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The Antioxidant defense Responses of Sea cucumber *Holothuria polii* Against Rickettsia-like organism (RLOs) Infection and Heavy Metal Pollution in Alexandria coast

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Abstract

Holothuria polii, the common black sea cucumber (Echinodermata, Holothuroidea), is abundant in the Mediterranean Sea along the Alexandria coast. This species was given great value commercially and for human consumption. The present study was objective to assess the biological effect of marine pollution on the sea cucumber, *Holothuria polii* collected from two locations in Alexandria coast; Abo-Qir station as an industrial area compared with Miami station as a reference area, using the oxidative stress biomarkers approach, heavy metals bio-accumulation, and Rickettsia-like organism (RLOs) infection. Sea cucumber samples were collected from both stations in Alexandria, during two seasons; winter (January) and summer (July) 2016. Atomic absorption spectrophotometer was used to detect Zn, Mn, Pb, Cu, and Cd in the body wall of the animal as well as in water samples.

The results showed a significant decrease in the antioxidant parameters; catalase (CAT), superoxide dismutase (SOD), Glutathione reductase (GSH), and glutathione transferase(GST) activities, while the Malondialdehyde (MDA) level was significantly increased in the body wall tissue of sea cucumber collected from Abo-Qir station as compared with Miami station. The concentrations of heavy metals (Zn, Mn, Pb, Cu, and Cd) in the body wall of sea cucumber and seawater samples at the two stations differed significantly, showed an increase in the industrial area (Abo-Qir station) compared with the Miami station. As parasites are ubiquitous in the marine pollutant environment, so the present study proved that the sea cucumber samples collected from the Abo-Qir station were infected by Rickettsia-like organism (RLOs), found in the digestive tract and the respiratory tree of this marine animal. The present results of pollutant biomarkers indicated that the degree of pollution increased in summer as compared with the winter season in the two locations.

This study also suggested that the sea cucumber was an ideal bioindicator animal for marine pollution. The suppression of antioxidant biomarkers in the tissue of sea cucumber compared with high values of MDA confirmed the presence of anthropogenic contaminants in the Abo-Qir station, which essentially due to the industrial rejections. Also, Rickettsia-like organism (RLOs) infection and the bio-accumulation of the heavy metals in the tissues of sea cucumber obstructed the antioxidant defense responses in the animal.

Keywords: Oxidative stress, Heavy metals, Rickettsia-like organism (RLOs), Marine Pollution, Alexandria.

Introduction

Sea cucumber *Holothuria polii* is a soft-bodied and worm-like marine invertebrate lives on the seafloor worldwide, but most abundant in tropical shallow-water coral reefs. It belongs to the Phylum Echinodermata; class Holothuroidea, order Aspidochirotida, and family Holothuriidae (Delle Chiaje, 1823). The commercial Sea cucumbers have been well recognized as a tonic and traditional remedy in Japan, China, and Malaysia for their benefits against hypertension, asthma, rheumatism, constipation, burns, and impotence (Hu *et al*., 2010). These medicinal benefits and health functions of sea cucumbers can be attributed to the presence of appreciable amounts of bioactive compounds, such as the triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan, sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, peptides, cerebrosides and lections (Bordbar *et al*., 2011).

In a marine environment, sea cucumbers may accumulate many contaminants such as heavy metals under chronic exposure to rather high levels of chemical stress (Luo *et al.* 2010). Heavy metals are considered critical contaminants since they are toxic, persistent, and non-biodegradable and would inevitably be accumulated and biomagnified in the food chain, exerting deleterious effects on the animals and human health when their concentrations are highly elevated (Spanopoulos-Zarco *et al.* 2014). In marine animals, it has been found that heavy metal accumulation is positively correlated with a metal concentration in diet and ambient water (Julshamn, Grahl- Nielsen, 1996). Water pollution is a serious problem in the global context, heavy metal pollution in the marine environment caused by diverse activities such as phosphate fertilizers, industrial effluent, foundry wastes, paints, mining and rock weathering (Muduli& panda, 2010). The bioaccumulation of contaminates by the tissues and organs of marine organisms has been extensively studied throughout the world and led to the adoption of the bioindicator concept for the environmental quality assessment (Langston & Spence, 1995). The origin of trace elements in the sampled water of the Egyptian Mediterranean sea is mainly the wastewater discharge (Radwan *et al.*,2014).

The importance of oxidative stress response as potential biomarkers of environmental pollution has been addressed by different experimental approaches (Ferreira *et al.*, 2005; Orbea *et al.*,2002). The biomarkers utilized include components of oxidative adaptative responses, such as antioxidant enzyme activities (catalase (CAT), superoxide dismutases (SODs), or glutathione peroxidase (GPX), or the estimation of oxidative damages in lipids, proteins, and DNA (Filho, 1996). When the defense mechanisms are unbalanced regarding the increased presence of ROS generated compounds, e.g. by the presence of pollutants, oxidative damage will occur, indicating a mechanism of toxicity in aquatic organisms. Ferreira *et al,* (2007) mentioned that antioxidant systems can be considered as non-specific biomarkers of exposure to pollutants, and also as an indicator of toxicity.

Rickettsia–like organisms (RLOs) are small, pleomorphic, rod-shaped coccid prokaryotes, most of which are obligate intracellular Gram-negative parasites (Edgerton *et al.*, 2002) some RLOs have been associated with diseases in mollusks and other marine animals (Lauckner, 1983). Despite their importance as causative agents of severe mortality outbreaks in farmed aquatic species, little is known about the life cycle of Rickettsia-like organisms and their host range (Ferrantini *et al.*2009). RLOs may have severe physiological implications in cultured organisms (Branson & Diaz-Munoz 2006), followed by massive mortalities.

This work aimed to describe the relationship between the infection with RLOs found in the sea cucumber *Holothuria polii*, pollution with heavy metals, and the antioxidant defense responses in the animal.

Material and methods:

1-Sampling sites:

Samples of sea cucumber were collected from 5-9m depth at Abu-Qir and Miami stations at Alex seawater (Fig1). Samples were collected seasonally (winter and summer).

2-Sample collection:

Collected samples were kept alive in containers filled with water. Samples of sea cucumber *Holothuria polii* were taken to the laboratory and transferred to well aerate aquaria. 40 specimens were collected from each station. The water temperature was $27c^{0}$ for water sampling. Samples were collected by hand-picking through forceps.

Fig.(1). Map showing the sites of collection. Google Maps.

3-Metal analysis:

The body wall of the sea cucumber was separated to remove all the internal organs before analysis and only the body wall was used for analysis after cleaning. The body walls were weighted (g). Ash samples of each specimen were weighed (g), dissolved in HCl (0.1 mol/L) and further treated with H2O2 (30%) till lucid solutions were formed, and then diluted by water. The 0.45 µm Whatman filter papers were chosen for filtration purposes. Cu, Cd, Zn, Mn, and Pb levels were determined by Perkin Elmer Analyst 700 Atomic Absorption Spectrophotometer, and the detection limits. The levels of different metals in the seawater were also analyzed three times by Atomic absorption spectrophotometer as described. Heavy metals in tissue were expressed as (mg/kg tissue) and in the seawater as (mg/L water).

4- Determination of the antioxidant parameters:

The tissue of the animal was separated and homogenized in TRIS buffer and prepared in ice-cold saline (0.9%), and the homogenized tissues were centrifuged at 3000 rpm at 4ºC for 30 min. The obtained supernatants were used for determination of malondialdehyde (MDA) as a measure of lipid peroxidation (Yoshioka *et al.*,1979), also some antioxidant parameters were measured such as; superoxide dismutase (SOD) activity was measured according to the method of (Beauchamp and Fridovich, 1971), Tissue catalase (CAT) activity was assayed using the method described previously (Claiborn 1986), Glutathione Transferase (GST) activity was measured according to the method described by (Habig *et al.*,1974), and reduced glutathione (GSH) was measured according to the method of (Beutler *et al.*,1963). The results were expressed as (Unit/100 g tissue).

5-Estimation of Total Protein:

Total protein content in the tissue of the animal was estimated according to a method described by (Lowry *et al.*,1951), data expressed as (mg /L).

6- Physicochemical analysis:

Table (1): The physicochemical parameters and the technique /tools used to analyze the Seawater.

Parameter	Technique / Tool
Water temperature (c)	Dry thermometer
Salinity (S%)	Conductivity Salinometer (Backman; model RS.10)
pH	Digital pH meter model 209
Dissolved oxygen (mg/L)	Modified Winkler method (Grasshoff,1976)

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7- Histological examination:

Part of the infected organs (respiratory tree and digestive gland) was fixed in 10% formalin solution. Fixed samples were dehydrated and embedded in paraffin wax (Bell and Lightner, 1988), then sectioned at 5-6µm in thickness using a microtome, mounted on glass slides, and stained using hematoxylin and eosin (H & E) and toluidine blue.

8- Transmission electron microscopy:

Respiratory tree and digestive gland of sea cucumber were fixed in 2.5% glutaraldehyde solution (pH 7.2, buffered 0.1 M phosphate buffer) for 2 -4 hrs at 4˚C and rinsed in 0.1 M phosphate buffer and then post-fixed in 1% osmium tetroxide (OsO4) solution for 2 hrs at 4˚C. After fixation, the specimens were washed with 0.1 M phosphate buffer 4 times for 2 hrs and dehydrated with ascending grades of ethanol, then were embedded in Epon 812, cut at ultrathin sections (70 nm in thickness), and placed on copper grids (200 meshes) to double stain with uranyl acetate and lead citrate. Specimens were examined using a TEM (Jem1200exII, Jeol, Japan).

9-Statistical analysis

Student t-test for comparing between the two seasons in each location was applied; the values are mean ± S.D. for 6 animals in each group. P-Values< 0.05 were considered as significant.

RESULTS:

Metal analysis:

Measurements of heavy metals in water samples in the investigated areas; Abo-Qir and Miami showed a significant increase in values of zinc (Zn), Manganese (Mn), lead (Pb), copper (Cu), and cadmium (Cd) in seawater collected from Abo-Qir during summer. The accumulation of these metals in tissue samples of sea cucumber collected from the same sites showed that the concentrations of metals in the order: Cu>Cd>Zn>Mn>Pb. The concentrations of Zn, Mn, Pb, Cd in tissue samples were significantly higher in the Abo-Qir station during the summer season, while the concentration of Cu showed a non-significant increase (table 2).

Table (2): Comparison between the two locations in each season according to heavy metals.

	Winter			Summer		
	Abo-Qir (n = 6)	Miami (n = 6)	p	Abo-Qir (n = 6)	Miami (n = 6)	p
Heavy metals in tissue (mg/kg tissue)						
Zn	4.6±0.2	4.3@±0.2	0.008*	5.0±0.2	4.6@±0.2	0.003*
Mn	6.2±0.4	4.2@±0.3	<0.001*	6.4±0.3	5@±0.4	<0.001*
Pb	1.9±0.1	0.6@±0	<0.001*	1.9±0.1	1@±0.2	<0.001*
Cu	2.7±0.4	2.4±0.2	0.063	2.9±0.2	2.6±0.2	0.059
Cd	1.2±0.2	0.9@±0.1	0.018*	1.3±0.2	1@±0.1	0.005*
Heavy metals in water (mg/L water)						
Zn	5.2±0.2	4.8@±0.1	0.009*	5.4±0.3	5@±0.2	0.017*
Mn	4.4±0.2	2.6@±0.2	<0.001*	4.2±0.3	3@±0.2	<0.001*
Pb	2.2±0.2	1.1@±0.2	<0.001*	2.4±0.2	1.3@±0.2	<0.001*
Cu	1.9±0.1	1.2@±0.2	<0.001*	1.9±0.1	1.3@±0.2	<0.001*
Cd	1.2±0.2	0.9@±0.1	0.003*	1.3±0.2	1@±0.1	0.008*

p_1: p-value for **Student t-test** for comparing between the two locations in each season
@ Statistically significant between the two locations in each season
*: Statistically significant at $p \le 0.05$

Antioxidant parameters investigation:

The current results showed a significant decrease in the antioxidant parameters; catalase (CAT), superoxide dismutase (SOD), Glutathione reductase (GSH), and glutathione transferase (GST) activities, while the Malondialdehyde (MDA) level was significantly increased in the body wall tissue of sea cucumber collected from Abo-Qir station as compared with Miami station. The protein content in tissue samples recorded an insignificant decrease in Abo-Qir compared with the Miami location (table 3 and Figs 2).

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Table (3): Comparison between the two seasons in each location according to different parameters

	Abo-Qir			Miami		
	Winter (n = 6)	Summer (n = 6)	p	Winter (n = 6)	Summer (n = 6)	p
MDA(Unit/100 g tissue)	47.5±2.4	53.3#±3.5	0.007*	35.7±2.8	41.7#±2.7	0.004*
CAT(Unit/100 g tissue)	80±3.9	70.8#±5.5	0.008*	136.3±7.8	122.7#±7.9	0.013*
SOD (Unit/100 g tissue)	19.7±2.1	17.5±1.9	0.086	34.5±4.5	28.5#±2.9	0.021*
GSH (Unit/100 g tissue)	25.5±3.1	21.5#±1.9	0.022*	52.8±4.9	41.5#±4	0.001*
GST(Unit/100 g tissue)	17.3±2.6	16.3±2.6	0.518	37.5±2.7	36.8±4.5	0.764
Total Protein(mg/L)	15.3±2.2	14.7±1.9	0.580	19.8±2.3	18.5±1.9	0.298

p_1: p-value for **Student t-test** for comparing between the two seasons in each location #: Statistically significant between the two seasons in each location
*: Statistically significant at $p \leq 0.05$

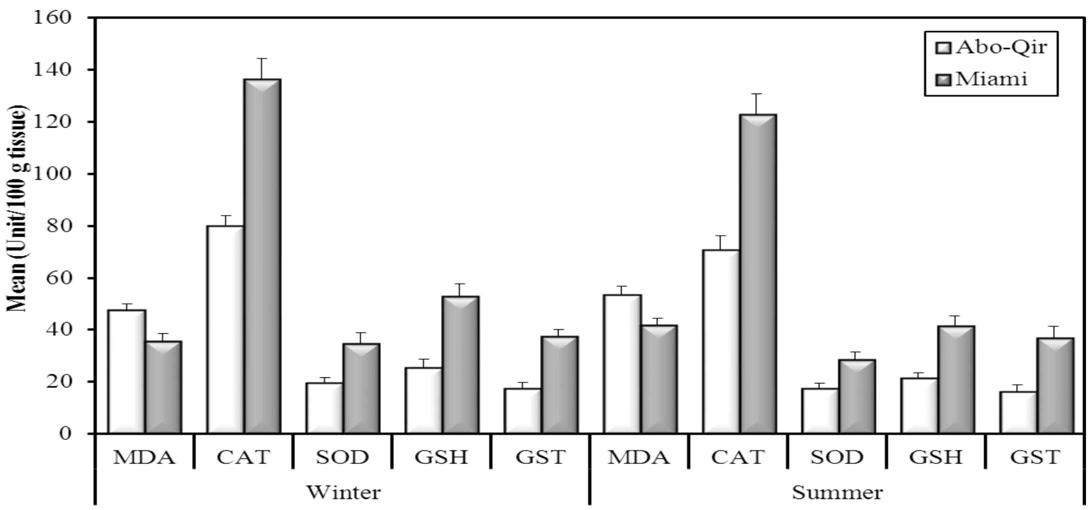

Figure (2): Comparison between the two seasons in each location according to different antioxidant parameters.

Physicochemical analysis:

The ecological investigation of water was done as (salinity, dissolved oxygen (DO), and PH). The results showed an insignificant increase in these parameters in seawater samples were collected from the studied areas Abo-Qir and Miami during summer and winter **(Table 4).**

Table (4): Comparison between the two locations in each season according to physicochemical parameters.

	Winter			Summer		
	Abo-Qir (n = 6)	Miami (n = 6)	p	Abo-Qir (n = 6)	Miami (n = 6)	p
Salinity(S%)	35.1 ± 1.4	34.5 ± 2.1	0.573	36.4 ± 1.2	35.2 ± 1.8	0.204
pH	8.5 ± 1.2	8 ± 1.3	0.505	8.4 ± 1.1	8.2 ± 1.3	0.780
Dissolved Oxygen (mg/L)	5.6 ± 0.6	6 ± 0.7	0.313	5.8 ± 0.9	6.4 ± 1.1	0.326

p_1: p-value for **Student t-test** for comparing between the two locations in each season
@ Statistically significant between the two locations in each season
*: Statistically significant at $p \leq 0.05$

Histology and Ultrastructure:

Histopathological study of the respiratory tree and digestive tract of sea cucumber *Holothuria polii* showed some abnormal changes like external melanized focal lesions of varying size and severity tissues under a light microscope revealed microcolonies of the round, basophilic to purple intra-cytoplasmic inclusion bodies are detected in the digestive tract (Fig.3) and respiratory tree (Fig.4) of Sea cucumber. On the other side, an examination of tissue using a transmission electron microscope revealed the presence of Rickettsia-like organism (RLOs) as round dense inclusion inside the respiratory tree and the connective tissue of the digestive tract (Fig 5&6).

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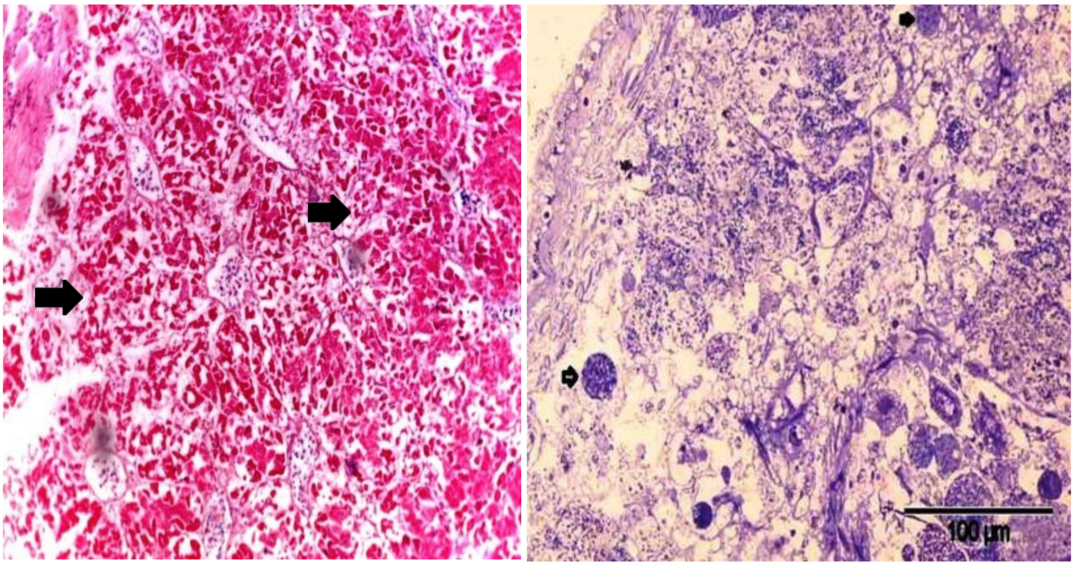

Fig (3): Photomicrograph showing Rickettsia-like organism (RLOs) in the digestive tract in the sea cucumber *Holothuria polii.*

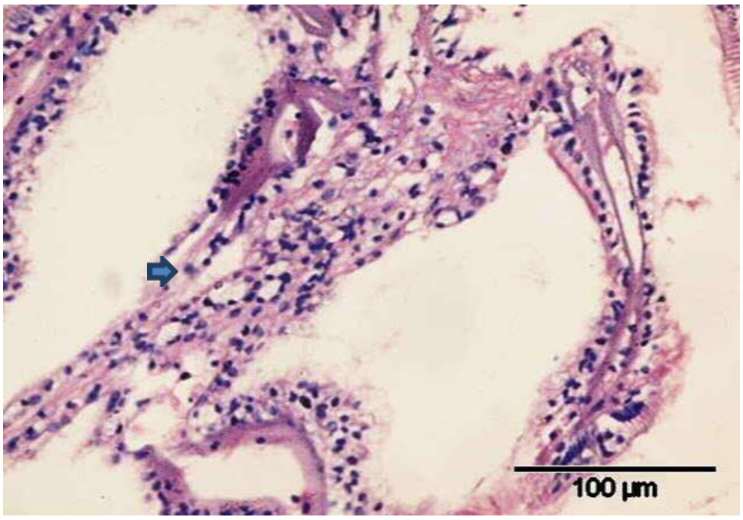

Fig (4): Photomicrograph showing Rickettsia-like organism (RLOs) in the epithelia of respiratory tree in the sea cucumber *Holothuria polii*.

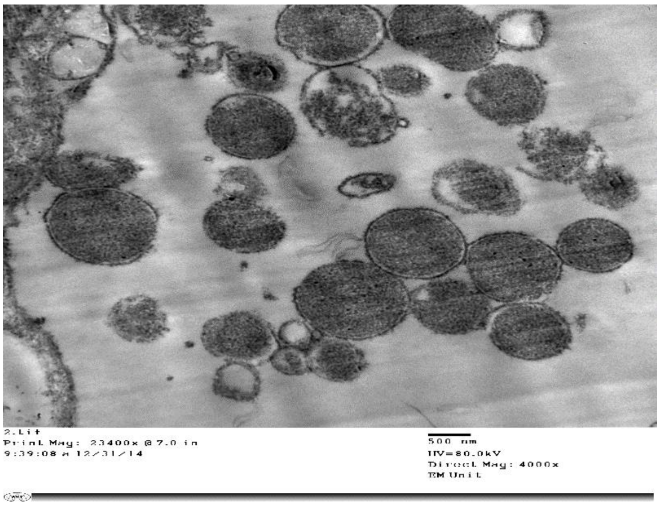

Fig (5): Transmission electron micrograph showing RLOs.in the respiratory tree of the sea cucumber *Holothuria polii*. Bar equal 500nm.

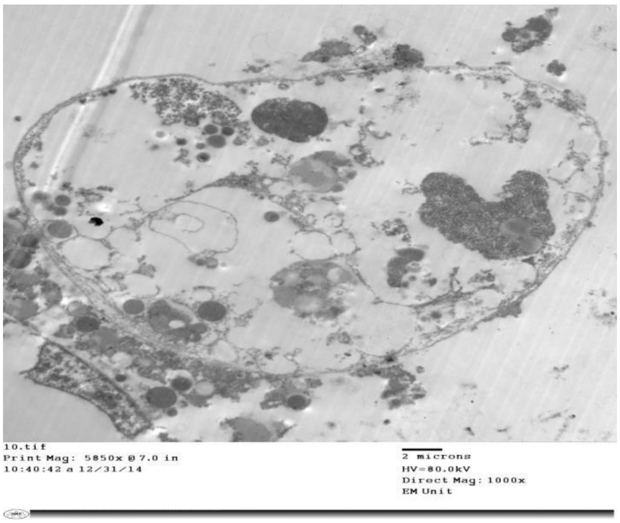

Fig (6): Transmission electron micrograph showing RLOs.in the digestive tract of the sea cucumber *Holothuria polii*.Bar equal 500nm.

DISCUSSION:

The toxicity of pollutants to the organisms are partially mediated by the production of reactive oxygen species (ROS). When the production of ROS exceeds the inherent capacity of an organism's antioxidant defense systems, oxidative stress ensues (Prevodnik *et al.*,2007). Heavy metal accumulation, which represents one of the most widespread and serious forms of environmental contamination, are known to induce oxidative stress by way of enhancement of intracellular reactive oxygen species (ROS) production, which often precludes the onset of alterations, such as protein carboxylation and DNA damage (Nunes *et al.*,2017).

The results of the present work indicated that the increased concentration of heavy metals (Zn, Mn, Pb, Cu, and Cd) in the tissue of sea cucumber, *Holothuria polii* collected from Abo-Qir station as an industrial area compared with Miami station as a reference location. The elevated bio-accumulation of the heavy metals evoked the antioxidant response in the tissue of the animal, but with increased intracellular ROS and lipid peroxidation production (measured by MDA) the antioxidant defense system have been obstructed. According to the current results, the level of malondialdehyde (MDA) was significantly increased in the body wall tissue of sea cucumber collected from Abo-Qir station as compared with Miami station. In contrast to MDA, the activities of the antioxidant parameters catalase (CAT), superoxide dismutase (SOD), Glutathione reductase (GSH), and glutathione transferase (GST), showed a significant decrease in the tissue of sea cucumber collected from the Abo-Qir station as compared with Miami station. The present finding is in agreement with those of previous studies (Pourahmad., *et al*.2001; Collen *et al.*,2003; Lee and Shin 2003) who stated that heavy metals pollution has been previously regarded as provoking factors of oxidative stress. Also, the current results agreed with Hu (2000) who concluded that the heavy metals, such as lead, cadmium, copper, zinc, mercury, and vanadium, could produce reactive oxygen species (ROS), resulting in lipid peroxidation and antioxidant enzyme alterations, leading to oxidative stress. The present pollutant biomarkers data indicated that the degree of pollution increased in Abo- Qir station compared with Miami station, and increased in summer as compared with the winter season in the two locations. This is maybe ascribed to the dumping of toxic industrial waste filled with heavy metals in the area of Abu Qir Bay compared to the open seawater of Miami station. Also, the number of resorts, the movement of industrial ships, it is cleaning, painting, and shedding their wastes increased in summer than winter.

The protein content of the sea cucumber was heavily influenced by environmental contaminants, where it non-significantly decreased in Abo-Qir station as an industrial area compared with Miami station. The current results were following Shacter *et al.*, (1994 a, b) who found that exposure to ROS is known to cause modifications to amino groups of proteins and to alter protein structure or function. One such modification is the formation of carbonyl moieties at amino acid side chains (Shacter *et al.*, 1994 a, b).

The physicochemical parameters (pH, temperature, and salinity) in the present study recorded no significant results. Temperature, salinity, diet, and individual variation are among other factors affecting the accumulation of heavy metals and consequently made cellular oxidative stress (Steniford, 2006). El Moselhy *et al.*, (2005) reported that different animals in the same community at the same trophic levels could accumulate pollutants differently due to differences in habitat physical and chemical properties. The present study indicated that the decrease in pH-value of water at Abo-Qir as (8.4± 1.1) related with the drop in oxygen content (5.8 ± 0.9) and this agreed with Nessim *et al.*, (2005) that recorded pH-values of water at the Abo-Qir site as (7.57-8.26). Salinity reflects the degree of contamination in an aquatic environment (Zyadah *et al.*, 2004). The present study showed that pollution reflects salinity variation in Abo Qir station and this agreed with (Abou-Taleb *et al.*, 2004) who stated that the El-Max Bay area exhibited wide fluctuation in salinity affected by a discharge of huge amounts of agricultural, sewage, and industrial wastewaters.

Rickettsia infections were also found to be significantly higher in deep-sea mussels exposed to petroleum seeps compared to shallow water individuals, although it has yet to be demonstrated that this difference is a direct reaction to contaminant exposure (Powell *et al.*, 1999). In the current study the histopathological examination of the respiratory tree and digestive tract of *Holothuria polii* collected from Abo-Qir in summer showed an infection with rickettsia-like organisms (RLOs) and this with an agreement with Romero *et al.*, (2000) who stated that the rickettsia-like organism (RLO) revealed the presence of different developmental stages and these included a rod-shaped and uniformly electron dense elementary body (EB) and an intermediate body (IB).

Rickettsia-like organism (RLO) colonies were observed inside small granular cells. Azevedo *et al.*, (2005) showed that the ultrastructural morphology of rickettsia–like organisms (RLOs) present in gill epithelial tissue of the oyster, *Crassostrea rizophorae*, from the estuarine region of the Parnaíba river, on the northeastern Atlantic coast of Brazil. Numerous rod-shaped RLOs formed microcolonies that were located in intracytoplasmic vacuoles up to 85 µm in diameter. These RLOs, which measured about 2 µm x 0.6 µm, had ultrastructural characteristics of prokaryotes that included a plasma membrane and a thin, Gram-negative type cell wall. Some non-dividing RLOs had a transversal constriction indicative of binary fission. Numerous free RLOs were seen following disruption of the vacuoles during host cell necrosis and degeneration. The presence of intracellular prokaryotes has been described in several marine invertebrates (Harshbarger *et al.*, 1977&Hine and Diggles 2002), is suggested in some occasions an association with mortalities (Gulka *et al.*,1983). Some authors indicated a key role in the appearance of disease and mortality (Gulka *et al.*, 1983&Villalba *et al.*, 1999), whereas other authors suggested that these microorganisms do not cause damages to the host cells or cause just a limited pathology.

The conclusion of this study could be summarized as, the decreased activity of the antioxidant defense system in the sea cucumber, *Holothuria polii* may be ascribed to both; the infection with rickettsia-like organisms (RLOs) and the bio-accumulation of the heavy metals in the animal tissues which may enhance the production of ROS disrupting the oxidative homeostasis in the animal tissues.

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