EnvironmentAsia
The international journal by the Thai Society of Higher Education Institutes on Environment

EnvironmentAsia 12(2) (2019) 164-171
DOI 10.14456/ea.2019.38
ISSN 1906-1714; ONLINE ISSN: 2586-8861

Metallothionein as a biomarker of heavy metal (Cd, Cu, Zn, Pb, Hg, Ni, Cr) pollution in hermit crab *(Clibanarius signatus)*

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Received: October 14, 2018; Revised: December 5, 2018; Accepted: January 18, 2019

Abstract

A field study was conducted to assess the potential use of metallothionein (MT) as a useful biomarker of heavy metal exposure. Hermit crab (*Clibanarius signatus*) were collected from ten sites from the northern coast of the Sea of Oman. Chemical analyses included the assessment of the heavy metal (Cd, Cu, Zn, Pb, Hg, Ni, Cr) in hermit crab hepatopancreas. MT levels were determined by Spectrophotometeric method. Zn had the highest concentration followed by Cu> Cr> Ni> Pb> Cd> and Hg. The results of spatial evaluation indicated that heavy metals and MT biosynthesis were significantly different ($p<0.05$) among sampling sites. In addition, significant correlations were found between heavy metal levels and selected biomarker ($p<0.01$). The obtained results suggest that MT in *C. signatus* was heavy metal (Cd, Cu, Zn, Pb, Hg, Ni, Cr) inducible and could be useful as a first investigation into the biological effects of heavy metal pollution in coastal areas.

Keywords: Biomarker; *Clibanarius signatus*; Metallothionein; Sea of Oman

1. Introduction

Technological advancement and the development of different industries have resulted in introducing large amounts of industrial and municipal wastewater with various chemical compounds into aquatic ecosystems, especially heavy metals. Heavy metals are considered as one of the most important environmental pollutants which enter the sea through coastal areas and rivers (Benkdad *et al*., 2011).

Heavy metals in the marine environment become bioavailable to crab and other marine organisms through the food chain (Valavanidis *et al*., 2008; Zuloaga *et al*., 2009). All kinds of crab respond to pollutants by adapting or altering their metabolic functions (Ali *et al*., 2008). Accordingly, crab has been used as bioindicator of the chemical contaminants. In this regard, biomarkers display aquatic health levels at lower biological levels whether biochemical or cellular levels (Tolosa *et al*., 2005; Sinaei *et al*., 2010).

Metallothionein is a low-molecular-weight protein (7-6 kDa), 75-57 amino acids, and is considered as a cysteine rich (18-20% in molecular weight) due to the lack of aromatic amino acids.This protein is known as the ultimate destination of some metal ions, which is combined with sulfur atom in the cystein groups leading to the formation of thiolate

clusters (Dabrio *et al.*, 2002; Amiard *et al.*, 2005). Thus, Metallothionein has been proposed as a sensitive biomarker of metal exposure and the prediction of potential detrimental effects induced by metal contamination (Ivankovich *et al.*, 2005; Sinaei *et al.*, 2010).

Hermit crabs are a group of marine crustacean belonging to the Paguroidea family which are ended up to protect their soft body from empty shells (Bilock and Dunbar, 2009; MacLaughlin *et al.*, 2010). These creatures are considered as important members of Macrozeobentic communities in the tidal and subtidal regions around the world (Turra *et al.*, 2002; Biagi *et al.*, 2006; Squires *et al.*, 2001).

Oman Sea is limited to 40,019 km^2 from the north by Makoran Mountains as one of the most important aquatic ecosystems in the world and one of the most important biological resources in Asia based on plant and animal resources, which are rich in genetic diversity. Oman Sea is under destruction due to specific ecological conditions, developmental programs on the coast, rapid development of various industries, especially aquaculture, the construction of harbors and ports, and subsequently an increase in shipping activities. Despite the documented pollution in the Oman Sea, little has been known about biological effects of pollutions in this ecosystem. Biomarker assessment is considered as a way for measuring biological effects in a polluted ecosystem. Thus, the present study aimed:

1: To determine heavy metal concentration in the hermit crab (*C. signatus*)

2: To study the potentiality of MT to detoxify heavy metals in the hepatopancreas of hermit crab (*C. signatus*)

3: To assess the relationships between the selected biomarker and heavy metal pollution in hermit crab (*C. signatus*)

2. Materials and methods

Ten different stations were chosen along the northern coast of Oman Sea (Figure 1). the locations were selected along a pollution gradient based on the earlier information available in literature about the local contaminant levels (Ravanbakhsh *et al.,* 2009). The biota (i.e., *C. signatus*) were collected in December 2017. The Hermit crab (n = 30) were collected from each of the ten sampling sites by using hand nets. The caught specimens were transferred to the laboratory under freezing conditions.

The crabs were immediately dissected and the hepatopancreas was quickly removed at the ice. A part of hepatopancreas tissues was frozen in liquid nitrogen and stored at -80° C until MT assessments. Finally, the remaining parts were

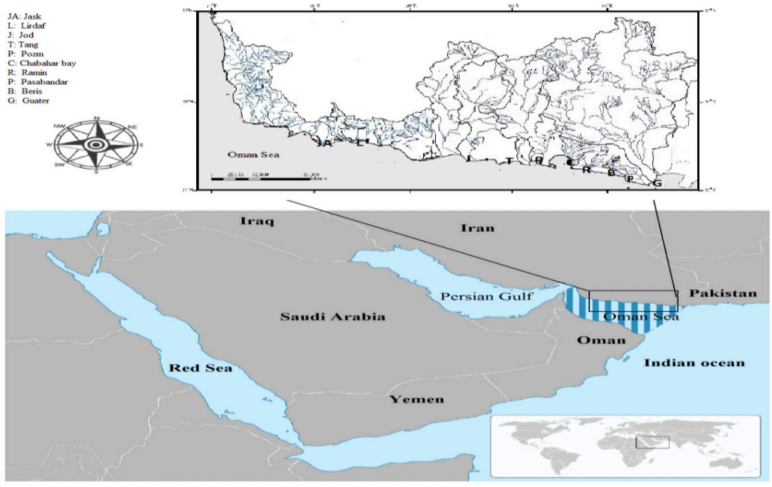

Figure1. Location of all sampling sites

stored at -20° C for the following heavy metals for pending analyses.

2.1 Tissue collection

The hermit crab were immediately dissected and the hepatopancreas was quickly removed at the ice. A section of hepatopancreas tissues were frozen in liquid nitrogen and stored at -80° C until the MT assessments. The remaining parts were stored at -20°C for the following heavy metals pending analyses.

2.2 Chemical analysis

The method proposed by Abuagla *et al.* (2017) and Sinaei and Bolouki (2017) was adopted to extract and purify heavy metals in the hepatopancreas tissue. Hepatopancreas samples were first freeze-dried for 73 hours at – 50°C and 39 $\times 10^{-3}$ mbar. Then, the powdered samples (0.30 g) were digested with quartz- distilled concentrated nitric acid (5 mL), and oxidized more in 10 ml of $KMnO_4$ saturation solution. In addition, a hot plate (HPA2235M) was used for complete digestion under established conditions after initial digestion at room temperature. Further, all samples were analyzed in triplicate by Atomic Absorption Spectrophotometer (Unicam, model 919)

Total mercury levels were determined using cold vapor analysis technique. Powdered samples (0.30 g) were digested in 20 ml of 3:1 concentrated redistilled HNO_3 and concentrated H_2SO_4 and then oxidized with 10 ml of saturated solution of $KMnO_4$. In addition, excess oxidizing agents and mercury ions were reduced by 10 ml of a reducing solution (3% NaBHa in 1% NaOH) in a hydride generator apparatus. Further, mercury was vaporized and measured in the atomic absorption spectrophotometer (Unicam, model 919).

2.3 MT analysis

In order to extract and purify MT, the methods introduced by Sinaei *et al.* (2010) and Andreani et al. (2008) were followed with minor modifications. MT was measured in triplicate for each sample. After thawing, the hepatopancreas samples were individually prepared by homogenization buffer (15 mM cold Tris–HCL pH=7.0) in which 10 mM M_2-mercaptoethanol and phenylmethanesulfonylfluoride (PMSF) as oxidation and protease inhibitors, respectively, were added in 1:3.0 (w/v) volumes by using a Teflon homogenizer (Sigma-Aldrich, Z659428) at 1,000 rpm. The homogenates were centrifuged (Lovibond, Model Z323K) at 12,000 g for 40 min at 4°C. In the next process, the supernatant was heated at 80°C for 10 min in order to denature the thermo-labile proteins and centrifuged again at 12,000 g for 40 min at 4°C. A volume of 1 ml cytosol from a total volume of 1.7 ml was exploited to a Sephadex chromatography column (0.9×90 cm) calibrated with rabbit MT (Abcam) as protein marker, and eluted with the

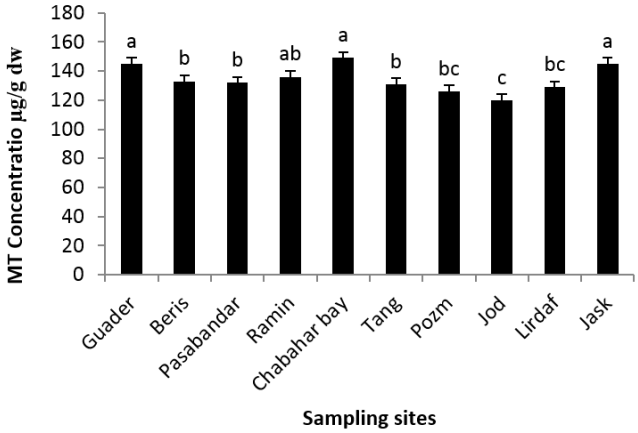

Figure 2. Mean and standard deviation values (µg/g of w.w) of biomarkers

Table.1. Mean and standard deviation values (µg/g of w.w) of Heavy metals (Cd,Cu,Zn,Hg,Ni,Cr,Pb) in the sediment and hermit crab (*C. signatus*).

	Guader	Beris	Pasabandar	Ramin	Chabahar bay	Pozm	Tang	Jod	Lirdaf	Jask
Cd	[a] 0.37±0.06	[b] 0.53±0.09	[c] 0.47±0.08	[d] 0.31±0.04	[e] 0.65±0.09	[a] 0.39±0.05	[f] 0.24±0.04	[g]0.19±0.02	[d] 0.30±0.07	[j] 0.60±0.06
Cu	[a]26.1±1.65	[a] 27.25±1.73	[b] 21.47±1.53	[b] 21.30±1.43	[c] 31.31±1.81	[a] 26.72±1.75	[a] 26.16±1.46	[a] 27.03±1.54	[c] 30.30±1.65	[a] 27.03±1.51
Zn	[a] 80.39±5.3	[b]27.80±1.04	[b] 27.19±1.43	[c] 18.40±1.3	[d] 85.96±5.3	[e] 22.24±1.03	[f] 15.36±1.00	[f] 13.52±0.93	[e] 21.21±1.16	[g] 89.36±4.5
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ni	[a] 11.00±0.93	[ab] 12.11±0.9	[c] 14.21±1.04	[d]7.34±0.68	[e] 18.76±1.3	[df] 8.42±0.93	[d] 7.46±8.9	[f]9.32±0.89	[af] 10.73±0.98	[b] 12.79±0.68
Cr	[a] 26.81±1.95	[b] 16.92±0.95	[c] 20.82±1.07	[d] 12.86±0.7	[a] 29.00±1.15	[e] 25.83±1.19	[bf] 16.31±0.95	[f] 15.24±1.00	[bf] 16.54+1.00	[g] 38.12±1.29
Pb	[a] 9.15±0.84	[bdc] 6.73±0.7	[bd] 6.83±0.6	[d] 7.97±0.85	[e] 13.32±1.5	[f] 11.11±0.83	[bc] 6.36±0.44	[c] 5.83±0.70	[bc] 6.00±0.69	[f] 11.83±0.92

Values followed by the same letter vertically are not significantly different ($p<0.05$)

Table.2. Pearson rank correlation between biomarkers and heavy metal concentrations in hermit crab (C. signatus).

	Heavy metals						
	Cd	**Cu**	**Zn**	**Ni**	**Cr**	**Hg**	**Pb**
MT	0.652 ($p<0.01$)	0.863 ($p<0.01$)	0.969 ($p<0.01$)	0.794 ($p<0.01$)	0.743 ($p<0.01$)	-	0.733 ($p<0.01$)

homogenizing buffer. The concentration of MT (μg/g dry weight) was analyzed in triplicate by Spectrophotometer and calculated based on the total metal eluting with the MT peak.

2.4 Quality control

Replicate samples, certified reference materials, and procedural blanks were used as quality control procedures. The procedural blanks were periodically analyzed for each batch of five samples. Quantitative analysis was done on a three-point linear calibration of heavy metal solution, obtained by diluting the certified standard mixture of heavy metal (TraceCERT® CRMs, Sigma Aldrich). It is worth noting that the values of the correlation coefficient R was above 0.99.

2.5 Statistical analysis

The collected data were analyzed by using Statistical Package for Social Sciences (SPSS) software, version 22. In addition, Microsoft Office Excel (2013) was applied to draw the diagrams and estimate the linear regression coefficient between heavy metals (Cd, Cu, Zn, Pb, Ni, Hg, Cr) concentration. The homogeneity of variance was observed among the data, which were normally distributed. ANOVA was run followed by a Tukey's test to compare the means ($p<0.05$) between heavy metals (Cd, Cu, Zn, Pb, Ni, Hg, Cr) contents determined in the hermit crab and sediment samples. Further, Pearson rank correlation was used to monitor the relationships between the selected biomarker and heavy metal pollutants.

3. Results

3.1 Heavy metals pollution

Heavy metals contents determined in the hemit crab tissue from ten sampling sites as well as the results of the related statistical analyses are displayed in Table 1. Fig 2 illustrates the mean concentration of heavy metals (Cd, Cu, Zn, Hg, Ni, Cr, Pb) in *C. signatus* samples of different sampling sites on the northern coast in Oman Sea.

3.2 MT results

The results of mean MT (μg/g w/w) in hermit crab (*C. signatus*) samples collected from various locations as well as the results of the related statistical analyses are presented in Fig 3. Spatial evaluation revealed the highest MT in hermit crab from Jask and Chabahar bay, compared to those of other sites.

3.3 Relationships between biomarkers and Heavy metal levels

Table 2 represents the results of Pearson rank correlations between MT measured in hermit crab and heavy metal concentration. As shown, a significant positive relationship was observed between the levels of MT activity and heavy metal concentration ($p<0.01$).

4. Discussion

The results indicated that Zn in the hemit crab (*C. signatus*) had highest concentration followed by Cu > Cr > Ni > Pb>Cd> and Hg.Further, the bio-accumulation pattern of metals in sediment and biota was not similar. Compared to other metals, high concentrations of Zn and Cu in the hepatopancreous tissue of hermit crab can be related to the high concentration of this metal in the environment, especially sediments. On the other hand, it can be argued that the bioavailability of this metal is higher than that of other metals for different reasons such as bacterial activity or higher volume of input into the environment (Wei and Yang, 2010). Cu is considered to be an essential element for enzymes such as amino acids, and cytochrome C oxidase (Duan *et al.,* 2014). Zn metal acts as a cofactor in a large number of enzymes and reducing proteins such as DNA and RNA polyester, carbonic anhydrase or alkaline phosphatase. Accordingly, one of the implications of this finding deals with the crucial role of these elements in the hermit crab (*C. signatus*) physiology and also the overcoming of metallic bonds of proteins such as metallothioneins.The same trend was observed in the results of the study related to the bioaccumulation of heavy metals in Astacus leptodactylus by Kurun et al. (2010).

The results indicated that hermit crab

at Chabahar bay and Jask had higher MT activity followed by Guader > Ramin > Beris > Pasabandar > Tang > Lirdaf > Pozm > and Jod. The gradient of environmental trace metal in the tissue of hermit crabs from the different sites is also reflected in biomarkers of exposure and effect. Further, the results indicated a strong relationship between heavy metals and MT levels in the hermit crab. The significant relationship between heavy metal and MT biosynthesis in the hermit crab allows crab to accumulate heavy metals without any destructive effect. Furthermore, the presence of elevated heavy metal concentrations in the tissue of hermit crabs result in creating cellular stress when cell damage occurs and is no longer reflected in raised MT levels. Based on the findings of this research, It could be concluded that metallothionein binding capacity is not completed in the hermit crab by the concentration of any heavy metal which results in enabling hermit crab for high heavy metal bioaccumulation. It is remarkable that hermit crab, responding efficiently when exposed to heavy metals, show high survival rate under heavy metal exposure (Sinaei *et al.,* 2010). It was reported that digestive gland *(*hepatopancreas) of crustaceans involved in metal uptake, storage and excretion have a high capacity of MT biosynthesis (Amiard et al., 2006). Results from the present study in the hermit crab (*C. signatus*) are in complete agreement with those previously reported for the crab *Pachygrapsus marmoratus* and *Carcinus maenas* (Legras *et al.*, 2000) and the Chinese crab *Eriocheir sinensis* (Silvestre *et al.,* 2005) and *Pachygrapsus marmoratus* (Mouneyrac et al., 2001).In addition, Brown *et al.*(2004) and Canli *et al.* (1997) reported the same results for MT hepatopancreas as a sensitive tool to detect metal contamination in crustacean. However, further studies are necessary to evaluate the effects of biotic and abiotic factors on hepatopancreas MT biosynthesis in hermit crab.

The obtained results in the current study indicated that MT biosynthesis is highly sensitive in the hermit crab (*C. signatus*). The results may be explained by the role of biomarker which can be for describing the biological effects of pollution and determining the bioavailability of pollution. The use of multiple biomarkers is recommended for biomonitoring heavy metal impact in hermit crab.

Zn was reported to be more important for MT biosynthesis due to more significant correlation followed by Cu > Ni > Pb> Cr> Cd> Hg. A possible justification for the findings is related to the Cu and Zn exposure gradients. Furthermore, Cu and Zn are essential trace metals and MT play a major role in their hemostasis in crabs.

5. Conclusion

The present study investigated the MT biosunthesis in the hepatopancreas of hermit crab (*C. signatus*) in the northern coast of Oman Sea. The findings indicated that MT biosynthesis is highly sensitive in *C. signatus* as the hermit crab. Thus, hermit crab is considered as appropriate sentinel organisms for heavy metal biomonitoring in aquatic systems. The significant relationship between heavy metal bioaccumulation with MT biosynthesis indicates that hermit crab (*C. signatus*) has a high capacity for MT biosynthesis as a detoxification mechanism and heavy metal bioaccumulation. These parameters could be extended for the use of MT in *C. signatus* as a biomarker of heavy metal pollution in marine ecosystems. Finally, the use of hermit crab (*C. signatus*) as a bioindicator of heavy metal pollution and multiple biomarkers are highly recommended for biomonitoring heavy metal, by considering the ease of analyzing the parameters.

Acknowledgments:

Thanks are due to Mr. Arbabi, Mr. Sultanpour, Mr. Hashemi, Mr. Barmoodeh, Mr. Hosseini, Chabahar and Jask Environment Protection office and Islamic Azad University Chabahar branch, Graduate school of Marine Science and Technology, Science and Research Branch, Islamic Azad University for supporting this work.

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