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Interactive Effects of Microplastics and Nickel on Subcellular Metal Partitioning and Biomarker Responses in Juvenile *Cyprinus carpio* L

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ABSTRACT

Aims: Microplastics (MPs) have emerged as persistent pollutants in aquatic environments, yet their interactions with dissolved metals and the resulting biological risks for fish remain poorly understood. This study aimed to evaluate the individual and combined effects of MPs and Nickel (Ni) on subcellular metal partitioning and biomarker responses in juvenile common carp (*C. carpio*).
Materials & Methods: Juvenile carp (n=390) were exposed for 21 days to graded concentrations of Ni (0.05, 0.15, 0.20, and 0.25 mg.L^{-1}), MPs(0.25, 0.50, 0.75, and 1.00 mg.L^{-1}), and their mixtures. Subcellular fractionation was performed to quantify Ni distribution among cytosol, debris, and granules. Biochemical indicators, including malondialdehyde (MDA), total antioxidant capacity (TAC), weight change, and the Biomarker Response Index (BRI), were assessed to determine physiological stress and mixture effects.
Findings: Ni predominantly accumulated in the cytosolic fraction, but co-exposure with MPs significantly increased its retention in cellular debris ($p<0.05$). Debris Ni levels are strongly correlated with whole-body burden ($r>0.8$), MDA, and TAC. While MDA and TAC indicated moderate oxidative stress, WL revealed severe physiological impairment, with alteration levels (AL%) exceeding 100% in most treatments. The results of the Effect Addition Index (EAI) were consistent with synergistic interactions across most Ni+ MPs combinations, except at the highest concentrations, where antagonism occurred.
Conclusion: Microplastics altered Ni subcellular partitioning and intensified physiological stress in juvenile common carp, with debris-associated Ni showing the strongest relationship with oxidative stress biomarkers. Overall, the results indicate that combined exposure effects are concentration-dependent and should be considered in ecological risk assessment.

Keywords: Biomarker Response Index (BRI); Carp; Microplastics; Nickel; Subcellular Partitioning.

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Introduction

Anthropogenic expansion of industrial, agricultural, and urban activities over recent decades has markedly increased contaminant loading into aquatic environments, making these ecosystems major reservoirs of environmental pollution and a significant threat to the health of living organisms . Among the various types of pollutants, two groups of contaminants have gained significant attention in recent years: heavy metals and microplastics [3]. Heavy metals such as Ni, Lead, mercury, and Cadmium are among the most persistent environmental pollutants due to their chemical stability and bioaccumulation in the food chain. These metals can easily enter surface and groundwater through industrial, agricultural, and transportation activities, accumulating in the bodies of living organisms [4]. Nickel, as one of the most common heavy metals in industrial sources, particularly in steel production, mining, and battery manufacturing, has garnered considerable attention. Prolonged exposure to Ni can lead to toxicity, damage to cellular membranes, structural changes in DNA, and even cell death in organisms [5].

In addition to heavy metals, MPs (plastic particles <5 mm in diameter) have emerged as a major environmental threat. These particles are categorized as primary (intentionally manufactured, e.g., microbeads) or secondary (resulting from the fragmentation of larger plastic debris). Composed of polymers like polyethylene (PE) and polypropylene (PP), MPs persist in aquatic systems due to their resistance to degradation .Microplastics are small plastic particles that result from the excessive use of single-use plastics and the degradation of larger plastic debris in the environment. These particles can readily enter aquatic ecosystems and serve as carriers of various pollutants, including heavy metals. Studies have shown that MPs can adsorb heavy metals from water, thereby facilitating their uptake and accumulation by aquatic organisms. Previous studies have reported that polystyrene microplastics can interact with Nickel and alter its mobility or adsorption behavior . These pollutants, both independently and synergistically, pose significant threats to environmental and public health. The ability of MPs to transport and concentrate toxic substances, such as heavy metals, raises concerns regarding their potential to exacerbate the effects of chemical pollutants in aquatic ecosystems [11, 12].

However, most research on environmental pollution has focused on the individual effects of heavy metals or microplastics, with relatively few studies addressing the combined effects of these pollutants in aquatic environments [13]. This gap in the literature arises because biological systems exposed to these pollutants may be affected not only by the individual properties of each pollutant but also by complex interactions among them, leading to nonlinear and sometimes unpredictable effects [14]. Specifically, when these pollutants co-occur in varying concentrations in the environment, they may interact synergistically or antagonistically, altering the toxicity profile of each substance. Such interactions complicate the assessment of the environmental risks posed by these pollutants, necessitating a deeper understanding of their joint toxicity [15, 16].

In this context, more comprehensive and detailed studies of the combined effects of MPs and heavy metals—particularly

their impacts on aquatic organisms-are essential[13]. One of the model organisms frequently studied in this area is *C. carpio* (common carp), which is an ideal model for assessing chemical toxicity due to its high sensitivity to pollutants and its position near the top of the aquatic food chain. The biological effects of these pollutants are often evaluated using biomarkers, including changes in antioxidant levels, oxidative stress, cellular damage, and physical alterations such as weight loss. These biomarkers provide valuable insights into organismal health and serve as tools for assessing the severity of environmental contamination [17].

This study aims to evaluate the combined effects of Ni and MPs on the biological health of *C. carpio*, using biomarkers such as MDA, TAC, and weight loss, and to examine subcellular Ni distribution patterns. These biomarkers serve as indicators of oxidative stress and cellular damage, which can directly or indirectly reflect the level of harm to tissues and cellular structures [18]. Furthermore, this research focuses on the distribution of Ni in three subcellular compartments: the cytosol, debris, and granules, which is crucial for understanding how these pollutants accumulate and interact at the cellular level. This study also explores how the presence of MPs may influence the distribution and bioavailability of Ni within the cells. The hypothesis is that microplastics could enhance metal bioavailability, thereby altering typical patterns of metal accumulation and potentially intensifying the biological impact of pollutants. The interaction between MPs and heavy metals has been shown to disrupt normal accumulation patterns, thereby influencing both the toxicity and distribution of metals within cells [19, 20].

Ultimately, this study investigates the combined effects of Ni and MPs on the biological health of *C. carpio* and explores the use of biomarker response indices (BRI) and effect addition indices (EAI) to assess the severity and interactions of these pollutants. The results from this research will contribute to a more nuanced understanding of the complex interactions between MPs and inorganic pollutants, highlighting the importance of considering their combined effects when evaluating environmental risks in aquatic ecosystems. These findings can also inform strategies for better environmental management and protection against the growing threat of exposure to multiple contaminants.

Materials & Methods

Microplastic Preparation and Characterization

Polystyrene microplastics were obtained as pellets from Tabriz Petrochemical Company (Tabriz, Iran). The pellets were embrittled in liquid nitrogen and pulverized using an industrial grinder at the Iran Polymer and Petrochemical Institute (Karaj, Iran) to produce irregular fragment-shaped particles. The resulting powder was sieved to obtain a particle size fraction of 20–40 µm. Before exposure, the microplastics were dispersed in deionized distilled water and sonicated in an ultrasonic bath (40 kHz) for 30 min to minimize aggregation and ensure a homogeneous suspension [21].

Fish Samples and Toxicity Testing

Juvenile common carp (*C. carpio*; n=390; initial weight: 35.27 ± 1.52 g) were obtained from a certified aquaculture facility with documented water quality (NH_3 <0.02 $mg.L^{-1}$, NO_2^- <0.01 $mg.L^{-1}$). Fish were acclimated for 14 days in 1000-L aquaria

in accordance with OECD guideline 215 [22]. Environmental conditions were maintained at 22 ± 1°C, pH 7.0–7.5, dissolved oxygen >6 $mg \cdot L^{-1}$ (continuous aeration), a 12:12 h light: dark cycle, and 30% water renewal every 2 days. Fish were fed commercial pellets (Behadaneh Co.) twice daily. Ammonia and nitrite levels were measured twice weekly and kept below detectable limits by regular water exchange. Thirteen experimental treatments (10 fish per tank, in triplicate) were established, including four Ni concentrations (0.05, 0.15, 0.20, and 0.25 $mg.L^{-1}$), four MPs concentrations (0.25, 0.50, 0.75, and 1.00 $mg.L^{-1}$), four combined Ni–MPs treatments, and one control group. Dose selection was based on preliminary LC_{50} values and reported environmental concentrations of Ni and microplastics in uncontaminated and contaminated habitats[23, 24]. Final exposure doses were sublethal yet sufficient to induce the study's targeted biomarker responses. Exposures lasted 21 days. At termination, livers were excised, frozen in liquid nitrogen, and stored at −80°C.

Preparation of Liver Tissue Plasma

Livers obtained from various treatments were homogenized with ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5; 0.25 M Sucrose; 1 mM EDTA, pH 7.5; 1:5 tissue weight to buffer volume ratio) using a homogenizer at 10,000 rpm for 40 seconds to obtain a tissue homogenate. The homogenate was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was diluted with deionized distilled water at a 1:4 ratio (supernatant to deionized water). The diluted supernatant (tissue plasma) was used to measure MDA levels and TAC [25, 26].

MDA and TAC Assays

Malondialdehyde levels, as an indicator of oxidative damage to cell membranes, were measured using the thiobarbituric acid (TBA) method proposed by Ohkawa et al. (1978), with minor modifications [27]. In this method, 500 µL of 20% trichloroacetic acid solution and 200 µL of 0.67% TBA solution in water were added to 100 µL of tissue plasma. The mixture was incubated in a water bath at 95°C for 30 minutes. After cooling to room temperature, the mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C.

Total antioxidant capacity was measured using the iron reduction method developed by Benzie and Strain (1996)[28] The basis of this method is the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by reducing molecules present in the biological samples, followed by the formation of a Fe^{2+} complex with the 4,2,6-tris(2-pyridyl)-1,2,5-triazine molecule, resulting in the production of a blue-colored complex. The intensity of this color change indicates the TAC. The working solution was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 10 mM 4,2,6-tris (2-pyridyl)-1,2,5-triazine in 40 mM hydrochloric acid, and 1 volume of ferric chloride (20 mM). A 1.5 mL volume of this working solution was added to the microtubes and incubated for 5 minutes at 37°C in a water bath. Then, 100 µL of tissue plasma obtained from the different treatments was added to each microtube and incubated for an additional 10 minutes at 37°C.

Malonaldehyde bis and $FeSO_4 \cdot 7H_2O$ served as the standards for MDA and TAC, respectively. The MDA and TAC concentrations were determined by measuring the absorbance at 532 nm and 593 nm, respectively, with a spectrophotometer (PG Instruments, UK). The calculations were based on a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}.cm^{-1}$.

Determination of Intracellular Localization of Metals

A fish sample was randomly selected from each treatment group under investigation. The liver tissue was excised from the abdomen and homogenized with an ice-cold buffer at a ratio of 1 (weight) to 5 (volume) using a homogenizer at 1000 rpm for 40 seconds. One milliliter of homogenized liver tissue was collected prior to the separation step to determine the total Ni content. The homogenized tissue was then centrifuged at 10,000 rpm for 30 minutes at 4°C, and the supernatant was collected as the first fraction (cytosol). The pellet, which contained granules, tissue fragments, cell membranes, and intact cells, was subsequently incubated in a boiling water bath for 2 minutes. Four milliliters of 0.5 M sodium hydroxide (NaOH) were added, and the mixture was incubated at 70°C for 1 hour. The solution was then centrifuged at 10,000 rpm for 10 minutes at 20°C. The supernatant was collected as the second fraction (debris), and the pellet was collected as the third fraction (granules). All fractions were stored at -80°C until analysis [29, 30].

For acid digestion of the solid fractions, 100 µl of 60% nitric acid and 40 µl of hydrogen peroxide were added to each milligram of the solid tissue. The samples were left at room temperature for 48 hours. Then, 1 mL of deionized water per milligram of solid tissue was added to the samples. Finally, the samples were completely acid-digested by heating at 110°C for 1.5 hours in an oven. A similar acid digestion procedure was applied to the supernatant samples, assuming that 1 mL of supernatant was equivalent to 1 mg of solid tissue [31]. Ni concentration was measured using an atomic absorption spectrometer (PerkinElmer model AA 700). A Ni standard solution was prepared from a 1000 $mg \cdot L^{-1}$ stock solution, and recovery rates ranged from 96% to 104%. Concentrations were reported as $mg.L^{-1}$ of the digested solution.

Biomarker Response Index (BRI)

The BRI comprises two primary parameters: the score (S), which reflects the Alteration level (AL), and the weight (W) of the biomarker [32].

Biomarker alteration levels are assessed using the BRI and classified as follows: slight (AL ≤ 20%; score = 4) with a BRI of 3.01–4.00, moderate (20% < AL ≤ 50%; score = 3) with a BRI of 2.76–3.00, major (50% < AL ≤ 100%; score = 2) with a BRI of 2.51–2.75, and severe (AL > 100%; score = 1) with a BRI of 1.00–2.50. It is determined using the following equation:

$$AL = \left(\frac{\text{Biomarker}_{\text{treatments}} - \text{Biomarker}_{\text{control}}}{\text{Biomarker}_{\text{control}}} \right) \times 100$$

Eq. (1)

The weights of the biomarkers were assigned based on their biological levels. Piva et al. (2011) report that the weights for TAC, MDA, and biomass reduction are 1, 1.2, and 2.4, respectively [33]. The calculation of BRI is determined using the following equation.

$$BRI = \frac{\sum(\text{Score of Biomarker n} \times \text{Weighting of Biomarker n})}{\sum \text{Weighting of Biomarker n}}$$

Eq. (2)

Based on the calculated BRI, the biological health status of fish can be classified as slight, moderate, major, and severe, as outlined above.

Joint Effect Evaluation

The combined impact of Ni and microplastic on integrated responses (BRI) was evaluated using the EAI as described by Zhang et al. (2012) [34]. The BRI value obtained was then

used to calculate the overall effect (E%) of the treatment:

$$E = \frac{\text{BRI in control group} \times \text{BRI in treatment group}}{\text{BRI in control group}} \times 100 \quad \text{Eq. (3)}$$

$$EAI = 1 - \frac{1-\left(1-E_{Ni-single}\right)\times\left(1-E_{MP-single}\right)}{E_{MP,Ni-mixture}} \quad \text{Eq. (4)}$$

$E_{Ni-single}$ Moreover, $E_{MP-single}$ refer to the total effects of Ni and microplastic when applied individually. $E_{Ni,MP-mixture}$ Refers to the total effect of Ni and microplastic mixture application. An EAI value of 0 indicates an additive effect (zero interaction or no deviation from the expected combined effect), EAI>0 indicates synergism, and EAI<0 indicates antagonism. A zero biological response, however, refers to the absence of a measurable effect relative to the control and should not be confused with EAI = 0.

Statistical Analyses

Data were analyzed using SPSS v.22. Normality and homogeneity of variances were checked using the Shapiro–Wilk and Levene's tests, respectively. The normality of the data was tested using the Shapiro-Wilk test. When necessary, data that did not meet the assumption of normality were appropriately transformed before analysis to satisfy the requirements of parametric statistical testing.

Homogeneity of variances was also assessed prior to conducting parametric tests. Since the datasets met the assumptions required for parametric analysis (after transformation where applicable), differences among experimental treatments were examined using a general linear model (GLM). Post-hoc multiple comparisons were performed using Tukey's honestly significant difference (HSD) test to identify significant differences between groups. In addition, Pearson's correlation analysis (two-tailed) was applied to assess relationships between measured variables. All results are presented as mean ± standard error (SE), and statistical significance was defined at $p < 0.05$

Findings

Subcellular Partitioning

Nickel distribution across subcellular fractions (cytosol, debris, granules) and recovery rates in the livers of juvenile common carp (*C. carpio*) are shown in Table 1. Total Ni bioaccumulation increased significantly with exposure concentration ($p<0.05$). Cytosolic Ni predominated across all treatments (cytosol > debris > granules), representing the highest percentage of recovered metal. Co-exposure with MPs significantly increased Ni retention in the debris fraction ($p<0.05$), shifting partitioning from cytosol toward debris.

Subcellular Fractions and Relationships with Biomarkers

A statistical association was observed between Ni concentrations in subcellular fractions and oxidative stress biomarkers, as indicated by Pearson correlation coefficients (Table 2). Significant positive correlations were found between debris-associated Ni and both MDA ($r=0.673$, $p<0.01$) and TAC ($r=0.587$, $p<0.01$), suggesting a strong statistical relationship between Ni partitioning in the insoluble fraction and these biomarkers. At the same time, total internal Ni burden also correlated significantly with MDA ($r=0.518$, $p<0.01$) and TAC ($r=0.374$, $p<0.05$). In contrast, Ni levels in the cytosol and granules did not show significant statistical associations with the measured oxidative stress markers ($p>0.05$).

Table 1) Distribution of Ni among subcellular fractions (cytosol, debris, granules) in the liver of juvenile *C. carpio* following a 21-day exposure to Ni (mg.L^{-1}) and Ni (mg.L^{-1})+ MPs (mg.L^{-1}) combinations (n=10 fish/tank, triplicate).

Fraction	Exposure Dose of Ni	Ni Value (mg.L^{-1})	Exposure Dose of Ni+Mps	Ni Value (mg.L^{-1})
Cytosol	Control	43±2.54^{a}	Control	43±2.54^{a}
Debris		52±1.68^{b}		52±1.68^{b}
Granule		14±0.30^{a}		14±0.30^{a}
Sum		109		109
Cytosol	0.05	52±6.26^{a}	0.05+0.25	47±5.23^{a}
Debris		45±2.5^{b}		50±2.1^{b}
Granule		15±0.37^{c}		15±0.47^{c}
Sum		112		112
Cytosol	0.15	56±5.27^{a}	0.15+0.50	49±4.32^{a}
Debris		47±1.72^{b}		53±2.62^{b}
Granule		16±0.4^{c}		16±0.3^{c}
Sum		119		118
Cytosol	0.20	55±4.73^{a}	0.20+0.75	51±4.62^{a}
Debris		51±3.14^{b}		55±1.62^{b}
Granule		10±0.6^{c}		13±0.5^{c}
Sum		116		119
Cytosol	0.25	58±4.53^{a}	0.25+1.00	43±6.74^{a}
Debris		50±3.56^{b}		56±2.43^{b}
Granule		9±1.16^{c}		12±0.5^{c}
Sum		117		111

Note: Different letters indicate statistically significant differences among treatments ($p<0.05$).

Table 2) Pearson's correlation coefficients between malondialdehyde (MDA), total antioxidant capacity (TAC), and Ni concentrations in subcellular fractions of the liver, including the cytosol (cyt), debris (deb), and granules (gran), as well as whole-body internal (int) concentration in juvenile *C. carpio*.

Fraction	MDA	TAC
Cyt	0.321	0.227
Deb	0.673**	0.587**
Gran	0.074	0.123
Int	0.518**	0.374

Note: **$p < 0.01$. n=30 per treatment group (10 fish/tank, triplicate).

Biomarker Response Index (BRI)

Biomarker Responses and Alteration Levels (Als)

Figures 1 and 2 show biomarker responses in *C. carpio* after 21 days of exposure to Ni (0.05–0.25 mg.L^{-1}), MPs (0.25–1.00 mg.L^{-1}), and their combinations. Single and combined exposures significantly altered all examined biomarkers compared with controls ($p < 0.05$).

Both biomarker responses and weight loss (WL) were normalized as % relative to the control (100% = control mean; Figures 1-2), so control bars are not shown separately, as they represent the 100% baseline. All treatments were compared against this control baseline.

The level of MDA in *C. carpio* exposed to Ni, MPs, and their mixture revealed a distinct dose-effect pattern for all three exposure modes, with the peak being detected at higher doses for Ni, MPs, and their combination, as shown in Fig. 1 (a). A higher significant ($p<0.05$) increase was observed under the dosage of 0.25 mg.L^{-1} for Ni; 1.00 and 1.00 mg.L^{-1} for MPs; and 0.25+1.00 mg.L^{-1} for binary exposure related to other treatments, and corresponding ALs (shown in Table 3) showed negligible (20%-50%); moderate (0%-20%) and negligible (20%-50%) deviation, respectively.

According to the data presented in Figure 1 (b), it can be observed that the TAC in *C. carpio* exhibited a significant increase ranging from 6% to 27%, 3% to 8%, and 8% to 35% in response to the individual exposure of Ni, MPs, and their combination, respectively, when compared to the control group ($p<0.05$). Additionally, the ALs of these substances demonstrated moderate (20% to 50%) for Ni and the combination of

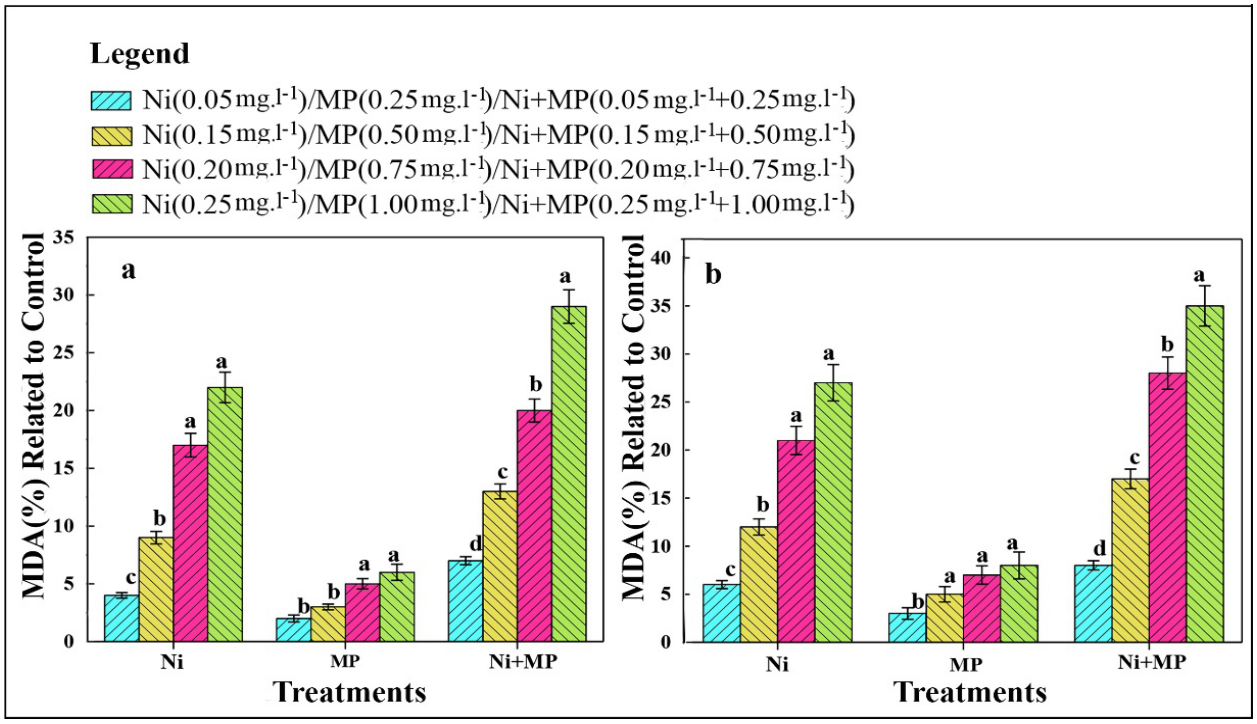

Figure 1) Malondialdehyde (MDA) and total antioxidant capacity (TAC) (normalized to control = 100%; no separate control bar shown) in the liver of juvenile *C. carpio* following a 21-day exposure to Ni (0.05, 0.15, 0.20, and 0.25 mg.L^{-1}), microplastics (MPs; 0.25, 0.50, 0.75, and 1.00 mg.L^{-1}), and and their combined treatments (n=10 fish/tank, triplicate). **Note:** Data are expressed as percentages of the control (control = 100%); therefore, no separate control bar is shown, and the horizontal reference line represents the control baseline. Different letters indicate statistically significant differences from the control ($p<0.05$).

both substances, and negligible (0% to 20%) for MPs, as indicated in Table 3.

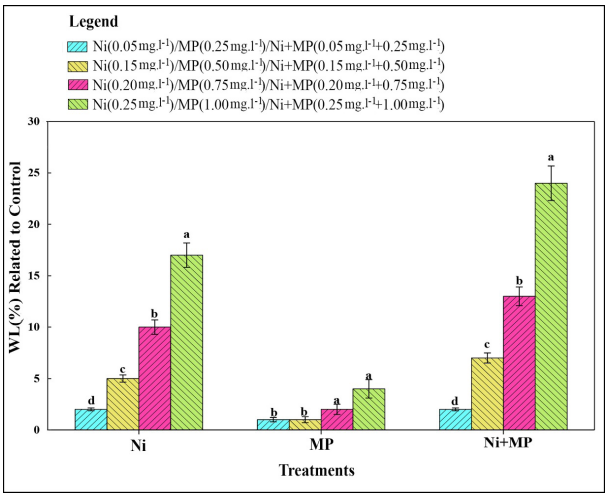

Figure 2) Changes in body weight (g) of juvenile *C. carpio* following a 21-day exposure to Ni (0.05, 0.15, 0.20, and 0.25 mg.L^{-1}), microplastics (MPs; 0.25, 0.50, 0.75, and 1.00 mg.L^{-1}), and combined Ni–MPs treatments. Data are presented as mean ± SD for 30 fish per treatment group (n=10 fish/tank, triplicate).

Note: Data are expressed as percentages of the control (control = 100%); therefore, no separate control bar is shown, and the horizontal reference line represents the control baseline. Different letters indicate statistically significant differences from the control ($p<0.05$).

Malondialdehyde (MDA) levels showed clear dose-response relationships across Ni, MPs, and Ni+MPs exposures, peaking at the highest concentrations (0.25 mg.L^{-1} Ni, 1.00 mg.L^{-1} MPs, 0.25 mg.L^{-1} Ni + 1.00 mg.L^{-1} MPs; Figure 1a). Significant increases occurred even at lower concentrations, indicating high sensitivity of lipid peroxidation to both individual and combined contaminants. Corresponding alteration levels (AL%) for MDA ranged from negligible (≤20%) to moderate (20–50%), with greater deviations under Ni+MPs co-exposure (Table 3).

Total antioxidant capacity (TAC) increased significantly above controls by 6–27% (Ni), 3–8% (MPs), and 8–35% (Ni+MPs) treatments (Figure 1b; $p < 0.05$). TAC enhancement was most pronounced under combined exposure, reflecting compensatory activation of antioxidant defenses against intensified oxidative stress. Alteration level analysis showed moderate deviations (20–50%) for Ni and Ni+MPs exposures, but negligible changes (≤20%) for MPs alone (Table 3).

Table 3) Biomarker alteration level (AL, %) and corresponding severity score (S) in juvenile *C. carpio* following a 21-day exposure. Biomarker alteration levels were classified as follows: **Slight** (AL ≤ 20%; score = 4), **Moderate** (20% < AL ≤ 50%; score = 3), **Major** (50% < AL ≤ 100%; score = 2), and **Severe** (AL > 100%; score = 1). T1-T4 represent Ni treatments (0.05, 0.15, 0.20, and 0.25 mg.L^{-1}) and microplastics (MPs) treatments (0.25, 0.50, 0.75, and 1.00 mg.L^{-1}), respectively.

Biomarker	Treatment Style	T1	S	T2	S	T3	S	T4	S
		AL%		AL%		AL%		AL%	
MDA	Ni	4	4	9	4	17	4	22	3
	MPs	2	4	3	4	5	4	7	4
	Ni+ MPs	7	4	13	4	20	4	29	3
TAC	Ni	4.95	4	10.89	4	19.80	4	25.74	3
	MPs	1.98	4	3.69	4	5.94	4	6.93	4
	Ni+ MPs	6.93	4	15.84	4	26.73	3	33.66	3
WL	Ni	158.17	1	614.29	1	1328.57	1	2328.57	1
	MPs	42.86	3	42.86	3	185.71	1	471.43	1
	Ni+ MPs	185.71	1	900	1	1757.14	1	3328.57	1

Table 4) Biomarker Response Index (BRI) and Environmental Alteration Index (EAI) in juvenile *C. carpio* following a 21-day exposure to Ni, microplastics (MPs), and combined Ni–MPs treatments. T1-T4 represent Ni treatments (0.05, 0.15, 0.20, and 0.25 $mg.L^{-1}$) and microplastics (MPs) treatments (0.25, 0.50, 0.75, and 1.00 $mg.L^{-1}$), respectively.

Treatment	T1	T2	T3	T4
Single Ni BRI (ENi-Single)	2.25 (1.25%)	2.25 (1.25%)	2.25 (1.25%)	1 (1%)
Single MP BRI (EMp-Single)	2.5 (1.5%)	2.25 (1.25%)	2.25 (1.25%)	2.25 (1.25%)
Combined Ni and MP BRI (ENi+MP-Mixture)	2.25 (1.25%)	2 (1%)	2 (1%)	1.75 (0.75%)
EAI	1	0.06	0.06	-0.25
Joint Effect	Synergism	Synergism	Synergism	Antagonism

Table 3 summarizes the alteration levels (AL%) and severity scores (S) for all assessed biomarkers. Among the endpoints evaluated, weight loss (WL) showed the most severe alterations, with AL% consistently exceeding 100% in the Ni and Ni+MPs treatments (S = 1), suggesting that WL is a highly sensitive physiological indicator in this exposure scenario. In contrast, oxidative stress biomarkers (MDA and TAC) exhibited lower severity scores (S = 3–4), indicating that although oxidative damage and antioxidant responses were evident, their magnitude remained moderate compared with whole-organism physiological responses, particularly under combined exposure. Weight loss in *C. carpio* displayed clear dose–response relationships across single Ni, single MPs, and combined Ni+MPs treatments (Figure 2). Notably, all AL% values for weight loss in Ni-containing treatments—both individual and combined—reached severe levels (>100%), as shown in Table 3.

BRI and EAI

Table 4 presents BRI values and EAI for *C. carpio* after 21 days of exposure to Ni, MPs, and Ni+MPs combinations. BRI values ranged from 1.00 to 2.50, indicating severe health alterations across all treatments (Table 3 classification). No consistent dose-response trend was observed in BRI values. EAI values ranged from -0.25 to 1.00, demonstrating synergistic interactions at lower concentrations (T1–T3; EAI > 0) and antagonism at the highest concentration (T4; EAI = -0.25). These results are consistent with the hypothesis of concentration-dependent joint toxicity in Ni+MPs mixtures.

Discussion

Biomarker Response and EAI

While a single biomarker cannot accurately predict the effects of stress, analyzing multiple biomarkers provides a more quantitative assessment. Indexes such as the BRI provide a more comprehensive assessment of stress on species [35]. In this study, the biological health of *C. carpio* was assessed using the BRI method, incorporating varying levels of Ni and MPs. Key indicators included MDA, TAC, and weight loss. Except for the weight-loss biomarker, the use of Ni, MPs, and their combination resulted in negligible to moderate alterations in *C. carpio.* However, when we examine it from the perspective of the biomass change biomarker, we find that

this score reflects the severity of the impact. This was due to the relatively high weight and importance of the weight loss biomarker in the BRI.

The results indicate that while MDA and TAC showed only minor to moderate changes, they did not strongly reflect the severity of stress in *C. carpio*. These biomarkers, typically used to assess oxidative stress, may not have been sufficiently sensitive in this case to detect significant physiological effects, possibly due to compensatory mechanisms in the fish [36]. In contrast, weight loss, a key component of the BRI, provided a clearer indicator of stress severity. As a direct measure of biomass change, it more accurately reflected the fish's overall health, highlighting the importance of incorporating weight loss into stress assessments. This suggests that while oxidative stress markers such as MDA and TAC can provide insights into biological responses, biomarkers of energy balance, such as weight loss, are more reliable for assessing the overall impact of environmental stressors like Ni and MPs. Similar to our study, Gashkina (2024) [37] and Naz and Chatha (2022) [38] reported that weight loss is a reliable marker.

Traditional methods for assessing the joint toxicity of chemical mixtures often rely on fixed-effect concentrations, such as LC_{50}, to characterize their impact. However, these approaches fail to capture the dynamic nature of toxicity interactions across varying exposure levels. To address this limitation, we introduced the joint effect index (EAI) as a more nuanced tool to examine the interaction between MPs and Ni across a spectrum of effect levels, allowing for a more comprehensive understanding of mixture toxicity. According to the EIA, the combined effects shifted from synergism to slight antagonism at higher doses, a transition that may result from multiple underlying mechanisms in the Ni-MPs co-exposure system. First, receptor saturation (e.g., metal transporters or MPs surface adsorption sites) at elevated concentrations could trigger competitive binding, reducing bioavailable fractions of toxicants. This aligns with reports by Li et al. (2022)[39] and Fortin et al. (2024)[40], who documented contrasting interaction patterns in different experimental systems. Second, metabolic feedback loops, such as upregulation of detoxification pathways (e.g., CYP450 enzymes), might accelerate pollutant degradation at high doses, diminishing cumulative effects.

The discrepancy between the anticipated Trojan horse effect and the observed antagonism could stem from altered uptake-excretion dynamics.

The synergistic interaction at low concentrations highlights the potential role of MPs as vectors that facilitate Ni bioavailability. This interpretation is supported by the shift in Ni partitioning toward the debris fraction in co-exposed fish, which is strongly associated with increased oxidative stress markers (MDA and TAC). While the internal detoxification system, such as metal-binding proteins, likely manages high-dose exposures—leading to the observed antagonism—the integrated BRI and EAI values provide a robust framework for assessing these complex interaction patterns.

For example, Liu et al. (2024) reviewed how MPs can influence the mobility, bioavailability, and toxicity of heavy metals, highlighting that the combined effects can be synergistic or antagonistic depending on environmental conditions and pollutant

concentrations [16]. Furthermore, Shi et al. (2025) emphasized that system-specific factors such as pollutant ratios, species sensitivity, and exposure duration critically determine whether synergism or antagonism dominates in co-exposure scenarios [41].

Consistent with our findings, other studies have also observed varying interactions between two different contaminants at different dosage levels. For example, Li et al. (2019) report clear antagonism at relatively low doses and synergism at higher doses [32]. Research on the combined toxicological effects of MPs and other pollutants is still in its infancy and needs further advancement. The combined toxicity of MPs and inorganic pollutants has been reported to result in both synergistic [42] and antagonistic [43, 44] effects.

Subcellular Partitioning

This study partitioned the total body Ni load into three compartments: intracellular cytosol (soluble), granules, and debris (insoluble). The recovery of the initial Ni homogenate concentration was nearly 100%, suggesting that a substantial amount of the metal was not lost during compartmentalization within intracellular sites. Recovery of initial metal concentrations during the intracellular compartmentalization process in fish is rarely reported in the literature.

The results of this study demonstrate the compartmentalization of Ni bioaccumulation in the model organism, with the highest levels observed in the cytosol, followed by debris and granules. This pattern suggests that the cytosol plays a crucial role in the storage and/or processing of metal ions, possibly due to Ni's solubility and bioavailability in this compartment. Several studies have indicated that the cytosol is the primary site of metal ion accumulation, which may be attributed to intracellular sequestration by metal-binding proteins such as metallothionein (MT). While MT concentrations were not quantified in the present study, its involvement represents a plausible explanation, given its well-established role in cytosolic metal detoxification and storage. [45, 46]. Additionally, the cytosolic compartment often serves as a buffer, helping the cell cope with metal stress by preventing toxic metal concentrations in more sensitive cellular structures [47]. Similarly, Giguère et al. (2006) found a higher concentration of Ni in the cytosol [48].

The lower levels of Ni found in the debris and granules compartments suggest that these areas may be less favorable sites for Ni accumulation, potentially due to lower metal affinity or distinct mechanisms of metal sequestration [49]. It is worth noting that granules, which are involved in cellular storage processes, might also be less efficient at retaining metals than the cytosol [30], or they may sequester metals in forms that are not easily detectable by our assay methods. The simultaneous exposure to Ni and MPs, which led to increased Ni accumulation in the debris compartment, warrants further exploration. Microplastics act as vectors for metal transport due to their large surface area and ability to adsorb various environmental contaminants, including heavy metals [50].

This pattern is consistent with the hypothesis that MPs may influence Ni distribution and bioavailability, potentially facilitating its retention in different cellular compartments. Furthermore, MPs have been shown to induce oxidative stress and alter cellular mechanisms of metal homeostasis, potentially influencing

the distribution and compartmentalization of metals such as Ni [51, 52].

The increased accumulation in the debris compartment suggests a potential shift in metal partitioning; however, whether this is due to changes in cellular metabolism or to Ni-MP interactions remains a hypothesis requiring further direct evidence. [53]. This finding aligns with recent studies suggesting that microplastic exposure can disrupt normal metal bioaccumulation patterns, thereby influencing their toxicity and distribution [53, 54].

The results of this study are consistent with the findings of Gefard et al. (2010), who reported a significant recovery of Ni in the debris fraction of *Gammarus* sp. cells [55]. Similarly, Giguère et al. (2006) also found high levels of Ni in the debris fraction of the yellow perch (*Perca flavescens*) cells[48].

Subcellular Partitioning and Relationship with Biomarkers

Our results demonstrate a significant correlation between the accumulation of Ni in the debris fraction (insoluble component) and increased MDA and TAC levels, both of which are markers of oxidative stress. This suggests a significant statistical association between Ni accumulation in the debris fraction and oxidative stress markers. While this link is strong, it may indicate that the debris fraction is a site where Ni is sequestered concurrently with cellular injury, rather than being the sole direct cause of it. Previous studies have shown that metal accumulation in insoluble cellular compartments correlates with enhanced oxidative stress [56]. This finding aligns with the notion that metal exposure in aquatic organisms leads to chronic oxidative stress when metals are not efficiently removed or sequestered [57].

In contrast, we did not observe significant correlations between Ni concentrations in the cytosol or granules and the oxidative stress biomarkers. This could be due to the distinct roles these cellular compartments play in metal detoxification and sequestration [58, 59]. The cytosol, which contains high-affinity metal-binding ligands such as metallothioneins, may act as a buffer, preventing metals from reaching more reactive forms that could induce oxidative damage. Studies have shown that metallothioneins bind metals such as Ni, thereby minimizing their toxicity by preventing free metal ions from interacting with cellular macromolecules [47]. Similarly, granules are thought to store metals in less bioavailable forms, such as metal–protein complexes or metal salts, reducing their ability to generate ROS and contribute to oxidative stress. The lack of correlation between Ni in these compartments and oxidative stress markers suggests that these regions may act as sequestration sites, where the metal is effectively immobilized, preventing it from inducing further cellular damage [60].

Study Limitations and Future Perspectives

While the present study offers valuable insights into the combined toxicological effects of Ni and MPs, certain methodological limitations warrant attention. A primary limitation is the lack of direct evidence regarding the adsorption–desorption kinetics between Ni and the MP surfaces. Because we did not perform direct measurements of Ni associated with MPs or assess the distribution of dissolved versus particle-bound metal ions, the proposed mechanism of enhanced bioavailability—often referred to as the 'Trojan horse effect'—remains a hypothesis in this study.

The synergistic interactions observed, particularly at lower concentrations, are consistent with this theory, but they do not provide definitive proof. Future research should prioritize kinetic studies and direct quantification of metal-microplastic complexes to validate these mechanistic pathways and more accurately distinguish between potential vector-mediated uptake and independent concentration-dependent toxicity.

Conclusion

In this study, we examined the combined effects of Ni and MPs on the biological health of *C. carpio* using biomarkers and a joint effect index (EAI). Our findings highlight weight loss as a sensitive indicator of stress. In addition, the EAI revealed a shift from synergistic to slightly antagonistic effects at higher Ni and MPs concentrations, suggesting dose-dependent toxicity interactions. The subcellular partitioning of Ni showed the highest accumulation in the cytosolic fraction, which may reflect intracellular sequestration processes; however, the possible involvement of metallothionein was not directly examined and remains to be confirmed. Increased Ni levels in the debris fraction under co-exposure suggest that MPs may influence Ni distribution and bioavailability within cells. Overall, these findings improve our understanding of Ni–MP interactions and support the need for further studies on the joint toxicity of microplastics and inorganic pollutants in aquatic organisms.

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