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# Biochemical and genetic modulations of *Rhabdosargus haffara* against high pollution marine environment with heavy metals

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## ABSTRACT

The current field study was performed between the high metal-polluted and the less metal-polluted areas at northern of Suez Gulf in Egypt during winter and summer seasons; to evaluate the impact of heavy metal pollution on Cytochrome c oxidase (COX) mRNA expression and alkaline phosphatase enzymatic activity in Red Sea haffara seabream *(Rhabdosargus haffara*) liver tissues. Elevated values of most studied metals concentrations were detected in fish tissues caught from the high polluted sites as compared to the less polluted sites. Alkaline phosphatase (ALP) activity recorded a non-significant difference at p-value = 0.4 in the liver tissue of *Rhabdosargus haffara* fish between the highly polluted and the less polluted areas. Higher activity values of ALP were recorded during the summer season as compared to the winter season, especially in the high polluted area at p-value = 0.001. Using qRT- PCR, COX relative quantitation (RQ) of the highly polluted area was up-regulated by 11.5-fold as compared to the less polluted area 0.98-fold with a highly significant difference at p-value = 0.001. Moreover, there was a high significant difference in COX expression between winter and summer seasons in the highly polluted areas (p = 0.001) and the less polluted areas (p = 0.03). As a result, COX can be utilized as a metal contamination biomarker for subsequent studies. Health risk assessment of heavy metals in the muscles (edible parts) of the fish indicated that, the provisional tolerable daily/weekly intake (PTDI/PTWI) was much less than the standard guidelines. In addition, target hazard quotient (THQ) values of the tested metals did not exceed the permissible level (less than 1.0) indicates that the potential exposure is within the degree of exposure that is considered safe or acceptable and there is no health risk for consumers.

## 1. Introduction

The molecular and biochemical biomarkers can be used as an accurate warning index of heavy metal accumulation in all living organisms. The firstly respond to heavy metals contamination is the biomarkers at the molecular level, followed by responses at the biochemical and physiological levels, finally at the morphological and histological levels [1]. The primary goal of ecotoxicological studies is to ensure that heavy metal pollution resulting from anthropogenic contamination not lead to adverse or harmful effects on living organisms and consumer health [2]. The aquatic environment is a definitive basin of metals and so that belonging organisms have a tendency for heavy metals accumulation, consequently it provides an indication into the aspect of toxicological stress initiated by these metals.

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Despite the presence of several storage and detoxifying systems, heavy metals could cause harmful effects to a broad range of marine organisms [3].

Metal ions are vital components in living systems; some of these elements may have poisonous or cancerous-causing effects. One of the most dangerous characteristics of heavy metals is their accumulation in the animals; even when their amounts are relatively little in the living environment, they can reach hazardous levels [4]. Heavy metals, in biological systems, have been proven to influence cellular organelles and components including nuclei, cell membranes, lysosomes, mitochondria, and many enzymes that have a role in detoxification, damage repair and metabolism [5]. Because of the elevated susceptibility of the aquatic environment for toxicants, fish has been introduced as the important biotic marker for the presence of toxicant exposure and the impact on molecular, cellular to physiological state [6, 7]. Utilization of fish in the toxicological studies plays a necessary role wherever consumption of metal-contaminated fish

seriously deteriorate the health of humans, including severe threats as liver damage, cardiovascular abnormalities, renal failure, and even death [8, 9].

*Rhabdosargus* (Haffara seabream) is biologically, financially, and monetarily significant fish species. Be that as it may, little exploration has been performed on the connection between this species and heavy metal contamination for the safeguarding of the marine climate and consumer health. In fish, liver is the main organ of the detoxification processes, therefore, the traits measured in liver could be a good indicators (e.g., gene expression, enzymatic activity) to estimate the impact of the xenobiotics on the fish; because it is one of the most affected organs by contaminants in the marine environment due to its multiple functions and blood supply [10, 11]. Adjustments in liver catalysts and quality articulation of proteins in sea-going creature liver tissues could be a consequence of tissue harm and brokenness prompted by heavy metals in a contaminated marine environment [12]. Consequently, we focused via our study on the liver as the most sensitive organ of pollutants.

As is well known, the cellular alterations and enzymatic reactions are one of the most sensitive biomarkers methods to monitor and track the impact of heavy metals in case of acute or chronic exposure toxicity [7]. One of these enzymes is Alkaline phosphatase (ALP, EC 3.1.3.1, optimum pH ≥ 8.0), which is an essential plasma- membrane metalloenzyme located in most animal cells [13]. ALP has also been studied as heavy metal bioindicator enzyme due to its sensitivity to metal pollution [14, 15, 16], whereas heavy metals can inhibit or activate its activity [17].

Accumulation of metals in the tissues of fish is stated to initiate and catalyze reactions that responsible for reactive oxygen species (ROS) generation [18], leading to environmental oxidative stress, and tissues, DNA, lipids, and proteins damage [19]. Cytochromes are most frequently studied about their role in the metabolism of xenobiotics and ROS limitation [20, 21]. The continuous exposure to environmental toxicants, e.g. heavy metals; induce a series of toxic effects on mitochondria [22, 23, 24]; which, originate mainly from mitochondrial respiratory chain dysfunction allowing the release of cytochrome c into the cytosol to excite apoptosis [25]. COX is the terminal enzyme of the mitochondrial respiratory chain, it's a metalloenzyme contains two heme groups and three Cu atoms. It is noticeable that COX needs more research that focuses on the relationship between its mRNA regulation in aquatic animals and metal pollution, especially in the field studies. Consequently, through the recent study, the heavy metal pollution influence on COX mRNA gene expression as well as liver ALP activity was highlighted. Furthermore, because of the temperature is an important environmental factor which has a significant role in metal aggregation [26], this study tried to display a comparative analysis between winter and summer seasons through the selected parameters.

## 2. Materials and methods

### *2.1. Study area*

The investigated area was divided according to the degree of sediment heavy metal pollution into two categories: highly polluted and less polluted sites at northern of Suez Gulf, Egypt as reported by El-Sikaily et al. [27]. The highly polluted area includes from the sites (1 to 6) and the less polluted area includes three stations at the site (7) as shown in Fig (1). The sites selected on the basis that they are hotspots of metal pollution in that industrial zone; as a result of many manufactures including batteries and electrical sources; pigments and paints; alloys and solders; pesticides; glass; fertilizer; refiners; plastic [28, 29, 30]. These growing industrial activities coupled with the fact that Suez Gulf represents the south entrance of the Suez Canal have resulted in the conversion of the whole Suez Bay into large harbour.

### *2.2. Fish sampling*

Fish species (*Rhabdosargus haffara*), which belongs to "Sparidae" family were collected during winter and summer (2017) from Suez Bay and also from Ain Sokhna coast. The mean of water temperature at the time of sampling was (18.68±0.7) ºC in winter and (29.76±0.3) ºC in summer. Fish samples were collected fresh and alive from fishermen working in the area of study. About 50 fish of similar weight (30 - 40 g) and average length (11.5-13.9 cm) were sampled from the less polluted area and a similar number was sampled from the highly polluted area in each season. Samples were dissected on ice plate and fish muscles, liver, and gills were abstracted and stored frozen at -20 °C for examination. A part of fresh liver samples was examined for enzyme assay within 8 hrs. from the collection; another part of liver samples was immersed immediately in RNA Later solution (BioFlux, China) for total RNA extraction and molecular analysis.

### *2.3. Determination of metal concentrations*

Preparations of subsamples and heavy metal analysis were performed according to UNEP/FAO/IAEA/IOC [31]. Fish liver, muscles, and gills were isolated, homogenized, then 1 g of each sample type was digested using 4 ml of annular nitric acid and perchloric acid for gills (4:1) in Teflon vessels, covered tightly and allowed predigesting at room temperature overnight followed by hot plat digestion at 80 ◦C until the solution become clear, and finally cooled at room temperature. The final solution was then completed to certain volume with distilled deionized $H_2O$ and analyzed for Cd, Mn, Pb, Fe, Ni, Zn, and Cu using the Atomic Absorption Spectrophotometer AAS model GPC A932 ver. 1.1 (Perkin Elmr, USA) working with an air/acetylene flame and D2 background correction. The obtained data were expressed as µg/g wet weight. All glassware were soaked in 10% $HNO_3$ and lastly swilled with distilled deionized $H_2O$ before use to avoid metal contamination and the used reagents were of analytical grade.

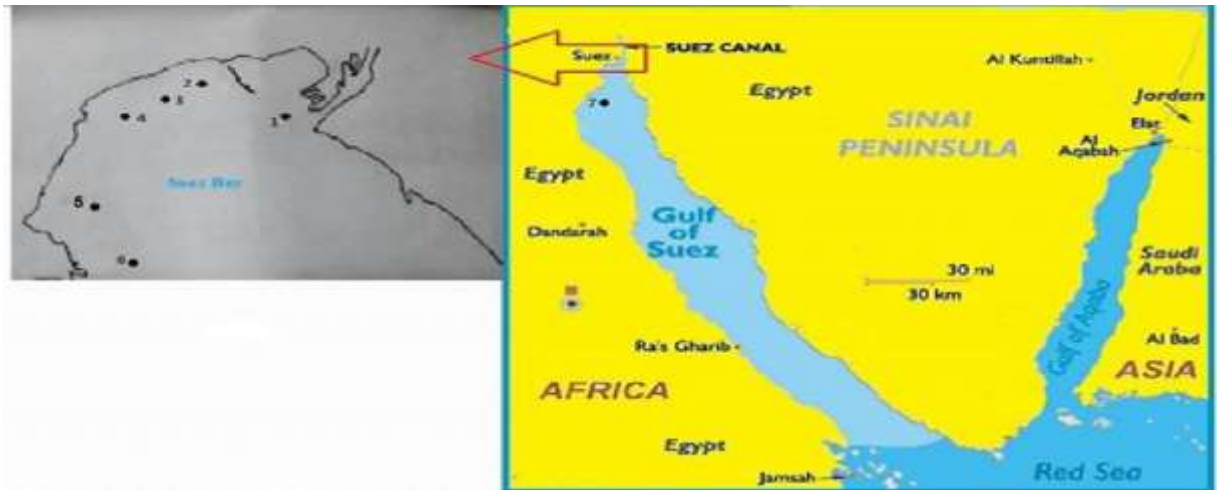


**Fig (1):** Positions of the sampling stations: stations 1, 2, 3, 4, 5, 6 of (Port Tawfiq, Suez Oil- Processing Company, El Cabanon, Etaqa Electric Station, Attaka Fishing Port, and Adabyia Port respectively; 3 sites at station 7 (Ain Sokhna Coast).

### *2.4. Human risk assessment analysis*

The human risk assessment was based on calculation of Estimated Daily Intake (EDI), Estimated Weekly Intake (EWI) and Target Hazard Quotient (THQ) allowing the evaluation of health risk from fish consumption. The EDI (mg $kg^{-1}$ $day^{-1}$bw) was calculated using the following equations:

$$EDI = \frac{C \times IR \times EF \times ED}{BW \times AT}$$

Where, $C_{fish}$ = average value of heavy metal concentration in fish muscle (mg/Kg wet weight); IR = the ingestion rate or fish consumption (0.0643 Kg $day^{-1}$ per person); EF= exposure frequency (365 days/year); ED= exposure duration (70 years for adult (lifetime exposure)]; BW = average body weight of Egyptian population (70 kg); AT= averaging time, or the period over which cumulative exposures are averaged (noncancerous/ lifetime = ED× 365 days/year).

$$THQ = \frac{EDI}{RfD}$$

Where, EDI = estimated daily intake; $R_f$ D = reference dose (mg $Kg^{-1}$ $day^{-1}$). However, a THQ of 1.0 for any element is used to assess acceptable exposure and is utilized as a reference point. A THQ that is less than or equal to 1.0 indicates that the potential exposure is within the degree of exposure that is considered safe or acceptable; THQ that is more than 1.0 indicates that adverse health effects are expected to occur. Estimated Weekly Intake (EWI) was calculated from EDI. The human risk assessment was estimated using the provisional tolerance daily/weekly intake (PTDI/PTWI), and reference dose ($R_f$ D) previously reported by the Joint FAO/WHO Expert Committee on Food Additives JECFA [32] and European Food Safety Authority (EFSA)[33] (Table 5).

### *2.5. Determination of Alkaline phosphatase activity*

The alkaline phosphatase activity in *Rhabdosargus haffara* liver was estimated at 25 °C with the methodology described by Bergmeyer [34] using SPECTRUM alkaline phosphatase assay kit, Egypt. ALP catalyzes the hydrolysis of p-Nitrophenyl phosphate (p-NPP) to p-Nitrophenol. The reaction is accompanied by an increase in absorbance at 405 nm. For 0.3 g of each liver organ, 1 mL of phosphate buffer saline (pH 7.4) was added and homogenized in an electric homogenizer (Wise stir Hs-30E, Germany) at 0 °C, followed by homogenate centrifugation at 5,000 rpm for 5 min in a refrigerated centrifuge (Sigma, Germany). Twenty microliters of supernatant were separated and 1000 µl of the reaction solution was added and mixed then incubated at 37 °c for one minute in the incubator (Heraeus, Germany). The absorbance was measured spectro-photometrically (JENWAY6100, UK) at 405 nm after color development during a three minutes reaction time. The calibration of blank was performed by using distilled water. Blanks were analyzed in duplicate and samples in triplicates. The enzymatic activity was expressed as U/L. Total protein (TP) was assessed using the DIAMOND diagnostics kit according to Burtis [35] to evaluate the specific activity of the enzyme.

### *2.6. Total RNA extraction from Rhabdosargus haffara liver tissue*

RNA extraction and purification were performed using a spin column kit purchased from (Bio Basic Inc., Canada) according to the manufacturer's instruction. This kit simplifies total RNA isolation by combining the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-based purification. Exactly 0.45 ml Buffer RLT (provided in kit) was added to about 25- 50 mg of liver tissues (that preserved previously in RNA later solution) and then homogenized. Ethanol is added to the lysate to provide optimal binding conditions. The lysate is then loaded onto the EZ-10 column with a silica membrane.

RNA binds to the silica membrane, all protein and other components are removed in the flow-through. The remaining contaminants and salts are efficiently washed away. Purified RNA is eluted in RNase-free water has OD260/OD280 ratios of 1.9-2.1 (measured in 10 mM Tris-HCl, pH 7.5) and is ideal for use in RT-PCR as recommended by the kit manufacturer .

### *2.7. First-strand cDNA synthesis*

The prepared RNA was used as a template for cDNA preparation using (Thermo Scientific RevertAid$^{TM}$ First Strand cDNA synthesis kit, USA) according to the manufacturer's instruction. The enzyme maintains activity at 42-50 °C and is suitable for the synthesis of cDNA up to 13 kb. Exactly 10 µl of template RNA was pipetted into each well of a 96-well reaction plate, and 10 µl of a master mix of the kit provided components were added with mixing, and then loaded into thermal cycler for incubation. The first-strand cDNA was used as templates for qRT-PCR with a pair of COX-1 specific primers from (Thermo Scientific, USA) designated as:

COX-1 Forward: 5'-GGT GCC CCA GAC ATA GCA TT-3'

COX-1 Reverse:5'-GCC TGC TAG GGG AGG ATA GA -3'

### *2.8. Determination of the COX-1 mRNA expression level by Real-time quantitative polymerase chain reaction (qRT-PCR)*

For real-time RT-PCR analysis, the β-actin housekeeping gene was used as an internal standard for normalization of target gene expression levels. The resulting cDNA was subjected to PCR analysis for 35 cycles (heating at 94 °C for 45 sec , annealing at 58 °C for 50 sec, extension at 72 °C for 90 sec, and final extension at 72 °C for 10 min ) using the respective primers. Data accession was achieved during the extension step and PCR reactions were repeated three times and the mean was recorded. The Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix kit (USA) (for each 25 µl reaction) along with four designed primers (for genes COX-1 and β-actin) were used for qRT-PCR and the results were computerized using (Plex Rotor- Gene Qiagen 5, Germany) machine. Melting curve analysis was carried out to verify the specificity and identity of the PCR product (amplicon size 141bp). *Rhabdosargus haffara* mitochondrial COX-1 gene has been deposited in the GenBank Database and assigned the accession number (LC543874.1).

### *2.9. Statistical analysis*

Statistical analysis was performed using SPSS V20. Comparisons of quantitative variables were conducted between groups using the Mann-Whitney *U* test for non-parametric data expressed as (medians or Interquartile ratio) and student *t-test* for parametric data expressed as (mean ± SD). The probability/significance value (P value) ≤ 0.05 was set as statistically significant and ≤ 0.001 as highly significant.

## 3. Results and Discussion

Many studies showed that metals assessment in living organisms could be more accurate indicator for water quality than sediment and water column chemical analysis [36, 37, 38]. The current study focused on the metal accumulation in *Rhabdosargus haffara* liver, muscles, and gills tissues in each of the highly-metal polluted and the less metal-polluted areas. A comparative analysis between the heavy metal concentrations in the fish tissues sampled from the less and the high polluted areas was given in Table (1), and a comparative analysis between summer and winter seasons for fish tissues sampled from the less and the highly polluted areas regarding the content of heavy metals was given in Table (2).

**Iron** concentrations in the less and the highly polluted areas followed the order of: liver > gills > muscles**.** Fe recorded a highly significant difference between the highly polluted and the less polluted areas in muscle tissues at p = 0.004. By comparison between summer and winter seasons, Fe showed a non-significant difference at p-value > 0.05 in liver, muscles and gills tissues in either less polluted or high polluted sites. Iron recorded the highest accumulation in fish tissues in the present study, and this could be attributed to the large quantity of Fe in water, which is mainly due to liberation of Fe as ferrous ions from sediments and industrial wastes effluents [39]. Fe levels in the muscle tissue of fish caught from the less and the highly polluted areas were less than maximum permissible limit (MPL) reported by WHO [40] and FAO/WHO [41].

**Manganese** is an essential metal which exists in limited concentrations in the aquatic organisms due to its role in physiological metallo-enzymes, and for normal functioning of nervous system [40]. Mn recorded its highest average values in gills > liver > muscles tissues. Data also showed that, Mn recorded a significant difference between the high polluted and the less polluted tissues in liver samples at p = 0.02. Between summer and winter seasons, Mn showed a non-significant difference at P > 0.05 in the liver, muscle and gills tissues that collected from the highly polluted sites, but recorded a significant difference at p = 0.05 in gills tissues collected from the less polluted sites. Levels of Mn in muscle tissues of fish caught from the highly polluted areas were higher than MPL (the maximum permissible limit) reported by WHO [40].

**Zinc** is an essential component in most cells, and it is utilized as a cofactor for several enzymes [42]. Zn concentrations in the highly polluted area recorded the following order: liver > muscles > gills tissues. Meanwhile, in the less polluted area, Zn concentrations followed the order: liver > gills > muscles tissues. Results showed that Zn recorded a highly significant difference between the high polluted and the less polluted areas in muscle tissues at p-value = 0.004, also showed a significant difference in gills tissues at p = 0.03. On the other hand, between summer and winter seasons, Zn showed a significant difference at p-value = 0.05 in liver and gills tissues that sampled from the less and the highly polluted sites. Meanwhile, Zn recorded a high significant difference at p-value ≤ 0.05 in liver, muscle and gills tissues collected from the highly polluted sites. Zinc levels in muscle tissue of fish caught from the highly polluted areas were higher than MPL reported by FAO [43].

**Copper** is an essential component for numerous oxidation reduction enzymes as (cytochrome oxidase, uricase and tyrosinase), but a very high intake can cause adverse health problems including liver and kidney damage [44]. Cu recorded its average values as the following order: liver > gills > muscles. Cu recorded a highly significant difference between the highly and the less polluted areas at p-value **=** 0.004 in liver, muscles, and gills. Cu recorded a significant difference between summer and winter seasons at p-value = 0.05 in liver tissue from the less polluted area. Cu levels in muscles did not exceed the permissible levels of WHO [40], FAO/WHO [41], FAO [43], and EU limits [45].

**Nickel** average concentrations in the highly and the less polluted areas followed the order: liver > gills > muscles. Ni showed a highly significant difference between the highly and the less polluted areas in the liver at p-value = 0.01, as well as in gills at p- value = 0.002. By comparison between summer and winter seasons, Ni recorded a significant difference at p = 0.05 in the liver examined from the less polluted stations, also recorded a significant difference at p = 0.05 in muscles examined from the highly polluted sites.

**Lead** is considered a toxic metal to aquatic life; it is known as one of the environmental contaminants which cause intensive deterioration to human health [46]. In the highly polluted area, Pb average concentrations followed the order: gills > liver > muscles. While, in the less polluted area, Pb followed the order: liver > gills > muscles. Results showed that Pb recorded a highly significant difference between the highly polluted and the less polluted areas in muscles and gills at p-values = 0.004 and 0.002 respectively, also recorded a significant difference in liver at p = 0.05. As for the comparison between summer and winter, Pb showed a significant difference at p = 0.05 in gills examined from the less polluted sites. Lead levels in the muscles of fish from the highly polluted area were higher than the MPL of FAO/WHO [41], FAO [43], EU limits [45], and EC limits [47]; while, Pb levels were higher than the MPL of EU limits [45], and EC limits [47] in the muscles of fish from the less polluted area.

**Cadmium** excessive exposure could increase reproductive, hepatic, skeletal, renal, and pulmonary toxicity consequences and cancer [48]. Cd may replace Zn in certain enzymes, and severely limited oxygen metabolism of mitochondria in the liver of fish [49]. In the high polluted area, Cd average concentrations followed the order: liver > gills > muscles. While, in the less polluted area, Cd average concentrations followed the order: liver > muscles > gills. Cd recorded a highly significant difference between the highly polluted and the less polluted areas in all tested tissues; liver, muscles and gills at p-values = 0.004, 0.004, and 0.002 respectively. Between summer and winter seasons, Cd showed a significant difference at p = 0.05 in the liver tissue of fish caught from the less polluted sites. Cd levels in muscles were higher than the permissible levels of FAO [43], EU limits [45], and EC limits [47], but less than the permissible levels of WHO [40].

**Table (1):** Comparative analysis between the heavy metals content in the fish tissues caught from the less and the high polluted areas (expressed as a total average from both seasons values).

| Metal (µg/g) | Liver tissue | | Muscle tissue | | Gills tissue | | Maximum Permissible Limit of heavy metals in fish muscles | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | | | | | | | | | | |
| | Less polluted | Highly polluted | Less polluted | Highly polluted | Less polluted | Highly polluted | FAO | FAO/WHO limit | WHO | EC limits | EU limits |
| **Cd** | 0.8±0.2 | 2.0±0.2 | 0.2±0.06 | 0.6±0.08 | 0.1(0.4)* | 0.7(0.8)* | 0.05 | 0.5 | 1 | 0.05 | 0.1 |
| | p=**0.004** | | p=**0.004** | | p=**0.002** | | | | | | |
| **Cu** | 4.5±2.3 | 12.0±1.3 | 1.5±0.6 | 4.7±1.5 | 2.2±0.6 | 8.4±0.9 | 30 | 30 | 30 | - | 10 |
| | p=**0.004** | | p=**0.004** | | p=**0.004** | | | | | | |
| **Fe** | 55.2(72)* | 131±59 | 8.1±2.4 | 24.2±12.5 | 29(34)* | 46(52)* | - | 100 | 100 | - | |
| | p=0.07 | | p=**0.004** | | p=0.07 | | | | | | |
| **Mn** | 4.2±2.5 | 9.5±2.7 | 1.0±0.2 | 2.9±1.3 | 5.4(5)* | 9.6(7)* | - | - | 1 | - | |
| | p=**0.02** | | p=0.06 | | p=0.06 | | | | | | |
| **Ni** | 1.5(2.8)* | 5.2(3)* | 0.3(0.6)* | 0.4(1.2)* | 0.5(0.8)* | 2.4(2.2)* | - | - | - | - | |
| | p=**0.01** | | p=0.1 | | p=**0.002** | | | | | | |
| **Pb** | 3.0±1.2 | 5.5±2.2 | 0.5(0.8)* | 2.7(3)* | 2.1(4)* | 8.3(4)* | 0.5 | 0.5 | 2 | 0.2 | 0.1 |
| | p=**0.05** | | p=**0.004** | | p=**0.002** | | | | | | |
| **Zn** | 39(45)* | 71(59)* | 2.3(1.5)* | 37(61)* | 7.2(10)* | 24(58)* | 30 | 40 | 100 | - | |
| | p=0.08 | | p=**0.004** | | p=**0.03** | | | | | | |

*: median values as data are not normally distributed in this group.

**Table (2):** Comparative analysis between summer and winter seasons for fish tissues caught from the less and the highly polluted areas regarding the content of heavy metals.

| Metal (µg/g) | Liver tissue | | | Muscle tissue | | | Gills tissue | | |
|---|---|---|---|---|---|---|---|---|---|
| | Less polluted areas (Median) | | | | | | | | |
| | Summer | Winter | p-value | Summer | Winter | p- value | Summer | Winter | p- value |
| **Cd** | 0.9 | 0.5 | **0.05** | 0.2 | 0.1 | 0.3 | 0.3 | 0.2 | 0.8 |
| **Cu** | 2.4 | 6.5 | **0.05** | 0.9 | 1.9 | 0.1 | 2.5 | 2.2 | 0.5 |
| **Fe** | 79 | 25±5 | 0.2 | 9.0±2.7 | 7.1±1.9 | 0.3 | 36 | 27 | 0.5 |
| **Mn** | 3.8 | 3.0 | 0.8 | 0.9 | 1.1 | 0.3 | 3.7 | 8.0 | **0.05** |
| **Ni** | 2.7±0.5 | 0.2±0.09 | **0.05** | 0.6 | 0.08 | 0.3 | 0.5 | 0.6 | 0.8 |
| **Pb** | 4.0±0.9 | 2.2±0.7 | 0.13 | 0.2 | 0.8 | 0.8 | 4.4±1.2 | 1.0±0.2 | **0.05** |
| **Zn** | 37 | 16 | **0.05** | 2.3±1.5 | 2.2±0.6 | 0.8 | 7.7 | 6.6 | **0.05** |
| Highly polluted areas (Median) | | | | | | | | | |
| **Cd** | 2.0 | 2.0 | 0.3 | 0.6 | 0.6 | 0.7 | 1.0 | 1.2 | 0.8 |
| **Cu** | 11.4±0.9 | 13±1.3 | 0.1 | 5.4±1.7 | 4.0±1.1 | 0.3 | 7.8±0.7 | 9.0±0.5 | 0.13 |
| **Fe** | 111 | 160 | 0.5 | 28 | 20 | 0.8 | 49 | 42 | 0.3 |
| **Mn** | 9.0±4.0 | 9.7±1.7 | 0.8 | 3.0±0.9 | 2.8±2.0 | 0.5 | 5.9 | 9.8 | 0.3 |
| **Ni** | 6.5 | 4.8 | 0.5 | 1.3 | 0.3 | **0.05** | 3.0±1.0 | 3.0±1.4 | 0.5 |
| **Pb** | 6.7±2.7 | 4.6±0.9 | 0.18 | 3.2±1.6 | 2.8±1.9 | 0.5 | 9.3±2.6 | 9.0±2.0 | 0.8 |
| **Zn** | 85 | 44 | **0.05** | 66 | 8.4 | **0.004** | 63 | 21 | **0.05** |

*mean* ±*SD* = value expressed for normally distributed data.

In general, the recent study indicated that all metals in the liver, gills, and muscles tissues tested of *Rhabdosargus haffara* fish collected from the highly polluted areas showed more concentrations compared to fish tissues from the less polluted areas. Cd, Cu and Pb recoded highly significant differences at $p \leq 0.01$. Between summer and winter seasons, Mn, Zn, Cu, Ni, Pb, and Cd recorded a significant differences at $p \leq 0.05$ in fish tissues collected from the less polluted and/ or the highly polluted areas. These findings maybe attributed to seasonal variations and water temperature with subsequent influence of detoxification rate and accumulation of toxicants [50].

Diversity in the tested organs ability for metals accumulation in the current results showed that Fe, Zn, Cd, Cu, and Ni were the highest in the liver. This is may be attributed to the fact that the liver is the major organ for any toxic substance and is responsible for detoxification and xenobiotic metabolism [11]. Therefore, it is the target organ for Cu, Zn, and Fe accumulation. Furthermore, the liver also recorded high concentrations of Cd which could be explained by the ability of cadmium in substitution the normally metallothionein (MT)-associated essential metals in liver tissues [54]. Similar findings of high Cd, Zn, and Cu in the liver tissues were recorded in several previous studies [37, 51, 52]. In the present study, liver tissues collected from both high and less polluted sites recorded the highest values of Fe as compared with gills and muscle tissues. In general, iron tends to be concentrated in hepatic tissues due to the liver physiological function in hemoglobin and blood components synthesis [53]. The variation in accumulation potential between tissues could be due to the activity of metallothioneins, which are metal-binding proteins that can restrict specific heavy metals and subsequently facilitate their accumulation in the tissue with a high extent [54, 55, 56]. In other words, the excessive aggregation of several metals in the hepatic tissues could be as a result of the high inclination of the elements to combine with the amino group, sulfur, nitrogen, oxygen or carboxylate of the mercapto group in the metallothionein protein, which exists in higher concentrations in the liver [57]. Furthermore, metallothioneins do not bind lead metal [56], which could demonstrate why the present study found lower levels of Pb in the liver tissues.

Gills are the prime path of metal ion exchange from water; because of having a very large surface area which facilitates the fast diffusion of metals [48, 59]. The present study indicated that only Mn showed higher concentrations in gills of fish collected from both less and high polluted sites than liver and muscle; meanwhile, Pb showed higher concentrations in gills of fish caught from the highly polluted sites. The reason of that the highest accumulation of Mn and Pb was in the gills tissues could be attributed to the metal complexion with the mucus; therefore, it is so difficult to be completely removed from the lamellae prior tissue preparing for analysis [60]. In addition, high loads of some metals in gills may be due to that the water as the major source of contamination, so it is believed that the accumulated metals in gills are principally concentrated from water [61]. High concentrations of Pb in gills tissues were reported in several studies [48, 62, 63, 64].

Muscles tissue, the primary edible fish part, can directly impact the consumer health. Muscles tissues showed the least concentrations of most of the analyzed metals.

**Table (3):** Comparative analysis between ALP activity, Total Protein (TP) content, Specific Activity (S.A) and COX-1 gene expression in fish liver caught from (the less polluted & the highly polluted areas) in both seasons.

| Parameter | Less polluted | Highly polluted | p-value |
|---|---|---|---|
| | Mean ± SD | | |
| **ALP (U/L)** | 945±136 | 900(898)* | 0.4 |
| **TP(g/dl)** | 8.4±2.7 | 7.7±2.0 | 0.4 |
| **S.A(U/mg protein)** | 121±35 | 121(99)* | 0.5 |
| **RQ (COX-1)** | 0.98(2.3)* | 11.5(19)* | **0.001** |

∗= the value is presented as median (Interquartile ratio); RQ: relative quantity.

The tested metals concentrations in muscle tissues were on reasonable average more than in liver and gill samples; but, in the polluted area, the levels of heavy metals in fish muscles have exceeded the permissible limits reported by some of world health organizations. However, the muscles tend to aggregate less metal, and this could be attributed to that muscles are not active tissues in accumulating heavy metals [58].

Phosphatases are recognized as sensitive enzymes for metal exposure and could be utilized to predict the toxicity of metals; any change in alkaline phosphatase (ALP) activity directly affects the fish metabolism. In the assessment of ecotoxicological effects, ALP enzyme could be used as biomarkers of heavy metal intoxication due to its sensitivity to metal contamination [14, 15]. As shown in Tables (3&4); ALP activity, Total protein, and Specific activity in the liver tissue of *Rhabdosargus haffara* fish recorded a non-significant differences at p-value > 0.05 between the less polluted and the highly polluted areas.

It was found that the high values were recorded during the summer season as compared to the winter season, especially in the highly polluted area that recorded a highly significant difference at p-value = 0.001 between the two seasons. Also, total protein content showed highly significant difference at p = 0.003 in the liver tissue of fish caught from the highly polluted sites during summer season. This could be related to the fact that during the summer season, the fish metabolic activities are high and the food availability increases so the fish growth increases. Since ALP enzyme is related to the growth in fish, as expected these higher values during the summer rather than the winter season [67]. On the other hand, in the less polluted area, Cd, Ni, and Zn metals recorded higher values in liver tissue collected in summer than those in winter with a significant difference at p = 0.05. Meanwhile, in the highly polluted area, only Zn metal recorded higher values in summer than those in winter with a significant difference at p ≤ 0.05. This may be indicated that Cd, Ni, and Zn metals could affect ALP activity.

ALPs as metalloenzymes have metal ions in their structures such as $Zn^{2+}$ and $Mg^{2+}$ existing at the active site pocket and thus playing a direct catalytic role, therefore this means that ALP is classically considered $Zn^{2+}$ and $Mg^{2+}$ dependent enzyme [66, 69]. Zinc ion catalyzes the phosphate esters hydrolysis by interacting with phosphate through oxygen bridges. Metal ions either interfere with calcium or zinc dependent processes or substitute for these ions in enzymes and regulatory proteins [65]. Consequently, most of divalent cations, in excessive amounts, especially ($Zn^{2+}$) can bind to an otherwise unreactive substrate thence in most phosphotransfer reactions bind to nucleoside 5- di- and tri-phosphates due to their inability for reacting in the metal-free patterns [68]. So, it is normal to say that ALP activity in *R. haffara* liver tissues could be strongly affected by Zn concentrations in that tissue.

Many studies focused on the relationship between ALP activity and heavy metal contamination in aquatic organisms; Sonawane [70] indicated that ALP showed a constant decrease in ALP activity in the bivalve *Lamellidens Marginalis* after the heavy metal stress, which is in agreement with Sharifian et al. [66] who showed that some metals had inhibitory effect on ALP activity, and Jiang et al. [16] who observed that ALP and ACP in the *Carassius auratus gibelio var* spleen were most sensitive to Cu stress, so could be used as desirable biomarkers for Cu contamination in the aquatic organisms.

**Table (4):** Comparative analysis between ALP activity, Total Protein (TP) content, Specific Activity (S.A) and COX-1 gene expression in fish liver caught from the polluted sites during summer and winter seasons.

| Parameter | Less polluted | | p-value | Highly polluted | | p-value |
|---|---|---|---|---|---|---|
| | Summer | Winter | | Summer | Winter | |
| | Mean ± SD | | | Mean ± SD | | |
| **ALP (U/L)** | 996±125 | 861±115 | 0.06 | 1169±132 | 316±66 | **0.001** |
| **TP(g/dl)** | 8.7±3.0 | 7.0±2.0 | 0.64 | 8.0±2.3 | 6.7±1.0 | **0.003** |
| **S.A(U/mg protein)** | 124±34 | 117±41 | 0.8 | 156±50 | 47±6.8 | **0.001** |
| **RQ(COX-1)** | 1.0±0.9 | 2.0±0.9 | 0.03 | 8.2 (5.7)* | 21.2(18)* | **0.001** |

∗= the value is presented as median (Interquartile ratio); RQ: relative quantity.

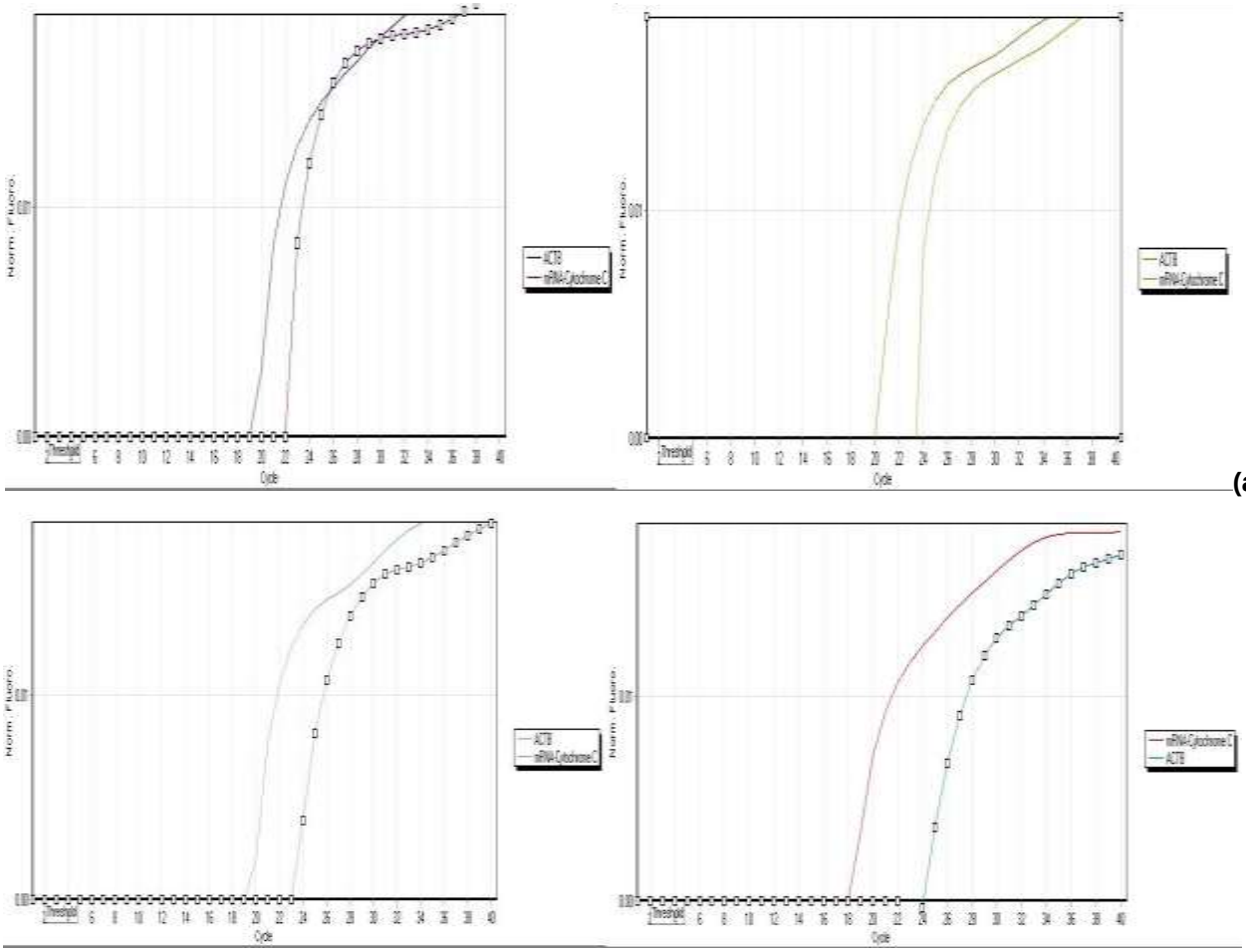

**Fig (2):** A comparison between the amplification plots of COX-1 in liver tissue caught from the less polluted area in summer and winter seasons (a) and the highly polluted area in summer and winter seasons (b).

**Table (5):** The comparison with recommended values and the EDI and EWI of metals in *Rhabdosargus haffara* fish edible part from Suez Gulf in Egypt.

| Metals | Average values | EDI[a] | EWI[b] | PTWI[d] | PTWI[e] | PTDI[f] | THQ[g] |
|---|---|---|---|---|---|---|---|
| **Cd** | 0.40 | $3.67\times10^{-4}$ | $2.57\times10^{-3}$ | 7 | 490 | 70 | 0.367 |
| **Cu** | 3.10 | $2.85\times10^{-3}$ | 0.019 | 3500 | 245000 | 35000 | $7.12\times10^{-3}$ |
| **Fe** | 16.15 | 0.015 | 0.105 | 5600 | 392000 | 56000 | 0.021 |
| **Mn** | 1.95 | $1.79\times10^{-3}$ | 0.013 | 980 | 68600 | 9800 | 0.013 |
| **Ni** | 0.35 | $3.22\times10^{-4}$ | $2.25\times10^{-3}$ | 35 | 2450 | 350 | 0.016 |
| **Pb** | 1.61 | $5.60\times10^{-4}$ | $3.92\times10^{-3}$ | 25 | 1750 | 250 | 0.280 |
| **Zn** | 19.7 | 0.018 | 0.126 | 7000 | 490000 | 70000 | 0.060 |

[a]Estimated daily intake (mg/day/70 kg body weight); [b]Estimated weekly intake (mg/week/70 kg body weight); [d]Provisional Tolerable Weekly Intake (mg/week/ kg body weight) [74]. [e]Provisional Tolerable Weekly Intake for 70 Kg adult person (mg/week/70 kg body weight). [f]Provisional Tolerable Daily Intake for 70 Kg adult person (mg/day/70 kg body weight).[g] Target Hazard Quotient (If the ratio is <1, there is no obvious risk).

On the opposite side, Ismail et al. [71] recorded significantly ($p < 0.05$) higher values of serum ALP activities in the Nile tilapia fish collected from the highly polluted locations than those in the samples from the locations with less grade of pollution. Anyway, heavy

metals can inhibit or activate their activities, in either cases, this causes undesirable effects [17].

Cytochromes are present at high levels in the liver, representing 1-2% mass of hepatocytes [20]. Cytochrome enzymes are one of the most intensively studied biomarkers, in both laboratory and field conditions. For example, cytochrome P 450 levels had been determined as an organism's response biomarker to the presence of pollutants in the aquatic environment in many studies [21, 72, 73, 74]. COX is a terminal key enzyme in the electron transport chain (ETC), located in inner mitochondrial membrane, any increase in COX protein and both elevated oxygen consumption potential followed by an increase in oxidative ATP production capacity [75]. Cytochrome oxidase gene was first recorded in the Egyptian Red Sea only in *L. corrugatus* crab [76]. COX subunit-1 mRNA expression was studied in fish species in previous research [77, 78, 79].

As shown in Table (3), COX-1 relative quantitation (RQ) mean of the highly polluted area was up-regulated by 11.5 - fold as compared to the less polluted area 0.98 - fold with a highly significant difference at p-value = 0.001. A possible explanation is that the up-regulation of COX-1 gene and potential subsequent increase in enzymatic activity could help in protecting and resisting the organism against the impact of its surrounding metal- contaminated environment and therefore induced the elevation of enzyme mRNA expression to enable the fish to survive. This prediction was in agreement with Lushchak [65] who reported that fish need high plasticity and adaptive potential to survive in such diverse environments, in addition, although aquatic conditions are principally more stable than terrestrial, environmental factors can still change significantly in many cases demanding adequate responses from fish. If fish adaptive potential is confused by external factors, organisms may enter stress conditions including changes in salinity, oxygen availability, temperature and ion composition, as well as exposure to pollutants (e.g. heavy metals) resulted from natural processes or human activity.

In addition, COX receives an electron from each of 4 cytochrome c (cyt c) molecules and transfers them to one $O_2$ molecule, converting the molecular oxygen to 2 molecules of $H_2O$. During this process it binds 4 protons from the inner aqueous phase to make 2 $H_2O$ molecules, and translocate another 4 protons across the membrane, increasing the transmembrane difference of proton electrochemical potential, which the ATP synthase then utilizes in ATP synthesis that is needed for all the biological processes in the fish to stay alive, as a way of fish adaptation [80]. The present results were compatible with Craig et al. [74] who exposed the soft water-acclimated Zebrafish to 8 and 15 µg/l Cu for 48 h, this exposure resulted in significant increases in gene expression of COX, associated with increased Cu concentrations in the liver and gills; in addition they recorded a progressive increase in liver COX mRNA expression with a high Cu exposure, with a significant eightfold increase in expression after 48 h and no changes in the gene expression of COX gene expression in the gills.

Reactive oxygen species (ROS), particularly superoxide anions, are produced invariably as byproducts of normal cellular metabolism and the mitochondria are the most significant site of ROS production. These ROS are highly reactive entities and can bring about damage to all kinds of molecules in the body; excessive production of ROS and the associated cytotoxic effects are generally called oxidative stress. Direct oxidative stress is caused by diverse substances directly entering the cellular redox cycles such as metal ions. However, the liver also possesses a high metabolic rate, which also makes it a target for oxidative damage [81].

Many of defensive mechanisms for counteracting the influence of ROS are reported in many aquatic animals, especially fish [82]. In eukaryotes, under aerobic conditions more than 90 % of oxygen consumed is reduced directly to water by cytochrome oxidase in the ETC using four-electron mechanisms without ROS release [83]. Although over 90 % of oxygen consumed by organisms is used to produce energy in the form of ATP (via coupling of the ETC with oxidative phosphorylation), much less than 10 % of oxygen consumed is reduced via one-electron successive pathways that begin with the conversion of molecular oxygen to the superoxide anion radical ($O_2^{\cdot-}$). The further reduction via a one-electron mechanism is the formation of hydrogen peroxide ($H_2O_2$), hydroxyl radical ($HO^{\cdot}$) and water. These intermediates possess unpaired electrons in external electron orbitals and thus are termed free radicals, while $H_2O_2$ does not possess such electrons and is not a free radical. All three species $O_2^{\cdot-}$, $H_2O_2$ and $HO^{\cdot}$ are more active than molecular oxygen and collectively are called ROS [65].

Hüttemann et al. [84] concluded that cyt c act as $O_2^{-}$ scavenger in the cell, so ROS neutralization and the redox capabilities of cyt c make it a perfect antioxidant for the cell. Very low cyt c concentrations in the cell strongly and specially inhibit the ROS formation under conditions of reverse electron transfer points to a functional significance of this process. However, the superoxide-oxidizing activity of cyt c can represent a line of the antioxidant defence of aerobic cell [85]. Although the exact molecular mechanisms responsible for enhancement of antioxidant potential are not fully established, it seems that up-regulation of the expression of certain genes is crucial [65].

The continuous exposure to environmental toxicants, e.g. heavy metals; induce a series of toxic effects on mitochondria [22, 23, 24], which originate mainly from mitochondrial respiratory chain dysfunction allowing the release of cyt c into the cytosol to excite apoptosis [25]. Cytochrome c induces apoptosis by allowing a tissue to be purified from those cells that still produce large amounts of ROS. It might be possible that such kind of effects contribute to development of apoptosis when strong pro-apoptotic signals initiate release of all the cyt c molecules from all the mitochondria in the cell [85]. From the foregoing, it became clear that Cyt c and COX play a

significant role in xenobiotics metabolism in general and particularly heavy metals.

As shown in Table (4), when comparing the seasons of winter and summer in each area, the present results showed that liver COX-1 RQ of the highly polluted area during summer was down regulated by (8.2-fold) as compared to winter (21.2-fold) with a highly significant difference at p-value = 0.001. In addition, COX-1 RQ of the less polluted area during summer (1.0- fold) was down regulated as compared to winter (2.0- fold) with a significant difference at p-value = 0.03 Fig (2). This may indicate that the temperature of the fish environment could affect COX mRNA gene expression. Biotic factors, such as metabolic and reproductive status of fish are season-related; which could affect by the environmental conditions (e.g. temperature of water, oxygen level, food availability, salinity, photoperiod, etc.) [86]. Many studies showed that the acclimation of fish to low temperatures induces a compensative increase of COX activity in fish tissues [87, 88]. Moreover, numerous species of fish respond to lower temperature by mitochondrial biogenesis motivation, reflected in an elevation in the activity of COX mitochondrial enzyme [89]. In conclusion, the present study assumed that heavy metal pollution may cause COX overexpression in fish liver with regard of seasonal variations.

The health risk assessment approach was performed to assess the current risk status related with the fish edible tissues consumption of the study area through estimated daily intake (EDI), estimated weekly intake (EWI), Provisional Tolerable Daily/weekly Intake (PTDI/ PTWI) and Target Hazard Quotient (THQ). The calculations showed that for 70 Kg Egyptian adult person, the values of EDI of fish muscle lead to metal consumption lower than the oral reference dose ($R_f$ D) guidelines for all studied metals, thus indicating no health risk due to consumption of metal-contaminated edible tissues in Red Sea *Rhabdosargus haffara*. The THQ values of Cd, Cu, Fe, Ni, Pb, Mn, and Zn for the muscle tissue did not exceed one, indicating that there was no health risk for consuming the investigated fish. Therefore, the consumption of muscle tissue of *Rhabdosargus haffara* at current consumption rate possesses no health problems for consumers.

In summary, the recent comparative study between the highly and the less metal polluted areas for fish tissue samples based on the source samples collection, and between summer and winter seasons indicated that; ALP activity in haffara seabream fish collected from the highly polluted sites and in the area with less degree of pollution revealed non-significant difference at p- value > 0.05. On the other hand, ALP activity recorded a highly significant difference at p- value = 0.001 between summer and winter seasons. Relative quantitation of COX-1 was significantly highly expressed (p- value = 0.001) in liver tissue sampled from the highly polluted area in response to heavy metal pollution as compared to the less polluted area for mRNA gene expression. Moreover, there was a significant difference in COX-1 between the two seasons in the highly polluted area at (p = 0.001) and in the less polluted area at (p = 0.03).

## 4. Conclusion

The current study suggested that the biochemical and genetic expression in *Rhabdosargus haffara* fish sampled from the highly metal polluted area could be due to the sediment and water pollution by heavy metals (Cd, Pb and Cu), and the probable effect of other organic pollutants that discharged into this hot spot industrialized area. Cd, Ni, and Zn metals may effect on ALP activity in the liver with temperature difference (summer and winter). Elevation in mRNA gene expression of COX-1 in the liver tissue of the highly metal-polluted sites indicated that such molecular parameters could be used as sensitive bioindicators for monitoring the heavy metals effect on aquatic organisms. COX can be utilized as a metal contamination biomarker for subsequent studies. The present study analysis was not intended to be exhaustive, only to highlight the probable impact of metal contamination on COX-1 expression in the field study, so it needs further work. Finally, seasonal variation could affect the enzymatic and genetic parameters in the field study analysis including fish.

Health risk assessment of heavy metals in the muscles (edible parts) of the fish indicated that THQ values of Cd, Cu, Fe, Ni, Pb, Mn, and Zn did not exceed the permissible level (less than 1.0) indicates that the potential exposure is within the degree of exposure that is considered safe or acceptable and there is no health risk for consuming the investigated fish. Therefore, the consumption of muscle tissue of Red Sea *Rhabdosargus haffara* at current consumption rate possesses no health problems for consumers.

### Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

### Conflict of interest

The authors declare that they have no conflict of interest.

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