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Article

The Duckweed, *Lemna minor* Modulates Heavy Metal-induced Oxidative Stress in Nile Tilapia

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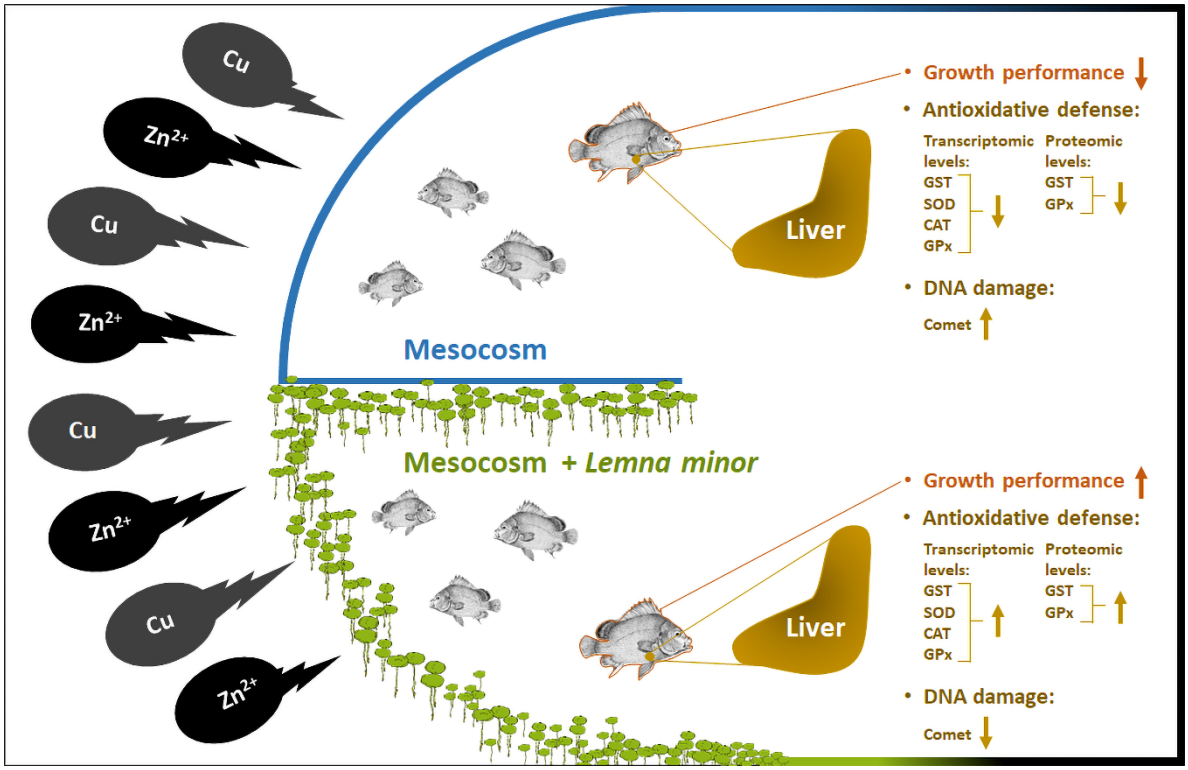
Graphical abstract:



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Abstract: A two-fold integrated research study was conducted; firstly, to understand effects of copper (Cu) and zinc (Zn) on the growth and oxidative stress in Nile tilapia, *Oreochromis niloticus;* secondly, to study the beneficial effects of the duckweed *Lemna minor* L. as a heavy metal remover from wastewater. Experiments were conducted in mesocosms with and without duckweed. Tilapia fingerlings were exposed to Cu (0.004 and 0.02 mg/L) and Zn (0.5 and 1.5 mg/L) and fish fed for four weeks. We evaluated the fish growth performance, the hepatic DNA structure using comet assay, the expression of antioxidative genes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx and glutathione-S-transferase, GST) and GPx and GST enzymatic activity. The results showed that Zn exhibited more pronounced toxic effects than Cu. Low dose of Cu did not influence the growth whereas higher doses of Cu and Zn significantly reduced the growth rate of tilapia compared to control, but addition of duckweed prevented weight loss. Further, in the presence of a high dose of Cu and Zn, DNA damage decreased, antioxidant gene expressions and enzymatic activities increased. In conclusion, results suggest that duckweed and Nile tilapia can be suitable candidates in metal remediation wastewater assessment programs.

Keywords: Nile tilapia; *Oreochromis niloticus*; liver; duckweed; *Lemna minor*; Cu; Zn; Glutathione Peroxidase; GPx; Glutathione-S-Transferase; GST; Superoxide dismutase; SOD; Catalase; CAT; remediation assessment

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1. Introduction

One of the major health concern worldwide is the massive release of toxic compounds into the natural environment including soil and water [1,2]. Many of these compounds are defined as heavy metals and are toxic even at minimal concentrations and which may be cytotoxic, carcinogenic and mutagenic in nature [3,4]. They occur in the environment from natural and anthropogenic sources [5,6]. Dietary contamination by these chemical elements give rise to a numerous adverse effects on human and animal physiology [1,2,7]. These compounds may seriously affect cellular processes [7]. Their toxicity involves the generation of reactive oxygen and nitrogen species, which disturb redox systems [8], and antioxidants [9–11]. An overexpression of free radicals production or a downregulation of radicals-scavenging activity alters cellular functions through direct modification of biomolecules and by alteration of signaling pathways [7].

The most effective antioxidative physiological defense systems are comprised of enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) and Glutathione-S-Transferase (GST), which are known as biomarkers of oxidative stress [8,12–15]. The SOD converts superoxides (O^{2-}) generated in peroxisomes and mitochondria to hydrogen peroxide detoxified by the CAT enzyme. The SOD & CAT systems provide the first mechanism for combating oxygen toxicity. The GPx catalyzes the reduction of hydrogen peroxide and lipid peroxides; GST acts as a catalytic agent in the biotransformation process by conjugation of metabolites as xenobiotic metabolites. Antioxidant enzymes have been shown to have different responses and significantly lower activities in the polluted sites [12,16,17].

Thus, to make the environment safer and healthier for humans as regards to food consumption, and to insure adequate fish growth performance, contaminated waters and lands need to be decontaminated to very much lower levels of heavy metals and trace compounds [18,19]. Several techniques are currently used to remove heavy metals. Most of them, in particular physico-chemical methods, become ineffective when heavy metals concentrations are under 100 mg/L [20]. In fact, metal salts are present in water in a dissolved form and can not be separated using physical approach [21]. Introduction of aquatic phytoremediation plant species and adsorbents should be performed in land management plans in order to reduce risks due to their contamination [22]. Therefore, plants represent an alternative remediation approach has escalated in the last few decades, [23–29].

The eco-friendly macrophyte *Lemna minor* (family Lemnaceae, genera *Lemna*), commonly known as duckweed, is present worldwide [30], and is used as a standard ecotoxicological test species [31]. As macrophytes are more sensitive than equivalent indicators which lack a vascular system, they are more environmentally protective, thus confirming this plant as an acceptable species for toxic metals remediation [32,33]. *Lemna minor* is also used for elimination organic matter, nutrients such as Phosphorus and Nitrogen, soluble salts, as well as the reduction of faecal coliform densities and suspended solids [34,35]. It is also well known as able to accumulate Cu and Zn from contaminated wastewater [36–39]. Specifically, *Lemna minor* can accumulate a wide range of pollutants in its root tissue [39,40], highlighting its use for remediation programs [5,33,41].

Fish, as aquatic organisms, are subject to a vast array of water pollutants and, as such, may serve as indicators for contamination assessment. Therefore, a series of biomarkers, including oxidative stress biomarkers, can be successfully applied for detection of biological impacts and for environmental quality assessment [42]. These biomarkers provide a clear and useful link between pollution exposure, tissue contamination, and early adverse effects in organisms [8,41,43].

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The Nile tilapia, *Oreochromis niloticus* is a domesticated fish species extensively used in environmental studies because: it is easily handled and maintained in the laboratory; it readily adapts to confinement; it is susceptible to various pollutants; it has economic importance. Moreover, the Nile tilapia and its primary tissue for detoxification, the liver, has been widely used for toxicity evaluation of several contaminants in aquatic ecosystems [44–46].

The chief aim of this research was to detect the low and high concentrations of copper and zinc effects on hepatic antioxidative biomarkers in tilapia and to examine the efficacy of the duckweed *Lemna minor* for their bioremediation in the environment.

2. Materials and Methods

2.1. Fish and mesocosm

Tilapia fish, *Oreochromis niloticus* (n= 810, body weight 36 ± 3.2 g, male), were requested to the National Research Centre farm in Nubaria, Egypt. Over an approximate two hours transport period the fish were transferred to the laboratory at National Research Centre in a fiberglass container (1 m^3 water capacity) supplied with battery-powered aerators for oxygen supply. The tilapia fish were treated with lidocaine, CHNO (5 mg/l), during the transportation, for stress reduction. In the laboratory, the fish undergo to 40 days of acclimatization in 40 L glass mesocosm (45 x 60 x 30 cm, N= 9 tanks with ten fish each), un-chlorinated, well aerated and tap water (27.2 ±1.8ºC and pH 7–8). A pelleted diet (32% protein ration) was provided daily at rate of 3% of fish body weight and the water was removed daily. This experiment followed the Egyptian ethical guidance for animal research of the Institutional Animal Care and Use Committee (IACUC), 2013.

2.2. Experimental design

Fish were distributed into nine experimental groups and were exposed to water with copper and zinc for four weeks as follows: the first fish group was exposed to regular, uncontaminated, water as a control. The second and third groups were exposed to water contaminated with low and high doses of copper sulfate of 0.004 mg/L (CuL) and 0.02 (CuH) mg/L, respectively. The fourth and fifth groups were exposed to water with the same doses of copper as in the previous groups plus one layer of duckweed, *Lemna minor*, covering the water surface. The sixth and seventh groups were contaminated with low (ZnL) and high (ZnH) doses of zinc acetate of 0.5 and 1.5 mg/L, respectively. The eighth and ninth groups were exposed to water with the same concentrations of zinc as in the previous groups plus one layer of duckweed covering the water surface. The applied doses of copper and zinc were based on the permissible concentrations in natural water [47] and the estimated levels in polluted areas in Egypt [48]. At the end of the four-week, fish were euthanized and dissected. Growth performance was evaluated in the mesocosms treated and untreated with metals, and with and without duckweed. Liver from fish within the different treatment groups were examined immediately for DNA structure analysis and the remaining samples stored at -80°C for the various biochemical and molecular analyses.

2.3. Growth performance

Growth performance was determined as follows: Weight gain =W 2 –W 1; where W 2 is final weight after the experimental periods (four weeks), W 1 is initial weight.

2.4. Analysis of DNA

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128 2.4.1. The Comet Assay

129 Comet assay followed the protocol establish by Blasiak et al., (2004) [49]. Images from 100
130 randomly selected cells (fifty counts on each duplicate slide) were analyzed for each sample. In each
131 comet class were calculated the mean score and standard deviation. Different classes distinction were
132 used as follow: class 0 (no visible tail), class 1 (low fluorescence, round head and low damage-tail
133 length not more than 30 μm), class 2 (equally brightly fluorescent for head and tail, medium damage
134 tail length between 30 and 50 μm), and class 3 (bright and head small and weakly fluorescent and
135 high damage-tail length between 50 and 70 μm). Comets with completely disintegrated head and
136 only visible tails were considered apoptotic and were not included in the analysis.

137 *2.5. Gene Expression analysis*

138 2.5.1. RNA extraction

139 RNA was extracted from tilapia liver tissues using TRIzol Reagent (Invitrogen, Germany). 1 ml
140 of TRIzol reagent buffer was used to homogenize 50 mg of liver at room temperature for 15 minutes.
141 0.2 mL of chloroform was subsequently added. The samples were vortex for 15 seconds, incubated
142 for 3 minutes and then centrifuged at 4°C at 12000 g. for 15 minutes. The upper aqueous layer was
143 transferred to a fresh tube and mixed to 0.5 ml isopropyl alcohol for RNA precipitation. Samples were
144 first incubated at 30°C for 10 minutes and then centrifuged at 4°C, 12000 g for 10 minutes. The RNA
145 pellet obtained was washed with 1 ml of 75% ethanol, centrifuged at 4°C, 7500 g for 5 minutes, air-
146 dried for 10 minutes, dissolved in 100μL of diethylpyrocarbonate (DEPC)-treated water and stored
147 at -80°C.
148

149 2.5.2. Reverse transcription (RT) reaction

150 The RNA from tilapia liver was transcribed in 20 μl of cDNA using RevertAid™ First Strand cDNA
151 Synthesis Kit (MBI Fermentas, Germany). The RNA (5 μg) was mixed to 50 UM- MuLV reverse
152 transcriptase, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity), 50
153 μM oligo-dT primer, 10 mM of each dNTP, 50 mM $MgCl_2$ and 5x reverse transcription (RT) buffer. The
154 RT thermal reaction program were 25°C for 10 minutes followed by one h at 42°C with a final heating
155 at 99°C for 5 minutes. The final reaction were cooled in ice and then used for quantitative real time-
156 polymerase chain reaction (qRT-PCR).
157

158 2.5.3. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

159 Reaction mixtures (35 μL) of qRT-PCR consisted of 5 μL of cDNA template, 5 μL 0.2 μM of each
160 primer, 12.5 μL of 1× SYBR® Premix Ex Taq TM (TaKaRa, Biotech. Co. Ltd.) and 7.5 μL d.H_2O was used.
161 The PCR was performed as follows: 95.0°C for 3 minutes, then 28 cycles of 95°C, 1 minutes; 60°C, 1
162 minutes; 72°C, 1 minutes then, 71 cycles at 60°C and then changed every 10 seconds at about 0.5°C until
163 reaching 95°C. By the end of each qRT-PCR, a melting curve analysis was carried out at 95°C to check
164 the quality of primers used in the reaction [50]. All reactions were performed using Step One Real-Time
165 PCR system (Applied Biosystems, USA), and each run contained distilled water as a control. The
166 expression level of the following antioxidant enzyme genes was quantified in liver tissues of tilapia fish:
167 SOD, CAT, GPx and GST. The primers were designed using Primer3 software
168 (http://bioinfo.ut.ee/primer3/) Table 1. The quantitative values of RT-PCR were normalized using to
169 housekeep genes β-actin [51]
170

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Table 1. Primer sequences for the Nile tilapia *Oreochromis niloticus* genes encoding antioxidant enzymes

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA
CAT	TCCTGAATGAGGAGGAGCGA	ATCTTAGATGAGGCGGTGATG
SOD	GGTGCCCTGGAGCCCTA	ATGCGAAGTCTTCCACTGTC
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC
β-actin	CAATGAGAGGTTCCGTTGC	AGGATTCCATACCAAGGAAGG

2.6. Biochemical measurements

2.6.1. Glutathione-S-Transferase (GST) activity

GST activity was estimated in tilapia liver tissues of each treatment group according to methods described by Habig et al. (1974) [52]. The GST was evaluated with spectrophotometer for 5 minutes at 25°C due to the conjugation of reduced glutathione with 1- chloro-2,4-dinitrobenzene (CDNB) at 1 mM final concentration, 1 mM 1- chloro-2,4-dinitrobenzene, and 100 mM potassium phosphate buffer (pH 6.5) considering the blank values. Bradford protein assay was used to determine the protein concentration, using bovine serum albumin (Sigma) as standard. GST activity was expressed as μM/min/mg protein.

2.6.2. Glutathione peroxidase (GPx) activity

GPx activity was measured in tilapia liver tissues of each treatment group according to methods described by Mannervik (1985) [53]. The enzymatic reaction was estimated using the consecutive glutathione reductase reaction, the oxidation of NADPH and the substrate t-butyl hydroperoxide. Bradford protein assay was used to determine the protein concentration, using bovine serum albumin (Sigma) as standard. In accord to Flohe` and Gunzler (1984) [54], a unit of GPx activity is defined as the amount of GPx needed to reduce initial glutathione concentration. The GPx activity was expressed as μM/min/mg protein

2.7. Statistical analysis

One-way ANOVA and when appropriate Scheffé post-hoc test were used to analyze multiple group data. Data are shown as mean ± standard error of the mean (SEM). The level of statistical significance was set at $p<0.05$.

3. Results

3.1. Effect of duckweed on growth performance

The results for fish weight-gain reported in Table 2 shows that tilapia fish exposed to a low dose of Cu did not have a significantly reduced final body weight compared to the control fish.

Table 2. Growth performance of Nile tilapia, *Oreochromis niloticus* exposed to heavy metals: Low and high dose Cu (CuL, CuH), and Low and High dose Zn (ZnL, ZnH) in mesocosm with or without the duckweed, *Lemna minor*.

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Treatment	Initial weight (g)	Final weight (g)
Control	36.2±2.4	99.3±3.2 [a]
CuL	37.1±3.2	88.1±4.1 [ab]
CuH	35.4±1.9	77.6±2.9 [bc]
CuL+L. minor	38.2±2.7	93.2±4.8 [ab]
CuH+L. minor	36.4±1.6	84.4±5.2 [b]
ZnL	36.2±1.5	81.5±3.7 [b]
ZnH	37.5±2.2	71.2±2.4 [c]
ZnL+L. minor	38.2±3.3	89.1±3.8 [ab]
ZnH+L. minor	36.6±2.1	80.3±3.1 [b]

Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$, Scheffé-Test).

However, a high dose of Cu resulted in significantly reduced final body weights of tilapia compared to control fish. Likewise, low and high doses of Zn reduced significantly the final body weight of tilapia compared with control fish.

3.2. Effect of duckweed against heavy metals induced DNA damage

The results for the percentage of DNA damaged cells reported in Table 3 revealed that fish exposed to Zn exhibited rates of DNA damage more significant than those exposed to Cu compared to control group. Furthermore, fish exposed to a low dose of Cu and Zn revealed relatively similar rates of DNA damage compared to those in control fish. However, the high dose of Cu and Zn induced higher frequencies of DNA damage with percentages of 17.4 and 19.6 for Cu and Zn, respectively, compared to the control group. Results for percentage of DNA damaged cells assessed in *Oreochromis niloticus* liver indicated less damage when *Lemna minor* was added with respect to treatment with both metals. Specifically, DNA damage reduction was 1.6% for CuL concentration and 6.2% for CuH concentration and 2.0% for ZnL and 7.2% for ZnH concentration.

Table 3. Total comets, class of comet and % DNA damaged liver cells in Nile tilapia, *Oreochromis niloticus* exposed to heavy metals: Low and high dose Cu (CuL, CuH), and Low and High dose Zn (ZnL, ZnH) in mesocosm with or without the duckweed, *Lemna minor* using the comet assay.

Treatment	Comet class

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	Total comets	0	1	2	3	DNA % damaged cells
Control	33	467	22	11	0	6.6±1.1 [c]
CuL	46	454	17	16	13	9.2±1.6 [bc]
CuH	87	413	26	32	29	17.4±2.4 [a]
CuL+L. minor	38	462	12	15	11	7.6±1.2 [c]
CuH+L. minor	56	444	16	21	19	11.2±1.6 [b]
ZnL	49	451	18	15	16	9.8±1.5 [bc]
ZnH	98	402	28	37	33	19.6±2.2 [a]
ZnL+L. minor	39	461	21	11	7	7.8±1.3 [c]
ZnH+L. minor	62	438	18	22	21	12.4±1.8 [b]

Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$, Scheffé-Test) (n = 5).

3.3. Effect of duckweed on antioxidants gene expression

Quantitative expression of antioxidant enzyme related genes including glutathione-s-transferase (GST), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) genes in liver tissues of Nile tilapia is summarized in Figure 1A-C. GST, SOD, CAT and GPx genes were significantly down-regulated in the liver tissues of tilapia exposed to a high dose of Zn (1.5 mg/L) and Cu (0.02 mg/L) compared to control group. In particular, even at low Zn dose (0.02 mg/L), GST, SOD and CAT ($P < 0.01$) were affected in comparison to control fish. Interestingly, SOD, CAT and GPx expression, which were reduced with high doses of Cu, was not affected in the presence of *Lemna minor*. While for the low Zn treatment, decreased expression of SOD and CAT was not observed for the same treatment with *Lemna minor* addition. Surprisingly, reduced CAT expression ($P < 0.01$) observed at high Zn exposure, remained at control levels in experiments where *Lemna minor* was added.

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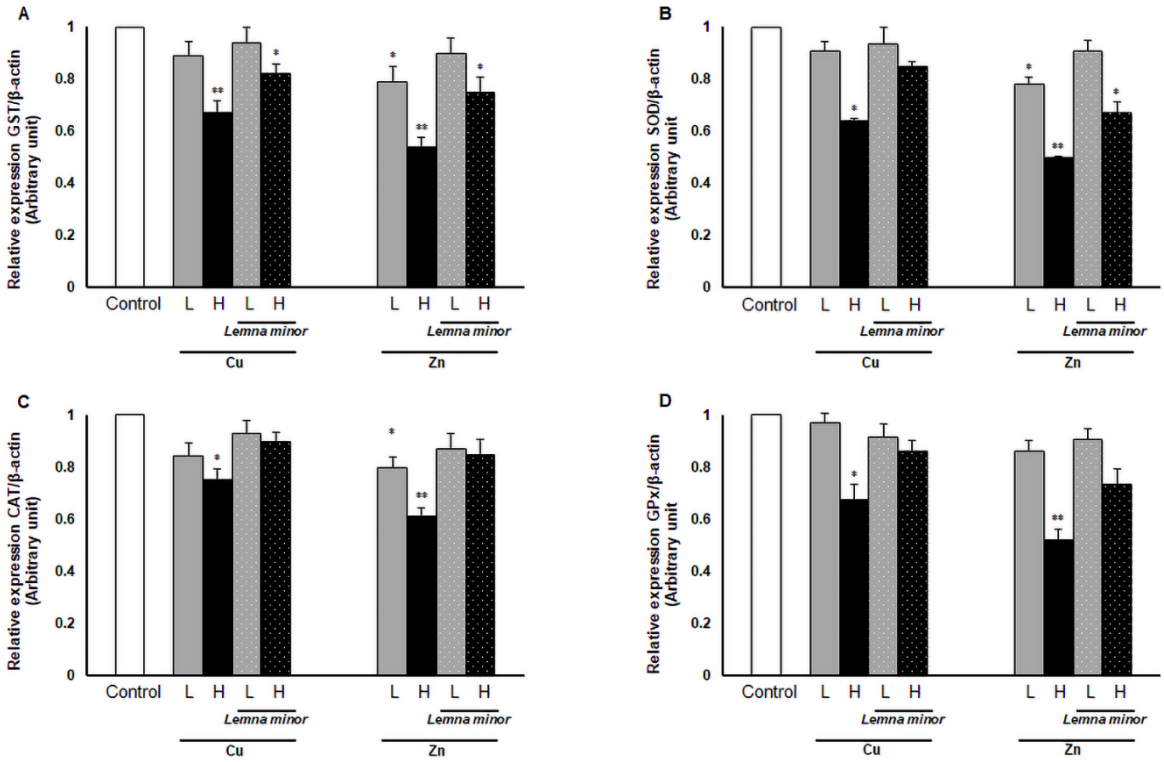

Figure 1. RTqPCR expression analysis of liver antioxidant enzyme genes (GST, CAT, SOD, GPx) of Nile tilapia, *Oreochromis niloticus*. The relative expression indicated in arbitrary units defines the expression change in comparison to that of the reference housekeeping β-actina rRNA gene in samples exposed to heavy metals, Cu and Zn in mesocosm with or without duckweed, *Lemna minor* (Cu L: 0.004 and H: 0.02 mg/l); Zn, L: 0.5 and H: 1.5 mg/l) with respect to samples without treatment used as control. *P <0.05 and ** P < 0.01 for the treated groups compared with control group.

3.4. Effect of duckweed on the GST and GPx activities

Results show damaged liver cells of Nile tilapia *Oreochromis niloticus* exposed to different concentrations (low, L and high, H) of heavy metals, Cu and Zn alone or combined with duckweed, *Lemna minor*. The applied doses of Cu and Zn were chosen based on estimated levels in polluted areas in Egyptian river water in the last [47,48] and recent assessment [55].

Biochemical measurements were performed to examine hepatic GST and GPx activities in *Oreochromis niloticus* (Fig 2). The results show a high dose of Cu (0.02 mg/L) and Zn (1.5 mg/L) induced significantly lower activity levels of GST and GPx. In particular, for both enzymes, Zn doses induced the lowest activity levels of the enzymes even at low concentrations (0.05 mg/L). Moreover, the significant decrease of GPx activity subjected to low Zn concentration (0.5 mg/L) was not affected in the presence of *Lemna minor* (Fig. 1B).

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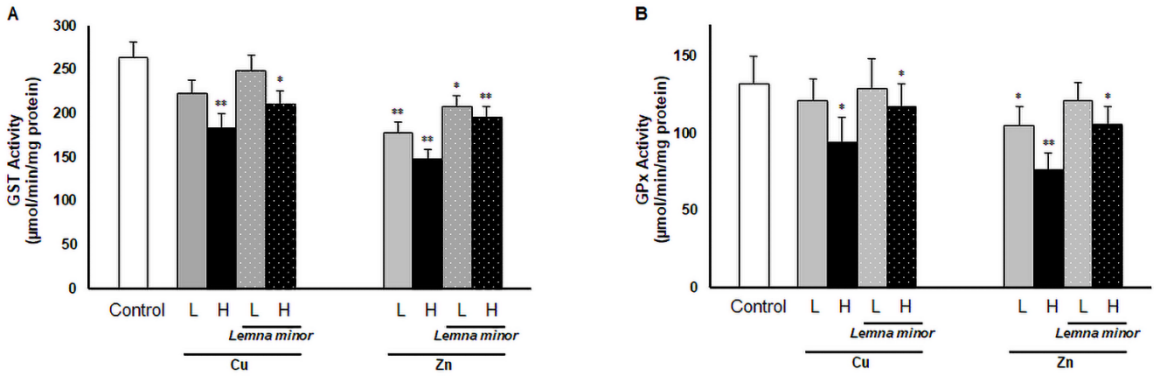

Figure 2. Activity levels of GST (A) and GPx (B) in the liver tissues of Nile tilapia, *Oreochromis niloticus* exposed to heavy metals (Cu and Zn) in mesocosm with or without the duckweed, *Lemna minor*. Cu = Copper exposure (L: 0.004 and H: 0.02 mg/L), Zn = Zinc exposure (L: 0.5 and H: 1.5 mg/L). Data are presented as mean ± SEM. *P <0.05 and ** P < 0.01 for the treated groups compared with control group.

4. Discussion

Heavy metals accumulation in fish has potentiality to induce toxicological effects [1,2] and cause oxidative damage to tissues determining cell functions loss [10,11,56]. Zinc and copper, in particular, are essential trace minerals for teleost fish and all vertebrates and are found in all organs, tissues, and fluids. These metals have structural and catalytic functions and also play a regulatory role in multiple metalloenzymes as a specific cofactor and catalyst. Their toxicity is often linked to the physiological processes disruption [42,57–59]. Nonetheless, it is also known that under normal conditions, these elements are essential micronutrients. Zn, in fact, is one of the most important essential trace elements involved in animal growth and the most widely used metal cofactor in many enzymes. Cu, acts as a catalyst in many enzyme systems mainly for cytochrome oxidase and the electron carrier plastocyanin and is actively taken up by liver mitochondria via an energy-dependent system. As ionic Cu and Zn inhibit a number of enzymes, it follows that the basis for their toxicity may be due to their diminished activity. In particular, it is well known that the liver, among all the tissues, is the site of multiple oxidative reactions and maximal free radical generation [60–62].

Thus, to reduce the excessive free radical production, in the present study, Nile tilapia were exposed for a period of four weeks to copper or zinc in the presence of duckweed *Lemna minor*. The concentrations used in the mesocosm water (Cu: 0.004 mg/L and 0.02 mg/L; Zn: 05 mg/L and 1.5 mg/L) match those estimated in polluted areas in Egypt [48]. However, at the proteomic level, after high Cu exposure and both low and high Zn exposure, the magnitude of hepatic activity of GST and GPx decreased, as has been reported in the liver of Nile tilapia [15,16,46]. Such changes in the antioxidative capacity in Nile tilapia could be attributed to metal ion of Cu and Zn concentration and duration of exposure [15,16,46].

Cu acts as a cofactor for a wide range of metal-binding enzymes, fluctuating between the oxidized and reduced copper forms. These forms, which have a high affinity for protein sites, act as potential ligands that lead to the displacement of essential metal ions from their active sites [63]. Furthermore, their excess lead to their involvement in the overexpression of free radicals able to damage DNA, lipids and proteins [64].

Zn is well known for its role as a cofactor for SOD, and it protects biological structures from damage caused by free radicals. But at high levels, Zn can also cause osmoregulatory disturbances in

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aquatic organisms [65], and may also cause cytotoxic effects in the presence of hydrogen peroxide [66]. In fact, a significant correlation between GST, GPx and Zn, as well as, Cu levels supports our results (Fig.1). Zinc exhibited more toxic effects than Cu in fish in terms of liver cell damage which led to reduced weigh gain of fish in the Zn exposed group compared to the Cu exposed group. Our results concur with other studies reported that although Zn may be present at allowable normal levels, it can be toxic at both conventional and at permissible high-level standards [65,67].

This is the first study wherein we have studied the impacts of duckweed (*Lemna minor*) on the hepatic oxidative/redox status of Nile tilapia in the presence of heavy metals in a mesocosm [68].

Our present results are in agreement with a previous study in which fish show liver SOD inhibition when exposed to 5 mg/L ZnONPs [69]. It has also been shown that copper oxide nanoparticles suppressed activity levels of GPx and GST and also inhibited levels of GSH and resulted in increased oxidative stress in the digestive gland of the freshwater snail [70]. Moreover, treatment of Nile tilapia with 1 and 2 mg/L ZnONPs resulted in suppression of antioxidants activity. ZnONPs also decreased the gene expression of SOD and CAT in the liver and gills of Nile tilapia [71].

At transcriptional levels, SOD, CAT, GPx and GST gene expression pattern have been validated as biomarkers of exposure to oxidative stress-inducing chemical pollutants and also to abiotic factors such as hyperthermia [11].

In our study, exposure to Cu and Zn caused the greatest reduction in SOD, CAT and GPx and GST transcription and an increase in DNA damage. However, Zn may have the more deleterious effect by notably decreasing enzymatic activity even at low concentration (Fig 2). These results are in accord with other studies on antioxidative mRNA expression, which in the hepatopancreas, gills and kidney were shown to be down-regulated by exposure to Cd, Cu and Zn [15,72,73]. Contrariwise, much research has shown an increase of hepatic gene expression in relation to toxic metals exposure [74–76]. Thus, it has been suggested that expression of antioxidant biomarkers can be enhanced or reduced depending on many factors as the chemical stress intensity and duration, as well as the investigated species sensitivity [11,57,58,74,77,78]. These studies of antioxidative expressions at transcriptional and translational levels can answer fundamental questions linked to the xenobiotic type, exposure times, data on seasonal time of sampling, and the gender and sexual maturity of fish [12,44,45,79].

Our results on fish growth performance and DNA structure together with our analysis of genes expression and biochemical measurements highlight the potential use of *Lemna minor for* reducing oxidative stress and enhancing the capacity for heavy metal tolerance in Nile tilapia. This is important because when antioxidative capacity is lowered, protection against cell damage is also impacted due to reduction in the scavenging ability for free radicals leading to increased oxidative stress.

In accordance with our DNA damage analysis (Table 3), it has been evidenciated that high concentrations of heavy metals, either individually or in combination [80,81,82] induced both sub-lethal and lethal effects in fish. The parameters most markedly affected include: tissue genotoxicity, immunity suppression, endocrine disruption, enzyme and vitamin degradation and morphological alteration in cells [5,83,84].

Interestingly, the expression levels of all examined genes were significantly increased, and the rate of DNA damage decreased in fish treated with duckweed *Lemna minor*, highlighting inhibition of the deleterious effect posed by Cu and Zn exposure in water. Thus, the consistency between the change of enzyme activities and gene mRNA abundance exposed to toxic substances underscores how activities of antioxidant enzymes could be regulated. This strengthens our data showing that the decrease in antioxidant activity reflects the reduction in the gene expression, and the addition of

duckweed *Lemna minor* prevents the alteration of enzymatic activity and gene expression previously diminished by metal exposure [74].

Finally, it was demonstrated that *Lemna minor* prevented decrease of the final body weight in fish exposed to low doses of Cu and Zn compared to control fish. This result confirms the duckweed *Lemna minor* as a successful treatment for preventing the deteriorating effects of water-borne metals, copper and zinc, on growth performance and health of Nile tilapia; effects which have been already amply demonstrated in the literature [18,19].

5. Conclusions

In summary, *Lemna minor* is a potential remediator for protection of one of the most important aquaculture species in Egypt and worldwide, the Nile tilapia *Oreochromis niloticus.* This remediation may be achieved by reducing oxidative stress and enhancing heavy metal tolerance of these fish. In this regard, tilapia can be introduced as an *in vivo* model through utilization of liver antioxidants as biomarkers for remediation screening.

Understanding relationships between stressors, stress responses, and the recovery process contribute to the effective management and restoration of aquatic ecosystems.

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