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Assessment of oxidative stress, genotoxicity and histopathological responses in the digestive gland of *Ruditapes decussatus* collected from northern Tunisian lagoons

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Summary: The aim of the present study was to investigate the combined effects of seasonality and anthropogenic pressure on a battery of oxidative stress, lipid peroxidation, protein oxidation, DNA damage and histological alterations in the native clam *Ruditapes decussatus* collected from a less contaminated area (LCA), Ghar El Melh, a moderately contaminated area (MCA), the North Lake, and a highly contaminated area (HCA), the South Lake, all located in the southern Mediterranean Sea. The accumulation of cadmium, lead, copper, iron and zinc was higher in the digestive glandsof clams collected from the MCA and the HCAthan in those from the LCA, particularly during the warm season. Our results reveal that metallothionein, lipid peroxidation, protein oxidation levels and antioxidant defence systems were higher, while acetylcholinesterase activity was lower, in clams from the MCAand HCAthan in those from the LCA. The results also indicate that clams from the MCA and the HCAare characterized by histological alterations and DNA damage. In conclusion, the evident changes of antioxidant defence systems and macromolecules between the studied lagoons reveal the perturbation of the physiological states of clams from polluted sites thatcope with seasonal changes and trace element accumulations.

Keywords: *Ruditapes decussatus*; digestive gland; trace element accumulations; redox status; macromolecule injuries; histoarchitecture alteration.

Evaluación del estrés oxidativo, genotoxicidad y respuestas histopatológicas en la glándula digestiva *Ruditapes decussatus* recolectada de las lagunas del norte de Túnez

Resumen: El objetivo del presente estudio es investigar los efectos combinados de la estacionalidad y la presión antropogénica en una batería de estrés oxidativo, peroxidación lipídica, oxidación de proteínas, daños en el DNA y alteraciones histológicas en la almeja nativa *Ruditapes decussata* recolectada de un área menos contaminada (Ghar El Melh «LCA») y de dos sitios con diferentes niveles de contaminación (la laguna norte «MCA» y la laguna sur «HCA» de Túnez) en el sur del mar Mediterráneo. La acumulación de cadmio, plomo, cobre, hierro y zinc fue mayor en la glándula digestiva de las almejas recolectadas de la MCA y la HCA en comparación con las de la LCA, particularmente durante la estación cálida. Nuestros resultados revelan que la metalotioneína, la peroxidación lipídica, los niveles de oxidación de proteínas y los sistemas de defensa antioxidante aumentaron, mientras que la actividad de la acetilcolinesterasa disminuyó en las almejas del área moderadamente y altamente contaminada en comparación con la menos contaminada. Los resultados también indican que las almejas del MCA y el HCA se caracterizan por varias alteraciones histológicas y daños en el ADN. En conclusión, los cambios evidentes de los sistemas de defensa antioxidante y las macromoléculas entre las lagunas estudiadas revelan la perturbación de los estados fisiológicos de las almejas de los sitios contaminados que hacen frente a los cambios estacionales y las acumulaciones de metales.

Palabras clave: *Ruditapes decussatus*; glándula digestiva; acumulaciones de metales; estado redox; lesiones de macromoléculas; alteración de la histoarquitectura.

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INTRODUCTION

Marine ecosystems, particularly lagoons, are of great ecological and economic importance becausethey support vital habitats for numerous marine organisms. However, they sustain several anthropogenic pressures (Barhoumi et al. 2014). In fact, increasing industrialization has led to a massive release of pollutants into these enclosed ecosystems (Di Salvatore et al. 2013). During the last few decades, the aquatic environment in Tunisia, especially the north coast, has been contaminated by trace element (TE) pollutants as a result of rapid industrialization and urbanization (Mzoughi and Chouba 2012, Daldoul et al. 2015). In recent years, the discharge of industrial wastewater containing a high level of TEs has increased (Ghribi et al. 2020). The TE content in sediments from the Tunisian gulfhas been observed to reach a considerable level that could be released into the water by flow changes or benthic agitation, causinga sustained impact on aquatic organisms and eventually on human beings through the food chain (Ennouri et al. 2016).

Among these contaminants, metals and metalloids are a particular concern because of their toxic effects and their persistence through bioaccumulation and biomagnification along the food chain (Leung et al. 2016, Faggio et al. 2018). From an ecotoxicological point of view, it has been demonstrated that TEs can adversely affect aquatic organisms mainly through an excessive generation of reactive oxygen species (ROS) (Telahigue et al. 2018, Yang et al. 2018). Indeed, ROS are proven to cause metabolic and oxidative homeostasis imbalances because of their high reactivity towards cellular components such as lipids, proteins and nucleic acids (Bejaoui et al. 2020). To protect cells against ROS, some specific antioxidants such as superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx), thiols as glutathione (GSH) and ascorbic acid (Vit C) are also elevated in the detoxification of free radicals (Barhoumi et al. 2014). Oxidative stress can also be reduced through the complication of free TEs by metallothionein (MT). The latter is an oxyradicalscavenger playing an important role inthe homeostasis of essential TEs and in the detoxification of non-essential ones (Gagné et al. 2008). Histopathology is also a powerful tool for monitoring anthropogenic contamination (El-Shenawy et al. 2009). According to Bejaoui et al (2020), the histological study could provide information on the adaptive response to environmental pollution and cell damage. In marine ecosystems, histopathological alterations have been associated either with deterioration of environmental conditions or with pollution (Fanta et al. 2003, Marchand et al. 2009).

During the last few decades, marine invertebrates, especially bivalve molluscs, have been widely used as sentinel species for aquatic pollutants associated with ROS generation (Tsangaris et al. 2016, Uluturhan et al. 2019, Bejaoui et al. 2020). These sessile filter-feeder organisms are commonly used to assess the biological effects of contaminated ecosystems (Cravo et al. 2012). The clam *Ruditapes decussatus* is among the most common marine molluscs in Tunisia and around the world. It is an important worldwide economic resource and has been widely used as a bioindicator for monitoring water quality in numerous environmental investigations (Campillo et al. 2013, Mansour et al. 2020, Bejaoui et al. 2020).

There are numerous studies focusing on TEs contamination in sediments and marine organisms from the northern coast of Tunisia (including the Tunisian Gulf and the Bizerte lagoon) (Chalghmi et al. 2016, Ghannem et al. 2016). However, investigation of the effect of TE contamination in bivalves from Ghar el Melh and the Northern and Southern Lagoons of Tunisia has so far been insufficient. Previous studies have integrated the toxicity responses provided by the clam *R. decussatus* and contamination to assess the quality of some Tunisian lagoons (Chalghmi et al. 2016). The current study is aimed at comparing the environmental quality of three lagoons with different contamination levels, a less contaminated area (LCA), a moderately contaminated area (MCA) and a highly contaminated area (HCA) on the northern Tunisian coastline by integrating water parameters, TE accumulation, biological responses, macromolecules and tissue injuries using the digestive gland of *R. decussatus*.

MATERIALS AND METHODS

Study areas

Three sites with different anthropic pressure levels were chosen to conduct our study (Fig. 1):

– Ghar El Melh (37°11′26.25″N 10°18′73.49″E) is considered one of the most important wetlands in Tunisia. It is situated in the extreme north of the Gulf of Tunis (Fig. 1) at the downstream end of the lower valley of the Mejerda River and is linked to the Mediterranean Sea through a dredged inlet (Ayache et al. 2009). This lagoon has an elliptical shape, covers an area of approximately 28.5 km^2 and has an average depth of 0.8 m (Moussa et al. 2005). The water column of Ghar el

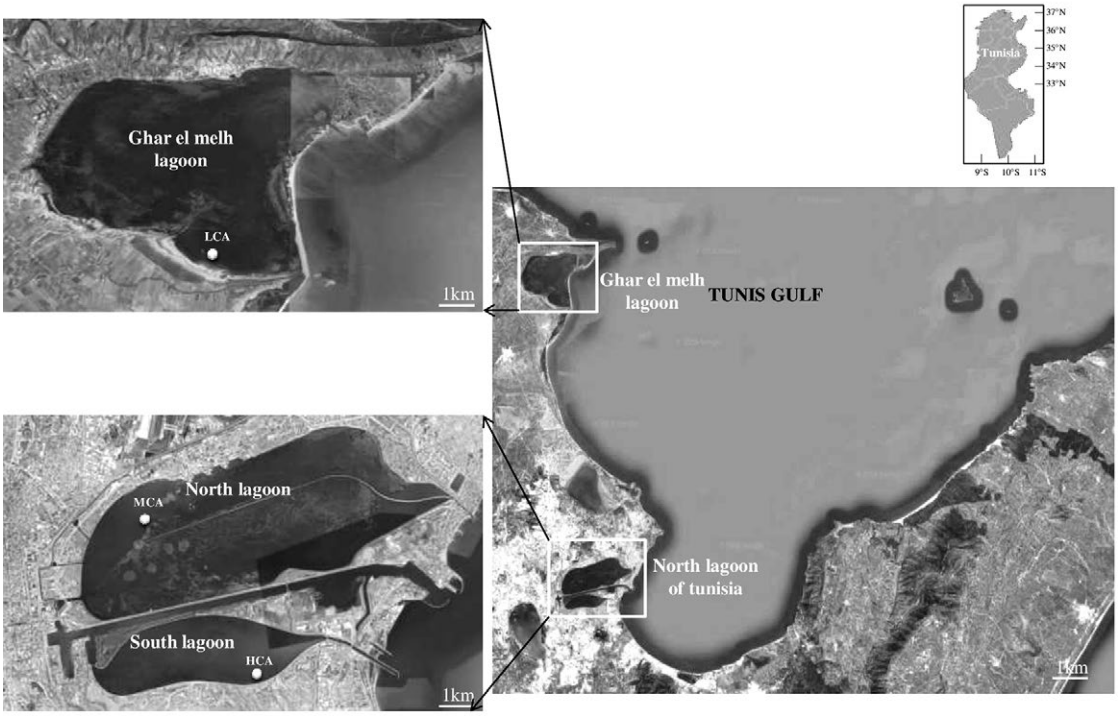

Fig. 1. – The location of sampling sites at Ghar El Melh (LCA) and the North Lake (MCA) and South Lake (HCA) of the northern Tunis lagoon.

Melh lagoon residues are well homogenized throughout the year thanks to wind-induced mixing. A narrow channel enables a restricted water exchange with the open sea (water residence time in the lagoon: 35 days; Rasmussen et al. 2009). Ghar El Melh Lagoon receives various discharges from the Mejerda River, which delivers 17 million t of sediment annually (Oueslati et al. 2010). The lagoon ecosystem has suffered progressive deterioration and is considered hypereutrophic (Rasmussen et al. 2009) because of human activities within the lagoon itself and in the surrounding area (such as discharges of domestic wastewater, industrial waste from the drainage system and fishing activity) (Moussa et al. 2005). Furthermore, natural disturbances may cause alterations to the hydrodynamics and sediments resulting in a hyper-eutrophication of the environment. According to Nourisson et al. (2013), Ghar El Melh is considered a moderately polluted site, because it is one of the world's wetlands awarded the Wetland City Label accredited by the international Ramsar Convention for the protection of wetlands (Ben Haj 2012). Thus, it was selected as the LCA site in the current study on the basis of other published works (Oueslati et al. 2010, Bejaoui et al. 2018).

– The Northern Lagoon of Tunis is a shallow submarine environment located at the bottom of the Gulf of Tunis, on the eastern side of Tunis City (36°37′N 10°11′E). It communicates with the sea via the Kheireddine channel (Fig. 1) and receives various influxes of anthropogenic contaminants. The length and the maximum breadth are 10 and 3 km, respectively, for a total surface area of 24 km^2 and an average depth of 1.5 m (Ben Maiz 1997). The hydrodynamics of this lagoon is semidiurnal, principally controlled by wind and tide (Jouini et al. 2005). Furthermore, the average water residence time in the lagoon varies from 6.6 to 8.2 days, with 2.57 million m^3 day^{-1} of total water exchange with the sea (Jouini 2003). The zones close to this lagoon are characterized by the presence of activities such as harbours, tourism, agriculture, urban developments and industries (Chalghmi et al. 2016). The Northern Lagoon of Tunis is part of the wetlands partially separated from the marine environment by a coastline, meeting both marine salty waters and continental fresh water (Ben Mosbah et al. 2010). Also, this lagoon is the largest water body of the Tunis conurbation. It is divided into two parts named the North Lake and the South Lake, separated by a navigation channel. In the current study, two remote sites from the two different parts of the lagoon were chosen to determine the effects of pollution on *R. decussatus*. The MCA (36°49′4.31″N 10°13′5.49″E) is located in the North Lake near the protected zone (Chikly Island). This site is characterized by a sandy substrate and is supposedly less polluted after the restoration works in 1985 to 1988, as reported by Barthel (2006) and Chouba et al. (2010). The HCA is located in the South Lake (36°47′12.40″N 10°13′12.88″E), in a highly polluted area with higher anthropic pressure than the other sampling sites (Chalghmi et al. 2016). Indeed, this site is characterized by black sticky mud and water stagnation, with a strong smell of hydrogen sulphide (H_2S), and is very rich in seaweeds with some fragments of shells (Kochlef 2003, Bejaoui et al. 2019). Previous studies have shown that *R. decussatus* is abundant in this lagoon (Tlig-Zouari and Maamouri 2008, Zamouri-Langar 2010, Fradi 2012). These studies showed that *R. decussatus* has a maturation period in spring and a partial spawning period commencing in summer, the reproductive cycle finishing with an inactive stage during winter after degeneration during the

autumn. The growth curves of these bivalves from the MCA were fitted to the von Bertalanffy equation as follows: Lt =57, 75($1^{-e\ -0,\ 22(t+1934)}$) (Zamouri-Langar 2010, Fradi 2012).

Clams and water sampling

A total of 300 clam individuals with a similar size were sampled at low tide from the LCA, the MCA and the HCA on about the 15th day of each month from March 2014 to February 2015. The clams were collected by hand or by scuba divers at depths greater than 1m. The samples were immediately placed in iced boxes and transported to the laboratory within two hours. Upon arrival, the specimens were dissected and the organs were removed. Then, ten digestive glands per month were analysed individually to obtain 30 samples per season. For each season, 30 (n=30 independent replicates) digestive glands were homogenized with Tris HCl buffer (20 mM, pH 7.4) and stored at liquid nitrogen until the biochemical analysis. Twenty digestive glands per season were pooled into six independent replicates (n=6) and frozen in nitrogen liquid for TE analysis. Also, nine independent digestive glands (n=9) were conserved in ethanol (70°) for DNA analysis and ten (n=10) others were fixed in buffered formalin (10%) for histological study. The choice of the digestive gland as a model organ for assessing the three study areas was based on the fact that this organ has the ability to accumulate and detoxify various xenobiotic substances, reflecting the state of an ecosystem, as reported previously for many bivalve species (Usheva et al. 2006, Faggio et al. 2018). Also, the digestive gland is known as the major site of oxyradical-generating biotransformation enzymes (Livingstone et al. 1992). The water parameters (temperature, salinity and pH) were measured in situ using a WTW portable multi-parameter probe (model WTW LF.325). Suspended matter and chlorophyll *a* were determined according to the method of Aminot and Chaussepied (1983) (see Supplementary Material Table S1).

Trace element analysis

Concentrations of zinc (Zn), copper (Cu), lead (Pb), iron (Fe) and cadmium (Cd) were measured in the digestive gland tissues of *R. decussatus* according to Carvalho et al. (2000). Firstly, the digestive glands were cleaned in order to remove all the epibiotic material and to prevent the interference of TE presence in the clams related to inorganic particles. Secondly, all the digestive glands were lyophilized and the dried samples of 0.5 g were finely ground in a porcelain mortar and digested with nitric acid (HNO_3, 60%) and hydrogen peroxide (H_2O_2, 37%) at 80°C. The mineralized solution was gauged with water at a volume of 50 mL until analysis. The TE content was determined with inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS was equipped with a graphite furnace using a dynamic reaction cell and had a detection limit of 0.002 ppm. Blank samples and certified reference materials (Mussel Tissue Standard Reference Material SRM 2976, National Institute of Standards and Technology) were processed to check the analytical accuracy. TE contents obtained for standard reference materials were continually within the 95% confidence interval of certified values.

Condition and gonad index analysis

The condition index (CI) was considered an indicator of the clams' physiological condition. The CI for the population of *R. decussatus* collected from the LCA, MCA and HCA was determined each season on a group of 40 individuals. After complete dehydration of the soft tissues and shells in the oven for 24 hours at a temperature of 105°C, the dry weight was determined using a 0.01 g precision balance. CI was estimated according to the Walne (1979) formula:

$$CI=(\text{dry weight of the organ or individual/weight of dry shell}) \times 100$$

The digestive gland index was estimated using the formula established by Campillo et al. (2013):

$$DGI = (\text{dry weight of digestive gland/weight of dry shell}) \times 100$$

Biochemical analysis

Metallothionein level measurement

Metallothioneins (MTs) are involved in the maintaining of the essential TE in homoeostasis and the detoxification of the non-essential one (Amiard et al. 2006). MT concentrations were determined in the digestive gland of *R. decussatus* according to the method developed by Viarengo et al. (1985). One mL of digestive gland supernatant was added to 1 mL of cold absolute ethanol and 80 µL of chloroform and centrifuged at 6000×g for 10 min. The resulting supernatant was mixed with absolute ethanol (3V) and incubated at –20°C for 1 h. After incubation, the mixture was centrifuged at 6000×g for 10 min and the pellet was cleaned with 87% ethanol and 1% chloroform. The pellet containing MTs was resuspended in 150 µL NaCl (0.25 M) and 150 µL HCl (1 N) containing EDTA (4 mM). Before centrifugation at 3000 g for 5 min, 4.2 mL of NaCl (2 M) containing DTNB (0.43 Mm) buffered with Na-phosphate (0.2 M; pH=8) was added to each pellet at room temperature. MT absorbance was read at 412 nm and the expression was presented as nmol of GSH/mg protein using glutathione (GSH) as a standard.

Malondialdehyde level measurement

Malondialdehyde (MDA) is a convenient index that is widely used to monitor the lipid peroxidation status in the body (Jamil 2001). MDA was determined spectrophotometrically according to the method of Draper and Hadley (1990). An aliquot of 500 µL was mixed

with 500 µL of trichloroacetic acid (TCA 30%). After centrifugation at 3500 g for 10 min at cold, 1 mL of TBA mixture (0.67 %; pH: 7.4) was added to 1 mL of supernatant and then incubated for 15 min at 90°C and cooled. The absorbance of the TBA-MDA complex was measured at 532 nm using a spectrophotometer. 1,1,3,3-tetraethoxypropane (TEP Sigma) was used as a standard and the MDA amount was expressed as nmol/mg protein.

Advanced oxidation protein product level measurement

The advanced oxidation protein product (AOPP) has been considered a reliable marker of oxidant-mediated protein damage (Wu 2015). AOPP levels were quantified according to Kayali et al. (2006). Briefly, 400 µL of the digestive gland supernatant was mixed with 0.8 mL of phosphate buffer (0.1 M; pH 7.4). After 2 min, 0.1 mL of 1.16 M potassium iodide (KI) was treated with the previous solution followed by 0.2 mL of acetic acid. The absorbance of the reaction mixture was registered at 340 nm. The AOPP level for each sample was calculated using the extinction coefficient of 261 and the results were expressed as nmol/mg protein.

Antioxidant assays

Glutathione is a crucial component of the antioxidant defence mechanism and it functions as a direct reactive free radical scavenger (Romao et al. 2006). Total GSH concentration was quantified by the reduced glutathione recycling assay (Ellman 1959). An aliquot of 500 µL of digestive gland homogenate was added to 3 mL of sulfosalicylic acid (4%) and then centrifuged at 1.600×g for 15 min. Five hundred mL of supernatant was taken and added to Ellman's reagent. The absorbance was measured spectrophotometrically at 412 nm after DTNB addition (10 mM). The level of GSH was calculated by a standard concentration and expressed as µg/mg protein.

Non-protein thiols react significantly faster with oxidizing species than other amino acid side-chains and thus contribute to antioxidant defence (Hansen et al. 2009). NPSH levels were determined by the method of Ellman (1959). A 500 mL aliquot of the homogenate was mixed with trichloroacetic acid (10%). After centrifugation, the –SH groups were determined in a pure supernatant. An aliquot of supernatant was added to potassium phosphate buffer (pH=7.4; 0.1 M) and DTNB (10 mM) 5,5-dithio-bis (2-nitrobenzoic acid). The absorbance of colorimetric reaction was measured at 412 nm and NPSH content was expressed as µmol of GSH/mg protein.

Vitamin C is known to directly scavenge ROS (Halliwell and Gutteridge 2001). Vit C was determined according to the method of Jacques-Silva et al. (2001). Protein was precipitated in a cold trichloroacetic acid solution (4 %), centrifuged for 10 min and incubated at 85°C for 30 min with DNPH (4.5mg/ mL) and CuSo4 (0.075mg/mL). The reaction was measured at 540 nm and expressed as nmol/mg protein.

Glutathione peroxidase participates in the detoxification of lipid hydroperoxides using glutathione (GSH) and consequently reducing the cellular pool of GSH (Winston and Digiulio, 1991). GPx was measured following the procedure of Flohe and Gunzler (1984). A 200 µL aliquot of digestive gland extract was mixed with 100 µL of phosphate buffer (pH=7.4) and 200 µL of glutathione (4 mM). This mixture was incubated for 10 min at 37°C and then 500 µL of H_2O_2 (5 mM) and 1 mL of TCA (5%) were added. The reaction was detected after the DTNB (10 mM) addition to the 100 µL of the mixture using spectrophotometric absorbance at 340 nm. GPx was expressed as nmol of GSH/min/mg protein.

Catalase activity is used as a marker involved in the primary defence against oxidative damage (Gutteridge 1995). The CAT activity was determined by the method of Aebi (1984) using H_2O_2 (0.5 M) as a substrate. The reaction was started by adding an aliquot of 20 µL of the homogenized digestive gland and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). The H_2O_2 decomposition level was followed by monitoring absorption at 240 nm. One unit of CAT was defined as µmol/min/mg of protein.

Acetylcholinesterase (AChE) is a key enzyme of the nervous system and one of the most commonly used biomarkers of neurotoxicity (Durieux et al. 2011). AChE activity was measured using a spectrophotometric method (Ellman et al. 1961). Acetylthiocholine iodide was used as a substrate in a concentration of 8.25 mM. The activity of AChE was expressed as nmol of substrate/min/mg protein and measured spectrophotometrically at 412 nm.

DNA degradation analysis

DNA extraction from the digestive gland was determined according to the Clarke (2002) method. The DNA was separated from the digestive gland with cetyltrimethylammonium bromide buffer, which was added to the sample and incubated for 1 hour at 55°C. Subsequently, 400 µL of the chloroform-alcohol mixture was added to the above solution, which was centrifuged for 15 minutes at 10000×g. The purified DNA obtained undergoes migration on an agarose gel (1%), which was observed in a dark room under an ultraviolet lamp and photographed. The molecular weight size marker (3 kb DNA ladder) was loaded. The DNA change was examined by a wavelength based on the method of Sambrook and Russell (2001) at 260 nm. Results were expressed as µg/g of tissue.

Histophatological analysis

For the histological study, tissues from digestive glands were fixed in formaldehyde 37% at ambient temperature. The dehydration was done using increasing ethanol concentrations and toluene. Histological sections of 5 µm were cut with a rotary manual microtome (Micros, Austria). Before coloration, sections were dewaxed, mounted on a glass slide with albuminous water and coloured with haematoxylin and

eosin (Reactifral: 33650 Martillac, France) (Martoja and Martoja-Pierson 1967) to visualize morphological structures and degraded tissues with a light microscope coupled to a CCD camera.

Data analysis

Index calculation

To order and to compare TE according to the overall spatial variability of the environmental levels along the Tunisian lagoons throughout the studied seasons, the Trace Element Spatial Variation Index (TESVI) was determined to compare each studied TE as described by Richir and Gobert (2014).

$$TESVI = [(Xmax / Xmin) / (\Sigma(Xmax / Xi) / n)] \times SD$$

Xmax is the maximum mean concentrations recorded among the sites; Xmin the minimum mean concentrations recorded among the sites; Xi the mean concentrations recorded at each site; n the number of sites; and SD the standard deviation from the mean ratio $\Sigma(Xmax/Xi)/n$.

The Target hazard quotient ratio (THQ) was determined to express the risk of non-carcinogenic effects of all the tested TEs. This index has been proposed for the estimation of the potential risks to human health caused by toxic TEs by the USEPA (2000).

$$THQ=[(E_F \times E_D \times F_{IR} \times C_m) / (W_m \times E_F \times R_{fD})] \times 10^{-3}$$

E_F is the exposure frequency (365 days/year).

E_D is the exposure duration, equivalent to an average lifetime of a Tunisian person (i.e. 60 years).

F_{IR} is the average level of bivalve consumers (17.86 g/person/day) (Jović and Stanković 2014).

C_m is the TE concentration in bivalves (mg/kg dry weight basis).

W_m is the average body weight of an adult person (60 kg).

R_{fD} is the oral reference dose based on 1 10^{-3} (Cd), 0.04 (Cu), 0.004 (Pb), 0.3 (Zn) and 45 (Fe), estimating the probable daily oral consumption of TEs in a human population relative to those frequently consumed over a lifetime without a considerable danger of harmful effects (USEPA 2000).

A THQ value above 1 means that contaminated food intake likely has some noticeable harmful effects on the exposed population.

A THQ value below 1 means that food intake is safe and appropriate for human consumption.

Statistical analysis

The results are expressed as means±SD (standard deviation) for each site. The level of significance was ascertained at 0.05. The results were first tested for normality using the Kolmogorov-Smirnov test and two-way ANOVA analysis was performed to assess significant effects for spatial and seasonal variations on the tested parameters. All statistics and the principal component analysis (PCA) were performed with R software version 2.15.2 (R Core Team 2017) using larger data sets. Herein, PCA is the tool most used to explore similarities and hidden patterns among samples and clarify the relationship between data and grouping.

RESULTS

Physicochemical parameters

The environmental parameters of the water in the study areas are summarized in Supplementary Material Table S1. Our data showed no significant variations in salinity and pH, whereas temperature, suspended matter and chlorophyll *a*, which showed significant differences between the sites ($p<0.01$; two-way ANOVA). Temperature and suspended matter were significantly higher in the MCA and the HCA ($p<0.01$; two-way ANOVA), while chlorophyll *a* concentration was far higher in the LCA ($p<0.001$; two-way ANOVA).

Biometric parameters

Results of the biometric parameters (weight (w), length (L), CI and gonadic index (GI)) are illustrated in Supplementary Material Table S2. Our results revealed a similar variation of *R. decussatus* weight and length in the three studied lagoons. As shown in Supplementary Material Table S1, CI was significantly lower in *R. decussatus* from the MCA and the HCA than in those from the LCA in each season ($p<0.05$, two-way ANOVA). The lowest values were recorded for *R. decussatus* collected from the HCA during the summer season (12.477%). Similar variations were observed for GI (see Supplementary Material Table S2). GI was significantly lower in clams collected from the MCA (36%) and the HCA (42%) than in those collected from the LCA, particularly in summer ($p<0.001$, two-way ANOVA). In summer, the CI and GI of *R. decussatus* from the LCA, MCA and HCA were significantly lower ($p<0.01$, two-way ANOVA).

Trace element concentrations

Site and seasonal variations of TE concentrations in *R. decussatus* digestive glands are reported in Table 1. Our results showed that the essential TE (Fe, Zn) exhibited higher concentrations than the non-essential ones (Cd, Pb and Cu). The mean Cd, Cu and Zn concentrations were lower than the certified reference materials recorded in bivalves; however, Fe and Pb concentrations were higher in *R. decussatus* digestive gland from the MCA and the HCA than the certified reference materials (Table 1).

Clams from the MCA and the HCA had higher concentrations of Cd, Pb, Cu, Fe and Zn than those from the LCA ($p<0.01$, two-way ANOVA). These significant concentrations were distributed among the seasons. In spring, MCA and HCA showed significantly higher accumulations of Cd (98% and 181%, respectively), Cu (86% and 162%, respectively), Zn (42% and 47%, re-

Table 1. – Comparison of trace element concentrations (in mg kg^{-1} of dry weight) in *R. decussatus* from Tunisian coasts with other bivalves from different Mediterranean coastal areas. Concentrations are also compared with maximum levels of TE admissible in shellfish flesh, as set by international organizations. LCA, Ghar el Melh; MCA, North Lake; HCA, South Lake. Values are presented as mean±SD (n=6 repetitions). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001. Metal determination in shellfish tissue based on IAEA452 (scallop tissue, standard reference material, dispersed by International Atomic Energy Agency reference materials); FAO (2003) (Food and Agriculture Organization of the United Nations); SRM 2976 (muscle tissue, National institute of Standards and Technology). Values of IAEA 452 and SRM 2976 are presented as means and standard deviation, while FAO (2003) values are presented as a range. NB:Zn is not declared in IAEA 452 and FAO (2003).

		Cd	Pb	Cu	Zn	Fe
Spring	LCA	0.34±0.08	0.08±0.01	1.69±0.31	43.47±7.93***	105.49±13.33
	MCA	0.69±0.13***	0.12±0.05**	3.16±0.38***	61.92±0.50***	191.25±3.69***
	HCA	0.80±0.10 ***	0.18±0.09***	4.46±0.22***	63.92±6.72***	252.75±11.80***
Summer	LCA	0.32±0.04	0.13±0.01**	1.69±0.22***	47.47±6.24***	107.74±0.60
	MCA	0.50±0.08***	1.29±0.21***	4.16±0.21 ***	51.78±4.82***	617.75±21.56 ***
	HCA	0.75±0.10 ***	1.34±0.22 ***	5.20±0.56***	63.43±6.67***	740.96±24.01 ***
Autumn	LCA	0.29±0.01**	0.10±0.01	1.34±0.14	46.47±1.49***	119.19±2.35
	MCA	0.61±0.06***	1.66±0.32***	2.16±0.22***	58.00±1.04***	692.97±19.70 ***
	HCA	0.67±0.12***	2.07±0.49***	5.37±0.27***	65.32±6.09***	772.27±56.08 ***
Winter	LCA	0.52±0.06***	0.13±0.01**	1.24±0.08***	48.97±3.46 ***	122.18±1.18
	MCA	0.72±0.12***	0.69±0.26***	1.74±0.08 ***	53.70±0.48***	463.06±10.78 ***
	HCA	0.81±0.10***	0.89±0.06***	1.79±0.16***	55.12±2.89***	640.42±66.55 ***
IAEA 452		29.6±3.7	0.37±0.01	10.80±1.30	166±21	ND
FAO (2003)		2	1-6	10-30	40-100	ND
SRM 2976		0.82±0.16	1.19±0.18	4.02±0.33	137±13	171±4.9
Figueira and Freitas (2013)		0.4	0.19-0.34	0.5-0.7	8 -10	ND
Chalghmi et al. (2016)		0.16±0.01-0.96±0.6	3.6±0.8-239±22	2.2±0.5-18±1.5	45±7-432±35	ND
Gabr et al. (2020)		1.5-3.0	3.0-4.3	1.6-2.7	2.9-4.2	ND
Bejaoui et al. (2020)		0.87±0.10-1.87±0.38	1.13±0.33-2.33±0.31	5.39±0.97-8.42±1.06	36.08±7.49-50.44±6.36	395±15.03-435.22±13.07

spectively) and Fe (81% and 140%, respectively) than the LCA. In summer, Pb, Fe and Cu concentrations in the digestive gland of specimens from the MCA and the HCA were significantly higher than those in the LCA (p<0.01, two-way ANOVA). However, only in individuals from the HCA were Cd and Zn concentrations significantly higher than in individuals from the LCA (p<0.01, two-way ANOVA). In autumn, clams from the HCA and the MCA had significantly higher Cd, Pb, Cu, Zn and Fe levels in their digestive gland than those from the LCA (p<0.01, two-way ANOVA). In winter, clams from the MCA and the HCA showed higher Pb, Cu and Fe than those from the LCA. Likewise, TE concentrations obtained for each sampled lagoon were significantly higher in summer and autumn (p<0.05, two-way ANOVA).

Table 2 represents the TESVI and THQ values of the studied TEs. Regarding the overall spatial variability of the three study sites and the five studied TEs, Zn concentration displayed the lowest variability of TESVI, with 0.010 (in summer). However, the highest value among seasons was recorded for Pb. The highest levels of the THQ index among seasons were recorded for Cd and the lowest for Fe.

Table 2. – Trace Element Spatial Variation Index (TESVI) and target hazard quotient (THQ) values calculated from trace element mean concentrations in *R. decussatus* from the Tunisian coast: Ghar el Melh, the North Lake and the South Lake. Numbers indicate the highest value of each TE per season. Numbers in bold indicate the TEs with the highest values.

		TESVI	THQ
Spring	Cd	0.027	0.461
	Pb	**0.074**	0.023
	Cu	0.047	0.053
	Fe	0.047	0.002
	Zn	0.023	0.134
Summer	Cd	0.018	0.417
	Pb	**0.063**	0.091
	Cu	0.014	0.065
	Fe	0.045	0.007
	Zn	0.010	0.125
Autumn	Cd	0.042	0.375
	Pb	**0.076**	0.222
	Cu	0.036	0.051
	Fe	0.023	0.008
	Zn	0.020	0.130
Winter	Cd	0.021	0.460
	Pb	**0.060**	0.099
	Cu	0.026	0.048
	Fe	0.031	0.006
	Zn	0.022	0.119

Metallothionein levels

Site and seasonal variations of MTs levels in *R. decussatus* digestive gland are given in Table 3. Our results revealed a significantly higher MT level in *R. decussatus* collected from the HCA and the MCA than in those collected from the LCA (p<0.01, two-way ANOVA).

MT levels in digestive glands from the HCA were significantly higher in spring (139%), summer (285%) and autumn (98%) than in those from the LCA (p<0.001, two-way ANOVA). However, only in summer did clams from the MCA exhibit a significantly higher MT level than those from the LCA (p<0.05, two-way ANOVA). Nonetheless, no significant differences were registered in winter (p>0.05, two-way ANOVA). The greatest difference in MT levels was recorded in summer, when levels in digestive glands collected from the MCA and the HCA were significantly higher (p<0.001, two-way ANOVA).

Malondialdehyde levels

Site and seasonal variations in MDA levels in digestive glands from the three sites are presented in Figure

Table 3. – Mean (±SD) biomarker responses in *R. decussatus* from Tunisian coast: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). MTs, metallothioneins; GPx, glutathione peroxidase; CAT, catalase; GSH, glutathione; NPSH, non-protein SH; Vit C, vitamin C. Values are expressed as means±SD (n=30). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001; a, nmol of GSH/ mg protein; b, nmol of GSH/min/mg protein; c, µmol/min/mg of protein; d, µg/mg protein; e, µmol of GSH/mg protein; f: nmol/mg protein.

		MTs [a]	GPx [b]	CAT[c]	GSH[d]	NPSH[e]	Vit C[f]
Spring	LCA	0.09±0.01	0.28±0.03	0.34±0.01	0.50±0.02	0.04±0.002	11.93±0.45
	MCA	0.12±0.02***	0.48±0.08***	0.48±0.01 ***	0.51±0.21***	0.06±0.02***	13.51±2.12***
	HCA	0.20±0.04***	1.17±0.14***	1.02±0.26 ***	2.71±0.57***	0.14±0.02***	18.85±3.68***
Summer	LCA	0.09±0.006	0.50±0.01***	0.30±0.01***	0.709±0.03	0.04±0.001*	6.24±0.39***
	MCA	0.18±0.03***	1.10±0.33***	0.40±0.10	1.04±0.30**	0.06±0.02***	7.00±0.25***
	HCA	0.35±0.06***	2.98±0.32***	0.96±0.05***	4.63±0.21***	0.19±0.04***	13.73±2.55***
Autumn	LCA	0.08±0.002	0.59±0.05	0.40±0.04**	0.50±0.01	0.03±0.006	10.84±0.67
	MCA	0.15±0.01***	0.94±0.19***	0.68±0.03 ***	0.57±0.06**	0.05±0.01***	12.38±2.00***
	HCA	0.188±0.05***	1.59±0.25***	1.40±0.43 ***	1.39±0.18***	0.07±0.01***	16.71±2.57***
Winter	LCA	0.08±0.005	0.42±0.10***	0.56±0.08***	0.33±0.009***	0.03±0.002	5.19±0.25***
	MCA	0.09±0.002	0.80±0.26***	0.91±0.24	0.42±0.07	0.05±0.05***	6.50±1.98***
	HCA	0.11±0.03***	0.93±0.34***	1.02±0.16***	1.11±0.33***	0.08±0.02***	7.00±1.82***

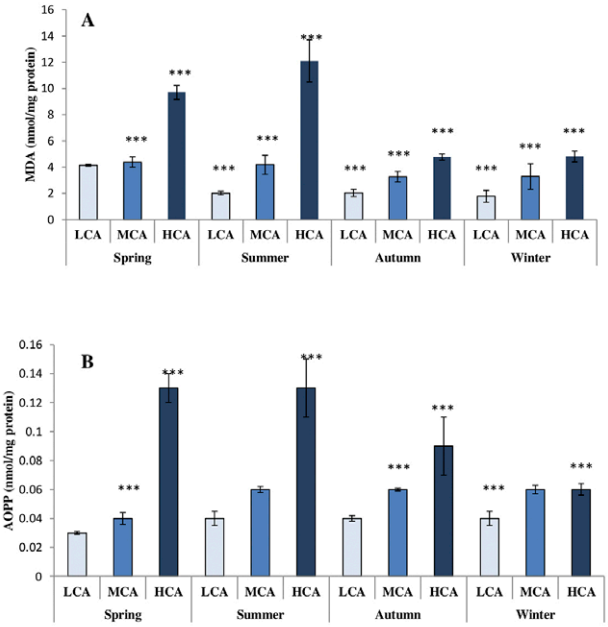

Fig. 2. – Seasonal variation of (A) malondhyaldehide (MDA) and (B) advanced oxidation of proteins product (AOPP) levels in the digestive gland of *R. decussatus* sampled from three coastal Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Results are presented by mean±SD. Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

2A. Higher MDA levels in clams from the MCA and the HCA than in clams from the LCA were observed in summer (106% and 496%, respectively) and winter (84% and 170%, respectively) (p<0.001, two-way ANOVA).

However, only clams from the HCA exhibited significantly higher MDA levels in spring and autumn than those from the MCA (120% and 45%, respectively) and the LCA (133% and 135%, respectively) (p<0.01, two-way ANOVA).

Advanced oxidation protein products levels

Site and seasonal variations of AOPP levels in the digestive glands are shown in Figure 2B. AOPP levels were significantly higher in clams from the HCA than in those from the LCA (p<0.01, two-way ANOVA). As shown in Figure 2B, AOPP levels appeared to be significantly higher in digestive glands from the HCA than in those from the LCA in spring (348%), summer (230%) and autumn (85%). However, similar variations in AOPP levels were observed in digestive glands collected from the LCA and the MCA.

Glutathione levels

Sites and seasonal variations in GSH levels are summarized in Table 3. *R. decussatus* collected from the HCA displayed higher levels of GSH than those from the LCA and the MCA (p<0.01, two-way ANOVA). Clams from the HCA showed significantly higher levels than clams from the MCA and the LCA in spring (169% and 339%, respectively), summer (345% and 553%, respectively), autumn (143% and 178%, respectively) and winter (164% and 236%, respectively). GSH levels were significantly higher in clams from the MCA than in clams from the LCA only in summer (p<0.01, two-way ANOVA).

Non-protein SH levels

Site and seasonal variations of non-protein-SH levels in *R. decussatus* digestive glands are summarized in Table 3. NPSH levels were significantly higher in digestive glands of clams from the MCA and the HCA than in those from the LCA (p<0.05, two-way ANOVA). Among seasons, *R. decussatus* from the HCA showed significantly higher NPSH levels in spring (250%), summer (375%), autumn (133%) and winter (166%) than those from the LCA (p<0.05, two-way ANOVA). A similar trend was observed in clams from the MCA, which in all seasons showed significantly higher NPSH levels (≤50%) than those from the LCA (p<0.05, two-way ANOVA).

Vitamin C levels

Site and seasonal variations in vitamin C level were slightly modified over the study period (Table 3). Clams from the LCA and the MCA showed signifi-

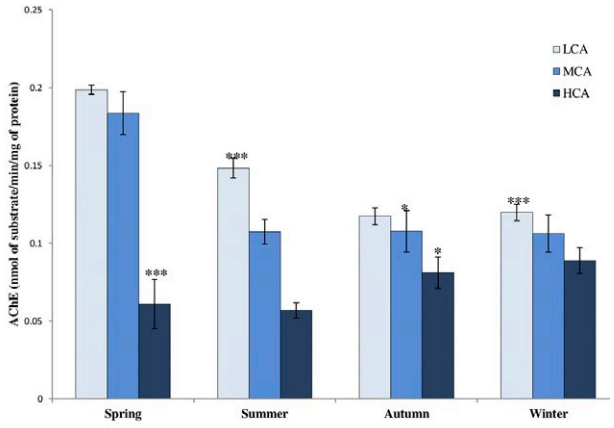

Fig. 3. – Seasonal variation of the acetylcholinesterase (AChE) activity in the digestive gland of R. decussatus sampled from three coastal Tunisian lagoons (Ghar el Melh: LCA, North lagoon: MCA and South lagoon: HCA). Results are presented as mean±SD. Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05; **p<0.01; ***p<0.001.

cantly lower Vit C levels than those from the HCA in spring (58% and 50%, respectively), summer (120% and 96%, respectively) and autumn (54% and 54%, respectively).

Glutathione peroxidase activity

Site and seasonal variations of GPx activities in *R. decussatus* from the sites are summarized in Table 3. GPx activities in clams sampled from the MCA and the HCA were significantly higher in spring (70% and 315%, respectively), summer (119% and 492%, respectively) and autumn (58% and 166%, respectively). However, no significant difference was observed in the GPx activities among sites in winter (p>0.05, two-way ANOVA). GPx activity was more pronounced in summer and autumn in clams collected from all three sites (p<0.01, two-way ANOVA).

Catalase activity

Site and seasonal variations in CAT activities in *R. decussatus* digestive glands from the LCA, the MCA and the HCA are shown in Table 3. CAT activity was significantly higher in digestive glands of clams from the MCA and the HCA than in those from the LCA (p<0.01, two-way ANOVA). The differences were of the order of ≤41% in all seasons (p<0.01, two-way ANOVA).

Acetylcholinesterase activity

Site and seasonal variations in AChE activity are documented in Figure 3. Significantly lower AChE activity was observed in digestive glands from the MCA and the HCA than in those from the LCA (p<0.05, two-way ANOVA). The lowest levels were observed in clams from the HCA in spring (69%), summer (62%) autumn (30%) and winter (25%). However, similar activity was recorded in clams from the MCA and the LCA in all seasons (p>0.05, two-way ANOVA), except in summer, when activity was 27% lower in clams from the MCA.

Principal component analysis

PCA is shown in Figure 4, which allowed us to retain the first two factorial axes that explained 68% of the total variance. The first one is the axis which

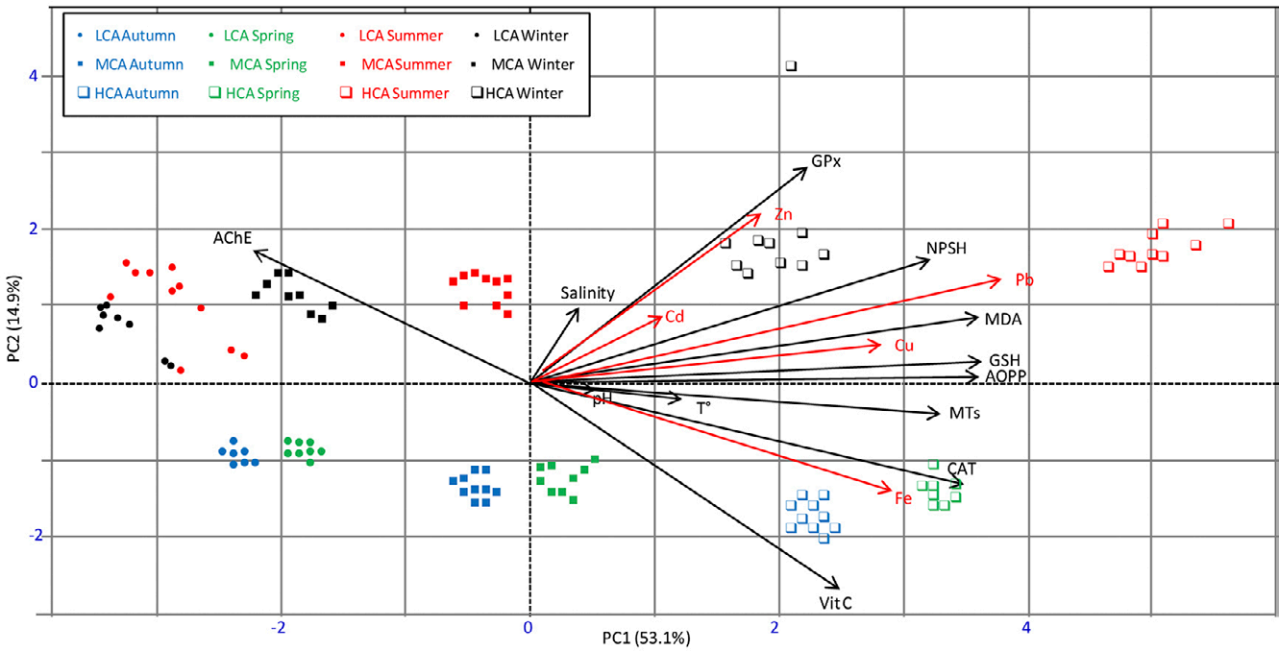

Fig. 4. – Principal analysis component results represented by two factors and produced by abiotic factor, biochemical and chemical variables measured in the digestive gland of *R. decussatus* sampled from three costal Tunisian lagoons.

is preserved by projection, for maximum dispersion of the initial cloud point (53.1% of the total dispersion): MDA, AOPP, GPx, GSH, NPSH, Vit C, GSH, MTs, Cd, Cu and Fe contributed negatively with this first axis (Fig. 4). Nevertheless, only AChE activity correlated positively with the first axis. The second axis was described by 14.9% of the total dispersion. This axis was characterized by a negative correlation with T°C, pH and chlorophyll *a*, while, Zn and CAT correlated positively with it.

Correlation matrix

Multivariate statistical analyses were performed to establish correlations between the environmental conditions (T°C, salinity, chlorophyll *a*, carotenoids and suspended matter), TE accumulations (Pb, Cu, Cd, Fe, and Zn) and the tested parameters of *R. decussatus* digestive glands (see Supplementary Material Table S4). Significant correlations were found between TE concentrations and almost all of the antioxidant responses and biometric parameters. The Pearson correlation matrix indicated that digestive gland AChE activity showed a significant negative correlation with the environmental parameters and the trace element

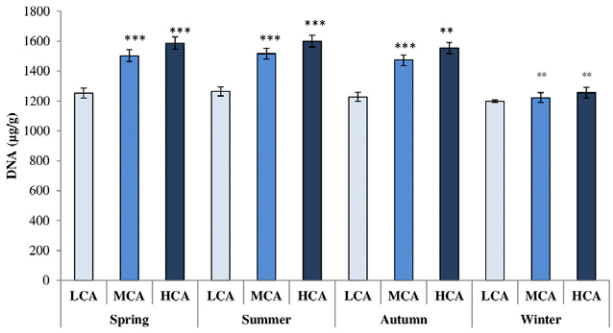

Fig. 5. – Seasonal deterioration among seasons of the DNA structure isolated from *R. decussatus* collected from Tunisian coastal lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Results are presented as mean±SD. Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

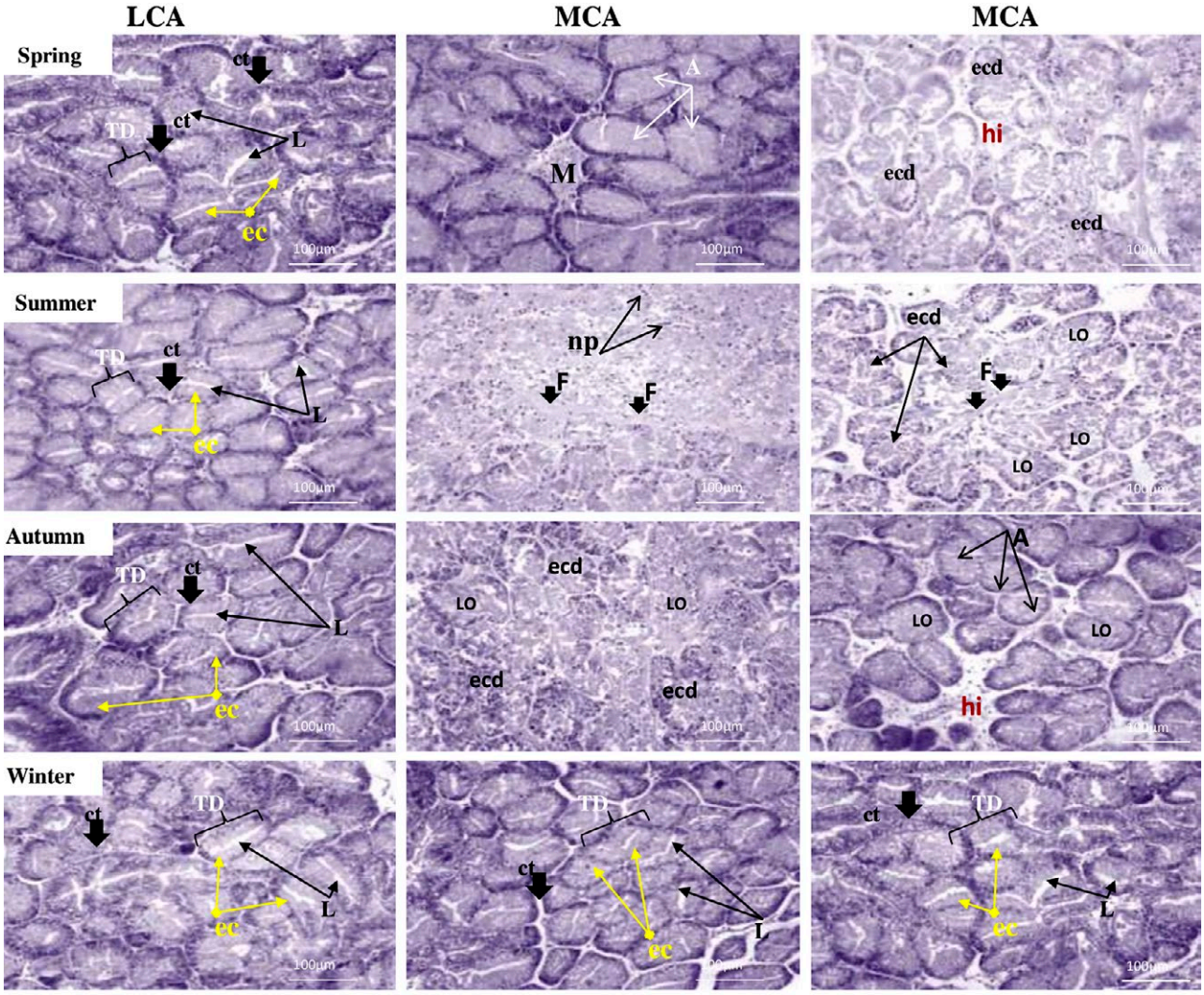

Fig. 6. – Histological sections of *R. decussatus* digestive glands from three coastal Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). A, atrophy; ct, connective tissue; ec, epithelial cells; ecd, epithelial cell degradation; F, fibrosis; hi, hemocyte infiltration; L, lumen; LO, lumen occlusion; M, melanin; np, neoplasic hemocytes; TD, digestive tubules. Optic microscopy: haematoxylin-eosin ×100.

concentrations. Also, chlorophyll *a* showed a significant and negative correlation with all the parameters tested, with a high value observed in MTs ($p<0.05$, $r=-0.535$) (see Supplementary Material Table S4). The results indicated that all tested parameters were positively correlated with temperature ($p<0.05$), salinity ($p<0.05$) and MES ($p<0.01$), but pH did not correlate with the biometric and biochemical parameters ($p>0.05$). Important positive relationships were observed between trace element concentrations in *R. decussatus* digestive glands and the tested parameters (including abiotic, biotic and biochemical) ($p<0.01$). Both enzymatic and non-enzymatic activities showed a strikingly positive relationship with Cd ($p<0.001$, $r\approx0.373$-0.771), Pb ($p<0.001$, $r\approx0.338$-0.733), Cu ($p<0.001$, $r\approx0.236$-0.853), Fe ($p<0.01$, $r\approx0.264$-0.806) and Zn ($p<0.01$, $r\approx0.406$-0.813).

DNA analysis

Site and seasonal variations in DNA damage in *R. decussatus* digestive glands from the LCA, the MCA and the HCA are summarized in Figure 5. In spring, summer and autumn, results showed significantly higher DNA damage contents in digestive glands of *R. decussatus* from the MCA and the HCA than in those from the LCA ($p<0.001$, two-way ANOVA). However, similar contents were observed in winter for *R. decussatus* digestive glands collected from all the studied lagoons. Nevertheless, among seasons, clams from the LCA showed similar contents of DNA damage (in the order of $\approx$1200 μg g^{-1}), however, MCA and HCA showed comparable contents of DNA damage in spring, summer and autumn, which decreased significantly in winter ($p<0.01$, two-way ANOVA). On the other hand, clams collected from the HCA were characterized by a significant reduction of DNA degradation in autumn and winter ($p<0.01$, two-way ANOVA).

Histological analysis

Site and seasonal variations in the histopathological alterations in the digestive gland of *R. decussatus* from the north lagoon of Tunisia are summarized in Figure 6. All the digestive glands of *R. decussatus* from the LCA were characterized by a normal digestive tubule with epithelial cells, a narrow or almost occluded tubule lumen and interstitial tissue between them (Fig. 6). However, clams from the MCA and the HCA were characterized by an infiltration of their haemocytes, lumen occlusion and degradation at the level of the epithelial cells. We also noted the presence of atrophic tubules, melanized haemocyte aggregate, epithelial cell degradation, tubule infiltration and fibrosis in spring, summer and autumn, revealing serious damage in these two populations. A neoplastic haemocyte intruding into a necrotic focus was observed in summer (Fig. 6). Nevertheless, similar digestive gland structures were observed in winter for clams from all sampling sites, showing less damage than in the other seasons (Fig. 6).

DISCUSSION

Owing to their sedentary nature and filter-feeding behaviour, bivalves are particularly exposed to anthropogenic contaminants that enter coastal environments. These organisms have a great ability to accumulate TEs in their tissues and some species may even thrive in highly polluted environments (Cantillo 1998). In the current study, the recorded levels of TEs (Zn, Cu, Pb, Fe and Cd) in *R. decussatus* digestive glands collected from three sites impacted by different human activities provide evidence for the ability/capacity of this organ to accumulate TEs. The inter-relationship for essential (Fe and Zn) and non-essential (Pb, Cd, and Cu) TEs accumulated in *R. decussatus* digestive gland strongly indicates a similar pathway of TE uptake. The results obtained showed that TE concentrations in *R. decussatus* were significantly higher in clams collected from the MCA and the HCA. Bivalves can absorb TEs directly from the surrounding environment, both by absorption and/or adsorption from interstitial water and by direct ingestion from sediment, and these TEs may influence their metabolism and other species via the food chain (Chen et al. 2007).

Several investigations have demonstrated that sediments from the HCA are highly impacted by TEs. Their accumulation rate in *R. decussatus* digestive gland must depend on their filtration, ingestion rates, gut fluid quality and detoxification strategies (Chouba et al. 2010, Bejaoui et al. 2020). Our data match well with previous studies that reported a major accumulation of TEs, especially Pb, in the sediments of the central area of the Gulf of Tunis (Ennouri et al. 2010, Chalghmi et al. 2016) and in others organisms such as green macroalgae (Chouba et al. 2010) and mollusks (Brahim et al. 2015, Lahbib et al. 2016, Bejaoui et al. 2017) following the discharge of TEs from several anthropogenic activities such as harbours, industrial and electrical discharges (Bejaoui et al. 2020).Similar results have demonstrated that TE accumulations in *R. decussatus* is associated with the anthropogenic pollution of the Tunisian coasts (Chalghmi et al. 2016, Bejaoui et al. 2020) and other Mediterranean areas (Figueira and Freitas 2013, Gabr et al. 2020).

The current study has also revealed that TE concentrations in the digestive glands of *R. decussatus* from the MCA and the HCA vary considerably depending on seasons, showing higher trends of Cd, Pb, Cu, Zn and Fe in summer and winter. Such findings could be explained by the fact that in summer the low hydrodynamics of these lagoons and the high evaporation rate might contribute to the concentration of TEs in the water column and their deposition on the surface sediments and consequently on the bivalve tissues (Ghribi et al. 2020). However, in winter the current and wave activity agitates the sediments and transports the TEs in the aqueous phase, leading to their bioavailability and their uptake by the bivalves. This increase in winter could be due to the possible discharge of TE concentrations into the waters of the MCA and the HCA or to runoff from adjacent farmland, because these sampling areas are located near the conurbation of Tunis city (Chouba et al.

2010, Chalghmi et al. 2016). Our results were confirmed by the characteristics of the lagoon, which is a laminar medium with an average bathymetry not exceeding one metre and prevailing drastic conditions, such as abrupt and very great spatiotemporal fluctuations of abiotic parameters. In addition, exchanges with the sea are weak and the hydrodynamics are highly attenuated, especially in the coves, where the waters are almost stagnant (Ben Mosbah et al. 2010). Furthermore, recent anthropogenic activities such as industrial wastewater discharges (e.g. CHOTRANA) and sediment excavation can also alter the hydraulic loading and TE source at these two sites (Ben Mosbah et al. 2010).

In addition, the environmental conditions such as temperature, salinity, pH and chlorophyll *a* are considered the main factors influencing TE concentrations in bivalves, affecting their speciation, solubility and reaction rates in the water column (Frías-Espericueta et al. 1999, Ghribi et al. 2020). As shown in our present work, TE showed positive correlations with T°C, salinity and suspended particulate matter ($p<0.01$; $r\leq0.451$) (see Supplementary Material Table S4). Similar works have reported on *Mytilus galloprovinciallis* from Algerian coasts (Rouane Hacene et al. 2015) and Bizerte lagoon (Kefi et al. 2015) and on *Arca noae* from Bizerte lagoon (Ghribi et al. 2020). The seasonal variations in TE concentrations could also be related to the physiological status of bivalves and their reproductive cycle (Bordin et al. 1992, Regoli and Orlando 1994). According to several studies, bivalves accumulate high TE contents during maturation and less during the spawning period. The reproductive cycle of *R. Decussatus* is characterized by a maturation phase followed by an extended spawning phase during summer (Fradi 2012). A new ripening phase takes place in winter, especially for the clams from the HCA, followed by a short massive spawning in late November and early December (Hmida 2004). These dynamisms of clam maturation may explain the higher accumulation of TEs during the summer and winter, especially for these collected from the HCA.

The bioaccumulation of TEs in bivalve species depends not only on direct exposure to the contaminant and its environment, but also on different physiological and biochemical activities through which a specific organism treats micronutrients (Nicholson and Lam 2005). Likewise, bivalves have seasonal changes in their biometric parameters, especially during the warm season. Previous findings on bivalves demonstrate that the decline of biometric indices, such as the CI, is a consequence of anthropogenic activities in the ecosystem (Nicholson and Lam 2005, Bejaoui et al. 2017). It appears that TE uptake could be one of several factors influencing the CI and GI of clams sampled from the MCA and the LCA. Our results agree with several studies carried out on TE accumulation in bivalve tissues (Sabatini et al. 2011, Marques et al. 2016).

The application of the TESVI and THQ could possibly demonstrate suitable tools for the monitoring programmes, in order to define the anthropogenic pollution sources of the study sites. In fact, the TESVI has been determined in *R. decussatus* for the first time, providing an operational system that highlights the most environmentally challenging elements and allows comparisons of TEs based on the overall spatial variability of their environmental levels throughout the study areas (Richir and Gobert 2014). Our results indicate that the high TESVI values recorded for Pb correlate with our previous data, showing that it has a larger spatial variation than the other TEs. Regarding the THQ, our data indicate that all *R. decussatus* investigated were safe for human consumption regarding the levels of Zn, Cu, Fe, Cd and Pb (which were below 1), as already stated by Vieira et al. (2011).

It has been widely reported that TEs in bivalves are capable of stimulating ROS production and inducing MT production (Cipro et al. 2017). MTs are low molecular weight peptides rich in cysteine, which work against the toxicity caused by TEs and are involved in detoxification processes (Viarengo et al. 1985). These metallo-proteins are known for their protective free radical scavenging activity, playing an active role in the capture of harmful oxidant radical species (Kumari et al. 1998, Telahigue et al. 2018). The observed induction of MTs in *R. decussatus* when exposed to environmental pollution probably shows an adaptive response of the digestive gland to the toxicological manifestation induced by TEs, especially Cd and Pb. Our results are in agreement with previous studies carried out by Rabei et al. (2018) on *Donax trunculus* collected seasonally from polluted sites in the gulf of Annaba and Bejaoui et al. (2020) on *R. decussatus* collected from Tunisian lagoons.

Stimulation of MTs is related to the protection against oxidative stress and macromolecule damage (Baltaci et al. 2017). TE accumulation generates an intracellular imbalance and stimulates ROS, leading to the appearance of lipids, proteins and DNA damage through the generation of hydroxyl and free radicals from H_2O_2 via the Fenton reaction (Thomas et al. 2013). As a result of massive free radical generation, MDA performs as second toxic messengers and can form during lipid peroxidation (Cravo et al. 2012, Telahigue et al. 2018). Our results showed an increase in the MDA level in the digestive gland of *R. decussatus* collected from the HCA and the MCA, particularly during the warm season. This increase appears to be due to TE uptake, which accelerates the ability to scavenge ROS through the extreme production of lipid peroxides in the digestive gland. In earlier studies, similar increases in MDA levels have been reported in *Fulvia fragilis* from the Bizerte lagoon (Mahmoud et al. 2010), *R. decussatus* from the Ria Formosa lagoon (Cravo et al. 2012) and *Aulacomya atra* from the South Atlantic Patagonian coast (Di Salvatore et al. 2013). Furthermore, many studies have established a clear link between lipid peroxidation and genotoxic diseases (Marnett 2002, Javed et al. 2016, Bejaoui et al. 2020). In fact, MDA appears to be an important contributor to DNA damage and mutations, as reported by Ayala et al. (2014). In our study, the increase in MDA levels may be considered a significant endogenous source of DNA damage. This finding was confirmed by a significant degradation of the DNA contents in the *R. decus-*

satus digestive glands collected from the MCA and the HCA. The alteration of the DNA in clams' digestive glands from the HCA indicates how bivalves are experiencing pollution stress, in view of the fact that this area has long been receiving untreated waters and harbour discharges (Ennouri et al. 2016). Similarly, high DNA alteration rates in aquatic organisms collected from different contaminated sites have been observed (Bejaoui et al. 2018).

The potential of TE accumulation causing adverse effects in the environment and its constituents has been well reported for the tissues of organisms (Mahmoud et al. 2010, Bejaoui et al. 2018). Proteins are one of the main targets of the effects of TEs (Bejaoui et al. 2020). Oxidation is the major consequence of protein damage both externally and within cells. The occurrence of protein oxidation in the digestive gland of *R. decussatus* was also confirmed by the level of AOPP, which reflects an excess of free radical generation (Prevodnik et al. 2007). The comparison between sites showed higher AOPP levels in the digestive gland of clams from the MCA and the HCA than in those from the LCA, suggesting the important role of TEs in the exertion of protein oxidation (Rabei et al. 2018).

Protein oxidation can lead to amino acid changes leading to the formation of carbonyl and other oxidized moieties, as well as to the failure of sulfhydryl groups (Bainy et al. 1996). These groups, including thiols, can react with free radicals and with products of lipid peroxidation to protect cells against the development of oxidative stress (Tandon et al. 2002, Carmeli et al. 2008). Among them, the glutathione status is one of the first lines of defence including GSH and NPSH. These antioxidants are the most abundant thiols in the cell and are considered the main cellular redox barrier. In our study, specimens from the MCA and the HCA exhibited higher GSH and NPSH levels over the year than those from the LCA. This increase can be explained by the new synthesis of GSH and NPSH as an adaptive response in addition to ROS generation. Our results are in accordance with those of previous studies carried out on bivalves and show the increase of the GSH and NPSH levels in relation to seasonal change and TE exposure (Mahmoud et al. 2010, Augustyniak et al. 2009).

Vitamin C is a powerful reducing agent and an important hydrophilic vitamin for animals and humans. It plays an important protective role in the damage induced by free radicals scavenging hydroxyls, radicals and singlet oxygen (Tandon et al. 2002). The increase in Vit C levels in the digestive gland of *R. decussatus* from the MCA and the HCA could be due to the crucial role of this antioxidant in protecting the physical status of organisms exposed to TE pollution (Chetoui et al. 2019). In line with our data, Bejaoui et al (2020) showed a similar variation in Vit C levels in *R. decussatus* sampled from the HCA. Conversely, Jena et al. (2009) showed a decrease in the Vit C level in *P. viridis* digestive gland collected from a polluted site. In fact, these authors explained the decrease in Vit C by the availability of glutathione to reduce the dehydroascorbate into ascorbate as a result of the high ROS generation.

To mitigate damage to the lipid membrane, bivalves are capable of enhancing their antioxidant defence systems, such as CAT and GPx enzymes (Regoli and Giuliani 2014). Several studies have demonstrated that antioxidant enzymes could protect bivalves from the toxicity of environmental pollutants. In the present work, clams collected from the MCA and the HCA exhibited increases in GPx and CAT activities. These findings highlight the ability of *R. decussatus* to quench ROS overproduction and to alleviate cellular injuries. Our results are in total agreement with the increase in CAT and GPx activities in *Tapes philippinarum* from the Adriatic Sea (Bocchetti et al. 2008), in *Aulacomya atra* from the Nuevo Gulf in Northern Patagonia (Giarratano et al. 2014) and in *Fulvia fragilis* in the Bizerte lagoon (Mahmoud et al. 2010) in relation to high TE concentrations and chemical pollutants.

The effect of environmental pollution on digestive gland mechanisms was also assessed through the AChE activity. In fact, 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). TEs show a noticeable affinity with the sulphur donor, which binds to the thiol residues of proteins (Viarengo 1985). Our results confirm that the metallic pollution found in the MCA and the HCA affected the cholinergic system, as demonstrated by a significant inhibition of AChE in the digestive gland of *R. decussatus*. Tankoua et al (2012) suggested that the decrease in AChE activities could be induced by the accumulation of TEs (such as Cd, Cu and Zn) in *Scrobicularia plana* on the French coast.

Oxidative stress responses in *R. decussatus* digestive glands resulting from the combination of TE accumulation and abiotic factors may cause injuries in tissues. The study of histological injuries induced by prolonged exposure to environmental conditions is considered a successful approach for the assessment of the environmental quality of an area (Cappello et al. 2013, Vajargah et al. 2018). It is well established that the enhancement of antioxidant responses in the digestive gland may trigger a cascade of events, eventually leading to cell death. Clams collected from each of the three sampling areas exhibited pathological changes. The occurrence and severity of abnormalities showed fluctuations throughout the sampling seasons. The investigation of the interaction between the location and pollution type showed that the rate of histopathological lesions in the digestive glands was highest in clams from the HCA moderate in clams from the MCA and lowest in clams from the LCA.

The two affected clam populations were characterized by the presence of haemocyte infiltration and epithelial cell degradation during winter and autumn. This phenomenon has been well described as one of the responses to diverse environmental stressors (Pirrone et al. 2018). It has previously been established that haemocyte infiltration is the cause of the inflammatory process due to a rapid release of pro-inflam-

matory and vasoactive mediators (Bouallegui et al. 2018). Furthermore, atrophic tubules were observed in the same seasons and were characterized by a decrease in the density of the epithelia followed by the extension of the digestive lumen. Also, necrosis and fibrosis were observed, confirming the cell membrane rupture and the inadequate DNA degradation in the digestive gland of clams from the HCA. These abnormalities are known to be induced by exposure to a variety of pollutants (such as polycyclic aromatic hydrocarbons and TEs) and untreated municipal wastewater (Schlacher et al. 2007, Sonawane 2015, Osman et al. 2017). These abnormalities could be correlated to TE uptake but also to seasonal variability of water parameters (Kolyuchkina et al. 2017). Our results were confirmed by the existence of several probable sources of contaminants within the HCA, including the watershed, a wastewater treatment plant, harbour activities and industrial and domestic waste (Ben Ayed et al. 2012, Ennouri et al. 2016). In addition, our results were similar to those of previous reports showing that the histological abnormalities can be harmful when the non-essential TE levels exceed those required for the normal metabolic function (Yee-Duarte et al. 2018). Similar studies reported changes in the histological structure of some bivalves in relation to TE pollution and seasonal variation (Costa et al. 2013, Bejaoui et al. 2020).

The general analyses of data by PCA indicate a significant separation among sampling periods and lagoons. This study established a clear seasonality of the abiotic factors, TEs and biological parameters tested as biomarkers in clams from different lagoons. Indeed, clams' digestive glands from the HCA were characterized by elevated concentrations of TE, confirming their metabolic responses in relation to TE accumulation. Homogeneity in response to TE may also be due to a combination of geomorphologic and hydrologic factors characterizing the HCA. Indeed, this lagoon is a semi-enclosed basin with a high input of anthropic discharges, backwater and relatively high water mixing between sites (Chouari 2015, Ennouri et al. 2016). Previous reports have described that the circulation of water in the HCA area is strongly limited by the shallow depth and by the very irregular shape of the banks (Chouari 2015). Furthermore, these important oxidative responses to TEs in the clam digestive glands from the HCA could be associated with the filtration rate of the sediment particles, which have a high level of TEs, brought from the Tunis Gulf by currents (Ennouri et al. 2010, Zaaboub et al. 2014). In addition, a marked effect of seasonality was detected for clams from the HCA, especially during the summer season. These results indicate that *R. decussatus* was more influenced by the seasonal variations in environmental parameters such as temperature, salinity and food availability (suspended matter, etc.). Indeed, the HCA was characterized by a black sticky mud with a strong smell of hydrogen sulphide and rich seaweeds with some fragments of shells and water stagnation (Kochlef 2003).

CONCLUSION

Based on the results of the investigated biochemical biomarkers (MDA, MTs, AOPP, GSH, NPSH and Vit C), we conclude that the oxidative stress generated in the digestive gland of *R. decussatus* is probably related to the seasonal variation in TEs and environmental parameters. However, in the future, similar studies should be conducted for other lagoons along the Tunisian coasts using *R. decussatus* as a sentinel species, reflecting the impact of organic and inorganic pollutants according to seasonal variation. Further investigations on the organic and inorganic pollutants in the sediments are still required to provide a global view of the health status of these coastal environments. These data could also provide us with a time-integrated picture concerning the toxicity of TEs in *R. decussatus* and the capacity of this bivalve to regulate or accumulate TEs.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available through the online version of this article and at the following link: http://scimar.icm.csic.es/scimar/supplm/sm05054esm.pdf

Table S1. – Mean (±SD) environment parameters recorded during four seasonsinthe three Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Values are presented as mean ±SD (n=3 repetitions). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

Table S2. – Mean (±SD) weight (W), length (L), condition (CI) and gonad (GI) indices of *R. decussatus* from Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Values are expressed as means ±SD (n=40 replications). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

Table S3. – Levels of trace elements in the water column and sediments of Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA).

Table S4. – Correlation matrix of non-parametric Spearman's rank correlation coefficients between *R. decussatus* biomarkers, trace element digestive gland tissue levels (Cd, Pb, Cu, Zn, and Fe) and environmental parameters measured in this study. AChE, acetylcholinesterase; CAT, catalase; AOPP, advanced oxidation proteins products; GPx, glutathione peroxidase; GSH, gluthatione; NPSH, non-protein SH; MDA, malondialdehyde; Vit C, vitamin C; MTs, metallothioniens; T, temperature; S, salinity; pH, hydrogen potential; SPM, suspended matter; Ch *a,* Chlorophyll *a*. The positive and significant correlation is presented in red and the negative and significant correlation is presented in green.

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Assessment of oxidative stress, genotoxicity and histopathological responses in the digestive gland of *Ruditapes decussatus* collected from northern Tunisian lagoons

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Supplementary material

Table S1. – Mean (±SD) environment parameters recorded during four seasons in the three Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Values are presented as mean ±SD (n=3 repetitions). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

		Temperature (°C)	Salinity (psu)	pH	Suspended matter (mg/L)	Chlorophyll *a* (µg/L)
Spring	LCA	28.51±2.50	37.62±1.15	8.11±0.43	175.27±15.51	2.04±0.30
	MCA	27.92±1.63	37.60±0.68	8.13±0.11	182.18±12.31**	1.91±0.28***
	HCA	29.34±2.41***	37.61±1.55	8.10±0.58	211.35±10.96***	1.87±0.24***
Summer	LCA	29.47±1.86	38.50±2.38	8.12±0.69	132.25±14.63***	1.58±0.11***
	MCA	30.21±4.28***	39.26±2.12	8.09±0.21	148.00±12.47***	1.56±0.18***
	HCA	31.10±1.76***	39.74±1.72	8.11±0.46	150.30±10.96**	1.43±0.16***
Autumn	LCA	22.30±1.31***	36.87±0.52	8.15±0.20	123.59±20.56***	1.39±0.15***
	MCA	23.19±1.67***	37.41±1.77	8.12±0.30	137.45±10.34***	1.27±0.31***
	HCA	22.85±2.53***	37.16±1.85	8.20±0.15	132.26±9.42***	1.00±0.27***
Winter	LCA	20.42±2.71***	35.27±2.72	8.13±0.69	120.20±7.11***	1.12±0.36***
	MCA	19.20±1.44***	34.72±1.92	8.11±0.24	142.34±12.56***	1.00±0.19***
	HCA	19.33±2.92***	35.05±2.04	8.15±0.70	141.73±5.70***	1.26±0.33***

Table S2. – Mean (±SD) weight (W), length (L), condition (CI) and gonad (GI) indices of *R. decussatus* from Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA). Values are expressed as means ±SD (n=40 replications). Significant differences are determined at 0.05 using two-way ANOVA: *p<0.05, **p<0.01; ***p<0.001.

		W (g)	L (cm)	CI	GI
Spring	LCA	41.96±3.02	12.80±2.23	23.22±1.06	8.70±0.22
	MCA	42.47±2.18	13.68±2.46	20.05±2.10**	7.85±0.61**
	HCA	40.42±4.34	11.22±4.16	17.00±1.04**	7.57±1.08**
Summer	LCA	44.05±3.18	13.44±2.34	18.34±1.08*	5.21±0.83
	MCA	44.59±2.29	14.37±2.59	15.38±2.30***	3.29±0.38***
	HCA	42.45±4.56	13.78±4.37	12.47±1.24***	3.00±0.44***
Autumn	LCA	42.70±3.96	13.98±2.85	20.55±2.26	6.33±0.82**
	MCA	40.49±2.76	14.61±2.48	16.93±2.45**	6.78±0.74***
	HCA	41.66±3.63	14.93±2.84	15.58±1.41***	5.90±0.96***
Winter	LCA	42.22±2.42	13.50±2.68	22.63±3.51*	7.54±0.34**
	MCA	40.67±2.47	13.66±3.32	20.93±2.73	6.97±0.66*
	HCA	40.78±3.08	13.26±2.21	15.90±2.33**	6.70±1.02*

Table S3. – Levels of trace elements in the water column and sediments of Tunisian lagoons: Ghar el Melh (LCA), the North Lake (MCA) and the South Lake (HCA).

		Pb	Cu	Cd	Zn	Fe	References
LCA	Water (nmol/l)	2-3500	4-1600	2-1000	3-9000	-	Oueslati et al. (2014)
	Sediments (mg/g)	8.82	3.32	50.31	6.01	2.14	Oueslati et al. (2017)
MCA	Water	-	-	-	-	-	-
	Sediments (mg/kg)	18.7-98.8	7.28-89.30	0.07-0.67	75-249	25.73-47.92	Ennouri et al. (2010)
HCA	Water	-	-	-	-	-	-
	Sediments (µg/g)	170-239	11-19	0.78-0.96	341-432	-	Chalghmi et al. (2016)

Table S4. – Correlation matrix of non-parametric Spearman's rank correlation coefficients between *R. decussatus* biomarkers, trace element digestive gland tissue levels (Cd, Pb, Cu, Zn, and Fe) and environmental parameters measured in this study. AChE, acetylcholinesterase; CAT, catalase; AOPP, advanced oxidation proteins products; GPx, glutathione peroxidase; GSH, gluthatione; NPSH, non-protein SH; MDA, malondialdehyde; Vit C, vitamin C; MTs, metallothioniens; T, temperature; S, salinity; pH, hydrogen potential; SPM, suspended matter; Ch *a,* Chlorophyl *a*. The positive and significant correlation is presented in red and the negative and significant correlation is presented in green.

	T°C	Spus	pH	MES	Ch *a*	Cd	Pb	Cu	Zn	Fe
CI	0.635	0.632	ns	0.854	-0.243	0.934	0.646	0.645	0.432	0.835
GI	0.579	0.631	ns	0.801	-0.429	0.756	0.801	0.596	0.863	0.905
AChE	ns	ns	-0.307	-0.241	0.220	-0.533	-0.499	-0.541	-0.780	-0.596
CAT	0.405	0.444	ns	0.601	-0.171	0.657	0.455	0.780	0.804	0.587
AOPP	0.340	0.444	ns	0.564	-0.302	0.752	0.564	0.767	0.801	0.700
GPx	Ns	0.225	ns	ns	-0.309	0.531	0.421	0.236	0.264	0.406
GSH	0.340	0.451	ns	0.549	-0.309	0.754	0.561	0.756	0.799	0.703
NPSH	0.319	0.401	ns	0.482	-0.333	0.754	0.606	0.620	0.639	0.660
MDA	0.310	0.406	ns	0.519	-0.342	0.771	0.598	0.733	0.763	0.726
Vit C	0.345	0.382	ns	0.427	-0.192	0.373	0.338	0.682	0.702	0.460
MTs	0.428	0.499	ns	0.413	-0.535	0.562	0.733	0.853	0.806	0.813
DNA	0.657	ns	ns	0.545	ns	0.405	0.375	0.603	0.345	0.426
T°C	ns	ns	ns	ns	ns	ns	ns	0.603	ns	ns
Spus	ns	ns	ns	ns	ns	ns	ns	0.630	0.239	ns
pH	ns	ns	ns	ns	ns	ns	0.241	ns	ns	0.374
MES	ns	ns	ns	ns	ns	0.404	ns	0.539	0.280	ns
Ch*a*	ns	ns	ns	ns	ns	ns	-0.735	-0.370	-0.353	-0.716