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Cytochemical responses of Hediste diversicolor (Nereidae, Polychaete) sampled from polluted sites along the Tunisian coast *

Respostas Citoquímicas em Hediste diversicolor (Nereidae, Polychaeta) de locais poluídos na Zona Costeira Tunisina **

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ABSTRACT

The polychaete worm *Hediste diversicolor* was collected in several sites from the Tunisian coast. The aim of our study was to study several cytochemical biomarkers in this species in response to a pollution gradient caused by various discharges a long the Tunisian coast. Worms were collected from six sites: Bizerta Lagoon, Gargour, Nakta, Mahres, Skhira and from Teboulba, witch is considered a reference site.

The biomarkers selected in this work were lysosomal membrane stability, lipofuscin and neutral lipid accumulations and levels of Ca^{2+}-ATPase activity analyzed in the intestinal cells. Chemical analyses of Cd, Cu and Zn were also carried out in sediment. The results obtained indicate significant changes in most of the parameters measured in *H. diversicolor*. They are consistent with the chemical analysis and that worms from Bizerta and Mahres have been submitted to high levels of pollution.

Keywords: Biomarkers, lysosomal membrane stability, neutral lipids, lipofuscin, Ca^{2+}-ATPase activity, *Hediste diversicolor*.

RESUMO

Com o objectivo de estudar vários marcadores citoquímicos em Hediste diversicolor ao longo de um gradiente de poluição foram colhidos indivíduos dessa espécie na costa Tunisina (Bizerta Lagoon, Gargour, Nakta, Mahres, Skhira e Teboulba como local de referência).

Os biomarcadores selecionados foram a estabilidade membranar dos lisossomas, lipofuscina e acumulação de lípidos e atividade Ca2+-ATPase analisados em células intestinais. Foram ainda analisados nos sedimentos Cd, Cu e Zn. Os resultados obtidos indicam alterações significativas na maioria dos parâmetros analisados em H. diversicolor. Estes resultados mostraram-se consistentes com as análises químicas e ainda com a maior exposição a poluentes nos locais Bizerta e Mahres.

Palavras-Chave: *Biomarcadores, estabilidade membranar dos lisossomas, lípidos neutros, lipofuscina, atividade Ca2+-ATPase, Hediste diversicolor.*

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1. INTRODUCTION

Estuaries and coastal waters are particularly at risk from anthropogenic pollution including industrial, agricultural and/or urban development, receiving toxic effluents. Compared to current practice in aquatic monitoring based on recording the concentrations of a small range of pollutants in biological matrices in a given environment, a probably more realistic way to assess the "health status" of the environment is to measure a suite of biomarkers. Chemical analyses reveal only the bioaccumulated concentrations of particular pollutants in the organisms. While an integrated response approach involving a suite of the *in situ* responses of populations at each particular site, demonstrates their interactive and combined effects with regard to the numerous environmental factors characterizing the location. It is always very difficult from only contamination body burden data to obtain information about their significance upon animal health. Therefore, techniques for measuring biological effects are critical for any pollution monitoring program. A large number of biomarkers have been tested and validated for their applicability to detect biological effects as indicators of chemical stress at different levels of biological organization (Amiard *et al.*, 2006; Magni *et al.*, 2006; Nigro *et al.*, 2006; Bouraoui *et al.*, 2009). However the use of enzyme activities, performs a risk of being inhibited by high contaminant loads (Narbonne *et al.*, 2005), and it is therefore important to also monitor the overall health status by making use of biomarkers of effect and stress. Several of the most sensitive cellular stress markers are lysosomal parameters, e.g. lysosomal membrane stability (LMS), lipofuscin (LF) and neutral lipid (NL) contents, lysosomal volume by assessing the lysosome/cytoplasm ratio (Lowe *et al.*, 1981; Moore, 1988; Viarengo *et al.*, 1991; Dondero *et al.*, 2006) and Ca^{2+}-ATPase activity. Sediments (both suspended and deposited) constitute the main reservoir for most of the chemicals introduced into aquatic environments by human activities. Then, it is necessary to develop the use of biomonitors more representative of the sedimentary compartment of aquatic habitats.

The polychaetes are the dominant species of macrofauna within fine sediments. In this group, many species seem to exhibit an extraordinary tolerance to various environmental contaminants, being also the most common invertebrates found in polluted areas (Eriksen *et al.*, 1988). *H. diversicolor* is a marine annelid which lives in estuary sediments rich in microorganisms and toxic agents resulting from pollution. It has been the subject of numerous studies, focusing on different aspects of its biology and ecology, including a range of pollution related subjects. This polychaete is characterized by a high physiological tolerance to extreme variation of many environmental parameters such as temperature and salinity (Bartels-Hardege & Zeeck, 1990; Ait Alla *et al.*, 2006). Therefore, the use of polychaete worms as bioindicators for estuarine ecosystems has proved efficiency to be a useful tool in the assessment of environmental quality, being not insensitive to stressful environmental conditions (Dean, 2008).

The aim of this work was to employ cellular stress markers in *H. diversicolor* to assess the marine environment quality along the Tunisian coasts.

2. MATERIAL AND METHODS

2.1. Sampling sites

Sampling sites along the Tunisian coast were chosen for the presence of a natural population of the polychaete *H. diversicolor* and their geographical locations near urban, industrial and agricultural areas (Fig. 1); they are constantly threatened by contamination due to their proximity to human settlements and high economic and industrial activities. Menzel Abdelrahmen is located in the north of Tunisia in the Bizerta Lagoon (S1), a Mediterranean lagoon covering roughly 15 km^2 that represents an economically important body of water due to a variety of fishing and aquaculture activities. The other four sites, Nakta (S2), Gargour (S3) and Mahres (S4) and Skhira (S5), are located in the gulf of Gabés, in the southeastern coast of Tunisia, which is considered as a great Tunisian aquatic resource, contributing to more than half of the national production. Important industrial activities, mainly crude phosphate treatments, chemical industries and tannery, are being developed along this region, possibly affecting this marine ecosystem (Hamza-Chaffai *et al.*, 1995; Boujelben, 1998; Banni *et al.*, 2005). The control worms were collected from Teboulba, located on the mid of Tunisia; this site is characterized with no apparent contamination sources and thus considered in many field works as reference site (Banni *et al.*, 2007; Jebali *et al.*, 2007; Bouraoui *et al.*, 2010).

2.2. Collection

Samples were collected in the intertidal zone at low tide from six sites in Tunisian coastal areas during September 2009; worms were sampled from Bizerta lagoon, Gargour, Nakta, Mahres, Skhira and from Teboulba (natural population). The surface oxygenated layer (a few mm deep and about 300 cm^2 area) of sediments destined for metal analysis was collected and placed in aluminum box. Once in the laboratory, the worms were examined (specimens with exoskeleton or skin infections were excluded) and flash-frozen in N-hexane, chilled in liquid nitrogen and then stored at -80 °C.

2.3. Metal analysis in sediments

Sediment were digested with 2:5 (V:V) 33% HCl and 65% HNO_3 (UNEP/COI/AIEA/FAO 1994). Cu, Cd and Zn were analyzed by atomic absorption spectrophotometry AAS (Zeeman effect) in these acid solutions after dilution with deionised water. The calibration was carried out using the following standards for all of the three metals: 125, 250, 500 $ng.ml^{-1}$. For each sediment sample, three replicates were analysed concomitantly. All metal concentration was reported in micrograms per gram dry weight of sediment.

2.4. Cytochemical analysis

2.4.1. Lysosomal membrane stability (LMS) assay

Pieces of excised worms were placed on two different aluminum cryostat chucks for a total of six individuals, and sections (10µM) were cut with a cryostat (Leica CM3050) and flash-dried by transferring them to room temperature. The cryostat sections were incubated at 37 °C in 0.1 M citrate

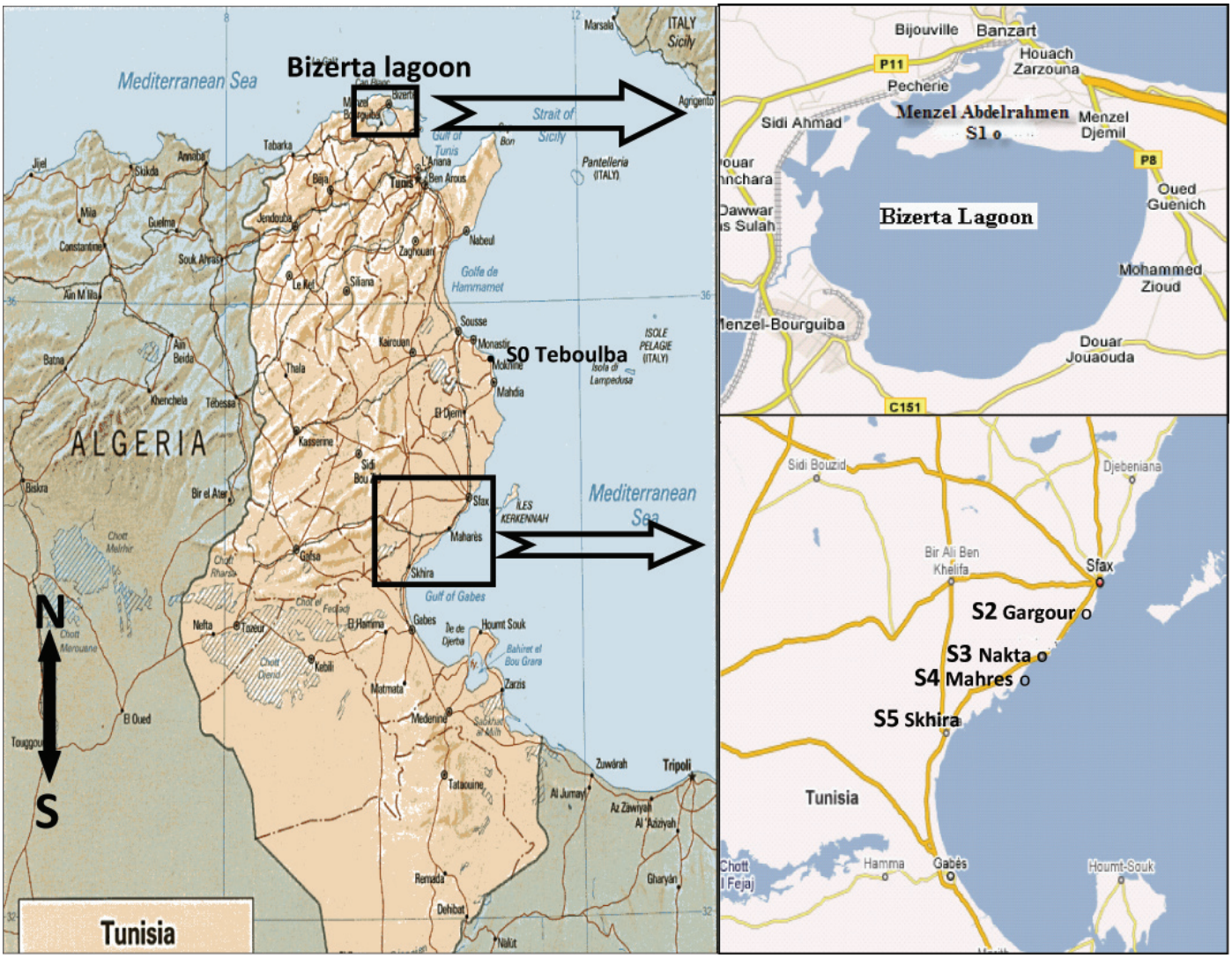

Figure 1. Location of the sampling sites: (S1): Bizerta lagoon; (S2): Gargour, (S3): Nakta, (S4): Mahres and (S5): Skhira situated in gulf of gabes; (S0): Teboulba considered as Control site.
Figura 1. *Localização dos locais de colheita: (S1): Bizerta lagoon; (S2): Gargour, (S3): Nakta, (S4): Mahres e (S5): Skhira situada no golfo de gabes; (S0): Teboulba (local de controlo).*

buffer containing 2.5% NaCl for various pre-treatment times (0, 2, 5, 10, 15, 20, 25, 30, 35 min) in order to labilise the lysosomal membrane. Then, sections were incubated at 37 °C in 50 mL of 0.1 M citrate buffer with 2.5% NaCl, containing 20 mg naphthol AS-BI-N-acetyl-β-glucosaminide previously dissolved in 2.5 mL 2-methoxyethanol and 3.5 g of polypeptide, rinsed at 37 °C in 3% NaCl, treated at room temperature with 1 mg/mL Fast Violet B in 0.1 M phosphate buffer and fixed for 15 min in Baker's fixative at 4 °C (Moore, 1976). Staining intensity of lysosomes was determined by studying the slide at 400×magnification with an inverted Axiovert microscope (Zeiss), connected to an Axiocam digital camera (Zeiss). Digital image analysis was carried out using the Scion Image software package (Scion Corp).

2.4.2. Neutral lipids content

This analysis was made from sections prepared the same as for the lysosomal membrane stability assay by fixing the sections in calcium-formaldehyde (2% Ca-acetate (w/v), 10% formaldehyde (v/v)) for 15 min at 4◦C, followed by a rinsing step with de-ionised water, and incubation with 60% triethylphosphate (TEP) for 3 min. The sections were then stained with Oil Red- O (1% in 60% TEP) for 30 s, rinsed with de-ionised water, and mounted in 20% (v/v) glycerol (Moore, 1988). Neutral lipid content was quantified by digital image analysis, as described for the LMS assay.

2.4.3. Lipofuscin content

Cryostat sections were fixed in calcium-formaldehyde and rinsed with de-ionised water, as described for the neutral lipid assay, followed by a 5 min incubation step with 1% Fe_2Cl_3, 1% potassium ferrocyanide in a 3:1 ratio (Moore, 1988). The sections were rinsed with 1% acetic acid and mounted in 20% (v/v) glycerol. Lipofuscin content was quantified by digital image analysis of stained sections, as described for the LMS assay.

2.4.4. Ca^{2+}-ATPase activity assay

This enzyme activity was quantified from cryostat sections obtained as for the LMS assay, using the histochemical method described by Pons *et al.* (2002). Cryostat sections were washed in 0.05M cacodylate buffer (pH 7.4), fixed in 1% paraformaldehyde (pFA) in 0.05 M cacodylate buffer (pH 7.4) for 30 min at 4 °C and washed again in 0.05M cacodylate buffer. Samples were then dehydrated in increasing acetone concentrations at 4°C and embedded in Technovit 7100 resin. Serial cross sections (2µm) were cut using a microtome, transferred onto glass slides and incubated for 6 h at room temperature in a medium containing 2.4 mM ATP, 18 mM $CaCl_2$, 8 mM levamisole, 0.2 mM ouabain,1 mM $Pb(NO_3)_2$, and 20 mM sodium barbiturate. After incubation, the medium was removed and slides washed in water and rinsed in an ammonium sulfide-saturated water solution (3 min) to reveal the brown lead sulfide. $Pb_3(PO_4)_2$ precipitates stained with ammonium sulfide was quantified on sections by digital imaging as described above.

2.4.5. Statistics

Cytochemical data (n = 6) were analyzed for difference between two groups, the reference site (Teboulba site) versus each other site, by the Student's t-test, using the Instat 4.0 software (Graph Pad, USA).

3. RESULTS

Considering the total determinations of metals in sediments (Table 1), Bizerta Lagoon and Mahres are the most heavily contaminated sampling site. In fact, sediments from the latter two sites showed the highest content of Cd, Cu and Zn, respectively 0.77, 116.6 and 1208.9 µg/g dry weight (dw) from Bizerta Lagoon and 0.63, 105.32 and 1016.11 µg/g dw in samples from Mahres. Relatively high concentrations of these same metals were also observed in samples from Skhira. Teboulba showed the lowest heavy metal contents with only a level of 0.08 µg Cd/g dw, 12.49 µg Cu/g dw and 176.9 µg Zn/g dw.

Evaluation of a stress syndrome in worms collected from Tunisian coasts was made by using a total of four biomarkers. Three of these are dedicated to the cellular level: lysosomal membrane stability (LMS), lysosomal contents of neutral lipids (NL) and lipofuscin (LF) and one to the tissue level: Ca^{2+}-ATPase activity. A significant decrease in LMS with respect to controls (Teboulba site) was observed in intestinal cells of worms collected from each site, except for Nakta site (Fig. 2). LMS in polychaetes sampled in Mahres site is approximately 74% less then reference individuals. Worms from Bizerta, Gargour and Skhira have also showed a significant reduce of LMS compared to controls by respectively 43%, 50% and 64%.

The results of neutral lipid (NL) and lipofuscin content in *H. diversicolor* are reported in figures 3 and 4.

Lysosomal contents of neutral lipids and lipofuscin showed similar trend. In comparison to polychaetes from the reference site, contents of both parameters increased in the individuals sampled at each site then peaked at Mahres and were still high at Bizerta site. The content of lysosomal lipofuscin proved a more sensitive parameter than neutral lipids, with significantly higher levels in individuals from all the study area. For neutral content, significantly higher value with respect to reference worms were measured in individuals from Mahres (483%), from Bizerta (271%) and from Skhira (248%). A not significant increase was recorded in specimens collected at Gargour and Nakta.

The results of Ca^{2+}-ATPase activity is reported in figure 5. It has significantly decreased only in worms from Mahres (44%) with respect to reference individual.

Table 1. Heavy metals contents (µg/g dry weight) in sediments from the different investigated sites.
Tabela 1. *Conteúdo em metais pesados (µg/g dry weight) nos sedimentos dos vários locais de colheita.*

Sites	Metals Cd	Cu	Zn
Bizerta lagoon	0.77±0.06	116.6±2.12	1208.9±4.33
Gargour	0.15±0.01	2.08±0.20	173.5±3.42
Nakta	0.15±0.04	37.43±1.54	236.28±5.56
Mahres	0.63±0.02	105.32± 3.5	1016.11±6.14
Skhira	0.59±0.07	89.5±4.3	897.51±6.34
Teboulba	0.08±0.002	12.49±1.21	176.9±2.51

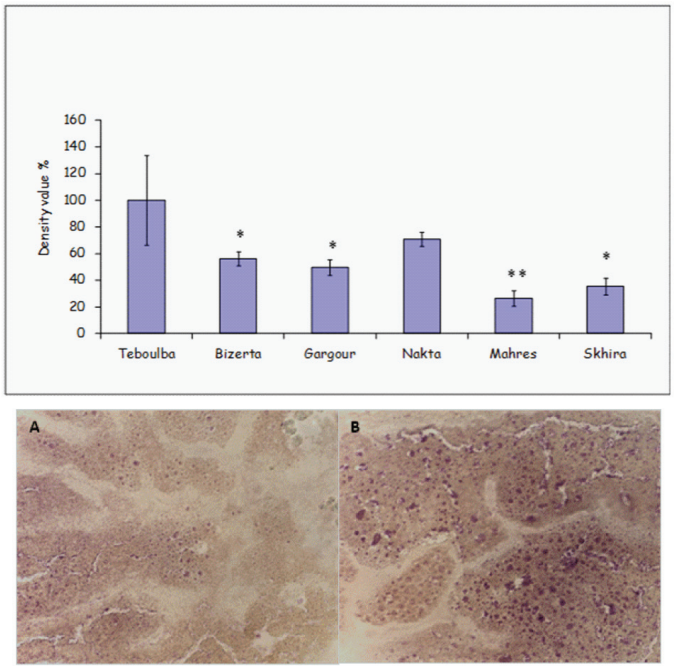

Figure 2. Lysosomal membrane stability in intestinal cells of *H. diversicolor* from the different sites. Data, expressed in percentage changes in optical density with respect to controls, are the mean±SD carried out on 6 different animals. *: p<0.05; **: p<0.01, Student's t test. Representative images in the cells of worms from Teboulba (A) and from Mahres (B) are reported.
Figura 2*. Estabilidade membranar dos lisossomas em células intestinais de H. diversicolor colhidos nos diferentes locais. Dados expressos em percentagem da densidade óptica em relação aos seus respectivos controlos: média±DP a partir de 6 indivíduos. *: p<0.05; **: p<0.01, Student's t test. Imagens representativas das células de indivíduos colhidos em Teboulba (A) e Mahres (B).*

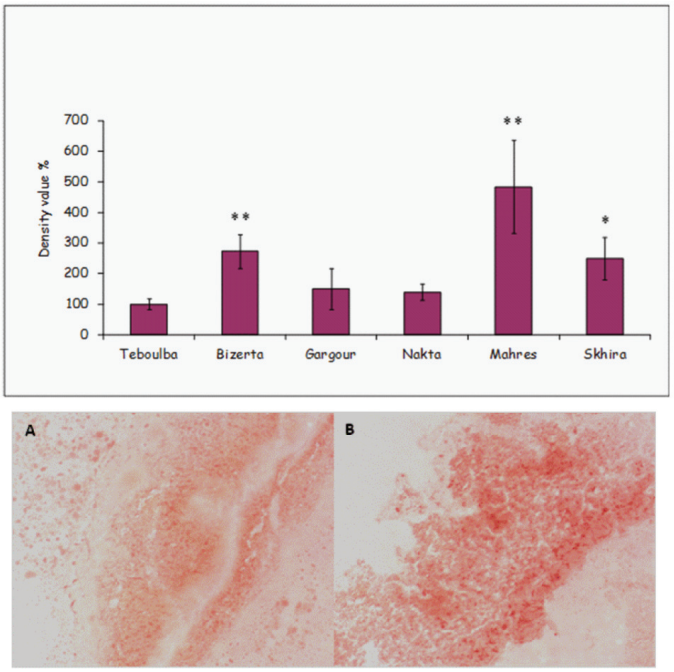

Figure 3. Neutral lipid (NL) accumulation in intestinal cells of *H. diversicolor* from the different sites. Data, expressed in percentage changes in optical density with respect to controls, are the mean±SD carried out on 6 different animals. *: p<0.05; **: p<0.01, Student's t test. Representative images in the cells of worms from Teboulba (A) and from Mahres (B) are reported.

Figura 3. *Acumulação de Lípidos Neutrais (NL) nas células intestinais de H. diversicolor colhidos nos diferentes locais. Dados expressos em percentagem da densidade óptica em relação aos seus respectivos controlos: média±DP a partir de 6 indivíduos. *: p<0.05; **: p<0.01, Student's t test. Imagens representativas das células de indivíduos colhidos em Teboulba (A) e Mahres (B).*

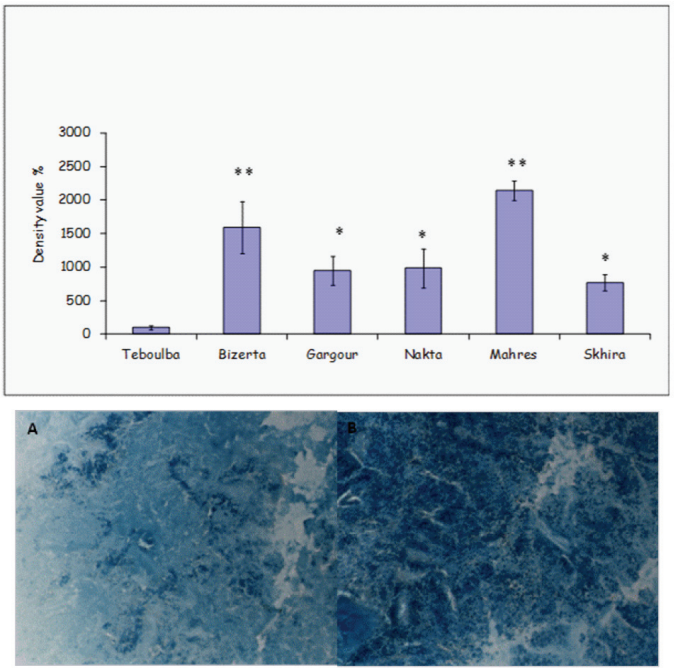

Figure 4. Lipofuscin accumulation in intestinal cells of *H. diversicolor* from the different sites. Data, expressed in percentage changes in optical density with respect to controls, are the mean±SD carried out on 6 different animals. *: p<0.05; **: p<0.01, Student's t test. Representative images in the cells of worms from Teboulba (A) and from Mahres (B) are reported.

Figura 4. Acumulação de lipofuscina em células intestinais de *H. diversicolor colhidos nos diferentes locais. Dados expressos em percentagem da densidade óptica em relação aos seus respectivos controlos: média±DP a partir de 6 indivíduos. *: p<0.05; **: p<0.01, Student's t test. Imagens representativas das células de indivíduos colhidos em Teboulba (A) e Mahres (B).*

4. DISCUSSION

The study of the biological responses of organisms to different environmental conditions and the quantitative evaluation of their physiological status are being considered as a successful approach for the assessment of environmental quality (Banni *et al.*, 2009, 2010; Dondero *et al.*, 2010). Invertebrates, such as polychaetes are suitable organisms for studying the biological effects of pollutants (Viarengo *et al.*, 2007; Carvalho *et al.*, 2013; Díaz-Jaramillo *et al.*, 2013). In fact, the use of *H. diversicolor* as an experimental model is widely advised. This polychaete is an endobenthic worm species widespread in brackish water environments throughout Tunisian coasts. It is thus among the first species exposed to terrestrial pollutants, and consequently, environmental contamination occurs earlier and could be considered more accentuated than in bivalve species (Scaps, 2002).

In this study, we employed a battery of four biomarkers to polychaetes *H. diversicolor* that had been collected from polluted site along the Tunisian coast (Fig. 1). We selected six sites characterized by different levels of heavy metal contamination. Our result clearly showed different degrees of heavy metal loads in the sediment sampled along the Tunisian sites (Table 1). Zinc content was higher at Bizerta lagoon, Marhes and Skhira. Cadmium content was very low in Teboulba and still low in Gargour and Nakta, the latter three showing also the lowest copper amounts. However Bizerta lagoon, Skhira and Mahres were characterized by relative high heavy metal loads (Cd and Cu), as previously reported by several study on the Tunisian coast (Hamza-Chaffai & Pellerin, 2003; Banni *et al.*, 2007; Bouraoui *et al.*, 2010; Jebali *et al.*, 2013). Based on the present chemical analysis and our previous results, we maintain Teboulba as a reference site and we confirm that Bizerta lagoon, Skhira and Mahres contains metal-tolerant populations of *H. diversicolor*. Numerous metals, among them Zn, Cu, and Cd, can be sequestered by invertebrates as electron-dense concretions. In invertebrates, these granules are present in all major phyla, including annelids (Bryan, 1974; Mouneyrac *et al.*, 2003).

The lysosomal system of many marine organisms such as molluscs, annelids, crustaceans and fishes, is known to be particularly sensitive to environmental perturbations and, for this reason, its alterations are widely used as indicators of physiological stress. Lysosomes, particularly in the digestive cells, are involved in various cellular processes including

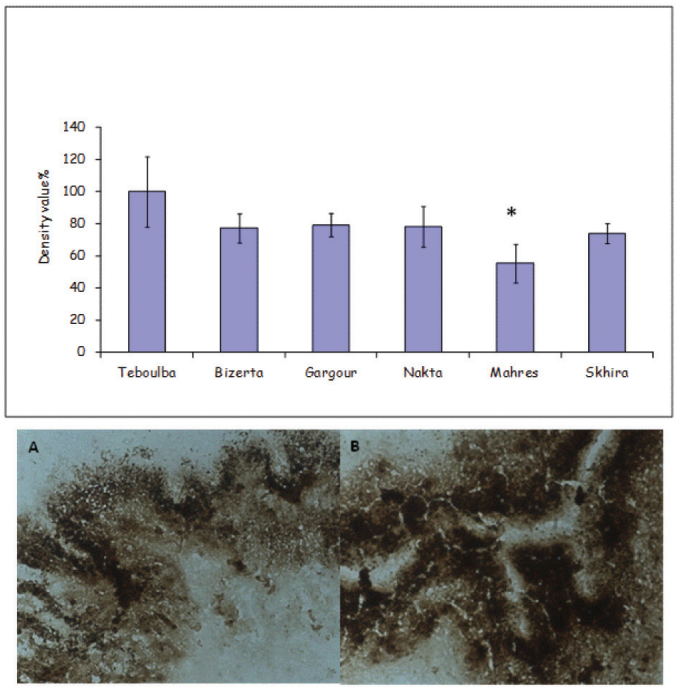

Figure 5. Ca^{2+}-ATPase activity in the intestinal cells of *H. diversicolor* from the different sites. Data, expressed as percent changes in optical density with respect to controls, are the mean±SD carried out on 6 different animals. *: p<0.05; **: p<0.01, Student's t test. Representative images in the cells of worms from Teboulba (A) and from Mahres (B) are reported.

***Figura 5**. Atividade Ca2+-ATPase analisada em células intestinais de H. diversicolor colhidos nos diferentes locais. Dados expressos em percentagem da densidade óptica em relação aos seus respectivos controlos: média±DP a partir de 6 indivíduos. *: p<0.05; **: p<0.01, Student's t test. Imagens representativas das células de indivíduos colhidos em Teboulba (A) e Mahres (B).*

digestion, defence, and reproduction (Moore, 1988; Viarengo *et al.*, 2007). Moreover, they are actively implied in the detoxification metabolisms, being involved in the sequestration and accumulation of a wide range of chemicals, such as metal ions, polycyclic aromatic hydrocarbons PAH, pharmaceuticals as well as nanoparticles (Moore, 2006; Koehler *et al.*, 2008).

Several reports have already demonstrated activation of lysosomes in response to environmental stress (Gastaldi *et al.*, 2007; Banni *et al.*, 2009; Catalano *et al.*, 2012). All the lysosomal parameters evaluated in poychaetes digestive cells, were clearly affected by a pollution gradient. A few reports have already demonstrated activation of lysosomes in response to environmental stress in polychaetes *H. diversicolor* (Catalano *et al.*, 2012; Moschino *et al.*, 2014). The effects on lysosomal membrane stability in *H. diversicolor* were evaluated in digestive cells following the reaction for N-acetyl-β-hexosaminidase using histochemical procedures applied on frozen tissue sections. This method is currently chosen by many researchers; for example it was selected in the BEEP EU project in the Baltic Sea as well as in the MARS project (Broeg *et al.*, 2002; Koehler *et al.*, 2002). However, while membrane destabilization is considered as a parameter of general stress, an increase in the contents of neutral lipids and lipofuscin indicates a situation of oxidative stress leading to lipid peroxidation of biological membranes (Moore, 1988; Gorbi *et al.*, 2012; Raftopoulou & Dimitriadis, 2012). In fact, Reactive Oxygen Species (ROS) produced in both physiological and stress conditions can stimulate the peroxidation of membrane lipids. The end-products accumulated within the lysosomes as insoluble pigments known as lipofuscins (LF) (Brunk & Collins, 1981). Measurement of lysosomal lipofuscin accumulation represents an index of peroxidation processes in a tissue (Moore *et al.*, 2006). Another general response to contaminant-induced stress is the alteration of fatty acid metabolism and the lysosomal accumulation of high levels of unsaturated neutral lipids (NL) that are internalised into lysosomes by autophagic uptake (Moore, 1985).

The global trend of lysosomal responses in the present study (LMS, NL and LF), clearly indicates accelerated oxidative stress in intestinal cells as a result of increasing metals loads as demonstrated by the chemical analysis. A previous study performed by our group on *H. diversicolor* sampled at field sites along the Tunisian coast and based on a biomarker battery included NADPH cytochrome C reductase, glutathione-S-transferase, catalase and malondialdehyde has reported the same trend in these studied areas (Bouraoui *et al.,* 2010). In fact, using the scale of classification based on biochemical markers established by Narbonne *et al.* (1999), our group showed that the most polluted site reflected by a higher multi-marker pollution index (MPI) is Mahres. The second MPI was recorded in Bizerta and Skhira and the lowest in Gargour and Nakta. Our Lysosomal responses data confirms those of other authors to emphasize the pollution statue of Sfax city coasted area due essentially to the presence of continuous discharge of heavy metals and also of organic compounds from local industrial activities (Smaoui- Damak *et al.*, 2004; Jebali *et al.*, 2007; Banni *et al.*, 2009). As for the region of Bizerta, our results are also consistent with those of Dellali *et al.* (2001) which reported a remarkable pollution status of this site by pesticides (organophosphorous and carbamates) and heavy metals.

Finally, another parameter of general stress, Ca^{2+}-ATPase was used. This enzyme is membrane SH-containing proteins that play a pivotal role in Ca^{2+} homeostasis. In the present work, Ca^{2+}-ATPase activity is the less significant parameter to discriminate between sites (Fig. 5). The Ca^{2+}-ATPase activity decreased levels of the intestinal cells of *H. diversicolor* from Mahres is, once again, an indication of oxidative stress induced by in field contaminant exposure. In fact, oxidative stress conditions and heavy metals such as Cu, Hg and Cd may lead to a partial inhibition of Ca^{2+}-ATPases in both aquatic invertebrates, such as mussels and seaworms (Viarengo *et al.*, 1998; Ermak & Davies, 2001; Burlando *et al.*, 2004, Catalano *et al.*, 2012) and terrestrial invertebrates such as earthworm *Eisenia andrei* (Gastaldi *et al.*, 2007).

5. CONCLUSION

Biomarkers have been assessed and evaluated in many field surveys. However, studies on lysosomal responses remain scarce. Our results provide an important mean for

characterizing the potential effects of contaminant impact on organisms living at polluted areas. This study has demonstrated that physiological effect, as represented by lysosomal biomarkers, is able to assess a stress syndrome and discriminate sites with various degrees of pollution. Our study may thus be used to better protect the health of Tunisian coasts to ensure sustainable management of coastal areas.

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