

Toxicity of Heavy Metals on Fish at Jeddah Coast KSA: Metallothionein Expression as a Biomarker and Histopathological Study on Liver and Gills

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Abstract: Metal pollution may damage marine organisms at the cellular level and possibly affect the ecological balance. Metallothionein (MT) is a low molecular weight protein that binds heavy metals in marine organisms, therefore, it is considered as biomarker of heavy metal pollution in aquatic environments. In this study, the expression of MT production and histopathological changes in Sleek Unicorn fish (*Naso hexacanthus*) was examined due to heavy metal pollution. Fish were collected from five sampling areas and two potentially non-contaminated (Control) areas, over the coast Jeddah on Red Sea. Cd, Cu, Zn and Pb concentrations were measured at studied areas. Liver and gills from samples were used for, histopathological, histomorphometrical examinations and MT-gene expression assays. Histopathological examination of the liver revealed hepatocytes vacuolation, cellular swelling, nuclear degeneration and congestion of blood vessels. Pathological changes of gills exhibit secondary lamellar disorganization, rupture in lamellar epithelium and epithelial lifting. Morphometric measurements showed significant decrease in both secondary lamella length (SLL), width (SLW) and interlamellar distance (ILD) in fish from contaminated areas. Gene expression of MT, resulted in significant increase in response to metal pollution. In conclusion, in this study using MT as biomarker revealed clear metal pollution in the studied areas of Red Sea near Jeddah coast which was proven by the histopathological assessment. Therefore, the problem of metal pollution is considered among the most serious once that face mankind in the twenty-one century. It is supposed to be one of the greatest national health problems with referring to peoples eating sea foods in KSA, it require special and intense effort at all level individual, groups, national, and international.

Key words: Metallothionein % Biomarker % Metal pollution % Histopathology % Red Sea

INTRODUCTION

Industrial development in the developing and developed countries has resulted in heavy metal contamination of local waters. Metal pollution may damage marine organisms at the cellular level and possibly affect the ecological balance. Exposure and ingestion of polluted marine organisms as sea foods can cause health problems in people and animals including neurological and reproductive problems [1]. Chemicals of industrial effluents and products of ships and boats such

as heavy metals which find their way into different water systems can produce toxic effects in aquatic organisms [2]. Petroleum products are one of the most relevant pollutants to aquatic ecotoxicology. Exposure to crude oil and derivatives can induce a variety of toxic symptoms in experimental animals. Petroleum hydrocarbons can act as a mediator in free radical generation in fish [3].

Jeddah is a major city with a population of over 2.6 million and an area 1,200 km². Samples of the Red Sea water were collected from 24 important locations near Jeddah and analyzed in the laboratory for various water

quality parameters [4]. Other previous studies revealed coastal pollution with several pollutants as well as heavy metals [5]. Saad and Fahmy [6] recorded four main pollution sources at Jeddah coast: the untreated domestic sewage wastes, oil pollution from oil refinery of factory Petromin, fish wastes from the big fish market of Bankalah region and probably desalination plant effluents. Recently, Badr's group [7] confirmed the heavy metal pollution at sea bed of Jeddah coast.

Fish species were recently suggested as environmental biomarkers [8-10]. Quantification of fish metallothionein transcript levels in absolute units has only recently been presented [11,12]. It also, considered as early warning for degradation of environmental quality, but also specific measures of the toxic, carcinogenic and mutagenic compounds in the biological materials [13]. Liver and gills as main organs for metabolism and respiration are target organs for contaminants accumulation as reported by many authors concerning structural damage to organs and tissues related to the exposure of fish to petroleum derivatives [14 - 16]. The gills [17], liver [18] and kidney [2] are commonly the primary target organs for pollution. Histopathological lesions and increase in size were reported in various fish exposed to heavy metals [19-25]. Chronic exposure of rainbow trout gill and liver cell lines showed a reduction in cell membrane integrity, mitochondrial activity and lysosomal function at high levels of Naphthenic acids [26]. Active fish with high oxygen requirements have larger gill surface areas than slow-moving benthic fish [27]. Furthermore, species known to live and tolerate hypoxic conditions have also been reported to have increased gill area [28-32].

Measuring heavy metals in aquatic organisms may be a bioindicators of their impact on organism and ecosystem health [33], but a true evaluation of the damage inflicted by heavy metals should come from comprehensive biomarker studies. Biomarkers are more telling than bioindicators as measurements of heavy metal contamination because they deal with chemical and physiological changes on the organism level and assess contamination based on a direct measure of change in the organism [1, 34]. Research over time has focused on various species and various biomarkers to determine the amount of heavy metal toxicity in aquatic environments as sea anemones [35], sea urchins [36], grass shrimp [37] and fish [38]. Biomarkers in marine organisms such as glutathione (GSH) and metallothionein are often used to evaluate heavy metal contamination [39]. The intracellular

fate of both essential and non-essential metal ions strongly depends on thiol-containing molecules, particularly metallothionein (MT) that afford protection from metal toxicity and oxidative stress in numerous organisms [40]. These properties have implicated MT to be an important factor in metal resistance in both aquatic invertebrates [41] and vertebrates [42, 43-46, 47]. Metallothioneins (MTs) comprise a class of inducible metal-binding proteins characterized by a low molecular weight of 6-10 kDa, a high content of cysteine (of about 30%), a lack of aromatic amino acid residues and a wide distribution in various organisms [48]. It is generally accepted that MTs play an important role in the detoxification of heavy metal ions such as Cd and Hg, this is referred to its strong binding ability to the heavy metals because everyone mole of MT binds 7 moles of cadmium or zinc [49]. MT also has a role in buffering changes in free metal ion levels in cells by binding essential metals such as Cu and Zn [50,51]. Also, there is a positive relationship between MT and heavy metal pollutants since overexposure to heavy metal contaminants can lead to overproduction of MT and consequently systemic damage to the organism [33, 52, 53-56]. Although many species produce metallothionein and can be tested for metal toxicity via MT measurements, fish and mussels have demonstrated higher rates of accumulation for metals than other species because of their filter feeding and sessile life histories. This has been shown to be especially true for cadmium [38, 57]. The expression and role of fish MTs have mostly been studied in organs that play a central role in metal uptake and accumulation, i.e., the liver, kidney and gills [58].

This study aimed to check the level of heavy metal-pollution at certain areas near Jeddah sea-shore by investigating the histopathological and morphometrical changes of gills and liver in Sleek Unicorn fish (*Naso hexacanthus*) fish exposed to heavy metals as well as semi-quantitatively expression of Metallothionein gene as a molecular biomarker.

MATERIALS AND METHODS

Study Sites: The current work was done in five sampling areas (Contaminated [6] and [7]) and two potentially non-contaminated (Control) stations, distributed over about 40-km stretch of Jeddah coast of the Red Sea with the following positions on Google earth map (Table 1).

Table 1: GPS-Positions for the studied areas according to Google earth map

| Sampling Area | | N : Position | E: Position |
|---------------|-----|----------------|----------------|
| Control | I | 21 °23' 31.87" | 39 °00' 04.94" |
| | II | 21 °22' 22.37" | 39 °01' 17.46" |
| Contaminated | I | 21 °23' 03.60" | 39 °05' 40.38" |
| | II | 21 °23' 58.01" | 39 °05' 14.45" |
| | III | 21 °24' 20.58" | 39 °05' 32.00" |
| | IV | 21 °24' 15.76" | 39 °06' 23.92" |
| | V | 21 °25' 25.69" | 39 °07' 09.54" |

Sampling stations were chosen according to previous records [5, 7, 59] and confirmed by the current environmental measurements in our study. Water quality parameters were determined during sampling days, mid of May 2009.

Fish Sampling: five fish per each area were collected from overnight pre-held pots (Standard mesh sizes were used). Weight and length of each sample were measured (Length averages = 22.22 ± 0.79 mm and Body weight averaged = 157.86 ± 0.9 g), samples then, were immediately dissected, gills and liver of each examined fish were rapidly excised, washed (in DEPC-Water) and divided into four equal parts/each. Three parts were frozen in liquid nitrogen and maintained at -80°C till processed for molecular analysis, the other part was fixed in buffered formalin for histopathological examinations. Individuals with the similar sizes were selected to ensure a uniform sampling. For other investigations, samples will be kept at -4°C .

Determination of Metal Pollution of Water Samples:

Water samples were collected from the study areas according to Saad and Fahmy [59] and later on, the laboratory measurements of heavy metal concentrations, were done by an axial inductively coupled plasma atomic emission spectrophotometer (ICP-AES, Varian Liberty Series II).

Histopathological Studies: The prefixed tissues samples were dehydrated in graded concentrations of ethanol before paraffin embedding. Sections were cut at $5\ \mu\text{m}$ and subsequently stained with counter stain (H&E). Images of gills and liver were picked up using Leica microscope equipped with digital camera. Image analyzer (Motic image plus 2.0 software) was used to measure morphometric parameters.

Total RNA Isolation: Total RNA was extracted from approximately 100 mg of fresh tissue using Trizol Reagent protocol (Gibco BRL) and according to method of Chromoczynski and Sacchi, [60].

Semi-Quantitive Reverse Transcriptase Polymerase Chain Reaction:

cDNA synthesis was done with random hexamers-primer and M-MLV-reverse transcriptase (from Quiagen). 299 base pair (bp) of MT-gene was amplified with primer sequences of 5'-atggaccgtgcgagtg-3' and 5'-ctgatgacaagtacaaaatggat-3'. Other primer pair of 5'-gagaggctacagcttcaccac-3' and 5'-atctccttctgcatcctgca-3' was used to amplify 366 (bp) β -actin fragment as an internal control. PCR reaction was carried out [91]. PCR products were resolved on 1.5% agarose, visualized by UV trans-illumination and digitized with a Gel Doc 1000 Gel Documentation system (Bio-Rad). Image-analysis of the amplified fragments was carried out by using Molecular Analyst software (Bio-Rad). To analyze semi-quantitatively the result of RT-PCR, the intensity of the PCR product was measured. This relies on the quantification of the amplification products on the basis of optical density of the detected bands. The relative intensity of MT gene to that of β -actin was determined. Corrected values were obtained by dividing the measured value for MT transcript by that of β -actin.

Statistics: The Mann-Whitney U-rank test was used to test for the differences in MT/ β -actin ratios among the tested groups of fish compared to the control group. In all the statistical tests, difference was considered significant when $P < 0.05$.

RESULTS

Ecological Results: Water temperatures at sampling areas were 23.3 ± 1.5 and $23.6 \pm 1.2^{\circ}\text{C}$, dissolved oxygen was 7.2 ± 1.9 and 6.9 ± 1.6 mg/L (for control and contaminated, respectively). The distribution of heavy metals in water samples of the studied areas were represented by the following histograms.

There were a significant difference ($p < 0.05$) in concentrations of heavy metals, especially in the contaminated area number III. Cadmium range was $2.155 \pm 0.05\ \mu\text{g/L}$ at control areas compared to $5.26 \pm 0.35\ \mu\text{g/L}$ in the contaminated areas (maximum significant concentration in Cd $5.7\ \mu\text{g/L}$ of area III). Copper range of concentration at areas I and II was 16.9 ± 0.9 compared to $26.76 \pm 3\ \mu\text{g/L}$ in the contaminated areas with non-significant average of 9.7 ± 0.3 compared to $21.4 \pm 1.45\ \mu\text{g/L}$ in the contaminated areas with significant maximum concentration of $22.9\ \mu\text{g/L}$. However, lead concentrations were the most to be in the average of 64.7 ± 0.3 against $82.04 \pm 5\ \mu\text{g/L}$ in the contaminated areas with significant maximum concentration of $90\ \mu\text{g/L}$.

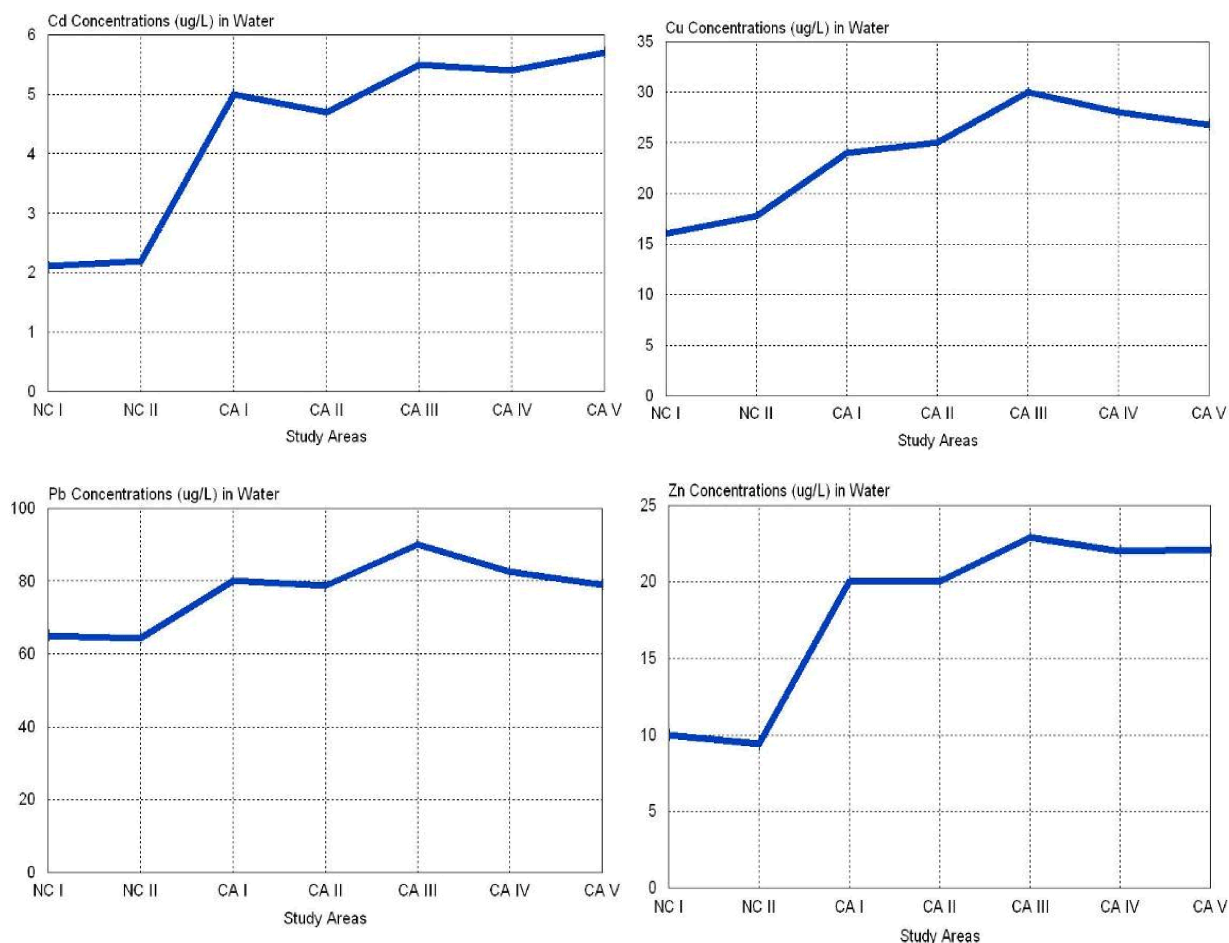


Fig. 1: Curves represent concentrations of heavy metals(Cd=Cadmium, Cu= Copper, Pb= Lead and Zn= Zinc) measured in ug/L (microgram of metal/Liter of water)

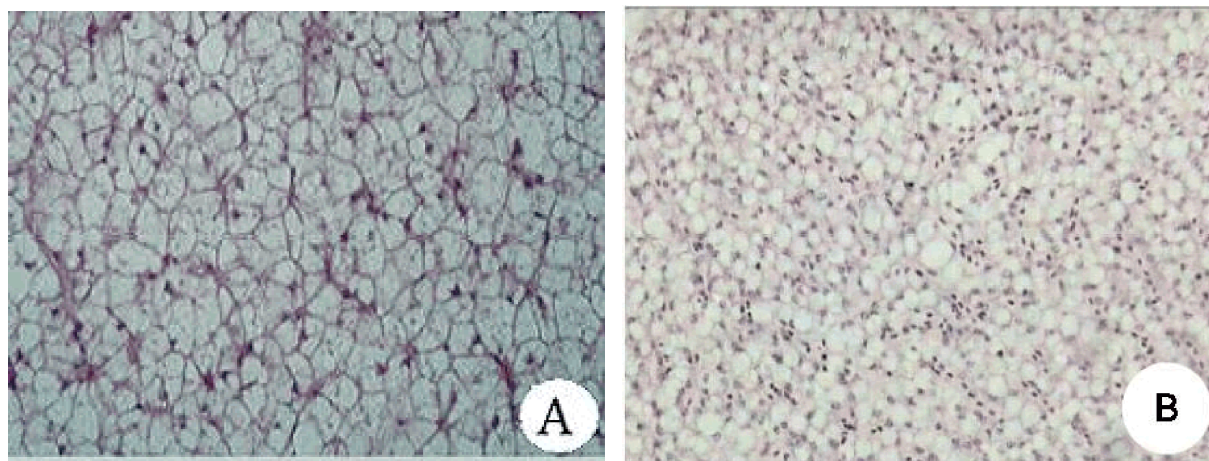


Fig. 2: Liver photomicrograph of studied areas showing; normal (A) and affected (B) liver sections showing cellular and nuclear degeneration and cytoplasmic vacuolation. (H&E, X400).

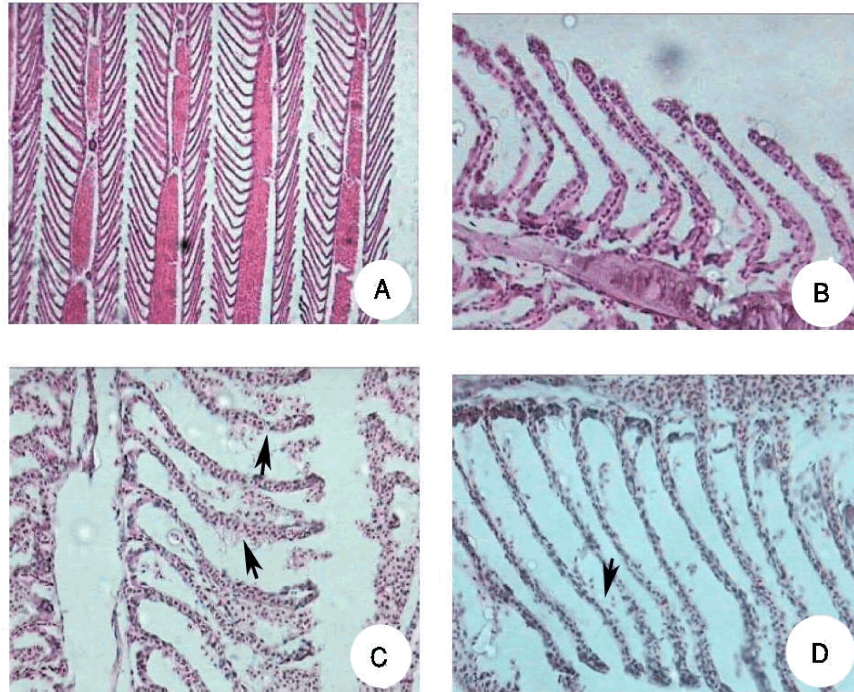


Fig. 3: Photomicrograph of gills from control area fish (A and B) stained with Hx/Eos and magnified at 40× and 400× respectively demonstrating normal epithelial cells of secondary lamellae (B); rupture of lamellar epithelium (arrow at C); severe degeneration of the epithelial cells and epithelial lifting (arrows at D)

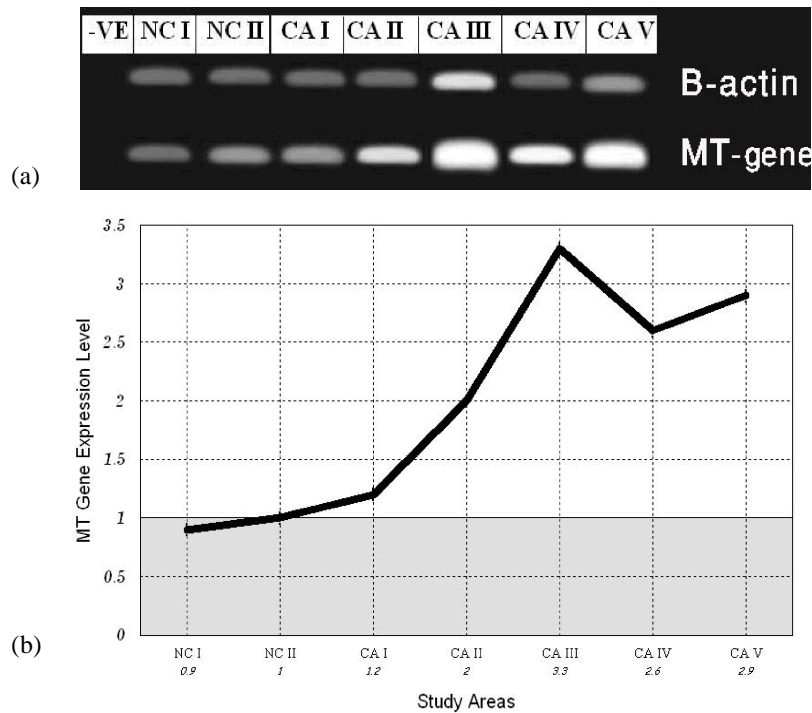


Fig. 4: Metallothionein Semi-quantitative Gene Expression: represented as (a) PCR-products of B-Actin (466 bp of *Beta-Actin* gene) and MT-gene (299 bp of *Metallothionein* gene). (b) level of MT-gene expression in relation to the expression of Beta-Actin (PCR-internal control), NC I and NC II (Non-Contaminated Areas) and CA I, CA II, CA III, CA IV. And CA V (Contaminated Areas I, II, III, IV and V respectively)

Table 2: Histomorphometrical measurements of secondary lamellar length (SLL), secondary lamellar width (SLW) and interlamellar distance (ILD) of different collected areas of the Slek Unicorn fish (*Naso hexacanthus*)

| Parameters | Control area (μm) | area 1(μm) | area 2 (μm) | area 3(μm) | area 4 (μm) | area 5 (μm) | Means of polluted area |
|------------|---------------------------|--------------|--------------|--------------|---------------|--------------|-----------------------------|
| SLL* | 308.54±15.76 ^a | 275.65±12.87 | 280.78±11.65 | 264.4 ±10.98 | 275.76± 13.95 | 279.43±12.54 | 275.22 ± 12.39 ^b |
| SLW* | 26.78± 3.76 ^a | 25.24 ± 4.21 | 21.77± 2.98 | 24.54± 3.65 | 23.76± 4.98 | 25.98± 3.56 | 24.25 ± 3.87 ^b |
| ILD* | 34.54± 5.21 ^a | 30.89 ± 4.67 | 28.65± 4.21 | 29.56± 3.93 | 27.65± 4.13 | 26.54± 3.98 | 28.65 ± 4.18 ^b |

The data expressed as Mean ± Standard deviation, n = 5 fishes

Histopathological Results

Liver: Microscopic examination of hepatocytes and their nuclei of fish from contaminated areas exhibit histopathological changes in comparison to the control ones due to contamination. Hepatocytes lose their normal boundaries (Figure 2). There were cellular and nuclear degeneration, cytoplasmic vaculation and in most regions inflammatory infiltration (Figure 2).

Gills Histopathology: Control fish had a normal morphological structures. Rows of secondary lamellae were in regular shapes, the epithelial cells covered secondary lamellae (Figure 3a and 3b). Gills of fish from contaminated areas showed secondary lamellar disorganization, rupture of lamellar epithelium (Figures 3c and 3d). However, those from other areas showed secondary lamellar disorganization, hemorrhage, rupture of lamellar epithelium and epithelial lifting (Figure 3d).

Gills Histomorphometry: Data of morphometrical measurements including secondary lamellar length (SLL), secondary lamellar (SLW) width and interlamellar distance (ILD) were obtained (Table 2). Data showed that there is a significantly decrease ($P < 0.05$) of secondary lamellar length (SLL) of polluted areas ($275.22 \pm 12.39 \mu\text{m}$) in comparison to the control group ($308.54 \pm 15.76 \mu\text{m}$). Secondary lamellar width (SLW) of polluted areas was also decreased ($24.25 \pm 3.87 \mu\text{m}$) in comparison to control one ($26.78 \pm 3.76 \mu\text{m}$) and difference was significant ($P < 0.05$). Data obtained described that interlamellar distance (ILD) of polluted areas fishes ($28.65 \pm 4.18 \mu\text{m}$) significantly decreased ($P < 0.05$) in comparison to the control one ($34.54 \pm 5.21 \mu\text{m}$).

Molecular Results (Semi-Quantitative Analysis of MT Gene Expression): Agarose gel electrophoresis showed that single PCR products were amplified. Results showed significant increase ($P < 0.05$) in the OD (Optical Density) of the PCR-products in comparison to that produced from samples of the control areas. Corrected values were obtained by dividing the measured value for MT transcript by that of β -actin.

Data showed a range of 0.95 ± 0.05 in expression of Metallothionein at the control areas, while, the contaminated areas have a range of 2.4 ± 1.02 with a significant maximum value of 3.3 at samples from the contaminated area number III (CA III).

DISCUSSION

Human activities in industry and the increasing use of metals in industry has lead to serious environmental pollution through effluents and emanations of large quantities of metals to localized area of the sea. In the present study, Slek Unicorn fish (*Naso hexacanthus*) from Red Sea over the coast of Jeddah were used. Weight and length of each fish were measured; gills and liver of each fish also were examined. The obtained data showed significant decrease in both secondary lamella length (SLL), width (SLW) and interlamellar distance (ILD) in fishes from contaminated areas. Both liver and gills revealed several histopathological lesions and the expression of metallothionein mRNA, resulted in significant increase in expression of MT in response to metal pollution. These results represent how the heavy metals in the contaminated water attach the marine organisms, it also, supported by most of the previous studies, dealing with the levels of heavy metal pollution in the coastal area of the Red Sea of Saudi Arabia concerning the central and northern parts of the coastal waters of Jeddah city. The concentrations of Mn, Zn, Cu, Cd and sediments in the water body of the area of Sharm Obhor, a creek situated just to the north of the area of Jeddah were studied. The data warned from the increase in concentrations of metals due to human impact and the sediments of this area are composed of biogenic carbonate and aragonite in varying proportions [59,61]. Most of the previous works were confined to the area of Jeddah which considered as the most industrialized area in the country during the last two decades [7]. Heavy metal pollution was also recorded as one of the major types of toxic pollutants commonly present in surface waters and highly toxic to marine and freshwater aquatic life [62]. Also, several histopathological lesions in fish liver were reported in this study and supported by

many studies deals with monitoring fish health and environmental pollution in natural water bodies [63]. Previous studies have shown that exposure to either cadmium or zinc caused histopathology of the kidney and epidermis [64], the gills [65] and the liver [66]. The exposure of fish to concentrations of heavy metals has correlation generally with an accumulation, more or less linear, in target organs, producing at the same time, histomorphological alterations. The results of this study showed a histological response in exposed specimens with the most prevalent histological characteristics identified being increased vacuolation associated with lipid accumulation, congestion of blood vessels, necrosis and cellular swelling of the liver. This results is accompanied with previous research related hepatocellular alterations in fish hepatic tissue to exposure to toxins [67]. Fatty change in the liver was characterized by increasing lipid deposits and a consequent loss of sinusoid space. Increased lipid content of the liver can be explained by either increased deposition of lipid in excess of nutritional requirements, or a failure to mobilize lipid stores during exposed to toxins [68]. Results of this work are similar to those obtained in juvenile yellow perch and rainbow trout exposed to 25 or 125 ng TCDD/g fish [69,70]. *Clarias gariepinus* remains an important aqua-cultural species in Africa and Asia [71] and are grown in areas heavily contaminated from copper mines [72]. Similar findings on the effect of pollutants were found in previous studies [73,74]. Gills are the main site for gas exchange and other important functions such as ionic and osmotic regulation. Most previous studies stated that the exposure of fish to heavy metals is associated with structural damages in gill epithelia. Most gill changes caused by pollutants of fish in this study were characterized by epithelial lifting an inflammatory response of the tissue. Results of this work is in agree with Arellano *et al.* [75] who observed lifting and peeling of the lamellar epithelium and rupture of capillaries as common lesions in the Senegal sole gills after copper exposure. Similar alterations have been described for other species contaminated with copper and other heavy metals such as Zn or Hg [76]. Reduction of secondary lamellar length, width is in accordance with Nero *et al.* [77] on Gills and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) when exposed to oil sands process-affected water. Generally, gill and liver pathological data suggest that degenerative changes were the most prevalent and sensitive changes observed following exposure of Sleek Unicorn, *Naso hexacanthus* to contaminated water that contains elevated levels of

heavy metals. The current work denotes to an increase in MT-gene expression in the level of mRNA synthesis due to metal pollution, which can be explained by gene amplification or induction. Gene amplification is a process in which specific DNA sequences are replicated to a disproportionately greater degree than other sequences in the genomes in which they reside and is most commonly observed under conditions where high level expression of certain gene products mediates resistance to toxic events [78]. Copper and cadmium-resistant mammalian cell lines have demonstrated that MT levels in these cells is increased and this increase has been attributed, in some cases, to amplification of the MT-1 and MT-2 genes [79 - 81]. Koropatnick and his coworkers [82, 83], had reported that MT-1 genes in adult mouse liver undergo a two-fold increase in average copy number within six hours of treatment with high levels of cadmium salts and the extra MT gene copies are both transcriptionally competent and inducible. The concentration of metal ions required to induce MTs and the time required to reach peak transcription levels, varies according to the inducing metal [84]. Zinc and cadmium are among the most potent inducers of MT transcription and protein synthesis. Transcriptional regulation of MT-1/MT-2 genes by heavy metals is conferred by metal response elements (MREs) [85] although MREs are also necessary for basal MT gene transcription [86]. However, Comparison of the human and mouse MT-1 and MT-2 promoters reveals differences in MRE distribution and orientation indicating that, although active MREs are critically necessary for MT gene transcription, their arrangement is not [87]. MTF-1 is now recognized as the critical transcription factor regulating inducible MT expression. In view of the capacity of MTF-1 to respond to zinc and only zinc, the precise mechanism by which multiple inducing agents and conditions activate MTF-1 is not resolved. One possibility is that the activity of Metal regulatory transcription factor (MTF-1) and possibly other zinc-requiring transcription factors and proteins, depends on a metallo-regulatory protein or proteins that do not, themselves, have DNA binding or trans-activation capacity [83]. Such a “zinc regulator” would respond to many inducing events by adding zinc and only zinc, from MTF-1. Metallothioneins are good candidates for such a role. They are the predominant single liganding species for zinc in cells untreated with other heavy metals and release zinc in response to induction with cadmium or copper [88]. Released zinc would then be available to mediate MTF-1 activity, especially if the newly released zinc were present in the nucleus. Such a metal-responsive

zinc carrier model would be part of a multiple factor signal transduction system and might be particularly important under conditions where zinc availability is limited [83, 89]. The capacity of MT to enhance zinc-requiring transcription factor activity in cultured cells has been observed directly. It was also observed that that a greatest expression in the contaminated area number III (a zone of most contamination record in our study). This may be due the Zn-concentration from industrial wastes, Suhy's group [90] reported that, mouse cell line had high MT mRNA expression due to extreme zinc deprivation.

In conclusion, the present field study provide a first estimate of the health of *Naso hexacanthus* in Jeddah coast, Red Sea combining two biomarkers, histopathological and molecular, the use of which in marine toxicology is increasing because of their potential use as diagnostic predictors of heavy metals pollution shifts. However, a possible effect on the health of people due to fishing and arise indirect economic concern by induce decrease in production of healthy fish.

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