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Evaluation of single and combined effects of mancozeb and metalaxyl on the transcriptional and biochemical response of zebrafish (*Danio rerio*)

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ABSTRACT

Mancozeb and metalaxyl are fungicidal agents frequently used in combination to control fungi in crops that may affect non-target organisms when entering ecosystems. This study aims to evaluate the environmental effects of Mancozeb (MAN) and Metalaxyl (MET), alone and in combination, on zebrafish (*Danio rerio*) as an experimental model. The oxidative stress biomarkers and the transcription of genes involved in detoxification in zebrafish (*Danio rerio*) were assessed after co-exposure to MAN (0, 5.5, and 11 μg L^{-1}) and MET (0, 6.5, and 13 mg L^{-1}) for 21 days. Exposure to MAN and MET induced a significant increase in the expression of genes related to detoxification mechanisms (*Ces2*, *Cyp1a*, and *Mt2*). Although *Mt1* gene expression increased in fish exposed to 11 μg L^{-1} of MAN combined with 13 mg L^{-1} of MET, *Mt1* expression was down-regulated significantly in other experimental groups ($p < 0.05$). The combined exposure to both fungicides showed synergistic effects in the expression levels that are manifested mainly at the highest concentration. Although a significant ($p < 0.05$) increase in alkaline phosphatase (ALP) and transaminases (AST and ALT), catalase activities, the total antioxidant capacity, and malondialdehyde (MDA) contents in the hepatocytes of fish exposed to MAN and MET alone and in combination was detected, lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT) activities, and hepatic glycogen content decreased significantly ($p < 0.05$). Overall, these results emphasize that combined exposure to MET and MAN can synergistically affect the transcription of genes involved in detoxification (except *Mt1* and *Mt2*) and biochemical indicators in zebrafish.

1. Introduction

Fungicides are pesticides widely used to prevent the growth of fungi and prevent fungal diseases (Huang et al., 2021). In recent years, the use of combinations of two or more pesticides has prevailed to eliminate pests more effectively (Cycoń et al., 2010; Damalas and Eleftherohorinos, 2011). This strategy can be very effective in controlling pests, but at the same time, it can exert serious environmental and biological consequences (Curchod et al., 2020; Xu et al., 2020; Sula et al., 2020a; Stara et al., 2021; Tresnakova et al., 2022b). The frequent use of fungicides at 10 to 15 day intervals to control fungal diseases in crops and vegetables has been reported to double the risk of environmental pollution (Garcia et al., 2020). This is especially relevant when the fungicide residues remain in the soil or travel to surface waters through runoff and agricultural drainage. Consequently, their increased bioaccumulation in terrestrial and aquatic ecosystems can negatively affect non-target organisms (Stara et al., 2019a, 2019b; Pagano et al., 2020; Stara et al., 2020; Fan et al., 2021; Banaee et al., 2022b; Barathinivas et al., 2022; Tresnakova et al., 2022a). Exposure of non-target organisms to MET has been found to cause chromosome abnormalities in lymphocytes, histopathological damage, oxidative stress, and reproductive disorders (Xie and Yang, 2018; Wu et al., 2019; Lerro et al., 2021).

Mancozeb (MAN) is a polymer composed of zinc (Zn) and manganese (Mn) ethylene-bis-dithiocarbamate (EBDC) salts. This agent is a broad-

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spectrum commercial fungicide used for the control of a wide range of diseases in agricultural, horticultural, and ornamental crops (Wang et al., 2021). Although the toxicity of this fungicide is low and relatively unstable with a short environmental persistence, the exposure of non-target organisms to MAN has been related to the overproduction of reactive oxygen species (ROS) and oxidative damage leading to hepatotoxicity and apoptosis (Fenga et al., 2016; Saber et al., 2019; Akthar et al., 2020; Leandro et al., 2021). MAN toxicity is mainly attributed to its metabolites, i.e., ethylene thiourea and propylene thiourea (Mandić-Rajčević et al., 2020). The traceable MAN concentration in surface waters ranges between 0.455 and 1.279 μg L^{-1} (Marques et al., 2016). The concentration of ethylene thiourea, as the most crucial metabolite of MAN, is found to be about 4.30 and 22.50 μg L^{-1} in underground and surface waters, respectively (Marques et al., 2016). The lethal concentration 50 (LC_{50} – 96 h) of MAN for rainbow trout, *Oncorhynchus mykiss*, is 1.9 μg L^{-1} (Bisson and Hontela, 2002), and for zebrafish embryos, it is 2.17 mg L^{-1} (Vieira et al., 2020). Exposure of zebrafish embryos to MAN led to oxidative stress, reduced hatching and survival rates, and morphological changes in larvae (Vieira et al., 2020). Behavioral changes and oxidative damage in zebrafish after exposure to MAN were also reported by Leandro et al. (2021). Murugasan and Barathi (2020) also found that exposure of zebrafish to MAN could alter the expression of genes involved in brain cell apoptosis. Moreover, significant toxic effects of MAN were detected in the rate of micronuclei and nuclear abnormalities in the red blood cells of *Astyanax jacuhiensis* (Goldoni and Da Silva, 2012) chromosomal abnormalities in the walking catfish, *Clarias batrachus* (Srivastava and Singh, 2013). Exposure of rainbow trout, *O. mykiss*, to MAN changed hematological parameters (Atamanalp and Yanik, 2003). Additionally, Marques et al. (2016) showed that exposure to European eel, *Anguilla anguilla* induced cytotoxic effects and endocrine disruption damage to DNA structure and chromosomes (Marques et al., 2016). Figueiredo-Fernandes et al. (2006) and Pariseau et al. (2009) reported that exposure to MAN could cause histopathological changes in the liver of Nile tilapia, *Oreochromis niloticus* (Figueiredo-Fernandes et al., 2006), and neoplasia in the *Mya arenaria* shell (Pariseau et al., 2009). During cell metabolism, Zn and Mn ions are released from MAN, which can also contribute to ROS production via the Fenton reaction, inducing oxidative stress in fish exposed to the fungicide (Kubrak et al., 2012).

Metalaxyl (MET) (methyl N-(2, 6-dimethylphenyl)-N-(methoxyacetyl)-DL-alaninate) is a systemic fungicide with high solubility in water usually used to prevent fungal diseases caused by Oomycetes (Yao et al., 2009). MET has a half-life of 106 days, and its concentrations in the surface water have been reported in the range from 0.022 to 0.288 μg L^{-1} (Bermúdez-Couso et al., 2013; Hamed et al., 2020).

The LC_{50} of MET in zebrafish, *Danio rerio*, has been determined with values between 227 and 242 mg L^{-1} (Yao et al., 2009). Yao et al. (2009) reported a disturbance in Na^+/K^+ ATPase activity in *D. rerio* exposed to sublethal MET levels. Moreover, a significant change was observed in the gene expression of enzymes involved in the hypothalamic-pituitary-gonadal axis and the hypothalamic-pituitary-thyroid axis of *D. rerio* embryos exposed to MET (Zhang et al., 2016). Zeng et al. (2022) found that exposure of zebrafish embryos to metalaxyl reduced hematopoietic stem cell numbers and suppressed the immune system.

Zebrafish are widely used as experimental models in numerous studies, especially in genetics, developmental biology, pharmacological physiology, and ecotoxicology (Ni et al., 2019; Faria et al., 2021; Jijie et al., 2021; Rashidian et al., 2021; Paduraru et al., 2021; Plhalova et al., 2020; Martyniuk et al., 2023). As a preferred organism for studying vertebrate gene activity, the zebrafish has grown in prominence. Because to their virtually transparent embryos and ability to expedite genetic studies by gene knockdown or overexpression, zebrafish are widely utilized in the detailed exploration of vertebrate gene function and, increasingly, in the research of human genetic problems. Almost 70 % of human genes have at least one obvious zebrafish orthologue, according to the comparison with the human reference genome (Howe et al., 2013). Zebrafish are valued by the scientific community for their peculiarities, being an inexpensive model for studying the toxicity of various substances (Blahova et al., 2020; Petrovici et al., 2020; Sehonova et al., 2019).

MET is usually used in combination with MAN to deal with fungi in crops. For this reason, the present study aimed to determine the lethal concentration and impacts of MET, alone and combined with MAN, in different concentrations by determining the existence of oxidative stress and the expression of antioxidant and detoxifying enzymes in zebrafish.

2. Materials and methods

2.1. Fungicides

A commercial-grade of Metalaxyl (as granules, G 5 %) and Mancozeb (as wettable powders, WP 80 %) were purchased from Gyahcorp., Iran.

2.2. Animals

One month old Zebrafish, *D. rerio*, an ideal vertebrate animal model (Plhalova et al., 2018; Porretti et al., 2022; Zicarelli et al., 2022; Ilie et al., 2022; Merola et al., 2022), with an average weight of 0.26 ± 0.02 g and an average length of 2.77 ± 0.09 cm, were purchased from a local breeder in Shiraz, Iran, and transferred in aerated containers to the aquaculture laboratory of the Natural Resources Faculty, Behbahan Khatam Alanbia University of Technology, Iran.

The specimens were adapted to the laboratory conditions for two weeks at 26 ± 2 °C, pH of 7.4 ± 0.2, with dissolved oxygen 7.0 ± 0.5 mg L^{-1}, and under a photoperiod (14 Light: 10 Dark) cycle in a plastic tank with de-chlorinated water (1000 L). During the acclimatization period, the zebrafish were fed ornamental fish feed twice a day (Beyza Feed Mill, Shiraz, Iran: 45–55 % protein, 10–11 % lipid, 20–30 % carbohydrate, 1.5–2 % fiber). The experimental procedure and methodology of this study have been approved by the Ethics Committee of the Behbahan Khatam Alanbia University of Technology, Iran (2484357-97-07-29).

2.3. Acute toxicity experiments

Acute toxicity tests were carried out following the Organization for Economic Co-operation and Development (OECD) guideline 203 (OECD, 2019). After two weeks of adaptation, four hundred-twenty fish were randomly transferred into two completely separate experimental groups consisting of forty-two tanks (80 L), with ten fish in each tank. Twenty-one of the tanks were allocated to MAN, and the other twenty-one tanks were assigned to MET. In the first group, zebrafish were exposed to serial concentrations of MAN 0.0, 37, 74, 111, 148, 185, and 222 μg L^{-1} for 96 h, whereas in the second group, serial concentrations of MET 0.0, 25, 50, 75, 100, 125, and 150 mg L^{-1} were applied. Each test was replicated three times, and fish were not fed during the acute toxicity trial according to OECD guideline 203. The acute toxicity was carried out as a semi-static renewal procedure, and 80 % of the water was exchanged daily to maintain water quality. Then, a new fungicide solution was added to tanks to hold fungicide concentrations near the nominal level. The physicochemical quality of the water was measured daily before and after replacement. Dead fish were collected, and mortality was recorded every 24 h. The mean lethal concentrations of MAN and MET for zebrafish were calculated separately using probit analysis (95 % confidence limits).

2.4. Sub-lethal toxicity experiments

At this stage, four hundred and five adult zebrafish were randomly transferred to twenty-seven 80 L plastic tanks (15 fish in each group) to carry out nine experimental treatments (with three independent replicas). The fish were divided into nine experimental groups and exposed to the 96 h LC_{50} of MAN at 0, 5, and 10 % and the 96 h LC_{50} of MET at 0,

5, and 10 %. The experimental groups were exposed to 5 % 96 h LC_{50} MET; 10 % 96 h LC_{50} MET; 5 % 96 h LC_{50} MAN; 10 % 96 h LC_{50} MAN; 5 % 96 h LC_{50} MET + 5 % 96 h LC_{50} MAN; 5 % 96 h LC_{50} MET + 10 % 96 h LC_{50} MAN; 10 % 96 h LC_{50} MET + 5 % 96 h LC_{50} MAN; 10 % 96 h LC_{50} MET + 10 % 96 h LC_{50} MAN; and control (0 % 96 h LC_{50} MET + 0 % 96 h LC_{50} MAN), respectively for 21 days. After changing the water, a fresh solution of the fungicide was made and added to the tanks to maintain their nominal concentrations every day. The fish were fed a commercial diet and starved 24 h before the sampling to ensure complete emptying of the digestive system (Chaklader et al., 2021). At the end of the experiment, fish were individually captured, and nine fish from each group were placed in liquid nitrogen (−196 °C) for genetic analysis (Amparyup et al., 2020; Sun et al., 2022). The remaining fish ($n = 36$) were anesthetized with a clove extract solution (100 mg L^{-1}) (Balamurugan et al., 2016), immediately dissected, and the liver was removed and rinsed with a saline solution (Hamidi et al., 2022). Samples were homogenized with a manual homogenizer in ten volumes (*w/v*) of phosphate buffer (PBS, pH 7.2) and Triton X-100 at 4 °C for 1 min. Following a 20-minute centrifuge (Thermo Scientific Sorvall ST16) at 15,000 ×g (~8827 rpm) and 4 °C, the supernatant was collected and maintained at −25 °C until biochemical analysis.

2.5. Biochemical parameters

Catalase (CAT) activity was measured following the method combining optimal enzymatic conditions with a spectrophotometric measurement of hydrogen peroxide based on the development of a stable complex between the compound and ammonium molybdate (Góth, 1991). The absorbance was measured at 405 nm and using hydrogen peroxide as a substrate. Results were expressed as U g^{-1} protein. The FRAP assay (Ferric Reducing Antioxidant Power Assay) method was used for measuring the total antioxidant capacity (TAO) at 593 nm (Benzie and Strain, 1996). Malondialdehyde (MDA) was determined using thiobarbituric acid, monitored at 532 nm, and the results were expressed as μmol per gram of tissue (Tsikas, 2017). Superoxide dismutase (SOD) activity was measured in hepatocytes using a commercial kit following the manufacturer's instructions at 545 nm (Biorexfars Co., Iran). Glycogen content in the liver was assessed by the technique suggested by Zhang (2012), and results were expressed as mg g^{-1} tissue. Pars Azmun Co. commercial kits and a UV/VIS spectrophotometer (Biochrom Libra S22) were used to measure the activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and gamma-glutamyl transferase (GGT) in tissue extracts (Moss and Henderson, 1999; Banaee et al., 2019). Results were expressed as U g^{-1} protein. Protein levels were determined using a total protein assay kit (Pars Azmun Co., Iran), and results were expressed as g dL^{-1} (Johnson et al., 1999).

2.6. RNA isolation and mRNA gene expression

The removed liver was cut into pieces and rinsed with a cold normal saline solution contains 0.9 % NaCl. These pieces were then soaked in Trizol reagent (RiboEx, South Korea), and the cellular RNA was extracted. In this stage, samples were treated with water containing diethyl pyrocarbonate (DEPC) to deactivate RNases (Thermo Scientific, USA). Then, 5 μg of total RNA were treated with DNase I to separate the samples from the genome,(Thermo Scientific, USA) for 30 min at 37 °C. The quantity of purified RNA was measured with a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000, USA) at 230, 260, and 280 nm. The quality of total RNA was evaluated using horizontal electrophoresis (Biorad, Minigel) on agarose gel (1 %) stained with loading dye. cDNA synthesis was carried out using 5 μg of total RNA, oligo (dT) as the primer, and reverse transcriptase based on Thermo Scientific kit instructions. Primer design of five genes, involved in antioxidant and detoxification processes (*P450, Met1*, *Met2*, and *Ges2*), was made by OLIGO Software and performed in NCBI (Table 1).

Table 1
Primer sequences, amplicon lengths, and annealing temperatures used in real-time PCR.

Gene name	Primer	Sequence (5-3)	Tm	Length (bp)
Ces2	Forward	ACCCTCCATCACAGTTGCCTC	62	116
	Reverse	CGGCCTTCACAAAACTGGGTC	62	
P450A	Forward	TCGCTCCGGGTTATTAAATCAGC	61	119
	Reverse	CGCATGAGCAGATACACCAAAC	61	
Met1	Forward	CTGTTCTTGTTGCCCGTCTG	59	110
	Reverse	ACAAAACATCACGTTGACCTCC	59	
Met2	Forward	ACTACCTGCAAGAAGAGTTGTTG	59	164
	Reverse	GCAGACGTGGAGTAGACAAAC	59	
β-Actin	Forward	CCGTGACATCAAGGAGAAGCT	58	201
	Reverse	TCGTGGATACCGCAAGATTCC	58	

The final volume of the solution in the real-time PCR reaction was 25 μL and consisted of 10 μL of SYBR Green Master Mix (2×) reagent, 2 μL of cDNA, 0.8 μL of each primer (10 μM), 0.4 μL of ROX dye, and 11 μL of DEPC water. There was an initial denaturation phase at 94 °C for 5 min to activate the enzyme, followed by 40 denaturation cycles at 94 °C for 30 s, annealing at 60 °C for 30 s, and polymerization at 72 °C for 30 s. An additional ramp phase from 95 to 65 °C was carried out for the melting curve. All reactions were done in 3 replications with a negative control and no samples (only having 1 μL of DEPC water). QRT-PCR analysis was accomplished according to the additional one-step real-time PCR. Levels of genes expressions were normalized using β-actin as housekeeping (Guo et al., 2013). β-Actin has been usually used in other studies on toxicity in *D. rerio* as housekeeping (Derikvandy et al., 2020; Gaaied et al., 2019; Chen et al., 2021; Xu et al., 2021). The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$ and the mRNA levels of control samples, which referred to as 100 % (Schmittgen and Livak, 2008).

2.7. Assessment of synergism and antagonism

The possible interaction between MAN and MET's effects was estimated according to the following mathematical models (Banaee et al., 2020). The synergism rate only occurs when the expected effect is greater than the observed effect.

1. Predicted effects of the endpoints of fish exposed to individual fungicides

$$\text{Predicted effect} = \frac{\text{MAN}}{\text{Control}} \times \frac{\text{MET}}{\text{Control}}$$

2. Observed effects of the endpoints of fish exposed to fungicides in combination

$$\text{Observed effect} = \frac{\text{The combination of MAN and MET}}{\text{Control}}$$

3. The synergistic effect

$$\text{Synergy ratio} = \frac{\text{Predicted effect}}{\text{Observed effect}}$$

2.8. Data analysis

Statistical analyses were carried out using the GraphPad Prism 8.0.2 software. Data normality was assessed by the Kolmogorov-Smirnov test. Data analysis was performed using two-way analysis of variance (two-way ANOVA) at 95 % confidence level ($p < 0.05$). The treatments analyzed in the two-way ANOVA were MAN and MET at different concentrations and their interactions. When significant effects were found,

the means were compared with Duncan's post hoc test. The results are presented as mean ± standard deviation (SD).

3. Results and discussion

3.1. Acute toxicity

Exposure of non-target species to fungicides can alter physiological processes (Sula et al., 2020b) and induce toxicity and, even, cell death if effective detoxification does not occur. The median lethal concentrations of MET and MAN were calculated in zebrafish, *D. rerio* after 24, 48, 72, and 96 h (Table 2). The LC_{50} of MET for zebrafish was 341.35 (24 h), 238.25 (48 h), 156.96 (72 h), and 132.09 mg L^{-1} (96 h), whereas the LC_{50} of MAN for zebrafish was 234.95 (24 h), 176.75 (48 h), 151.47 (72 h), and 111.39 μg L^{-1} (96 h) (Table 2).

The results of the acute toxicity test showed that MAN is highly toxic to *D. rerio*, while the toxicity of MET is lower and can be considered moderately toxic. Therefore, the ecological risk associated with chronic MAN exposure was very high for zebrafish, whereas the chronic ecological risk of MET can be considered low.

Yao et al. (2009) reported that the 96-h LC_{50} values of MET for adult Zebrafish, *D. rerio*, were 227–242 mg L^{-1}. The 96 h LC_{50} of MET was higher than 100 mg L^{-1} in *C. carpio*, and the 24 h LC_{50} of rac-MET and R-MET were 258.47 and 237.67 mg L^{-1} for zebrafish embryos, respectively (Wu et al., 2019). The 96 h LC_{50} of MAN was 9 mg L^{-1} in adult goldfish, *Carassius auratus* (Atamaniuk et al., 2014), 2.2 mg L^{-1} in rainbow trout, *O. mykiss* (Atamanalp and Yanik, 2003), 28.58 mg L^{-1} in juvenile Asian catfish, *Clarius batrachus* (Srivastava and Singh, 2013), and 8.03 mg L^{-1} in juvenile common carp, *C. carpio* (Simakani et al., 2018).

3.2. Gene expression

In the present study, several biomarkers related to oxidative stress and the detoxification system were measured in zebrafish exposed to MAN and/or MET to understand their toxicity rates. The initial step in detoxifying xenobiotics is carried out by phase I detoxification enzymes such as carboxylases and cytochrome P450A. These enzymes facilitate the excretion of pesticides from the body through their biotransformation into more water-soluble compounds. The effects of MET and MAN exposure on transcriptional gene levels of enzymes involved in detoxification are presented in Figs. 1–4. The *Cyp1a* gene expression significantly ($p < 0.05$) increased in the hepatocytes of zebrafish exposed to MET (13 mg L^{-1}) and MAN (5.5 and 11 μg L^{-1}). The expression of *Ces2* was up-regulated in the hepatocytes of fish exposed to MET (13 mg L^{-1}) and MAN (11 μg L^{-1}). There was a significant rise in *Ces2* and *Cyp1a* mRNA levels in the hepatocytes of zebrafish co-exposed to 6.5 mg L^{-1} of MET and 11 μg L^{-1} of MAN with respect to the control group. Following the co-exposure of MET (13 mg L^{-1}) combined with 5.5 and 11 μg L^{-1} of MAN, there was a significant increase in the gene expression of *Ces2* and *Cyp1a* ($p < 0.05$) (Fig. 1).

The significant up-regulation of *Cyp1a* and *Ces2* expression, which encode cytochrome P450A and, carboxylesterase, respectively, after exposure to MET alone, and in combination with MAN might be the consequence of an activated cellular detoxification system. An elevated *Ces2* gene expression was reported in the zebrafish embryos following the exposure to 2.15 μM diuron for 96 h (Velki et al., 2017). Similarly, increases in the expression of *Cyp1a* have been described in different species after exposure to different types of xenobiotics. In this sense, higher mRNA levels of *Cyp1a* were observed in the Western clawed frog (*Silurana tropicalis*) after exposure to 46.6 μg L^{-1} bitumen for 72 h (Lara-Jacobo et al., 2019). Burkina et al. (2018) provided evidence that zebrafish exposure to pharmaceutical effluents could increase *Cyp1a* gene expression (Burkina et al., 2018). Zebrafish exposure to polycyclic aromatic hydrocarbons (PHAs) resulted in increased *Cyp1a* gene expression (Geier et al., 2018). *Cyp1a* gene expression significantly increased in the liver of goldfish (*Carassius auratus*) after exposure to polyvinyl chloride microplastics (Romano et al., 2020). Increased *Cyp1a* mRNA expression was detected in the Atlantic cod, *Gadus morhua* (Søfteland et al., 2010), *Hypophthalmichthys molitrix*, *H. nobilis*, and *Ictiobus cyprinellus* (Amberg et al., 2012), *D. rerio* (Gaaied et al., 2019), and *C. carpio* (Agus et al., 2015) exposed to beta-naphthoflavone, 50 μg L^{-1} rotenone, and 0.8 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and di-n-butyl phthalate, respectively.

Metallothioneins (MTs) are cytosolic proteins involved in the detoxification of metals, which is essential in maintaining metal ion homeostasis (Banaee et al., 2015). MTs are found in two different isoforms of MT-1 and MT-2 in all types of soft tissues. Therefore, any changes in the expression level of MTs genes can alter a defense mechanism against oxidative stress and excess metals. In the hepatocytes, MET and MAN alone down-regulated the mRNA levels of *Mt1* in comparison with the control group ($p < 0.05$). Moreover, co-exposure to 13 mg L^{-1} of MET and 11 μg L^{-1} of MAN increased *Mt1* gene expression, whereas 13 mg L^{-1} of MET and 5.5 μg L^{-1} of MAN did not affect *Mt1* gene expression ($p > 0.05$).

Results showed that *Mt2* expression was up-regulated in the hepatocytes of fish exposed to 13 mg L^{-1} of MET. No significant change was observed in the *Mt2* expression in the hepatocytes of fish exposed to 5.5 and 11 μ L^{-1} of MAN when compared to the control ($p > 0.05$). However, *Mt2* gene transcription was increased after co-exposure to 13 mg L^{-1} of MET combined with 5.5 and 11 μ L^{-1} of MAN (Fig. 1). Decreased expression of *Mt1* genes may be related to the increased formation of ROS and reactive nitrogen species (NOS) (Ebrahimi-Kalan et al., 2011). Therefore, the reduction of *Mt1* gene expression may increase the sensitivity of liver cells to the zinc metal present in MAN (Pei et al., 2023). Moreover, MTs may protect cells from apoptosis through intracellular metal ion modulation and free radical scavenging (Rodrigo et al., 2020). Therefore, the reduction of *Mt1* gene expression in fish hepatocytes exposed to MAN and MET may increase the rate of programmed cell death (Pei et al., 2023). However, combined exposure to the highest dose of fungicides led to increased gene expression.

In contrast, an increase in the expression level of MTs genes can be a defense mechanism against oxidative stress and excess metals. Metallothionein-2 can weakly bond with biological metals such as copper and zinc as an antioxidant to neutralize hydroxyl radicals. Therefore, the observed increase in the expression of the *Mt2* gene may respond to MAN's zinc residues. Elevated expression of the *Mt1* and *Mt2* genes can be a bio-indicator of oxidative stress (Derikvandy et al., 2020). Also, the effect of some pesticides with metal residues on MTs gene expression has been reported in Nile tilapia, *O. niloticus* (Ghazy et al., 2017). This study showed that fish exposure to MET combined with MAN could lead to a suppressive effect on *Met1* and *Met2* mRNA levels.

The results obtained in the study revealed that MET combined with MAN might have a synergic effect on *Cyp1a* gene expression, while *Ces2*, *Cyp1a*, *Mt1*, and *Mt2* results indicated the inverse pattern (Fig. 4).

Table 2
Median lethal concentrations (LC50) values of mancozeb and metalaxyl.

Time	LC50 MET (mg L^{-1})	LC50 MAN (μg L^{-1})
24 h	341.4 (213.9–544.7)	235.0 (178.6–309.1)
48 h	238.3 (146.4–387.7)	176.8 (127.1–245.8)
72 h	157.0 (107.6–229)	151.5 (113.5–202.1)
96 h	132.1 (95.1–183.5)	111.4 (87.9–141.2)

Slightly toxic (10–100 mg L^{-1}); Moderately toxic (1–10 mg L^{-1}); Highly toxic (0.1–1.0 mg L^{-1}); Extremely toxic (<0.1 mg L^{-1}) (Banaee, 2012).

3.3. Oxidative stress biomarkers

Usually, the biomodification of xenobiotics is associated with ROS production (Valon et al., 2013; Klotz and Steinbrenner, 2017). In these situations, the cellular antioxidant defense system tries to re-establish

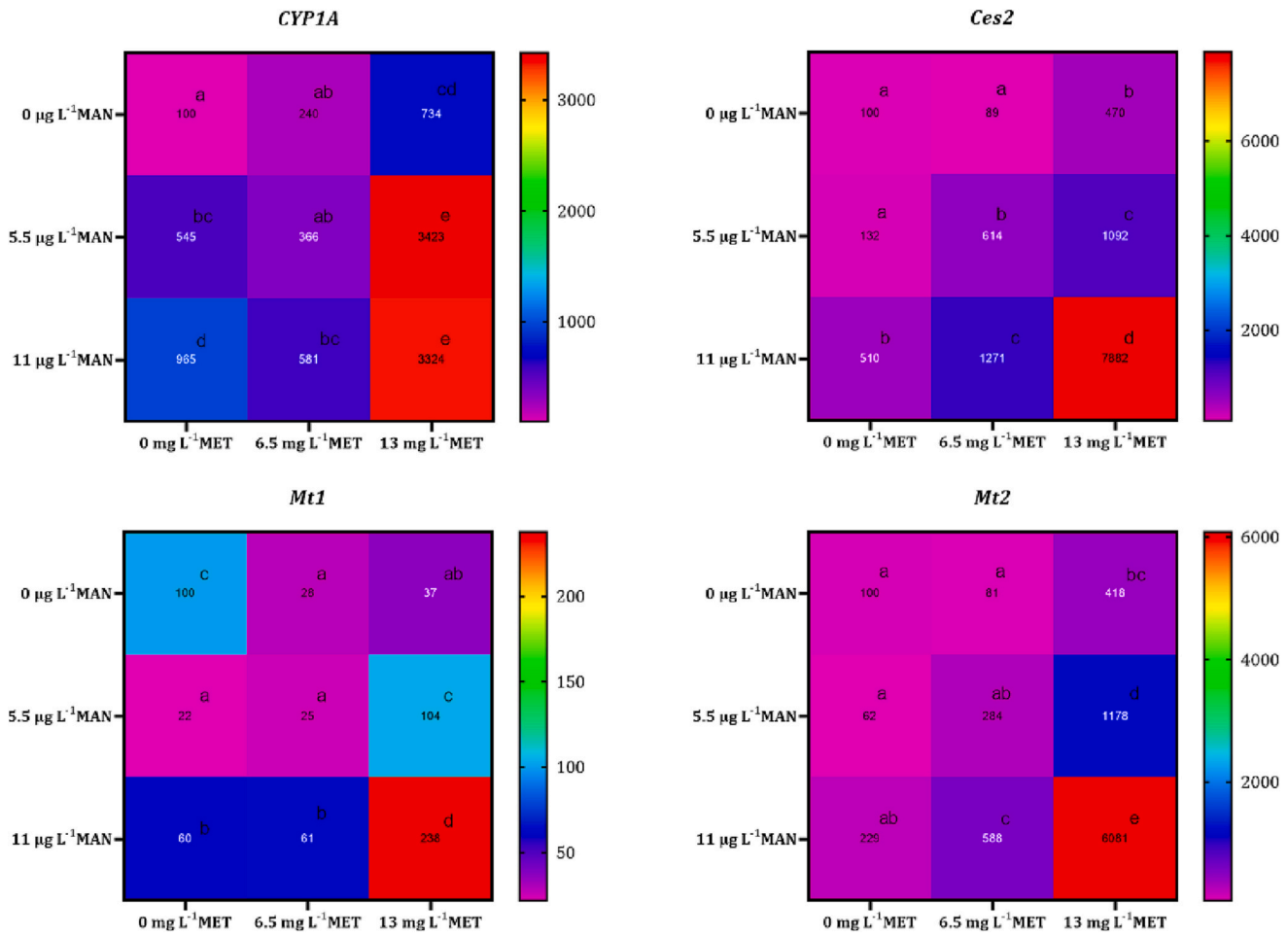

Fig. 1. Changes in *Met1*, *Met2*, *Ces2*, and *Cyp1a* gene expression in the hepatocytes of zebrafish (*Danio rerio*) after exposure to MET and MAN for 21 days. Significant differences between groups were identified by alphabetical characters ($P < 0.05$).

the balance between oxidants and antioxidants. However, if the antioxidant system cannot detoxify the excessive ROS production, it can lead to oxidative stress (Sharifinasab et al., 2016; Aliko et al., 2018; Ibrahim et al., 2021; Banaee et al., 2022a, 2022b). Sies et al. (2017) showed that excessive challenge of cells with oxidants and free radicals could damage biomolecules.

Superoxide dismutase is the first enzyme at the forefront of the antioxidant defense system, acting on the superoxide anion. A significant increase in SOD activity can be explained by its antioxidant catalytic activity, which allows the dismutation of the superoxide radical in O_2 and H_2O_2. Fish exposed to MET and MAN alone showed a significant increase in SOD activity. Regarding SOD activity, a significant increase was observed in fish exposed to the highest concentrations of MET and MAN (Fig. 2). Atamaniuk et al. (2014) also showed a significant increase in SOD activity in the liver and kidney of goldfish after treatment with MAN (Atamaniuk et al., 2014). Similar results were observed in the European flounder, *Platichthys flesus*, exposed to cadmium (Sheader et al., 2006), and in the Asian clam, *Corbicula fluminea*, exposed to heavy metals (Xie and Yang, 2018). Some authors have reported changes in the antioxidant system caused by MAN exposure in goldfish (*Carassius auratus*) (Kubrak et al., 2012) or alterations in SOD and CAT activities in rat spleen lymphocytes (Medjdoub et al., 2011).

Catalase (CAT) and glutathione peroxidase (GPx) are the main enzymes responsible for detoxifying hydrogen peroxide into water and oxygen (Hamidi et al., 2022). The increased CAT activity in the liver of zebrafish exposed to MAN and MET could indicate an excessive amount of hydrogen peroxide in hepatocytes related to the detoxification process. A significant ($p < 0.05$) increase in the CAT activity was observed in the hepatocytes of fish exposed to MET and MAN alone with respect to the control group. These findings showed that exposure to MAN and MET, alone or in combination, could increase CAT activity in the hepatocytes of zebrafish. The maximum increase in CAT activities was observed in the hepatocytes of fish exposed to 13 mg L^{-1} of MET combined with 5.5 and 11 µ L^{-1} of MAN (Fig. 2). Moreover, the increased CAT activity may reflect a proliferation in *Cat* gene expression after fish exposure to MAN and MET. Mao et al. (2020) found that zebrafish exposure to the fungicides kresoxim-methyl and pyraclostrobin increased CAT activity. The expression of the *Cat* gene was raised in the liver of *D. rerio* after treatment with 0.8 mg L^{-1} 2,4-D (Gaaied et al., 2019), and 100 µg L^{-1} atrazine (Jin et al., 2010).

MDA levels and cellular TAO capacity significantly ($p < 0.05$) increased after fish exposure to MET and MAN. Also, co-exposure to MAN and MET significantly ($p < 0.05$) increased MDA and TAO contents in the hepatocytes of zebrafish (Fig. 2). Increased levels of the total antioxidant capacity indicated the hepatocytes' physiological response to counteract the cytotoxicity of fungicides. However, this generalized increase in antioxidant defenses is not enough to avoid the appearance of oxidative damage evidenced by increased levels of MDA after exposure to fungicides. Similar results were evidenced in the adult cichlid *Australoheros facetus* exposed to the fungicide azoxystrobin with elevated CAT activity and MDA (Crupkin et al., 2021). A synergistic oxidative effect was observed when MET was used in combination with MAN.

The potential synergistic effects of MET and MAN on oxidative stress biomarkers were presented in Fig. 4.

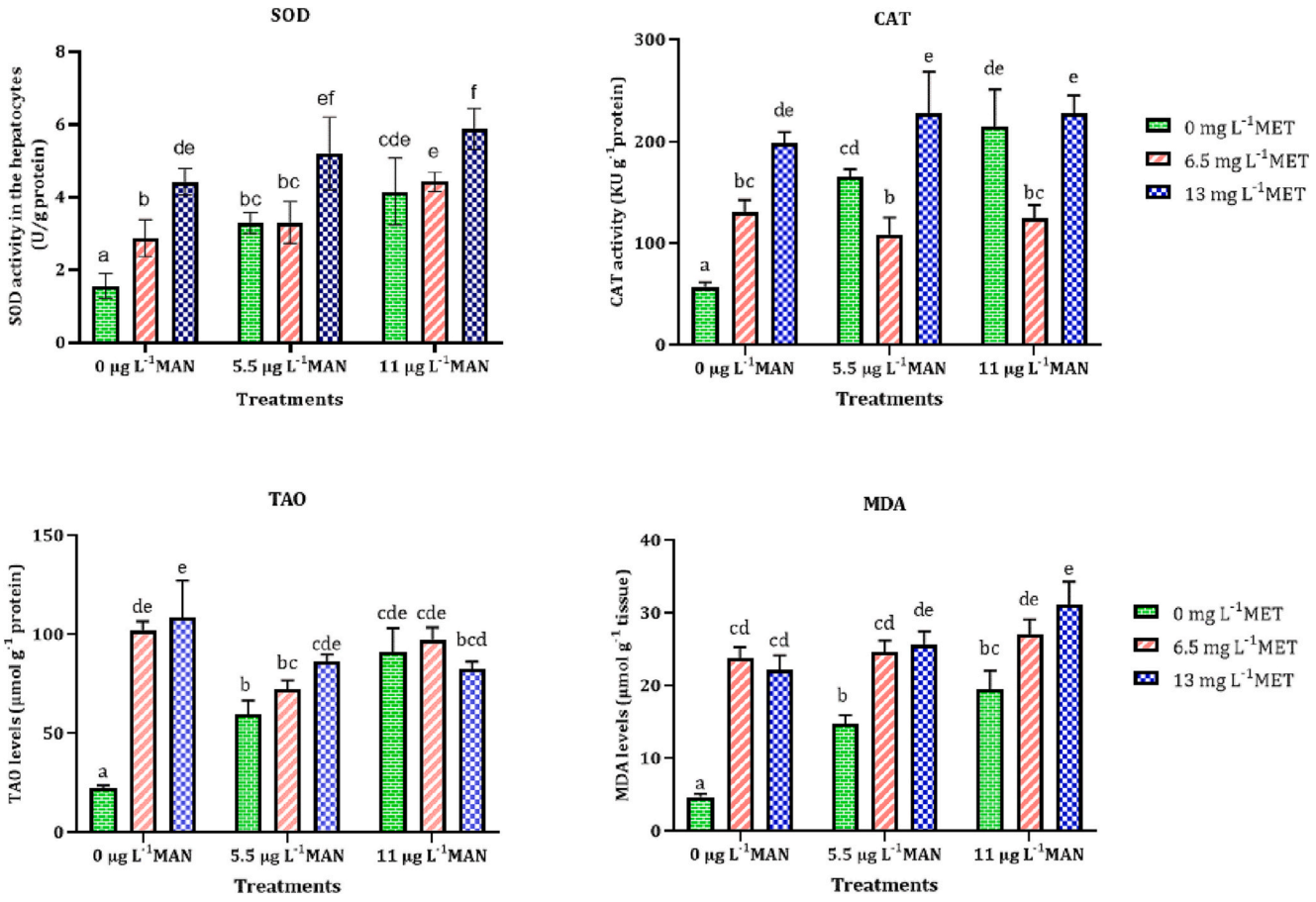

Fig. 2. Changes in oxidative biomarkers in the hepatocytes of zebrafish (*Danio rerio*) after MET and MAN exposure for 21 days. Results are illustrated as mean ± standard deviation (S.D.). Different letters indicate statistical differences between groups ($P < 0.05$).

3.4. Biochemical parameters

Although no significant ($p > 0.05$) changes in the AST activity were found in fish exposed to 5.5 and 11 µg L^{-1} of MAN, the AST activity in the hepatocytes of fish was significantly increased following exposure to 6.5 and 13 mg L^{-1} of MET. AST activity was significantly ($p < 0.05$) increased after fish treatment with 6.5 mg L^{-1} of MET combined with 5.5 and 11 µg L^{-1} of MAN. Furthermore, AST activity was significantly ($p < 0.05$) increased in the hepatocytes of fish exposed to 13 mg L^{-1} of MET combined with 5.5 and 11 µg L^{-1} of MAN. Similarly, significant ($p < 0.05$) increases in the ALT activity were detected in the hepatocytes of fish exposed to MET and MAN alone. Exposure to 6.5 and 13 mg L^{-1} of MET combined with 11 µg L^{-1} of MAN significantly increased the ALT activity (Fig. 3). Increased AST activity and ALT activity may have been related to increased transaminase levels in cells involved with fungicides. Therefore, increased AST and ALT activities may be a metabolic response to fungicides. Bao et al. (2020) also showed that zebrafish exposure to the fungicide carbendazim had been shown to increase AST and ALT activity (Bao et al., 2020). Furthermore, AST and ALT are involved in the metabolism of amino acids and gluconeogenesis, which provide energy to cope with the stress of pesticide exposure (Falco et al., 2020). Therefore, increased AST and ALT activities could be a metabolic response to MET and MAN toxicity. Elevated AST and ALT activities were observed in the liver of *Pangasianodon hypophthalmus* exposed to triclosan (Paul et al., 2019), and the hepatopancreas of the Chinese mitten crab (*Eriocheir sinensis*) exposed to deltamethrin (Zhang et al., 2019).

A significant ($p < 0.05$) decrease in GGT activity was observed in the hepatocytes of fish exposed to MET and MAN. Co-exposure to MET and MAN also caused a significant reduction in GGT activity in the hepatocytes of fish (Fig. 3). GGT is located in the cell membrane and plays an essential role in the biodegradation of extracellular GSH and the supply of cysteine to regenerate cellular GSH (Zhang et al., 2005). Furthermore, GGT is involved in the metabolism and deglutamylation of glutathione-conjugated compounds (Baumann et al., 2014). Therefore, decreased GGT activity could lead to a reduction in the rate of extracellular glutathione recycling.

LDH is an enzyme involved in carbohydrate metabolism and is used as a diagnostic biomarker of cytotoxicity. MET and MAN alone significantly inhibited the activity of LDH in the hepatocytes of fish. A significant ($p < 0.05$) decrease in LDH activity was detected in the hepatocytes of fish exposed to 6.5 mg L^{-1} of MET combined with 5.5 and 11 µ L^{-1} of MAN. Also, 13 mg L^{-1} of MET combined with 11 µ L^{-1} of MAN caused a significant reduction in LDH activity (Fig. 3). Reduced LDH activity may be due to the interference of aerobic and anaerobic metabolism in hepatocytes after exposure to MET and MAN (Abhijith et al., 2016). Moreover, Tripathi and Shasmal (2011), and Abhijith et al. (2016) found that the decrease in LDH activity in the liver of fish exposed to pesticides could be caused by the binding of pesticides and their metabolites to LDH (Tripathi and Shasmal, 2011; Abhijith et al., 2016). Allen et al. (2013) also reported inhibition of LDH activity by dieldrin analogues in dopaminergic cells (Allen et al., 2013).

The present study showed that exposure of fish to MET and MAN alone inhibited LDH and GGT activities. Moreover, MAN and MET, in combination, synergistically decreased LDH and GGT activities (Fig. 4). Decreased LDH and GGT activities may be due to their denaturation as a result of interactions with fungicides (Cattani et al., 2014).

Individual and combined treatment with MET and MAN caused a

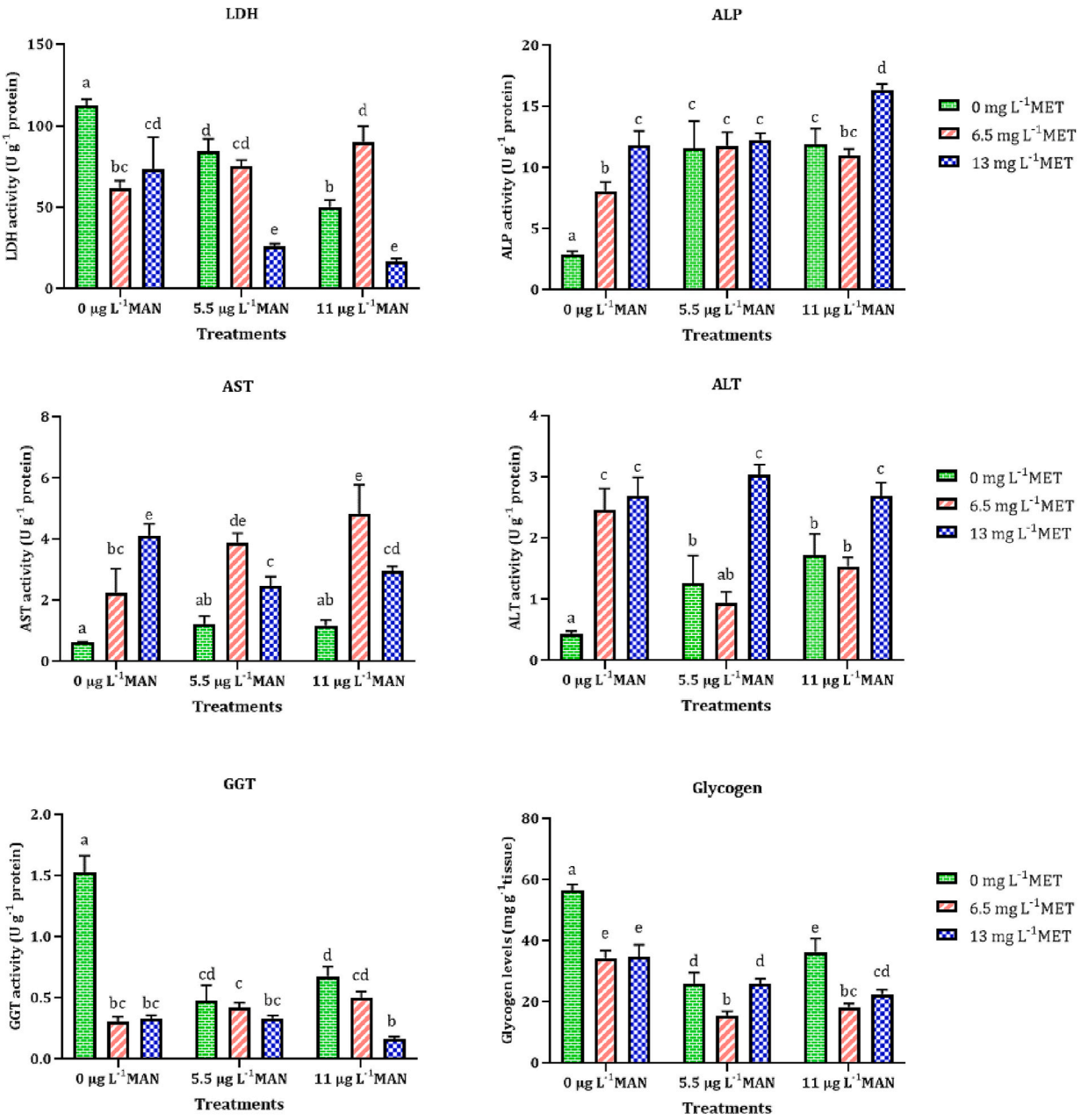

Fig. 3. Changes in biochemical parameters in the hepatocytes of zebrafish (*Danio rerio*) after MET and MAN exposure for 21 days. Results are illustrated as mean ± standard deviation (S.D.). Different letters indicate statistical differences between groups ($P < 0.05$).

significant ($p < 0.05$) increase in the ALP activity when compared with the control group. The highest ALP activity was observed in the liver of exposed fish to 13 mg L^{-1} of MET combined with 11 µ L^{-1} of MAN (Fig. 3). MET and MAN exposure also resulted in a remarkable increase in ALP activity. The increase in ALP in the hepatocytes may be due to damage to bile ducts (Banaee, 2020). A similar result was observed in the *E. sinensis* after exposure to deltamethrin (Zhang et al., 2019). The combination of MET with MAN resulted in a synergic effect on AST, ALT, and ALP activities.

A significant ($p < 0.05$) decrease in glycogen content was found after exposure to MET and MAN. Furthermore, the combination of these two fungicides induced a significant reduction in glycogen levels (Fig. 3). Decreased glycogen stores in the liver of fish indicated an increase in glycolysis rate to provide energy to counteract the metabolic stress induced by fungicides. This result indicated a suppressive effect of MAN alone and in combination with MET on the hepatic glycogen levels. Delahaut et al. (2019) showed that reducing glycogen storage in the hepatocyte of fish is a physiological mechanism to provide energy to deal with stress caused by environmental pollutants (Delahaut et al., 2019). The reduction of glycogen storage in the muscles and liver of carp, *C. carpio*, exposed to heavy metals is similar to the present study results (Delahaut et al., 2019). Depleted glycogen contents were also observed in the liver of freshwater snail, *Galba truncatula* exposed to dimethoate and cadmium (Banaee et al., 2019).

Results also showed synergistic effects between the toxicity of MET and MAN on biochemical parameters, except for GGT activity (Fig. 4).

4. Conclusion

Although the combined use of two or more fungicides can be a suitable technique to control fungal diseases in plants, their environmental impact on non-target organisms should be considered.

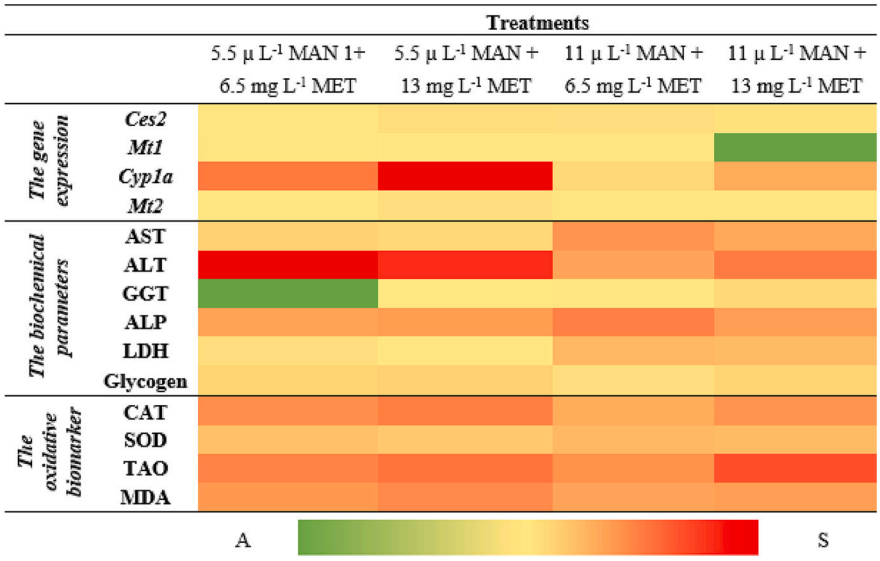

Fig. 4. Evaluation of synergism of MAN and MET concerning the gene expression, the biochemical and oxidative biomarkers after 21 days. Synergistic effect: S; Suppressive effect: A.

Toxicological results showed that MAN and MET were individually toxic to zebrafish, and the fish responded to both fungicides with increases in the expression of antioxidant and detoxification enzymes and altered biochemical parameters. Zebrafish exposure to combined concentrations of MET and MAN altered blood biochemical parameters to a greater extent and increased the levels of liver enzymes, suggesting a disruption of biochemical homeostasis in hepatocytes. Moreover, the co-exposure to fungicides induced oxidative stress that led to the activation of antioxidant defense mechanisms that, however, were not enough to prevent the increase in lipid peroxidation. In conclusion, these findings revealed that the combination of MEN and MAN had a synergistic toxicity effect on zebrafish that was much higher compared to individual effects, which should be considered when using fungicides in combination as they can affect non-target species.

CRediT authorship contribution statement

Mahdi Banaee: Assistance Professor (Aquaculture and Ecotoxicology); Contribution: Supervisor; Investigation, Project administration, Validation, Formal analysis, Writing - Original Draft

Antoni Sureda: Professor (Biochemistry); Contribution: Writing - Original Draft, Review & Editing

Shiva Sagvand: Master science student (Aquaculture); Contribution: Investigation, Project administration

Mohammad Amini: Assistance Professor (Aquaculture and Fish biology); Contribution: Supervisor; Cooperation in project implementation

Behzad Nematdoost Haghi: Instructor (Aquaculture and Ecotoxicology); Contribution: Cooperation in project implementation

Caterina Faggio: Writing - Original Draft, Review & Editing

Mentor Sopjani: Writing - Original Draft, Review & Editing

Declaration of competing 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.

Data availability

The authors do not have permission to share data.

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Ethics approval

International, national, and institutional guidelines for the care and use of animals were followed. Experimental protocols were done following the Iranian animal ethics framework under the supervision of the Iranian Society for the Prevention of Cruelty to Animals and Behbahan Khatam Alanbia University of Technology (2484357-97-07-29).

Consent to publish

In the present study, there was no individual person's data in any form (including any individual details, images or videos).

Consent to participate

In this study, none of the authors used human beings as research subjects.

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Availability of data and materials

The data that support the findings of this study are available from Behbahan Khatam Alanbia University of Technology but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Behbahan Khatam Alanbia University of Technology.

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