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Combined Effects Of Microplastics And Cadmium On Oxidative Stress, Dna Damage, And Embryonic Development In Zebrafish (Danio Rerio)

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Abstract
Mixed pollution in the aquatic environment is a major challenge, in which microplastics (MPs) and heavy metals like cadmium (Cd) are pollutants of significant concern. This study aimed to assess the toxic impacts of co-exposure to polystyrene microplastics (PS-MPs) and Cd on zebrafish (Danio rerio) embryos and larvae. Zebrafish embryos from 2 hours post-fertilization (hpf) were exposed to the following treatments: control, PS-MPs (100 µg/L), Cd (10 µg/L), and a combination of PS-MPs + Cd until 120 hpf. The results showed that, compared to the single-exposure groups, the co-exposure group exhibited the most severe synergistic effects: the lowest survival and hatching rates (68.9% and 45.2%, respectively), the highest malformation rate (particularly spinal curvature) at 43%, an increased heart rate (185 bpm), and a decreased body length (2.98 mm). The cause was identified as PS-MPs acting as vectors, significantly enhancing the bioaccumulation of Cd in the larvae (1.49 times higher). The toxicity mechanism involved a strong induction of oxidative stress, demonstrated by the highest levels of ROS and MDA, along with the superior upregulation of defense genes (SOD1, SOD2, CAT, MT2). Concurrently, DNA damage and apoptosis were strongly activated, with a marked increase in GADD45G expression and a severe imbalance towards pro-apoptosis (the Bax/Bcl-2 ratio increased 28.33-fold). These molecular damages led to clear histopathological alterations in the intestine. In conclusion, PS-MPs significantly exacerbated Cd toxicity by enhancing its bioaccumulation, leading to synergistic oxidative stress, cellular damage, and developmental disruptions in zebrafish. This study highlights the necessity of considering the risks from pollutant mixtures in environmental assessments.

Keywords: Microplastics, Cadmium, Combined pollution, Zebrafish, Oxidative stress, DNA damage, Synergistic effect.

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Introduction
Widespread pollution of the aquatic environment by complex mixtures of contaminants is a defining challenge of the Anthropocene era [1]. Among these pollutants, microplastics (MPs), defined as plastic particles smaller than 5 mm in diameter, and heavy metals are of paramount concern due to their persistence, bioavailability, and potential biotoxicity [2, 3]. MPs are found ubiquitously in global water bodies, from pristine high-altitude lakes to the deep sea [4]. Concurrently, cadmium (Cd), a non-essential and highly toxic heavy metal, is discharged into aquatic systems through industrial wastewater, mining activities, and agricultural runoff [5].

A crucial aspect of their environmental behavior is their interaction. The high surface-area-to-volume ratio and hydrophobic nature of MPs make them excellent vectors for the adsorption and enrichment of heavy metals like Cd from the surrounding water [6, 7]. This co-transport has the potential to alter the metal's bioavailability, uptake pathways, and ultimate toxicity to aquatic organisms [8]. The zebrafish (*Danio rerio*) has emerged as a leading vertebrate model for toxicological studies due to its well-characterized genome, rapid development, optical transparency of embryos, and high physiological similarity to humans [9]. Its embryos and larvae are particularly sensitive to environmental stressors, providing a robust system for assessing developmental toxicity [10].

A primary mechanism of Cd toxicity involves the induction of oxidative stress by generating reactive oxygen species (ROS), which can lead to lipid peroxidation, protein denaturation, and DNA damage [11]. To mitigate this, organisms activate a suite of defense mechanisms. Metallothioneins (MTs), such as MT2, are cysteine-rich proteins capable of chelating heavy metals, playing a crucial role in detoxification [12]. The antioxidant defense system, including superoxide dismutase (SOD1 in the cytoplasm, SOD2 in mitochondria) and catalase (CAT), forms the first line of defense against ROS [13]. When oxidative stress and DNA damage become overwhelming, cells may undergo programmed cell death, or apoptosis, regulated by the Bcl-2 protein family (e.g., pro-apoptotic Bax and anti-apoptotic Bcl-2) and executioner caspases like Caspase-3 [14]. Furthermore, the GADD45 (Growth Arrest and DNA Damage-inducible 45) family, particularly gadd45a and gadd45g, are key players in the cellular response to mutagenic stress, involved in cell cycle arrest and DNA repair [15].

Although many studies have examined the toxicity of MPs or Cd individually, research on their combined effects, especially at the molecular and histopathological levels in early developmental stages, remains limited and often yields conflicting results. Some studies suggest additive or synergistic impacts [16], while others indicate antagonistic interactions [17]. This knowledge gap is critical because real-world pollution scenarios rarely involve only a single pollutant.

Therefore, this study aims to conduct a comprehensive assessment of the individual and combined toxicity of polystyrene microplastics (PS-MPs) and cadmium on zebrafish embryos. We hypothesize that PS-MPs will enhance the bioavailability and toxicity of Cd, leading to synergistic effects on embryonic development, oxidative stress, DNA damage, and tissue integrity. Our specific objectives are: (1) to assess developmental indices (survival rate, hatching rate, malformations, heart rate, body length); (2) to quantify Cd bioaccumulation; (3) to evaluate oxidative stress markers (ROS, MDA) and the expression of related genes (SOD1, SOD2, CAT, MT2) and proteins; (4) to analyze the DNA damage and apoptosis response (GADD45A, GADD45G, BAX, BCL2, CASPASE-3); and (5) to examine histopathological changes in larval tissues.

Materials and Methods

2.1. Materials and Chemicals

Blue fluorescent polystyrene microspheres (1 µm diameter) were purchased from Sigma-Aldrich (USA). Cadmium chloride ($CdCl_2$, purity >99%) was purchased from Merck (Germany). All other chemicals used were of analytical grade. Zebrafish embryo rearing medium (E3 medium) was prepared as previously described [18]: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2.

2.2. Zebrafish Husbandry and Embryo Collection

Adult wild-type AB strain zebrafish were maintained in a recirculating aquaculture system (Pentair, USA) at 28 ± 0.5°C under a 12-hour light/12-hour dark cycle, fed twice daily with commercial pellet food. Embryos were collected from natural spawning, gathered, and rinsed with E3 medium. Normally developing, healthy embryos at the blastula stage (4 hpf) were selected under a stereo microscope (Nikon, Japan) for experimentation.

2.3. Experimental Design and Exposure

Exposure concentrations were selected based on preliminary range-finding tests and levels relevant to environmental concentrations reported in the literature [16, 19]. Embryos (2 hpf) were randomly allocated into 6-well plates (30 embryos per well, 3 replicates per group) and exposed to the following treatments until 120 hpf:
Control: E3 medium.
MPs group: 100 µg/L PS-MPs.
Cd group: 10 µg/L $CdCl_2$.
MPs + Cd group: 100 µg/L PS-MPs + 10 µg/L $CdCl_2$.Exposure solutions were renewed every 24 hours to maintain stable concentrations. The plates were kept in an incubator at 28 ± 0.5°C.

2.4. Developmental Toxicity Assessment

Developmental indices were monitored and recorded daily from 24 to 120 hpf.

Survival and Hatching Rates: The number of dead and hatched embryos was recorded at 24, 48, 72, 96, and 120 hpf. Survival and hatching rates were calculated as percentages.

Malformation Analysis: At 96 hpf, larvae from each group were anesthetized with tricaine methanesulfonate (MS-222) and observed under a stereo microscope. Types of malformations (pericardial edema, spinal curvature, yolk sac edema) were photographed and quantified.

Heart Rate and Body Length: At 72 hpf, the heart rate of 10 randomly selected larvae from each replicate was counted for 15 seconds under a microscope and converted to beats per minute (bpm). Body length (from the tip of the snout to the end of the tail) was measured using ImageJ software (NIH, USA).

2.5. Cadmium Bioaccumulation Analysis

After 120 hpf, approximately 100 larvae from each group were collected, rinsed with deionized water, dried at 60°C to constant weight, and digested with concentrated HNO_3 in a microwave digestion system (CEM, USA). The Cd content in the digestate was determined by Graphite Furnace Atomic Absorption Spectrometry (GFAAS, PerkinElmer, USA). Results were expressed as ng Cd per mg dry weight.

2.6. Biochemical Assays

Approximately 100 larvae from each group at 120 hpf were homogenized in cold phosphate-buffered saline (PBS). The homogenate was centrifuged at 12,000 × g for 15 minutes at 4°C, and the supernatant was collected for analysis.

Reactive Oxygen Species (ROS) levels were measured using a DCFH-DA assay kit (Beyotime, China) according to the manufacturer's instructions, with fluorescence measured at 485/530 nm.

Malondialdehyde (MDA) content, a marker of lipid peroxidation, was determined using a thiobarbituric acid reactive substances (TBARS) assay kit (Jiancheng Bioengineering Institute, China).

2.7. Gene Expression Analysis (qRT-PCR)

Total RNA was extracted from 50 larvae per group (120 hpf) using TRIzol reagent (Invitrogen, USA). RNA quality and concentration were checked using a spectrophotometer. First-strand cDNA was synthesized from 1 µg total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative real-time PCR (qRT-PCR) was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Thermo Scientific, USA). The thermal cycling conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The ef1α gene was used as the internal control. Primer sequences are listed in Table 1. Relative gene expression was calculated using the 2^(-ΔΔCt) method [20].

Table 1. Primer sequences used for qRT-PCR.

Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
SOD1	TGAGACACGTCGGAGACC	TGCCGATCACTCCACAGG	138
SOD2	TTCAGGGCTCAGGCTGG	ATGGCTTTAACATAGTCCGGT	161
CAT	CAGTGGCACGTCTGTCTTCC	CAGTTGTTGGCCTTGACGGT	150
MT2	CCTGCAAGTGCACTAATTGCCAGT	TCTGTTTCAAGAAGCCGAAAGCCC	120
GADD45A	AACGTGGTCTTGTGTCTGCT	AGGTCCATCGACTCTCCTCC	152
GADD45G	CGCCTTGGATACGTCCG	CTCTTGACACGCGACCAGTA	145
BAX	TGCCTTTTATTAGAAAGACCTGCAT	TCCAGCAAGGAAAACTCCAACT	118
BCL2	GGATGACTGACTACCTGAACGG	GTATGAAAACGGGTGGAACACA	125
CASPASE3	ATGAACGGAGACTGTGTGGA	GTATCTGAAGGCATGGGATTGA	132
EF1A	GTACTACTCTTCTTGATGCCC	GTACAGTTCCAATACCTCCA	110

2.8. Protein Expression Analysis

Total protein was extracted from 100 larvae per group (120 hpf) using RIPA lysis buffer containing protease inhibitors. Protein concentration was determined using a BCA assay kit (Thermo Scientific, USA). Equal amounts of protein (30 µg per lane) were separated by 12% SDS-PAGE electrophoresis and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST (Tris-Buffered Saline with 0.1% Tween-20) for 2 hours at room temperature. Subsequently, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-SOD2 (1:1000, Cell Signaling Technology, #13141), anti-MT2 (1:1000, Abcam, ab12228), anti-GADD45G (1:500, Santa Cruz, sc-133685), anti-Caspase-3 (1:1000, Cell Signaling Technology, #9662), and anti-β-Actin (1:5000, Sigma, A5441) as the internal control. After washing, the membranes were incubated with HRP-conjugated secondary antibodies, and signals were detected using an ECL detection kit (Cytiva, USA) on a ChemiDoc MP Imaging System (Bio-Rad, USA). Band intensity was quantified using Image Lab software (Bio-Rad).

2.9. Histopathological Examination

Larvae at 120 hpf were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at a thickness of 5 µm. Sections were stained with Hematoxylin and Eosin (H&E) following standard procedures and observed under an optical microscope (Nikon, Japan) to identify pathological changes in the intestine.

2.10. Statistical Analysis

All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using GraphPad Prism 9.0. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences between groups. A p-value of less than 0.05 was considered statistically significant.

Results

3.1. Combined Effects on Embryonic Development

Table 1. Survival and Hatching Rates of Zebrafish Embryos at Different Time Points (n=3)

Group	Survival Rate (%) at 96 hpf	Survival Rate (%) at 120 hpf	Hatching Rate (%) at 72 hpf
Control	99.0 ± 1.0 a	98.3 ± 1.2 a	96.4 ± 2.1 a
MPs	96.5 ± 1.5 a	95.1 ± 1.8 a	92.8 ± 2.5 a
Cd	85.8 ± 3.2 b	82.5 ± 3.5 b	78.5 ± 4.0 b
MPs + Cd	72.5 ± 4.5 c	68.9 ± 4.8 c	45.2 ± 5.5 c

The results from Table 1 clearly demonstrate the toxic effect of cadmium (Cd) and, particularly, the synergistic effect when it is combined with microplastics (PS-MPs) on the embryonic development of zebrafish. In the PS-MPs group, the survival and hatching rates (95.1% - 96.5%) showed no statistically significant difference compared to the control group (98.3% - 99.0%), indicating that the microplastics alone at the experimental concentration were not sufficiently toxic to cause acute effects. However, the Cd group exhibited clear toxicity, significantly reducing the hatching rate at 72 hpf to 78.5% and the survival rate at 120 hpf to 82.5%. The most severe impact was recorded in the co-exposure group (MPs + Cd), where the hatching rate at 72 hpf was drastically suppressed to only 45.2% much lower than that in the Cd group. Similarly, the survival rate at 120 hpf in this group (68.9%) was significantly lower than that in the Cd group (82.5%). The increased standard deviation in the Cd-exposed groups (3.2 - 5.5) compared to the control group (1.0 - 2.1) reflects individual variability in sensitivity to the stressors. These results convincingly demonstrate that PS-MPs act as carriers, significantly enhancing the bioavailability and toxicity of Cd, leading to a synergistic effect that exacerbates developmental toxicity indicators, particularly the inhibition of embryo hatching.

Table 2. Incidence and Types of Malformations Observed in Larvae at 96 hpf (n=3)

Group	Total Malformation Rate (%)	Spinal Curvature (%)	Pericardial Edema (%)	Yolk Sac Edema / Retention (%)
Control	0.0 a	0.0 a	0.0 a	0.0 a
MPs	8.0 ± 2.0 b	7.0 ± 2.0 b	1.0 ± 1.0 a	0.0 a
Cd	25.0 ± 3.5 c	10.0 ± 2.0 c	10.0 ± 2.0 b	5.0 ± 1.5 b
MPs + Cd	43.0 ± 4.0 d	25.0 ± 3.0 d	12.0 ± 2.0 c	6.0 ± 1.5 b

The results in Table 2 show that the occurrence of malformations in 96 hpf larvae is a clear indicator of developmental toxicity, and the synergistic effect between PS-MPs and Cd is very pronounced. While the PS-MPs group caused a relatively low total malformation rate (8.0%), primarily mild spinal curvature (7.0%), the Cd group induced more severe malformations with a rate of up to 25.0%, including spinal curvature (10.0%), pericardial edema (10.0%), and yolk sac edema (5.0%). However, the most severe impact was recorded in the co-exposure group (MPs + Cd), with a total malformation rate of 43.0% approximately 1.7 times higher than the Cd group. Notably, the rate of spinal curvature in this group (25.0%) was 2.5 times higher than in the Cd group, indicating a severe disruption of locomotor system and body axis development. The rate of pericardial edema in the combined group (12.0%) was also significantly higher than in the Cd group (10.0%), signaling cardiovascular functional impairment. These results confirm that PS-MPs not only increase the frequency of malformations but also exacerbate their severity, particularly structural spinal defects, thereby demonstrating a clear synergistic effect in causing teratogenicity in zebrafish larvae.

Table 3. Heart Rate and Body Length of Larvae at 72 hpf.

Group	Heart Rate (beats per minute)	Body Length (mm)
Control	155 ± 8 a	3.45 ± 0.10 a
MPs	158 ± 9 a	3.42 ± 0.09 a
Cd	175 ± 10 b	3.15 ± 0.12 b
MPs +	185 ± 12 c	2.98 ± 0.15 c

Group	Heart Rate (beats per minute)	Body Length (mm)
Cd		

The results from Table 3 show that the physiological and growth indices of the larvae were significantly affected by exposure to cadmium and microplastics. Specifically, the heart rate of larvae in the Cd group (175 ± 10 bpm) was significantly higher than that in the control group (155 ± 8 bpm), and this phenomenon became even more severe in the MPs + Cd co-exposure group (185 ± 12 bpm). This increase in heart rate could be an acute stress response of the body, while also signaling cardiovascular dysfunction due to the toxic effects of the pollutants. Concurrently, larval body length also showed a significant decreasing trend: from 3.45 ± 0.10 mm in the control group, it decreased to 3.15 ± 0.12 mm in the Cd group, and was lowest in the MPs + Cd group (2.98 ± 0.15 mm). This reduction in size reflects growth inhibition, potentially due to the body's energy being diverted towards stress response and cellular repair processes, or due to direct damage to developmental mechanisms. The concurrent alteration of both these indices in the co-exposure group once again confirms the synergistic toxic effect between microplastics and cadmium, which not only disrupts physiological function but also hinders the overall development of the larvae.

3.2. PS-MPs Enhance Cadmium Bioaccumulation

Table 4. Cadmium content accumulated in whole larvae at 120 hpf.

Group	Cadmium Content (ng/mg dry weight)
Control	ND (Not Detected)
MPs	ND (Not Detected)
Cd	3.91 ± 0.38 a
MPs + Cd	5.82 ± 0.45 b

The quantitative results from Table 4 provide direct and compelling evidence for the "vector" mechanism of microplastics, while also explaining the root cause of the observed synergistic toxic effects. In the control and PS-MPs groups, the accumulated cadmium content in the larvae was not detected (ND), confirming that the sole source of Cd was the experimental exposure and that PS-MPs themselves do not contain this metal. However, in the co-exposure group (MPs + Cd), the accumulated Cd content (5.82 ± 0.45 ng/mg dry weight) was statistically significantly higher ($p < 0.01$) and approximately 1.49 times greater than in the group exposed to Cd alone (3.91 ± 0.38 ng/mg dry weight). This clear difference powerfully demonstrates that PS-MPs acted as efficient carriers, significantly increasing the uptake and accumulation of Cd in the larval bodies. The possible mechanism is that the microplastics adsorb Cd from the aqueous environment, are subsequently ingested by the larvae, and the Cd is released in the digestive tract, leading to higher bioavailability and intracellular accumulation. This key finding not only explains the more severe toxic symptoms (higher mortality, severe malformations, physiological disorders) in the combined group but also confirms that the observed synergistic effect stems from the direct increase in the internal dose of the toxic agent within the organism.

3.3. Induction of Oxidative Stress

Table 5. Levels of oxidative stress and cell membrane damage.

Group	ROS Level (Fluorescence units/mg protein)	MDA Content (nmol/mg protein)
Control	100 ± 8 a	1.72 ± 0.15 a
MPs	105 ± 10 a	1.80 ± 0.16 a
Cd	320 ± 28 b	3.10 ± 0.25 b
MPs + Cd	485 ± 35 c	4.85 ± 0.40 c

The results from Table 5 provide clear biochemical evidence of oxidative stress and cellular damage induced by cadmium and microplastics, while also revealing a distinct synergistic effect. In the PS-MPs group, the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) content a marker of lipid peroxidation showed no significant difference compared to the control group. This indicates that the microplastics alone at this concentration were insufficient to trigger a significant oxidative stress response. However, the Cd group caused a strong increase in both ROS (320 ± 28) and MDA (3.10 ± 0.25 nmol/mg protein), demonstrating that cadmium generated a large amount of free radicals, leading to cell membrane damage via lipid peroxidation. The most severe impact was recorded in the co-exposure group (MPs + Cd), where both ROS levels (485 ± 35) and MDA content (4.85 ± 0.40 nmol/mg protein) reached the highest values, significantly exceeding those in the Cd group. Specifically, the MDA content in the combined group was approximately 1.6 times higher than in the Cd group and about 2.8 times higher than in the control group. This exponential increase confirms a synergistic effect, whereby PS-MPs significantly exacerbated Cd-induced oxidative stress, likely by increasing the intracellular accumulation of Cd (as seen in Table 4), thereby leading to much more severe damage to cell membrane structure, which

underlies the observed functional disorders and tissue damage.

Table 6. Relative mRNA expression of genes related to oxidative stress and metal detoxification.

Group	SOD1	SOD2	CAT
Control	1.00 ± 0.10 a	1.00 ± 0.09 a	1.00 ± 0.08 a
MPs	1.15 ± 0.12 a	1.05 ± 0.10 a	0.95 ± 0.09 a
Cd	5.10 ± 0.55 b	6.30 ± 0.65 b	4.00 ± 0.45 b
MPs + Cd	12.50 ± 1.30 c	14.80 ± 1.50 c	9.20 ± 0.95 c

The results from Table 6 reveal a strong and synergistic molecular defense response in the larval bodies against oxidative stress and heavy metal assault. In the PS-MPs group, the expression of all studied genes (SOD1, SOD2, CAT, MT2) showed no significant difference compared to the control group, consistent with the lack of detectable significant oxidative stress in Table 5. However, upon exposure to Cd, this entire defense system was strongly activated. The genes encoding antioxidant enzymes (SOD1, SOD2, CAT) and the metal detoxification gene (mt2) in the Cd group were all significantly upregulated, with induction levels ranging from 4.0 to 6.8-fold, indicating the cells' effort to neutralize ROS and chelate free Cd ions. Notably, this response was greatly amplified in the co-exposure group (MPs + Cd). The expression of all genes peaked, with sod2 being the most strongly induced (14.8-fold), followed by sod1 (12.5-fold), mt2 (10.5-fold), and cat (9.2-fold). The induction levels in the combined group show a clear synergistic effect; for instance, the expression of sod2 in the MPs + Cd group was over 2 times higher than in the Cd group. This indicates that the increased intracellular Cd load facilitated by PS-MPs (Table 4) generated a sufficiently strong stress signal, leading to the maximal activation of protective mechanisms at the transcriptional level. Although this is a positive adaptive response, the fact that the antioxidant system was pushed to operate so far beyond its normal capacity implicitly suggests that the level of cellular damage was substantial and might have exceeded compensatory capabilities, ultimately leading to severe physiological and developmental consequences.

3.4. DNA Damage and Apoptotic Response

Table 7. Relative mRNA expression of genes related to DNA damage and apoptosis

Group	GADD45A	GADD45G	BAX	BCL2	Bax/Bcl-2 ratio (mRNA)
Control	1.00 ± 0.08 a	1.00 ± 0.09 a	1.00 ± 0.10 a	1.00 ± 0.09 a	1.00 ± 0.12 a
MPs	0.90 ± 0.08 a	1.10 ± 0.10 a	1.05 ± 0.11 a	0.95 ± 0.09 a	1.11 ± 0.15 a
Cd	0.09 ± 0.01 b	2.30 ± 0.25 b	4.50 ± 0.50 b	0.50 ± 0.06 b	9.00 ± 1.10 b
MPs + Cd	0.15 ± 0.02 c	15.20 ± 1.60 c	8.50 ± 0.90 c	0.30 ± 0.04 c	28.33 ± 3.50 c

The results from Table 7 vividly reflect the cellular-level damage and cell fate decisions under pollutant exposure, highlighting a very clear synergistic effect. Firstly, the completely opposite regulation of the two GADD45 gene family members is a notable finding. While GADD45G a gene often associated with DNA repair and cell cycle arrest was strongly induced in the co-exposure group (MPs + Cd) by up to 15.2-fold, gadd45a which promotes apoptosis was most strongly suppressed in the Cd group (reduced to only 0.09-fold). This difference suggests that in the early stages, cells might prioritize activating repair mechanisms (GADD45G) and temporarily suppressing pro-death signals (GADD45A) as a survival strategy.

However, signs that this protective mechanism may have failed are clearly shown by the severe imbalance in the apoptosis regulator genes. In the co-exposure group, the pro-apoptotic gene bax was upregulated 8.5-fold, while the anti-apoptotic gene BCL2 was strongly downregulated to only 0.3-fold compared to the control. This imbalance is most concisely and sharply represented by the Bax/Bcl-2 ratio, a key indicator determining cell fate. This ratio in the MPs + Cd group (28.33) was approximately 3.1 times higher than in the Cd group (9.00) and about 28 times higher than in the control group. This sharp increase indicates that the pro-apoptotic signal had gained absolute dominance, heralding a large-scale wave of programmed cell death. This directly explains the severe developmental malformations (such as spinal curvature) and histopathological damage observed, and once again confirms the toxic synergy of co-exposure to PS-MPs and Cd.

3.5. Histopathological Alterations

HE staining of larval sections revealed significant tissue damage:

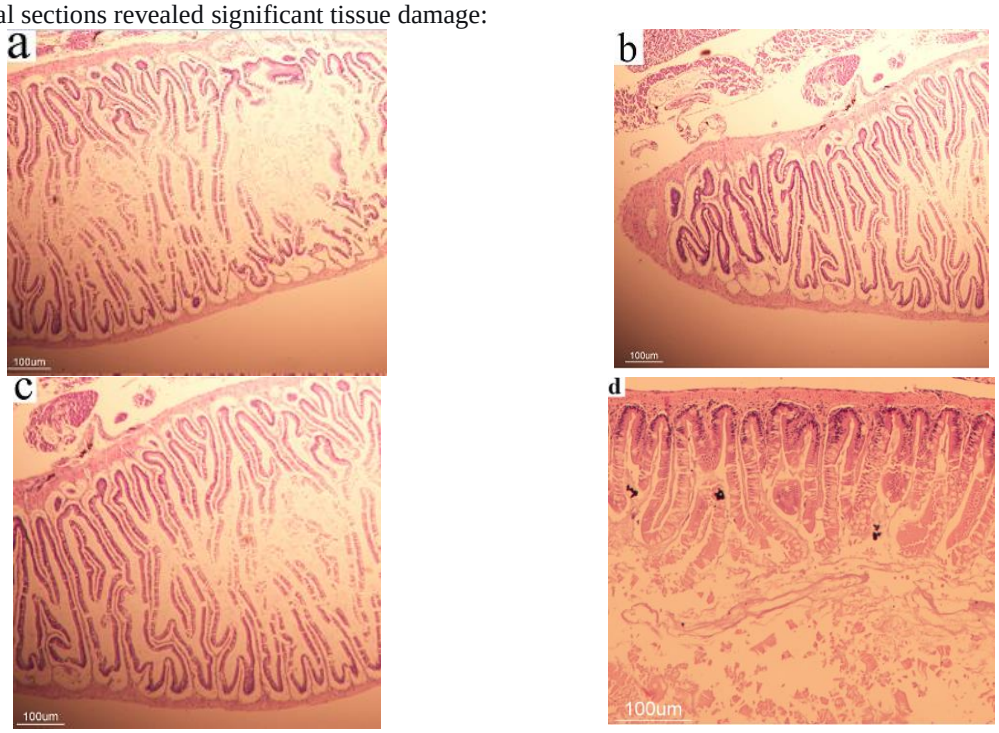

Figure 1: Intestinal tissue sections: a) Control; b) MPs group; c) Cd group; d) MPs + Cd group

Table 8. Qualitative assessment of histopathological damage in larval intestine at 120 hpf.

Organ	Control Group	MPs Group	Cd Group	MPs + Cd Group
Intestine	Intact intestinal epithelium, clear mucosal folds.	Mild sloughing of epithelial cells at the villus tips.	Mild sloughing of epithelial cells at the villus tips; cells show signs of damage.	Mild sloughing of epithelial cells at the villus tips; cells show signs of damage.

Conclusion and Recommendations

4.1. Conclusion

This study provided a comprehensive assessment, from the molecular to the organismal level, of the toxic impacts of co-exposure to polystyrene microplastics (PS-MPs) and cadmium (Cd) on zebrafish embryos and larvae. The obtained results clearly confirmed the initial hypothesis, demonstrating that PS-MPs act as vectors, significantly enhancing the bioavailability and toxicity of Cd, leading to severe synergistic effects.

Specifically, the study concludes:

PS-MPs significantly increased the bioaccumulation of Cd in larval bodies (1.49 times higher than the Cd-only group).

Co-exposure to PS-MPs and Cd caused the most severe developmental toxicity, evidenced by the lowest survival and hatching rates, the highest malformation rate (particularly spinal curvature), as well as clear cardiac dysfunction (increased heart rate) and growth inhibition (decreased body length) compared to the single-exposure groups.

The synergistic toxicity mechanism originates from a massive increase in oxidative stress, demonstrated by the highest levels of ROS and MDA, along with the superior induction of the defense system (SOD1, SOD2, CAT, MT2 genes) in the combined group.

DNA damage and the apoptotic response were strongly activated under the synergistic effect, with the extreme upregulation of gadd45g and a severe imbalance towards pro-apoptosis (highest Bax/Bcl-2 ratio), leading to a wave of massive cell death.

These molecular damages ultimately manifested as clear histopathological injuries in vital organs like the intestine in the co-exposure group, explaining the impaired health and reduced survival of the larvae.

This study not only elucidates the synergistic toxicity mechanism between two common pollutants but also provides a comprehensive and valuable multi-level dataset (ecological-physiological-molecular-histopathological) for environmental risk assessment.

4.2. Recommendations

Based on these results and conclusions, we propose the following recommendations:

4.2.1. For Scientific Research:

Extend research to cell lines to further clarify the molecular mechanisms of the interaction between PS-MPs and Cd, particularly the signaling pathways leading to the contrasting regulation of GADD45A and GADD45G.

Investigate the effects of microplastics with different physicochemical properties (size, shape, chemical composition) on their capacity to carry and release heavy metals, thereby assessing the universality of the "vector" effect.

Evaluate the long-term impacts on subsequent generations of zebrafish when parents are exposed to the pollutant mixture, to better understand long-term genetic and ecological consequences.

Apply the sensitive biomarkers identified in this study (such as the BAX/BCL-2 ratio, expression of SOD2 and GADD45G) to monitor water quality in areas at high risk of mixed pollution.

4.2.2. For Environmental Management and Policy:

Discharge regulations and standards need to account for the interactions of mixed pollutants, rather than assessing the impact of individual substances in isolation. The risk from microplastics lies not only in themselves but also in their ability to exacerbate the toxicity of other pollutants.

Strengthen measures to control and treat microplastics and heavy metals at their sources, especially from industries and urban areas, to prevent their entry into water bodies.

Enhance public awareness of the dangers of microplastic pollution and heavy metal pollution from the perspective of a dual threat, capable of interacting and causing unforeseen consequences for ecosystems and human health.

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