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ORIGINAL ARTICLES

Heavy Metal Pollution and Metallothionein Expression: A Survey on Egyptian Tilapia Farms

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ABSTRACT

This study constitutes a survey on the Egyptian tilapia fish farms. In this study, tilapia fish (*Oreochromis niloticus*) were collected from three different fish farms in Egypt; Al-Abbasa, Kafr El-Sheikh and El-Fayoum to assess the heavy metal pollution. Cadmium (Cd), Copper (Cu), Zinc (Zn) and Lead (Pb) concentrations were measured in water and fish muscles from these studied areas. Liver enzymes, total protein levels were determined in fish serum and the expression of MT-gene was examined. Water and tissue concentrations of heavy metal recorded its highest values in Al-Abbasa followed by El-Fayoum then Kafr El-Sheikh fish farms. Analysis of GOT and GPT revealed altered values, a clear decrease in the total protein levels in association with high contamination by heavy metals was observed. Obvious increase in the expression of MT gene was observed in response to heavy metal pollution. In conclusion, this study revealed a clear heavy metal pollution in the studied Egyptian tilapia fish farms which was indicated by the high concentrations of heavy metals in water and fish tissues, altered liver enzymes; serum protein and MT gene expression.

Key words: Tilapia fish farms; heavy metals; Metallothionein and biochemical analyses

Introduction

Aquaculture has grown very rapidly and is presently undergoing rapid changes in response to pressures from globalization and growing demand for aquatic products in both developing and developed countries. In Egypt, during the past 25 years, different forms of fish culture have been developed, among them, earthen pond culture and rice-fish culture, which made popular by various organizations and are now widespread.

Rearing of fish is not only concerned with quantitative growth, but also with improving the quality of the product. It is necessary to have a sufficient supply of good quality water for the construction of ponds (Agoz *et al*., 2005). Commercial aquaculture facilities require abundant clean water with oxygen, pH and nutrient levels at a suitable level to support the farmed species (Wallace, 1993), Variations beyond acceptable ranges for these water parameters lead to stress, impaired health by disrupting physiological functions as ionic regulation, liver and kidney functions (Conte, 2004) and mortality. Thus, optimal fish health is best achieved by rearing fish in a good environment, with good nutrition, and a minimum stress.

The major benefit of farmed fish is that they provide a good and low-cost source of polyunsaturated fatty acids which can enhance cardiovascular health in humans. However, consumers have been surprised to find that several natural and man-made toxic substances are at higher concentrations in farmed than wild fish (Cole *et al.*, 2009). This situation is pronounced in Egypt, where Ministry of Irrigation prohibits the use of irrigation water in fish farms, and always recommends use of drainage water. According to the Egyptian Law No. 124/1983, only brackish and marine water, and infertile land that is not suitable for agriculture, can be used in aquaculture. Water supply should be restricted to water from lakes and drains, and the use of fresh (i.e. irrigation) water is prohibited.

Agricultural drainage waters containing high concentrations of different pesticides, fertilizers runoff and heavy metals (Alne-na-ei, 2003; Khallaf *et al.,* 2003; Tayel *et al*., 2007; Authman, 2008; Authman *et al*., 2008). Heavy metal pollution is one of the greatest national health problems with referring to peoples eating fish foods, it requires special and intense effort at all levels; individual, groups, national, and international. Heavy metals are inorganic chemicals that are non- biodegradable, cannot be metabolized and will not break down into harmless forms since they leave biological cycles very slowly. Elements such as cadmium, copper, lead and zinc are considered most dangerous in the ecotoxicological aspect (Golovanova, 2008). Metal ions can be incorporated into food chains and concentrated in aquatic organisms to a level that affects their physiological state and causes drastic environmental impact on all organisms. Such health risk may over shadow the cardiovascular benefits from the consumption of certain farmed fish, Moreover, aquaculture products are sometimes banned due to rejection of export consignments.

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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 transition metals via thiolate bonding (Dunn et al 1987). The change in the expression level of metalothionein gene in fish has been used as a biomarker for water pollution with heavy metals (Sturve et al., 2005). It also, considered as early warning for degradation of environmental quality and specific measures of the toxic, carcinogenic and mutagenic compounds in the biological materials (Verlecar *et al*, 2006).

Understanding of the relationships between Metallothionein (MT mRNA) gene expression and exposure to exogenous stresses will undoubtedly contribute to improve strategies for use of MT in assessing aquatic organism health. For this to be achieved there needs to be a better understanding of MT function and the dynamics of MT mRNA induction.

Because pollution pose probably the greatest threat to the success of intensive aquaculture worldwide and contribute to be the main impediment to aquaculture production, the present study investigates heavy metal pollution in association with metalothionine gene expression as a biomarkers for heavy metal pollution in three Egyptian tilapia farms; Al-Abbasa, Kafr El-Sheikh and El-Fayoum to build a data base help in the plane of improving aquaculture fish productivity.

Materials and methods

Studying areas:

Three fish farms Al-Abbasa fish farm, Sharkia, Kafr-El-Sheikh fish farm and (El-Fayoum fish farm were chosen during 2011 for the studying the status of aquaculture and heavy metal pollution (Fig. 1).

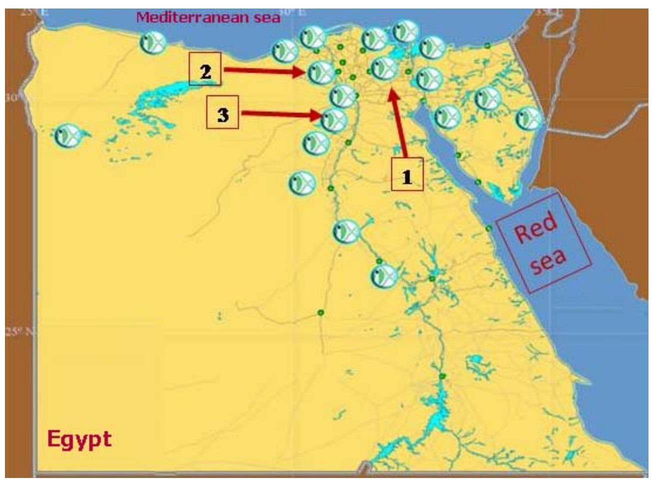

Fig. 1: Distribution of the main aquaculture production sites in Egypt showing the three studied locations (Google Egypt map, 2010). 1: Al-Abbasa ,Sharkia; 2: Kafr-El-Sheikh and 3: El Fayoum

Water samples and analysis:

A total of three water samples were collected from each studied pond for studying some physical and chemical characteristics of water; pH, temperature, salinity, dissolved oxygen and Ammonia (APHA,1998). Determination of some heavy metals (Cu, Zn, Cd and Pb) was carried out by flame atomic absorption Spectrophotometer according to APHA (1998). All metal concentrations were expressed in terms of µg / L dry weight.

Fish:

Random fish samples (50 samples) of *Oreochromis niloticus* were caught from the same locations of water samples.

3.1 Blood sampling:

Blood samples were taken from fish from the caudal vein by sterile syringe rinsed with heparin. Serum was separated by centrifugation of blood at 3000 rpm for 5 min and stored at -20^{o}C until analyses.

3.2 Biochemical analyses:

Total protein level in serum was determined according to Cannon *et al*. (1974). Activities of aspartate amninotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957). All previous analyses were carried out using kits supplied from Spectrum, Egypt.

3.3 Molecular study:

3.3.1 Total RNA Isolation:

Total RNA was extracted from livers of fish with Trizol reagent (Invitrogen, Carlsbad, CA, USA), then was purified using a spin column kit purchased from Fermentas life science Co. (Invitrogen Corporation) (Van Allen Way, Carlsbad, Canada) according to the manufacturer's instructions. RNA samples were treated with DNase I (Ambion, UK) to remove contaminating genomic DNA and repurified by spin column. Then RNA samples were stored in –80 o C until the process of reverse-transcriptase.

3.3.2 SMART cDNA synthesis and Semi- quantitative RT- PCR for tissue expression of MT gene:

*T*wo µg RNA was reverse transcribed with Revert Aid First Strand cDNA Synthesis Kit™ (purchased from Fermentas life science Co., Invitrogen Corporation, using hexanucleotides. The cDNA mixture was stored at −20 °C until its use in real-time PCR reaction.

3.3.3 Quantitation of MT by Real-time PCR:

Real-time PCR was performed by methods described previously by Zhang *et al*., (2008). Amplification and quantitation of cDNA were performed in the Applied Biosystems 7300 Real-Time PCR, The 18S rRNA served as an internal control for normalization. Each sample was run in triplicate and mean values were reported. Normalized gene expression values were obtained using Light Cycler Relative Quantification software. Relative gene copy numbers were derived using the formula 2ΔCT where ΔCT is the difference in amplification cycles required to detect amplification product from equal starting concentrations of fish liver RNA.

3.4 Heavy metals residues:

Muscle samples were separated, weighed and dried at 70ºC to constant weight. Samples were grinded and analyzed for heavy metals according to UNEP/FAO/IAEA/IOC (1984).

An exact weight of dry sample (triplicate, each of 0.5 g) was placed in Teflon vessel and 4 ml of nitric acid (AR- grade) was added. The vessels were tightly covered and allowed to predigest at room temperature over night. The digestion block was placed on a preheated hot plate at 80ºC for three hours. The samples were cooled at room temperature and then were transferred to 25 ml volumetric flask. All digested solutions were analyzed by Flame Atomic Absorption Spectrophotometer (Perkin-Elmer, Model 2380). All metal concentrations were expressed in terms of µg/kilogram dry weight.

4. Statistical analysis:

Descriptive statistical analysis (Mean±SD) was performed using a computer program of SPSS Inc. (version 17.0 for Windows).

Results:

Water analysis:

The data (Table 1) showed that Hydrogen ion concentrations (pH) recorded at El-Fayoum fish farm (8.3) was relatively higher degree than those detected in Al-Abbasa and Kafr-El-Sheikh farms. The salinity appeared in its highest value in El-Fayoum farm (18.7 PPM). Ammonia was also higher in El-Fayoum fish farm (2.21 mg/L) compared with the other two farms.

The obtained results of heavy metals in water samples showed some variations in their concentration among different fish farms as shown in Table 2. It is clear from the obtained results that zinc, copper and lead showed the highest values at Al-Abbasa farm (111.3, 33.7 and 32.1 µg/l, respectively) while cadmium showed its highest level in El-Fayoum farm (8.3 µg/l). In addition, the water of Kafr el sheik maintained generally lower metal contents than Al-Abbasa and El-Fayoum farm waters except for zinc concentration.

Table 1: The Physico-chemical parameters in the studied fish farms.

Parameter	Al-Abbasa	Kafr el sheikh	El -Fayoum
pH	6.95 ± 0.26	7.85 ± 0.39	8.3± 0.83
Temperature (°C)	22.00 ± 0.68	23.00 ± 0.12	25.00 ± 1.43
Salinity (PPM)	11.6 ±17.72	14.54 ±17.72	18.7± 0.81
Dissolved Oxygen (mg/L)	6.86 ± 0.32	7.82 ± 0.07	6.53 ± 3.12
Ammonia (mg/L)	1.27 ± 0.05	1.1± 0.92	2.21± 0.32

Data are represented as mean ± SD.

Heavy metal residues in muscles:

Muscles of Tilapia showed a pronounced high accumulation value of heavy metals (Table 2). Cadmium has the highest value in El-Fayoum followed by Kafr-El-Sheikh and finally Al-Abbasa fish. The highest zinc, lead and Copper contents in Tilapia muscles was recorded in Al-Abbasa farm with observation that Copper content was within permissible limit fluctuated between 0.27 and 2.17.

Table 2: Heavy metal concentration in water (µg/l) and fish muscle (µg/kg dry wt) samples from the studied locations.

Metal		Al-Abbasa	Kafr El-Sheikh	El-Fayoum	FAO, 1983
Zinc (Zn)	Water	111.3±0.77	96.3±1.67	66.4±3.63	
	Muscle	33.13 ± 7.4	28.37 ± 4.7	21.7 ± 0.8	30
Cadmium(Cd)	Water	4.9±1.54	4.1±0.87	8.3±1.26	
	Muscle	2.27 ± 2.02	2.33 ± 0.4	4.27 ± 0.4	0.5
Lead (Pb)	Water	32.1	17.9	28.2±4.23	
	Muscle	18.8 ± 11.3	6.27 ± 3.4	8.63 ± 2.4	0.5
Copper (Cu)	Water	33.7±2.71	27.8 ±5.2	31.6±4.23	
	Muscle	2.17 ± 1.8	0.6 ± 0.5	0.27±0.05	30

Data are represented as mean ± SD

Biochemical analysis:

It was noticed that the highest values of AST was pronounced in Kafr-El-Sheikh followed by Al-Abbasa and El-Fayoum respectively, where as the ALT was of highest value in Al-Abbasa followed by El-Fayoum and Kafr-El-Sheikh. The total protein level reached the highest value in El-Fayoum farm and the lower and comparable values were in Al-Abbasa and Kafr-elsheikh respectively (Table 3).

Table 3: Biochemical parameters in *Oreochromis niloticus* from the studied three locations.

Parameter	Al-Abbasa	El-Fayoum	Kafr elsheikh
AST (U/ml)	77.8±3.74	76.6±25.3	83.8±34.4
ALT (U/ml)	16.3± 2.27	14.5±9.2	14.3±9.5
Total Protein (g/dl)	0.8±0.33	1.78±0.46	0.77±0.34

Data are represented as mean ± SD

Metalothionine gene expression:

Using real time quantitative PCR assay, livers of tilapia cultured in the three farms under study revealed obvious higher expression of metallothionein gene in the three locations (Al-Abbasa, Kafr-El-sheikh and El-Fayoum) respectively compared to the internal control 18sRNA (Fig. 2).

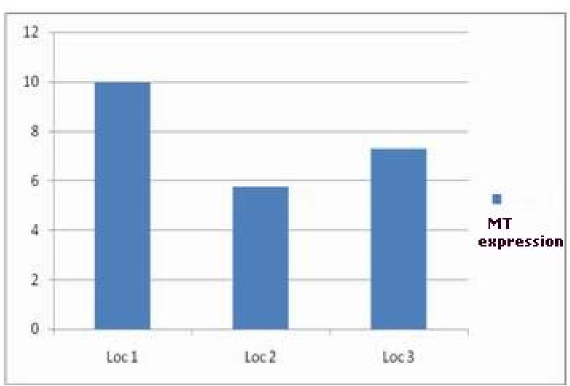

Fig. 2: Graphical analysis of Metalothioneine (MT) gene expression levels in *Oreochromis niloticus* liver from three locations (Al-Abbasa, El-Fayoum and Kafr-El-Sheikh respectivly). Data are expressed as mean ± standard deviation.

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Discussion:

Aquaculture is considered as one of the most important sources of animal protein production. Countries that have over population problems like Egypt have an increasing demand for protein production of fishes. So, realize the maximum yield of all useable resources for production food is vital. These attempts can be realized by increasing the area of cultured fish ponds and using the facilities of aquaculture developmental methods and different programs of fighting and controlling of diseases as it is the main impediment for aquaculture productivity.

Most environmental problems of concern today are attributed to the production and release of toxic chemicals capable of interacting with the environment and disrupting the ecosystem.

The current study was carried out on fish farms in three different locations: Al-Abbasa, Kafr El-Sheikh and El-Fayoum. In this investigation, water quality was analyzed as water quality is still an abstract concept for fish farming; the criteria behind it have never been adequately described. Hydrogen ion concentrations (pH) recorded at El-Fayoum fish farm (8.9) was relatively higher than that detected in Al-Abbasa and Kafr El-Sheikh (7.85 & 6.95) respectively. pH changes in surface water result from the interaction of various biotic and abiotic processes. The highest pH values at El-Fayoum fish farm which was characterized by high salinity also, the salinity contents at El-Fayoum were generally higher than other farms due to seepage from Lake Qarun as it is near to the hyper saline water.

From the heavy metal analysis of water and fish muscle samples in the three locations under investigation it was elucidated that location 1 (Al-Abbasa) showed higher concentrations of Zn, Cu and Pb compared to Kafr El-Sheikh and El-Fayoum (Location 2 & 3), where the higher concentrations of Cd appeared in El-Fayoum fish farm. Comparing the heavy metal concentrations with the standard water limits, Al-Abbasa and Kafr El-Sheikh farms showed high concentrations of Zn, Cd and Pb which exceeded the permissible limits indicated by FAO, 1983; in contrast to Cu concentration which was within the normal levels. The higher levels of Pb, Zn and Cu in Al-Abbasa fish farm may be explained as these farms depend on El-Ismailia canal which in turn receives its water carrying different industrial pollutants from Mostorod area, where many factories, especially battery factories, fertilizers and pesticides factories are present. Also there are a large number of industrial facilities including textile, ceramic, glass, electrical cable, battery and metallurgical factories surrounding Al-Abbasa fish farm. These facilities constitute potential sources of heavy metals, which might reach fish either through direct drainage or atmospheric deposition.

In El-Fayoum farm cadmium was the dominant and the highest concentration member of heavy metals compared to FAO permissible limit (0.5) in 1983, this may be attributed to the heavy use of fertilizers where this fish farm is surrounded by the agricultural lands around the fish ponds.

In Egypt, most of fish farms are depending on agriculture drainage water mixed with industrial, herbicides and the phosphate fertilizers which are considered the main source of cd in the environment (Osman, et al., 2009); these types of environmental pollution are considered an important source of cadmium. With special emphasis that the concentration of the other heavy metals in El-Fayoum is lower than Al-Abbasa, which may be elucidated as the ponds in El-fayoum is near Qarun lake where the salinity is high and this factor facilitate precipitation of heavy metals onto the sediments (Luoma, 1990). Also Ali and Abdel-Satar, (2005) in their study revealed that Dayer El-Berka and El-Wadi drains represent the main water feeder sources of all fish farms in El-Fayoum Province and also received the output wastewater of these fish farms.

The activity of AST and ALT enzymes in blood may also be used as a stress indicator. The changes in the activities of these enzymes in blood plasma indicate tissue impairment caused by stress (James *et al*., 1991; Svoboda, 2001). In a study of Shalaby, (2007) he found that sub-lethal concentration of Cd caused significant increases in AST (from 51.49±1.79 to 99.27±1.91) and ALT (from 32.96± 2.58 to 53.93 ±0.76) in *Oreochromis niloticus* after 7 days exposure. The increase in concentration of AST and ALT in blood plasma indicates impairment of liver, in addition, the increase of plasma AST and ALT may be attributed to the hepatocellular damage or cellular degradation by heavy metal.

In the present study, if we compared our data for the AST and ALT with those of Shalby, (2007), it could be confirmed that there were an increase in AST (83.8, 77.8 and 76.6) with the highest value appeared in Kafr El-Sheikh followed by Al-Abbasa and El-Fayoum). In contrast, there was a decrease in the ALT activities in the plasma of fish collected from the three locations (16.3, 14.5 and 14.3) with the lowest value observed in Kafr El-Sheikh. Studying the effects of heavy metal concentrations suggested that liver damage might have induced production of mitochondrial enzymes were subsequently released into the blood. The toxic effects could be lipolytic in nature, as a result of which the cell membrane, lysosomal membrane, and other organelles underwent dissolution, releasing the enzymes into the blood. Hence, the increased AST enzyme activities in the present investigation may be a result of liver cell damage.

Several heavy metals stimulate internal activity and plasma corticosteroid and glucose level in fish (Pratap and Wendelaar Bonga, 1990). Hyper secretion of adrenalin and cortisol considered as primary stress response which trigger a broad suite of biochemical and physiological alterations include catabolism of muscle protein and altered blood levels of protein (Wendelaar Bonga, 1997).

In various fish species, proteins are of importance as structural compounds, biocatalysts and hormones for control of growth and differentiations. So variation in fish proteins could be used as bioindicator for monitoring physiological status of the tested fish (Begum and Vijayaraghavan 1996).

Data of protein levels observed from this study (0.8, 1.78 and 0.77 g/dl) indicated a decreased levels of serum protein in the three locations under study (Al-Abbasa, El-Fayoum and Kafr El-Sheikh respectively) compared to protein results of Shalaby, 2007 who found also that *Oreochromis niloticus* serum protein level decreased in case of exposure to cd metal (from 1.856 to 1.653 g/dl) and explained this result on the bases that the heavy metal pollution causing protein breakdown (Shalaby, 2007).

Protein depletion could be attributed to change in the water quality as a result of the pollution including heavy metals in the agricultural drainage. (Zaghloul, 2000). This may be explained as the exposure to metals (as Cu and Zn) may lead to high accumulation in gills that cause a structural damage and a reduction in oxygen consumption causing sharp reduction in the metabolic rate of fish and consequently decrease protein contents in tissues. Moreover, decreased tissues protein in fish living in polluted environment may be a result of decreased insulin level caused by metal toxicity (Zaghloul, 2001). Insulin is known to have profound effects on the proteogenic pathways in fish; it stimulates the inward cellular transport of amino acids, particularly in muscle, leading to intracellular accumulation of amino acid with subsequent decrease of protein contents (Reda *et al*., 2002).

The change in gene expression is used as biomarker for exposure to pollutants. The expression level of metalothionein gene in fish has been used as a biomarker for water pollution with heavy metals (Sturve *et al*., 2005). To identify relationships between physiological stressors like heavy metal pollution and MT mRNA expression, characterization of a model system using a sentinel species must be performed with an evaluation of specific inducers. For utilization in potential environmental monitoring projects, RT-PCR has been shown to provide the ability to sensitively and accurately quantitative levels of MT mRNA expression in target tissues of the fish and various other aquatic species. However, heavy metals in the contaminated water attach to the water organisms meanly to the MT protein in aquatic organisms, which makes the up-regulation of MT gene could be a biological indicator for heavy metal pollution in water (Sturve et al., 2005). George et al., 1996 in his study observed a significant increase in gill MT mRNA expression in killifish exposed to Cd compared with controls, this result supports our study with tilapia that have demonstrated an obvious increase MT mRNA expression in heavy metal polluted areas.

In several species of fish, MT levels have been demonstrated to increase in a dose-responsive and/or time responsive manner after intra-peritoneal administration of heavy metals. Ueng *et al*., 1996 reported that single intra-peritoneal administration of CdCl2 at 3, 4, and 10 mg/ kg resulted in 12-13 fold increase in hepatic MT in tilapia. Studies on metal induction of tilapia MT gene expression *in vivo* showed tilapia MT mRNA is responsive to the studied metals including cadmium (Cheung *et al*., 2004). Chandrasekera1 *et al.,* , 2008 in their study showed that hepatic MT in Nile tilapia is a sensitive biomarker to indicate moderate to high levels of waterborne heavy metal exposure in the environment. Montaser *et al.,* 2010, denoted to an increase in MT-gene expression in the level of mRNA synthesis due to metal pollution in *Naso hexacanthus* fish at Jeddah Coast.

All the previous findings support our results which indicated higher levels of MT gene expression in the heavy metal polluted ponds under study compared to the internal control 18sRNA using the real time quantitative PCR assay. The expression data was related to the concentration of heavy metals in water and fish tissue; location 1 (Al-Abbasa) showed high concentration of zinc, lead and copper, this was indicated by lower Ct cycles in fish liver; high expression of the MT gene. Locations 2 and 3 (Kafr-El-Sheikh and El-Fayoum) showed comparable concentrations of heavy metals, this also was related to their Ct cycles for metalothionein expression consequently similar levels of gene expression in the fish.

In conclusion, the present field study provide a first estimate of the cultured fish health of Egyptian aquaculture combining biochemical and molecular biomarkers, the use of which in aquaculture toxicology is increasing because of their potential as diagnostic predictors of heavy metals pollution shifts, however, a possible effect on the health of people and arise economic concern by induce decrease in production of healthy fish.

Conflict of Interest:

The authors declare that there is no conflict of interest.

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