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Distribution of Trace Metals (Cd, Hg, Pb, Cu) and Polycyclic Aromatic Hydrocarbons (PAH) in Loggerhead Turtles (Reptilia: Testudines: Cheloniidae: *Caretta Caretta* (Linnaeus, 1758)) Tissues Stranded Along the North Tunisian Coasts

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Abstract

Cadmium, mercury, Lead, Copper and Polycyclic Aromatic Hydrocarbons, in liver, kidney, muscle and heart samples of 5 loggerhead turtles *Caretta caretta* (Linneaus, 1758) stranded along the north Tunisian coast were measured. Analyses were performed by using the Atomic Absorption Spectrometry (AAS, VARIAN 220Z) for trace metals and the mass chromatography for PAH. The results demonstrated that the kidney and the liver are the main concentrated organs and muscles generally display the lowest trace metals and PAH concentration. By comparing with some bibliographic data, the mean trace metals concentrations are about identical to loggerhead turtles' tissues of others localities in Mediterranean Sea. In addition, obtained results show that the kidney is relatively the most concentrated organ in cadmium, lead and PAH, the liver and the heart are relatively the two most organs concentrated in copper and the highest concentrations of mercury were found in the liver. This is the first assessment into metal and polycyclic aromatic hydrocarbons distribution in tissues of loggerhead turtles from Tunisian Mediterranean Coastline.

Key words: Trace metals; polycyclic aromatic hydrocarbons; loggerhead turtles; stranding; Tunisia;

Introduction

Three species of marine turtles: the leatherback turtle *Dermochelys coriacea* (Vandelli, 1761), the loggerhead turtle *Caretta caretta* (Linnaeus, 1758) and the green turtle *Chelonia mydas* (Linnaeus, 1758) are encountered in the Mediterranean. The leatherback turtle is a visitor from the Atlantic and can be found all over the basin, although it does not breed in the Mediterranean [9,24]. The other two species reproduce in the Mediterranean and have evolved local populations with a genetic divergence from the Atlantic populations (Casale & Margaritoulis 2010, and references therein). The main identified threats at sea to these two Mediterranean populations are incidental catch, collision and intentional killing while the impact of other potential threats like chemical contaminants and debris is not clear yet [28].

All three marine turtle species mentioned were reported in Tunisian waters and were protected by several international conventions [25]. Loggerhead turtles, the most common sea turtles found on the marine habitats off the Tunisian coasts [22], are the only species that nest mainly in the Kuriat Islands [10,22,23].

Since 1988, the data of first record of the loggerhead sea turtles nesting in Tunisia, a Tunisian Sea Turtle Program (TunSTP) included in the activities of the National Institute of Sea Sciences and Technology (INSTM) was launched in order to identify appropriate conservation measures for these species, which are listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [15]; this program include (i) the monitoring of the loggerhead turtles nesting sites, (ii) the by-catch surveys and (iii) the national stranding network [10]. Moreover, a specialized sea turtle rescue centre has been established in the National Institute of Marine Sciences and Technologies at Monastir in 2004.

Following industrialization, high quantities of chemical pollutants have been released and continue to be released into the sea altering the natural biological equilibrium [20] and the sanitary of aquatic animals, particularly those long living species such as marine mammals, sea birds and sea turtles which have the potential to accumulate organic and inorganic contaminants from food, sediment and water in their tissues [4,18]. Therefore, the concentrations of these contaminants may vary depending on biological factors such us age and migration habits.

Trace elements are natural components of rocks and soil. The erosions favour their release in aquatic ecosystem. These elements occur naturally in very low concentrations in the environment.

They are not necessarily toxic but many anthropogenic activities increase their natural concentrations causing pollution [37]. Some of them persist in the tissues of animals and can biaccumulate over time. Trace elements can be either non essential (e.g. Cd, Pb, Hg and Cs) or essential (e.g. Co, Cu, Fe, Mn) to living organisms [3] and above a threshold concentration, they are considered toxic.

Polycyclic Aromatic Hydrocarbons (PAH) are compounds containing two or more fused aromatic rings in linear, angular or clustered arrangements. PAH have high molecular weight and low volatility and are known to be highly persistent in the environment [35]. Hydrocarbons may arise from petroleum hydrocarbons pollution and incomplete combustion of organic materials such as wood, coal and oils. The health hazard posed by these compounds has been extensively studied by several authors [6,42]. Several polycyclic aromatic hydrocarbons were studied, including sixteen that are reported in the priority list of pollutants of the US Environmental Protection Agency (EPA). PAHs are bio available to fish and other marine organisms through the food chain, as waterborne compounds and from contaminated sediments. PAHs uptake always depends on their bioavailability as well as the physiology of the organisms [31]. Such compounds have adverse effects on health (carcinogenic and/or mutagenic activity) and ecosystem [27]. Numerous studies indicate that one-, two- and three-ring compounds are acutely toxic, while higher molecular weight are considered to be genotoxic [32,33].

Despite the extensive literature regarding the concentrations of contaminants in loggerhead turtles in the Mediterranean Sea [1,17,21,30,37], there is currently no available information on chemical elements in sea turtles stranded along Tunisian coast.

Considering the importance of pollution as a threat to sea turtles [1,11,14,29,38], the present study aims to assess levels of four trace metals Cadmium (Cd), Mercury (Hg), Lead (Pb), Copper (Cu) and Polycyclic Aromatic Hydrocarbons (PAH), in the tissues of the loggerhead turtles from the coast of Tunisia and to compare these data with those reported from other locations in Mediterranean Sea.

Materials and Methods

Sample collection

Tissues samples were taken during necropsies of five loggerhead turtles stranded along the north east Tunisian coasts (Figure 1) between July 2006 and November 2008. The Curved Carapace Length (CCL), measured to the nearest 1 cm using a flexible meter tape. Necropsies were performed and samples were taken only from fresh dead or slightly decomposed individuals. Sex was determined via visual examination of the gonads and in case of doubt confirmed by histology. Liver, kidney, muscle and heart were sampled and stored in bottles to avoid any contamination. All organs of all individuals were frozen at -20 °C until the chemical analysis was carried out.

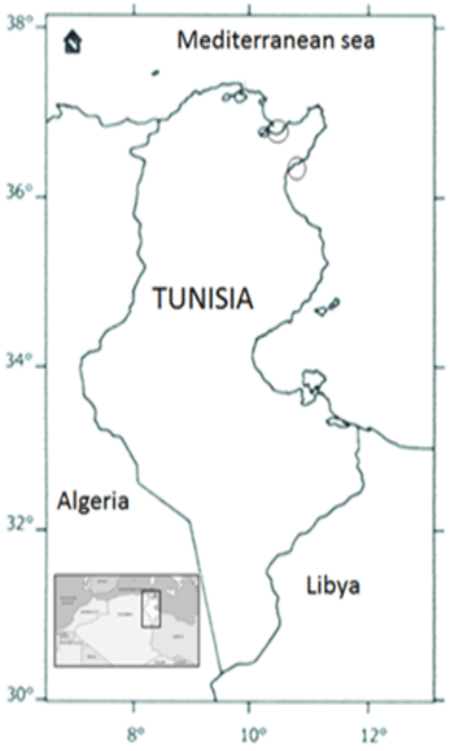

Figure 1: Map of Tunisia showing the sampling locations (circles)

Chemical analysis

Trace elements: Once in the laboratory, samples were freeze-dried and then grounded on an agate mortar to obtain a fine homogeneous powder. Separate sub-samples were analysis in three replicates. All element concentrations (µg/g) were expressed on a dry weight basis.

Digestion: To analyse Cd, Pb and Cu, the digestion of 200 mg of each organ was carried out in a Teflon bombs with 5 ml HNO_3 using a microwave (Millestone type Ethos) digestion at 100% power with pressure set at 120 psi for 20 min, the overall digestion time for the one cycle was 40 min. Blank acid mixtures were digested in the same way [39]. Mercury was treated separately using 300 mg biota samples in 5 ml HNO_3 using a water bath at a temperature of 90°C to dryness. The samples were then diluted to 50 ml with milli Q water [40].

Atomic Absorption Spectrometry (AAS): The dilutions of the acid extracts from both procedures were analysed by Atomic Absorption Spectrometry (AAS, VARIAN 220Z) using the graphite furnace technique and back ground corrections for cadmium and lead. The mercury concentration was determined by cold vapor technique using stannous chloride as a reducing agent and VGA-AAS (Varian AA10) system.

The instrument was calibrated with diluted solution prepared from known stock solutions of each element. Accuracy of the determinations was checked by the analysis of the international

standard reference material Nist 2976. The analytical precision was generally better than 5%.

Polycyclic Aromatic Hydrocarbons (PAH): Analysis of tissue samples was performed according to the technique proposed by Villeneuve, 1995. Appropriate blanks were analysed with each set of analyses. Sample comparisons were made with reference material (IAEA 406) for quality control purposes. Recoveries are ranged from 71% to 89% and the method detection limits are ranged from 0.05 to 0.25 ng g^{-1}. Extraction of tissue was realised by a Soxhlet extractor during 8 h with Methanol. Internal standards 9, 10-dihydroanthracene, n-octadecene (C18:1) and n-dotriacontane (n-C32) were added to tissue samples before Soxhlet extraction to determine the recoveries during the analytical procedure. A saponification step was realised with addition of potassium hydroxide in a Soxhlet extractor during two hours to eliminate fatty acids. The mixture of KOH/MeOH was extracted twice with hexane into a glass separating funnel with a Teflon stopcock and then concentrated. Separation of PAH was performed with silica gel and alumina column. A mixture of hexane and dichloromethane (90:10 v/v) was used to obtain the PAH fraction. Analysis of PAH was performed by gas chromatography coupled with high resolution mass spectrometry (GC-HRMS) Varian 4000. All trace elements and PAH concentrations were expressed as (µg/g) dry weight (dw).

Statistical analysis

Mean trace elements and PAH concentrations were expressed with coefficients of variation. The comparison of the concentrations between organs for each metal was made with comparison t-test. Significant differences were considered at p < 0, 05.

Results and Discussion

Turtles' size ranged from 65 to 93 of CCL (80, 9 cm ± 16.12). All turtles were male except one individual for which it was not possible to realize histological section. Due to the small sample size, sex or size dependent variation in pollutant concentrations was not examined. Previous studies based on the analysis of a larger number of individuals failed to detect any correlation between size and the element concentrations probably because of the confounding effect of trophic and physiological status on tissue contamination levels [30].

Cd, Hg, Pb, Cu and PAH mean concentrations (dry weight) are presented in (Table 1). The results show that kidney and liver are the main target tissues. In fact, the highest concentrations of mercury have been found in liver. The heart and the liver are relatively the most concentrated organs in copper. The highest concentrations of cadmium, lead and PAH have been found in the kidney and the muscle is the lowest concentrated organ in trace metals and PAH. The highest copper level has been detected in the heart but this result would be taken with caution because derived from the analysis of a single individual.

Table1: Trace metals (µg/g dry weight) and polycyclic aromatic hydrocarbons concentrations (ng/g dry weight) (mean ± standard error, n < 30 and & = 0.05) in loggerhead turtles samples.

Elements (Wt)	Liver	Kidney	Heart	Muscle
Cd (µg/g)	8.31 ± 3.22 (n=5)	78.13 ± 59.6 (n=2)	1.07 ± 0.2 (n=4)	0.8 ± 1.13 (n=4)
Hg (µg/g)	1.15 ± 0.8 (n=5)	0.61 ± 0.39 (n=3)	0.15 ± 0.1 (n=4)	0.12 ± 0.13 (n=4)
Pb (µg/g)	0.23 ± 0.01 (n=5)	0.67 ± 0.51 (n=3)	0.18 ± 0.07 (n=4)	0.26 ± 0.3 (n=4)
Cu (µg/g)	9.56 ± 8.1 (n=2)	6.07 ± 5.07 (n=2)	10.09 (n=1)	4.35 ± 0.98 (n=2)
PAH (µg/g)	151.02 ± 107.04 (n=5)	329.2 ± 619.51 (n=2)	ND	81.95 ± 20.05 (n=4)

ND= not determined

*PHA are the sum of 24 compounds: naphthalene, 1-methylnaphtalene, ethylnaphtalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, 2-methylphenanthrene, 1-methylphenanthrene, 3,6-Dimethylphenanthrene, fluoranthene, pyrene, 1-methylpyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-cd) pyrene, dibenzo (a,h) anthracene and benzo (ghi) perylene.

Statistically, there has been no statistical difference in mercury, copper and PAH concentrations between organ samples. However, cadmium levels have been significantly higher in kidney than in liver, in heart and in muscle (p=0, 000004 < 0, 05) and lead levels have been also significantly higher in kidney than in liver, in heart and in muscle (p = 0, 01< 0, 05).

It 'is known that metal concentration in loggerhead sea turtles is mainly determined by enviromental exposure and may vary depending on biological factors such as age, sex, migration habits. So, by comparing the results of this study with others conducted in Mediterranean sea, we can't determine the most or the least contaminated area because studies have not been performed at the same time and the samples were generally not enough representative. However we can deduce that the results we have obtained on samples taken in north east of Tunisia are nearly in the range of variation of the studies cited in (Table 2). With the exception of cadmium concentration in the kidney which is higher than those reported by these studies that also show that the level of this metal is always higher in the kidney.

Table 2: Trace elements (µg/g dry weight) and polycyclic aromatic hydrocarbons mean concentrations (ng /g dry weight) in loggerhead turtles samples in different areas of the Mediterranean.

	Liver	Kidney	Heart	Muscle	Reference
Cd (µg/g)	8.31 ± 3.22 (n=5)	78.13 ± 59.6 (n=2)	1.07 ± 0.2 (n=4)	0.8 ± 1.13 (n=4)	Present study (North east Tunisia)
	23.38 ± 53.66 (n=16)	31.47 ± 70.75 (n=19)	-	0.20 ± 0.14 (n=16)	Antonio et al. 2009 (Southern Spain)
	2.4 ± 0.4 (n=11)	5.8 ± 1.1(n=9)	2.2 ± 0.2 (n=3)	0.81 ± 0.04 (n=10)	Andreani et al. 2008 (Mediterranean)
	-	57.2 ± 34.6 (n=19)	-	0.2 ± 0.0.2 (n=26)	Maffucci et al. 2005 (West Italy)
	2.58 ± 4.12 (n=7)	13.3 ± 13.6 (n=5)	-	0.08 ± 0.05 (n=21)	Caurant et al. 1999 (West France)
	7.60 ± 6.05 (n=12)	24.23 ± 21.40 (n=12)		0.55 ± 0.63 (n=12)	Storelli et al. 1998 (East Italy)
Hg (µg/g)	1.15 ± 0.8 (n=5)	0.61 ± 0. 39 (n=3)	0.15 ± 0.1 (n=4)	0.12 ± 0.13 (n=4)	Present study (North east Tunisia)
					Antonio et al. 2009 (Southern Spain)
	-	-	-	-	Andreani et al. 2008 (Mediterranean)
	-	0.9 ± 0.7(n=20)	-	0.4 ± 0.3 (n=26)	Maffucci et al. 2005 (West Italy)
	-	-	-	-	Caurant et al. 1999 (West France)
	1.68 ± 1.04 (n=12)	0.65 ± 0.34 (n=12)	-	0.69 ± 0.46 (n=12)	Storelli et al. 1998 (East Italy)
Pb (µg/g)	0.23 ± 0.09 (n=5)	0.67 ± 0.51(n=3)	0.18 ± 0.07 (n=4)	0.26 ± 0.3 (n=4)	Present study (North east Tunisia)
	2.75 ± 1.64 (n=16)	0.52 ± 0,49 (n=19)		0.26 ± 0.23 (n=20)	Antonio et al. 2009 (Southern Spain)
	0.1 ± 0,08 (n=11)	0.1 ± 0,07 (n=9)	BLD (n=3)	BLD (n=3)	Andreani et al. 2008 (Mediterranean)
	-	-	-	-	Maffucci et al. 2005 (West Italy)
	-	-	-	-	Caurant et al. 1999(West France)
	1.23 ± 1.0.1(n=12)	0.70 ± 0.35 (n=12)	-	0.54 ± 0.17 (n=12)	Storelli et al. 1998 (East Italy)
Cu (µg/g)	9.56 ± 8,1(n=2)	6.07 ± 5.07 (n=2)	10.09 (n=1)	4.35 ± 0.98 (n=2)	Present study (North east Tunisia)
	21.60 ± 8.03 (n=16)	3.77 ± 3.50 (n=19)		5.04 ± 1.93 (n=20)	Antonio et al. 2009 (Southern Spain)
	17.5 ± 2.44 (n=11)	5.56 ± 0.96 (n=9)	8.96 ± 2 (n=3)	2.4 ± 0.24 (n=10)	Andreani et al. 2008 (Mediterranean)
	-	2.6 ± 0.7 (n=19)	-	2.7 ± 1.4 (n=26)	Maffucci et al. 2005 (West Italy)
	8.25 ± 6.59 (n=7)	2.21 ± 0.46 (n=5)	-	0.73 ± 0.45 (n=21)	Caurant et al. 1999 (West France)
	-	-		-	Storelli et al. 1998 (East Italy)
PAH (µg/g)	151.02 ± 107.04 (n=5)	329.2 ± 619.510 (n=4)	ND	81.95 ± 20.05 (n=4)	Present study (North east Tunisia)

ND: not determined, BLD: Below the detection limit, n: number of samples.

Cadmium is known to accumulate with increasing age [7] and to cause damage to the vertebrate kidney, but there is little evidence to suggest that the cadmium levels are high enouph to cause harm to sea turtle. In marine mammals like dolphin, seals and porpoises, the kidney is also the organ where Cadmium is preferentially accumulated. The combination of several physiological factors explains the accumulation of cadmium in the kidneys of these animals [26].

In addition, we find that both the results of the present study than other studies reported in (Table 2), the lowest concentrations of trace elements in organs were obtained with lead and mercury. As lead accumulates preferentially in the skeleton and disturbs bone development [8], in future, it would be suggested to analyze this metal in bone.

It is difficult to interpret the significance of these results because so little is known about baseline levels and physiological effects of trace elements on marine turtle populations [43]. Nevertheless, the relatively high amounts of trace elements mainly in kidney and liver should suggest the involvement of metallothionein proteins which provide protection against metal toxicity in loggerhead turtles [1].

PAH tend to accumulate more in marine organisms than in other matrices such as sediment [31]. However, marine organisms can rapidly convert up to 99% of the PAHs to metabolites within 24 h of uptake. In addition, the half life of PAHs, contrary to other organic pollutants class, as polychlorinated biphenyls, is generally very short, ranging from six to nine days for fluorene, phenanthrene, anthracene and fluoranthene [31].

According to our knowledge, studies on PAH concentrations in marine turtles are rare. [2] Showed that turtle eggs contained detectable amounts of PAHs and Camacho and his collaborators have found PAHs in loggerhead turtles plasma samples with

predominance of Di- tri-cyclic PAHs suggesting petrogenic origin rather than urban sources of PAHs.

Coherently with bibliographic data obtained from fish, total PAH concentrations are lower in muscle samples (Table 2) while higher in liver which is considered the best indicator of hydrocarbon contamination in marine ecosystem [36]. The inter individual variation in PAH levels may be explained by different food regime of the analysed individuals or by the diverse exposure to these pollutants. Values reported here are comparable to the average background values for biological tissue (range from 0.01 to 1 µg kg-1) reported in literature for individual PAH [16]. Benzo (a) pyrene, a general indicator of total PAH in a given sample, whose concentration should not exceed a limit of 10 µg kg-1 [34] was not detected in all samples studied.

The PAHs toxicity depends on form of the contaminant, route of exposure and stage in life of the organism [19]. Exposure to PAHs on the egg reduces survival of snapping turtle embryos and causes developmental abnormalities [41]. Considering the toxic and carcinogenic effects of PAH and their impact on organizations, it would be interesting to study further the bioaccumulation of these products in sea turtles.

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