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# Seasonal assessment of some heavy metal pollution and Metallothionein gene expression in cultured *Oreochromis niloticus*

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## Abstract

**Background:** The present study was planned to estimate the heavy metal concentrations in water samples and fish tissue residue (liver and muscle) of cultured *Oreochromis niloticus* as well as metallothionein (MT) gene expression in fish liver. Fish samples were collected from different private fish cultures in Kafr El-Sheikh governorate during April 2018–April 2019 in order to assess the public health risks associated with consuming cultured fish. Therefore, we investigated the concentrations of four metal elements (cadmium [Cd], copper [Cu], lead [Pb], and zinc [Zn]) in both fish tissues (muscle and liver) and water samples during the four seasons. Other water parameters (pH value, chemical oxygen demand, dissolved oxygen, alkalinity, total hardness, ammonia, and nitrite) were also determined.

**Results:** NH<sub>3</sub> values were above recommended limits mostly along the year. The trend of metal mean contents found in the fish were in decreasing order of Zn > Cu > Pb > Cd, and the liver showed greater accumulation than muscle. The highest amounts of metals accumulated in fish liver and muscle were recorded in winter and autumn, respectively, while the lowest amount was recorded in summer. Regarding fish muscle which is the edible part consumed by human, the concentration of studied metals was within the safe limits for seafood except for Pb. MT showed a significant high level in response to metals accumulated in fish liver. A positive correlation occurred between MT levels and Cu and Pb concentrations across different seasons.

**Conclusion:** In conclusion, MT expression levels seem to be sensitive to the heavy metals in natural habitats making it a powerful biomarker of heavy metals pollution in fish cultures. In addition, cultured fish in this study could be unfit for human consumption due to a high level of Pb in the edible part of fish. Therefore, greater attention should be set to Pb sources accumulated in cultured fish in Kafr El-Sheikh governorate.

**Keywords:** Metallothionein, Gene expression, Heavy metals, *Oreochromis niloticus*, Liver, Muscle

## Introduction

Metal pollution is an important worldwide problem, which is growing at an alarming rate. Despite progress made in environmental waste management, heavy metals still pose immense health hazards to humans and biota. Unlike other classes of pollutants, which can be biodegraded and destroyed, metals are non-biodegradable (Wepener et al. 2001) and can neither be created nor destroyed. An accelerated release of heavy metals into the aquatic environment poses serious water pollution problems because of their toxicity (Tulasi et al. 1989),

persistence, and bioaccumulation in food chains (Rishi and Jain 1998). Heavy metal accumulation in the environment could be because of acid mine drainage, industrial emissions, traffic, domestic sewage, storm water, atmospheric deposition, and building materials (Xia et al. 2011). In developing countries, the rapid increase in domestic, agricultural, and industrial activities add heavy metals loads in the air, water, and soil (Solaraj et al. 2010). The accumulation of heavy metals in fish gill, muscle, and liver has a great attention because of the potential toxic effects of such substances not only on fish, but also on human consumption (Burger and Gochfeld 2005). So, heavy metal load in fish has become an important worldwide threat, not only for fish but also for human as a result of the health risks associated with fish consumption (Begum et al. 2013).

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Metallothioneins (MTs) are a family of low molecular weight cytosolic proteins that contain highly conserved cysteinyl residues. These residues allow MT to bind, transport, and store various essential (Zn and Cu) and non-essential (Cd and Hg, mercury) heavy metals via thiolate bonding (Isani and Carpenè 2014). The change in the expression level of metallothionein gene in fish is used as a biomarker for heavy metal pollution in the ecosystem (Struve et al. 2005). MT also is considered as an indicator for degradation of environmental conditions and specific measures of the toxic, carcinogenic, and mutagenic compounds in the biological materials (Verlecar et al. 2006).

Therefore, our aim to estimate the heavy metal concentration in water samples and fish tissues (liver and muscle) of cultured *Oreochromis niloticus* as well as MT gene expression in fish liver in order to assess the public health risks associated with consuming cultured fish.

## Methods

### Study area

Random fish samples (200) were collected from private fish cultures in Kafr El-Sheikh governorate, Egypt, along the four seasons (autumn, winter, spring, and summer).

### Sampling collection and preparation

#### Water sample and analysis

Twelve water samples were collected during the study period (April 2018–April 2019). One liter of water samples at each pond was placed in polyethylene bottles previously washed with acid (0.01 N HNO<sub>3</sub>) and rinsed by distilled water, then placed in a cooler at 4 °C and transferred to the lab for further analysis.

#### Physico-chemical analysis of water samples

The investigated physico-chemical analysis was pH, chemical oxygen demand (COD), dissolved oxygen, alkalinity, total hardness, total kjeldahl nitrogen (TKN), ammonia (NH<sub>3</sub>), and nitrite (NO<sub>2</sub>). Detection of some heavy metals (Cd, Cu, Pb, and Zn) was done using flame atomic absorption spectrophotometer. All analyses were carried out according to the American Public Health Association for the Examination of Water and Wastewater (APHA 2012).

#### Heavy metal residues

Liver and muscle tissues were taken, then placed in polyethylene bags and stored at –20 °C until analysis. About 1 g from previously oven-dried muscle or liver tissues was ignited and digested with concentrated HNO<sub>3</sub> and HCl according to procedures recommended by AOAC (2005) using Flame Atomic Absorption Spectrophotometer (Thermo Electron Corporation S Series AA Spectrometer).

#### Metal pollution index (MPI)

The metal pollution index (MPI) was used to compare the total metal contents of muscle and liver of the studied fish in different year seasons. MPI was calculated with the formula:  $MPI = (M1 \times M2 \times M3 \times \dots \times Mn)^{1/n}$ , where Mn is the concentration of metal expressed in microgram per gram of wet weight, according to Usero et al. (1997).

#### Metallothionein gene (MT) expression analysis

##### RNA extraction and cDNA synthesis

Total RNA (ribonucleic acid) was extracted from liver tissue samples using Easy red total RNA extraction kit (Intronbio, Korea) according to the manufacturer's instructions. RNA was run on 1.5% formaldehyde denaturing agarose gel to assess its integrity. RNA purity and concentration were measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNase-free DNase kit (Promega) was used to remove any DNA (deoxyribonucleic acid) contamination from extracted RNA. DNase-treated RNA was reverse-transcribed into First-strand cDNA (complementary DNA) using HiSenScript™ cDNA kit (Intronbio, Korea) in a 20 µl reaction containing 1000 ng total RNA.

##### Real-time PCR analysis

Metallothionein gene, as a biomarker for water pollution with heavy metals, and beta actin (β-Actin), as internal control, were used in the present study; primers were obtained from Macrogen (Grand Island, NY), and sequences are shown in Table 1. TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics) was used to perform qRT-PCR (polymerase chain reaction) with 1 µl cDNA (total 50 ng RNA) in a 20 µl reaction mixture using Stratagene Mx3000P Real-Time PCR System (Agilent Technologies, USA). PCR conditions included 15 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, at 60 °C for 15 s, and 72 °C for 30 s. Melting curve analyses were performed following the end of each reaction to confirm specific PCR products. Metallothionein gene expression levels normalized to β-actin were calculated using the 2<sup>-ΔΔCt</sup> method (Livak and Schmittgen 2001).

##### Statistical analysis

The results were represented as mean ± standard error (SE) values. Statistical analysis of data was carried out using SPSS 14 software package. Data analysis was done using the one-way analysis of variance (ANOVA) with post hoc Tukey HSD analysis to compare the data among seasons at the level of significance ≤ 0.05. Correlation between hepatic MT expression levels and metal concentrations in fish across different seasons were evaluated using the Pearson correlation coefficient.

**Table 1** Name, accession number, primer sequence, and product size for genes used for real-time PCR analysis

Gene	Accession number	Nucleotide sequence 5'-3'	Size of PCR product (bp)
Metallothionein	XM_003447045.5	CTGCAACTGCCGAGGA GGTGTCGCATGTCTTT CCTT	136
$\beta$ -Actin	XM_003443127.5	TGTGTGGTGTGTGGTT GTTTTG CAGCAAGCAGGAGTAC GATGAG	136

## Results

### Water parameters and heavy metal concentration

Seasonal variations in water parameter and heavy metal concentrations are presented in Table 2 in reference to the permissible limits of water parameter and heavy metal concentrations (mg/l): pH 7–8.5, COD  $\leq$  10, D.O  $\geq$  5, alkalinity 20–150, NH<sub>3</sub>  $\leq$  0.5, Cd  $\leq$  0.003, Cu  $\leq$  1, Pb  $\leq$  0.5, and Zn  $\leq$  2 (Egyptian Environmental Law 48-decision 92, 2013). pH did not vary noticeably between summer, spring, and autumn, but is higher in winter (8.03). A detectable elevation in chemical oxygen demand (COD) was recorded in summer (80 mg/l). Autumn showed the highest nitrite (NO<sub>2</sub>) concentration (1.2 mg/l) compared with other seasons. Ammonia exceeded the permissible limits in all seasons, in summer (4.8 mg/l), spring (3.6 mg/l), autumn (3.2 mg/l), and winter (1.1 mg/l). The pattern of alkalinity and total hardness in different seasons was in decreasing order as follow: autumn > summer > spring > winter. The highest value of dissolved oxygen (DO) was recorded in winter (5.05 mg/l), and the lowest value recorded in summer (4.3 mg/l). The maximum values of total kjeldahl nitrogen were recorded in summer (15.7 mg/l). All heavy

metal concentrations in water were under permissible limits.

### Heavy metals in muscle tissues

As shown in Table 3, the results revealed that the highest levels of Zn were recorded in autumn (50  $\mu$ g/g) that exceeded the permissible limits and the least levels were recorded in summer (10.7  $\mu$ g/g). Cd showed extremely low levels ( $<$  0.05  $\mu$ g/g) across the four seasons. The highest levels of Pb and Cu were recorded in autumn followed by winter and spring (1.8, 1.2, and 1.15  $\mu$ g/g, respectively). Pb levels were above the permissible limits (except for summer) while Cu levels were below limits across the four seasons.

### Heavy metals in liver tissues

As shown in Table 4, Zn showed low levels across seasons except for autumn, where the levels (60.1  $\mu$ g/g) exceeded permissible limits. It was noticeably that Pb levels exceeded permissible limits across seasons (except for summer), where the highest levels for Pb were recorded in autumn (2.1  $\mu$ g/g) followed by spring and winter (1.2 and 1.6  $\mu$ g/g, respectively). Cu levels were markedly high and exceeded permissible limits in spring (68.8  $\mu$ g/g) followed by winter (36.3  $\mu$ g/g), while they were below limits in summer and autumn (15.8 and 28.6  $\mu$ g/g). Cd showed extremely low levels, except in winter (5.6  $\mu$ g/g) which exceeded permissible limits.

### MPI in liver and muscle tissue

As revealed in Fig. 1, the highest metal pollution index (MPI) in the liver was that for fishes collected in winter and in muscle were in autumn, while the least MPI in both tissues was that for fishes collected in summer. On

**Table 2** Water parameters (mg/l) and seasonal variations of heavy metals concentrations (mg/l) in three water samples per season of fish cultures in Kafr El-Sheikh governorate

Water parameter/heavy metal	Winter	Spring	Autumn	Summer	Permissible limits
pH value	8.03	7.33	7.62	7.5	7–8.5
Temperature	18 + 0.5 °C	20 + 0.5 °C	22 + 1 °C	25 + 1 °C	
Chemical oxygen demand (COD) (mg/l)	10	20	30	35	$\leq$ 10
Dissolved oxygen (DO) (mg/l)	5.05	4.59	4.6	4.3	$\geq$ 5
Alkalinity (mg/l)	140	120	110	90	20–150
Total hardness (mg/l)	70	90	175	230	30–180 (Santhosh and Singh 2007)
Ammonia (NH <sub>3</sub> ) (mg/l)	1.1	3.6	3.2	4.8	0.5
Nitrite (NO <sub>2</sub> ) (mg/l)	0.42	0.3	0.4	0.3	$<$ 0.2 (OATA 2008)
Cadmium	$<$ 0.001	$<$ 0.001	$<$ 0.001	$<$ 0.001	$\leq$ 0.003
Copper	$<$ 0.01	$<$ 0.01	$<$ 0.01	$<$ 0.01	$\leq$ 1
Lead	$<$ 0.001	$<$ 0.001	$<$ 0.001	$<$ 0.001	$\leq$ 0.5
Zinc	$<$ 0.001	$<$ 0.001	$<$ 0.001	0.59	$\leq$ 2

Data represented as an average of three samples

**Table 3** Seasonal variations of heavy metals concentrations ( $\mu\text{g/g}$  dry weight) in muscle tissue of *Oreochromis niloticus*

Heavy metal	Winter	Spring	Autumn	Summer
Cadmium	< 0.05	< 0.05	< 0.05	< 0.05
Copper	1 $\pm$ 0.02 <sup>b</sup>	0.97 $\pm$ 0.05 <sup>b</sup>	2.2 $\pm$ 0.1 <sup>a</sup>	< 0.05
Lead	1.2 $\pm$ 0.08 <sup>b</sup>	1.15 $\pm$ 0.8 <sup>b</sup>	1.80.05 <sup>a</sup>	< 0.05
Zinc	17.9 $\pm$ 0.6 <sup>bc</sup>	21.4 $\pm$ 0.9 <sup>b</sup>	50 $\pm$ 2 <sup>a</sup>	10.7 $\pm$ 0.32 <sup>c</sup>

Data are shown as mean  $\pm$  SEM; (n=5); mean values with unlike superscript letters were significantly different ( $p \leq 0.05$ )

FAO (1983) limits in fresh muscle (mg/kg): Cd (0.5), Cu (30), Pb (0.5), and Zn (30)

EOS (1993) MPL limits in fish (mg/kg): Cd (0.5), Cu (20), Pb (2), and Zn (40)

the other hand, MPI in liver tissue was greatly higher than MPI in muscle.

### MT expression levels in the liver

The mRNA expression levels of MT gene relative to the  $\beta$ -actin gene were shown in Fig. 2, where the melting curves showed single and sharp peaks confirming the specificity of the primers and accuracy of results. The results from the Pearson correlation (Table 5) displayed a significant positive correlations ( $p < 0.05$ ) for MT-Cu and MT-Pb, but exceptional for MT-Zn.

Real-time PCR results (Fig. 3) also showed that liver tissue of *Oreochromis niloticus* displayed a significant increase ( $p < 0.05$ ) in MT expression levels during all seasons, except summer. The highest MT mRNA levels were recorded in spring and winter followed by autumn. Meanwhile, liver tissue of fish collected in summer showed the least MT mRNA levels.

### Discussion

According to the results, heavy metals in water of fish culture were found to be below detection limits. In addition, in all seasons, the pH of the water was measured to be slightly alkaline which is considered as unfavorable conditions for the solubility of metals so, it was not expected to find metals in water samples, while the heavy metals were deposited in the sediments and accumulated in fishes (Ayas et al. 2007).

**Table 4** Seasonal variations of heavy metals concentrations ( $\mu\text{g/g}$  dry weight) in liver tissue of *Oreochromis niloticus*

Heavy metal	Winter	Spring	Autumn	Summer
Cadmium	5.6 $\pm$ 0.23 <sup>a</sup>	< 0.05	< 0.05	< 0.05
Copper	36.3 $\pm$ 1.3 <sup>b</sup>	68.8 $\pm$ 1.9 <sup>a</sup>	28.6 $\pm$ 0.17 <sup>b</sup>	15.8 $\pm$ 2.6 <sup>c</sup>
Lead	1.6 $\pm$ 0.01 <sup>b</sup>	1.2 $\pm$ 0.05 <sup>c</sup>	2.1 $\pm$ 0.006 <sup>a</sup>	< 0.05
Zinc	3.8 $\pm$ 0.01 <sup>c</sup>	18.8 $\pm$ 0.3 <sup>b</sup>	60.1 $\pm$ 1.5 <sup>a</sup>	14.8 $\pm$ 1.7 <sup>c</sup>

Data shown as mean  $\pm$  SEM (n = 6), mean values with unlike superscript letters were significantly different ( $p \leq 0.05$ )

FAO (1983) limits in fresh muscle (mg/Kg): Cd (0.5), Cu (30), Pb (0.5) and Zn (30)

EOS (1993) MPL limits in fish (mg/Kg): Cd (0.5), Cu (20), Pb (2) and Zn (40)

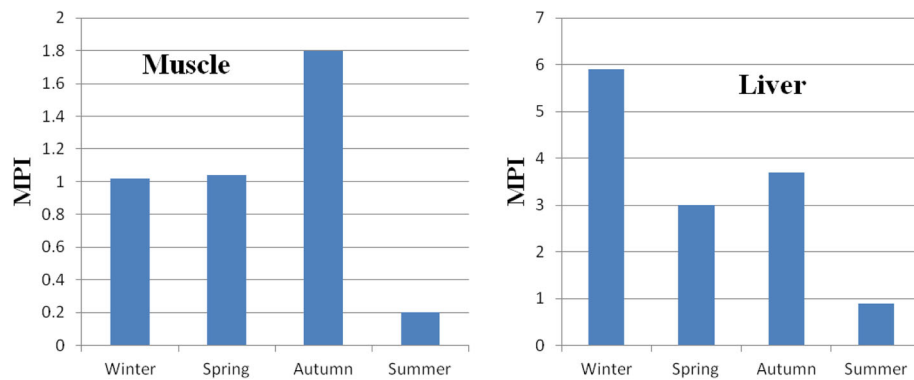
Also, concerning this aspect, He et al. 2012 found that the alkaline environment favors heavy metal accumulation in sediment. Water concentration of ammonia exceeds the permissible limits, and this may be attributed to the use of agricultural drainage water. In addition, the elevation of toxic unionized ammonia could explain the elevation of toxic nitrite, which is recorded in this study.

The elevated level of COD and the decreased level may be attributed to high organic matter, which can be deduced from the recorded increase in both unionized toxic ammonia and nitrite. According to the present study, it is found that total hardness values in summer were higher than those in winter. The increasing total hardness values at summer may be ascribed to the increase of salinity (Ali et al. 2016) as a result of evaporation of water due to the high temperature during this season.

Unionized ammonia (NH<sub>3</sub>) is extremely toxic substance to aquatic animals that retard fish growth and may increase their susceptibility to other unfavorable conditions in fish culture system (Boyed and Tucker, 1992). The concentration of ammonia in water exceeded the permissible limits. According to our results, heavy metal accumulation is generally more evident in liver than in muscle, as active metabolite organ accumulates higher amounts of heavy metal than muscle (Ben Salem et al. 2014). That in agreement with measurements obtained by Bayomy et al. (2015). The liver is known to be the active central site of metal uptake and storage, and it plays an important role in detoxification and excretion (Kim and Kang 2004). Moreover, the lowest concentrations of metals found in the muscle than in the liver may be due to the little blood supply to the muscular tissues (Kalkan et al. 2015). Thus, the liver is the most recommended tissue as an environmental indicator of water pollution (Henry et al. 2004).

As the muscle is the main edible part of fish and can directly influence human health, so heavy metal assessment in muscle is essential given that the flesh is destined for human consumption. The essential metals, such as zinc and copper, are in higher values, presumably due to their function as co-factors for the activation of a number of enzymes and regulated to maintain a certain homeostatic status in fish (Kumar et al. 2011). Although they are needed for the organisms' growth, they will be toxic if their concentrations exceed the allowable limit. In the present study, Cu and Zn (except in autumn) concentrations in fish muscle are below the permissible limits of the FAO, while Cu concentrations exceeded these limits in liver tissue in spring and winter.

Pb and Cd are nonessential elements, described as xenobiotic, for living organism. Pb possesses various effects such as neuro- and nephro-toxicity, rapid behavioral malfunction, and decreases the growth, metabolism, and

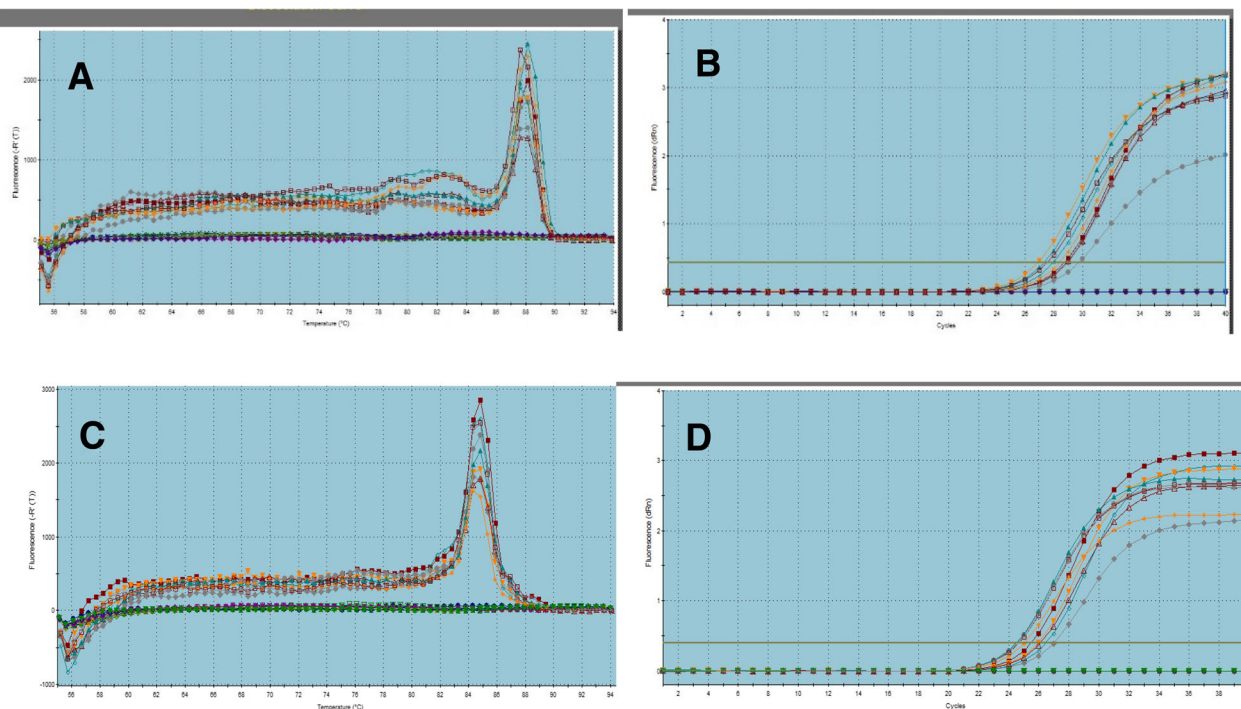


**Fig. 1** Seasonal variations of metal pollution indices (MPI) in liver and muscle tissues of *Oreochromis niloticus*

survival rate, alteration of social behavior in some mammals (Garcia-Leston et al. 2010). According to our results, Pb was found to exceed the limits in both fish tissues (liver and muscle), coincide with Rashed (2001) who found that elevated Pb level in fishes obtained from freshwater ecosystem affected by extended agriculture, poultry farms, textile, industrial, and other activities. However, regarded sediments could be the major sources of Pb contamination. Excessive Cd exposure may give rise to renal, pulmonary, hepatic, skeletal, and reproductive toxicity effects and cancer (Rajeshkumar et al. 2018). Although Cd concentration in fish muscle in our study was found to be

less than limits, a great attention should be taken as Cd may accumulate in humans via food chain magnification (Ben Salem et al. 2014).

According to the results obtained here, metal concentrations in fish liver exhibited seasonal variations in which higher metal pollution index (MPI) was that for fish collected in winter followed by autumn and spring and the lowest MPI was for fish collected in summer. These results are supported by El-Batrawy et al. (2018) and Bayomy et al. (2015), who recorded that average metal concentration in *Oreochromis niloticus* tissues in winter is higher than in summer. Also, Abd-El-Khalek et al. (2012)



**Fig. 2** Melting curve analysis showed a single peak for MT gene (a) and  $\beta$ -actin (c) gene. Representative amplification plot for MT gene (b) and  $\beta$ -actin gene (d)

**Table 5** Pearson correlation between hepatic MT expression levels and metal concentrations in fish in different seasons

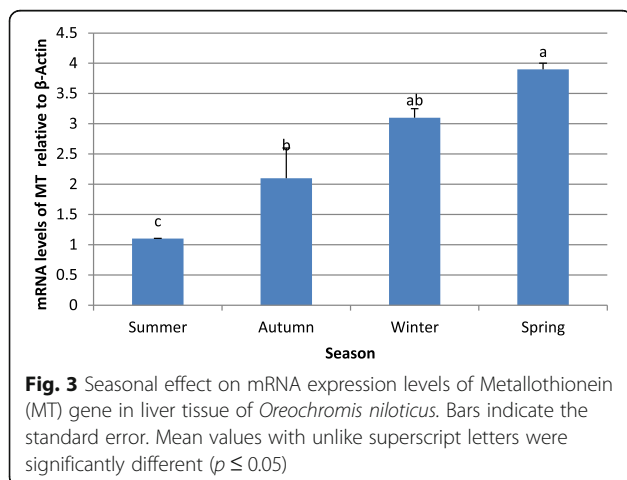
Metal	Correlation coefficient with MT expression	<i>p</i> value
Pb	0.609**	< 0.001
Zn	0.037	0.87
Cu	0.752**	< 0.001

\*\*Correlation is significant at the 0.01 level (two-tailed)

reported higher heavy metal accumulation in autumn than in summer. Conversely, Bahnasawy et al. (2009) demonstrated that heavy metal levels in summer were higher than in winter. Seasonal variations can be attributed to the increase or decrease of drainage water discharged into the farm (Bahnasawy et al. 2009).

On the other hand, different season-dependent conditions such as salinity, pH and hardness, dietary factors, and growth and reproductive cycles are also influential on metal accumulation in fish tissues (Yilmaz et al. 2010 and Saei-Dehkordi and Fallah 2011). A negative relation between hardness and alkalinity and heavy metal concentration in fish tissue. This result is in accordance with a study by Malcolm (1995), who revealed that at higher values of these variables the metals bind to form hydroxide and carbonate complexes which are considered less toxic to fish rather than the metal ions.

MT has been suggested to be a suitable biochemical indicator and its expression changes in fishes used as a biomarker for detecting the metal pollution in water. Although MT induction in fish is known to be high in tissues involved in metal uptake, storage and excretion, such as gills, liver, kidney, intestine, and muscle (Min et al. 2016), the highest concentration of MT was induced in the liver (Sevcikova et al. 2013). Liver measurement remains the most used since it reflects early exposure to contaminants because the liver is the main detoxification organ of the body (Jakimovska et al. 2011). Therefore, in the present study, the mRNA levels were evaluated in the liver of the



collected fish and since MT are involved in metal storage and detoxification processes, it is relevant to compare collected fish exposed to heavy metal pollution in their environment with fish living in sites considered to be unpolluted. MT normally occurs in tissues in trace amounts; however, exposure to metals induces its formation (Rajeshkumar et al. 2018). Our results reflected a significant increase in the mRNA levels of MT in liver tissue of fish collected from farms relative to the control that may be due to the heavy metals accumulation.

A few reports were conducted in Egypt regarding the use of MT gene expression in fish as a biomarker for detecting the metal pollution, such as Abumourad et al. (2014) who reported that Metallothionein was significantly highly expressed in liver tissue in response to heavy metal pollution. MT transcript and protein are induced in fish by a variety of bivalent metals, including cadmium, zinc, copper, and lead (Carginale et al. 1998; Riggio et al. 2003).

The elevation of MT concentration in fishes by zinc exposure coincides with those of Zhang and Wang (2005) and that occurs only in higher exposure of Zn. However, Butler and Roesijadi (2001) verified that Cu and Cd are the most inducers of MT than Zn. According to our results, Cu and Pb concentration differences were found to be significantly correlated with hepatic MT expression. This emphasized the role of MT as a biomarker for exposure of Cu and Pb in *Oreochromis niloticus*. In agreement with Juhari (2014), a significant increase in MT mRNA levels in *Oreochromis niloticus* induced by Cu and Pb.

As proposed previously, the binding affinity of MT for Cu ions is bigger than other metal ions (Sakulsak 2012). As well Boeck et al. (2003) found that MT induction was partially attributed to the Cu regulation process and MTs may be in favor of Cu storage. Additionally, a positive correlation between the extent of Cu accumulation and MT concentration in black sea bream (Dang et al. 2009). It was also observed an increase in hepatic Cu conjugated to MT proteins in perch exposed to Cu (Wang et al. 2014).

## Conclusion

Our results indicated that MT gene expression seems to be induced by metal pollution especially Cu and Pb; therefore, the appropriateness of MT as a marker of metal exposure in fish may be confident under field conditions. The results proved that the heavy metal accumulation in fish may vary depending on changes in the environmental conditions, that means that seasonal sources of heavy metals in fish is alarming as it will directly come into the human food chain and will affect the human health. In addition, cultured fish in this study could be unfit for human consumption due to a high

level of Pb in the edible part of fish. Therefore, greater attention should be set to Pb sources accumulated in cultured fish in Kafr El-Sheikh governorate.

#### Abbreviations

ANOVA: Analysis of variance; CD: Cadmium; COD: Chemical oxygen demand; Cu: Copper; DNA: Deoxyribonucleic acid; DO: Dissolved oxygen; ESO: Elder scrolls online; FAO: Food and Agriculture Organization; HCl: Hydrogen chloride; MPI: Metal pollution index; MT: Metallothionein; NH<sub>3</sub>: Ammonia; NO<sub>2</sub>: Nitrite; Pb: Lead; PCR: Polymerase chain reaction; RNA: Ribonucleic acid; TKN: Total kjeldahl nitrogen; Zn: Zinc

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#### Authors' contributions

SMG designed this work, participated in laboratory analysis, and wrote with critical revision of the manuscript. DMM participated in laboratory analysis and wrote the manuscript. MIH completed the sample collection in the field and laboratory analysis and wrote the manuscript, AA was involved in reviewing the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

The authors declare that all data and materials are available.

#### Ethics approval and consent to participate

Did not do the ethics process for this work. All authors declare consent to participate.

#### Consent for publication

All authors declare that this manuscript is consent for publication.

#### Competing interests

The authors declare that they have no competing interests.

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