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Hepatic transcriptomic and histopathological responses of common carp, *Cyprinus carpio*, to copper and microplastic exposure

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ABSTRACT

The combined effects of copper and polyvinyl chloride (PVC) microparticles were investigated on the metal accumulation, histopathological biomarkers, and targeted transcriptomics in *Cyprinus carpio* liver. The fish were exposed to 0.25 mg/L copper and/or 0.5 mg/L PVC microparticles over a 14-d period. The results showed that hepatic copper accumulation is facilitated by the PVC microparticles presence in water. All treatments induced significant hepatic stress and inflammation; however, the transcriptional responses involving in detoxification pathways and apoptotic mechanisms were mixed and often down-regulated in the fish exposed to copper and/or PVC microparticles. Exposure to copper and/or PVC microparticles induced hypermeia, leukocyte infiltration and increase in melanomacrophage centers number and area. Generally, the severity of the lesions was in the following order: PVC microparticles < copper < copper+ PVC microparticles. In conclusion, PVC MPs act as a copper vector, facilitating accumulation of copper in the fish liver and increasing the tissue damage.

Keywords: PVC microparticle, bioaccumulation, co-exposure, gene expression, hepatic damage

1 INTRODUCTION

Plastic pollution is a pervasive and growing global threat (Borrelle et al., 2020). Estimates suggest that in 2016, 19 to 23 million metric tons of plastic (11% of the plastic generated) entered aquatic ecosystems, with annual emissions expected to keep increasing and potentially reaching 53 million metric tons by 2030 (Borrelle et al., 2020). Once in the natural environment, plastics undergo physical, photodegradation and biodegradation processes, forming microplastic (MP). This fragmentation not only makes their identification and removal more difficult, but also increases their bioavailability (Botterell et al., 2019; Min et al., 2020; Ali et al., 2021). While the effects of larger plastics on wildlife are well known (e.g. suffocation, entanglement, blockage of alimentary canals, indigestion and starvation), the eco-toxicological impacts of MPs have been remained much less understood (Moreschi et al., 2020; Pannetier et al., 2020; Ragusa et al., 2021). Of worrying concern is the capacity of MPs to adsorb different chemicals such as heavy metals, which may result in enhanced toxicity (Lu et al., 2018; Fred-Ahmadu et al., 2020; Magadini et al., 2020). Whilst a growing body of literature has recently focused on understanding the combined effects of different metals and MPs on fish physiology, there are still important knowledge gaps (Naqash et al., 2020). Particularly, literature shows contrasting results depending on the type of MPs, metal species and form and fish species studied, suggesting complex interactions that require further study. For example, polystyrene (PS) MPs increases cadmium accumulation in zebrafish, *Danio rerio* (Lu et al., 2018), but shows an opposite effect in discus fish, *Symphysodon aequifasciatus* (Wen et al., 2018). Another study has revealed that whilst low concentrations of PS-MPs decrease cadmium toxicity in zebrafish larvae, the toxicity is enhanced at higher PS-MPs concentrations (> 0.1 mg/L) (Zhang et al., 2020). In the case of copper, polyethylene (PE) MPs has no significant effects on hepatic copper accumulation and subsidizes the copper-induced oxidative stress and DNA damage in *Prochilodus lineatus* (Roda et al., 2020). In contrast, it has been found that co-exposure of zebrafish larvae to polymer MPs and copper induces neurotoxicity, alters behaviors and decreases their growth and survival (Santos et al., 2020; Santos et al., 2021b; Santos et al., 2021a).

To date, most of the studies investigating MPs toxicity effects on fish have been performed using light density MPs such as PS and PE (Alomar et al., 2017; Karbalaei et al., 2021; Umamaheswari et al., 2021; Tongo and Erhunmwunse, 2022). However, polyvinyl chloride (PVC) MP, which is not only one of the most used plastics but also due to its higher density it tends to sink and thus be more available for benthic organisms, has received much less attention. Despite some recent studies showing that PVC MPs induce negative effects (such as oxidative stress and histopathological lesions) in fish (Espinosa et al., 2018; Espinosa et al., 2019; Boyle et al., 2020; Romano et al., 2020),

no studies have yet evaluated the combined toxicity of PVC MPs and metals in fish (or most aquatic animals). In fact, only one recent work has investigated the combined effect of PVC MPs and cadmium in marine nematodes and shows that combined treatments displays less toxicity than the separate exposure (Wakkaf et al., 2020).

In this work, we set to investigate the combined effect of copper and PCV MPs on metal accumulation and toxicity in common carp, *Cyprinus carpio*. Copper is a well-known toxicant in aquatic life, particularly in fish, with a variety of negative effects including induction of oxidative stress, apoptosis, inflammation, and histopathological lesions (Al-Bairuty et al., 2013; Pereira et al., 2016). The interaction between MPs and adsorbed metals such as copper is extremely relevant in aquatic (both coastal and freshwater) ecosystems, which are often exposed to high quantities of pollutants due to their proximity to industrialized and urban areas (Wong et al., 2020). Wild common carp is a bentho-pelagic fish found both in brackish waters and freshwater rivers (Ghelichpour et al., 2013). Common carp is widely used as a fish bioindicator due to its widespread distribution, easy availability and sensitivity to xenobiotics (Saucedo-Vence et al., 2017; Chen et al., 2019). Due to its omnivorous, sediment-dwelling behaviour, this species tend to accumulate more heavy metals than pelagic fish (Alam et al., 2002) and might be more exposed to high-density plastics (such as PVC) that could exacerbate metal and pollutant accumulation. The present study aimed at testing the hypothesis of whether PVC MPs can facilitate copper accumulation in carp and intensify its negative effects on the fish liver. To test this, common carps were exposed to copper and/or PVC MPs for 14 days and hepatic copper accumulation, histopathological lesions, and transcriptomic responses were investigated.

2 MATERIALS AND METHODS

2.1 MP and copper sources

In the present research, PVC meal (CAS 9002-86-2) were purchased from Arvand Petrochemical Co. (Khuzestan province, Bandar-e-Mahshahr, Iran). Scanning electron microscope (SEM- MIRA3-XMU-TESCAN, Brno, Czech Republic) were used to investigate the morphology and distribution size of PVC MPs. In order to determine the size distribution of the particles, 40 particles were randomly measured under the SEM. Accordingly, the particles exhibited relatively spherical shape with smooth surface and average diameter of 140.7 ± 5.11 μm (mean ± SE) (Fig. 1). Using this PVC MP source, a stock solution of 10 g/L (pH 7.23) was prepared in the test water for the fish exposure trial. The solution was stirred for 10 min on a magnetic stirrer to achieve a homogenous suspension (with a milky appearance), immediately before adding to the test water.

Copper sulfate pentahydrate (CAS 7758-99-8; Merck KGaA, Darmstadt, Germany) was used as copper source and dissolved in the test water to have a final concentration of 5 g/L. This stock was used for the aquaria inoculation.

2.2 Experimental protocol

This experiment was conducted after consultation with the Scientific Board of the Inland Waters Aquatic Resources Research Center, Gorgan Iran. Four-month old common carp (37.8 ± 1.01 g; 11.8 ± 0.42 cm standard length; mean ± SE), offspring of domestic broodstock, were purchased from a local farm (Behshar, Mazandaran Province, Iran) and stocked into 12 aquaria (40 L) at a density of seven fish per aquarium. Total number of the fish in this experiment was 84 specimens that were randomly (simple randomization) distributed into the 12 aquaria. They were acclimatized to the experimental conditions for ten days, during which they were fed a commercial diet (Beyza Feed Mill, Shiraz, Iran) based on 2% of biomass per day. The aquaria were continuously aerated and the water renewal rate of 50% was done daily. After the acclimation, the aquaria were numbered and randomly (simple randomization) assigned into four triplicate groups. Each triplicate group was consider as a treatment: Control (fish were kept in clean water); copper (fish were kept in water containing 0.25 mg/L copper); PVC-MP (fish were kept in water containing 0.5 mg/L PVC-MP); and copper+PVC-MP (fish were kept in water containing 0.25 mg/L copper + 0.5 mg/L PVC-MP). The fish were exposed to water copper and PVC MPs over a 14-day period because this period was found enough to induce negative effects in fish (Hoseini et al., 2016; Ding et al., 2018; Yin et al., 2018). The concentration of copper was based on a previous study on common carp (Dautremepuits et al., 2004), whereas, the concentration of PVC MPs was an environmentally-relevant one according to previous reports (Lasee et al., 2017; Romano et al., 2020). To adjust the water copper and PVC MPs levels, 2 mL of the copper and PVC MPs stock solution were inoculated into the aquaria at the first day of the exposure. During the exposure, 50% of the aquaria water was replaced with clean tap water (dechlorinated by intense aeration for 24 h) and the concentrations of copper and PVC MPs were adjusted by adding 1 mL of the stock solutions to the aquaria. The fish were fed as the acclimation period during the exposure to copper and PVC MPs. At the end of the experiment, six fish per treatment were sampled for histopathological analysis, copper assay and gene expression assay. The fish were dissected and the hepatic pieces were removed by a scissors and washed with distilled water. The samples of histopathological analysis were fixed immediately in buffered formalin (10%) for one month at 16-20°C. A piece of the fish liver were dried at 70°C (24 h), kept at 4°C (2 days) and used for copper assay. For gene expression assay, the samples were immediately frozen in liquid nitrogen and transferred to -70°C until analysis.

Water temperature, pH, dissolved oxygen, hardness, unionized ammonia, and copper levels were 23.5 ± 1°C, 7.79 ±0.65, 6.39 ± 0.45 mg/L, 195 ± 8.99 mg $CaCO_3$/L, 0.01 ± 0.005 mg N/L, and 0.002 ± 0.000 mg/L, respectively. The copper concentrations of the aquaria were measured four times during the experiment; accordingly, water copper contents of Control, copper, PVC-MP, and copper+PVC-MP treatments were 0.0035 ± 0.001, 0.242 ± 0.022, 0.0055 ± 0.001, and 0.250 ± 0.020 mg/L, respectively.

2.3 Water physicochemical analyses

Water physicochemical parameters were measured once a day, before the aquaria water renewal. Water temperature, pH and dissolved oxygen levels were investigated using a portable digital probe (Hach portable probe; Model HQ40D, Loveland, Colorado, USA). Water ammonia (indophenol salicylate method; 0-1 mg/L) and total hardness (EDTA and eriochrome black method; 0-500 mg/L) were determined using a customized photometer (Palintest photometer; Model 7100, Palintest House, Kingsway, Team Valley, Gateshead, Tyne & Wear, NE11 0NS, United Kingdom).

2.4 Hepatic histopathological examination

The fixed hepatic samples were used to prepare cross sections for histopathological examination. The samples were collected in 25-mL tubes containing formalin, blindly numbered and used for sectioning. Each sample (approximately 5 mm) was embedded in one paraffin block. The samples were dehydrated in ethanol before embedding in paraffin. Three sections (at 200 µm intervals) with 4-µm thickness were prepared from each sample, mounted on slides and stained by hematoxylin and eosin. Sample of each fish were mounted on one slide. The slides were blindly-examined under light microscopy (Nikon Eclipse 50i, NY 11747-3064, USA) equipped with a camera and LCD (Model: Digital Sight DS-L2, Nikon, NY 11747-3064, USA) and histopathological lesions were defined qualitatively and quantitatively. Nine fields (400 × 300 µm; approximately 75% of each section area) were observed on each slide (at 20x magnification) and number of melanomacrophage centers (MMCs) in the hepatic parenchyma were counted. MMCs number per mm^2 of hepatic section was calculated and average of MMCs number per mm^2 of hepatic sample was used for statistical analysis (Supplementary data 1). The dimensions of MMCs were recorded using the LCD software and used for MMCs area calculation. The sum of MMCs are per section was calculated and averages area of three sections per sample were used for statistical analysis (Supplementary data 2). Moreover, 20 pancreas tissues per slide were examined and percentage of pancreas with MMC was used for statistical analysis. Other lesions were also checked in the same nine fields and presented semi-quantitatively. Briefly, nine fields per section of a sample were observed and number of the fields exhibiting the lesions were recorded. Based on these data, four scales of severity

were adjusted, including none (lesion not presented), mild (1-9 out 27 fields exhibiting the lesion), moderate (10-18 out 27 fields exhibiting the lesion), and severe (19-27 out 27 fields exhibiting the lesion).

2.5 Water and hepatic copper assay

The water and hepatic samples were collected in separate tubes, which were numbered blindly. The water samples were mixed with equal volume of concentrated nitric acid before injection to a graphite atomic absorption spectrophotometry (Agilent 240z, Santa Clara, California, USA). For tissue copper content determination, 100 mg of dried samples were mixed with 2 mL concentrated nitric acid and digested over 24 h at 65°C in an oven to achieve a clear solution. After the completion of digestion, 2 mL of distilled water was added to the samples and the final mixture was injected to the graphite atomic absorption spectrophotometry. Appropriate dilutions were made, if necessary, and the samples were double-assayed. Copper assays were performed blindly.

2.6 Gene expression analysis

The liver samples were blindly collected in plastic tubes. The tubes were numbered and used for gene expression analysis. In the liver samples, the expressions of glutathione-s-transferase (*gst*), heat shock protein 70 (*hsp70*), tumor necrosis factor alpha (*tnfa*), interleukin 1 beta (*il1b*), caspase 3 (*cas3*), caspase 9 (*cas9*), and Cytochrome P450 1A1 (*cyp1a1*) were assessed. Primer of the genes were designed using Geneious IR9 and Oligoanalyzer software (Table 1).

The liver samples were treated with a commercial kit (Dena Zist Asia Co., Mashhad, Iran) for RNA extraction. The products were treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove DNA contamination and RNA quality was checked using a NanoDrop (Thermo Scientific, 2000c, Waltham, MA, USA) at 280/260 nm. Then, cDNA was synthetized using a commercial kit (SMOBIO Technology, Hsinchu City 30075, Taiwan). Gene expressions were assayed using quantitative RT-PCR (Applied biosciences, Step One, Foster City, California, USA). Reaction mixtures contained 1µL cDNA, 0.5 µL primer and 5 µL SYBR Green (Ampliqon A/S, Stenhuggervej 22, 5230 Odense M, Denmark), which filled up to 10 µL by addition of Diethyl pyrocarbonate (Bio Basic Inc., Markham, ON, Canada). Forty cycles consisting initial denaturation (120 s, 94°C), denaturation (15 s, 94°C), annealing (30 s, 58°C), and expansion (20 s, 72°C) were run per sample. Each reaction was run in duplicate and gene expression was normalized based on a housekeeping gene (beta-actin) of the Control treatment. Gene expression was calculated according to ΔΔCt method (Livak and Schmittgen, 2001). RT-PCR processes were performed blindly.

2.7 Statistical analysis

Comparison of the *cas3* among the treatments was performed by one-way ANOVA and Tukey tests (mean of six replicates) after confirmation of normal distribution (Shapiro-Wilk test) and variance homogeneity (Levene test). Comparison of the other data among the treatments was conducted by Kruskal-Wallis and Mann-Whitney U tests, as they failed to meet ANOVA assumptions. $P < 0.05$ was considered as significance and the data were presented as mean ± SE. All analyses were performed in SPSS v.22.

3 RESULTS

3.1 Hepatic copper content

Hepatic copper content exhibited significant differences among the treatments ($P < 0.001$). There was no significant difference in hepatic copper content between the Control and PVC-MP treatments. Hepatic copper contents of copper and copper+PVC-MP treatments were significantly higher than that of the Control; moreover, the hepatic copper content in copper+PVC-MP was significantly higher than that of Copper treatment (Fig. 2).

3.2 Hepatic histopathological assessment

Hepatic histopathological lesions are presented in Table 2 and Fig. 3 and 4. Exposure to copper and/or PVC-MP significantly increased hepatic hyperemia and leukocyte infiltration. The severity of these lesion was as following order: PVC-MP < copper < copper+PVC-MP. There were significant differences in the number ($P = 0.008$) and area ($P = 0.001$) of MMC in the hepatic parenchyma and percentage of pancreas tissues with MMC ($P = 0.003$). Number of MMCs significantly increased in copper and copper+PVC-MP treatments, compared to the Control. Exposure to copper, PVC-MP, and copper+PVC-MP significantly increased area of MMCs and percentages of pancreatic tissues with MMCs. The highest MMCs area was observed in copper and copper+PVC-MP treatments; whereas, the highest percentages of pancreatic tissues with MMCs was observed in copper+PVC-MP treatment.

3.3 Targeted transcriptomics

hsp70 ($P = 0.001$), *tnfa* ($P < 0.001$) and *il1b* ($P = 0.001$) gene expressions were significantly affected by copper and/or PVC MPs exposure. Copper, PVC-MP and copper+PVC-MP treatments showed significant up-regulations in hepatic *hsp70* gene expression compared to the Control and the highest expression was related to copper treatment. Copper, PVC-MP and copper+PVC-MP treatments exhibited significant up-regulations in hepatic *tnfa* and *il1b* gene expression, compared to the Control. The highest expression of *tnfa* was observed in copper treatment and the highest expressions of *il1b* were related to PVC-MP and copper+PVC-MP treatments (Fig. 5).

There were significant effects of copper and/or PVC MPs exposure on hepatic *cyp1a1* ($P = 0.003$) and *gst* ($P = 0.001$). Whereas PVC-MP and copper+PVC-MP exhibited no changes in hepatic *cyp1a1* gene expression, compared to the Control, copper treatment showed a significant down-regulation (Fig. 5). Hepatic *gst* exhibited a significant down-regulation in copper treatment, but significant up-regulations in PVC-MP, compared to the Control (Fig. 6).

Significant effects of exposure to copper and/or PVC-MP were found on hepatic *cas3* expression ($F = 15.7$; $P < 0.001$). Hepatic expression of *cas3* exhibited a significant up-regulation in PVC-MP treatment, and down-regulation in copper+PVC-MP treatment, compared to the Control treatment. The gene expression in copper treatment was comparable to the Control (Fig. 6). Exposure to copper and/or PVC MPs significantly affected hepatic *cas9* expression ($P = 0.002$). Copper and copper+PVC-MP treatments exhibited significant down-regulations in hepatic *cas9* gene expression, compared to the Control. Nevertheless, the gene expression in PVC-MP treatment was comparable to the Control (Fig. 7).

4 DISCUSSION

4.1 PVC MPs as copper vector

The liver is the main organ responsible for detoxification, transformation and storage of toxic compounds in fish (Bawuro et al., 2018). Metal accumulation (e.g. copper and cadmium) has been previously observed in fish liver, and was associated to the high concentration of metallothionein proteins, which play essential roles in the regulation and detoxification of intracellular metals (Das et al., 2006; Ghedira et al., 2010). Here, we demonstrated that copper accumulation was facilitated by environmental PVC MPs, with copper levels were 2-fold higher in the combined treatment (copper+PVC-MP) than in the copper treatment. This could be explained by involuntary ingestion of MPs with adsorbed copper on their surfaces. Then, once in the gut, the copper (or perhaps the MPs with copper) could be transferred to the liver via the blood stream. Facilitation of metal uptake and accumulation in different fish tissues by MPs have been previously shown (Lu et al., 2016; Lu et al., 2018).

4.2 Histopathological damage induced by copper and PVC MPs

The observed liver histological changes (increases in MMCs, hyperemia, and leukocyte infiltration) in the three treatments are commonly associated with hepatocyte response to toxic substances (Ghelichpour et al., 2017; Macêdo et al., 2020), with the combined treatment (copper+PVC-MP) displaying the highest liver damage. It has been reported

that exposure to toxicant persuades immunological responses (Agius and Roberts, 2003), which explains increased number/volume of MMCs and leukocyte infiltration in the present study. MMCs are the sites of concentration of cellular debris, formed during toxicant exposure (Agius and Roberts, 2003). Moreover, MMCs have immunological roles and increase in their population in the fish hepatic tissue might be due to inflammation caused by copper and/or PVC-MP exposure (see *hsp70*, *tnfa*, and *il1b* gene expressions). Inflammation results in recruiting leukocyte to the damaged tissue, which explains higher leukocyte infiltration in the liver of the exposed fish. Besides, blood flow increases within the inflamed tissues, which explains presence of hyperemia in the exposed fish. Such pathological changes have been previously reported in fish exposed to copper (Mela et al., 2013; Paulino et al., 2014; Yancheva et al., 2014) or MPs (Jabeen et al., 2018; Santana et al., 2022). Our results not only show that both treatments separately lead to liver alterations but that the co-exposure exacerbates these alterations. A plausible explanation for the higher damage in fish co-exposed to copper and PVC MPs could be related to the higher availability (i.e. bioaccumulation) of copper as proposed for earthworms co-exposed to MPs and cadmium (Huang et al., 2021). However, co-exposure of copper and MPs could also display synergetic effects at different subcellular levels. For example, Qiao et al. (2019) suggested that higher toxicity in zebrafish co-exposed with copper and PS-MPs was attributed to the inhibition of Cu-ion transport and the synergetic copper and MPs effect on oxidative stress. Similarly, Eom et al. (2021) also observed that mysid co-exposure to different metals and PS MPs increased metal toxicity and impaired the antioxidant defense and cholinergic systems.

4.3 Transcriptional changes induced by copper and PVC MPs

Toxicity of MPs and metals in cells is mainly due to the generation of reactive oxygen species (ROS) (Dazy et al., 2009; Gauthier et al., 2014; Jeong et al., 2016; Alomar et al., 2017), which triggers different cellular responses to protect the organisms from ROS-associated damage, such as stress-induced signaling pathways (e.g. heat-shock proteins), pro-inflammatory responses (e.g. *tnfa* and *il1b*), detoxification mechanisms (*gst* and *cyp1a1*), and apoptosis (Gao et al., 2013; Morcillo et al., 2015; Morcillo et al., 2016). Our results showed that all treatments induced significant hepatic stress and inflammation, as evidenced by significant overexpression of *hsp70*, with fish exposed only to copper displaying the highest *hsp70* values. We also observed that all treatments induced inflammation, with expression of *tnfa* being highest in fish exposed to copper and *il1b* being highest in PVC-MP and copper+PVC-MP treatments. Such inflammatory responses have been well-documented in fish exposed to copper [reviewed by Pereira et al. (2016)]. It has been previously reported that PVC MPs induce no significant changes in *hsp70*, *il1b* and another

pro-inflammatory cytokine, interleukin 8 (*il8*), when administered through dietary route in European seabass, *Dicentrarchus labrax* (Espinosa et al., 2019), and gilthead seabream, *Sparus aurata* (Espinosa et al., 2017). On the other hand, inflammatory transcriptomic responses were observed in fish exposed to water contaminated with other MPs. For example, exposure to polystyrene MPs induced transcription of *tnfa*, *il1b*, *il8*, and interferon gamma in Nile tilapia, *Oreochromis niluticus* (Ahmadifar et al., 2021). Such inconsistent results might be due to the difference in MPs concentration, type and size, and target species and organ, which arise the need for further studies to illustrate the inflammatory effects of MPs in fish.

Cytochrome P450 1A1 and glutathione-s-transferase belong the first and second phases of detoxification process, respectively (Bengtson Nash et al., 2014). While, the processes is normally activated upon exposure to toxicants, copper has shown to have an inhibitory effect on *cyp1a1* and *gst* expression in fish including common carp (Jiang et al., 2014; Jiang et al., 2015), *Oryzias javanicus* (Woo et al., 2009), and rainbow trout, *Oncorhynchus mykiss*, hepatocytes (Faverney et al., 2000; Risso-de Faverney et al., 2000). In the case of PVC MPs, the results are in line with those found in goldfish, *Carassius auratus*, as no change in *cyp1a1* and elevation in *gst* were observed in different organs of the fish (Romano et al., 2020). Such results were, also, observed, when other MPs were test in fish; for example polyethylene MPs in sheepshead minnow, *Cyprinodon variegatus* (Choi et al., 2018), and low density polyethylene MPs in zebrafish (Rainieri et al., 2018). Overall, it seems that PVC MPs and copper have distinctive effects on detoxification process in fish, as copper has inhibitory effects. Such effects are harmful to fish and this might explain higher histopathological lesions in the copper and copper+PVC-MP treatments.

Caspases (3 and 9) are vital molecules in regulating apoptotic mechanisms (Gao et al., 2013). Previous works have shown that different pollutants including copper can enhance *cas3* and *cas9* and induce apoptosis in fish (Gao et al., 2013; Jiang et al., 2015; Zhang et al., 2018). Here, we observed a significantly down-regulation of *cas3* and *cas9* in fish exposed to copper and copper+PVC-MP. Although these results are in contrast with some previous literature, Krumschnabel et al. (2005) showed that copper-induced apoptosis was dependent on mitochondrial permeability transition (MPT) induction, and some trout hepatocyte sub-populations responded differently, some of which displayed reduced MPT potential, with decreases apoptosis and increased necrosis. Although, the origin of caspases down-regulation in this study remains puzzling, perhaps some procedures similar to those described in Krumschnabel et al. (2005) might apply. However, as caspases play key roles in protecting organisms from stress signals, their

inhibition in fish exposed simultaneously to copper and PVC MPs could play a role in the higher toxicity and damage in this group.

5 CONCLUSION

In summary, the present results show that PVC MPs act as a copper vector, facilitating accumulation of copper in carp liver and increasing the tissue damage in fish co-exposed to copper and PVC MPs. Our results suggest that co-exposure to copper and MPs not only lead to higher toxicity due to copper bioaccumulation, but also through subcellular interactions such as inhibition of apoptosis and detoxification pathways and increases oxidative stress.

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494 FIGURE CAPTIONS

495 Fig. 1: SEM micrograph of PVC MPs at different magnifications (a, b and c) and particle size distribution histogram
496 (d).

497 Fig. 2: Hepatic copper content in different treatments after 14 days exposure to 0.25 mg/L Copper and/or 0.5 mg/L
498 PVC MPs. Different letters indicate significant differences among the treatments (Kruskal-Wallis and Mann-Whitney
499 U tests; $n = 6$; $P < 0.001$).

500 Fig 3: Hepatic section of the fish. A and B: control fish (A: 20x; B: 40x); C-E: Copper treatment (C: 20x; D: 40x; E:
501 20x); F-H: PVC-MP (F: 20x; G: 40x; H: 20x); I-K: Copper+PVC-MP (I: 20x; J: 40x; K: 20x). PN: pancreas; HN:
502 hepatocyte nucleus; ER: erythrocyte; HR: hyperemia; MMC: melanomacrophage centers; LI: leukocyte infiltration
503 (scale bar is 25 μm).

504 Fig. 4: Number and area of MMC within the hepatic parenchyma and percentage of pancreatic tissue with MMC in
505 different treatments. Different letters above the bars show significant differences among the treatments [Kruskal-
506 Wallis and Mann-Whitney U tests; $n = 6$; number of MMC within the hepatic parenchyma ($P = 0.008$), area of MMC
507 ($P = 0.001$); percentage of pancreas with MMC ($P = 0.003$)]

508 Fig. 5: Hepatic *hsp70*, *tnfa* and *il1b* gene expressions (relative to *beta-actin*) in different treatments after 14 days
509 exposure to 0.25 mg/L Copper and/or 0.5 mg/L PVC MPs. Different letters indicate significant differences among the
510 treatments [Kruskal-Wallis and Mann-Whitney U tests; $n = 6$; *hsp70* ($P = 0.001$), *tnfa* ($P < 0.001$) and *il1b* ($P = 0.001$)].

511 Fig. 6: Hepatic *cyp1a1* and *gst* gene expressions (relative to *beta-actin*) in different treatments after 14 days exposure
512 to 0.25 mg/L Copper and/or 0.5 mg/L PVC MPs. Different letters indicate significant differences among the treatments
513 [Kruskal-Wallis and Mann-Whitney U tests $n = 6$; *cyp1a1* ($P = 0.003$) and *gst* ($P = 0.001$)].

514 Fig. 7: Hepatic *cas3* and *cas9* gene expressions (relative to *beta-actin*) in different treatments after 14 days exposure
515 to 0.25 mg/L Copper and/or 0.5 mg/L PVC MPs. Different letters indicate significant differences among the treatments
516 (*cas3*: ANOVA and Tukey tests; $n = 6$; $F = 15.7$; $P < 0.001$; *cas9*: Kruskal-Wallis and Mann-Whitney U tests; $n = 6$;
517 $P = 0.002$).

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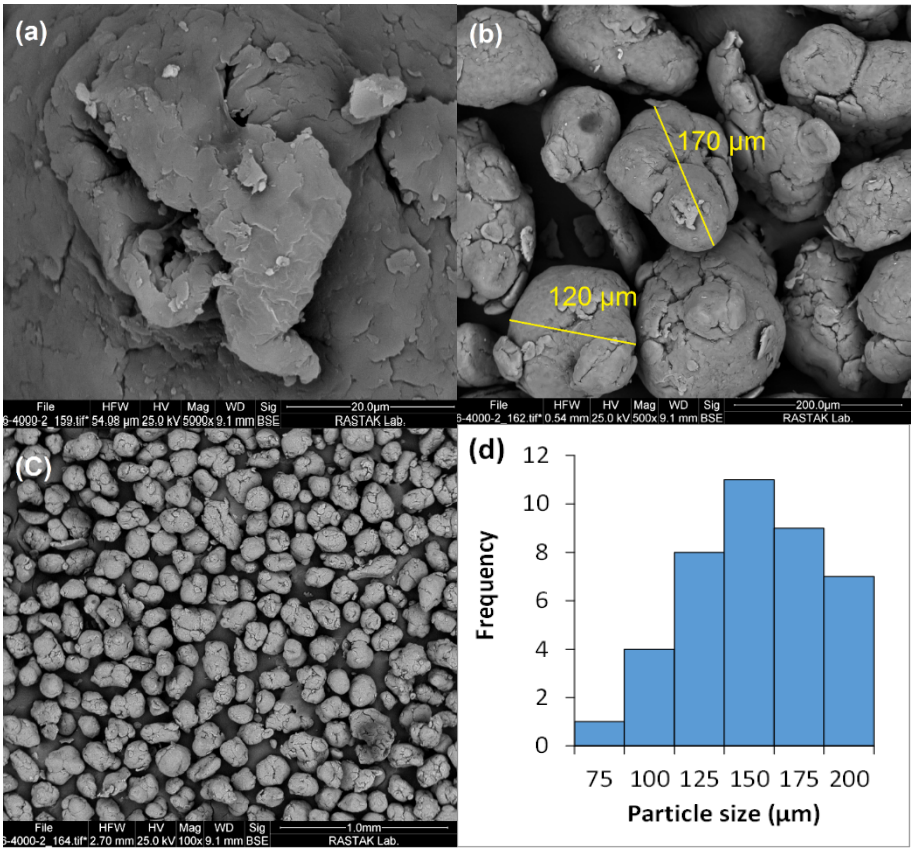
(a)
(b)
170 µm
120 µm
(C)
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Frequency
Particle size (µm)
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2
4
6
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10
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75 100 125 150 175 200

Fig. 1:

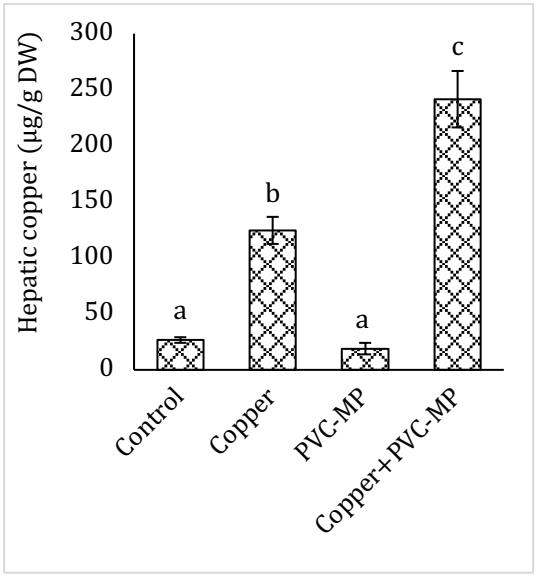
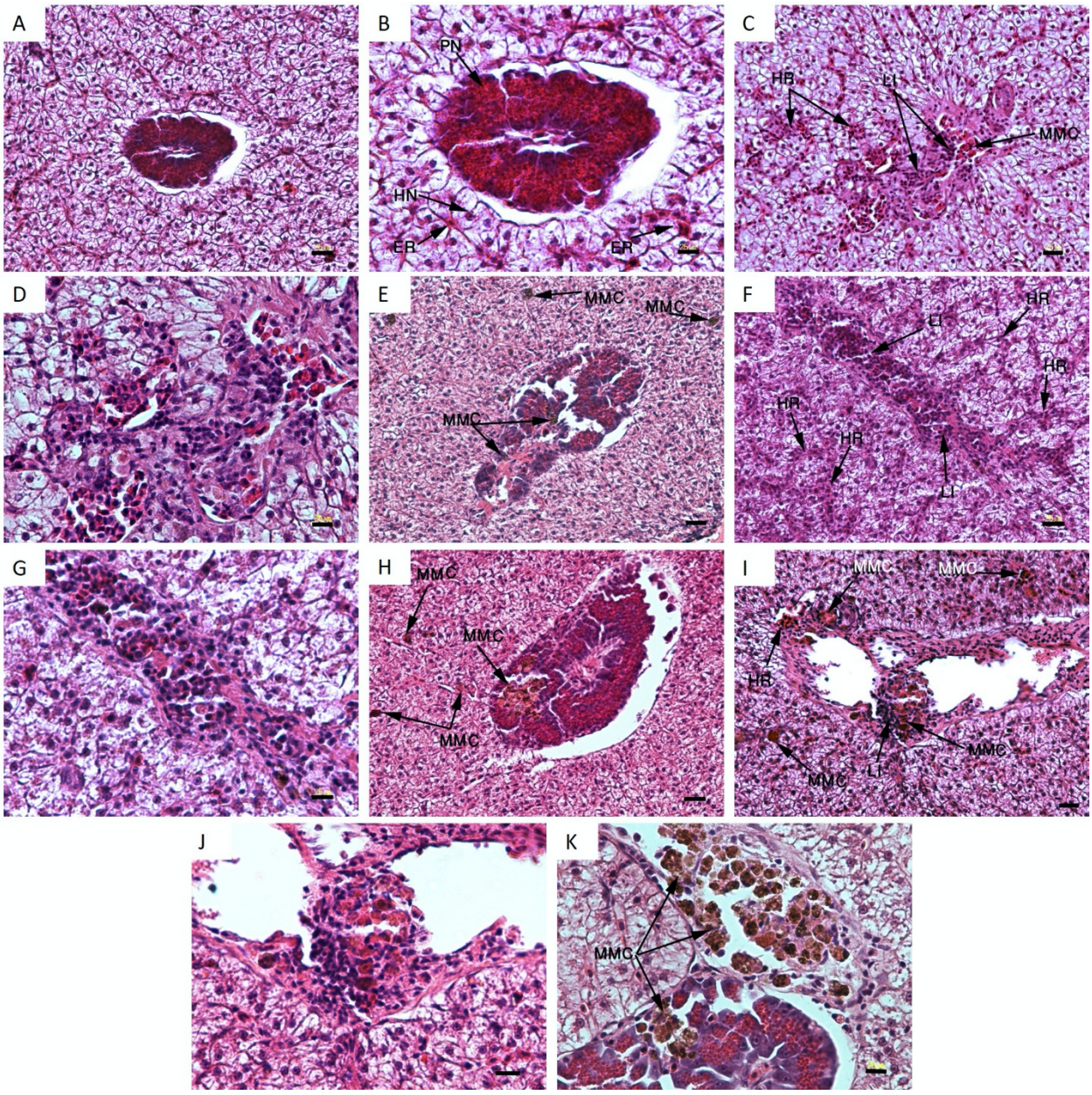

Hepatic copper (µg/g DW)
0
50
100
150
200
250
300
a
b
a
c
Control
Copper
PVC-MP
Copper+PVC-MP

Fig. 2:



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529 Fig. 3

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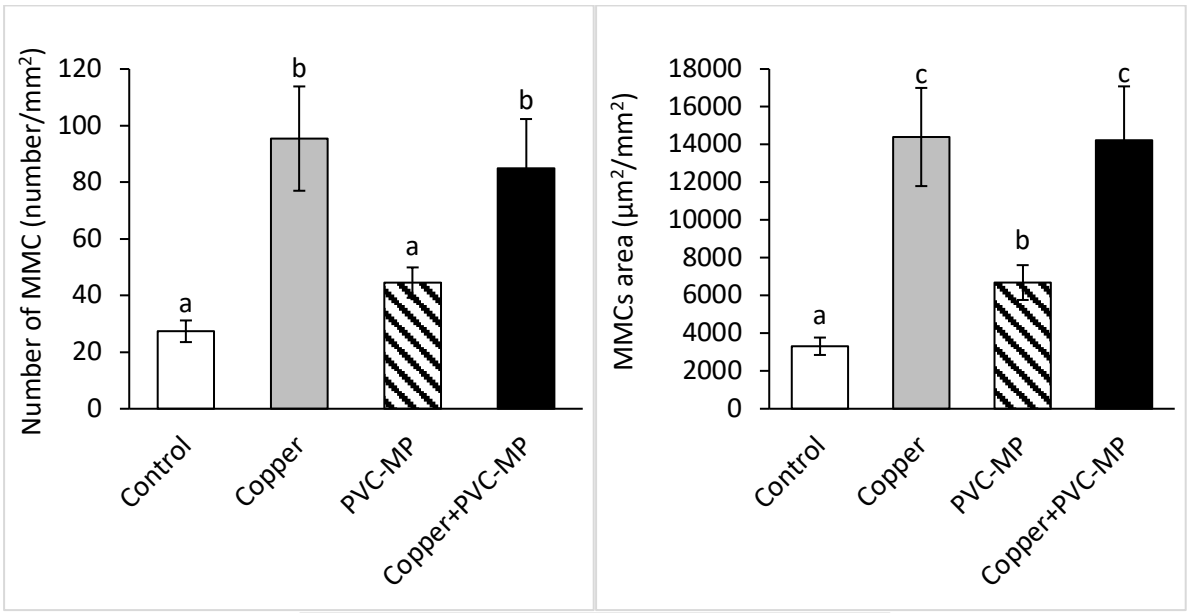

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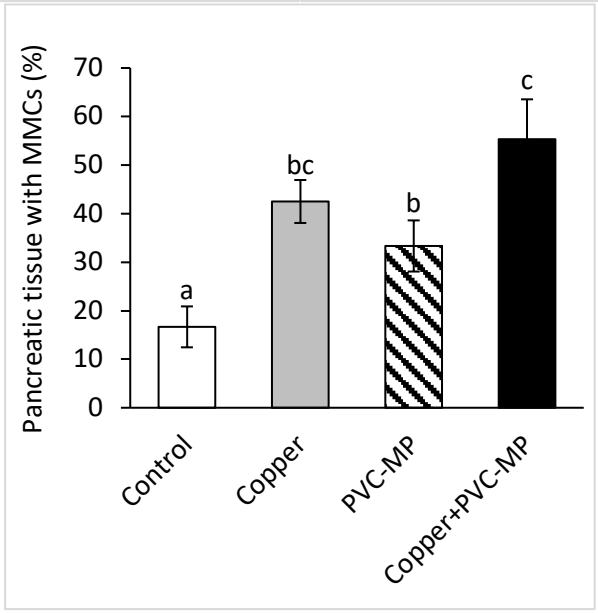

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533 Fig. 4

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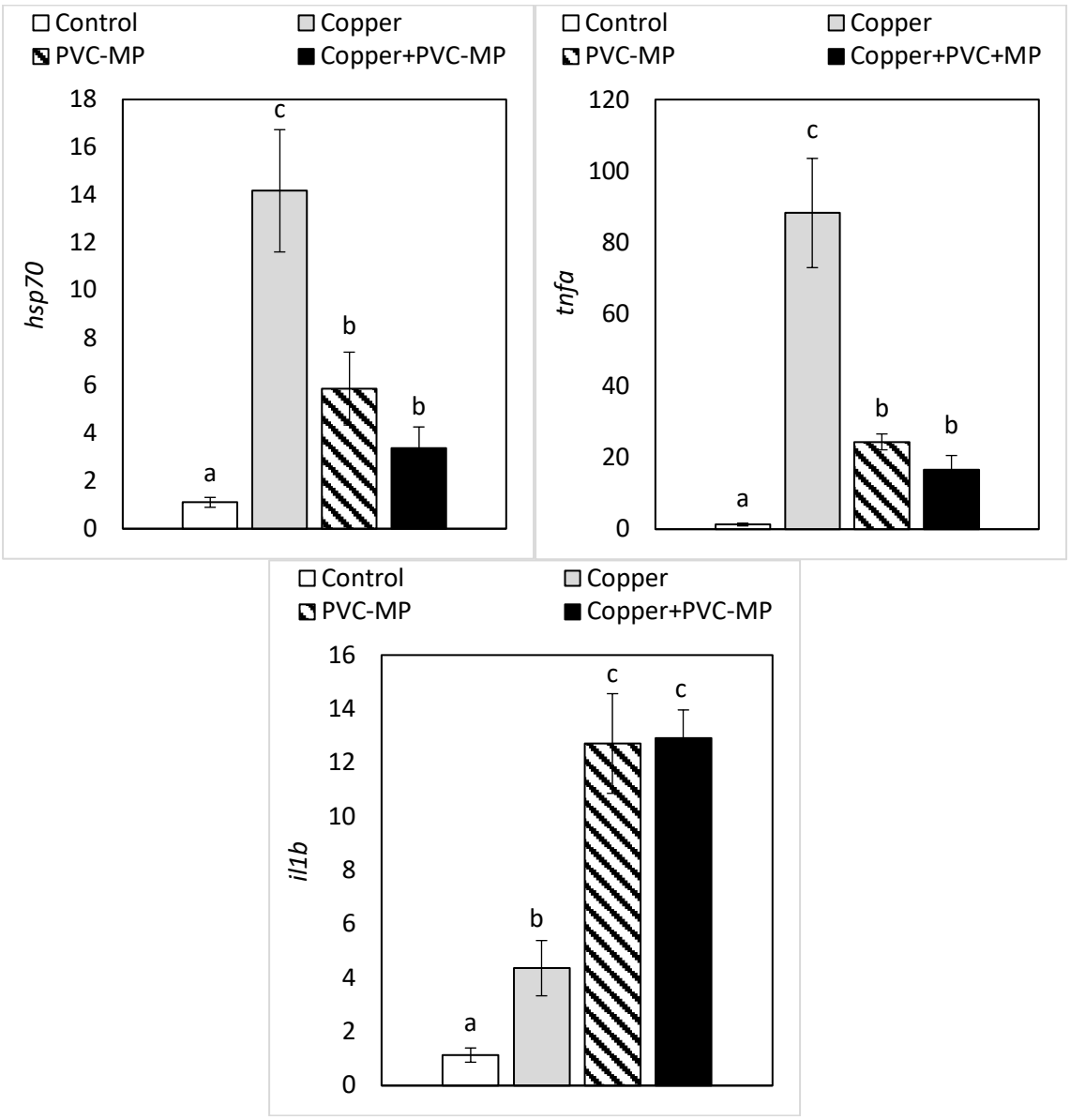

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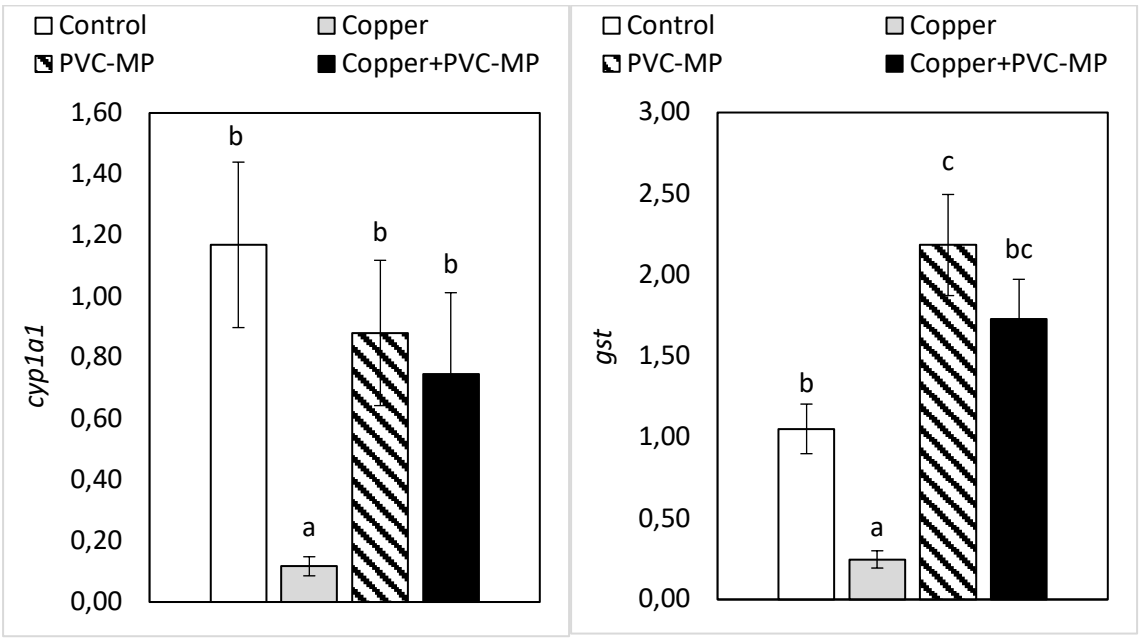

Fig. 6:

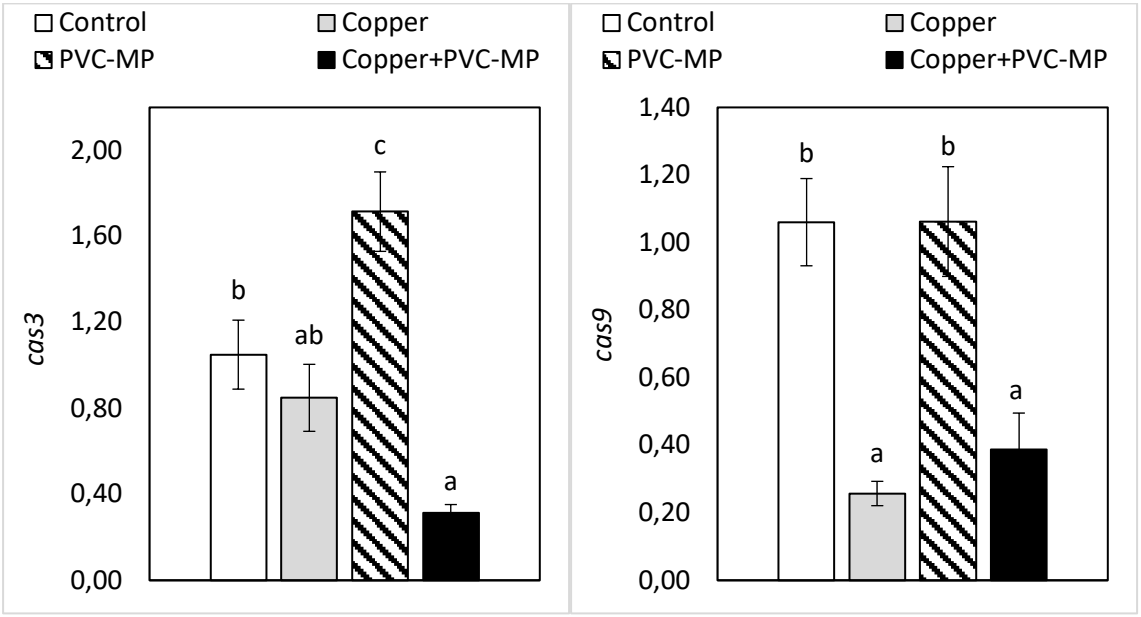

Fig. 7:

Table 1: Sequences of the primers used in this experiment

Primer	Sequence (5-3)	length	Tm	Amplicon (bp)	Accession no.

hsp70	F:ATGTTGCCTTCACAGACACTG	21	60	120	XM_019074376.1
	R:GGTCATCAAACTTTCTGCCGA	21	60		
cyp1a1	F:GAAGAAGTTCGTGGCCATCAA	21	60	101	XM_019064218.1
	R:TGATGTCTCGGATGTTGTCCT	20	60		
gst	F:AGGCCAAAGATCGCTTTCTTC	21	60	127	XM_019105124.1
	R:AGTAACTCCTGCAGCATCAGA	21	60		
cas3	F:AGCGGTTCTTGGTTCATTCAG	21	60	148	XM_019110173.1
	R:TCCTAGCATCAAAGACTGGCT	21	60		
cas9	F:TTGGGTGGGATAGATGACCAG	21	60	132	XM_019066459.1
	R:GGTTGAGTAGGACACCAGGAT	21	60		
tnfa	F:GAACAATCAGGAAGGCGGAAA	21	60	128	XM_019088899.1
	R:GGGTTTCTGTGGACACTTCAG	20	60		
il1b	F:CATTGCTTGTACCCAGTCTGG	21	60	121	XM_019111089.1
	R:TCTGAAGAAGAGGAGGCTGTC	21	60		
beta-actin	F: TCTGCTATGTGGCTCTTGACT	21	60	118	XM_019106214.1
	R: AACCTCTCATTGCCAATGGTG	21	60		

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549 Table 2: Prevalence and severity of hepatic hyperemia and leukocyte infiltration of the fish exposed to copper and/or

550 PVC-MP for 14 d.

		Control	Copper	PVC-MP	Copper+PVC-MP
	Number of examined fish	6	6	6	6
Hyperemia					
	Number affected fish	0	6	6	6
	Mild	0	2	3	1
	Moderate	0	3	3	1
	Severe	0	1	0	4
Leukocyte infiltration					
	Number affected fish	0	5	5	6
	Mild	0	1	4	2
	Moderate	0	4	1	4
	Severe	0	0	0	0

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