films, disposable packaging, and medical supplies. However, several biodegradable plastics, while theoretically degradable, are difficult to degrade in real-world environments. With their extensive application and emission, their ecological threats have become a new concern [1]. Previous research of polylactic acid (PLA), the most extensively applied biodegradable plastic, found that micrometer-sized PLA particles exerted toxic influences on mice, fish, plants, and other organisms, raising concerns about other biodegradable plastic types [2].

Polybutyleneadipate-co-terephthalate (PBAT), synthesized through the copolymerization of butanediol adipic acid and terephthalic acid, is another vastly employed biodegradable plastic. PBAT combines good mechanical characteristics with biodegradability, and its ductility, flexibility, and processability closely match those of low-density polyethylene (LDPE). It has been extensively used in agricultural mulch films, packaging films, disposable tableware, *etc*. [3, 4]. Currently, knowledge on the occurrence, degradation, transport, and ecological threats of PBAT in real environments remains limited. Reports have indicated slow degradation of PBAT in cold and arid environments, potentially taking years in ambient soil, and even longer under deep-sea conditions. In coastal environments, the degradation rate of PBAT declines significantly and under mechanical forces, light exposure, and microbial action. PBAT becomes more prone to fragmentation, aging, and release of micro/nanoplastics. During photohydrolysis, PBAT releases dissolved organic carbon (DOC) into seawater, such that release rate initially increases and then decreases over prolonged light exposure, potentially causing adverse ecological influences. PBAT and its degradation products can act as potential electron acceptors with inherent toxicity, which can impair photosynthetic systems in plants and inhibit their growth [4-6]. Therefore, despite high expectations for PBAT as a biodegradable plastic, the potential toxicity of its degradation products and environmental release behaviors suggest that biodegradable MPs may still pose risks to different environments and ecosystems.

Zebrafish (*Danio rerio*) is a classical model organism in toxicology, useful in the analysis of toxic effects on both aquatic organisms and humans, because they share 85% genetic homology with humans. Their neural system development mechanisms and processes as well as their responses to drugs are very similar to those of humans. Hence, research findings based on zebrafish are of significant reference value for higher animals [7-9]. Behavioral studies on zebrafish have resulted in the development of several classical methods such as social behavior, swimming behavior, and avoidance behavior enabling successful quantitative analysis and evaluation of the toxicity of various hazardous substances [10-14]. This research aimed to explore the behavioral toxicity influences of micrometer-sized PBAT on zebrafish based on different indicators including swimming, shoaling, anxiety-like and stimulus avoidance behaviors to improve the understanding of the safety of biodegradable plastics and provide a theoretical basis to control and regulate their emission.

Materials and methods

Experimental subjects and materials

PBAT material with particle size of about 100 µm was purchased from Zhonglian Plastics Technology Co., Ltd. (Jiangmen, Guangdong, China). Male zebrafish of wild type AB-strain with the ages of about 3-months old along with their specialized feed were obtained from Shanghai Feixi Biotechnology Co., Ltd. (Shanghai, China). Transparent acrylic fish tanks of 42 cm in length, 15 cm in width, and 20 cm in depth were adopted for fish feeding and behavioral testing. Breeding conditions for zebrafish were at pH 7.3, DO value 8.3 mg/L, hardness 157 mg/L, and temperature 27°C with feeding frequency of twice a day [2].

Sub-chronic exposure experiments of PBAT

A total of 100 zebrafish were adopted for one week of adaptive feeding, and then, 80 healthy zebrafish were selected for 21 days of PBAT subchronic exposure experiments. Three exposure groups along with one control group were included in the research with 20 zebrafish in each group. The doses of PBAT for all groups were 0, 0.2, 2.0, and 20.0 mg/day for control and experimental groups, respectively. PBAT powder was administrated by mixing it with feed. The performance of the zebrafish was observed and recorded daily throughout the experimental period.

Swimming behavior observation

For each group, 5-minute video recordings were captured using Zebrafish Behavior Analysis System (Baihuan Biotechnology Co., Ltd., Guangzhou, Guangdong, China) to analyze the locomotion patterns of zebrafish. Three key parameters of horizontal movement range, velocity, and vertical position were measured with five measurements being performed for each metric. The mean values were calculated and reported. Swimming depth was measured as the shortest linear distance from the centroid of the fish to water surface. The speed and range of swimming were calculated as follows.

$$S = \sqrt{L^2 + W^2} / t \quad (1)$$

where S was the swimming speed (cm/s). L was the swimming length (cm). W was the swimming width (cm). t was the time (s).

$$R = L \times W \quad (2)$$

where R was the swimming range (cm^2). L was the swimming length (cm). W was the swimming width (cm).

Shoaling behavior observation

The shoaling behavior experiment was set up with five zebrafish being restricted to the right compartment of the tank using a clear divider (Figure 1). Individual test subjects from various treatment groups were introduced to the left section and their social behaviors were monitored in terms of the three metrics during 4-minute observation periods, which included frequency of boundary crossings, latency to depart from the group, and cumulative isolation time [15].

Anxiety-like behavior observation

Following tank transfer, the zebrafish showing quick descent to lower regions were considered as stressed, while those displaying gradual movement patterns with extended trajectories in upper water layers were classified as calm. Behavioral parameters in this test were frequency of midline transitions, initial latency to cross the central axis, and cumulative time spent in upper zones [15, 16].

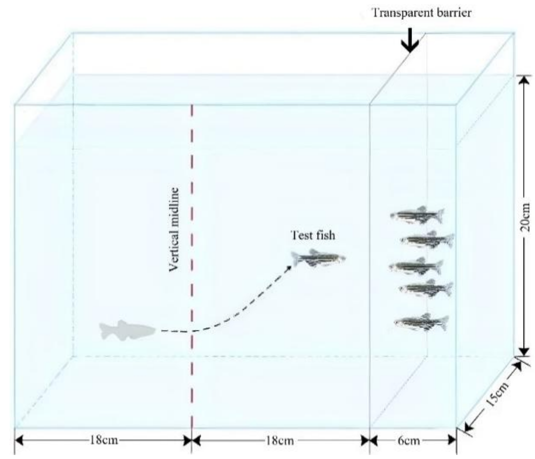

Figure 1. The evaluation of shoaling behavior.

Avoidance behavior

When synchronized swimming behavior was observed among all zebrafish, mechanical stimulation was performed through tank tapping to generate vibrational cues. Immediate (1-second) responses were captured in video recordings, which categorized individuals by their avoidance intensity (highly, moderately, or weakly sensitive) [2]. Tapping was conducted on the end of the tank towards which the zebrafish was swimming with the force causing significant water shaking. The tapping action was performed by the same person to ensure similar force. Repetitive testing was performed at least 3-minute intervals to ensure that the zebrafish was in a similar relaxed state. Each experiment was performed five times with mean response frequencies being calculated for each reaction category. The zebrafish completing turning within 1 second, exhibiting reduced speed, or showing no response were defined as highly sensitive, moderately sensitive, and weakly sensitive individuals, respectively.

Statistical analysis

SPSS v13.0 (IBM, Armonk, NY, USA) was employed for statistical analysis of this study.

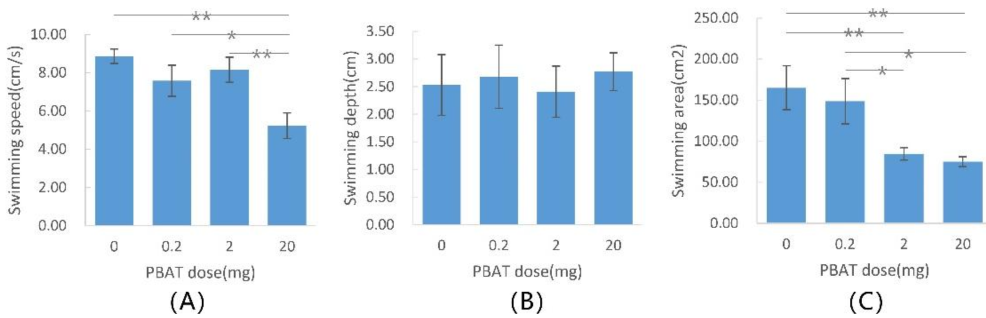

Figure 2. The swimming behavior of the zebrafish in each group. **A.** swimming speed. **B.** swimming depth. **C.** swimming area. *: $P < 0.05$. **: $P < 0.01$.

Statistical significance between PBAT-exposed and control groups was analyzed using one-way ANOVA Duncan's multiple comparison test. The *P* value less than 0.05 was defined as significant difference, while *P* value less than 0.01 was defined as extremely significant difference.

Results

Swimming behavior

The results showed that the swimming speeds of zebrafish in 0, 0.2, 2.0, and 20 mg/day groups were 8.85 (± 0.38), 7.58 (± 0.82), 8.15 (± 0.65), and 5.23 (± 0.66) cm/s, respectively. The swimming speed in high-dose group was significantly lower than that in the control group ($P < 0.01$), low-dose group ($P < 0.05$), and medium-dose group ($P < 0.01$) (Figure 2A). Zebrafish swimming depths in 0, 0.2, 2.0, and 20 mg/day groups were 2.53 (± 0.55), 2.68 (± 0.57), 2.41 (± 0.46), and 2.77 (± 0.34) cm, respectively. However, these differences were not statistically significant (Figure 2B). Zebrafish swimming areas in 0, 0.2, 2.0, and 20 mg/day groups were 165.01 (± 26.66), 148.63 (± 27.58), 84.55 (± 7.46), and 75.12 (± 6.16) cm^2, respectively. The swimming areas in all PBAT exposed groups were significantly lower than that in the control group ($P < 0.01$), while the swimming areas in the medium-dose and high-dose group were both significantly lower than that in the low-dose group ($P < 0.01$) (Figure 2C).

Shoaling behavior

The approaching times of 0, 0.2, 2.0, and 20 mg/day PBAT dose groups were 168.00 ± 24.06, 159.67 ± 20.43, 146.67 ± 20.01, and 110.00 ± 14.42 seconds, respectively (Figure 3A). The approaching time of the high-dose group was significantly lower than that of the control group and the low-dose group ($P < 0.05$). Departure times of 0, 0.2, 2.0, and 20 mg/day PBAT dose groups were 72.00 ± 24.06, 80.33 ± 20.43, 93.33 ± 20.01, and 130.00 ± 14.42 seconds, respectively (Figure 3B). The departure time of the high-dose group was significantly higher than that of the control group and the low-dose group ($P < 0.05$). The shift times of 0, 0.2, 2.0, and 20 mg/day PBAT dose groups were 10.33 ± 2.31, 11.00 ± 2.65, 8.67 ± 1.53, and 18.33 ± 2.08 times, respectively (Figure 3C). The shift time of the high-dose group was significantly higher than that of the control group and the low-dose group ($P < 0.05$) and as extremely significantly higher than that of medium-dose group ($P < 0.01$). However, no significant differences were observed among control, low-dose, and medium-dose groups, which were similar to the results shown in Figures 2A and 2B.

Anxiety-like behavior

The latency to first midline crossings in zebrafish exposed to 0, 0.2, 2.0, and 20 mg/day PBAT groups were 57.67 ± 16.17, 63.67 ± 7.02, 76.33 ± 8.50, and 119.67 ± 27.06 seconds, respectively (Figure 4A). The latency to first midline crossing in the high-dose group was significantly higher than that in the control group and the low-dose group ($P < 0.05$). No differences were observed

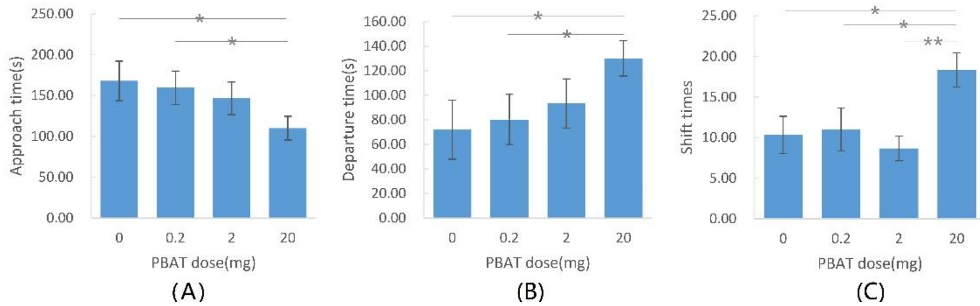

Figure 3. The shoaling behavior of the zebrafish in each group. **A.** approaching times. **B.** departure times. **C.** shift times. *: *P* < 0.05. **: *P* < 0.01.

among control, the low-dose, and the medium-dose groups. The total durations spent below midline by zebrafish in 0, 0.2, 2.0, and 20 mg/day PBAT groups were 155.67 ± 19.60, 140.00 ± 28.21, 198.33 ± 30.66, and 240.00 ± 21.28 seconds, respectively (Figure 4B). The duration spent below midline in the high-dose group was significantly higher than that in the control group ($P < 0.05$) and extremely significantly higher than that in the low-dose group ($P < 0.01$). No differences were observed among control, low-dose, and high-dose groups.

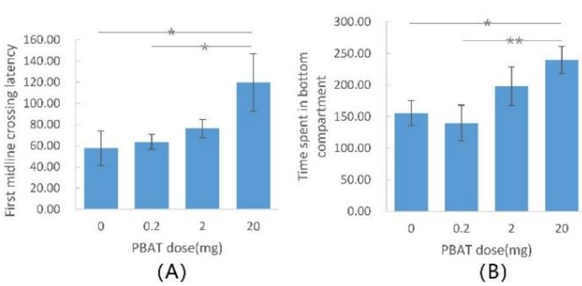

Figure 4. The anxiety-like behavior of the zebrafish in each group. **A.** the latency to first midline crossing. **B.** the total duration below midline. *: *P* < 0.05. **: *P* < 0.01.

Avoidance behavior

The numbers of high-sensitive zebrafish in 0, 0.2, 2.0, and 20 mg/day PBAT groups were 11.67 ± 2.08, 11.00 ± 3.61, 11.67 ± 1.53, and 7.33 ± 1.53, respectively (Figure 5A). The high-sensitive number in the high-dose group was lower than that in the control group and the medium-dose group. No differences were observed among control, low-dose, and medium-dose groups. The numbers of moderately sensitive zebrafish in 0, 0.2, 2.0, and 20 mg/day PBAT groups were 6.00 ± 1.00, 6.67 ± 1.53, 7.67 ± 1.53, and 9.33 ± 2.08, respectively (Figure 5B). Statistical analysis revealed no significant differences among treated and control groups. The numbers of low-sensitivity zebrafish in 0, 0.2, 2.0, and 20 mg/day PBAT groups were 2.67 ± 2.08, 2.33 ± 2.52, 0.67 ± 0.58, and 3.67 ± 2.08, respectively (Figure 5C). No significant differences were observed among treated and control groups.

Discussion

Biodegradable plastics are mainly divided into two categories of biobased and petrochemical based. Biobased plastics include PLA, polyhydroxyalkanoates (PHA), and starch-based plastics, while petrochemical based plastics are represented by PBAT, polybutylene succinate (PBS), and carbon dioxide copolymer (PPC). Currently, PLA has the largest share in biodegradable plastic market, accounting for over 45% [1], which is extensively applied in food packaging and 3D printing. However, it has poor brittleness and heat resistance. PHA is synthesized by microbial fermentation and has complete biodegradability, but it is expensive and is mainly employed in high-end medical fields. Starch-based plastics have the lowest cost but suffer from poor mechanical properties and are mostly applied in disposable tableware. Among petroleum-based plastics, PBAT has become very popular, accounting for about 30% of the total market due to its excellent flexibility

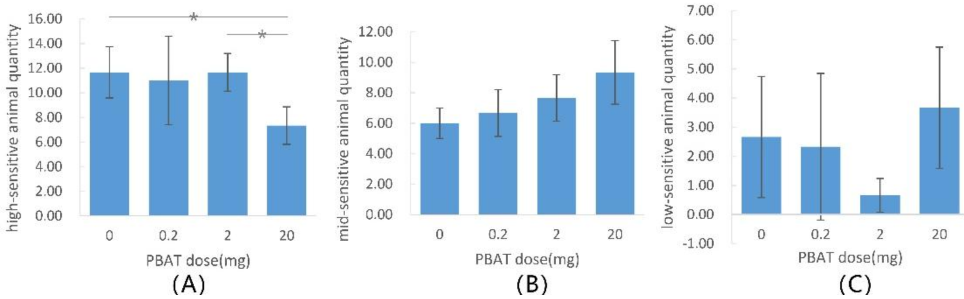

Figure 5. The avoidance behavior of the zebrafish in each group. **A.** high-sensitive zebrafish quantity. **B.** mid-sensitive zebrafish quantity. **C.** low-sensitive zebrafish quantity. *: $P < 0.05$.

and degradation performance and is especially suitable for agricultural films and packaging materials. During its degradation process, it releases 74.7% lower MPs compared to traditional plastics [1]. PBAT has become the most commonly applied material in the industry due to its balanced performance and large-scale production capacity. China is the main producer of PBAT in the world, accounting for 68% of the global production capacity in 2023. Its global market size is expected to exceed 5 million tons by 2030, which makes it essential to comprehensively explore the potential impacts of PBAT on the environment. Few research works have been performed on PBAT toxicity on animals. Boughattas *et al*. found that grinding different plastic particles including PBAT and adding them to organic agricultural soil containing *Eisenia fetida* at a concentration of 100 μg/kg for 7 and 14 days with the particle size below 100 μm gave rise to significant ingestion of these MPs by earthworms and created various abnormalities in several physiological and biochemical indicators. However, this research did not investigate the extent of contribution specifically attributable to PBAT [17]. Xie *et al*. reported that PBAT mixed with thermoplastic starch (TPS) particles significantly decreased zebrafish embryo survival rates and prolonged their hatching periods. The study applied a combined exposure approach and failed to isolate the specific contribution of PBAT [6]. Zhang *et al*. investigated PLA-PBAT combined exposure and confirmed its induction of abnormal behavior in zebrafish. However, like the aforementioned research works, the study could not delineate the precise contribution of PBAT [2]. This research demonstrated for the first time that high-dose subchronic exposure to pure PBAT microparticles could induce abnormalities in different behaviors of developing zebrafish including anxiety-like behavior, swimming behavior, social interaction, and avoidance responses. The primary manifestations involved decreased locomotor capacity, reduced social willingness, diminished responsiveness, and intensified anxiety-like behaviors. These findings indicated the potential ecological threat of PBAT. However, toxic effects were dose-dependent. The low-dose group presented no significant abnormalities in any of the investigated behaviors, while the medium-dose group only presented a significant reduction in swimming range indicator of the swimming behavior. In the high-dose group, all four behavior types involving 8 out of 11 secondary indicators were changed. Significant difference was observed in swimming range between medium-dose and the low-dose groups at different doses. Differences were observed in three behavior types including swimming, shoaling, and anxiety-like behaviors between high-dose and low-dose groups. Differences in three secondary indicators including swimming speed, shit times, and zebrafish proportion with high sensitivity avoidance ability were observed between high-dose and medium dose groups. Currently, no evidence is available proving that rivers in natural environments have reached or will reach such high levels of PBAT microparticle pollution.

Therefore, excessive concern about the likelihood of such severe scenarios is unwarranted. However, it is essential to note that biodegradable plastics are not absolutely green and harmless, and their potential ecological threats warrant attention.

The effects of MPs on zebrafish behaviors are mainly related to their neurotoxicity. Zebrafish share vast homology with humans in terms of brain patterns as well as the structures and functions of several physiological and neural systems. Growing evidence confirms that zebrafish and humans share conserved brain regions with analogous functions. Similar to humans, the central nervous system of zebrafish controls their complex behaviors and emotions. Sarasama *et al*. showed that zebrafish exposed to MPs presented disrupted circadian activity with significant changes in aggression, motor activity, predator avoidance behavior, and shallow behavior [18]. Some MPs can also damage the brain tissue of zebrafish by mechanisms related to neuronal damage and oxidative stress, finally causing behavioral disorders. Research has found that MPs 20 nm in diameter can enter zebrafish brain and generate excessive reactive oxygen species in the biologically accumulated brain regions, creating behavioral disorders and brain damage. The MPs-exposed zebrafish may exhibit seizure behavior, decreased motor ability, and reduced motor activity. LeMoine *et al*. found that MPs exposure downregulated the expression of genes related to neuronal differentiation, neuronal function, and axonal formation in fish [19]. MPs can lead to neurotoxicity by disrupting lipid peroxidation and interfering with neuro-related enzymes in zebrafish. MPs-exposed zebrafish can interfere with the transmission of various neurotransmitters such as acetylcholine, dopamine, melatonin, aminobutyric acid, vasopressin, oxytocin, serotonin, and kispeptin in their bodies. Acetylcholinesterase activity serves as a principal biomarker to evaluate neurotoxic effects among various neurotransmitter systems, indicating damage to cholinergic nerves in the body. Through its esterase activity, acetylcholinesterase (AChE) guarantees timely neurotransmitter clearance at cholinergic synapses, thereby maintaining appropriate neuromuscular function. Acetylcholine (ACh) acts as the main neurotransmitter mediating cholinergic signaling across both central synapses and neuromuscular junctions. Experimental evidence has demonstrated that MPs impaired AChE catalytic function in zebrafish models, disrupting neurotransmission and consequent neurophysiological impairments [18]. The blockade of acetylcholinesterase enzymatic activity has been found to give rise to supraphysiological ACh concentrations within neural tissue, compromising synaptic precision. Such neurotransmitter accumulation can induce the overstimulation of pathological receptor due to impaired clearance mechanisms, hinder neural signal transmission, and result in the loss of motor function and death in zebrafish. These findings have provided insights to explore the toxicological mechanisms of PBAT in the future. The neurotoxic effects of conventional MPs primarily depend on particle dimensions and exposure duration, while presenting no significant correlations with polymer composition, biological species, physical form, or environmental concentrations. Beyond the well-documented effects on acetylcholinesterase function, emerging research has correlated MP exposure to multifaceted neurobehavioral alterations through different mechanisms such as redox imbalance, neuronal injury, gut microbiome dysbiosis, and gut-brain axis disruptions. Research on zebrafish has further revealed MP-induced programmed cell death and neural circuit reorganization, finally manifesting as observable modifications in behaviors. Current understanding of the toxicological mechanisms of PBAT in fish remains sparse. Zhang *et al*. investigated the neurobehavioral toxicity mechanisms in zebrafish after co-exposure to PLA-PBAT, revealing widespread dysregulation of thousands of genes in brain tissue. The affected pathways encompassed protein synthesis/modification, immune responses, cellular signaling including signal transduction and cytokine interactions, and cytochrome P450 metabolism. Considering the predominant immune association of enriched pathways, the behavioral toxicity of PLA-PBAT nanoparticles has been found to be related to neuroimmune dysregulation. Crucially, validation of ko04060 cytokine-cytokine receptor interaction pathway confirmed strong correlations among gene expression changes and several behavioral parameters [2]. Regardless, further research is needed to elucidate the toxicological mechanisms by which PBAT affects zebrafish behavior, a key issue that remains to be addressed in this field. Limited by available resources, this study only conducted a preliminary toxicity assessment. Many closely related issues remain unaddressed such as establishing a comprehensive dose-response curve, conducting molecular-level mechanistic analysis, and incorporating environmental factors such as temperature fluctuations into experimental design. These areas require further experimental research to clarify.

Conclusion

Exposure to PBAT affected four behaviors of zebrafish in a dose-dependent manner. Subchronic exposure to PBAT MPs at 20 mg/day dose caused significantly degraded swimming behavior with lower swimming speeds and shorter swimming ranges, shoaling behavior with longer durations of straying from the group, weaker shoaling willingness, and higher shuttling frequency across the midline, anxiety-like behavior with increased time spent in the bottom zone and prolonged initial anxiety duration, and avoidance behavior with reduced sensitivity to external stimuli. The 2.0 mg/day dosage only decreased swimming area. Significant differences were also observed among different dose groups. The high-dose group differed from the low-dose group in swimming, shoaling, and anxiety-like behaviors, and from the medium-dose group in swimming, shoaling, and avoidance behaviors. Despite being promoted as an environmentally friendly material, the long-term ecological risks of PBAT in real-world environments required careful assessment. This research provided important behavioral toxicology evidence for the environmental safety evaluation of biodegradable plastics, while also highlighting the requirement for establishing a more comprehensive ecological risk assessment system to address this emerging environmental challenge.

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