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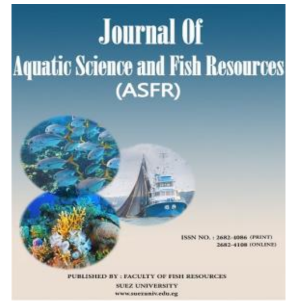

Pre-Exposure of Common Carp (*Cyprinus carpio)* to Ambient Copper and Microplastic Changes the Gill Ionoregulaion- Related Transcripts During Saltwater Exposure

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Abstract

In this study, common carp (*Cyprinus carpio*) were exposed to 0.25 mg/L copper alone (Cu treatment) or in combination with 0.5 mg/L polyvinyl chloride microparticles (Cu-MP treatment) for 14 days, followed by a 72-h challenge in brackish water (13 ppt NaCl). After 14 days of exposure, gill copper concentrations were significantly elevated in both the Cu and Cu-MP treatments relative to the control group (P = 0.008), with no significant difference between the two treatments. Gene expression analysis of gill tissue following the exposure period revealed substantial upregulation of the glucocorticoid receptor (*gr*) and Na^+/K^+-ATPase α1a subunit (*nakα1a*) genes in the Cu treatment. In contrast, the Cu-MP treatment showed only modest upregulation of *gr* and significant downregulation of *nakα1a*. After the subsequent brackish-water challenge, *nakα1a* expression was significantly downregulated in control fish, whereas *gr* expression remained unchanged. Overall, these gill gene expression patterns suggest that copper exposure disrupts ionoregulatory homeostasis in common carp, but co-exposure to polyvinyl chloride microparticles does not exacerbate these effects.

1. Introduction

Water pollution poses a significant threat to the aquaculture industry, adversely impacting fish health and the quality of fillets **(Sonone *et al.*, 2020; Das *et al.*, 2025)**. Among the primary pollutants affecting surface and seawater are microplastics (MPs) and copper, which have detrimental effects on fish well-being (**Malhotra *et al.*, 2020; Kim *et al.*, 2021**). Research has shown that these pollutants can induce oxidative stress, cause histopathological damage, and lead to overall stress in fish **(Gopi *et al.*, 2019; Naz *et al.*, 2021)**. Furthermore, microplastics (MPs) strongly tend to adsorb other waterborne pollutants, acting as carriers for metals and facilitating their transfer into fish through the oral route **(Banaee *et al.*, 2019; Owais *et al.*, 2025)**. Consequently, the presence of MPs in aquatic environments may exacerbate metal accumulation in fish tissues, heightening the toxic effects of these metals. Recent studies have increasingly focused on the combined impacts of microplastics and metals on fish health **(Lu *et al.*, 2018; Wen *et al.*, 2018; Jinhui *et al.*, 2019; Roda *et al.*, 2020; Asad *et al.*, 2024)**. Common carp, *Cyprinus carpio*, is a widely favored species in the global aquaculture industry, mainly due to its cultural significance and resilient nature. This hardiness makes it an excellent candidate for cultivation in waters with varying physicochemical properties. Although primarily a freshwater species, common carp can thrive in natural brackish water environments **(Barus *et al.*, 2001)**. Consequently, fish culturists often consider rearing fish in brackish water ponds. When common carp are transferred from freshwater to brackish water, they experience stress and activate various physiological pathways that help them achieve homeostasis in hyperosmotic conditions **(Ghelichpour *et al.*, 2018)**. The fish gill has pivotal roles under such conditions, as it is the main organ of osmoregulation and ionoregulation in the fish. The gill Na/K-ATPase is an important enzyme in osmoregulation **(Ghelichpour *et al.*, 2020)**. The enzymes have several subunits with different roles; among them, subunits α1a and α1b have higher activities in freshwater and saltwater **(McCormick *et al.*, 2009)**. The enzyme activity is controlled by cortisol through glucocorticoid receptors (gr) **(McCormick *et al.*, 2008)**. Along with this role in controlling Na/ Na/K-ATPase activity, cortisol has a significant role in fish metabolism under stressful conditions such as osmotic challenges **(Aluru & Vijayan, 2009)**; as a consequence, it is involved in energy supply during the stress **(Brun *et al.*, 2019)**. Therefore, it is interesting to determine the relationships between copper/MPs and the exposure of these proteins to saltwater in fish. This study investigates the effects of water pollution caused by copper, both alone and in combination with MPs, on common carp during exposure to saltwater conditions. Understanding these effects is crucial, as pollution from MPs is widespread, and copper is frequently employed in carp pond management as a therapeutic agent despite its status as a standard water pollutant. Therefore, there is a pressing need to evaluate the toxic impacts of copper and MPs on common carp when subjected to saltwater challenges, particularly in light of the growing interest in rearing this species in brackish waters. It was hypothesized that pre-exposure to water copper impairs osmoregulation in common carp, and co-exposure to copper and MPs worsens such an impairment.

2. Materials and Methods

1. Experimental protocol

Common carp (average weight: 37.5 ± 3.24 g) were placed in nine 40-liter aquaria with a stocking density of seven fish per tank. The fish underwent a ten-day acclimatization period during which they were fed a commercial sinking diet from Beyza Feed Mill (Shiraz, Iran) at a rate of 2% of their biomass daily. Continuous aeration was provided, and to maintain optimal water quality, half of the water in each aquarium was replaced with clean water daily **(Hoseini *et al.*, 2022)**. Following the acclimation period, the aquaria were divided into three treatment groups, each with three replicates. One treatment was exposed to 0.25 mg/L copper (Cu), one treatment was exposed to 0.25 mg/L copper and 0.5 mg/L polyvinyl chloride MPs (Cu-MP), and one treatment served as a control (not exposed to contaminants). Characteristics of copper and MPs used in this study have been reported before **(Hoseini & Al Sulivany, 2024; Asad *et al.*, 2025)**. Polyvinyl chloride MPs (CAS 9002-86-2) was obtained from Arvand Petrochemical Co., located in Bandar-e-Mahshahr, Khuzestan province, Iran. The morphology of the polyvinyl chloride MPs particles was characterized using a scanning electron microscope (SEM-MIRA3-XMU, TESCAN, Brno, Czech Republic). The particles showed a relatively spherical shape with smooth surfaces and an average diameter of 140 μm. A stock solution of 10 g/L was prepared in test water for use in the experiments.

Copper sulfate pentahydrate, obtained from Merck KGaA (Darmstadt, Germany), served as the source of copper. A stock solution of 5 g/L was prepared from this compound for the experiments.

The exposure lasted 14 days, during which 30% of the aquarium water was replaced with clean water. The desired amounts of copper and MPs were added to the water to maintain the initial concentrations. After 14 days of exposure to the contaminants, all fish were exposed to salt water (13 g/L sodium chloride) for 72 h. Gill samples were collected after 14 days of exposure to the contaminants, as well as 24 and 72 hours after the fish were subjected to saltwater conditions. For sampling, two fish from each aquarium were euthanized by a sharp blow to the head; their gills were then excised and rinsed with distilled water. Six gill samples were dried at 70°C for 24 hours and subsequently analyzed for copper content (n = 6), while three additional gill samples were immediately frozen in liquid nitrogen for gene expression analysis.

Throughout the study, water quality parameters were monitored using digital devices (Hach HQ40D portable probe, Colorado, USA; Palintest 7100 photometer, NE11 0NS, United Kingdom). The recorded measurements included temperature at 23.5 ± 1°C, pH at 7.79 ± 0.65, dissolved oxygen at 6.39 ± 0.45 mg/L, hardness at 195 ± 8.99 mg $CaCO_3$/L, and unionized ammonia levels at 0.01 ± 0.005 mg N/L **(Abdulrahman & Al Sulivany, 2025; Yousefi *et al.*, 2025; Hoseini *et al.*, 2025)**.

Copper concentrations in the aquaria water were measured four times during the experiment. The concentration in control, Cu, and Cu-MP treatments was 0.0035 ± 0.001, 0.242 ± 0.022, and 0.250 ± 0.020 mg/L, respectively.

2. Water and gill copper analysis

Copper concentrations in water and gill samples were determined using a graphite atomic absorption spectrophotometer (Agilent 240z, Santa Clara, California, USA). Water samples were digested with concentrated nitric acid at a 1:1 (v/v) ratio for the analysis. In contrast, gill samples were digested at a 1:20 (w/v) ratio before being injected into the atomic absorption spectrophotometer (Agilent 240z, Santa Clara, California, USA).

3. Gill transcript analysis

Specific primers for common carp were designed based on species data from GenBank, utilizing Geneious IR9 and Oligo-analyzer software (Table 1). RNA was extracted from gill samples using a commercial kit (Dena Zist Asia Co., Mashhad, Iran) and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to eliminate DNA contamination. Complementary DNA (cDNA) synthesis was performed using a commercial kit from SMOBIO Technology (Hsinchu City 30075, Taiwan). Gene expression levels were quantified via real-time PCR (Applied Biosystems, Step One, Foster City, California, USA). The reaction mixtures consisted of 1 μL cDNA, 0.5 μL primer, and 5 μL SYBR Green (Ampliqon A/S, Stenhuggervej 22, 5230 Odense M, Denmark), with the total volume adjusted to 10 μL by adding diethyl pyrocarbonate (Bio Basic Inc., Markham, ON, Canada). Each sample was analyzed in duplicate, and normalization was performed using the housekeeping gene *beta-actin*. The ΔΔCt method was used to calculate expression levels, which were log2-transformed and expressed as fold changes relative to control fish in freshwater.

Table 1: Forward and reverse sequences, length, amplicon size, and accession number of the selected genes' primers

Primer	Sequence (5-3)	length	Tm	Amplicon (bp)	Efficiency (%)	Accession no.
nakα1a-F	CCACTCTTGCTTCTGGTCTTG	20	60	131	98.5	JX570881.1
nakα1a -R	ACCAAGGATCAGGGAGAGAAC	21	60			
gr-F	GGTGTCAATGTGTCCGCTTTA	21	60	126	96.7	AJ879149
gr -R	AGCTGGTTCTTGTGTGTTACG	21	60			
beta-actin-F	TCTGCTATGTGGCTCTTGACT	21	60	118	93.5	XM_019106214.1
beta-actin-R	AACCTCTCATTGCCAATGGTG	20	60			

4. Statistical analysis

The gill copper content data did not satisfy the assumptions required for ANOVA (as confirmed by Shapiro-Wilk and Levene tests); therefore, the data were $\log_{10}$-transformed prior to conducting one-way ANOVA. Gene expression data underwent $\log_2$ transformation and were analyzed using two-way ANOVA (considering factors of toxicant exposure and saltwater challenge). Significant differences among treatment groups were determined using Tukey's test. A significance level of $P < 0.05$ was adopted, and results are presented as mean ± standard error (SE). All statistical analyses were conducted using SPSS version 22.

3. Results

There was no mortality in the treatments during the experiment. The copper content in the water for the control, Cu, and Cu-MP treatments was 0.0035 ± 0.001, 0.242 ± 0.022, and 0.250 ± 0.020 mg/L, respectively. Cu and Cu-MP treatments exhibited similar gill copper contents and significantly ($P = 0.008$) higher gill copper contents than the control treatment after 14-d exposure (Fig. 1).

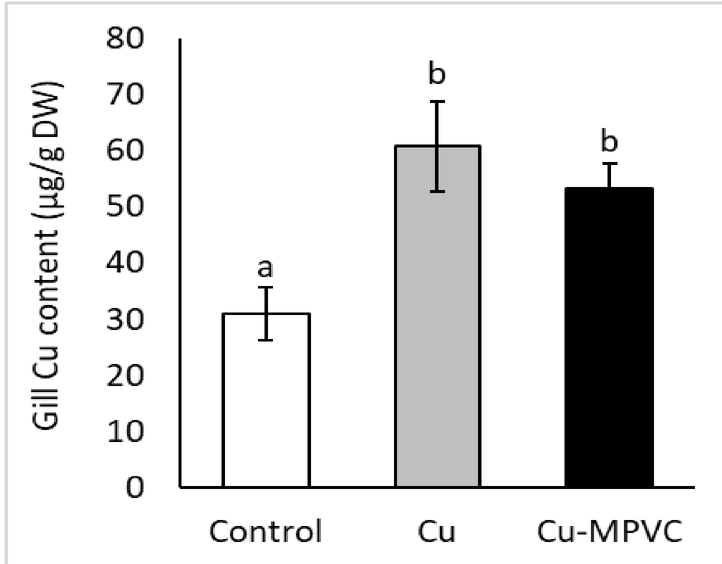

Figure 1. Gill copper content following 14 days exposure to 0.25 mg/L copper alone or in combination with 0.5 mg/L polyvinyl chloride MPs. Different letters above the bars indicate significant differences among the treatments (Tukey; n = 6).

Exposure to copper and polyvinyl chloride microparticles and saltwater challenge induced a significant interaction effect on gill *gr* gene expression ($P < 0.001$). After 14-day exposure to Cu or Cu-MP, the gill gr gene expression significantly increased in the Cu treatment group. The control fish exhibited no significant changes in the gill *gr* gene expression during 24 and 72 h saltwater exposure. The gill *gr* gene expression decreased in Cu and Cu-MP treatments after 24 h saltwater exposure and remained unchanged until 72 h (Fig. 2A).

Exposure to copper and polyvinyl chloride microparticles, along with a saltwater challenge, induced a significant interaction effect on gill *nakα1a* gene expression ($P < 0.001$). There was no significant difference in the gill *nakα1a* gene expression between the control and Cu-MP fish after 14-day exposure; however, the gene expression of Cu fish was significantly higher than the other treatments at this time. The gill *nakα1a* gene expression significantly decreased in all treatments after the 24-hour saltwater challenge; at this time, the control and Cu-MP treatments showed similar gene expressions and were significantly lower than that of the Cu treatment. 72 h after saltwater exposure, the gill nakα1a expression significantly increased in the control and Cu-MP treatments, compared to 24 h. However, the Cu fish exhibited a further down-regulation in the gill *nakα1a* expression after a 72-h saltwater challenge. At this time, all treatments exhibited lower *nakα1a* gene expression compared to the control fish before the saltwater challenge (Fig. 2B).

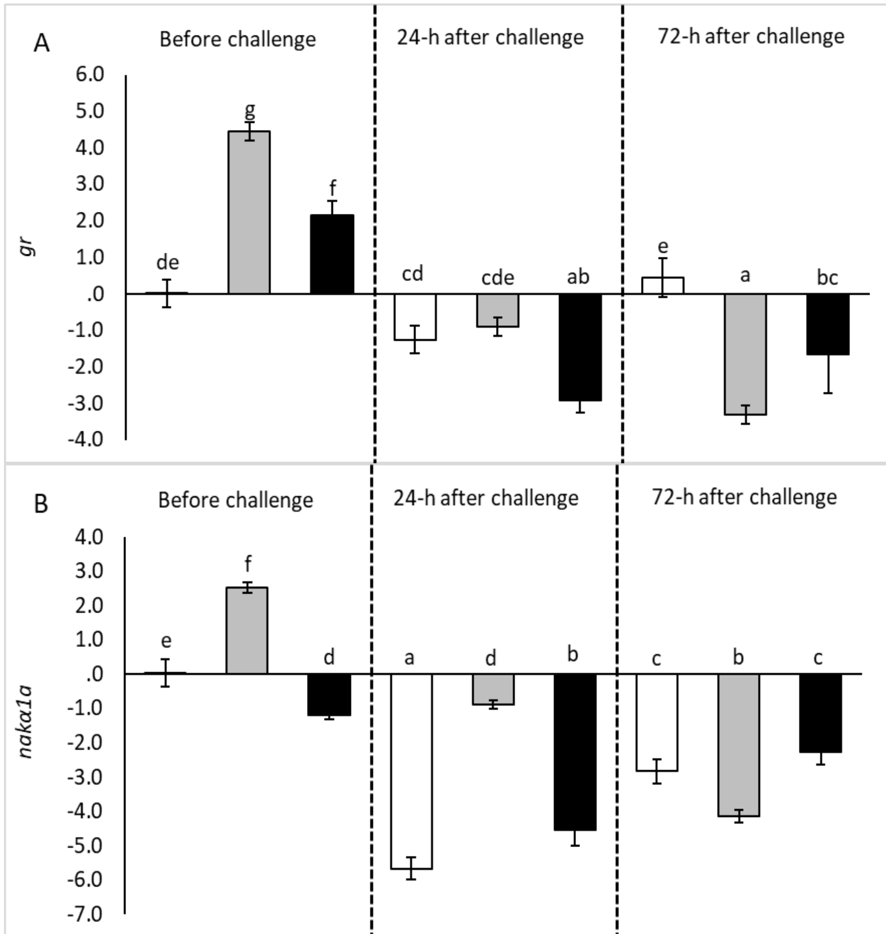

Figure 2. Expression of *gr* (A) and *nakα1a* (B) in the fish gill after 14 days exposure to 0.25 mg/L copper alone or in combination with 0.5 mg/L polyvinyl chloride MPs, followed by 24 and 72 h saltwater exposure. White bars: control fish; gray bars: Cu fish, and black bars: Cu-MP fish. Different letters indicate significant differences among the treatments (Tukey; n = 3).

4. Discussion

MPs serve as vectors for metals in aquatic environments, facilitating their accumulation in fish. The current findings align with the research by **Roda *et al.* (2020)**, which indicated that polyethylene MPs do not enhance copper accumulation in the gills of *Prochilodus lineatus*. In contrast, **Lu *et al.* (2018)** reported that polystyrene MPs promote cadmium accumulation in the liver, gut, and gills of zebrafish Danio rerio after a three-week exposure period. Furthermore, **Wen *et al.* (2018)** demonstrated that polystyrene MPs can reduce whole-body cadmium accumulation in discus fish, *Symphysodon aequifasciatus*, potentially due to lower levels of metallothionein in fish co-exposed to both MPs and cadmium. Given these findings, it is essential to investigate whether the lack of change in gill copper accumulation between copper-only and copper-MP exposures is related to metallothionein levels in the tissue.

Cortisol is necessary under stressful conditions or exposure to toxicants because it protects organs against necrosis by inducing apoptosis and regulating ion transportation **(Kiilerich *et al.*, 2007a; Hoseini *et al.*, 2022)**. *gr* is a mediator of cortisol effects on cells and is up-regulated under stress **(Kiilerich *et al.*, 2007a; Brun *et al.*, 2019)**. It has been documented that copper exposure increases *gr* expression in fish **(Chao Dang *et al.*, 2000)**, but there is no such data about the MPs. However, *gr*-mutant zebrafish exhibit higher whole-body cortisol levels in response to plastic nanoparticles than wild fish exposed to the same nanoparticles. The mutant fish had lower glucose levels than the wild fish, suggesting the importance of *gr* in cortisol-mediated energy supply during plastic exposure **(Brun *et al.*, 2019)**. Therefore, up-regulation of *gr* in the fish gill in response to copper or polyvinyl chloride microparticles is a defensive mechanism to protect the organ against the harmful effects of the toxicants. It has been demonstrated that the stability of the hormone-receptor complex, along with the receptors' richness and affinity, plays a pivotal role in mediating the effects of the hormone on target cells **(Shrimpton & McCormick, 1999)**. This may explain why the control fish did not change in *gr* expression during the saltwater challenge. The present results are in contrast to previous studies showing saltwater exposure increases *gr* expression in the fish gill **(Marsigliante *et al.*, 2000; Bury *et al.*, 2003; Kiilerich *et al.*, 2007b)**; however, they are in line with **Flores and Mark Shrimpton (2012),** who found no change in the gill *gr* expression in rainbow trout transferred to saltwater or ion-poor freshwater. According to the present results, Cu and Cu-MP exposure led to changes in the gill *gr* transcript in response to saltwater exposure. Such a down-regulation in the gill *gr* expression can disturb ion regulation, as the central role of cortisol in fish saltwater tolerance is the regulation of Na/ Na/K-ATPase pump, and such effects are mediated by *gr* **(McCormick *et al.*, 2008).**

nakα1a is a crucial protein that regulates ion concentration in the fish body. The enzymes have several sub-units and isomers with different functions. The isomer α1a was found to have higher activity than α1b when fish are in freshwater, and vice versa. The isomer α1a seems responsible for ion influx in the hypo-osmotic environment **(Bystriansky *et al.*, 2006; McCormick *et al.*, 2009)**. Copper toxicity has been known to cause sodium loss in fish, a mechanism mediated by a copper-induced decrease in *nakα1a* activity in the basolateral membrane of the gills **(Grosell *et al.*, 2007)**. According to the present results, the up-regulation in *nakα1a* expression in Cu treatment may be a response to lower enzyme activity and internal sodium loss. On the other hand, Cu-MP fish showed a down-regulation in the *nakα1a* gene expression, which might be due to the combined toxic effects of copper and microplastic on the transcription processes. Measurement of internal ions' concentrations may better reflect the mechanisms behind such changes. The control fish exhibited a significant down-regulation in the gill *nakα1a* expression after the saltwater challenge, indicating the role of nakα1a in a hypo-osmotic medium **(Bystriansky *et al.*, 2006; McCormick *et al.*, 2009)**. Therefore, the gene's down-regulation in Cu and Cu-MP treatments after the saltwater challenge is explained by increased electrolyte levels in the fish body, which lead to the activation of the α1b subunit. This is an exciting topic that should be pursued in the future.

5. Conclusion

Exposure to copper disrupted gill ion regulation in common carp, increasing gr and nakα1a gene expression. However, when combined with polyvinyl chloride microplastics (Cu-MP), the effect on nakα1a was reduced, suggesting a mitigating influence. Saltwater conditions further influenced gene activity, revealing the carp's adaptive mechanisms to osmotic changes. Notably, microplastics did not intensify copper's impact on ion balance. These results reveal intricate pollutant-salinity interactions, stressing the importance of deeper investigation into contaminant co-exposure in aquaculture to safeguard fish health and industry sustainability.

Ethical statement

All experimental procedures involving animals were approved by the Ethics Committee of the Inland Waters Aquatic Resources Research Center, Gorgan, Iran (Reference Approval Number: ...) and were performed in accordance with relevant guidelines and regulations

Author Contributions

Both authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Seyyed Morteza Hoseini, Basim S.A. Al Sulivany

Acquisition, analysis, or interpretation of data: Seyyed Morteza Hoseini, Basim S.A. Al Sulivany

Drafting of the manuscript: Seyyed Morteza Hoseini, Basim S.A. Al Sulivany

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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