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Potential Effects of Heavy Metals Bioaccumulation on Oxidative Stress Enzymes of Mediterranean clam *Ruditapes decussatus*

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ABSTRACT

The bivalves have capability to accumulate the toxicant substances as heavy metals in their body tissues, therefore, they might be used as a good bio-indicators of water contamination. The present work aimed to measure the concentration of Cd, Cu, Pb, Mn, and Zn in the soft tissues of *Ruditapes decussatus* which collected between December 2018 and February 2019 from two sites of Mediterranean Sea. One from Alexandrian Port (Site I) and the other from Port Said (Site II), Egypt, as well as to estimate the potential physiological change of the clam affected by these pollutants. Samples from Site I gives comparatively higher water salinity and metals concentration in soft tissues. The statistical analysis shows significant increase in the level of malondialdehyde (MDA) while superoxide dismutase (SOD) and glutathione peroxidase were found decreased in the *R. decussatus* soft tissue collected from Site I. The correlation coefficient of physicochemical parameters, heavy metals and oxidative stress biomarkers in Site I shows that glutathione peroxidase and superoxide dismutase have positive correlation with acetylcholinesterase (r=0.912) and (r=0.929), respectively. SOD, on the other hand, was having negative correlation with MDA (r= -0.886). The reported values in this study are considered as basic data in monitor of the anthropogenic activities in future along the coast, as well as it is starter point in assessment of pollution that maybe effect on the aquatic organisms in the Mediterranean marine environment.

Keywords: Bivalves, Environment, Heavy metals, Oxidative stress, Protein.

INTRODUCTION

The human activities lead to increase of the environmental pollutants that can penetrate and accumulate in the aquatic organisms, thus effecting their reproduction and health (Andreia *et al*., 2019). These pollutants including, the different forms of released chemicals from industrial works, xenobiotic and pesticides which might be increase the oxidative stress through the reactive oxygen species (ROS) yielding (Zhang *et al*., 2016; Wu *et al*., 2017). The overproduction of ROS caused by toxic heavy metals is considered the main problem as they might lead to changing in the activities of antioxidants enzymes or induce genotoxic effects in the aquatic flora (Nordberg and Arner, 2001). The important roles of the oxidative stress inside the aquatic organism bodies are they have a potential biomarker of the environmental pollutions (Verdelhos *et al*., 2015; Murtala *et al*., 2012). In order to understand the heavy metals toxic effects at the molecular and cellular levels in the aquatic organisms, the multiple antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase should be determining their activities (Bakhtawar *et al*., 2016), in addition to, the genotoxicity, DNA damage and immunotoxicity also required to be identified (Almeida *et al*., 2007; Marisa *et al*., 2016). Similarly, the heavy metals can be accumulated inside the nervous system leading to necrosis and degeneration effects on the marine aquatic species (Gao *et al*., 2012; Mannan *et al*., 2018).

Aquatic biota has been utilized as bio-indicators of environmental pollution due to their capability of pollutants accumulation when exposed to high concentrations of these contaminants such as heavy metals, the different organs of aquatic organisms may accumulate some amounts of these heavy metals (Campillo *et al*., 2013). The aquatic filter feeders such as clams, oysters and mussels due to their tolerance against pollutants are expected to use as bio-indicators and marine biomonitoring (Sayka and Vladimir, 2019). Due to the release of large quantity of untreated sewage and wastewater every year into Alexandria beachfront region through nearby sewage framework (El Khodary *et al*., 2018), the biological organisms such as *R. decussatus* which available in the area under study can be used as bio-indicator for detection the polluted area with high concentration levels of different heavy metals and other toxicants (Abd El Ghany, 2017; Hazrat *et al*., 2019). The main target of the current study is to investigate the concentrations of Cd, Cu, Pb, Mn, and Zn in *R. decussatus* tissues and assess its effects on the biomarkers and antioxidants enzymes.

MATERIALS AND METHOD

Study areas and sample collection

The present investigation was carried out at two sites in the Mediterranean Sea, Egypt. The first site is Alexandria Port (Site I) and the second one is Port Said (Site II). A total of 100 adult *R. decussatus* clam and 30 sea water samples were collected from each site between December 2018 and February 2019 from definite depth consistent to the clam habitation from each location, while, dead or damaged *R. decussatus* clam samples were excluded and only those with identical shell size (3.5-3.8mm) were used. Thereafter, it transported immediately in ice to the laboratory. The

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body soft tissues of clams were isolated from the shell, washed many times with deionized water, dried, kept in sterile plastic bags and saved at −80°C before further working.

Figure (1): The study sites locations map.

Physicochemical analysis of sea water

Determination of physicochemical parameters (salinity, dissolved oxygen and pH) was measured in sea water samples. Salinity was determined using Inductive Salinometer (Beckman mode) according to Grasshoff, (1976). The pH of water samples was measured using the pH meter (JENWAY, 3410 Electrochemistry Analyzer). The dissolved oxygen was measured according to the method of Grasshoff, (1976).

Heavy metal analyses

The concentration levels of Copper (Cu), Zinc (Zn), Mangenese (Mn), Cadmium (Cd) and lead (Pb) were measured in seawater samples and soft tissues of *R. decussatus* using the Flame Atomic Absorption Spectrometers Perkin Elmer-3300 (USA). The obtained results were expressed as µg/l of seawater samples and as µg/g of dry weight of soft tissues (El-Sikaily *et al*., 2004). Before starting the digestion, the tissue samples were dried in air oven at 60-70° C for 72 hours and then grinded by mortar. Twenty ml of both $HClO_4$ and HNO_3 were added to ten grams of the dried samples. The content was digested at 160° C, 5 ml of 20% HCl was added to the residue, filtered with Whatman filter paper No.1, completed to 100 ml with deionized water, then injected for measuring of the concentration (APHA, 1989).

Biomarker measurements

The determination of biochemical markers of oxidative stress in the soft body tissue of *R. decussatus* occurred by measurement of the lipid peroxidation and Malondialdehyde (MDA) (Yoshioka *et al*., 1979), while, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined by using the method of Beauchamp and Fridovich, (1971) and Rao (1996) respectively. Acetylcholinesterase (AChE) activity was estimated by counting the increasing of the sample absorbance at 412 nm for 5 min with using 1 mM of acetylthiocholine as a substrate (Ellman *et al*., 1961). In addition, the total protein content was determined by using the method described previously by Lowry et al., (1951).

Genomic DNA extraction, purification and quantification

The total DNA was extracted and purified from soft tissues by using the DNeasy® Blood & Tissue Kit according to manual's instructions (Qiagen, Valencia, CA, USA). The quality and quantity of DNA samples were measured at wavelength of 260 and 280 nm using Thermo Scientific NanoDrop 2000™ spectrophotometer.

Histopathology

After removing the clams from shells, it fixed in Davidsons' solution (Humason, 1972) for 24 hours and kept in 70 % ethanol. Sections of gonads were put in Bouin's fixing solution, dehydrated through subsequent portions of ethyl alcohol, clearing in terpineol and inserted in paraffin wax and transverse sections 5µm were taken, stained with Gill's heamatoxylin and eosin (Steven, 1990). Sex determination and maturity stages were examined under standard Lietz Dialux 20 EB research microscope.

Statistical analysis

The data were statistically analyzed using the column statistics and one-way ANOVA with Newman-Keuls Multiple Comparison. Data are expressed in mean ± S.D.

RESULTS

Physicochemical analysis of sea water

The physicochemical parameters obtained from analysis of 30 seawater samples collected from the two studied sites; Site I and Site II were indicated in Figure 2. The indicated results show, the highest mean value of salinity as 32.0±0.3 in samples collected from Site I at 2m depth while the highest mean value of dissolved oxygen was 9.8 ± 0.1 mg/l at 1.5m depth in Site II. In addition to, the pH value was recorded 7.84 ± 0.1 for Site I and 7.75 ± 0.06 for water collected from Site II.

Trace elements concentrations in water and soft tissues

The results in Figure 3 represent the heavy metals concentrations in water sampling from Site I and comparing with those of Site II. The mean concentration levels of Cd, Cu, Pb, Mn and Zn in seawater of Site I were reported as 3.0±0.2, 2.6±0.1, 3.9±0.5, 3.6±0.2 and 3.8±0.2 µg/l, respectively while in Site II their respective concentrations were found as 1.6±0.2, 1.6±0.1, 2.5±0.2, 2.3±0.2 and 2.7±0.1 µg/l (Figure 3). These levels of heavy metals detected in Site II were found in acceptable limits as reported by FAO, (1983).

The results show the presence of heavy metals with high concentration in Site I compared to Site II. The mean concentrations level of Cd, Cu, Pb, Mn and Zn (µg/g dry weight) of clam collected from Site I were 3.2±0.3, 4.0±0.2, 4.4±0.8, 4.9±0.1 and 5.2±0.3 respectively (Figure 4). The collected data from Site II

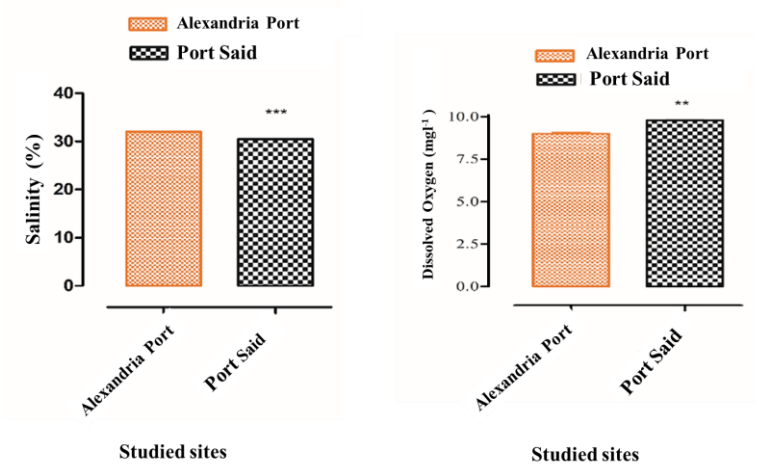

Figure (2): The concentration level of physicochemical characteristics of water samples collected from the two sites. Data expressed in mean ±SE. **, statistically significant at $p \leq 0.05$; ***, statistically significant at $p \leq 0.001$.

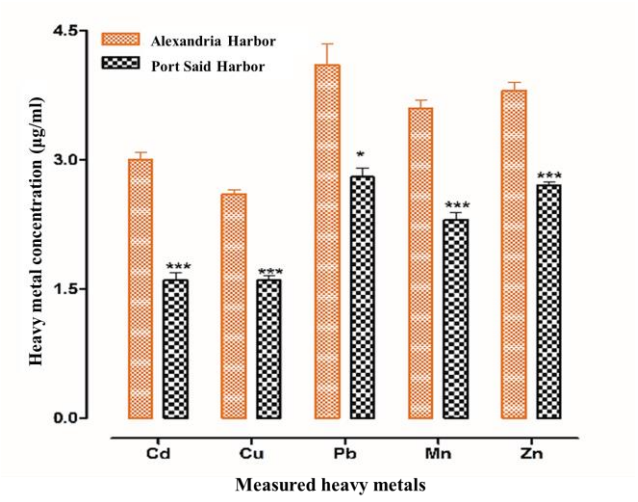

Figure (3): Heavy metal concentration (µg/ml) in sea water of the two studied sites, Alexandria and Port Said harbors. Data are represented in mean ±SD. *, statistically significant at $p \leq 0.05$; ***, statistically significant at $p \leq 0.001$.

indicated the heavy metals in clam tissue were 1.6±0.5, 2.6±0.4, 2.6±0.5, 2.6±0.3 and 3.8±0.8 respectively. The present result reported that the clam collected from Site I had more heavy metals accumulation in clam tissues than that collected from Site II.

Biochemical biomarkers responses

In the current study, the levels of MDA in tissues collected from Site I and Site II reported as 12.9±0.3 U/g and 9.7±0.2 U/g, respectively and this indicates the clams in the Site I were under much stress of pollution than those of Site II. The mean activity values of GPx in Site I was 10.6±0.3 U/g and that of Site II was 11.6±0.3 U/g. The mean activity values of SOD in Site I was 1.8±0.1 U/g whereas, that of the other location was 1.1±0.0 U/g. The total protein in Site I was 5.5±0.6 mg/g while that of Site II was 6.5±0.2 mg/g. Moreover, the AchE in Site I reported as 446.2±1.9 U/l where that of Site II reported as 468.0±1.9 U/l (Table 1).

The RNA /DNA in the Site I was reported as 4.5±0.2 and that of Site II was as 2.9 ± 0.1. The data show significant difference between the sites under investigation at p<0.001.

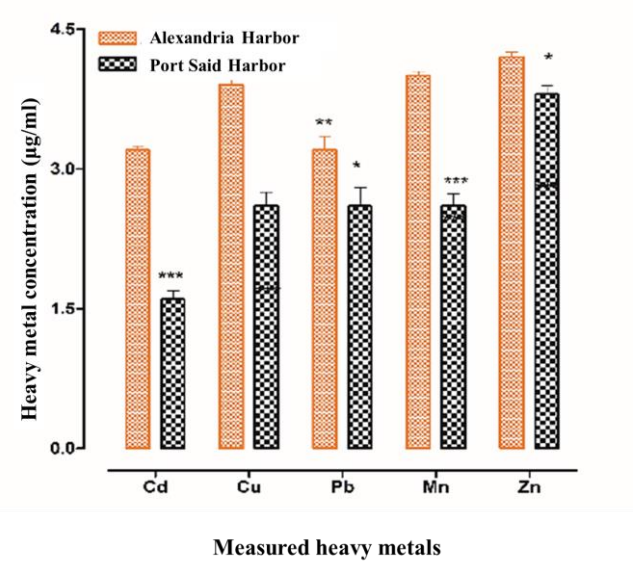

Figure (4): Mean and standard deviation (Mean± SD) of heavy metals in water (µg/l) in the two sites.
Data was expressed by using mean ± S.E. Statistically significant at ***$p \leq 0.001$, $^{ns}p \geq 0.05$.

Histopathology

The parasites were infected the digestive glands causing hypertrophy and necrosis of the host cells (Figure 5 A & B). It infected also, the stomach epithelial cells of the clam as cleared in Figure (5 D & F). The infected cells are lysed the lumen of the digestive tubules and a wide spread evidence of necrosis of the digestive tissues with destruction of large regions of the digestive tissues (Figure 5 B). In the healthy clam tissue, the nuclei were basically located and the individual cells were easily distinguishable (Figure 5 C & E).

Table (1): Antioxidant activity and damage biomarkers in Clam *Ruditapes.* Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities, Total protein content, Acetylcholinesterase (AchE), RNA/DNA and lipid peroxidation (malondialdhyde, MDA level) of *Ruditapes decussatus* collected from the both sites.

Studied Sites	Biochemical Parameters†				
	GPx (U/g)	SOD (U/g)	Total protein (mg/g)	AchE (U/l)	MDA (U/g)
Alexandria Harbor (Site I)	10.6±0.3	1.8±0.1	5.5±0.6	446.2±1.9	12.9±0.3
Port Said harbor (Site II)	11.6±0.3	1.1±0.0	6.5±0.2	468.0±1.9	9.7±0.2
P*	0.001*	<0.001*	0.008*	<0.001*	<0.001*
t-test	5.315*	11.501*	3.532*	18.167*	20.032*

† Data expressed in mean ± SE. *statistically significant at $p \leq 0.05$.

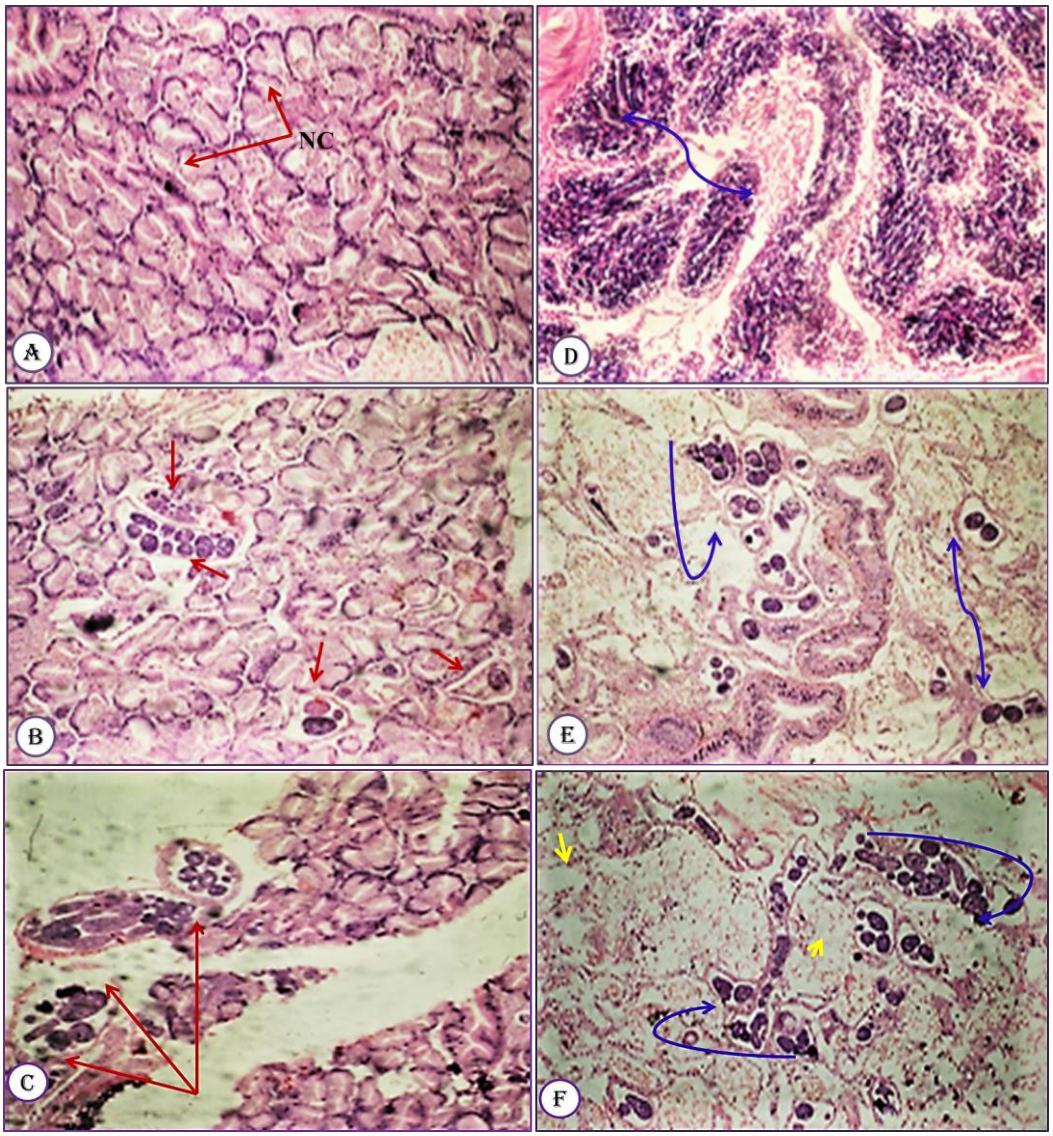

Figure (5): Photomicrograph of transverse section through the digestive gland (A, B and C) and testes (D, E and F) of *Ruditapes decussatus*, (A), normal uninfected digestive gland (NC); (B), mild infected digestive gland with cercaria larvae (arrows); C, heavy infections by cercaria larva causing cell autolysis (arrow); D, normal tissue of testes of the clam; E and F, mild to sever infection that showing the presence of trematode sporocyst with germ cells of different types of sporocyst containing cercariae (blue arrows) and sever infection causes cell autolysis (yellow arrows).

DISCUSSION

The heavy metals maybe accumulated in the aquatic environments comes from different sources such as urban overflow, stations of sewage treatments, industrial chemical wastes, mining industries, ship movement, local waste junkyards and agricultural pesticides overflow (Alemdaroglu *et al*., 2003). Earlier studies demonstrated that, the increasing of pollution sources maybe increase the heavy metal accumulation by aquatic organisms and fishery products (Chandrasekhar *et al*., 2004; Zhang *et al*., 2007).

The current results indicated, the increasing level concentrations of Pb, Cd, Zn, Mn and Cu in seawater and soft tissues of Site I were higher than Site II and this may be due to increasing in the contamination resources, which mainly from the industrial effluents, domestic, sewage effluents and wastewater, that effect directly or indirectly on the physiological functions of aquatic organisms and human being health (Arafa and Ali, 2008). Early studies reported that, the heavy metals are entering into water effluents through industrial, agricultural and municipal wastes and affects the rate of reproductive system of aquatic organisms leading to a gradual extinction of their generations; also, disturb their physiological functions (Shenai *et al*., 2017; García-Medina *et al*., 2017).

The toxic of heavy metals effect at both of cellular and molecular levels were identified through determination the antioxidant biomarkers in the living organisms (Bejaoui *et al*., 2018). The obtained results show a significant increase in MDA concentration in clam soft tissues collected from Site I compared to that of Site II. The heavy metals may lead to increasing of the ROS production and causing the oxidative damage through inhibition of the antioxidant enzymes activity (Dayem *et al*., 2017).

One study reported that, lead positively correlated with the MDA levels and the increasing damage of the cell membrane and subsequently enhances the oxidative stress (Chalkiadaki *et al*., 2015). In addition, the cell damage exhibited positive correlation with the antioxidant enzymes and toxic substances leakage and it frequently accompanied by increasing of the cell membrane permeability (Fernandez *et al*., 2010). The obtained results show high significant decreasing in the total protein and GPx activity of clam collected from Site I compared to those of Site II and this result was in agreement with Parate and Kulkarnim, (2003) who found, at protein content decreasing, this might be due to the proteins degraded into free amino acids which converted to α keto acid and participate the tri-carboxylic acid cycle for ATP production. Furthermore, the decreasing in the total proteins maybe reflect also the decreasing in the oxidative enzymes activity. The exposure to heavy metals was previously reported to decrease the activity of GPx (Maria and Bebianno, 2011) and reduce the capacity to scavenge H_2O_2 (Saidani *et al*., 2019). The contamination of water sources by genotoxic compounds is a worldwide problem (Sahayanathan *et al*., 2018). The probability of using changes in DNA integrity as markers of exposure effects of Geno-toxicants materials has been previously estimated in different aquatic organisms using cytogenetic analysis (Letelier *et al*., 2005).

The poisonous metals are equipped for aggravating the regular oxidation lessening balance in the cells through different components originating from their own particular complex redox responses with endogenous oxidants and consequences for cell cancer prevention agent frameworks (Wiem *et al*., 2019). The subsequent oxidative stress causes DNA harm which may add to metals danger (Tlili *et al*., 2010).

The results of the current research indicate the samples of Site I represented 5% of the samples were parasitized whereas, Site II samples represented only 3% of the samples were parasitized. Environmental stress such as parasites and heavy metal pollutants may have negative effects on the physiological properties of the host organisms, leading to high response to diseases causing agents (Radwan *et al*., 2018).

The data of correlations coefficient in Site I concluded that, the dissolved oxygen showed highly negative correlation with Zn, as r=-0.881 and presence of a positive correlation between Zn and Pb in sea water samples expressed as r=0.891. While, Zn was also positively correlated with Pb in clam tissue and expressed as r =0.975. In addition to, Pb is negatively correlated with AchE as r =0.950 and Zn has positive correlation with RNA/DNA as r =0.905. Finally, Cd is negatively correlated with AchE as r =0962. While, the data of correlations coefficient in Site II show there was high positive correlations among S% and DO as r =0.910. In addition, there was a high negative correlation between Cu and SOD as r=-0.965. Cu has positive correlation with MDA as r= 0.934, whereas, Cd is positively correlated with Cd in the tissue of the clam as r=0.962. Mn is negatively correlated with Zn in the tissues of the clam as r =0.953. GPx has positive correlation with AchE activity as r =0.912 and SOD is also positively correlated with AchE as r=0.929. Finally, SOD is negatively correlated with MDA as r=-0.886.

CONCLUSION

The current study was evaluated the toxic effects of the heavy metals on *R. decussatus*. It reduces and destroys the oxidative stress enzymes of *R. decussatus* soft tissues. Additionally, there are different positive correlation between the oxidative enzymes activity and the inhibition of AChE activity in the soft tissues. Finally, the current results are considered as the baseline data in the biomonitoring of the pollutants along the Egyptian coast and we need more research studies for complete the assessment process of pollution effectors on the aquatic organisms.

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التأثيرات المحتملة للتراكم الحيوي للمعادن الثقيلة على إنزيمات الإجهاد التأكسدي لمحار البحر الأبيض المتوسط من النوع *Ruditapes decussatus*

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الملخص العربي

تتمتع الحيوانات الرخوية البحرية مثل المحّار بالقدرة على تجميع المواد السامة والملوثات مثل المعادن الثقيلة في أنسجة أجسامها، لذلك يمكن استخدامها كمؤشرات حيوية جيدة لتلوث المياه. ولذلك هدفت هذه الدراسة إلى قياس تركيز بعض العناصر الثقيلة مثل الزنك، المنجنيز، الرصاص، النحاس والكادميوم في الأنسجة الرخوة لمحار *R. decussatus* والتي تم تجميعها في الفترة بين ديسمبر 2018 وفبراير 2019 من موقعين في البحر الأبيض المتوسط وهما الإسكندرية (المكان 1) وبورسعيد (المكان 2)، مصر، بالإضافة الى تقدير التغيرات الفسيولوجية المحتملة لأنسجة المحار نتيجة تأثره بتلك الملوثات. واثبتت النتائج ان ملوحة الماء وتركيزات العناصر الثقيلة في انسجة المحار كانت أعلى في العينات المأخوذة من المنطقة الأولى. وكذلك أوضحت التحليلات الإحصائية زيادة معنوية في تركيز المالونديهيد بينما انخفضت مستويات كل من انزيم السوبر اوكسيد ديسميوتيز وانزيم الجلوتاثيون بيروكسيديز في انسجة المحار في المنطقة الأولى. كذلك عند قياس كل من معامل الارتباط للمعلمات الفيزيائية الكيميائية، العناصر الثقيلة والمؤشرات الحيوية لمضادات الاكسدة، كما أظهرت النتائج انه في المنطقة الأولى أعطى كل من انزيم السوبر اوكسيد ديسميوتيز وانزيم الجلوتاثيون بيروكسيديز علاقة إيجابية معنوية مع انزيم الاسيتيل كولين استيريز (r=0.912) و(r=0.929) على التوالي. على الجانب الاخر اعطى انزيم السوبراوكسيد ديسميوتيز علاقة سلبية مع المالونديهيد (r= -0.886). ولهذا تعتبر نتائج هذه الدراسة بيانات أساسية في إمكانية رصد الأنشطة البشرية مستقبليا على طول ساحل البحر الأبيض المتوسط، فضلاً عن أنها نقطة بداية لتقييم التلوث الذي قد يؤثر على الكائنات المائية في البيئة البحرية في حوض البحر الأبيض المتوسط.