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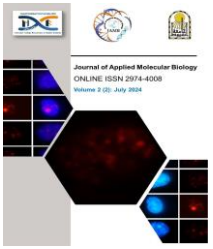

Histopathological and Molecular Responses of Goldfish (*Carassius auratus*) Exposed to Sodium and Manganese Sulfates

Walaa M. Shaalan[1,2,*], Axel Mosig[2] and Aya Elshaer[1]
[1]Zoology Department, Faculty of Science, Benha University, 13518 Benha, Egypt
[2] Bioinformatics Group, Faculty for Biology and Biotechnology and Center for Protein Diagnostics, Ruhr-University Bochum, Bochum 44801, Germany
[*]Corresponding Author: walaa.shalan@fsc.bu.edu.eg

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ABSTRACT

Aquatic environments are increasingly subjected to anthropogenic pollutants, including sulfate as sodium sulfate ($Na2SO_4$) and manganese sulfate (MnSO4), which can have deleterious effects on aquatic organisms. Our study investigates the histological alterations and gene expression changes in goldfish (*Carassius auratus*) exposed to $Na2SO_4$ and MnSO4. Goldfish with an average size of 20.42±3.25 g were divided into seven groups and reared for 96 hours. Group 1 served as the control group. Groups 2, 3, and 4 were exposed to MnSO4 at 3.76 mg/L, 7.52 mg/L, and 11.28 mg/L, respectively. Groups 5, 6, and 7 were exposed to $Na2SO_4$ at 1.598 g/L, 3.196 g/L, and 4.794 g/L, respectively. These doses correspond to 20%, 40%, and 60% of their respective LC50 values. Histological examinations of gill, liver, and muscle tissues were conducted to assess structural changes. Additionally, gene expression profiling for myogenic differentiation protein1 (MyoD), muscle RING Finger 2 (MURF2), and insulin like growth factor (IGF-1), was performed to elucidate molecular responses to the exposure. Preliminary findings reveal notable histological alteration, including gill epithelial damage, hepatic alterations, and renal changes, suggesting organ-specific sensitivity to $Na2SO_4$ and MnSO4 exposure. Furthermore, the differentially expressed genes associated with stress response, detoxification mechanisms, and inflammation pathways were identified by gene expression analysis of MyoD, MURF2, IGF-1. The results of this study shed light on potential mechanisms underlying the observed histological changes and molecular responses. Understanding these impacts is crucial for effective aquatic ecosystem management and the development of strategies to mitigate the adverse effects on aquatic organisms.

1. INTRODUCTION

Pollution in aquatic ecosystems poses significant threats to the delicate balance of natural environments and the organisms that inhabit them [62, 18]. With rapid industrialization, urbanization, and agricultural intensification, various pollutants find their way into rivers, lakes, oceans, and other water bodies [71]. These pollutants can be classified into several categories, including chemical contaminants, nutrient pollutants, and physical pollutants [1, 16]. The impact of pollution on aquatic ecosystems is profound and multifaceted [22, 49]. Chemical contaminants can bioaccumulate in aquatic organisms, leading to toxicity and affecting entire food webs [5, 6]. Nutrient pollution can cause harmful algal blooms and oxygen depletion, disrupting aquatic habitats and leading to mass fish kills [26]. Physical pollutants like plastics can persist for decades, harming marine life through ingestion and entanglement [9].

Sodium is an important mineral that is essential to the health of fish. It is crucial for fish due to taking part in various biological processes as it can keep balance of fish in the environment, absorb, and transport nutrients across the cell membrane [59]. On the other hand, it was found that different concentrations of NaCl cause growth inhibition in *Clarias gariepinus* [72]. Manganese is an essential element for normal development and growth of fish. It plays an important role in various biochemical processes including enzyme activation and energy metabolism [40]. Adequate levels of manganese are necessary for maintaining overall fish health. It is involved in the formation of bone and cartilage, as well as in the synthesis of certain enzymes and proteins [56]. Excessive levels of manganese can be toxic to fish. High concentrations of manganese salts in water can lead to various adverse effects, including reduced growth, impaired reproduction, and behavioral changes [77]. The effects of manganese on fish health are closely related to water quality. In some cases, manganese toxicity in fish can be a result of poor water quality, particularly when manganese levels are naturally high or when pollution introduces excessive manganese into aquatic environments [27, 34]. Different fish species may have varying tolerances to manganese. Some species are more sensitive to manganese toxicity than others, and the effects can also depend on the life stage of the fish [39]. In addition, sulfates derived from the decomposition of rocks and can cause toxic environment for the aquatic organisms [82].

Previous study on Marine Medaka revealed that the muscle of the fish is considered from the target tissues of pollutants [25]. A key role is taken by the myogenic differentiation protein1 (MyoD) gene, which is the myogenic regulator in the process of muscle formation and differentiation [75]. MyoD belongs to the family of basic helix-loop-helix (bHLH) transcription factors and is an important regulator of the route leading to muscle differentiation [35, 73]. MyoD stimulates the expression of a number of muscle-specific genes, such as those that code for structural proteins like myosin, which are crucial for the development and operation of muscles [70]. MyoD is involved in muscle regeneration in addition to embryonic development. When the muscle cell is

injured, MyoD is turned in satellite cells, or the muscle tissue's stem cells. The injured tissue is subsequently repaired by these satellite cells differentiating into new muscle cells [36, 58]. The growth and function of muscles may be affected by mutations or dysregulation of the MyoD gene. Furthermore, MyoD is being studied associated with muscle disorders and potential therapies for muscle regeneration [15]. The MURF2 gene, often referred to as muscle RING Finger 2, is a crucial gene involved in the control, upkeep, and growth of muscles [46, 81]. It belongs to the family of RING finger proteins, which also contains other proteins that frequently serve as E3 ubiquitin ligases and are involved in the degradation of proteins and cellular signaling [32]. MURF2 gene is predominantly expressed in muscular tissues and has a role in the control of muscle growth, upkeep, and contractile activity [50]. MURF2 gene has been linked to the decrease of muscular mass and function, or muscle atrophy. Muscle breakdown occurs when MURF2 and other E3 ubiquitin ligases are activated [13, 52], which happens when muscles tissues are exposed to environmental pollution [20]. Different studied provoked the correlation between pollution and sarcopenia [80].

The IGF-1 gene, referred to as Insulin-Like Growth Factor 1, plays a critical role in growth, development, and control of a number of physiological processes [3, 78], and is crucial for metabolism, tissue repair, and cell proliferation [2]. IGF-1 affects cell development, proliferation, differentiation, and survival by acting as both a hormone and a growth factor [30]. It is generated by a number of organs, including the liver, and when growth hormone (GH) is stimulated, it encourages cell division and growth [40, 29]. It contributes to bone lengthening and general body development [42]. Growth problems can result from IGF-1 gene mutations or dysregulation [31]. On the other hand, high levels of IGF-1 have been associated with diseases including acromegaly [74]. IGF-1 was reported to be mediated by the transmembrane receptor IGF-1R that has the activity of tyrpsine kinase [21]. IGF-1R activates the (PI3K/Akt) pathway and that have a role in muscle cell proliferation and maintenance of body muscle mass. The mitogen-activated protein kinase (MAPK/ERK1/2) pathway and the PI3K/Akt pathway were reported to mediate the activity of IGF-1 on myoblast cells [25]. In addition, Akt- encourage the promotion of protein synthesis and muscle development by the effect of IGF-1 [60].

Fish are the main organisms in the food chain of the aquatic ecosystem that concentrate the largest quantities of minerals in their body from the surrounding environment [8]. Goldfish (*Crassius auratus*) is one of the most well-known and generally recognized ornamental fish species. They are extremely simple to care for, that makes commercial goldfish aquaculture is a common activity across the world [43]. Goldfish are susceptible to several illnesses, much as any species used in aquaculture [12, 59]. It is crucial to feed fish in aquaculture environments a balanced diet that contains enough sodium, Mn, Mg, Calcium, and other vital minerals. Overall, it is essential to keep salt levels within a range for fish to maintain their life [40]. However, increasing their concentration may lead to harmful effects [54].

In our present study, we investigate the effect of different increased concentrations of $MnSO_4$ and Na_2SO_4 on the health and growth of *Carassius auratus*. In addition, we study their side effect on muscle fillet growth and quality. Measuring their effect on certain studied genes that are considered as biomarkers for aquatic pollution.

2. MATERIALS AND METHODS

The experiment was approved by the institutional ethics committee at faculty of science Benha university, protocol No. (BUFS-REC-2024-112Zoo). As concluded in figure 1, goldfish (*Carassius auratus*) fingerlings with average size 20.42±3.25 g and average length of 8.21±1.20 cm were obtained from fish shop at Benha city and were acclimated for 7 days before the start of experiments. The fish were divided into seven groups and reared for 96 hours. Group 1 served as the control group with no treatment. Groups 2, 3, and 4 were exposed to three different doses of $MnSO_4$ (3.76 mg/L, 7.52 mg/L, and 11.28 mg/L), while Groups 5, 6, and 7 were exposed to three different doses of Na_2SO_4 (1.598 g/L, 3.196 g/L, and 4.794 g/L). These doses correspond to 20%, 40%, and 60% of their respective LC50 values [7, 53]. The experiment was conducted in triplicate tanks (fish/ 2 Liter tank). Each tank contained 18 goldfish. Fish were manually fed a commercial fish diet (provided from Commercial fish market) at a ratio of 5–7% of fish body weight twice per day. Cleaning the tanks was done daily to get rid of feces and remaining food particles. The water temperature was 26 ±0.03, pH was7.6 to 8, and the dissolved oxygen was 7-9 ml/l. After the end of the experiment, overdose of MS222, Syncaine, Syndel, Canada was used to euthanize the fish [69]. Muscle, gills and liver samples were collected and stored in Ethyl alcohol for histological examination. Other muscle, gill and liver samples were stored in liquid nitrogen and immediately stored at − 80 °C.

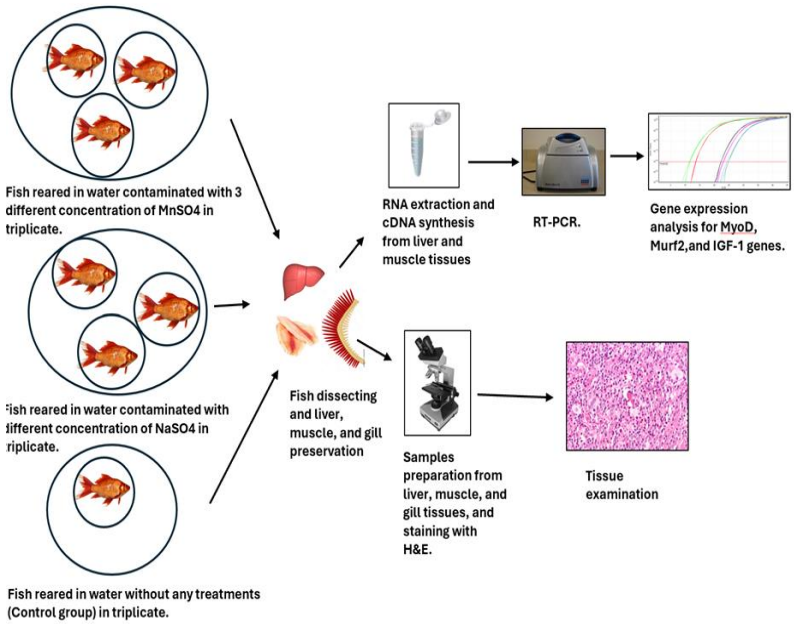

Figure 1. Schematic representation of the experimental set up

Histopathological analysis

For histopathological analysis, 1.5 x 1.0 x 0.5 cm samples of gill, liver, and muscle from eight fish per group were fixed in 10% formalin. Samples were processed through graded ethanol, cleared with xylene, embedded in paraffin, and sectioned at 5 μm using a microtome. Tissue sections were stained with Hematoxylin and Eosin (H&E) and examined under a high-resolution microscope with a digital camera [67, 79].

2.1. RNA isolation

The liver and muscle samples were randomly collected from 8 fishes/each tank, and were used for the total RNA extraction using TRIzol protocol [66]. The measurement of RNA concentration for each sample was done at 260nm absorbance using a Nanodrop spectrophotometer. Then the RNAs integrity can be performed by gel electrophoresis.

2.2. cDNA library preparation

Using a random primer and a verso cDNA synthesis kit (Thermo Scientific, Germany), cDNA was synthesized in accordance with the manufacturer's instructions. Buffer that is included in the kit was used to remove genomic DNA contamination. Using a Nanodrop, the concentrations of the cDNA samples were determined.

2.3. Real-time qPCR, calculations, and statistical analysis

The real-time qPCR primers for the MUFR2, MyoD, and IGF-1 genes were designed using primer3 software [38]. Table 1 shows the cDNA accession numbers for each gene from the NCBI as well as the sequences of the forward and reverse primers of studied genes. The Bio-Rad iCycler PCR detection device was used to perform qPCR on duplicated cDNA samples that had been diluted eight times. SYBR green master mix (Thermo Scientific, Germany) as well as forward and reverse primers (10 μM/μl), were added to the cDNA templates (0.006 μg/μl) that are prepared for the real-time qPCR experiment [65]. The β-actin gene of tilapia was used as housekeeping gene [37]. In the initial denaturation stage, the cycling process was conducted at 95°C for 7 minutes. Subsequently, each gene underwent 40 amplification cycles, which involved denaturing at 95°C for 0.1 minutes, annealing at temperatures between 57°C and 64°C, and concluding with a final extension step at 60°C for 5 minutes. The formula 2^−ΔΔCT was used to calculate and determine the relative gene expression of the treated samples compared to the control samples [64]. The fold change of studied genes between the treated groups and control one were used to determine the upregulation or downregulation of he genes. Statistical analysis was done using t-test to determine the significant differences between the treated groups and the control group at ($p \leq 0.05$).

Table 1. The forward and reverse primers of genes of interest

Gene name	Accession number	primer	
IGF-1	**GU583648.1**	Forward	TTCAAGTGTACCATGCGCTG
		Reverse	ACCGTCTTGAATTAGGCCCA
Murf2	**XM_026237960.1**	Forward	ATGAAGTTGTGTTGGACCGC
		Reverse	TAAGAGGGGCCACTTCACAG
MyoD	**XM_026239323.1**	Forward	GGGAGAGGAGAAGACTGAGC
		Reverse	CGCTGTAATGTTCCAGGACG
B-actin	**AB039726.2**	Forward	CAAAGCCAACAGGGAGAAGA
		Reverse	TGAAGCATACAGGGAGAGCA

3. RESULTS

3.1. Histopathological examination

The examined liver (Figure 2A) of fish in control group revealed normal hepatic parenchyma formed from polyhedral hepatocytes with central nuclei and eosinophilic cytoplasm [24]. While the microscopic examination of the liver of fish exposed to Na_2SO_4 toxicity revealed different histopathological changes according to the amount of Na_2SO_4 in water. The examined liver of fish exposed to low Na_2SO_4 concentration exhibited only small areas of degenerative changes in the form of vacuolar and hydropic degeneration (Figure 2D), while the liver of fish exposed to medium Na_2SO_4 concentration showed centrilobular hepatic degeneration and apoptosis with disappearance of the nuclei of some of hepatocytes (Figure 2G). Moreover, congestion of the portal vessels with diffuse degeneration of hepatocytes and bile ductal hyperplasia were recorded in the liver of fish exposed to high Na_2SO_4 concentration (Figure 2J).

The microscopic examination of the gills of fish in control group showed normal gill arch and primary lamellae with central cartilaginous support and secondary lamellae with central capillary network (Figure 2B). The examined gills of fish exposed to low and medium Na_2SO_4 concentration revealed nearly similar histopathological lesions characterized by edema, congestion of blood vessels of gill arch and filaments with focal desquamation and/or hyperplasia of the lining epithelium of gill lamellae resulting in partial and complete fusion of some secondary lamellae (Figure 2 E&H). Moreover, similar microscopical changes in addition to lamellar disruption with necrosis of secondary lamellar epithelium and focal aggregation of inflammatory cells in the gill arch and tips of gill filaments were recorded in gills of fish exposed to high Na_2SO_4 concentration (Figure 2K).

The microscopic examination of the skeletal muscles of fish in control group showed unbranched muscle fibers with flatted or oval nuclei at the periphery of these myofibers (Figure 2C). In addition, loose collagenous fibers with blood capillaries were seen in-between these muscle fibers. While the examined the skeletal muscles of fish exposed to low Na_2SO_4 concentration reveled focal areas of edema and mild mononuclear inflammatory cellular aggregation in the intermuscular connective tissue (Figure 2F).

Aggregation of melanomacrophage cells mainly around blood vessels together with necrosis of some skeletal muscles characterized by loss of striation, cytoplasmic eosinophilia and absence of the nuclei with focal areas of interstitial edema mixed with few inflammatory cells were prevalent in the examined muscles of fish exposed to medium Na_2SO_4 concentration (Figure 2I). Moreover, the examined muscles of fish exposed to high Na_2SO_4 concentration revealed similar microscopic changes in addition to presence of multiple areas of coagulative necrosis. Some of these necrotic areas were replaced by fibrous connective tissues and infiltrated with mononuclear inflammatory cells (Figure 2L)

The examined gills of fish in the control group exhibited normal structures of gill arch, primary and secondary lamellae (Figure 3A). While the microscopic examination of gills of fish exposed to low $MnSO_4$ concentration showed congestion of blood vessels of gill arch and filaments with proliferation of the epithelium of gill filaments and secondary lamellae and clubbing of secondary lamellae (Figure 3D). Inflammatory cellular infiltration of gill arch and filaments with focal desquamation and hyperplasia of the lamellar epithelium resulting in thickening of some secondary lamellae were recorded in gills of fish exposed to medium $MnSO_4$ concentration (Figure 3G). Moreover, lamellar disorganization with extensive necrosis of lamellar epithelium and presence of desquamated cells mixed with inflammatory exudate in-between gill filaments were found in gills of fish exposed to high $MnSO_4$ concentration (Figure 3J).

The microscopic examination of liver of fish in control group revealed normal liver histoarchitecture formed from the hepatocytes arranged in hepatic lobules radiating around central veins and separated by sinusoids (Figure 3B). While the microscopic examination of the liver of fish exposed to low and medium $MnSO_4$ conc. exhibited focal areas of hepatic degenerative changes in the form of hydropic degeneration of hepatic cells with pyknosis of some nuclei (Figure 3 E&H). Moreover, severe diffuse hepatic degeneration of hepatocytes with absence of the nuclei of many hepatic cells were prevalent in the liver of fish exposed to high $MnSO_4$ concentration (Figure 3K).

The examined skeletal muscles of fish in control group showed unbranched muscle fibers with flatted nuclei and thread-like myofibrils running parallel to each other with loose connective fibers in-between these muscle fibers (Figure 3C). While the examined skeletal muscles of fish exposed to low $MnSO_4$ concentration revealed focal areas of hyaline degeneration and cavitation of some muscles with intermuscular and perivascular edema mixed with mononuclear cells (Figure 3F). Focal aggregation of melanomacrophage cells particularly around blood vessels was also detected in this group. The examined skeletal muscles of fish exposed to medium $MnSO_4$ concentration showed extensive fibrosis replaced degenerated and necrotic muscles (Figure 3I). Moreover, similar microscopic changes in addition to massive destruction and cavitation of the muscles were found in the skeletal muscles of fish exposed to high $MnSO_4$ concentration (Figure 3J).

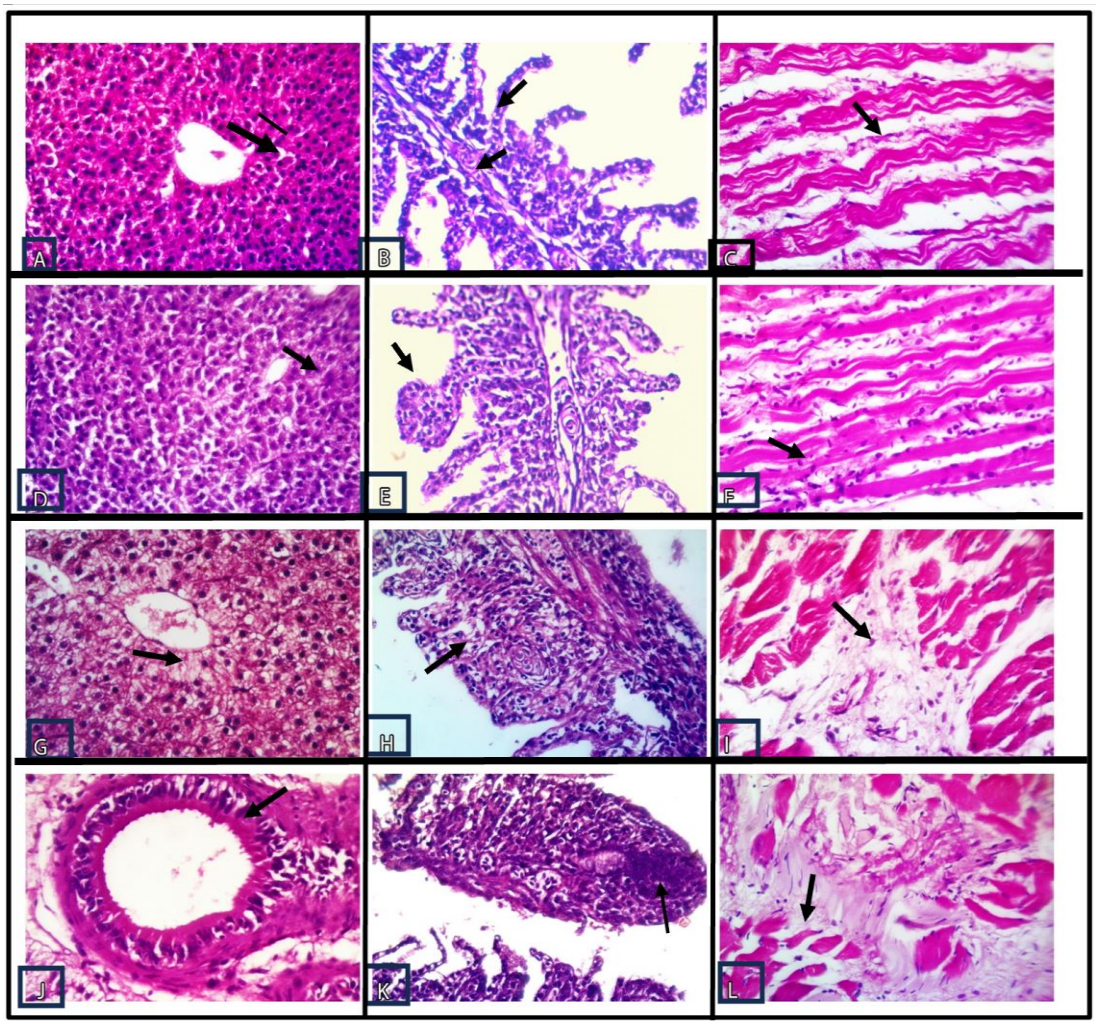

Figure 2. Photomicrograph of liver, gills and skeletal muscles of fish in control (A-C), low Na2SO4 concentration (D-F), medium Na_2SO_4 concentration (G-I) and high Na2SO4 concentration(J-L) groups. (A) liver with normal polyhedral hepatocytes (B) Normal gill showing gill filaments with central cartilaginous support and secondary lamellae (C) skeletal muscles showing unbranched muscle fibers with oval nuclei at the periphery (D) hydropic degeneration of some hepatocytes (E) focal hyperplasia of the lining epithelium of gill lamellae (F) mild mononuclear inflammatory cellular aggregation (G) centrolobular hepatic degeneration (H)focal desquamation and hyperplasia of the lining epithelium of gill lamellae(I) vacular degeneration of muscle bundels (J) bile ductal hyperplasia (K) focal aggregation of inflammatory cells at the tip of gill filament (L) Atrophy of muscle bundels. H&E stain X400.

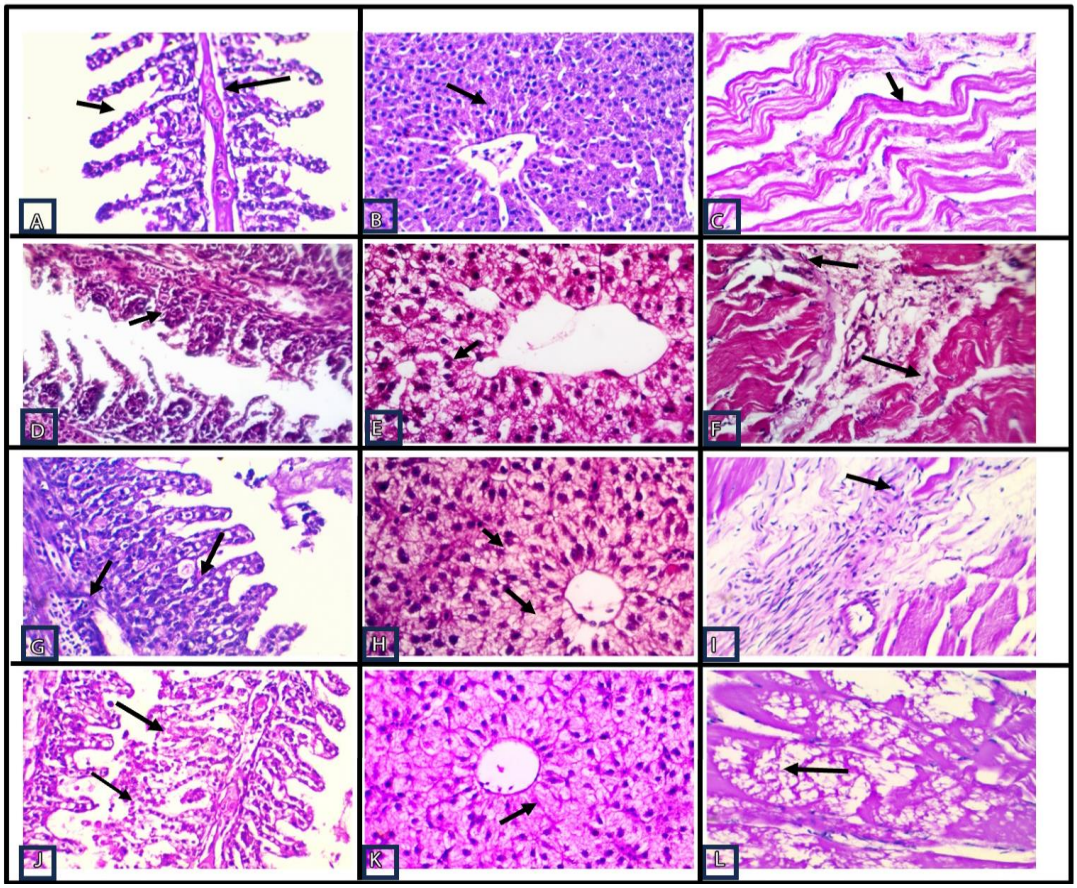

Figure 3. Photomicrograph of gills, liver and skeletal muscles of fish in the control (A-C), low MnSO4 concentration(D-F), medium MnSO4 concentration(G-I) and high MnSO4 concentration(J-L) groups. (A) Normal gill filament and secondary lamellae (B) liver showing normal hepatocytes (C) skeletal muscles showing unbranched muscle fibers (D) proliferation of the epithelium with clubbing of secondary lamellae (E) hydropic degeneration of some hepatic cells (F) hyalinization of some muscles fibers and perivascular inflammatory edema (G) inflammatory cellular infiltration of gill filament and thickening of the secondary lamellae (H) vacuolar degeneration of hepatic cells (I) replaced degenerated and necrotic muscles (J) gills showing lamellar disorganization (K) extensive degeneration of hepatocytes (L) extensive cavitation in muscle. H&E stain X400

3.2. Real time PCR:

It was recorded for fish exposed to low, medium, and high concentrations of $MnSO_4$ in muscle tissue to have significant downregulation of IGF-1 relative to the control group as depicted in figure 4a and 4b. The fold change decreased with increasing the dose of $MnSO_4$ in the water tank. The same pattern was recorded in fish muscle exposed to different concentrations of Na_2SO_4. The IGF-1 was downregulated in the different groups exposed to the Na_2SO_4 doses. IGF-1 of liver tissue was significantly gradually upregulated ($p \leq 0.05$) in low, medium, and high concentrations of Na/ Mn salts relative to the control group as recorded in figure 5a and 5b. MyoD gene was significantly downregulated by 0.95-fold in the low dose of $MnSO_4$ compared to the control group. It was significantly down regulated by 0.79 and 0.29 folds in medium and high doses of $MnSO_4$, respectively, compared to the control group as shown in figure 6a. on studying

the effect of different concentration of Na_2SO_4 on goldfish. It was reported significant downregulation ($p \leq 0.05$) by 0.35, 0.32, and 0.24 folds in low, medium, and high doses, respectively as presented in figure 6b. The expression of the MURF2 gene of muscle tissue was measured (Figure 7a,b). There was a significant upregulation of MURF2 ($p \leq 0.05$) in all treated groups relative to the control group. MURF2 was significantly overexpressed by 1.74 folds in the group exposed to the lowest dose of $MnSO_4$ relative to the control group. It was recorded a significant increase in the MURF2 gene expression in groups exposed to Medium and high doses by 1.92 and 2.4 folds, respectively, relative to the control group ($p \leq 0.05$). For the Na_2SO_4 treatment, it was showed a significant increase in MURF2 gene expression by 1.65, 2.8, and 4.3 folds ($p \leq 0.05$) in low, medium, and high concentration, respectively, relative to the control group.

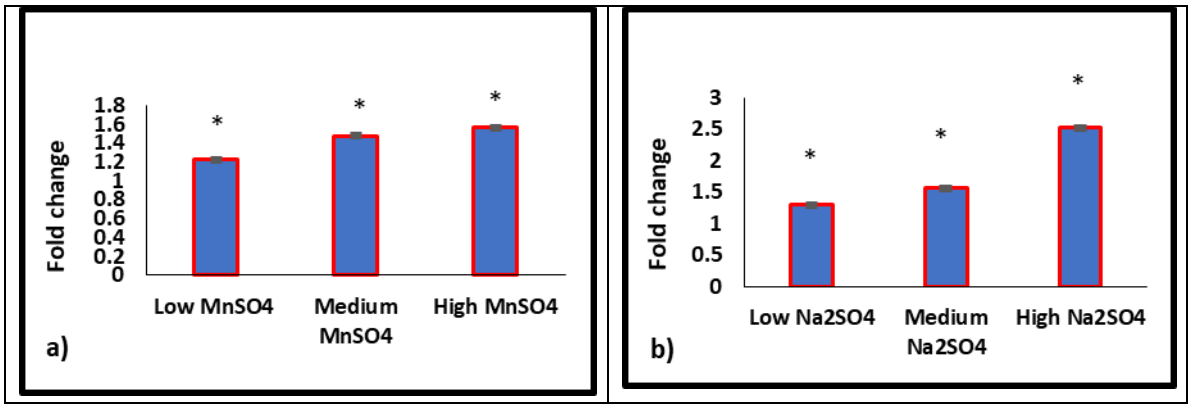

Figure 4. a) Differential gene expression of the IGF-1 gene of liver tissue in the low, Medium, and high concentrations of MnSO4 versus control groups. b) Differential gene expression of the IGF-1 gene of liver in the low, Medium, and high concentrations of Na2SO4 versus control groups. Fold change between treated group vs control group ± standard deviation, was used to present the gene expression, (*) mean the significance difference between treated and control group, where n = 7 and $p \leq 0.05$.

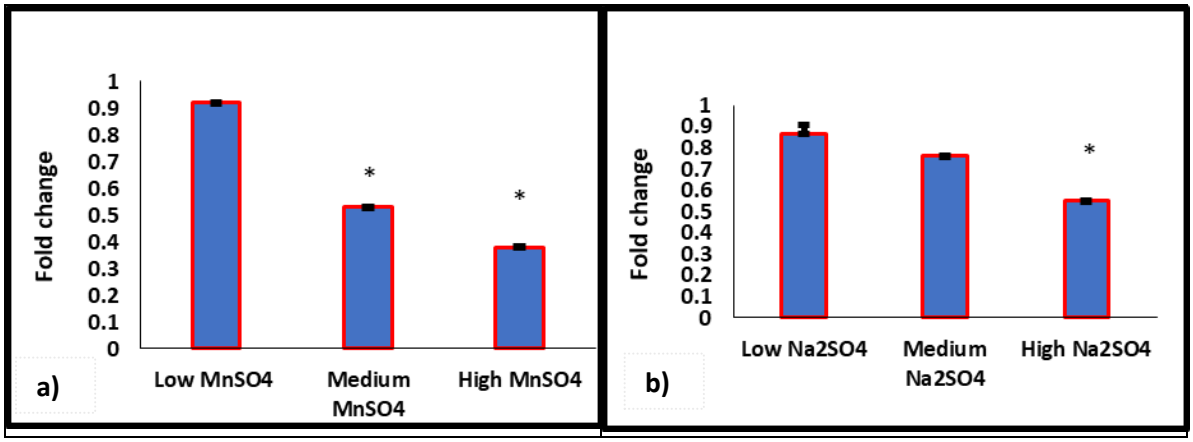

Figure 5. a) Differential gene expression of the IGF-1 gene of muscle tissue in the low, Medium, and high concentrations of MnSO4 versus control groups. b) Differential gene expression of the IGF-1 gene of muscle tissue in the low, Medium, and high concentrations of Na2SO4 versus control groups. Fold change between treated group vs control group ± standard deviation, was used to present the gene expression, (*) mean the significance difference between treated and control group, where n = 7 and $p \leq 0.05$.

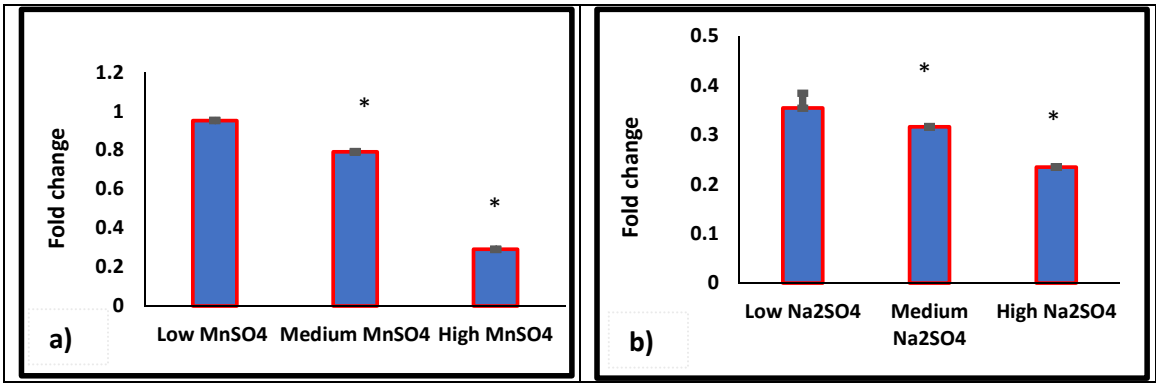

Figure 6. a) Differential gene expression of the MyoD gene of muscle tissue in the low, Medium, and high concentrations of $MnSO_4$ versus control groups. b) Differential gene expression of the MyoD gene of muscle tissue in the low, Medium, and high concentrations of Na_2SO_4 versus control groups. Fold change between treated group vs control group ± standard deviation, was used to present the gene expression, (*) mean the significance difference between treated and control group, where n = 7 and $p \leq 0.05$.

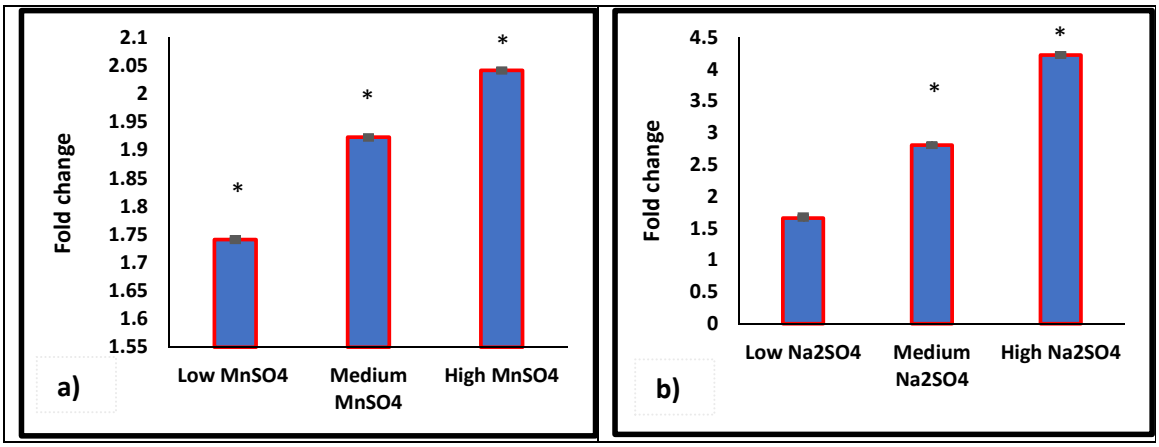

Figure 7. a) Differential gene expression of the MURF2 gene of muscle tissue in the low, Medium, and high concentrations of $MnSO_4$ versus control groups. b) Differential gene expression of the MURF2 gene of muscle in the low, Medium, and high concentrations of Na_2SO_4 versus control groups. Fold change between treated group vs control group ± standard deviation, was used to present the gene expression, (*) mean the significance difference between treated and control group, where n = 7 and $p \leq 0.05$.

4. DISCUSSION

4.1.Histopathological examination

Degeneration is considered to be an early stage of cell deterioration due to toxic pollutants that are entered the cell [55]. At this stage the cell can be recovered when the exposure to these pollutants is stopped. On the other hand, Hydropic degeneration reaches a degenerative stage where the cell cytoplasm contains water because of degeneration of the cell membrane. The presence of this water in the cytoplasm can decrease the oxidative phosphorylation, that leads to a decline in ATP supply which makes depletion in Na pump work [48]. It was reported previously for the liver of the tilapia exposed to a chemical toxin to have hepatic vacuolated cytoplasm that reflects the main function of liver organ in detoxification [47]. It was observed that hepatocyte degeneration and transformation to masses of eosin without nuclei or deeply stained

pyknotic nuclei in fish exposed to low dose of Mn and medium doses of Na and Mn salts. It was recorded in previous study on Labeo rohita fish that exposed one week to chromium exposure to have significant liver alterations including necrosis, vein dilation, blood vessel congestion, hepatocyte degeneration, presence of melano-macrophage centers, pigmentation changes, and hemorrhaging [19]. It was observed for fish exposed to different metals to have liver exhibited disorganization of hepatic cells, hypertrophy of hepatocytes, severe degradation of the liver parenchyma, and necrosis [4]. Hepatic fibrosis resulted from increasing cell damage and was accompanied by dilation of the bile duct (bile duct hyperplasia) as reported for medaka fish [14].

Gills are used to give indication for studying the environmental impacts and the water quality [61]. The histopathological changes from normal case to edema and lesion resulted from the exposure to the low and medium concentration of Na salts that was compatible with the results reported for the mosquito fish exposed to mercuryII [33]. It was recorded for the yellowfin seabream exposed to sublethal concentration of $HgCl_2$ to have gills with filament epithelium thinking and shortened or disappeared lamellar epithelium and may extend to edema [28]. Exposure to high concentration of Mn salts resulted in hyperplasia that leads to fusion of the secondary lamellae. Epithelial lining of Prochilodus lineatus gills and other fishes exposed to 7 days in disturbed urban stream, other metal contaminants, respectively, recorded epithelial lifting and fusion of lamellae [17, 45 and 51]. The fish gill damage and some changes in the blood vessels may occur as a result of exposure to high concentration of Na_2SO_4 of $MnSO_4$. In addition, it caused pillar cell injury and therefore the blood flow inside the lamellae was forced to increase, which leads to blood congestion. These findings are well in line with previous studies [33, 57].

The exposure to low Na_2SO_4 concentration revealed edema and mild mononuclear inflammatory cellular aggregation in muscle tissue. On the other hand, muscle exposed to low $MnSO_4$ concentration showed hyaline degeneration in addition to muscle with intermuscular and perivascular edema e necrosis in the muscle tissue. previous studies show that different stressors as infection can cause severe necrosis accompanied by oedema [44]. Melanomacrophage cells aggregations with necrosis in addition to a few inflammatory cells were found in the examined fish muscles exposed to the medium Na_2SO_4 or $MnSO_4$ concentrations. Similar changes were reported for fish muscle exposed to high concentration of Na_2SO_4 or $MnSO_4$. In addition, fibrous connective tissue may replace the necrotic areas with infiltration and presence of mononuclear inflammatory cells, followed by massive destruction of muscles. It was reported previously for muscle tissue of the Ctenopharyngodon idella exposed to 6.83 ppm copper for 7 days to have necrosis, oedema, and degeneration in muscle fibers. After longer exposure, this led to the destruction of muscle bundles that finally cause the muscle atrophy [67]. It was observed in the muscle tissue of Oreochromis niloticus that was exposed to aqueous and

ethanolic extracts of Ipomoea aquatica leaf, to have mild lesions, necrosis, inflammation and finally the muscle degeneration [10].

4.2. RT-PCR

Results on gene expression can reveal biological instances related to fish and reflect whether fish have been exposed to pollutants. Thus, qRT-PCR was used to investigate the interacting impact of $MnSO_4$ and Na_2SO_4 residues on the expression level of the IGF-1, MyoD, and MURF2 genes. The upregulation of expression of IGF-1 in liver tissues resulted from the exposure to the low, medium , and high concentration of $MnSO_4$ or Na_2SO_4 indicate their negative effect on fish immune system. The IGF-1 gene in liver was measured to be 1.23, 1.47, 1.56-folds in fish groups exposed to low, medium, and high dose of $MnSO_4$, respectively, compared to the control group. Additionally, it was reported expression values for low, medium ,and high dose of Na_2SO_4 to be 1.3, 1.56, and 2.53 folds, respectively, relative to the control group. Pesticides were reported to exert stimulation to the thyroid hormones to induce the expression of hepatic IGF-1 [63]. On the other hand, the significant decreased expression of the IGF-1 at ($p \leq 0.05$) in muscle tissues exposed to increased concentration of $MnSO_4$ or Na_2SO_4 gives an indication of the role of increased concentration of $MnSO_4$ or Na_2SO_4 on the muscle atrophy. Previously, it was reported that the increased concentration of pollutants as pesticide in muscle tissue resulted in the downregulation of IGF-1 in muscle tissue of cultured Mugil capito [23]. MyoD gene was significantly downregulated ($p \leq 0.05$) in all tested groups of $MnSO_4$ and Na_2SO_4, compared to the control group. The expression decreased with increasing the concentration of the $MnSO_4$ or Na_2SO_4. Previous studies reported that the expression of MyoD is an important upstream transcriptional factor for myogenesis. The increase of its expression was reported in the increase of myoblasts proliferation [68]. Previous study on stringing catfish reported muscle atrophy resulted from exposure to heavy metal pollutants. It was revealed that the muscle is like the gills are in close contact with the pollutants in the surrounding ecosystem. MuRF2 gene is one of the MuRF genes that was described to be ubiquitin E3 ligases. It was reported to have different regulatory mechanisms to muscle degradation [11]. The expressions on MnRF2 gene of goldfish were significantly upregulated ($p \leq 0.05$) in the different studied doses relative to the control group. It was recorded to be 1.74, 1.92, and 2.04 folds in fish groups exposed to the Low, medium ,and high doses of $MnSO_4$, respectively, relative to the control group. In addition, the highest expression value was measured in fish exposed to the high dose of Na_2SO_4 that was 4.2 fold relative to the control group. MuRF2 gene was reported to play a role in rainbow trout skeletal muscle degradation [76]**.**

CONCLUSION

The findings of this study reveal the chronic sublethal effects of water contamination with Na_2SO_4 and $MnSO_4$, particularly highlighting the histological changes in gill, liver, and muscle tissues. The study observed distinct histological alterations in these tissues

when exposed to varying doses of Na_2SO_4 and $MnSO_4$. These results underscore the significance of monitoring histological changes and the expression of specific genes involved in muscle proliferation and degradation. Such genes can serve as biomarkers for muscle exposure to different environmental stressors and can be valuable tools in pollution monitoring programs within aquatic ecosystems. This study contributes to the literature by providing insights into the comparative impacts of Na_2SO_4 and $MnSO_4$ on fish histology and genetic expression, thereby offering new potential biomarkers for assessing environmental stress in aquatic life.

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ETHICAL STATEMENT

The experiment was approved by the institutional ethics committee at faculty of science Benha university, protocol No. (BUFS-REC-2024-112Zoo)

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AUTHOR CONTRIBUTIONS

Walaa Shaalan designed the experiment. Walaa Shaalan and Axel Mosig analyze the data and writing the manuscript. Aya Eshaer has conducted the experiment and made sampling. All authors read and accept the submitted manuscript.

CONFLICT OF INTEREST

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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