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Assessment of trace element accumulation on the Tunisian coasts using biochemical biomarkers in *Perinereis cultrifera*

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Summary: Our study aimed to evaluate the effect of trace element pollution in the polychaete *Perinereis cultrifera* (Grube, 1840) from two Tunisian coasts (the port of Rades, S1; and the Punic port of Carthage, S2). To this end, we used an approach based on proximate composition, biomarker responses and trace element bioaccumulation. Our results showed a decreasing order of metals concentrations (Zn>Cu>Cd>Pb) in *P. cultrifera* from S1 and S2. The accumulation of Cd, Cu and Zn was significantly higher in S1 than in S2, especially in summer. Lipid, protein and glycogen content also changed significantly between S1 and S2 in relation to trace metal accumulation and environmental conditions. The results revealed a higher level of thiobarbituric acid in *P. cultrifera* from S1 than from S2. In addition, the enzymatic and non-enzymatic antioxidant defence system (catalase, glutathione-S-transferase, superoxide dismutase, glutathione and metallothionein) was enhanced and acetylcholinesterase activities decreased in *P. cultrifera* in S1 in comparison with S2. A principal component analysis showed that *P. cultrifera* from S1 exhibited a clear disruption of oxidative stress responses and trace element bioaccumulation among seasons. Overall, these findings revealed the sensitivity of those organisms to environmental conditions.

Keywords: polychaete; coastal pollution; Gulf of Tunis; oxidative stress; environmental conditions; metallic pollution.

Evaluación de la acumulación de elementos traza en las costas tunecinas mediante el uso de biomarcadores bioquímicos en *Perinereis cultrifera*

Resumen: Nuestro estudio tenía como objetivo evaluar el efecto de la contaminación por elementos traza en el poliqueto *Perinereis cultrifera* (Grube, 1840) de dos costas tunecinas (S1: el puerto de Rades; S2: el puerto púnico de Cartago). Para ello, hemos utilizado un enfoque basado en la composición proximal, las respuestas de los biomarcadores y la bioacumulación de elementos traza. Nuestros resultados mostraron un orden decreciente de las concentraciones de metales (Zn>Cu>Cd>Pb) en *P. cultrifera* de S1 y S2. Nuestros datos mostraron una acumulación significativa de Cd, Cu y Zn en *P. cultrifera* recolectada en el puerto de Rades (S1), especialmente durante la temporada de verano, en comparación con las del puerto púnico de Cartago (S2). Asimismo, los contenidos de lípidos, proteínas y glicógenos cambiaron significativamente entre S1 y S2 en relación con la acumulación de metales y las condiciones ambientales. Los resultados obtenidos revelaron un aumento del nivel de ácido barbitúrico (TBARS) en *P. cultrifera* de S1 en comparación con S2. Además, se observó un aumento significativo del sistema de defensa antioxidante enzimático y no enzimático (catalasa CAT, glutación-S-transferasa: GST, superóxido dismutasa: SOD, glutatión: GSH y metaloproteína: MT) y una disminución de las actividades de la acetilcolinesterasa (AChE) se observaron en *P. cultrifera* del puerto de Rades (S1) en comparación con las del puerto púnico de Cartago (S2). El análisis de componentes principales (PCA) mostró que la *P. cultrifera* de S1 presentaba una clara alteración de las respuestas al estrés oxidativo y la bioacumulación de elementos traza entre las estaciones. En general, estos resultados revelaron la sensibilidad de estos organismos frente a las condiciones ambientales

Palabras clave: poliquetos; contaminación costera; golfo de Túnez; estrés oxidativo; condiciones ambientales; contaminación metálica.

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INTRODUCTION

The daily discharge of pollutants from human activities such as industry, agriculture, shipping, ports, domestic waste and anthropogenic pressure is a serious problem that has harmful effects on aquatic organisms (Paraskevopoulou et al. 2014). Many animals and plants living in polluted environments are often exposed to complex mixtures of chemical contaminants (Sureda et al. 2011), and these species have been proposed as a biomonitor of pollutants in coastal areas (Bodin et al. 2011). Polychaetes (Annelida) have been used as sentinels in coastal studies for monitoring purposes, especially in soft-bottom habitats (Li et al. 2012). These worms are one of the most important groups in terms of diversity and abundance in macro-benthic communities in coastal and marine environments, (Chouikh et al. 2019). They play a vital role in ecosystem functioning, not only because of their dominance, but also because of their different feeding modes and reproductive strategies (Han et al. 2016).

Scientists and environmental managers are currently working for a more specific concept of biomonitoring of the marine environment based on the study of the biological response by analysing bioaccumulation of metals, which are of particular concern due to their toxic effect, their persistence and their tendency to bioaccumulate and spread throughout food chains (Yi et al. 2008). Due to the complex stress responses influenced by metals, metalloids and their mixtures, it is important to create a battery of biomarkers that represent different roles and biological levels, such as the variation of oxidative stress (Di Salvatore et al. 2013, Bejaoui et al. 2017) and trace element concentration (Di Salvatore et al. 2013, Sureda et al. 2011), which can be linked to whole-organism responses and ecological responses to indicate the presence of harmful substances in the marine environment (Cravo et al. 2012). The toxicity of metals has been attributed mainly to their capacity to increase the generation of reactive oxygen species (ROS), which are thought to be the main cause of lipid and protein peroxidation (Chetoui et al. 2019). To neutralize free radicals and counteract the deleterious effects of ROS, cells possess an antioxidant defence system (Halliwell 2007).

In this study, the marine polychaete *Perinereis cultrifera* (Grube, 1840) was used as a biological model. It is present on the northwestern coasts of Europe, the Mediterranean Sea, the Atlantic, and the Indian and Pacific Oceans (Durchon 1957, Wu et al. 1985). In Tunisia, it is widespread but poorly documented (Zghal and Ben Amor 1989). According to the literature, *P. cultrifera* is an intertidal species, inhabiting rocky coasts under stones and boulders, usually where there is an accumulation of sediment (Scaps et al. 1992). Other studies have shown that this species can live in muddy sediments (Ghirardini et al. 1999). The mode of reproduction of *P. cultrifera* differs widely according to the geographical situation of the populations. It has a lifespan of three years and reproduces by epitokous or atokous (Scaps et al. 1992, Rouabah and Scaps 2003). In Tunisia, no studies on the reproduction of *P. cultrifera* have been carried out.

Several studies have reported that *P. cultrifera* is a suitable bioindicator species of marine pollution (Guemouda et al. 2014, Snani et al. 2015, Meghlaoui et al. 2015). However, no study has been carried out on *P. cultrifera* responses to anthropogenic pollution on the Tunisian coast. The present study is the first to evaluate the effect of environmental trace element bioaccumulation on the redox status system (lipid peroxidation, neurotoxicity and biomarker responses) and the biochemical reserves in whole tissues of *P. cultrifera* from two sites in relation to seasonal variation.

MATERIALS AND METHODS

Study areas

In the current study, sampling sites were chosen for the presence of *P. cultrifera* and the potential impact of pollution in the Gulf of Tunis. This gulf is located between the western Mediterranean basin and the western boundary of the eastern basin. The area is characterized by dynamic and diverse benthic communities, which have often been the subject of environmental studies (Antit 2012). For a few years, the littoral of the Gulf of Tunis has been the focus of intensive agriculture, industry, urban development and tourism. Consequently, the coastal zone of the Gulf of Tunis has been submitted to the impact of many chemical and physical stressors (Tlili et al. 2013). Two sites in the Gulf of Tunis differing in their level of contamination were selected for this study (Fig. 1). The port of Rades (S1) (36°48.6'N, 10°17.1'E) is considered a polluted area because of its location in front of the industrial complex of Tunis Bay (chemical and metallurgic industries) and its intense maritime traffic (Antit 2012, Tlili et al. 2013). According to Souissi et al. (2000), the port of Rades is generally eutrophic because of the nutrient enrichment caused by urban and industrial discharges through the Rades canal and the influence of land-based inputs. However, the Punic port of Carthage (S2) (36°50'41.28''N, 10°19'37.92''E) is considered to have undergone eutrophication, which is caused by increased nutrient loading as a result of rapidly expanding human population growth in the region since 1990 (Aissaoui et al. 2014).

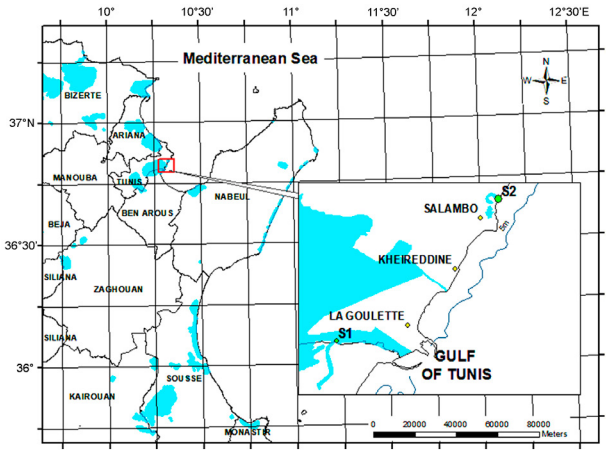

Fig. 1. – Map of the sampling sites in the Gulf of Tunis (Tunisia): S1, port of Rades; S2, Punic port of Carthage. Blue colour signifies the sea.

Sampling species and treatments

About 40 individuals (n=40) of *P. cultrifera* (S1, 9.09±1.3 cm length and 0.58±0.05 g weight; S2, 10.04±0.98 cm length and 0.63±0.02 g weight) were seasonally collected by hand from the two study sites in 2017-2018. They were collected in a sandy-muddy substrate where the species lives under sandy stones or even in the mud (upper 20 cm depth). After collection, specimens were transported to the laboratory. *P. cultrifera* (n=6 of 20 pooled individuals each season) were immediately frozen in liquid nitrogen for trace element quantification. All procedures of biomarker quantification were carried out at 0 to 4°C. As the influence of weight on biomarkers in different aquatic species has been previously reported, animals in the same tissue weight range were selected for the entire sampling period and for each site (Mouneyrac et al. 2000). For each sampling period and site, specimens (n=20 individuals) were homogenized by hand in liquid nitrogen with a porcelain mortar and pestle. The powder obtained was then homogenized with a motorized grinder with 1.5 mL citrate buffer at pH 5.0 for analysis for glycogen and lipids. Prior to protein analysis, specimens were ground in Tris-HCL (Trizma-base hydrochloric acid) buffer (0.8 M, pH 7.2) and centrifuged at 9000 g for 30 min at 4°C. The obtained supernatants were stored at –80°C until analysis.

Physico-chemical parameters

The physico-chemical parameters of the sampling areas were measured three times each season. Seawater temperature (T°C) was measured with a thermometer (model WTW.LF.325), salinity (S psu) was measured with salinity-conductivity (model WTW.LF.325) and pH was measured using a pH meter (model WTW.LF.325). The amounts of suspended particulate matter (SPM) and chlorophyll *a* (Chl *a*) were determined according to the method of Aminot and Chaussepied (1983).

Trace element analysis

Concentrations of cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) were determined in dried tissue for 48 h at 60°C. Sample powders were mineralized in a closed microwave (Milestone Inc.) using nitric acid and hydrogen peroxide mineralization. The resulting digestates were used to determine Cd, Cu, Pb and Zn by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer Inc.). The analytical procedure was checked using CRM-278R certified standard reference material. Results are expressed as mg per kg of dry weight. The extraction efficiencies for the tested trace elements were as follows: Cd=93%; Cu=94%; Pb=94%; and Zn=95%.

Biochemical analysis

Protein quantification

The protein content in the supernatant was determined according to the method of Bradford (1976) using Coomassie Blue as a reagent. Results are detected at 700 m using a microplate reader (Corning 9017) and are expressed as mg per g of tissue.

Lipid quantification

Total lipid concentration was analysed according to the method of Frings et al. (1972), using a sulfophosphovanillin reaction. The *United States* grade of olive oil (Sigma, St. Louis, USA) was used as a standard. Results were calculated by reference to a standard curve and expressed as mg/g of ww.

Glycogen quantification

Glycogen concentrations were estimated in two aliquots of the homogenate, one of which was submitted to enzymatic digestion by amyloglucosidase according to Carr and Neff (1984). Data were expressed as mg/g of ww.

Lipids and glycogen values were measured using colorimetric spectroscopy on a 96-well microplate reader (Corning 9017) at 540 and 420 nm, respectively.

Biomarker analysis

Thiobarbituric acid reactive substances

The concentration of thiobarbituric acid reactive substances (TBARS) was measured spectrophotometrically according to the method described by Knight et al. (1988). An aliquot of 0.5 mL of tissue extract supernatant was mixed with 1 mL of trichloro-acetic acid solution and centrifuged at 2500× g for 10 min. An aliquot of thiobarbituric acid (TBA) solution and 0.5 mL of supernatant was incubated for 45 min at 90°C and cooled. The absorbance of TBA-MDA complex was determined at 532 nm using a spectrophotometer. Lipid peroxidation was expressed as nmol of TBARS, using 1,1,3,3-tetra-ethoxypropane as standard. Results are expressed as nmols of MDA per mg of protein.

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined according to the method described by Ellman et al. (1961). The method is based on a coupled enzyme reaction involving acetylthiocholine as the specific substrate for AChE and 5,5′-dithio-bis-2-nitrobenzoate as an indicator for the enzyme reaction. The enzymatic reaction rate was measured spectrophotometrically at 412 nm. Results are expressed as nmol of substrate per min per mg of protein.

Enzymatic antioxidants

Glutathione S-transferase (GST) activity was determined according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene as a substrate and glutathione (GSH) in 100 mM potassium phosphate buffer, pH 6.5. Absorbance was measured at 340 nm, and activities were expressed as nmol of conjugated product formed per min per mg of protein.

Catalase (CAT) activity was measured following the method described by Aebi (1984) as a result of the decrease in absorbance at 240 nm due to the consumption of hydrogen peroxide (H_2O_2). The results are expressed as µmol of H_2O_2 consumed per min per mg of protein.

Superoxide dismutase (SOD) activity was evaluated by spectrophotometrically methods at 560 nm based on Beauchamp and Fridovich (1971). The obtained results were expressed as U per mg of protein.

Non-enzymatic antioxidants

Metallothionein (MT) level was estimated based on the method described by Viarengo et al. (1997) and modified by Petrovic et al. (2001). The absorbance was determined at 412 nm. The results were expressed as µmol of GSH per mg of protein.

Reduced GSH level in the *P. cultrifera* tissue was analysed at 412 nm by the Ellman (1959) method modified by Jollow et al. (1974), and the results were expressed as nmol per mg of protein.

Data analysis

Risk assessment trace metal indices

To compare trace element levels in each sampled individual for overall spatial and temporal variability, the metal pollution index (MPI) was determined as described by Usero et al. (2005):

$$MPI = (Cf1 \times Cf2 \ldots Cfn)\ 1/n \qquad [1]$$

where Cfn is the concentration of TEs n in the *P. cultrifera* tissue.

Statistical analysis

The results are expressed as means ± standard deviation (sd). The experimental data were initially tested for normality and homogeneity to meet the statistical demands. The statistical analysis between the tested population and seasons was performed using one-way ANOVA and Duncan's test and the observed difference at $p<0.05$ was considered significant. The whole data set of the biomarker responses, metal accumulation and biochemical contents in *P. cultrifera* was processed statistically by principal component analysis (PCA) and correlation matrix using STATISTICA software (version 13.2).

RESULTS

Abiotic parameters

The physico-chemical parameters are presented in Table 1. The most variation was recorded in summer, as evidenced by significantly lower T°C, SPM and Chl *a* in S2 than in S1 ($p<0.05$; one-way ANOVA). Additionally, only SPM was significantly lower in S2 in spring ($p<0.05$, one-way ANOVA) and autumn ($p<0.01$, one-way ANOVA). However, in winter, no change was noticed between the sampling sites. Summer exhibited the highest values of T°C and SPM and the lowest values of Chl *a* for both the sampling sites ($p<0.01$, one-way ANOVA).

Trace element concentrations in *P. cultrifera* tissues

The metal concentrations recorded in *P. cultrifera* are presented in Table 2. Zn exhibited the highest content followed by Cu, Cd and Pb. There was a significantly higher accumulation of Cd, Zn and Cu in spring in *P. cultrifera* from S1 than from S2 ($p<0.01$, one-way ANOVA). Also, Zn and Cu were significantly higher in *P. cultrifera* collected from S1 in summer ($p<0.01$, one-way ANOVA). Only Cd and Zn were higher in the *P. cultrifera* from S1 than from S2 in the cold seasons (autumn and winter, respectively). The greatest accumulation was observed in summer for both *P. cultrifera* populations ($p<0.05$, one-way ANOVA).

Table 1. – Seasonal variation of the physico-chemical parameters of sampling sites S1 (the port of Rades) and S2 (the Punic port of Carthage) in 2017/2018.

	Sites	Spring	Summer	Autumn	Winter
Temperature (°C)	S1	35.93±5.77	41.83±2.56$^{\#\#}$	34.43±4.06	27.41±2.91
	S2	35.18±1.23	38.31±1.08$^{**\#}$	34.25±1.47	27.61±2.45
Salinity (psu)	S1	32.83±0.28	34.40±0.52	30.33±0.57	28.50±0.50
	S2	32.41±0.52	35.06±0.30	30.16±0.25	28.10±0.26
pH	S1	8.03±0.01	8.10±0.09	8.13±0.05	8.13±0.11
	S2	8.01±0.02	8.05±0.05	8.05±0.05	8.00±0.01
Suspended matter (mg/L)	S1	907.66±79.47	1003.33±55.75$^{\#\#}$	738.50±33.75	595.66±46.50
	S2	813.33±73.71*	900.00±45.82$^{***\#\#}$	660.00±62.44**	563.33±47.25
Chlorophyll *a* (µg/cm3)	S1	1.94±0.19	1.09±0.20$^{\#\#\#}$	1.49±0.16	1.57±0.14
	S2	1.92±0.07	1.02±0.01$^{**\#\#\#}$	1.55±0.14	1.55±0.04

The results are represented as mean ± sd (n=3).
The statistical differences between sampling sites are represented at 5%: *p<0.05; **p<0.01; ***p<0.001 using one-way ANOVA.
The statistical differences between seasons are represented at 5%: #p<0.05; ##p<0.01; ###p<0.001 using one-way ANOVA.

Table 2. – Seasonal variation of trace element concentrations (Cd, cadmium; Cu, copper; Pb, lead; Zn, zinc) in *P. cultrifera* tissues collected from S1 (the port of Rades) and S2 (the Punic port of Carthage).

µg g^{-1} of DW	Sites	Spring	Summer	Autumn	Winter	CRM 278R
Cd	S1	0.37±0.01	0.34±0.11	0.43±0.10##	0.21±0.03	0.34±0.00
	S2	0.19±0.03***	0.51±0.03###	0.22±0.09**	0.30±0.10*	
Zn	S1	79.97±0.02	89.67±0.03#	71.41±2.01	71.03±0.02	83.100±1.7
	S2	56.42±1.81***	53.49±0.81***	74.39±3.31###	52.35±1.99***	
Cu	S1	5.70±0.22	6.88±0.03	6.78±0.01	5.76±0.02	9.45±0.13
	S2	4.95±0.49**	3.39±0.19**##	6.79±0.29	5.98±0.60	
Pb	S1	0.06±0.02	0.06±0.01	0.06±0.02	0.06±0.006	2.00±0.04
	S2	0.06±0.005	0.06±0.02	0.06±0.005	0.06±0.01	

The results are represented as mean ± sd (n=6 pooled individuals).
The statistical differences between sampling sites are represented at 5%: *p<0.05; **p<0.01; ***p<0.001 using one-way ANOVA.
The statistical differences between seasons are represented at 5%: #p<0.05; ##p<0.01; ###p<0.001 using one-way ANOVA.

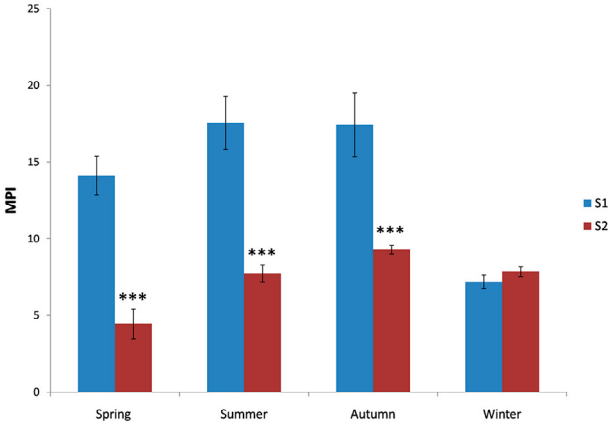

Fig.2. – Seasonal variation of the metal pollution index (MPI) in *P. cultrifera* tissues collected from the port of Rades (S1) and the Punic port of Carthage (S2). (The results are represented as mean ± sd [n=6]). The statistical differences between sampling sites are represented at 5%: *p<0.05; **p<0.01 and ***p<0.001 using one-way ANOVA. The statistical differences between seasons are represented at 5%: #p<0.05; ##p<0.01 and ###p<0.001 using one-way ANOVA.

Risk assessment of trace metals in *P. cultrifera* tissues

MPI values are presented in Figure 2. Our results demonstrated that this index was significantly higher in *P. cultrifera* from S1 in spring, summer and autumn (p<0.001, one-way ANOVA). However, no change was recorded in winter.

Proximate composition in *P. cultrifera* tissues

Glycogen contents

Glycogen content was significantly higher in S2 than in S1 in spring and autumn (p<0.05, one-way ANOVA), but no significant variation was observed in summer and winter. A significant decrease was observed in summer in both S1 (p<0.01, one-way ANOVA) and S2 (p<0.001, one-way ANOVA) (Fig. 3A).

Lipids contents

Lipid content was higher in S2 than in S1 in spring, summer and winter (p<0.01, one-way ANOVA). However, no significant variation was observed in autumn. Lipid content was highest in winter for both populations, while only S1 showed a significant decrease in lipid content in summer (p<0.05, one-way ANOVA) (Fig. 3B).

Protein contents

Protein content was significantly higher in S2 than in S1 (Fig. 3C) across seasons, but was significantly lower in summer for both populations (p<0.001, one-way ANOVA).

Biomarker responses

Thiobarbaturic acid level in P. cultrifera *tissues*

TBARS levels in *P. cultrifera* collected from S1 and S2 are presented in Table 3. Our results showed significantly higher TBARS levels in spring (53%), summer (65%) and autumn (64%) in S1 than in S2. However, in winter the levels of TBARS remained invariable between the populations. The highest levels were recorded in summer in S1 (5.01±0.14) and S2 (3.63±0.32) (p<0.05, one-way ANOVA).

Acetylcholinesterase activity in P. cultrifera *tissues*

Seasonal variations in AChE activity in *P. cultrifera* tissues are presented in Table 3. Our results revealed significantly lower AChE activities in S1 than in S2 in spring, summer and winter (p<0.05, one-way ANOVA). However, a similar trend of AChE activity was observed between S1 and S2 in autumn. AChE activity appeared to be significantly lower in summer and autumn in both populations (p<0.001, one-way ANOVA).

Enzymatic antioxidants

Glutathione S-transferase activity in P. cultrifera *tissues*

GST activity in *P. cultrifera* tissues is presented in Table 3. GST activity was significantly higher in summer (36%) and winter (89%) in S1 than in S2. However, no significant change was observed in spring and autumn. GST activity higher in both populations was significantly in summer than in the other seasons ($p<0.001$, one-way ANOVA).

Catalase activity in P. cultrifera *tissues*

CAT activity in both studied populations is summarized in Table 3. CAT activity was significantly higher in S1 than in S2 in all seasons ($p<0.05$, one-way ANOVA). CAT activity in both populations was significantly higher in summer ($p<0.001$, one-way ANOVA) than in the other seasons ($p<0.01$, one-way ANOVA).

Superoxide dismutase activity in P. cultrifera *tissues*

SOD activity was significantly higher in S1 than in S2 in spring (48%), summer (11%) and autumn (15%) (Table 3). SOD activity was significantly higher in spring and summer in both populations ($p<0.05$, one-way ANOVA).

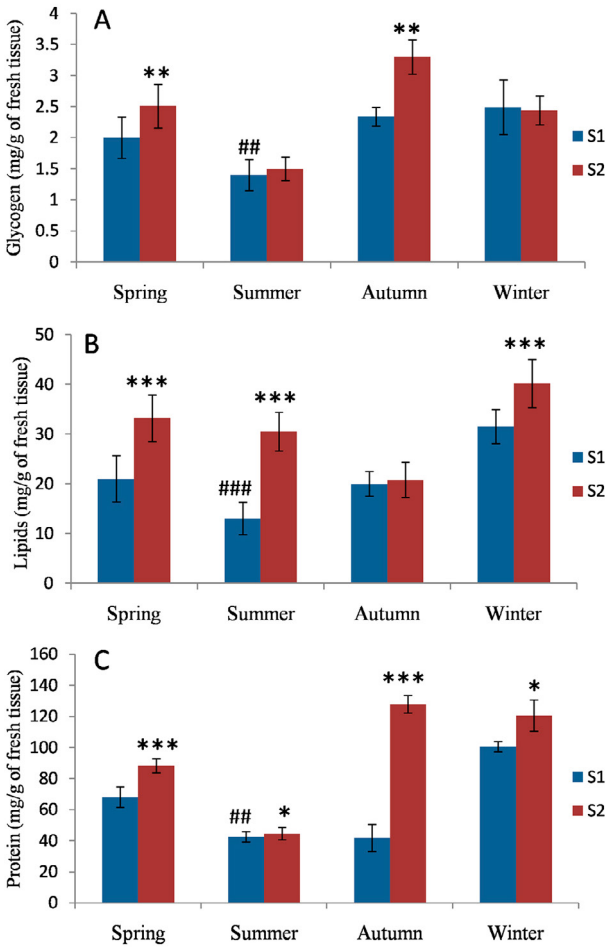

Fig.3. – Seasonal variation in the proximate fraction (mg/g ww) of *P. cultrifera* tissues collected from the port of Rades (S1) and the Punic port of Carthage (S2). (The results are represented as mean ± sd [n=20]). The statistical differences between sampling sites are represented at 5%: *$p<0.05$; **$p<0.01$ and ***$p<0.001$ using one-way ANOVA. The statistical differences between seasons are represented at 5%: #$p<0.05$; ##$p<0.01$ and ###$p<0.001$ using one-way ANOVA.

Non-enzymatic antioxidants

Metallothionein (MT) level in P. cultrifera *tissues*

The MT level was significantly higher in summer than in the other seasons for both populations ($p<0.05$, one-way ANOVA). The MT level was 40% and 21% higher in S1 than in S2 in spring and summer, respectively (Table 3). However, no significant difference was observed between the sites in autumn and winter.

Glutathione level in P. cultrifera *tissues*

Summer showed the highest GSH level in both populations ($p<0.05$, one-way ANOVA). The results for GSH levels of *P. cultrifera* tissues are presented in Table 3. Although GSH levels were higher in S1 than in S2 in spring (40%) and summer (20%), they were not statistically different in autumn and winter.

Principal component analysis

The results of the PCA are presented in Figure 4, which has input from key factor axes and shows the correlation circle and the projection of individuals (seasons) on the factorial design (1: 2). This analysis allowed us to retain the first two factorial axes, which explained 68.81% of the total variance. The first axis contributed the maximum dispersion (50.49% of the total) and correlated negatively with CAT, GST, TBARS, GSH, MTs, SOD, Cd, temperature, salinity, and SPM (Fig. 4). However, only protein, glycogen and Chl *a* were correlated positively with the first axis. The second axis was described by 18.32% of the total dispersion. This axis showed a positive correlation with Zn, Cu and pH and a negative correlation with lipid content. The screening of individuals (sites/seasons) on the same factorial showed different clusters. The first one, which included specimens taken from S1 and S2 in summer, coincided with the higher levels of TBARS, GSH, GST, CAT, Cd, Zn, salinity, temperature and SPM. Also, specimens taken from S2 in winter were showed a high lipid content and AChE activity. However, data observed at both S1 and S2 in spring, autumn and winter (only for S1) showed the highest activity of Cu, glycogen, Chl *a* and protein.

Correlation matrix between trace metals and tested parameters in *P. cultrifera* tissues

The statistical analyses were performed between the tested parameters and the study sites (Table 4). The correlations were recorded between Cd, Zn Cu, Pb and all of the redox status parameters. AChE activity and protein, glycogen and lipid content showed significant negative correlations with temperature ($p<0.05$), salinity ($p<0.01$), suspended matter ($p<0.01$) and trace metals accumulation ($p<0.05$) in *P. cultifera* from the Tunisian harbours. All tested parameters (CAT, GST, TBARS, GSH, MTs and SOD) were positively correlated with the abiotic parameters and the trace metal concentrations ($r\geq 0.320$; $p<0.05$).

Table 3. – Seasonal variation in the oxidative stress biomarkers in *P. cultrifera* tissues collected from the port of Rades (S1) and the Punic port of Carthage (S2).

Parameters	Sites	Spring	Summer	Autumn	Winter
TBARS	S1	2.39±0.12	5.01±0.14$^{###}$	1.71±0.26	1.79±0.41
	S2	1.58±0.31*	3.63±0.32*	1.02±0.13	1.33±0.46
AChE	S1	29.39±2.90	17.55±1.91	18.58±1.99	52.78±2.53$^{###}$
	S2	36.19±1.16	23.56±3.1*	19.32±1.97	57.50±2.77
MTs	S1	0.07±0.00	0.14±0.01$^{##}$	0.07±0.00	0.04±0.00
	S2	0.05±0.00***	0.11±0.00***	0.06±0.00*	0.04±0.00
GSH	S1	0.40±0.05	0.55±0.09$^{###}$	0.32±0.08	0.17±0.042
	S2	0.24±0.02***	0.44±0.06*	0.28±0.03	0.15±0.015
SOD	S1	5.12±0.87	6.93±0.56	3.27±0.46	1.90±0.352$^{###}$
	S2	2.61±0.57***	6.14±0.64*	2.76±0.35*	1.67±0.184
CAT	S1	53.73±2.66	101.90±3.78$^{###}$	46.80±9.49	41.49±1.05
	S2	49.10±9.02*	83.17±6.78**	33.71±8.60*	30.16±2.43*
GST	S1	57.58±2.82	190.30±6.09$^{###}$	33.49±1.45	41.41±1.69
	S2	48.98±3.85	138.93±4.58***	29.12±8.92	21.83±9.95

The results are represented as mean ± sd (n=20 individuals).
The statistical differences between sampling sites are represented at 5%: *p<0.05; **p<0.01; ***p<0.001 using one-way ANOVA.
The statistical differences between seasons are represented at 5%: #p<0.05; ##p<0.01; ###p<0.001 using one-way ANOVA.
TBARS, thiobarbituric acid; AChE, acetylcholinesterase; MTs, metallothionein; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GST, glutathione-S-transferase.

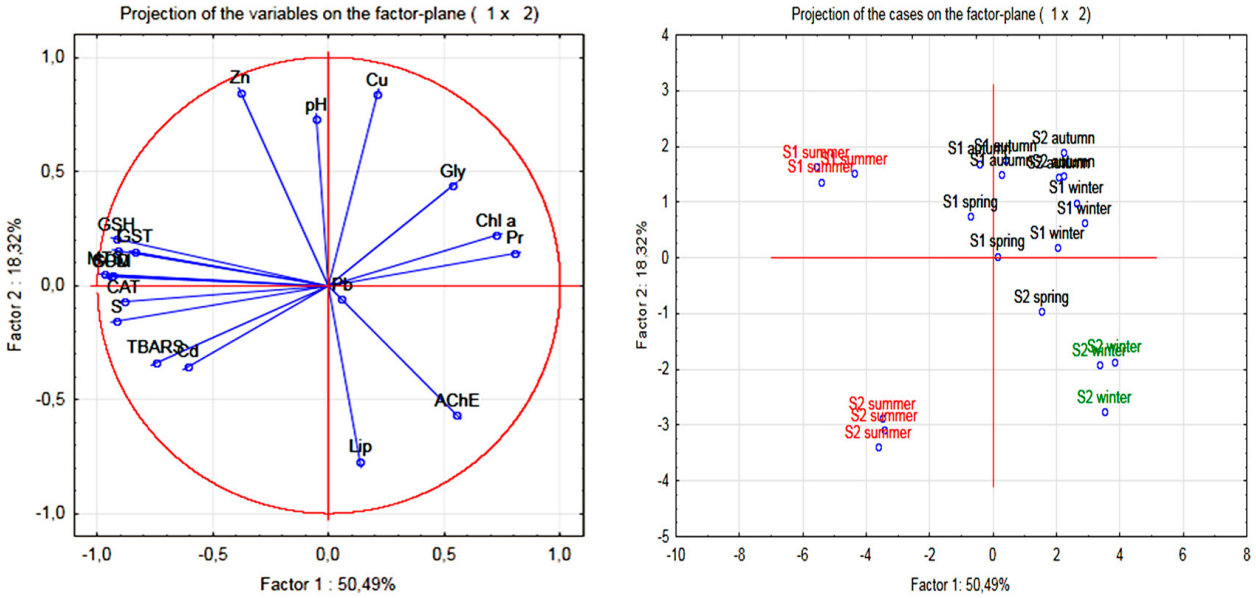

Fig. 4. – Results of PCA represented by two factors (F1=50.49% and F2=18.32%) and produced by biochemical variables and trace element concentrations in *P. cultrifera* from two sites in the Gulf of Tunis. Pr, proteins; Lip, lipids; Gly, glycogen; TBARS, thiobarbituric acid; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; GST, glutathione-S-transferase; AChE, acetylcholinesterase; MT, metallothionein; Cd, cadmium; Zn, zinc; Cu, cooper; Pb, lead; T, temperature; S, salinity; Chl *a*, chlorophyll *a*. (A) Projection of the variables on the factor-plane 1-2. (B) Projection of the cases on the factor-plane 1-2.

Table 4. – The matrix of correlation between the analysed parameters and the sampled *P. cultrifera.*

	T°C	S psu	SPM	Cd	Zn	Cu	Pb
CAT	0.327*	0.327*	-	-	-	-	-
AChE	-0.529**	0.544**	0.364*	-	-0.540**	-0.590*	-
GST	0.520**	-	-	-	0.458**	-	-
TBARS	0.641**	0.320*	-	0.451*	-	-	-
CSH	0.588**	0.509**	0.405*	0.536**	0.557**	-	-
MTs	0.607**	0.666**	0.555**	0.510**	0.418*	-	-
SOD	0.689**	0.478**	0.601**	0.532**	0.558**	-	-
Proteins	-	-	-0.678***	-0.547**	-	-	-
Lipids	-0.736***	-0.523**	-0.772***	-	-0.644***	-0.443*	-
Glycogen	-0.451*	-	-0.582**	-	-	-	-

TBARS, thiobarbituric acid; AChE, acetylcholinesterase; MTs, metallothionein; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GST, glutathione-S-transferase; T°C, temperature; S psu, salinity; SPM, suspended matter; Cd, cadmium; Zn, zinc; Cu, cooper; Pb, lead.
The significant correlation is presented by *p<0.05; **p<0.01 and ***p<0.001.

DISCUSSION

Over the past decades, aquatic ecosystems have suffered from an increase in anthropogenic pollution due to the presence of toxic contaminants (hydrocarbons, pesticides and heavy metals) and other environmental factors (hypoxia, changes in temperature and salinity) (Campillo et al. 2013). Several studies have revealed the effectiveness of marine invertebrates such as *P. cultrifera* as a bioindicator and sentinel organisms for toxicological studies in coastal waters (Guemouda et al. 2014, Snani et al. 2015, Meghlaoui et al. 2015). They are known to accumulate metals in their tissues and survive in contaminated environments, and they are herbivores, have sensitivity and resistance to contaminants and are relatively easy to collect, handle, culture and transport (King et al. 2004).

Spatial and temporal variations in metal concentrations were observed between the two sampling sites of the port of Rades (S1) and the Punic port of Carthage (S2), most likely resulting from the high temperature and evaporation, because in summer metal accumulation shows a significant positive correlation with water parameters such as temperature ($p<0.05$). The difference in accumulation of metals in the two populations probably indicated the anthropogenic discharges of industrial activities and maritime traffic in the port of Rades. In agreement with this hypothesis, several studies have shown a high accumulation of metals in polychaetes during the warm season. Rouhi et al. (2007) demonstrated that in spring and summer metals were accumulated mostly in the whole soft tissues of two polychaete species (*Sabellaria alveolata* and *Arenicola grubii*) collected from the coastline of Jorf Lasfar, Morocco. These findings are probably related to environmental factors and the physiological activity of the animals. Furthermore, Bat et al. (2019) showed a significant difference of metal accumulation between seasons in polychaetes on the shores of Sinop in the Black Sea.

As a metal binding protein, metallothionein (MT), a non-enzymatic antioxidant rich in cysteine and with a low molecular weight, plays a key role in cell defence in addition to ROS generation (Viarengo et al. 1999). Our data showed a higher MT level in S1 than in S2, probably reflecting an adaptative response of the sampled species to the toxic effects induced by trace metal accumulation. This result was confirmed by a positive correlation between MT level and the tested metals in *P. cultrifera* tissue (Cd: 0.510; Zn: 0.418). Our results were correlated with those of Suriya et al. (2012) and Won et al. (2008), who demonstrated that trace metal accumulation can generate ROS in *Capitella capitela* and *Perinereis nunta*, which in turn contributes to an enhancement of MT level.

Environmental agents such as metals generate intracellular imbalance and stimulate ROS, causing oxidative stress. The biological targets for these highly reactive oxygen species are lipids and proteins, which cause oxidations through the generation of hydroxyl and free radicals from H_2O_2 via the Fenton reaction, and can adversely affect cells by producing lipid peroxidation of intracellular membranes (Bejaoui et al. 2019). There is considerable evidence that lipid peroxidation, as measured by TBARS levels, could be proposed as a biomarker of membrane lipid damage because it is considered a by-product of lipid oxidative damage (Freitas et al. 2012). Our data show lower lipid content in S1 than in S2, which correlates with the increase in the TBARS level mainly in summer. This increase in the TBARS level and decreases in lipid content appears to be due to metal uptake, which accelerates the ability to scavenge ROS by extreme production of lipid peroxides resulting from alteration of membrane fluidity and integrity (Chetoui et al. 2019). Previous studies conducted on polychaetes such as *Hediste diversicolor*

from contaminated sites on the southwestern Iberian coast (Gomes et al. 2013) and the Oued Souss estuary in the Bay of Agadir, Morocco (Ait Alla et al. 2006) revealed similar increases in TBARS levels. This may be linked to several factors, including metal concentration (Cd, Pb, etc.) and environmental changes (temperature, salinity, etc.), as described by Sun and Zhou (2008) and Freitas et al. (2015) on *Hediste diversicolor* and *Diopatra neapolitana*, respectively. Also, Zhao et al. (2013) found that a high salinity level may increase the potential ecological risk of metal accumulation by generating chemical mobility, thus crossing the biological barriers and interacting with the components of the cellular membranes, causing alterations in their permeability and lipid peroxidation. Our results also showed a significant positive correlation between TBARS levels and Cd (0.735), salinity (0.420) and temperature (0.641) for polychaetes collected from S2.

Several studies have demonstrated that trace metal accumulation in marine organisms alters their biochemical composition, including proteins and glycogen (Bejaoui et al. 2019). Among these macromolecules, proteins are abundant in biological tissues, having high rate constants for reaction with many species, which is why these proteins are the main target for biological oxidants (Wang et al. 2019). Therefore, oxidant formation is the main consequence of protein damage both externally and within cells. The occurrence of protein reduction in the *P. cultrifera* tissues from S1 was probably due to excess free radical generation under metal accumulation. Both high lipid peroxidation and protein oxidation may be due to metal contamination, as reported in *Mytillus galloprovincialis* by Bouki et al. (2013), who suggested that metal contamination affects the amount of protein and increases TBARS levels. Our data show a similar glycogen content between the two sampled harbours in summer and winter, whereas in spring and autumn content was higher in S2. Our results were in accordance with those of previous studies carried out on aquatic organisms, which showed that proximate composition decreased when species were exposed to anthropogenic pollution (Douhri and Sayah 2009, Bejaoui et al. 2019).

The effect of environmental metal pollution on *P. cultrifera* tissue mechanism was also assessed through AChE activity. AChE is present in the neuromuscular junctions and cholinergic synapses of the central nervous system and terminates the signal transmission by hydrolysing acetylcholine (ACh), a neurotransmitter that conducts nerve impulses across neuromuscular junctions (Lodish et al. 2000). Its inhibition can be seen as an early warning biomarker of contaminant risks (Cravo et al. 2012). Our results showed inhibition of AChE activity in specimens taken from the port of Rades. Snani et al. (2015) suggest that the decrease in AChE release could be induced by the accumulation of trace metals in *Perinereis cultrifera* from the eastern coast of Algeria. Similar observations have been reported in *Hediste diversicolor* and *Patella vulgate* collected from polluted sites of Bay of Tangier in Morocco (Douhri and Sayah 2009). In addition, our results showed increases in AChE activity in winter and decreases in summer. Moulton et al. (1996) showed that high environmental temperature caused the inhibition of AChE activity in *Elliptio complenata* mussels. Additionally, Scaps and Borot (2000) demonstrated that salinity and temperature can also affect acetylcholinesterase activity in *Hediste diversicolor*.

At the cellular level, the functional changes in redox statistics induced by ROS production leads to alterations in cellular metabolism. This damaging action greatly increases the responses of enzymes antioxidants such as SOD, GST CAT and GSH levels (Cravo et al. 2012). SOD, CAT and GST are three major antioxidant enzymes in the cells. #SOD is the primary defence line against oxygen-derived free radicals and catalyses the dismutation of the superoxide anion (O_2^{-}) into hydrogen peroxide (H_2O_2), which changes into H_2O and O_2 through CAT# (Ma et al. 2017). CAT is one of the enzymes involved in reduction of H_2O_2 via the Fenton reaction (Chance et al. 1979). GST protects cells against trace element-induced toxicity by deactivating the cytotoxic and genotoxic compounds, catalysing the S-conjugation and reducing GSH (Salinas and Wong 1999).

We found higher SOD and CAT activity in S1 than in S2, most probably reflecting an adaptive response of those species to remove the harmful effects of ROS generation against environmental stressors, as reported by Carregosa et al. (2014). In addition, increased activities have been reported in several fish and invertebrate species collected from polluted sites (Jebali et al. 2007, Bejaoui et al. 2020). As shown in the study of Ait Alla et al. (2006), these enzymatic activities increased in *Hediste diversicolor* collected from the Oued Souss (Bay of Agadir, Morocco) before implantation of wastewater treatment. Douhri and Sayah (2009) also reported an increase in CAT activity in two marine invertebrates, *Hediste diversicolor* and *Patella vulgate*, taken from polluted sites of the Bay of Tangier in Morocco. Our findings are also similar to those obtained for *P. cultrifera* collected from three sites impacted with different levels of anthropogenic pollution on the eastern coasts of Algeria (Guemouda et al. 2014). The increase in enzymatic activities could also be explained by the higher concentration of bio-accumulated metals, which causes oxidative stress and an antioxidant response (Gaete et al. 2017). However, a significant correlation was observed between Cd, Zn and SOD activity as well as Zn and GST activity (Table 4), suggesting that these metals are bioavailable and responsible for the variation in enhancement of the activities of these antioxidants.

The results of seasonal variations in GST activity at each site showed that it was highest in summer and higher in the port of Rades (S1) than the Punic port of Carthage (S2) throughout the study period ($p<0.05$). In addition, this enzyme plays a role in cellular protection against oxidative stress, which can be triggered by pollutants such as metals, PCBs and PAHs (Michel et al. 1998). These findings are similar to those obtained for the worms *Hediste diversicolor* collected from the polluted estuary of the Seine (Durouet al. 2007). To substantiate the mechanisms underlying trace metal accumulation, GSH was also examined. This non-en-

zymatic antioxidant is the most important cytosolic sulfhydryl compound that acts as a reducing and protective agent against intensive toxic substances, including trace metals through their –SH groups (Das et al. 2017). Our results showed a higher GSH level in S1 than in S2, which could be a result of increased synthesis and neutralization of ROS via the thiol group. Dissimilar studies on polychaetes have shown a decrease in GSH level in relation to trace metal accumulation (Lv et al. 2016). According to the above results, the significant relationship between antioxidant biomarkers (mainly GSH, SOD and GST) and the concentrations of metals (mainly Cd and Zn) in *P. cultrifera* tissue suggest the activation of the mechanisms of detoxification due to the generation of ROS. These findings are similar to those obtained by Cataldo et al. (2011) in earthworms at sites contaminated by metals and by Gaete et al. (2017) in *Perinereis gualpensis* from estuaries of central Chile.

CONCLUSION

In conclusion, in different seasons and geographical locations *P. cultrifera* shows different physiological responses. In this study, we have assessed the effects of anthropogenic pollution in terms of metal accumulation, antioxidant response and alteration in proximate composition in relation to seasonal change in *P. cultrifera* tissues. The results clearly show that S1 is more affected by anthropic pollution than S2. Our study has identified some insights into anthropogenic pollution at the harbour sites, but further studies on metal accumulation in sediments and exposure of *P. cultrifera* to metals are still required to provide general information about the health status of these coastal harbours.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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