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The Effect of Single and Combined Stress of Microplastics and Heavy Metals on Growth, Biochemical Components and Antioxidant Activity of *Lemna gibba* (Duckweed)

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ABSTRACT
The increasing presence of microplastics (MPs) and heavy metals (HMs) in aquatic ecosystems poses a growing concern due to their potential ecotoxicological effects. While the individual toxicity of MPs and HMs has been widely investigated, limited attention has been given to their combined effects on aquatic macrophytes. In this study, we aimed to evaluate the single and combined impacts of two types of MPs [polypropylene (PP) and acrylonitrile butadiene styrene (ABS)] at concentrations of 25, 50, and 100 mg L^{-1}, along with three typical HMs (Zn^{2+}, Cu^{2+}, and Ni^{2+}), on the growth, biochemical components, and antioxidant activity of the model macrophyte *Lemna gibba* under laboratory conditions over a 7-day exposure period. The results revealed that both contaminants alone negatively impacted growth and biochemical performance, but the combined application caused a more pronounced decrease, suggesting a synergistic inhibitory effect on plant metabolism. The simultaneous application of ABS-MP with nickel, copper, and zinc resulted in more pronounced adverse effects on *L. gibba* growth parameters, photosynthetic pigments, and carbohydrate content compared to single-pollutant exposures. Co-application of copper and nickel induced pronounced oxidative stress in plant tissues, as evidenced by increased malondialdehyde levels. Furthermore, significant reductions were observed in total protein, total phenolic, and total flavonoid content across all treatments. Conversely, total antioxidant activity showed variable results dependent on the specific contaminant and concentration applied. These findings provide preliminary evidence that co-occurring MPs and HMs may exert additive or synergistic stress effects on aquatic macrophytes. In particular, the comparative evaluation of ABS and PP microplastics, along with Cu, Zn, and Ni treatments, highlights polymer- and metal-specific toxicity patterns and integrated antioxidant response profiles that have not been previously reported for *L. gibba*.

Keywords: *Duckweed bioassay*, Microplastic–metal interaction, Synergistic stress, Biochemical response

1. Introduction

Plastics, widely used as basic components in industrial and commercial applications due to their durability, economy and ease of use, have become an increasing environmental problem worldwide due to intensive production and improper disposal (Fayshal 2024). Microplastics, defined as small pieces of plastic waste ranging from 1 µm to 5 mm in size that decompose in the aquatic environment or microbeads and resin particles in commercial facial cleansers, have attracted worldwide regard due to their ubiquity in the ecosystem and unidentified dangers to living things (So et al. 2018; Frias & Nash 2019).

Plastic polymers such as polyvinyl chloride (PVC), polyolefin (PO), polyethylene (PE) and polypropylene (PP) are the most widely used thermoplastic family. Thermoplastics are recyclable polymers that can be melted and re-hardened after cooling. Different types of plastics are produced, especially as a result of the polymerization of ethylene and propylene. These are the most widely produced synthetic plastic polymers today due to their versatility (Anonymous 2024a). Acrylonitrile Butadiene Styrene (ABS) is another recyclable plastic polymer that is widely used as an engineering material in the automotive and home appliance industries, refrigeration equipment, toys, and sporting goods due to its combination of high strength and high impact resistance (Anonymous 2024b). Microplastics such as polyethylene (PE), polypropylene (PP), acrylonitrile butadiene styrene (ABS) and polystyrene (PS) have large specific surface areas and functional groups. This situation negatively affects the lives of living things by easily transferring microplastics to the food chain (Bradney et al. 2019; Zhang et al. 2020). Microplastics can absorb and accumulate pollutants in the aquatic environment due to their small size, porosity, large specific surface areas and high hydrophobicity. Recently, the retention of heavy metals, especially by microplastics, has become a remarkable issue studied and investigated by researchers (Gao et al. 2019; Dong et al. 2020; Santos-Echeandía et al. 2020; Santos- Gao et al 2021). Since microplastics can act as carriers of heavy metals on their own, the effect of these two pollution factors on living things becomes more complicated due to the interaction. Studies have shown that microplastics form microbial biofilms. This formation provided

an ecological niche for microorganisms. In addition, it has been determined that these biofilms may affect the absorption of chemical pollutants such as heavy metals or microplastics. In short, the effects of MPs on HMs are still a subject that is being researched because they are not fully understood (Tu et al. 2020; Yang et al. 2020).

Heavy metals, another pollutant commonly found in the environment, such as microplastics, are natural, anthropogenic, or industrial pollutants. Due to their non-degradable structure, they can constantly enter water areas and are enriched by recycling in the aqueous environment (Vinodhini & Narayanan 2009). Heavy metals such as copper, nickel, cadmium and zinc, which are toxic or carcinogenic in nature, pose a threat to living beings and the environment by negatively affecting photosynthesis, chlorophyll production, pigment synthesis, nervous system and enzyme activity in plants, animals and humans (Cao et al. 2025). Studies on MPs and HMs contamination, mostly caused by industrial emissions, have proven the presence of this pollution in the ocean, sea, lake, river, soil and atmosphere. As a result of these pollutants, aquatic organisms may slow their growth or even die. In addition, these pollutants can threaten human health through nutrition and drinking (Senturk & Yildiz 2016; Cao et al. 2021; Luo et al. 2021; Yu et al. 2024).

However, the ability to adapt to pollution stress may develop throughout the experiment between the pollutant and the organism used to clean the pollutant. *Lemna* sp. (Duckweed, Linnaeus 1753, *Lemnaceae*), a perennial aquatic plant widespread almost everywhere from temperate to tropical regions, is a flowering macrophyte frequently used for eco-environmental toxicity assessment as it is free-floating, fast-growing, high in nutritional value (proteins, vegetable fibres, fats, polysaccharides, flavonoids, amino acids, phenolic acids, vitamins), small in size and easy to cultivate. In addition, duckweed serves as a food source for waterfowl and a shelter for aquatic invertebrates (Tkalec et al. 1998; Vladimirova & Georgiyants 2014). As it is known, heavy metals, microplastics and other pollutants cause oxidative stress by affecting the metabolic functions of aquatic plants. Changes in the amount of total antioxidant enzymes, growth rate, total protein, malondialdehyde (MDA) and total carbohydrate reflect the unstable state of the cell under pollutant stress, and these parameters can be used to determine oxidative stress in aquatic plants (Banu Doganlar 2013; Celekli & Bulut 2020; Liu et al. 2021; Celekli & Alkan 2024). Despite the existing literature establishing the individual toxicity of MPs and HMs, a significant knowledge gap persists regarding the interactive ecotoxicological effects of diverse polymer types and key heavy metals prevalent in aquatic environments. Specifically, studies comparing the toxicity of Acrylonitrile Butadiene Styrene (ABS), a widely used but less-studied polymer, against common types like Polypropylene (PP) under co-exposure scenarios remain scarce. Furthermore, few studies simultaneously rank the comparative toxicity of multiple heavy metals (Ni, Cu, Zn) alongside MPs under identical experimental conditions while integrating a full suite of physiological, metabolic, and antioxidant defense endpoints. Therefore, this study aims to address these deficiencies and investigate the single and combined toxic effects of PP and ABS microplastics with Zn, Cu, and Ni on the growth and comprehensive biochemical profiles of the model macrophyte *L. gibba*. We pose two primary research questions: (1) Do different MP polymers (ABS vs. PP) exhibit distinct and differential effects on HM toxicity? and (2) Does the co-exposure of MP and HM result in an additive or synergistic toxic effect on *L. gibba*'s metabolism? We hypothesize that the combined exposure will lead to a synergistic inhibition, primarily driven by ABS microplastics acting as a vector to enhance the bioavailability and subsequent oxidative stress induced by the heavy metals.

2. Material and Methods

2.1. Organism, Medium and Pretreatment

Our study was carried out using an aseptic population of whole *L. gibba* plants were collected in April (2024) from the Yagcilar area of Manisa province, Türkiye (38°40′31″ N, 27°17′31″ E). It was separated from other plants and placed into glass sterile aquariums containing Swedish standard (SIS) sterile culture medium described in the OECD guideline 221 (2006), supplemented with 2% (w/v) sucrose (molar concentrations of aforementioned media are shown in Table 1). pH of media was set to 6-7 prior autoclaving (121 °C, 1 kg cm^{-2} s^{-1} for 20 min). Approximately 200 g of fresh mass was transferred 10 L glass container in an SIS growth medium. Plants were cultivated in a controlled environment room at 25 °C ± 2 °C with a day-to-night cycle of 16:8 h (photon flux 50 µmol m^{-2} s^{-1}) for approximately 1 week. After cultivation, the plants were treated with different concentrations of heavy metal and microplastic aqueous solution for 7 experimental days. A 7-day exposure period was chosen to capture early responses of *L. gibba* to metal and microplastic stress, allowing assessment of acute to sub-chronic effects. The frond diameter and root hyphae length of *L. gibba* were determined to be approximately 2-3 mm and 1.5-2 mm, respectively. The duckweed biomass was harvested at the end of the experiment using a strainer to remove fronds and then analyzed for recording growth and biochemical characteristics (Figure 1).

Table 1- Composition of Swedish standard (SIS) medium solution utilized for the cultivation of *L. gibba*

Medium composition	*Final concentration (1 L)*
$NaNO_3$	0.305 M
$K_2HPO_4.3H_2O$	6.13 mM
$CaCl_2.2H_2O$	29.9 mM
$MgSO_4.7H_2O$	60.85 mM
$MgCl_2.6H_2O$	60.0 mM
H_3BO_3	3.07 mM
$NaHCO_3$	178.8 mM
$Na_2MoO_4.2H_2O$	30.17 µM
$FeCl_3.6H_2O$	0.761 mM
$MnCl_2.4H_2O$	0.805 mM
$ZnCl_2$	0.024 mM
$CuCl_2.2H_2O$	0.070 µM
$CoCl_2.6H_2O$	5.884 µM
Na_2EDTA	0.806 mM

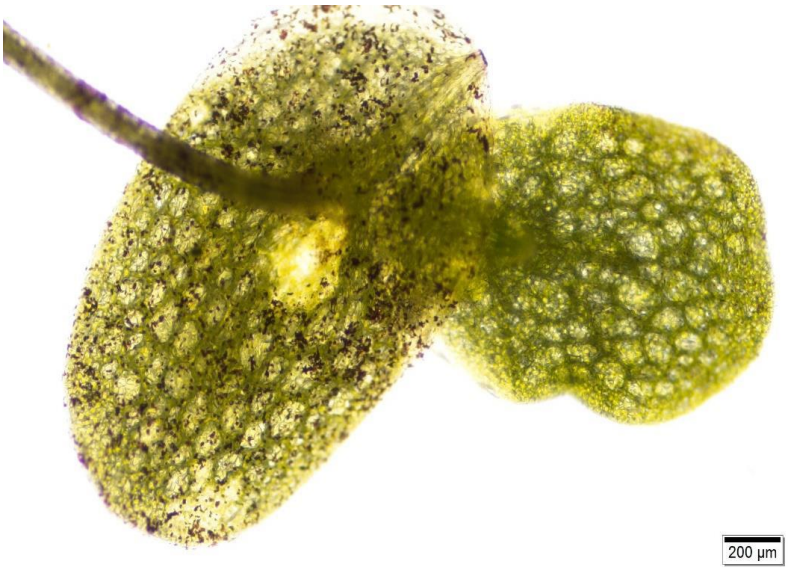


Figure 1- The roots and fronds of *L. gibba* under the stereo microscope (Olympus CX21) with an objective magnification of 4x.

2.2. Cultivation of duckweed

Pure ABS (size range: 100-350 µm) and PP plastics (size range: 100-150 µm) of different sizes were purchased from RC Mold Design (Manisa, Türkiye). Then, the ABS-PP particles were washed with 70% ethanol twice and double-distilled water to remove the dust and other contaminants, and dried at 40 °C overnight before the experiments (Chen et al. 2020). Zinc (Zn^{2+}), copper (Cu^{2+}) and nickel (Ni^{2+}) stock solutions (1000 mg L^{-1}) were prepared by diluting $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $NiSO_4.6H_2O$ (Sigma-Aldrich) in deionized water.

For each treatment, 5 g ± 0.5 (fw) of *L. gibba* was transferred into 200 mL of SIS medium containing the designated pollutant concentrations. Each treatment was performed in three independent biological replicates (n = 3), each consisting of ten healthy fronds randomly selected from the stock culture. The containers were randomly arranged within the growth chamber and repositioned daily to minimize positional bias. Three environmentally relevant concentrations (25, 50, and 100 mg L^{-1}) were selected based on previous reports on MP and HM contamination levels in aquatic environments (Ogo et al. 2022; Rozman et al. 2022; Ceschin et al. 2023), representing low to moderately polluted conditions frequently detected in industrial and urban effluents. Plants were cultivated under controlled conditions (25 ± 2 °C, 16:8 h light–dark cycle, 50 µmol m^{-2} s^{-1} photon flux) for seven days. Growth, pigment, carbohydrate, protein, and antioxidant parameters were then analyzed as described below.

Microplastic sizes and heavy metal weights were measured using a precision electronic balance (Mettler Toledo AL 204, Switzerland, Zurich) and a binocular microscope (Olympus CX21 with an objective magnification of 40x). Before exposure, mixed MP–HM solutions were pre-equilibrated for 24 h under continuous agitation (150 rpm) to allow for potential adsorption equilibrium between particles and metal ions. The pH of all media was adjusted to 6.5 ± 0.2 using 0.1 N NaOH and monitored throughout the experiment to prevent pH-induced osmotic artifacts. The study was divided into the following 22 groups: blank (Control), Zn, Cu, Ni, PP, ABS groups and Zn-PP, Zn-ABS, Cu-PP, Cu-ABS, Ni-PP and Ni-ABS groups (1:1 ratio). MPs and HMs doses used in the study are environmentally relevant. Based on the preliminary tests, we determined the concentration of heavy metals and microplastics to be applied together. 100 mg L^{-1} concentration was chosen in the experiment because the

growth of the organism exposed to high concentrations of heavy metals and microplastics was negatively affected. The specified pollutant concentrations were chosen to be low and high levels of pollutants.

2.3. Relative growth rate (RGR d%)

After 7 experimental days, all the treated *L. gibba* fronds were collected, and washed with ultra-pure water, and absorbent paper (filter 122) was used to remove the water on the surface of duckweed samples. The growth of *L.gibba* was estimated by fresh weight changes using a precision electronic balance (Mettler Toledo AL 204, Switzerland, Zurich). The relative growth rate (RGR) was calculated as follows (Glenn & Doty 1992).

$$RGR\ (d\%) = (\frac{In\ M_t - In\ M_0}{t}) \times 100$$

Where M_0 (g fw) is the initial fresh algal biomass; M_t (g fw) is the fresh mass after t days of cultivation and t is the exposure time (day).

2.4. Photosynthetic pigment analysis (mg g^{-1})

0.1 g (fw) of plant material was homogenized in a porcelain mortar with 10 mL of 96% (v/v) acetone. The resulting extract was filtered using filter paper. The final volume comprised the amount of acetone added at the beginning. Pigment extraction was completed by leaving it in a dark environment of 4 ºC for 24 h. At the end of the period, the samples were centrifuged at 10000 rpm for 4 min. Supernatants were calculated by measuring at 665, 649 and 470 nm wavelength in the spectrophotometer (Varian Cary 50 UV–Vis spectrophotometer) (Song et al. 2006). Chlorophyll-a (Chl-*a*), Chlorophyll-b (Chl-*b*) and total carotenoids (Cd) values were calculated with the absorbance values obtained (Lichtenthaler 1987) and expressed as mg of chl-*a* or chl-*b*/g of fw.

$$Chl - a\ =\ (13.95\ \times\ A665) - (6.68\ \times\ A649)$$

$$Chl - b\ =\ (24.96\ \times\ A649) - (7.32\ \times\ A665)$$

$$Cd\ =\ [(1000\ \times\ A470) - (2.05\ \times\ Chl - a) - (114.8\ \times\ Chl - b)]/245$$

2.5. Total carbohydrate analysis (mg mL^{-1})

Total carbohydrate contents (CH) were measured using the phenol-sulfuric acid assay, a simple and rapid colorimetric method to determine total carbohydrates (DuBois et al. 1956). Briefly, 5% (w/v) phenol solution was prepared in distilled water. 0.1 g (fw) of test samples and 5 mL of concentrated sulfuric acid were added to 1 mL of phenol solution. The mixture was stirred for 30 min. 1 mL aliquots of the cultures were used to quantify spectrophotometrically at 490 nm. The D-glucose (0.05, 0.10, 0.20, 0.40 and 0.80 mg mL^{-1}) concentration scale was used to construct the standard curve by Prado et al. (2000).

2.6. Malondialdehyde (MDA) analysis (nmol MDA g^{-1} fw)

MDA content, the indicator of lipid peroxidation, was measured according to Draper & Hadley (1990). 0.5 g (fw) of plant samples homogenized with the reaction mixture containing trichloroacetic acid (20% TCA) and thiobarbituric acid (5% TBA) were kept in a hot water bath at 95 °C for 30 min (3 mL in total), and then cooled in an ice bath. The samples were transferred to Eppendorf tubes and centrifuged at 10000 rpm for 10 min. Supernatants were calculated by measuring at 532 and 600 nm wavelengths in the spectrophotometer (Rao & Sresty 2000). A unit of MDA is determined as 1 nmol product per g fw. MDA was calculated as follows:

$$MDA\ (nmol\ g^{-1}) = (\frac{A_{532} - A_{600}}{155}) \times 10^6$$

2.7. Total protein analysis (mg g^{-1} fw)

Protein content was assayed using the modified Bradford Method according to Kruger (2009). To compare the results, the Bovine Serum Albumin (BSA) standard was prepared between 10-200 µg mL^{-1} and measurements were made at 595 nm within 5-60 min and the results were calculated according to the following formula:

$$\text{Crude Protein Content (CPC, mg } g^{-1}) = \frac{\text{ControlA x SampleA}}{\text{ControlA}}$$

Where; ControlA is the standard absorbance value (mg g^{-1} fw); SampleA is the sample absorbance value (mg g^{-1} fw). The amount of crude protein was found by calculating the concentration corresponding to the absorbance values.

2.8. Total phenolic (TPC) and total flavonoids (TFC) content (mg g^{-1} fw)

Folin-Ciocalteu was used as a reagent and gallic acid was used as a standard (1 mg mL^{-1} stock solution). The reaction conditions are briefly as follows: 200 µL of the crude extract in different concentrations was taken and 250 µL of Folin-Ciocalteu diluted in a 1:1 ratio was added and left for approximately 1 min. Then, 10% 1 mL of Na_2CO_3 (5 g in 50 mL DH_2O) was added and the total reaction volume completed to 4.45 mL with 3 mL of DH_2O and the reaction was left for 95 min. After the reaction period was completed, the color change in the reaction solution was determined at 760 nm absorbance and the results were calculated as gallic acid equivalents (mg g^{-1}) by comparing it with the standard absorbance subjected to the reaction under the same conditions (Saeed et al. 2012).

TFC was determined by the standard colorimetric method with minor amendments, using DH_2O solutions of 10% $AlCl_3$ and 1 M sodium acetate as reagents. The reaction conditions are as follows: 200 µL of methanol extract was taken and 2 mL of methanol was added to it. Then, 100 µL $AlCl_3$ and 100 µL sodium acetate were added and allowed to react for 30 min. After the incubation time was completed, absorbance measurements were completed at 415 nm with quercetin stock solution (1 mg mL^{-1}) as a standard prepared in different concentrations under the same reaction conditions. TFC of the extract was expressed as quercetin equivalents per gram of sample (mg g^{-1}) (Zhishen et al. 1999).

2.9. Antioxidant activity

Antioxidant activity was determined using the standard method with 1,1-diphenyl-2-picrylhydrazyl (DPPH) at a concentration of 0.002% (2 mg 100 mL^{-1} methanol). Briefly, 0.1 g of the duckweed sample, which was pre-treated by crushing with liquid nitrogen, was extracted with methanol at a ratio of 1:1 for 24 h and different concentrations (0.1-1 mg mL^{-1}) of the resulting extract were prepared. This mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The results were evaluated using ascorbic acid (10-100 µg mL^{-1}) as a standard. 2 mL of DPPH was added to each tube, mixed well and incubated for 50 min in the dark. Thereafter, DPPH radical scavenging activity was quantified at 517 nm and the percentage of DPPH reduction was calculated as:

$$\text{DPPH free Radical scavenging activity (RSA)} = \frac{\text{ControlA517} - \text{SampleA517}}{\text{ControlA517}} X100$$

Where; ControlA517 and SampleA517 refer to absorbance values of the DPPH methanol solution and sample at 517 nm, respectively (Prakash et al. 2007).

2.10. Statistical analysis

All experiments were performed in triplicate, and data were expressed as mean ± SD. In addition to one-way ANOVA followed by Tukey's HSD test, a two-way factorial ANOVA was conducted to assess the main effects of metal type, microplastic type, and their interaction (HM-MP) on each biochemical parameter. A significant interaction term ($P<0.05$) was interpreted as evidence of non-additive (synergistic or antagonistic) effects, whereas non-significant interaction indicated additivity. Statistical analyses were performed using SPSS v26.0 and verified with OriginPro v2023.

3. Results and Discussion

3.1. Effects of MPs and HMs on growth rate of L. gibba

The present study demonstrated that exposure of *Lemna gibba* to heavy metals (Zn, Cu, Ni) and microplastics (PP, ABS), both individually and in combination, significantly inhibited relative growth rate (RGR) compared to the control. While single stressors already caused measurable reductions in biomass accumulation, combined exposures generally produced more pronounced inhibitory effects, suggesting a synergistic interaction between heavy metals and microplastics.

During the experimental period, 13.99% growth was detected in the control group. After treatment of the organism with Zn, slight growth was observed at low heavy metal concentrations (at 25 and 50 mg L^{-1}). However, a 10% decrease in growth was detected at high concentrations of Zn exposure (100 mg L^{-1}). The analysis showed that Zn had no negative effect on the relative growth rate of *L. gibba* until it reached an extremely high concentration ($P>0.05$). In copper and nickel treatment, there was a statistically significant decrease (from 8 to 22% and from 8 to 11%, respectively) in relative growth rate in parallel with the heavy metal concentration ($P<0.05$).

A decrease in RGR was observed after microplastic treatment on *L. gibba* frond, but this decrease was not as much as the decrease in growth rate seen after heavy metal treatment. An approximately 1 - 2% decrease in growth was detected following exposure of *Lemna* to PP microplastic. PP microplastic exposure was determined to be the least effective pollution experimental group on

the growth of *Lemna*. As a result of treatment with ABS microplastic, a decrease in growth was observed from 7 to 15% compared to PP (Table 2). The concentration of 100 mg L^{-1} MPs and HMs was chosen for the combined adsorbate study because this was the only MPs and HMs concentration that had a statistically significant effect on the frond of *L. gibba* compared to the control.

Table 2- The effect of different microplastic (ABS, PP) and heavy metal (Zn, Cu, Ni) treatments on *L. gibba* growth rate. Asterisk (*) and apostrophe (') expressed as inhibition and uninhibition values compared to the control group, respectively. Data represent mean ± SD (n = 3). Different letters indicate statistically significant differences (P<0.05).

Concentration (mg L^{-1})		*RGR (g fw)*	*RGR (d%)*
Zn	25	$2.47^{a} \pm 0.02$	6.76^{a}'
	50	$2.41^{a} \pm 0.01$	5.88^{a}'
	100	$1.30^{ba} \pm 0.01$	10.02^{ba}*
Cu	25	$1.41^{ba} \pm 0.04$	8.49^{ba}*
	50	$0.62^{b} \pm 0.04$	19.72^{b}*
	100	$0.43^{b} \pm 0.02$	22.49^{b}*
Ni	25	$1.43^{ba} \pm 0.02$	8.14^{ba}*
	50	$1.24^{ba} \pm 0.02$	10.83^{ba}*
	100	$1.23^{ba} \pm 0.01$	11.06^{ba}*
PP	25	$1.87^{a} \pm 0.01$	1.79^{a}*
	50	$1.86^{a} \pm 0.02$	1.96^{a}*
	100	$1.86^{a} \pm 0.01$	1.97^{a}*
ABS	25	$1.50^{ba} \pm 0.02$	7.20^{ba}*
	50	$1.28^{ba} \pm 0.04$	10.26^{ba}*
	100	$0.92^{b} \pm 0.02$	15.47^{b}*
MPs-HMs[a]	Zn-PP	$1.31^{cb} \pm 0.01$	10.87^{cb}*
	Zn-ABS	$1.41^{cb} \pm 0.01$	15.36^{cb}*
	Cu-PP	$1.21^{c} \pm 0.04$	23.22^{c}*
	Cu-ABS	$1.21^{c} \pm 0.01$	17.22^{c}*
	Ni-PP	$1.39^{cb} \pm 0.02$	11.74^{cb}*
	Ni-ABS	$1.12^{cb} \pm 0.01$	17.50^{cb}*
Control		**$2.98^{a} \pm 0.002$**	**13.99'**

[a]: The concentration of MPs-HMs was used as 100 mg L^{-1} (1:1 ratio)

However, as a result of the treatment of PP with three other heavy metals (Zn, Cu and Ni), an approximately 10% decrease in the growth of *L. gibba* was determined. That is two pollution factors synergised the growth of the *L. gibba* (Adeleye et al. 2024). The findings obtained in this study will be different from the findings obtained in the studies of Kalcíková et al. (2017) and Mateos-Cárdenas et al. (2019) conducted on the related species *L. minor*, exposed for 7 days to microplastics, in which no significant effects on biomass and RGR were recorded. Conversely, Ceschin et al. (2023) have determined that *L. minuta* plant growth was negatively affected depending on different polystyrene comethylmethacrylate MP concentrations and exposure times.

Martinez et al. (2019) compared binary mixtures of Ni and Zn. They reported significant phytotoxic effects on *L. gibba* growth and biomarker responses even at relatively low concentrations underscoring strong interactive toxicity in HM mixtures.

The inhibitory effect of heavy metals on duckweed growth observed here is consistent with previous studies reporting the sensitivity of *Lemna* species to metal contamination. For instance, *L. minor* exhibited EC_{50} values as low as 0.47 mg L^{-1} for Cu and 1.29 mg L^{-1} for Ni, highlighting its high vulnerability to metal stress (Khellaf & Zerdaoui 2009). Similarly, Ni exposure has been associated with impaired chlorophyll biosynthesis and decreased biomass in aquatic plants (Di Marzio et al. 2024). Our findings confirm that Cu and Ni in particular are strong inhibitors of *L. gibba* growth.

Microplastics alone also suppressed growth, with ABS showing a stronger inhibitory effect than PP at equivalent concentrations. This aligns with recent studies where cadmium and polyethylene (PE) induced oxidative stress, disrupted photosynthetic activity, and reduced growth in *Lemna minor* (Yang et al. 2023). The differences among polymer types may be related to their physicochemical properties, such as hydrophobicity and surface charge, which influence their interaction with plant tissues.

Interestingly, in some earlier reports, microplastics were suggested to mitigate metal toxicity by adsorbing metal ions and reducing their bioavailability (Narwal et al. 2024). However, our results do not support this hypothesis; instead, combinations such as Cu–PP and Ni–ABS caused stronger inhibition than individual treatments. This discrepancy may be attributed to differences in experimental conditions (e.g., particle size, exposure time, and plant species) and suggests that under certain scenarios, microplastics may influence the bioavailability or toxicity of heavy metals, rather than mitigate their effects. A similar trend was observed by Xue et al. (2025), who reported that polyethylene microplastics enhanced cadmium toxicity in *L. minor* through increased oxidative stress and enzyme inactivation. The enhanced growth inhibition observed under Cu–ABS and Ni–

ABS exposure could be related to changes in metal ion availability or stress signalling in *Lemna gibba*, although this mechanism was not directly measured in the present study.

Microplastics alone produced modest reductions in RGR, with ABS exerting stronger negative effects than PP–an observation that warrants further investigation into polymer-specific phytotoxicity. Importantly, the combined exposure of microplastics and heavy metals at 100 mg L^{-1} produced significant suppression of plant growth, supporting the notion of enhanced toxicity under co-contamination scenarios (An et al. 2024).

3.2. Effects of MPs and HMs on chlorophyll and total carotenoids content of L. gibba

After treatment of the organism, pigments were measured and mg g^{-1} values were calculated. HMs had a statistically significant negative effect on the chl-*a* content of *L. gibba* except Zn exposure at concentrations of 25 and 50 mg L^{-1} ($P<0.05$). At the same time, chl-*b* and carotenoid contents decreased significantly ($P<0.05$) depending on the concentration in all heavy metal treatments. Chl-*a*, b and Cd values of the control group were determined as 0.5734, 0.1853 and 0.1763 mg g^{-1} fw, respectively. These values were determined as 0.2827, 0.1340 and 0.1362 mg g^{-1} fw after heavy metal treatment and as 0.2938, 0.1489 and 0.1376 mg g^{-1} fw after microplastic treatment, respectively (Figure 2). Zn and Ni also caused significant pigment declines, although less severe than Cu. Zn showed moderate effects, while Ni displayed concentration-dependent reductions. Microplastics (PP and ABS) induced decreases in pigment levels as well, but their effects were weaker than those of metals. Between the two, ABS exerted a stronger reduction than PP. Overall, the toxicity ranking in single exposures was Cu > Zn ≈ Ni > ABS > PP.

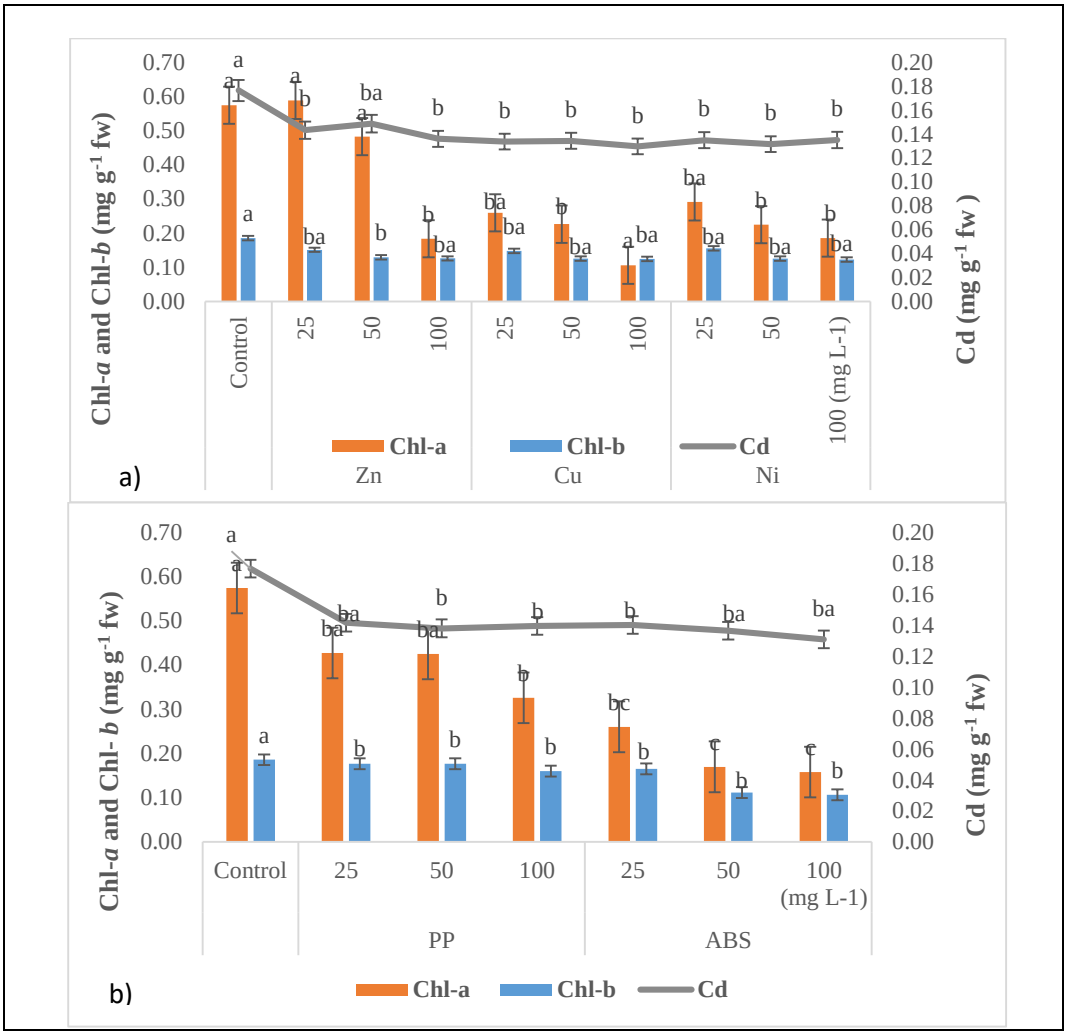


Figure 2- The effect of different concentrations of heavy metal (a) and microplastic (b) treatments on *L. gibba* pigment content (Chl-*a*, Chl-*b* and Carotenoid) compared to the control group. Standard deviations were presented by error bars. Different letters indicate statistically significant differences ($P<0.05$)

The experimental data show a significant reduction in chl-*a*, chl-*b*, and carotenoid content in *Lemna gibba* exposed to both heavy metals and microplastics compared to the control group. Among the heavy metals, Cu exerted the most pronounced effect, leading to more than 60% reduction in chl-*a* at high concentrations. Zn and Ni also induced declines in pigment contents, but the extent of reduction was less severe than Cu, particularly at lower concentrations. For microplastics, both PP and ABS treatments

caused decreases in chl-*a* and b levels, with ABS exerting a slightly stronger effect. Carotenoids showed reductions across all treatments, although their decline was less pronounced compared to chlorophyll, suggesting a possible protective role against oxidative stress.

The observed reduction in pigment contents highlights the higher toxicity of Cu compared to Zn and Ni, which is consistent with previous studies reporting Cu as one of the most potent inhibitors of photosynthesis in *Lemna* species (Singh et al. 2022; Matešković 2024). Cu is a redox-active metal that can catalyze the formation of reactive oxygen species (ROS), leading to damage in thylakoid membranes and inhibition of chlorophyll biosynthetic enzymes (Zeng et al. 2021). In contrast, Zn and Ni are essential micronutrients, and their effects are more concentration-dependent; they may support metabolic functions at low levels but cause toxicity at elevated doses (Lilay et al. 2024). Microplastics showed moderate effects on pigment reduction, with ABS causing more pronounced changes than PP. Recent reports confirm that MPs generally induce weaker pigment alterations than heavy metals but may trigger oxidative stress, shading, and physical damage (Nava & Leoni 2021; Zhao et al. 2023). Moreover, MPs can interact with metals, enhancing their bioavailability and synergistically intensifying toxicity (Xiong et al. 2024). The relatively lower decline in carotenoids compared to chlorophyll may indicate their antioxidative function, mitigating ROS accumulation under stress conditions. Overall, the results suggest that while both heavy metals and microplastics impair photosynthetic pigments in *Lemna gibba*, Cu poses the greatest threat among the tested pollutants.

As a result of the combined application of all microplastics and heavy metals at a concentration of 100 mg L^{-1}, a statistically significant decrease was detected in three pigment values ($P<0.05$). In addition to the separate application of heavy metals and microplastics on living organisms, the combined application of these two pollution factors dramatically reduced all pigment substances in the organism in general (Figure 3). Combined HM-MP exposures (100 mg L^{-1}, 1:1 ratio) showed stronger impacts on pigment contents compared to individual exposures. The Zn-PP and Zn-ABS combinations caused greater pigment reductions than Zn alone, with ABS combinations being more toxic. Cu-PP and Cu-ABS produced the strongest inhibitory effects overall, with Cu-ABS nearly halving Chl-*a* and Chl-*b* levels. Ni-PP and Ni-ABS also enhanced pigment reductions compared to Ni alone, again with ABS being more harmful than PP. Carotenoids declined under all combined treatments, but less severely than chlorophylls, indicating their antioxidant role in mitigating oxidative stress. The results suggest synergistic toxicity between HMs and MPs, particularly when Cu and ABS are combined.

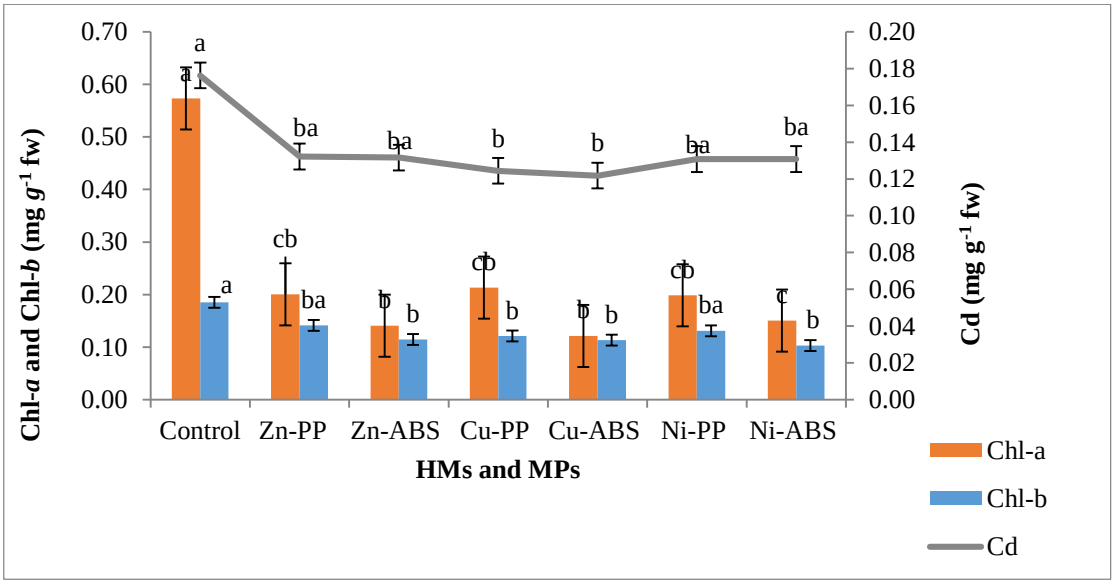


Figure 3- The combined effect of heavy metal-microplastic treatments at 100 mg L^{-1} concentration (1:1 ratio) on *L. gibba* pigment content (Chl-*a*, Chl-*b* and Carotenoid) compared to the control group. Standard deviations were presented by error bars. Different letters indicate statistically significant differences ($P<0.05$)

The comparative evaluation of single versus combined treatments demonstrates that while heavy metals alone exert substantial toxicity, their interaction with microplastics amplifies pigment loss. This is consistent with the role of MPs as vectors for metals, enhancing their bioavailability and toxicity in aquatic plants (Xiong et al. 2024). The stronger impact of ABS compared to PP aligns with previous findings showing that polymer type influences toxicity due to differences in hydrophobicity and chemical additives (Dhameri et al. 2025). Cu consistently emerged as the most harmful heavy metal, in agreement with reports that it disrupts chlorophyll biosynthesis and induces oxidative stress (Riyazuddin et al. 2021; Singh et al. 2022). The relatively smaller reduction in carotenoids supports their role as protective antioxidants (Rahman et al. 2023). Overall, the combined exposures highlight the ecological risk of multi-pollutant interactions in aquatic ecosystems. Both heavy metals and microplastics impair photosynthetic pigment content in *Lemna gibba*, with Cu being the most toxic metal and ABS being more harmful than PP. Combined HM-MP exposures caused stronger reductions than single treatments, indicating synergistic toxicity. These results emphasize the importance of considering pollutant mixtures rather than individual contaminants in aquatic risk assessments.

3.3. Effects of MPs and HMs on total CH and MDA content of L. gibba

The results of the total CH values showed that there was no statistically significant difference between control and HMs and MPs treatments during the whole duration of the experiment (P>0.05). The total CH of the organism was determined as 1.0308 mg mL^{-1} fw. The average values per each Zn, Cu, Ni, PP, and ABS treatment were 1.0227, 1.1129, 0.7350, 0.8882 and 0.9290 mg mL^{-1} fw, respectively (Figure 4). The experiment on the organism performed at three different concentrations of all microplastics and heavy metals detected a decrease by 9.7% in total CH content of *L. gibba*, except exposure of Cu-HM at 25 and 50 mg L^{-1} concentration and exposure of Zn-HM at 25 mg L^{-1} concentration.

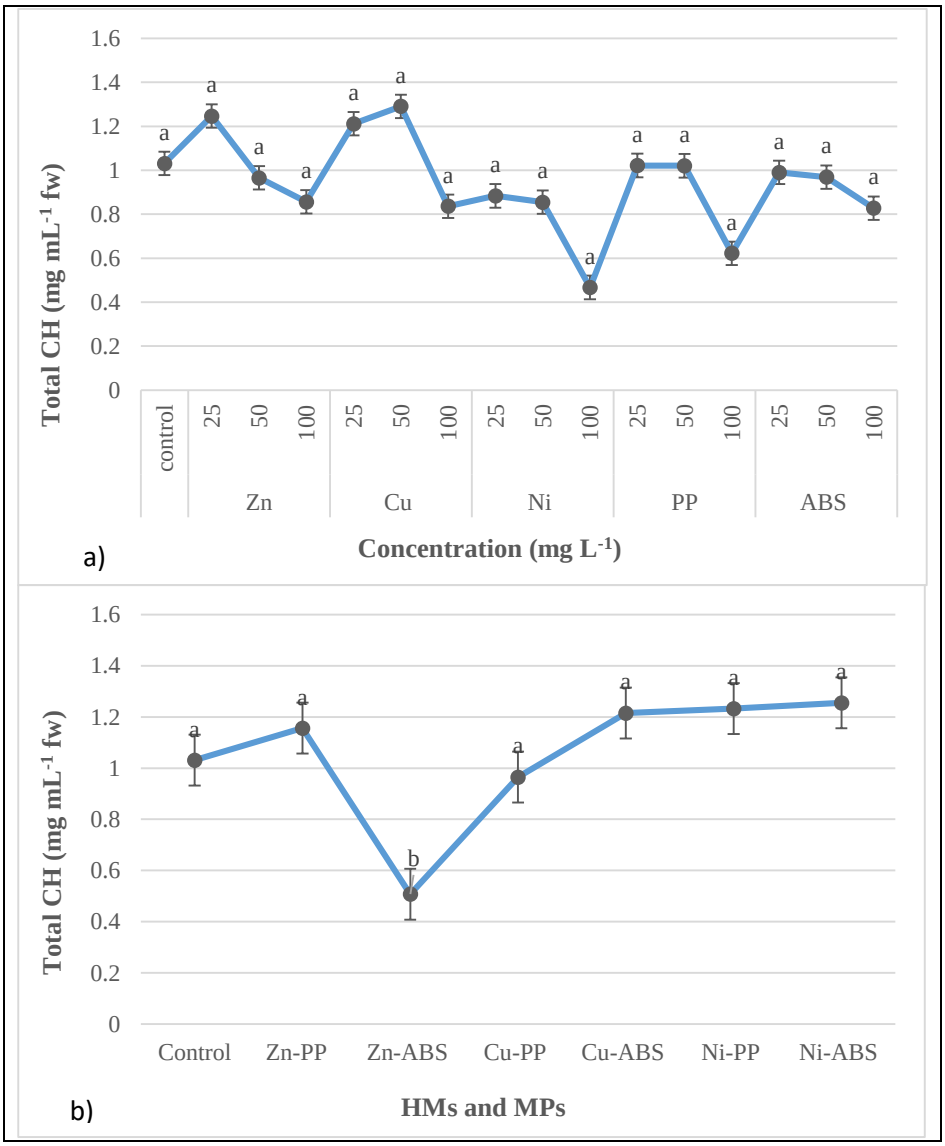


Figure 4- The individual (a) and combined (b) effect of heavy metal-microplastic treatments on *L. gibba* total carbohydrate content (CH) compared to the control group. Standard deviations were presented by error bars. Different letters indicate statistically significant differences (P<0.05)

Experiments conducted on *L. gibba* at a concentration of 100 mg L^{-1} for all MPs and HMs revealed no significant changes in mean CH values compared to the control group (P>0.05) in all experimental groups except for Zn-ABS exposure (P<0.05).

The MDA content of *L. gibba* is presented as average values and the control value was determined to be 2.34 nmol MDA g^{-1} fw. MDA content showed increasing changes in all experimental groups (P<0.05), except for both microplastic treatment groups of zinc. Compared to the control group, the average MDA values obtained as a result of Zn, Cu, Ni, PP, ABS and microplastic-heavy metal treatment were 2.61; 3.70; 2.97, 2.81, 3.49 and 3.55 nmol MDA g^{-1} fw, respectively (Figure 5).

The mean MDA values per each Cu treatment and combined Cu-MPs treatment showed statistically significant differences between treatments (P<0.05) compared to the control group. The MDA content was on average increased by 58.11% and 51.70% of Cu and combined Cu-MPs treatment, respectively (Figure 5).

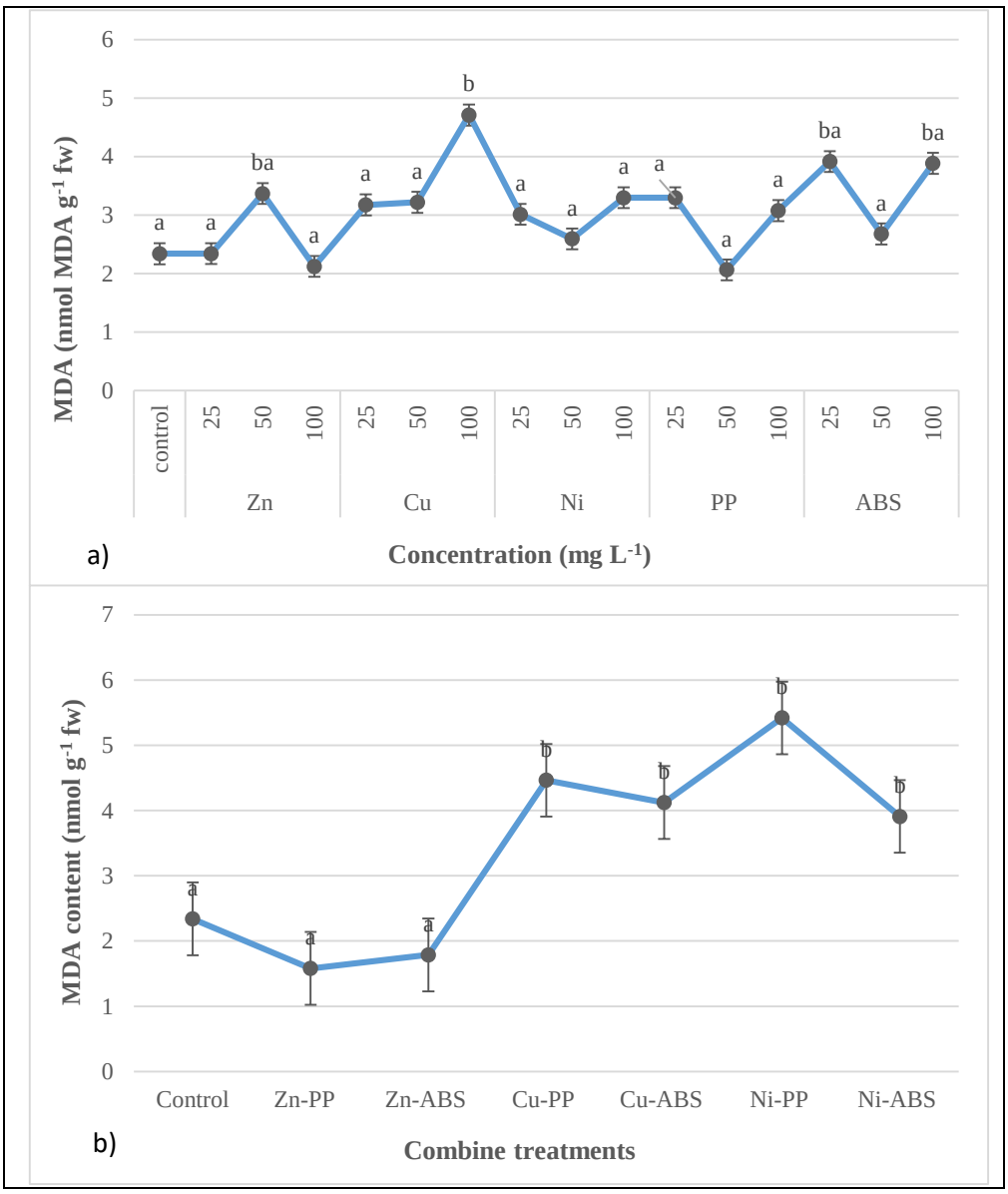


Figure 5- The individual (a) and combined (b) effect of heavy metal-microplastic treatments on *L. gibba* malondialdehyde content (MDA) compared to the control group. Standard deviations were presented by error bars. Different letters indicate statistically significant differences (P<0.05)

A decrease in total carbohydrate amount was determined in all experimental groups except copper and zinc exposure. However, during the Zn-ABS and Cu-PP treatment, heavy metals and microplastics showed a synergistic effect and had a negative effect on total carbohydrates. In contrast, a significant increase by 33.3% in MDA content was observed in almost every experimental group compared to the control group. The increase in MDA shows the extent of membrane lipid peroxidation, which indicates oxidative stress in plants (Blokhina et al. 2003). Oxidative damage to the *L. gibba* bio-membrane increased by 58.11%, particularly under cu exposure, compared to the control. This response may be associated with the ability of transition metals especially copper to catalyze the formation of hydroxyl radicals and to interfere with the plant's antioxidant defense system, thereby promoting oxidative stress. Although metal uptake and membrane interactions were not directly quantified in this study, these processes are likely contributors to the observed increase in MDA levels, as suggested in previous reports (Blokhina et al. 2003; Panda & Choudhury 2005).

3.4. Effects of MPs and HMs on total protein, phenolic, flavonoid content and antioxidant activity of L. gibba

Table 3 represents the analytical data for total protein, phenolic, flavonoid content and antioxidant activity of the aqueous extract of *L. gibba*. Data clearly show a considerable amount of total protein content in aqueous extract of *L. gibba*. The average total protein amount of *L. gibba*, which was exposed to three different concentrations of microplastics and heavy metals during 7 experimental exposure days, was determined as 73.06 mg g^{-1} fw. Similarly, under the different concentrations of HM and MP treatments, the average total phenolic, flavonoid and antioxidant activities (DPPH) were determined as 2.93, 0.51 mg g^{-1} fw and 49.5% while the control values were 9.7, 1.88 mg g^{-1} fw and 71.1%, respectively. When *L. gibba* was exposed to all HM and

MP groups, a slight decrease in total protein, phenolic, flavonoid contents and antioxidant activities occurred compared to the control group (P>0.05).

Table 3- Effects of individual and combined exposure of Zn, Cu, and Ni with polypropylene (PP) or acrylonitrile butadiene styrene (ABS) microplastics on biochemical parameters of *L. gibba*. Data represent mean ± SD (n = 3). Different letters within columns indicate significant differences (P<0.05)

	Concentration (mg L^{-1})	*Total Protein (mg g^{-1} fw)*	*Total Phenolic (mg g^{-1} fw)*	*Total Flavonoid (mg g^{-1} fw)*	*Radical Scavenging Activity (RSA%)*
Zn	25	18.7 ± 0.25^{d}	3.5 ± 0.007^{bc}	0.47 ± 0.001^{cd}	47.3 ± 0.026^{bc}
	50	109.4 ± 3.80^{c}	4.1 ± 0.007^{bc}	0.72 ± 0.001^{bc}	63.2 ± 0.026^{ab}
	100	120.0 ± 6.82^{bc}	5.9 ± 0.014^{b}	0.52 ± 0.002^{c}	58.3 ± 0.026^{b}
Cu	25	46.9 ± 1.00^{d}	2.1 ± 0.007^{c}	0.67 ± 0.001^{bc}	47.3 ± 0.039^{bc}
	50	7.74 ± 0.10^{de}	2.3 ± 0.005^{c}	0.14 ± 0.001^{d}	44.9 ± 0.014^{bc}
	100	134.06 ± 4.47^{b}	3.4 ± 0.014^{bc}	1.02 ± 0.004^{b}	45.4 ± 0.014^{bc}
Ni	25	81.7 ± 1.69^{cd}	2.7 ± 0.005^{c}	0.65 ± 0.001^{bc}	51.5 ± 0.014^{bc}
	50	28.0 ± 2.03^{d}	2.2 ± 0.017^{c}	0.44 ± 0.002^{c}	51.5 ± 0.014^{bc}
	100	100.8 ± 1.86^{c}	4.4 ± 0.098^{b}	0.55 ± 0.001^{c}	49.6 ± 0.054^{bc}
PP	25	26.6 ± 0.40^{d}	2.3 ± 0.007^{c}	0.50 ± 0.001^{c}	40.4 ± 0.026^{c}
	50	99.7 ± 3.79^{c}	2.0 ± 0.014^{c}	0.37 ± 0.001^{cd}	50.3 ± 0.014^{bc}
	100	77.01 ± 3.22^{cd}	2.0 ± 0.005^{c}	0.40 ± 0.001^{cd}	54.7 ± 0.026^{bc}
ABS	25	58.5 ± 2.75^{d}	2.6 ± 0.012^{c}	0.44 ± 0.001^{c}	40.9 ± 0.080^{c}
	50	107.5 ± 2.17^{c}	2.5 ± 0.017^{c}	0.51 ± 0.001^{c}	50.4 ± 0.054^{bc}
	100	79.3 ± 3.05^{cd}	2.0 ± 0.001^{c}	0.37 ± 0.001^{cd}	47.1 ± 0.067^{bc}
Combined*	Zn -PP	142.8 ± 2.32^{b}	2.3 ± 0.007^{c}	0.58 ± 0.001^{c}	52.8 ± 0.014^{bc}
	Zn-ABS	44.6 ± 0.53^{d}	2.4 ± 0.012^{c}	0.37 ± 0.001^{cd}	55.8 ± 0.054^{b}
	Cu-PP	43.6 ± 3.17^{d}	1.2 ± 0.002^{cd}	0.12 ± 0.001^{d}	55.1 ± 0.014^{b}
	Cu-ABS	170.1 ± 4.39^{ab}	0.9 ± 0.005^{d}	0.07 ± 0.001^{de}	50.3 ± 0.026^{bc}
	Ni-PP	86.7 ± 1.26^{cd}	2.0 ± 0.005^{c}	0.20 ± 0.001^{cd}	49.7 ± 0.014^{bc}
	Ni-ABS	100.5 ± 1.71^{c}	2.3 ± 0.098^{c}	0.42 ± 0.001^{cd}	43.2 ± 0.014^{c}
	Control	**194 ± 4.13^{a}**	**9.7 ± 0.017^{a}**	**1.88 ± 0.001^{a}**	**71.1 ± 0.026^{a}**

*: The combined effect of HMs and MPs treatments at 100 mg L^{-1} concentration (1:1 ratio)

On the other hand, the average total protein amount of *L. gibba*, which was exposed to 100 mg L^{-1} concentrations of combined HMs-MPs exposure during 7 experimental days, was determined as 98.05 mg g^{-1} fw. Besides, the average total phenolic, flavonoid and antioxidant activities (DPPH) were determined as 1.85, 0.29 mg g^{-1} fw and 51.15% after combined adsorbate exposure (Table 3).

Results show a significant decrease in total protein, phenolic, flavonoid content and antioxidant activity of the aqueous extract of *L. gibba* both in separate application and co-application of the pollutants. Plants detoxify by increasing the production of reactive oxygen species (ROS) under environmental stress factors such as drought, metal exposure and salt stress (Parlak & Yilmaz 2012). Especially as a result of the combined application of heavy metals and microplastics, the percentage of antioxidant activity increased compared to the application of pollution factors individually. Similar results were reported in other organisms exposed to MPs and HMs (Tanyolaç et al. 2007; Parlak 2016; Taş 2022).

Factorial ANOVA indicated significant main effects of both metal type (P<0.001) and microplastic type (P<0.001) on total protein content (Table 4). Both metal and microplastic types significantly influenced protein levels (P<0.0001), and factorial ANOVA revealed a strong MetalxMP interaction, indicating non-additive combined effects (P<0.001). Compared with the control, all metal-only treatments caused a notable decline in protein content, with Cu exposure showing the greatest reduction. However, the presence of microplastics modulated these responses: protein content under Zn-PP and Ni-ABS co-exposures increased slightly compared to the corresponding single-metal treatments, suggesting a potential mitigation effect. In contrast, Cu-PP exposure further decreased protein levels, pointing to a synergistic inhibitory interaction. These findings suggest that protein metabolism is highly sensitive to both individual and combined stressors, with interaction effects dependent on the specific metal polymer pair.

Total phenolic content significantly decreased under all single and combined exposures compared with the control (9.7 mg g^{-1} fw). The factorial ANOVA indicated significant main effects of metal ($F_{2,12}$ = 381.2, P<0.0001) and microplastic type (P< 0.0001), as well as a strong MetalxMP interaction (P<0.0001). The lowest phenolic level was observed under Cu-ABS co-exposure (0.9 mg g^{-1} fw), corresponding to an approximately 90% reduction relative to the control, suggesting a possible synergistic oxidative stress response. These results indicate that phenolic compounds key antioxidants in plants were strongly suppressed by combined metal and microplastic stress.

Flavonoid content followed a trend similar to phenolics, showing a significant decline across all treatments (P<0.0001). Factorial ANOVA showed highly significant main effects of both metal and microplastic type and a strong MetalxMP interaction (P<0.0001). Flavonoid concentration decreased most sharply under Cu-ABS co-exposure, followed by Cu-PP. Since flavonoids are important components of the non-enzymatic antioxidant defense system, their pronounced reduction suggests that prolonged or synergistic oxidative stress may have exceeded the plant's biosynthetic capacity. The differences between PP and ABS groups imply that polymer chemistry may alter metal-induced oxidative processes by modifying surface adsorption or metal bioavailability.

DPPH radical scavenging activity, a key indicator of overall antioxidant potential, varied significantly among treatments (P<0.0001). The highest DPPH inhibition was recorded in the control, while all treatments led to significant reductions, consistent with impaired antioxidant defense. Factorial ANOVA confirmed strong main effects and an extremely significant MetalxMP interaction (P<0.0001). Among the combinations, Ni-ABS and Ni-PP treatments showed the greatest suppression of DPPH activity, indicating synergistic oxidative damage. In contrast, Zn-ABS exposure retained a relatively higher antioxidant capacity, suggesting that certain polymer metal combinations may exert less severe oxidative impacts.

Overall, these findings highlight the complex nature of MP–metal co-exposure, where interactions can shift from additive to synergistic depending on the specific pair and endpoint.

Table 4- Results of two-way factorial ANOVA showing the main and interaction effects of heavy metal type (HM: Zn, Cu, Ni) and microplastic type (MP: PP, ABS) on the biochemical parameters of *L. gibba*. Significant HMxMP interaction terms (P<0.05) indicate non-additive (synergistic or antagonistic) effects under co-exposure conditions

Parameter	*Source*	*df*	*F*	*p-value*	*Interpretation*
Total Protein	HM	2	$1.23x10^{2}$	<0.0001	Significant main effect
	MP	1	$2.91x10^{2}$	<0.0001	Significant main effect
	HMxMP	2	$6.23x10^{3}$	<0.0001	*Strong interaction (non-additive)
Total Phenolic	HM	2	$1.40x10^{3}$	<0.0001	Significant main effect
	MP	1	$4.86x10^{1}$	<0.001	Significant main effect
	HMxMP	2	$9.24x10^{1}$	<0.0001	Significant interaction (non-additive)
Total Flavonoid	HM	2	$1.92x10^{5}$	<0.0001	Significant
	MP	1	$7.53x10^{2}$	<0.0001	Significant
	HMxMP	2	$6.30x10^{4}$	<0.0001	*Strong interaction
Antioxidant Activity (DPPH)	HM	2	$3.89x10^{5}$	<0.0001	Significant
	MP	1	$1.30x10^{5}$	<0.0001	Significant
	HMxMP	2	$1.44x10^{5}$	<0.0001	*Strong interaction

Abbreviations: df, degrees of freedom; F, statistic; p-value, probability value indicating statistical significance.

4. Conclusions

Overall, the results of this study demonstrate the individual and combined interactions of MPs and HMs on the floating aquatic macrophyte *L. gibba*. Compared to all other groups, the combined applications of ABS-Ni, ABS-Cu, and ABS-Zn had more negative effects on growth, chlorophyll, carotenoid and total carbohydrate content, respectively. In contrast, the combined application of Cu and Ni caused oxidative stress in plant tissue and increased malondialdehyde levels. The results clearly show a significant decrease in total protein, phenolic, flavonoid contents, and antioxidant activity in the aqueous extract of *L. gibba*, both after separate and co-application of pollutants. The findings partially support our hypothesis that combined MP-HM exposure induces stronger adverse effects than individual treatments, suggesting potential synergistic interactions under co-contamination scenarios. These findings highlight the need to incorporate multiple stress applications into ecotoxicological risk assessments, as aquatic plants in natural ecosystems are rarely exposed to pollutants alone. The present study indicated that

microplastics may influence heavy metal uptake and oxidative stress pathways, although these mechanisms were not directly measured in the experiments. Future research should aim to test the mechanistic basis of these interactions.

Author Declarations

Authors' contributions: Concept: T.Ş., M.O., Design: T.Ş., M.O., Data collection: T.Ş., M.O., Analysis: T.Ş., M.O., Writing: T.Ş., M.O.,

Conflict of interest/Competing interests: The authors declare no conflict of interest.

Ethics approval/declarations: Ethical approval is not required for this study because it does not contain any studies with human or animal subjects.

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Consent for publication: No need/ Not applicable.

Data availability: Data are available on request due to privacy or other restrictions.

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