

Metallothionein biosynthesis as a detoxification mechanism of heavy metals (Hg,Cd,Pb,Cu,Zn) in green sea turtles (*Chelonia mydas*)

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Abstract

In this study, heavy metals (Hg,Cd,Pb,Cu,Zn) and metallothionein (MT) biosynthesis were measured in blood of green sea turtles (*Chelonia mydas*) nesting on the northern coast of the Sea of Oman. Heavy metals concentrations in the sea water, the sediments, the blood samples ranged between 0.02-8.43 µg/l, 0.06-10.32 µg/l, and 0.16-36.78µg/g (ww), respectively. The highest concentration of heavy metals were measured at Ahmad rizeh followed by Ramin>Lipar >Kacho> and Tang Provinces. The concentrations of heavy metals in the coastal water and sediment were as follows; Zn>Cu>Pb>Cd>Hg. There were no significant differences between concentrations of heavy metals in different clutches laid in a nesting season. The lowest MT biosynthesis was recorded (115.7 ± 18.12 µg/g dry weight) at the Tang sampling sites, while the highest was registered (276.4 ± 22.18 µg/g dry weight) at the Ahmadrizeh. The relationship between metallothionein biosynthesis and heavy metals in the blood samples, the sea water and the sediment were significant ($p < 0.05$). Significant relationship was found between biomarker and heavy metals in this study, which indicates that green sea turtle (*C. mydas*) have high capacity in rapid response and detoxification of heavy metals on the northern coast of the Sea of Oman. The biomarkers measured in this study were useful as a first investigation into the biological effects of heavy metal pollution, as well as in determining the bioavailability of pollution.

Keywords: Sea water, Sediments, Blood, Pollution, bioavailability, *Chelonia mydas*

1. Introduction

Populations of green sea turtles (*Chelonia mydas*) are distributed throughout the world's tropical and subtropical marine habitat, face serious anthropogenic threats including poaching, fisheries impacts, pollution and habitat loss and consequently classified as

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endangered or threatened (as like other species of sea turtles are listed in Appendix I of the Convention of International Trade in Endangered Species) (IUCN, 2003; Ehsanpour et al., 2014).

Heavy metals enter sea turtles' bodies mainly through their food and water. Female sea turtles drink considerable amounts of water to decrease their body temperature during the nesting season and egg

production (Kenyon et al., 2001). This phenomenon causes increasing of concentrations of heavy metals in their blood. Studying blood provides more comprehensive information about toxicokinetics of essential and non-essential metals in turtle body during the nesting season (Sinaei and Bolouki., 2017).

Sea turtles are good indicators of environmental health status because of their longevity, position and place in the food chain, mobility and movement (Camacho et al. ,2014). Most of the studies on pollution levels in various species of sea turtles have been performed by collecting tissue samples of dead animals which may not reflect realistic contaminant levels and health status of turtles (Ehsanpour et al., 2014). A nonlethal method for sampling blood from sea turtles was developed by Owens and Ruiz (1980). The development of non-lethal methods such as collecting blood samples from live sea turtles has been considered as an appropriate tool to evaluate health status and level of pollution (Van de Merwe, 2010; Day et al., 2007).

Metallothionein with low molecular weight of 6–8 (kDa) among proteins class and containing 20 cysteine groups in its structure confers a unique metal-binding property (Dabrio et al., 2002; Chan et al., 2002; Sinaei et al., 2010). Metallothionein that acts as an absorber of toxic (Cd and Hg) and trace metals (Cu and Zn) has been proposed as a sensitive biomarker in the assessment of metal exposure and prediction of potential detrimental effects induced by metal contamination (Ivankovich et al., 2005; Sinaei et al., 2010).

There are more than 52 known habitats for marine turtles used for foraging, mating, and nesting on the northern boundaries of the Persian Gulf and the Sea of Oman (Pritchard et al., 1999; Sinaei and Bolouki., 2017). Green and hawksbill turtles (*Eretmochelys imbricata*) have a large number of nesting sites in the regions of the northern coasts of the Persian Gulf and the Sea of Oman (Askari Hesni et al. 2015; Tollab et al. 2015). The Sea of Oman includes important food and reproduction areas for Green turtles during

different life stages, but rare number of green turtle come to beach for nesting, So, nesting ecology and toxicology information about green turtle is rare. During the last three decades, intensive efforts have been made to conserve these sea turtles that nest in large groups on the northern coast of the Persian Gulf, however, there is a lack of information on migrations to the northern coast of Sea of Oman (Sinaei and Bolouki., 2017). Populations of the green sea turtle in this area are facing low fertilization and hatching success (Mohammadizadeh and Soltanpour, 2014). It has developed as an important free zone for Iran import and export, economy, urbanization and industries have also grown in the area parallel to economic development (Amini-Ranjbar and Miraki, 2006). So, Heavy metals discharged into the marine ecosystem of the Sea of Oman have the potential to negatively impact the hatching success of these population. Several studies have been performed in the field of ecotoxicology in various species around the Sea of Oman (De Mora et al., 2004; Gochfeld 2003; Yi et al., 2008; Bazzi, 2014). Overall, there is a clear need to improve knowledge of green turtles in Iranian territorial waters of the Sea of Oman. In line with these trends of research, this study was conducted: 1) to determine heavy metals (Hg,Cd,Pb,Cu,Zn) concentration in the green sea turtle, sediment and water; 2) to study the potential of MT activity in blood of the green sea turtle, 3) to evaluate MT activity as a biomarker of heavy metal pollution in the green sea turtle.

2. Materials and Methods

2.1. Study Area

Field trips were made to the sampling sites during the years 2014–2015. Female nesting green turtles were captured from their main nesting sites on the northern coast of the sea of Oman (see Table 1 and Fig 1).For this study, five sampling sites along the beach were chosen, which are frequented by turtles.

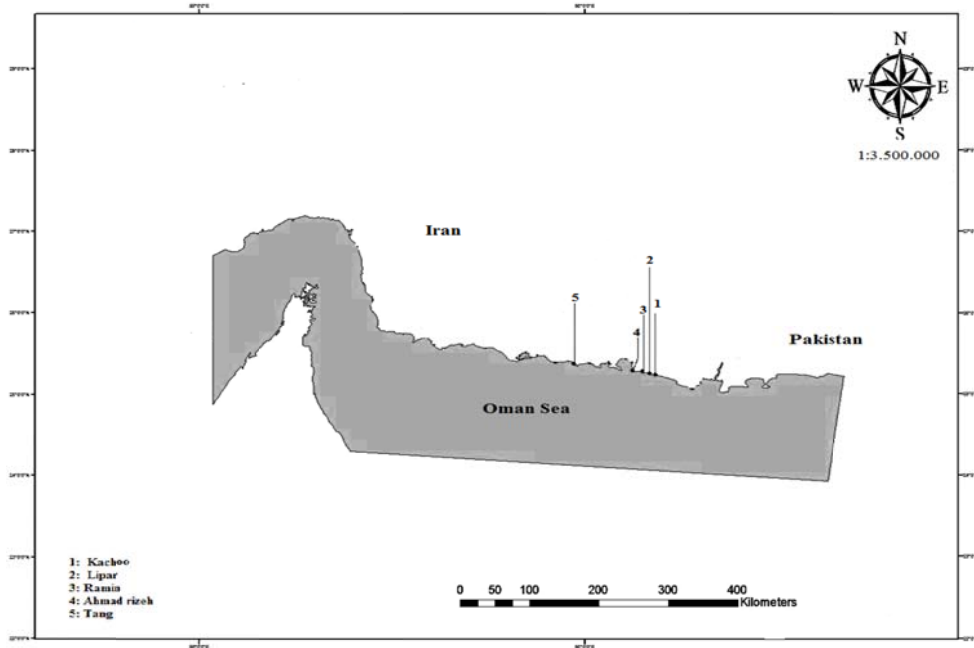


Fig. 1: Location of sampling sites.

Table 1: Sampling sites in the Northern coast of the sea of Oman.

Sampling sites	Coordinates	
	Longitude	Latitude
Ahmad Rizeh	25°63'12.0	61°15'32.00
Kachoo	25 13 53.5	60 54 40.00
Tang	25°23'38.00	59°50'49.45
Lipar	25 14 55.61	60 50 35.15
Ramin	25°15'44.08	60°46'38.49

2.2. Sample and Data Collection

Nesting females were approached approximately 10 min after egg laying activity ceased. A complete visual physical examination was performed (n=18) and curved carapace length (CCL) was measured. Health status of female turtles was rated based on nest-building behavior and general body condition (Deem et al. 2006; Perrault et al., 2012). Turtles were tagged, according to the methodology described by the National Marine Fisheries Service/Southeast Fisheries Science Center (2008). Blood was collected from the interdigital vein of the hind flipper via a dorsal approach with the use of an 18-gauge, 3.7-cm needle and a 15-ml syringe pre-coated with sodium

heparin (heparin sodium injection, USP, PPC, Inc., C504730, Canada). Blood tubes were kept on wet ice in a cooler during the time researchers were on the beach collecting samples (range 20 min to 2 hr). A total of 18 blood samples were analyzed in this study. Immediately after transportation to the laboratory, the samples were stored at -80°C for MT analysis. Samples of seawater (0.5 meters depth from the seawater surface) and sediments (about 5–10 cm) were collected from the intertidal zone of five stations in the northern coast of the Sea of Oman.

2.3. Chemical Analysis

Concentrations of heavy metals (Hg, Cd, Pb, Cu

and Zn) were measured in the sea turtle blood, seawater and sediment samples. Heavy metals in seawater samples were extracted by the APDC–MIBK procedure (Brewer et al., 1969; APHA, 1989). Sediments were dried in the oven at 70° C and kept in polyethylene until analysis (Amini Ranjbar, 1998). Sediment samples of 0.5 g were digested in Teflon vessels for 2 h with a mixture of 3: 2:1HNO₃, HClO₄ and HF acids, respectively, according to the method described by Origioni and Aston (1984).

Blood samples were freeze-dried (72 h at – 49°C and 133 9 10⁻³ mbar) and then powdered. Powdered samples (0.25 g) were digested with quartz-distilled concentrated nitric acid (5 mL) in hot plate equipment (HPA2235M) under established conditions (MESL, 1997). All samples were analyzed in triplicate by Atomic Absorption Spectrophotometer (Lovibond 712005, Vermont, United States) (Sinaei and Bolouki., 2017).

Total mercury levels were determined using cold vapor analysis technique. Powdered samples (0.25 g) were digested in 20 ml of 3:1 concentrated redistilled HNO₃ and concentrated H₂SO₄ and then oxidized with 10 ml of saturated solution of KMnO₄. Excess oxidizing agents and mercury ions were reduced by 10 ml of a reducing solution (3% NaBH₄ in 1% NaOH) in a hydride generator apparatus (Lovibond 712005, Vermont, United States).

2.4. MT Analysis

In order to extract and purify MT, the methods of Sinaei et al., (2010) and Andreani et al., (2008) were followed with minor modifications. Measurement for MT was performed in triplicate for each sample. After thawing, the blood samples were prepared individually by homogenization buffer (15 mM cold Tris–HCL pH=7.0) in which 10 mM M2-mercaptoethanol and phenylmethanesulfonyl fluoride (PMSF) as oxidation and protease inhibitors, respectively, were added in volumes of 1:3.0 (w/v) using a Teflon homogenizer (Sigma-Aldrich, Z659428) at 1,000 rpm. The homogenates were centrifuged (Lovibond, Model Z323K) at 12,000g for 40 min at 4°C. The supernatant

was heated at 80°C for 10 min in order to denature the thermo-labile proteins and then centrifuged again at 12,000g 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 homogenizing buffer. The concentration of MT (µg/g dry weight) was analyzed in triplicate by Spectrophotometer and calculated on the basis of the total metal eluting with the MT peak.

2.5. Quality Control

The procedural blanks were periodically analyzed for each batch of 5 samples. Quantitative analysis was done on a three-point linear calibration of heavy metal solution, obtained by dilution of the certified standard mixture of heavy metal (TraceCERT® CRMs, Sigma Aldrich). Values of the correlation coefficient R was above 0.99.

2.6. Statistical Analysis

Statistical analyses of the data were conducted by using Statistical Package for Social Sciences (SPSS) software, version 20. All data are reported as mean ± standard deviation. Additionally, Microsoft Office Excel (2010) was applied to draw the diagrams and to estimate the linear regression coefficient between heavy metals (Hg,Cd, Pb, Cu and Zn) concentration and metallothionein measured in green sea turtles (*C.mydas*). The data possessed the homogeneity of variance and were normally distributed. One-way analysis of variance (ANOVA) was run followed by a Tukey's test to compare the means (p<0.05) between heavy metals (Hg,Cd, Pb, Cu and Zn) contents determined in the green sea turtles blood samples.

3. Results

3.1. Heavy Metals Concentrations in Blood Sample

Concentrations of heavy metals (Hg,Cd, Pb, Cu and Zn) determined in blood samples of green sea

turtles from the northern coast of the Sea of Oman are presented in Table 2. Results showed that there are higher quantities of essential heavy metals (Cu, Zn) in blood of turtles compared to non-essential heavy metals (Cd, Hg, Pb). Concentrations of Cd, Zn and Hg did not exhibit significant differences in the various clutches in a single nesting season.

3.2. Heavy Metals Pollution of the Sediment and Water Sample

Heavy metals contents determined in the sediment and water from five sampling sites in the northern coast of the Sea of Oman as well as the results of the related statistical analyses are displayed in Table 3. Among heavy metals, levels of Zn in the sediment samples represented the highest concentration among all sampling sites. The highest concentration of heavy metals was measured at Ahmad rizeh followed by Ramin>Lipar >Kacho > and Tang. The concentrations of heavy metals in the coastal water and sediment could be arranged as the following sequence: Zn>Cu>Pb>Cd>Hg. When comparing concentration of heavy metals among sites, there were only significant differences between Lipar, Kacho and Tang for Zn concentrations in the sediment and water samples ($p < 0.05$) and also between Lipar, Kacho and Tang for Pb concentrations in the sediment samples ($p < 0.05$). However, there was no significant

difference between the other sites compared two by two against each other ($p > 0.05$).

3.3. Metallothionein Biosynthesis

The results of MT biosynthesis in blood samples of *C. Mydas* are illustrated in Figure 2. The lowest MT biosynthesis was recorded ($115.7 \pm 18.12 \mu\text{g/g}$ dry weight) in the Tang sampling sites, while the highest was identified ($276.4 \pm 22.18 \mu\text{g/g}$ dry weight) in the Ahmadrizeh. No significant difference was found between MT biosynthesis in different samples ($p > 0.01$).

3.3. Relationships between MT and Heavy Metal Levels

The results of Pearson rank correlations between heavy metal concentration and MT biosynthesis measured in green sea turtles (*C. mydas*) blood samples are summarized in Table 4. There was a strong significant positive relationship between the levels of blood MT biosynthesis and heavy metal level ($p < 0.01$). Significant correlations were also found between heavy metals level determined in both sediments and water and the selected biomarker ($p < 0.01$). Significant correlations were detected between the local distribution of metals in sediments with that of the water ($p < 0.01$).

Table 2: Mean and standard deviation values of Heavy metals (Cd, Cu, Zn, Pb and Hg) in the water ($\mu\text{g/g}$) and the sediment ($\mu\text{g/g}$).

Heavy metals Site ↓	Hg		Zn		Pb		Cu		Cd	
	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment
Ahmad rizeh	0.06±0.01 ^a	0.10±0.01 ^a	8.43±0.61 ^a	10.32±0.91 ^a	1.91±0.11 ^a	2.82±0.36 ^a	1.71±0.11 ^a	2.61±0.12 ^a	0.12±0.01 ^a	0.25±0.01 ^a
Ramin	0.05±0.01 ^a	0.10±0.01 ^a	8.11±0.56 ^a	10.11±0.76 ^a	1.68±0.16 ^a	2.51±0.26 ^a	1.61±0.12 ^a	2.52±0.11 ^a	0.11±0.01 ^a	0.18±0.01 ^a
Lipar	0.04±0.01 ^a	0.09±0.01 ^a	7.61±0.43 ^{ab}	9.37±0.73 ^{ab}	1.19±0.13 ^a	2.01±0.11 ^{ab}	1.32±0.12 ^a	2.32±0.14 ^a	0.10±0.01 ^a	0.17±0.01 ^a
Kacho	0.03±0.01 ^a	0.08±0.01 ^a	6.67±0.41 ^{ab}	8.87±0.61 ^{ab}	1.07±0.11 ^a	1.83±0.11 ^{ab}	1.23±0.13 ^a	2.22±0.13 ^a	0.09±0.01 ^a	0.16±0.01 ^a
Tang	0.02±0.01 ^a	0.06±0.01 ^a	5.81±0.32 ^b	7.12±0.62 ^b	1.02±0.10 ^a	1.45±0.12 ^b	1.1±0.10 ^a	2.2±0.10 ^a	0.08±0.01 ^a	0.14±0.01 ^a

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

Table 3: Heavy metals concentration (mean ± SD, $\mu\text{g/g}$ dry weight) in the blood of *C. Mydas*

CV%	Pb	%CV	Zn	%CV	Cu	%CV	Cd	%CV	Hg	Samples
15.9	0.77± 0.20 ^c	8.7	36.78±3.20 ^a	11.44	2.01± 0.23 ^a	5.4	0.37± 0.02 ^a	25	0.16± 0.04 ^b	Blood

Coefficient value : CV; Values followed by the same letter vertically are not significantly different ($p < 0.05$).

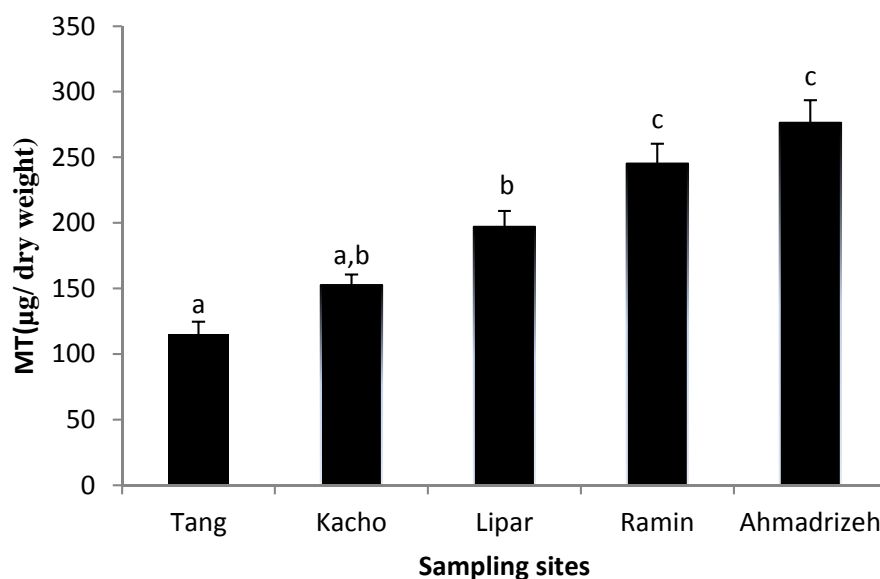
Fig. 2: MT biosynthesis in the blood sample of *C. Mydas*.

Table 4: Spearman rank correlation between MT biosynthesis and heavy metals (Pb,Hg,Cd,Cu,Zn) concentrations in the water, sediment and blood.

MT	Pb			Hg			Cd			Cu			Zn		
	B	W	S	B	W	S	B	W	S	B	W	S	B	W	S
	0.743 (<i>p</i> <0.01)	0.675 (<i>p</i> <0.01)	0.612 (<i>p</i> <0.01)	0.841 (<i>p</i> <0.01)	0.761 (<i>p</i> <0.01)	0.701 (<i>p</i> <0.01)	0.772 (<i>p</i> <0.01)	0.642 (<i>p</i> <0.01)	0.987 (<i>p</i> <0.01)	0.694 (<i>p</i> <0.01)	0.623 (<i>p</i> <0.01)	0.589 (<i>p</i> <0.01)	0.579 (<i>p</i> <0.01)	0.541 (<i>p</i> <0.01)	0.512 (<i>p</i> <0.01)

B: Blood; W: Water; S:Sediment

Table 5: Heavy metals concentration (mean \pm SD, $\mu\text{g/g}$ dry weight) in blood of marine turtles from different locations.

Reference	Location	Hg	Zn	Pb	Cu	Cd	Species
Guirlet <i>et al.</i> (2008)	French Guiana	0.011 \pm 0.003	11.1 \pm 0.28	0.18 \pm 0.05	1.34 \pm 0.28	0.08 \pm 0.03	<i>D. coriacea</i>
V-van de Merwe <i>et al.</i> (2010)	Australia	0.51 \pm 0.05	-	22.18 \pm 5.83	-	35.47 \pm 9.52	<i>C. mydas</i>
Paoez-Osuna <i>et al.</i> (2010)	Mexico	-	58.4 \pm 4.7	-	2.28 \pm 0.4	0.45 \pm 0.2	<i>L. olivacea</i>
Ehsanpour <i>et al.</i> , 2014	Persian gulf	0.18 \pm 0.05	37.6 \pm 3.98	0.56 \pm 0.25	1.89 \pm 0.78	0.34 \pm 0.08	<i>E. imbretica</i>
Guirlet <i>et al.</i> , 2008	French Guiana	0.011 \pm 0.003	11.10 \pm 0.28	-	1.34 \pm 0.28	0.08 \pm 0.03	<i>D.coriacea</i>
Current study	oman Sea	0.16 \pm 0.04	36.78 \pm 3.20	0.77 \pm 0.20	2.01 \pm 0.23	0.37 \pm 0.02	<i>C. mydas</i>

4. Discussion

The non-essential heavy metal concentration results in green sea turtle's blood and their comparison with those found by other researchers (Table 5) indicate that concentrations were similar and in the low ranges of contamination suggesting no acute effects for fetuses. The results achieved in the present study could be explained by the low concentrations of these metals in the environment or from the short duration of time that these turtles were present in the region

during the nesting season (Guirlet *et al.*, 2008).

There were variations in the heavy metal concentration in the water, the sediment and the biota samples. The heavy metal concentration at each site was found to be different even when the discharges were almost similar. These concentrations of heavy metals may be attributed to sewage and wastes discharged from the industrial activities related to Chabahar Free Trade-Industrial Zone, shipping activity (such as: repairing, fueling, greasing and painting of fishing ships) and boats. This may also

have natural origin. The geology of southeastern Iran (Makoran zone) is rich in ophiolites and metalliferous sediments of marine origin. The Makran ophiolites contain chromite and various nickel sulfide minerals (Jacob and Quittmeyer, 1979, Mcall, 1997, Farhoudi and Craig, 1977). Thus, the high metal concentrations are most likely due to the local mineralogy, and are natural, rather than pollution.

The results indicated that the accumulation of heavy metals is predominant in sediments rather than of seawater. This can be interpreted as sediments act as reservoir for all the contaminants and dead organic matter descending from the ecosystem above.

Results indicated that an increase in the number of clutches laid in a nesting season would reduce the Cu concentration in the blood. This can be due to lower entry of contaminants through food because turtles feed little or not at all during the nesting season (Andreani et al., 2008). Contrary to Cu concentration, the results represented that concentration of Pb increases with an increase in the number of clutches laid in a nesting season. A possible justification for our findings might be due to the replacement of calcium (Ca) during egg formation (Bilinski et al., 2001). Since, Pb and Ca have similar kinetics, Pb can be transferred to blood together with Ca (Fossette et al., 2008; Caut et al., 2007). As a result of replacement of the Ca required for more than 300 eggs during the nesting season, considerable quantities of Pb enter the blood.

This data indicated a good correlation between heavy metals and MT levels in the blood of green sea turtle. Anan et al., (2002) and Andreani et al., (2008) came to the same conclusions about relationship between MT and heavy metals in sea turtles. Significant correlation between heavy metal and MT levels in the blood of green sea turtle allows turtle to accumulate heavy metals without any detrimental effect. Accordingly, it is indicated in this study, metallothionein-binding capacity is not completed by any heavy metals concentration, so this provides a mechanism for high heavy metal bioaccumulation capacity of green sea turtle. It is remarkable that

green sea turtle, responding fast and bearing efficient organ during exposure, show the best survival rate under heavy metal exposure (Sinaei et al., 2010).

The present study provides useful initial information concerning concentrations of heavy metals (Cd, Cu, Zn, Pb and Hg) in the sediment, water and green sea turtles of the northern coasts of the Sea of Oman. The concentration of heavy metals varied among different sampling sites. The highest concentration of heavy metals was detected for Zn followed by Cu>Pb>Cd>Hg. The low concentrations of heavy metals in green sea turtles may be due to their diet, because they consume large quantities of algae and plants from the low levels of the food chain. Significant correlations were found among accumulated metal concentrations of heavy metals in sediment, water and blood samples and MT protein levels. The findings showed that MT biosynthesis is highly sensitive in the green sea turtle (*C. Mydas*). One of the possible risk assessment implications of this study is that biomarker can be applied not only to characterize biological effects of pollution exposures, but also to determine the bioavailability of pollution in aquatic systems. The use of multiple biomarkers is especially recommended for biomonitoring heavy metal impact in sea turtle, considering the ease of analyses of the parameters.

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