Metal body burden and tissue oxidative status in the bivalve *Venerupis decussata* from Tunisian coastal lagoons

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HIGHLIGHTS

- The health status of bivalves reflects natural and anthropogenic stressors
- Boughrara and South Tunis lagoons are the most impacted transitional waters
- Higher temperature and metal content enhance oxidative and toxicopathological traits
- Multivariate PCA discriminates environmental status of Tunisian transitional waters

ABSTRACT

Coastal transitional waters are exposed to many anthropogenic threats. This study aims to assess the trace metals´ pollution status of transitional waters by evaluating its biological effects in the clam *Venerupis decussata*. Among the studied sites along the Tunisian littoral, South Tunis and Boughrara were the most impacted, since clams from these two lagoons presented significant differences in: (i) trace metal contents, (ii) in-cell hydrogen peroxide, (iii) enzymatic and non-enzymatic defenses, (iv) damage to lipids and proteins, and (v) protein post-translational modifications. These changes related to evident histopathological traits. PCA showed a clear separation between the digestive gland and gills tissues and illustrated an impact gradient in Tunisian coastal lagoons. Water temperature was revealed as an added natural stressor that, when concurring with high pollution, may jeopardize an ecosystem´s health and contribute to the accumulation of hazardous metals in organisms.

Keywords:

Clams; Histopathology; Molecular biomarkers; Oxidative stress; Temperature; Trace metals; Transitional waters

Abbreviations: AOPP, advanced oxidation protein product; BG, Boughrara Lagoon; BZ, Bizerte Lagoon; CAT, catalase; DNPH, 2,4-dinitrophenylhydrazine; EL, Ellouza; GPx, glutathione peroxidase; GSH, reduced glutathione; mBBr, monobromobimane; MDA, malondialdehyde; NPSH, non-protein thiols; NT, North Tunis Lagoon; PAHs, polycyclic aromatic hydrocarbons; PCA, principal component analysis; PCO, protein carbonyl; PRS, protein redox state; ROS, reactive oxygen species; SOD, superoxide

25 dismutase; SPM, Suspended particulate matter; ST, South Tunis Lagoon; TBP,
26 tributylphosphine; TCA, trichloroacetic acid.

1. Introduction

Transitional waters are those situated between the land and the sea, including fjords, estuaries, lagoons, deltas and rias. Due to their strategic location, coastal lagoons usually exert vast ecological and economic impacts on their surrounding areas. However, lagoons are characterized by being relatively isolated from the open sea, which makes them highly vulnerable to anthropogenic pollution, as they are exposed to urbanization, industrialization and intensive agricultural activities (Alves Martins et al., 2015; Bilgin and Uluturhan-Suzer, 2017). The Tunisian coastline contains several of these ecosystems that are exploited for fishery and shellfish farming, particularly Bizerte, North and South Tunis, and Boughrara lagoons (Alves Martins et al., 2015; Lahbib et al., 2018).

As a consequence of an intense human activity, transitional water-bodies accumulate increasing amount of toxic chemicals, most of them highly persistent (Bald et al., 2005; Carreira et al., 2013). As marine pollution increases worldwide, there is a concomitant need to develop strategies to monitor the biological effects of contaminants.

Ecosystem pollution can be assessed by using sentinel organisms as bioindicators. Bivalve mollusks are used worldwide as marine pollution sentinels due to their sessile nature, filter-feeding habits, and ability to concentrate pollutants (Bebianno et al., 2004; Costa et al., 2013; Chalghmi et al., 2016a; Funes et al., 2006). The *Venerupis decussata* clam (Linnaeus, 1758) (also known as *Ruditapes decussatus* and *Tapes decussatus*) lives in the muddy sand sediments of coastal environments (Parache, 1982). *V. decussata* is widely used in fisheries and aquaculture in southwestern Europe and Mediterranean areas, where this mollusk has a high economic impact (Carreira et al., 2013). Due to its wide distribution, and sensitivity to contaminants, *V. decussata* is

also a common bioindicator of environmental pollution, particularly in confined coastal environments (Bebianno et al., 2004; Costa et al., 2013).

Trace metals constitute one of the most dangerous sources of pollution in marine ecosystems due to their toxicity, persistence, and non-degradability (Park et al., 2017). As many other toxicants, a well-described effect of exposure to transition metals is oxidative stress, by catalyzing the generation of highly hazardous and mutagenic reactive oxygen species (ROS), such as: hydroxyl radical ($HO^{\cdot}$), superoxide anion radical ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2) (Park et al., 2017; Roberts et al., 2009; Sies, 1986). Organisms respond to oxidative threats by activating protective mechanisms that include enzymatic and non-enzymatic antioxidants. As first line defense antioxidants, superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, while catalase (CAT) and glutathione peroxidase (GPx) convert H_2O_2 into water and diatomic oxygen. GPx also reduces reactive lipid-hydroperoxides using reduced glutathione (GSH), thus avoiding the formation of malondialdehyde (MDA) (Funes et al., 2006; López-Barea, 1995; Rodriguez-Ortega et al., 2002; Sies, 1986). Thiol-containing compounds are a very important class of non-enzymatic antioxidants. Among low-Mr non-proteins thiols, GSH plays a pivotal protective role against electrophilic xenobiotics and oxidative stress (Pompella et al., 2003). The antioxidant properties of ascorbic acid (vitamin C) are attributed to its capacity both to directly interact with oxidizing radicals, and to regenerate other small antioxidant molecules, including GSH (Ahmad et al., 2012; Gebicki, 2016). Among the thiol-containing proteins, metallothioneins (MTs) stand out as very sensitive metal-pollution biomarkers. MTs are small proteins with a high Cys content involved in the homeostasis of essential metals, sequestration and detoxification of trace metals, and scavenging of ROS (Amiard et al., 2006; Romero-Ruiz et al., 2008; Viarengo et al., 1999). When the ROS production rate exceeds that of the quenching

77 antioxidant detoxification mechanisms, damage can reach different biomolecules
78 impairing many physiological processes (Park et al., 2017; Sies, 1986). MDA is a
79 highly reactive by-product of lipid peroxidation (Draper and Hadley, 1990), while
80 formation of irreversible carbonyl groups (PCO) and advanced oxidation protein
81 products (AOPP) are major forms of protein oxidation (Kalousova et al., 2005; Kayali
82 et al., 2006; Yan and Forster, 2011). Because of their abundance and high reactivity,
83 proteins are the main targets in oxidative stress scenarios since they absorb around 70%
84 of ROS (Davies, 2005). As a consequence of ROS exposure, proteins are regulated by
85 post-translational modifications (PTMs) that influence their structural conformation,
86 biological activity, protein-protein interactions, turnover rates, and targeting to
87 subcellular localizations (Cabiscol and Ros, 2006; Eaton, 2006; Klomsiri et al., 2011;
88 Sheehan et al., 2010).

89 Changes at the molecular level constitute rapid, early-warning and reversible
90 responses to environmental alterations, so they have high toxicological relevance
91 (López-Barea, 1995). At a higher level, histopathological analyses in biomonitoring
92 programs provide additional relevant information on the health status of the organisms
93 (Costa et al., 2013).

94 Besides water and sediment pollution, mainly of anthropogenic origin, marine
95 organisms are also exposed to natural stressors, such as: fluctuations in temperature,
96 salinity or pH, desiccation, pathogens, and changes in O_2 or CO_2 levels. Synergistic
97 interactions between the effects of various natural stressors and pollutants are the most
98 common phenomena (Holmstrup et al., 2010; Sokolova and Lannig, 2008).

99 The aim of the present study is to develop a multivariate approach for the
100 evaluation of the trace metal contamination status of transitional waters, the generated
101 oxidative stress, and its biological effects. The clam *V. decussata* has been selected as

bioindicator, and several lagoons along of the Tunisian coastline were chosen as case studies. To implement this approach, we have carried out: (i) physico-chemical measurements in waters, and (ii) trace metal content, (iii) reactive oxygen species generation, (iv) enzymatic and non-enzymatic antioxidative defenses, (v) oxidative damage to biomolecules, (vi) global post-translational modifications, and (vii) histopathological analysis in clam tissues.

2. Materials and methods

2.1. Areas of study

Widely distributed areas of bivalve mollusks production along the Tunisia coastline have been studied in this work (Fig. 1). Ellouza (EL), located in the western coast of the Gabes gulf (Sfax city), was used as the reference site because previous studies have shown that it is a low impacted open-sea coastal area (e.g.: Annabi et al., 2013; Chalghmi et al., 2019; Kessabi et al., 2012). The other sites studied are lagoons, closed or semi-closed transitional water ecosystems with a great ecological value (Bilgin and Uluturhan-Suzer, 2017; Carreira et al., 2013). At the same time, lagoons might be subjected to a high anthropogenic pressure. Thus, the Bizerte Lagoon (BZ), with an extension of over 128 Km^2 and an average depth of 7 m, is surrounded by many industries (oil and its derivatives, plastic, clothing, agri-food, etc.), agricultural activities and urban areas (Alves Martins et al., 2015; Bejaoui et al., 2017). The Tunis Lagoon (40 Km^2) is divided into two parts, North (NT) and South (ST), separated by the Tunis-Goulette road and a navigation channel. NT receives urban discharges daily, while ST is mostly affected by fishing and navigation, port and industrial activities (Chalghmi et al., 2019). The Boughrara Lagoon (BG) is an exceptional site for both terrestrial and marine biodiversity, although its ecological future is in jeopardy. This lagoon is characterized by high salinity and temperature (Khedhri et al., 2017). BG is strongly impacted by many activities such as aquaculture farms and fishing boat traffic, thus receiving an important amount of organic matter. Moreover, high levels of phosphogypsum have been discharged into this lagoon, disturbing its quality, biodiversity and all ecosystem functions (Rekik et al., 2012).

2.2. Physicochemical analysis

132 Surface water samples were analyzed *in situ* at the different sites where clams were
133 collected. Temperature, salinity, pH and suspended particulate matter were measured in
134 February 2017, using a WTW multi-parameter probe (model WTW LF.325).

135 *2.3. Specimens and sample preparation*

136 *Venerupis decussata* clams were collected in February 2017 at the different
137 sampling sites shown in Fig. 1. Animals were immediately transferred alive to the
138 laboratory on ice and selected those with similar shell dimensions and weight
139 (Supplementary Table 1). The specimens were briefly rinsed with ultrapure water to
140 eliminate any exogenous materials, and then dissected on ice for excision of the
141 digestive gland and gills.

142 For metal analysis, the tissues of twenty clams per sampling site were pooled
143 into six independent replicates and freeze-dried. Fresh tissue samples were excised for
144 histopathology and fixed immediately, as described below (Section 2.9). For
145 biochemical analyses, individual tissues were snap-frozen, ground with a Freezer/Mill®
146 Grinder (SPEX Sample PreP) and stored at −80°C until pooled in eight independent
147 samples (twenty clams per pool). After that, the pooled samples were homogenized in
148 50 mM Tris-HCl buffer (pH 7.4, 5 mL/g) containing 1 mM EDTA and 1 mM PMSF,
149 using an electrically driven Potter-Elvehjem glass homogenizer and a Teflon pestle. The
150 homogenates were then centrifuged at 16,200 *g* for 15 min. The remaining supernatants
151 were aliquoted and stored at −80ºC until used for the biochemical analyses. The protein
152 content was determined in the supernatants by dye-binding (Bradford, 1976), using
153 bovine serum albumin (BSA) as standard.

154 *2.4. Trace element analysis*

155 Freeze-dried organ samples (0.5 g) were digested in a mixture of 6 mL of HNO_3
156 and 1 mL of H_2O_2 in Teflon reactors using a programmable microwave Touch Control
157 Terminal 320 system (Milestone, type Ethos). The first 5 min consisted of a heating
158 ramp up to 175 ± 5°C at 1200 W. After heating, the residue obtained was re-dissolved
159 in 5 mL of HNO_3, and the solution heated for a further 30 min at 150°C. After cooling
160 to room temperature, the solutions were transferred to new flasks and their final volume
161 was adjusted to 50 mL with ultrapure water. Concentrations of cadmium (Cd), copper
162 (Cu), lead (Pb), zinc (Zn), iron (Fe), nickel (Ni) and aluminum (Al) were determined by
163 Inductively Coupled Plasma-Mass Spectrometry (Triple Quadrupole ICP-MS, Thermo
164 Fisher), including blanks and internal standards. The metal contents are expressed as μg
165 g^{-1} sample dry weight. The reference material NIST SRM 2976 (trace elements and
166 methylmercury in mussel tissue) was analyzed using the same procedure, with recovery
167 rates being found to be within 95% of the certified range. The quality control of
168 standard of each tested element was as follows: Cd, 0.81 ± 0.11; Cu, 4.01 ± 0.31; Pb,
169 1.18 ± 0.15; Zn, 138 ± 12; Fe, 171.0 ± 4.9; Ni, 0.94 ± 0.10; Al, 134 ± 34 μg g^{-1}.

170 *2.5. Measurement of hydrogen peroxide*

171 The concentration of H_2O_2 in the digestive gland and gills of *V. decussata* was
172 determined spectrophotometrically at 560 nm following the Ferrous Oxidation in
173 Xylenol orange (FOX) reaction (Ou and Wolff, 1996). Standard solutions of H_2O_2 were
174 used to calibrate the assay.

175 *2.6. Enzymatic and non-enzymatic antioxidant defenses*

176 Enzymatic and non-enzymatic antioxidants were determined to evaluate the
177 defensive responses to oxidative stress. Cuvette-based colorimetric assays were used for
178 the different determinations. Catalase (CAT) was determined by recording the
179 breakdown of H_2O_2 at 240 nm (Aebi, 1984). Glutathione peroxidase (GPx) was

measured using GSH as a conjugation substrate coupling the assay to NADPH consumption, determined at 340 nm (Flohe and Gunzler, 1984). Superoxide dismutase (SOD) was determined spectrophotometrically at 580 nm (Beauchamp and Fridovich, 1971), based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxide anion.

Due to the relevant role of thiolic compounds as antioxidants, they were quantified as metallothionein-like proteins (MTLPs) after precipitating proteins from the extracts used for biochemical analysis (Section 2.3), and as non-protein thiols (NPSH) on the remaining soluble cytosol. MTLPs and NPSH were determined spectrophotometrically at 412 nm (Ellman, 1959; Petrovic et al., 2001; Viarengo et al., 1999). For both, the concentration was estimated using GSH as a reference standard. Vitamin C (Vit C) was determined at 529 nm by the DNPH and copper sulfate assay (Jacques-Silva et al., 2001). The ascorbic acid concentration was estimated from a previously prepared standard curve.

2.7. *Biochemical oxidative damage*

The level of thiobarbituric acid-reactive substances was assayed to assess malondialdehyde (MDA) content, a known marker of lipid peroxidation (Draper and Hadley, 1990). The levels of MDA were determined spectrophotometrically at 532 nm, according to a standard curve generated from serial dilutions of 1,1,3,3-tetraethoxypropane (TEP), an MDA precursor.

The oxidative damage to proteins was evaluated by determining advanced oxidation protein products (AOPP) and protein carbonyl (PCO) levels. The AOPP levels were determined spectrophotometrically at 340 nm (Kayali et al., 2006). PCO was determined by a method based on the reaction of carbonyls groups with 2,4-

dinitrophenylhydrazine (DNPH), and determined at 370 nm (Reznick and Packer, 1994).

2.8. *Electrophoresis-based evaluation of post-translational modifications*

All protein separations were performed by SDS-polyacrylamide gel electrophoresis on 4% (w/v) stacking gel and 12% resolving gel using the Laemmli buffer system. The samples were separated at 200 V constant in a Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were scanned using a ChemiDoc™ MP Imaging System (Bio-Rad). Image Lab software (Bio-Rad) was used for the acquisition of gel images and all subsequent image analyses.

The Pro-Q® Diamond gel stain (Molecular Probes®, Invitrogen) was used to selectively stain the phosphoproteins in polyacrylamide gels. 50 µg of protein was analyzed by SDS-PAGE and, after electrophoresis, the manufacturer´s recommendations were followed for the specific staining with Pro-Q Diamond of the phosphoproteins in the gels, that were scanned at 555/580 nm excitation/emission detection wavelengths to show the phosphorylated proteins. The gels were then stained with SYPRO Ruby (Bio-Rad) and rescanned at 532/555 nm excitation/emission detection to show total proteins. Determining the ratio of Pro-Q Diamond dye to SYPRO Ruby dye signal intensities provided a measure of the phosphorylation level normalized to the total amount of protein.

A fluorescence-based electrophoretic assay was used to quantify both the global level of reversibly oxidized and reduced thiols in proteins whose ratio indicates their redox status. Cysteinyl protein thiols were directly labeled with monobromobimane (mBBr) for native reduced groups. For oxidized thiol detection, all free thiol groups were first blocked with iodoacetamide (IAM), reversibly oxidized thiols were then reduced with tributylphosphine (TBP), and finally the reduced thiols generated were

labeled with mBBr. For the reduced thiol groups, 100 µg protein was denatured by incubating for 10 min at 37°C in 50 µl Tris-HCl 0.1 M, pH 7.4, and 1% SDS. mBBr was then added to 200 µM final concentration and the samples were incubated for 30 min at 37°C in the dark. After adding Laemmli sample buffer, the proteins were loaded and separated on 12% SDS-PAGE gels, avoiding light. To minimize the background, the gels were washed with ethanol:acetic acid (50:3) before scanning at 395/490 nm excitation/emission detection wavelength to show the fluorescent labeled proteins. For normalization, the gels were re-stained with Coomassie blue, and then the image analysis was carried out as indicated above. For determination of the oxidized thiol groups, the initial reduced groups in 200 µg protein extracts were blocked by incubating for 30 min at 37°C in Tris-HCl 0.1 M, pH 7.4, 1% SDS and 200 mM IAA. The proteins were precipitated with cold 20% TCA (w:v) followed by three washes with ethanol:ethyl acetate (50:50, v:v) to remove excess IAA. The final pellets were resuspended in Tris-HCl 0.1 M, pH 7.4, 1% SDS and 1 mM TBP to reduce the oxidized thiols, and then the procedure continued as for the initially reduced thiol groups. Approximately 20 µg of protein was loaded on the gels for both the reduced and oxidized thiol determinations.

2.9. Histopathological analysis

The preparation of the samples (5 gills and 6 digestive glands per site) for the histopathological appraisal follows the standard methods previously described (Martoja and Martoja-Pierson, 1967). In brief, tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E). Histopathological lesions and alterations were identified according to specific literature on *V. decussata* histology and histopathology (Costa et al., 2013).

2.10. Statistical analysis

Data are expressed as mean ± SD of six replicates for the trace elements, eight for the other biochemical analyses, and three for the post-translational modifications and physico-chemical parameters. The results were analyzed using the Statistica software (Version 8). The normality of the data was evaluated using the Kolmogorov–Smirnov and the Shapiro–Wilk W tests, while homogeneity of variance was verified using Levene's test. The comparison between the groups was made through one-way ANOVA followed by Tukey's test (for normal data), or through Kruskal-Wallis test (for non-normal data). Principal component analysis (PCA) was performed using R (Ihaka and Gentleman, 1996). The Spearman correlation matrix was also calculated to study the relationship between the different parameters analyzed using the GraphPad Prism 8.0.2 software.

3. Results

3.1. Physico-chemical characteristics of the transitional waters

Physico-chemical characteristics were determined in the surface water at the different locations (Table 1). Although no significant variations were observed in salinity and pH, compared to the EL reference site, marked increases were observed in temperature and suspended matter. Thus, the temperature was significantly higher in ST ($p < 0.05$) and BG ($p < 0.01$), while the concentration of suspended particulate matter greatly increased at all the lagoons studied ($p < 0.001$).

3.2. Accumulation of trace metals in clam tissues

Table 2 shows the results of the metal concentrations in the gills and in the digestive gland of clams collected from five locations along the Tunisian coastline. The lowest metal concentrations were found in the tissues from specimens collected at Ellouze (EL), considered here as the reference site. Otherwise, the highest metal levels were found at ST and BG, where the differences were statistically significant for Al (gills), Zn and Fe (digestive gland), and Cd, Pb, Cu and Ni (both tissues). Compared to the reference site, we wish to highlight the ≥ 2-fold increase in Pb, Cu and Ni (gills), Cd, Pb and Ni (digestive gland) contents at BG, and in Pb (digestive gland) and Ni (both tissues) levels at ST. Even though the ST and NT water bodies are connected, the ST specimens accumulated more metals than those from NT, where only Ni (gills) and Pb (digestive gland) increased significantly. Finally, clam tissues from BZ showed levels slightly superior to the reference site, although the differences were of little significance ($p < 0.05$) for Ni in gills, and Pb in both tissues.

3.3. Oxidative stress and antioxidant defensive responses in clam tissues

288 Table 3 shows the levels of H_2O_2 determined in the gills and in the digestive
289 gland of clams. In both tissues, significantly higher levels of H_2O_2 were detected in the
290 clams from BZ, ST and BG compared to EL, the reference site.

291 Table 3 shows the levels of enzymatic and non-enzymatic antioxidative
292 responses in the gills and in the digestive gland of *V. decussata* clams. In agreement
293 with the accumulation of trace metals in their tissues, the clams from BG and ST
294 presented significant increments in all the antioxidative responses in both tissues,
295 compared to the EL reference site. The most relevant changes were found for CAT
296 activity in ST and BG (around 70% increase in both tissues), for GPx in the digestive
297 gland, which increased 46% (ST) and 84% (BG), and for MTLPs in the gills, with a
298 91% and 74% rise at BG and ST, respectively. This last parameter also increases
299 approximately 55% in the digestive gland of the specimens from ST and BG.
300 Additionally, around 50% (NPSH) and 30% (SOD and Vit C) increments were obtained
301 in both tissues of the clams from these two sites. Finally, significantly higher levels of
302 CAT and MTLPs ($p < 0.01$) in the digestive gland, and GPx ($p < 0.05$) in the gills, were
303 detected in the clams from BZ.

304 *3.4. Oxidative damage and post-translational modifications in the clam tissues*

305 Fig. 2A shows the evaluation of the oxidative damage to the lipids and proteins
306 in the gills and in the digestive gland of clams captured at the different sites studied.
307 Malondialdehyde (MDA) content is a consequence of the oxidative damage to lipids.
308 According to their metal contents, MDA levels show top values in both tissues of the
309 ST and BG clams, with significant increases of up to 64% above the levels found at the
310 EL reference site. Meanwhile, lipid peroxidation levels in the BZ and NT specimens
311 were similar to those determined in the EL animals.

312 Global protein damage was determined by measuring both the protein carbonyl
313 (PCO) derivatives, and the advanced oxidation protein products (AOPP) (Fig. 2A). PCO
314 levels showed no differences between the two tissues analyzed, although again, in both
315 tissues, samples from the ST and BG lagoons presented significant increments in
316 protein oxidation ranging from 21 to 35 %, respectively, in comparison to those from
317 EL. Otherwise, AOPP levels increased in both tissues of clams from all the sampling
318 sites in comparison to EL, even though, again ST and BG were the two locations
319 showing the highest levels of protein oxidative damage.

320 Global protein phosphorylation status is shown in Fig. 2B. Our results show that:
321 (i) the digestive gland presented lower protein phosphorylation levels than the gills, and
322 (ii) in both tissues, phosphorylation was significantly reduced in the clams from BZ, ST
323 and BG compared to those from the EL reference site. As also shown in Fig. 2B, the
324 clams from ST and BG presented significant changes in their protein redox status.
325 Protein Cys were significantly more oxidized in the two tissues analyzed from both sites
326 compared to the reference site, an opposite trend to their phosphorylation levels.

327 *3.5. Histological alterations in the clam tissues*

328 The general histopathological state of the digestive gland of the clams collected
329 at different locations along the Tunisian coast is shown in Fig. 3A. The clams from EL
330 (Fig. 3A-EL), the reference site, essentially presented a normal digestive gland
331 architecture, comprising many digestive tubules lined by a single layer of epithelial cells
332 and a narrow lumen. The tubules are separated by intertubular connective tissue. In the
333 clams from the different lagoons, pathological traits were evident. Specifically, the
334 lesions in the BZ (Fig. 3A-BZ) and NT (Fig. 3A-NT) clams were scarce and of
335 relatively low biological significance, consisting mostly of minor foci of haemocyte
336 infiltration and lumen occlusion. However, the clams with the higher levels of metals

337 and molecularly more impacted, *i.e.* ST (Fig. 3A-ST) and BG (Fig. 3A-BG), presented
338 additional and more significant alterations. These included necrotic tubules and foci of
339 intertubular tissue necrosis, with moderate infiltration of haemocytes, as well as
340 epithelial cell vacuolization (most obvious in the BG clams) and atrophic tubules (in the
341 digestive gland of the clams from ST).

342 The gills of the clams from the reference site (EL) mostly presented the normal
343 structure of the frontal, intermediate and abfrontal zones (Fig. 3B). Ctenidia presented a
344 well-defined structure with a regular arrangement of filaments and lamellae. The
345 lamellar epithelial cells in the frontal zone presented intact cilia. The haemolymphatic
346 sinuses presented few haemocytes. Cells containing lipofuscin-like substances, like
347 rhogocytes, were sparse. Similar observations were made from the gills of the
348 specimens collected at the NT, BZ and ST lagoons (Fig. 3B-BZ, 3B-NT, 3B-ST).
349 However, the histological sections of the gills of the clams containing the highest metal
350 levels (BG) revealed moderate abrasion of ctenidia, foci of lamellar deformation and
351 diffuse epithelial desquamation (Fig. 3B-BG).

352 *3.6. Relationships between the abiotic and biotic parameters*

353 Principal Component Analysis (PCA) yielded Principal Component 1 (PC1)
354 accounting for 46.0% of the variability amongst the data while PC2 accounted for
355 12.9% (Fig. 4). Samples from the different tissues, the digestive gland and gills,
356 grouped differently mainly related to PC2. The locations presented the pattern that
357 has been perceived from all the results described. Thus, NT and BZ sites present
358 similar trends and the analysis of their data places them close to the reference site
359 EL. On the other hand, the ST and BG lagoons analyses overlap in both tissues and
360 are grouped differently from the other sites. Thus, the sites studied follow a gradient
361 along the PC1 axis: EL<NT<BZ<ST<BG (Fig. 4).

362 The Spearman correlation coefficients were also determined for cross-
363 validation of the relationships between the different variables (Supplementary Table
364 2). Most physico-chemical parameters, such as SPM, salinity and pH, were either
365 only weakly, or not related with the other variables analyzed. However, a high
366 correlation was found between temperature and the content of metals, including Cd
367 ($p < 0.0001$), Al ($p < 0.0002$), Pb, Cu and Ni ($p < 0.0021$). Significantly, a very
368 strong correlation ($p < 0.0002$) was found between temperature and in-cell H_2O_2, a
369 very important ROS consequence of oxidative stress. Accordingly, a high
370 correlation was found between H_2O_2 and metal concentrations, highlighting Cd ($p <$
371 0.0001), Pb, Cu, Al and Ni ($p < 0.0021$). Strong correlations were found among the
372 oxidative threats (temperature and trace metals), hydrogen peroxide, and the
373 molecular consequences of the oxidative stress generated (antioxidative defenses,
374 damage to biomolecules and PTMs). It should be emphasized that perfect
375 correlations ($p < 0.0001$) were stablished between H_2O_2 and SOD/MTLP, between
376 MDA and Zn/Fe, and between PCO and Vit C. Additionally, very strong
377 correlations ($p < 0.0002$) were found between temperature and CAT, Cd and
378 MTLP/AOPP/PRS, Pb and MTLP, Cu and PSR, Al and CAT/PCO. Finally, it is
379 noteworthy that significant negative correlations were obtained between protein
380 phosphorylation and several variables.

4. Discussion

Transitional waterbodies are important ecological systems and highly diverse environments in terms of socio-economic drivers, physico-chemical status, geophysics and nature of anthropogenic impacts (Bald et al., 2005; Bilgin and Uluturhan-Suzer, 2017; Carreira et al., 2013). The approach used here was designed to assess the status of metal pollution in transitional waters and its biological effects on the organisms at the biochemical and tissue levels. For that purpose, several lagoons along the Tunisian coastline were evaluated, using the bivalve mollusk *V. decussata* as the bioindicator organism.

In environmental studies, choosing an appropriate reference site is quite a difficult task, coupled with the difficulty of establishing the physico-chemical elements which correspond to low or undisturbed/non-impacted conditions (Bald et al., 2005). The clams from Ellouze (EL) presented the lowest levels of trace elements (Table 2), which was considered here as an appropriate reference area, in agreement with previous reports (Annabi et al., 2013; Chalghmi et al., 2019; Kessabi et al., 2012). Since EL is in an open-sea coastal area, the suspended particulate matter in the water was significantly lower than in the four lagoons studied. Lagoons are relatively isolated from the open sea, and that makes them more vulnerable to the impacts of pollution (Bilgin and Uluturhan-Suzer, 2017). Many studies have provided information about the presence of high metal contents in the sediments of different Tunisian lagoons. However, those studies focus on local restricted areas, particularly BZ, and the degrees of pollution described vary among reports (Barhoumi et al., 2016; Ben Said et al., 2010; Ghedira et al., 2016; Ghedira et al., 2011; Hellal Mel et al., 2011; Zaaboub et al., 2016; Zakhama-Sraieb et al., 2019). Furthermore, several other pollutants have been detected in the BZ lagoon, including polycyclic aromatic hydrocarbons (PAH), pesticides and

406 polychlorinated biphenhyls (Bancon-Montigny et al., 2019; Ben Said et al., 2010; Hellal
407 Mel et al., 2011; Mhadhbi et al., 2019; Triki et al., 2017). Nevertheless, when studied,
408 elevated levels of metals have also been identified in ST (Chalghmi et al., 2016a;
409 Chalghmi et al., 2016b), NT (Oueslati et al., 2018) and BG (Kharroubi et al., 2012),
410 and, additionally, a recent study describes ST as being moderately to highly PAH-
411 contaminated (Chalghmi et al., 2019).

412 Trace metal levels determined here were compared with the soft tissue
413 accumulation levels in the same species found at other contaminated areas in previous
414 studies. Although the concentration of most metals, Pb, Cu, Zn, Fe, Al and Ni,
415 determined here were similar or even lower to those found in other areas, the levels of
416 Cd determined were much higher than those found in all other polluted areas (Cravo et
417 al., 2012; Chalghmi et al., 2016a, 2016b; Bilgin and Uluturhan-Suzer, 2017). Cd is an
418 increasingly common contaminant of great ecological and human concern, due to its
419 widespread industrial use and because it is one of the most hazardous known substances
420 (Ghedira et al., 2011; Serafim and Bebianno, 2007). Significant ecological effects at the
421 population level have been shown in freshwater bivalves (*Pyganodon grandis*)
422 chronically exposed or transferred to a Cd gradient. Increased Cd concentration leads to
423 elevated mortality rates and to decreased population densities, growth, biomass,
424 secondary production, turnover ratio, and reproductive success, which results in the
425 impairment of population health status (Perceval et al., 2004, 2006). In a recent study,
426 lower condition and gonad indices were found in *V. decussata* clams from BG than
427 those from the Ghar El Melh Lagoon (northeast Tunisia), in agreement to the highest
428 levels of Cd and Pb determined in BG clams´ tissues (Bejaoui et al., 2020).

429 It must be emphasized that the levels of metals act, in this study, as a mere
430 surrogate of global contamination, in the absence of comprehensive data on organic

pollutants in the studied areas. However, it cannot be assumed that aquatic organisms employ the same strategies to accumulate different classes of pollutants, even among trace metals. In aquatic environments, trace metal species are distributed among different water-soluble agents, colloids, suspended matter and sedimentary phases. In general, these pollutants are retained in the sediments, and their mobility and bioavailability can be influenced by many factors including pH, temperature, salinity, redox potential, organic matter, other contaminants or even microbial activity (Filgueiras et al., 2004). Some of the water physico-chemical parameters determined in this study, such as salinity or pH, did not show differences when lagoons were compared with EL. However, the water temperature was higher in the different lagoons, especially at ST and BG (Table 1). BG is a shallow lagoon (4 m average depth) and is therefore highly influenced by the local dry and sunny climate (Khedhri et al., 2017). Among other environmental factors, temperature plays a key role, significantly in polluted coastal and transitional waters (Cabral et al., 2019; Lannig et al., 2006; Nardi et al., 2018; Sokolova and Lannig, 2008). It has been shown that an increase in temperature facilitates the solubility and mobilization of trace metals, which finally results in a higher bioavailability, thus, increasing uptake rates and bioaccumulation in aquatic ectotherms (Mubiana and Blust, 2007; Nardi et al., 2017, 2018; Richards and Chaloupka, 2009; Sokolova and Lannig, 2008). Due to the favored rate of metal uptake and bioaccumulation, an elevated temperature tends to enhance the toxic effects of metals on organisms, resulting in elevated mortality rates (Lannig et al., 2006; Sokolova and Lannig, 2008). Here, we found a high correlation between temperature and the metals contents such as: Cd, Al, Pb, Cu and Ni. Significantly, a perfect correlation ($R = 0.960$, $p < 0.0001$) was found between temperature and Cd (Supplementary Table 2). Thus, specimens from the ST and BG lagoons, where the water temperature is the

456 highest, accumulated the highest levels of metal in their tissues, suggesting that these
457 are the most impacted of all the ecosystems analyzed. Otherwise, the clams from BZ
458 and NT presented moderate levels of trace metals, with significant increases only for Pb
459 and Ni (Table 2).

460 In ectotherms, such as bivalves, the body temperature readily changes with the
461 ambient temperature, resulting in alterations of the rates of most physiological, cellular
462 and biochemical processes (Sokolova and Lannig, 2008). Additionally, when combined
463 with an elevated environmental temperature, pollution has a direct, synergistic impact
464 on these processes (Cabral et al., 2019; Lannig et al., 2006; Nardi et al., 2017, 2018;
465 Sokolova and Lannig, 2008). The gills and the digestive gland play crucial roles in
466 balancing gas and ions and digestion plus nutrient absorption, respectively. Whereas the
467 gills are a major organ of apical entry for waterborne toxicants, such as dissolved
468 metals, the digestive gland absorbs ingested toxicants and it is most likely involved in
469 their detoxification and storage, as an analogue of the vertebrate liver (Louiz et al.,
470 2018). In any case, both organs may have to cope with toxicological challenges and
471 their consequences, such as oxidative stress and cell-level alterations due to metabolic
472 impairment, when the levels of toxicants overcome the organism's defense mechanisms.
473 However, establishing cause-effect relationships between adverse effects and exposure
474 to specific toxicants is challenging. Still, the current histological studies in *V. decussata*
475 show a global pattern that is consistent with increasing contamination at ST and BG,
476 where specimens accumulated the highest concentrations of trace metals in their tissues.
477 Overall, the changes observed range from foci of mild reversible lesions (like foci of
478 haemocytic infiltration) to severe alterations (such as necrosis) that may heavily impair
479 organ functioning if becoming diffuse. Our results are comparable with those observed
480 in clams collected from low-moderately contaminated sites in the Mediterranean basin

481 or adjacent areas, such as Tunisia and Southern Portugal (Costa et al., 2013; Chalghmi
482 et al., 2016b). Altogether, the histopathology of both the gills and the digestive gland
483 suggest that moderate but persistent exposure to mixed environmental toxicants results
484 in chronic adverse effects. These lesions may, in time, reduce the fitness of the
485 population in the area, thus rendering the clams more susceptible to environmental
486 changes and parasites. With this respect, the importance of the digestive gland and gills
487 in detoxification and gas/ion balance must be highlighted, as these processes alter all
488 downstream metabolism.

489 Damage to the respiratory surfaces in the gills results in a less efficient oxygen
490 uptake and an associated greater need for active ventilation, which in turns leads to an
491 increased uptake of metals. Further, oxygen transport can also be impaired by exposure
492 to metals and elevated temperatures, which interferes with the normal O_2 delivery to
493 metabolizing tissues (Sokolova and Lannig, 2008). Besides the limited oxygen supply,
494 it must be added that trace metals strongly affect mitochondrial function by reducing the
495 activity of the electron transport chain and ATP production, which results in an increase
496 in mitochondrial ROS production (Sokolova and Lannig, 2008). A higher H_2O_2 content
497 was found here at BG and ST, followed by BZ (Table 3), in agreement with the
498 temperature levels and metal concentrations, such as Cd, Pb, Cu, Al, Ni, and to a lesser
499 extent Zn and Fe (Supplementary Table 2). In fact, very strong ($R = 0.935$; $p < 0.0002$)
500 and perfect ($R = 0.964$; $p < 0.0001$) correlations were found between H_2O_2 and
501 temperature, and H_2O_2 and Cd, respectively. Cd is a potent disruptor of mitochondrial
502 function, whose sensitivity is strongly enhanced at elevated temperatures, suggesting
503 synergism between both environmental stressors (Cherkasov et al., 2006a; Cherkasov et
504 al., 2006b; Cherkasov et al., 2010).

505 Metal-induced formation of free radicals and the consequent oxidative stress is
506 the basis of the toxicity of many trace metals. Thus, while some metals, such as Fe and
507 Cu, undergo redox-cycling reactions, in a second group, including Cd, Pb and Ni, the
508 mechanism is through the depletion of glutathione and bonding to sulfhydryl groups of
509 proteins (Stohs and Bagchi, 1995; Valko et al., 2005). Antioxidants provide protection
510 against deleterious free radical attacks (López-Barea, 1995; Sies, 1986). This includes
511 primary enzymatic (CAT, GPX and SOD) and non-enzymatic (MTLPs, NPSH and Vit
512 C) antioxidants, that significantly increased in the clams from ST and BG with the
513 higher metal loads in their tissues (Table 3). Thus, high correlations were found
514 between trace metals, the nonradical species H_2O_2, and most antioxidants (Fig. 4 and
515 Supplementary Table 2). Significantly, metallothionein-like proteins (MTLP) showed a
516 very strong correlation (R = 0.924 and 0.900; $p < 0.0002$) with metals such as Cd and
517 Pb, and a high correlation (R = 0.888, 0.796 and 0.827; $p < 0.0021$) with Cu, Zn and Fe.
518 MTs play crucial biological roles in the detoxification of trace metals (Ag, Cd, Co, Fe,
519 Hg, Ni, Pb) and in the homeostasis of essential metals (Cu, Zn) (Kägi, 1991).
520 Furthermore, a perfect correlation (R= 0.954; $p < 0.0001$) was found between MTLP
521 and H_2O_2, according to the relevant protective role of these proteins against oxidative
522 threats (Kägi, 1991; Viarengo et al., 1999; Viarengo et al., 2000). Protective
523 mechanisms, including antioxidant, detoxifying and damage-repairing proteins,
524 contribute to elevated metabolic rates and maintenance costs in metal-exposed
525 organisms, especially at elevated temperatures (Cherkasov et al., 2006a; Sokolova and
526 Lannig, 2008). The extra-energy expenditure for emergency maintenance, might
527 seriously threaten essential energetically demanding processes (*i.e.:* growth,
528 reproduction, immunity) and, as a consequence, the whole-organism´s physiology and
529 survival (Cherkasov et al., 2006a; Lannig et al., 2006; Sokolova and Lannig, 2008).

If toxic chemicals exceed pollutant-elicited defense mechanisms, especially in limited energy supply conditions, key biomolecules are damaged (Alhama et al., 2017). Oxidative damage to lipids (MDA) and proteins (PCO and AOPP) was evident here in the specimens with the highest metal concentrations in their tissues, those from BG and ST (Fig. 2A). The perfect correlation between MDA, and Zn and Fe should be highlighted, as well as the very strong correlation between PCO and Al, and between AOPP and Cd (Supplementary Table 2). Proteins are major targets of oxidative stress, which leads to post-translational modifications (PTMs) that can alter their structure and modify their function, and are important in cell signaling (Cabiscol and Ros, 2006; Davies, 2005). Reversible changes in the redox state thiols and in the phosphorylation level of proteins showed an opposite trend, both in the digestive gland and gills. Thus, the most oxidatively-threatened clams, those from BG and ST, presented the highest oxidized redox state in their protein cysteines. On the contrary, specimens from those areas, and those from BZ, presented low phosphorylation levels. We had previously described similar patterns in *Mus spretus* testis exposed to the persistent pollutant *p,p´*-DDE (Alhama et al., 2018). Damage and modifications of key biomolecules may reach higher organizational levels when faced with large and long-lasting deleterious effects from shifts in the internal oxidative status, with an emphasis on proteins, which form the primary structure and metabolic machinery of cells. Thus, several histopathological traits were evident in the clams with the highest levels of trace metals which were also more molecularly impacted, corresponding to samples from ST and BG digestive gland, and BG gills.

PCA analysis was performed to obtain a global vision of the results. Multivariate analysis grouped differentially the digestive gland and gills. In both tissues, sampling sites along the Tunisian littoral were separated following a pollution gradient:

555 EL<NT<BZ<ST<BG. This gradient is associated with the increasing concentration of
556 most trace metals (Cd, Pb, Cu and Ni) and with temperature, which results in higher
557 levels of oxidative stress biomarkers (H_2O_2, MTLP, AOPP and SOD).
558
559 **5. Concluding remarks**
560 The clam *Venerupis decussata*m has shown its usefulness as biomonitor
561 organism for detecting the impact of pollution in lagoon aquatic ecosystems. Our work
562 demonstrates that multivariate analysis is efficient to separate among sampling sites
563 along the Tunisian littoral, either using the digestive gland or gills tissues. Clams from
564 the different sites are differentially impacted following a pollution gradient:
565 EL<NT<BZ<ST<BG. The battery of biochemical parameters measured correlates with
566 trace metal pollution. The synergistic interaction between temperature and trace metals´
567 bioaccumulation may elicit oxidative stress that, if severe and long-lasting, may
568 overcome the antioxidant capacity of the clams and trigger histopathological traits in
569 their tissues, the digestive gland and gills. Altogether, this study highlights the
570 importance of conducting multifaceted in-field analyses to understand the effects of
571 chemical stressors in complex marine ecosystems, and puts the spotlight on
572 temperature, as an added natural stressor to be considered when organisms face seasonal
573 changes and in a global climate change scenario.
574
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585 **Figure Legends**

586 **Fig. 1.** Map showing the areas studied along the Tunisian coast. The location of the
587 sampling sites and the main polluting activities are indicated.

588 **Fig. 2.** Oxidative damage and post-translational modifications in the clam tissues.
589 **A.** Changes in malondialdehyde (MDA), and advanced oxidation protein products
590 (AOPP) and protein carbonyl (PCO) levels, as biomarkers of oxidative damage to
591 lipids and proteins, respectively. **B.** Electrophoresis-based evaluation of post-
592 translational modification levels, phosphorylation (arbitrary fluorescence units,
593 AUF) and the redox state of cysteine residues (oxidized/reduced thiols ratio), in
594 proteins. Determinations made in the gills (blue, left) and in the digestive gland
595 (red, right) of *V. decussata* are shown. Bars represent the mean ± SD of at least
596 eight independent determinations (A) or of three independent determinations (B)
597 from twenty pooled clams. Site abbreviations are as follows: Ellouze (EL) and
598 Bizerte (BZ), North (NT) and South Tunis (ST), and Boughrara (BG) lagoons.
599 Statistically significant compared to the EL reference site are expressed as: *, $p <$
600 0.05, **, $p < 0.01$, ***, $p < 0.001$.

601 **Fig. 3.** Histological alterations in the clam tissues. Micrographs of the digestive gland
602 and gill sections of *V. decussata* sampled from the reference site Ellouza (EL), and
603 four Tunisian lagoons suffering different degrees of anthropogenic pollution,
604 Bizerte (BZ), North Tunis (NT), South Tunis (ST) and Boughara (BG), are shown.
605 Six digestive glands and five gills were analyzed per site. **A.** The digestive gland of
606 the clams from Ellouza were characterized by a normal morphoanatomy of the
607 digestive glands, which consisted in digestives tubules (DT) with well-
608 distinguishable lumen (L), formed by a single layer of epithelial cells (EC). The
609 tubules are surrounded by interstitial (connective-like) tissue (IT). The digestive

610 gland of the clams from the four potentially impacted lagoons were mostly
611 characterized by lumen occlusion (LO), haemocyte infiltration within intertubular
612 tissue (HI), atrophic tubules (AT), focal necrosis (NF) and epithelial cell
613 vacuolization (TV). **B.** The clams from the reference site were characterized by a
614 normal morphoanatomy of gills, which consisted in: frontal (F), intermediate (I) and
615 abfrontal (AF) zones; regular filaments arrangements (FA), normal structure of cilia
616 (C), lipofuscin granules (LG) and haemolymphatic sinuses (HS). The most
617 significant alterations detected in the specimens from Boughrara, the potentially
618 most impacted area, were lamellar deformation (LD) with epithelial desquamation
619 (ED) and abrasion of ctenidia (AC).

620 **Fig. 4.** Principal component analysis (PCA). Plot of physico-chemical parameters in
621 the water, and metal contents and biochemical determinations (green) in the gills
622 (blue) and in the digestive gland (red) of *V. decussata* clams, PC1 *vs* PC2, are
623 represented. Ellipses show 95% confidence intervals. Sites are represented as
624 follows: the Ellouza (●), and Bizerte (◆), North (■) and South (❋) Tunis, and
625 Boughrara (✖) lagoons. Biomarker abbreviations are as follows: AOPP, advanced
626 oxidation protein products; GPx, glutathione peroxidase; CAT, catalase; MDA,
627 Malondialdehyde; MTLP, metallothionein-like proteins; NPSH, non-protein
628 sulfhydryls; PCO, protein carbonyl levels; PP, protein phosphorylation; PRS,
629 protein redox state; SOD, superoxide dismutase; SPM, suspended particulate
630 matter; Vit C, vitamin C.

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632

References

Aebi, H., 1984. Catalase in vitro. Methods in Enzymology 105, 121-126.

Ahmad, I., Mohmood, I., Coelho, J.P., Pacheco, M., Santos, M.A., Duarte, A.C., Pereira, E., 2012. Role of non-enzymatic antioxidants on the bivalves' adaptation to environmental mercury: Organ-specificities and age effect in *Scrobicularia plana* inhabiting a contaminated lagoon. Environmental Pollution 163, 218-225.

Alhama, J., Fuentes-Almagro, C.A., Abril, N., Michán, C., 2018. Alterations in oxidative responses and post-translational modification caused by *p,p´*-DDE in *Mus spretus* testes reveal Cys oxidation status in proteins related to cell-redox homeostasis and male fertility. The Science of the Total Environment 636, 656-669.

Alhama, J., Michán, C., López-Barea, J., 2017. New Trends in Aquatic Pollution Monitoring: from Conventional Biomarkers to Environmental Proteomics, in: Gómez-Ariza, J.L, García-Barrera, T. (Ed.), Environmental Problems in Marine Biology: Methodological Aspects and Applications. Science Publishers (CRC Press/ Taylor & Francis Group).

Alves Martins, M.V., Zaaboub, N., Aleya, L., Frontalini, F., Pereira, E., Miranda, P., Mane, M., Rocha, F., Laut, L., El Bour, M., 2015. Environmental quality assessment of Bizerte Lagoon (Tunisia) using living foraminifera assemblages and a multiproxy approach. PloS one 10, e0137250.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquatic Toxicology (Amsterdam, Netherlands) 76, 160-202.

Annabi, A., Said, K., Messaoudi, I., 2013. Heavy metal levels in gonad and liver tissues-effects on the reproductive parameters of natural populations of *Aphanius facsiatus*. Environmental Science and Pollution Research International 20, 7309-7319.

Bald, J., Borja, A., Muxika, I., Franco, J., Valencia, V., 2005. Assessing reference conditions and physico-chemical status according to the European Water Framework Directive: a case-study from the Basque Country (Northern Spain). Marine Pollution Bulletin 50, 1508-1522.

Bancon-Montigny, C., Gonzalez, C., Delpoux, S., Avenzac, M., Spinelli, S., Mhadhbi, T., Mejri, K., Hlaili, A.S., Pringault, O., 2019. Seasonal changes of chemical contamination in coastal waters during sediment resuspension. Chemosphere 235, 651-661.

Barhoumi, B., Elbarhoumi, A., Clerandeau, C., Al-Rawabdeh, A.M., Atyaoui, A., Touil, S., Driss, M.R., Cachot, J., 2016. Using an integrated approach to assess the sediment quality of an Mediterranean lagoon, the Bizerte Lagoon (Tunisia). Ecotoxicology (London, England) 25, 1082-1104.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44, 276-287.

Bebianno, M.J., Geret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Romeo, M., 2004. Biomarkers in R*uditapes decussatus*: a potential bioindicator species. Biomarkers 9, 305-330.

Bejaoui, S., Bouziz, M., Ghribi, F., Chetoui, I., El Cafsi, M., 2020. Assessment of the biochemical and nutritional values of *Venerupis decussata* from Tunisian lagoons submitted to different anthropogenic ranks. Environmental Science and Pollution Research 27, 1734–1751.

Bejaoui, B., Solidoro, C., Harzallah, A., Chevalier, C., Chapelle, A., Zaaboub, N., Aleya, L., 2017. 3D modeling of phytoplankton seasonal variation and nutrient budget in a southern Mediterranean lagoon. Marine Pollution Bulletin 114, 962-976.

Ben Said, O., Goni-Urriza, M., El Bour, M., Aissa, P., Duran, R., 2010. Bacterial community structure of sediments of the Bizerte Lagoon (Tunisia), a southern Mediterranean coastal anthropized lagoon. Microbial Ecology 59, 445-456.

Bilgin, M., Uluturhan-Suzer, E., 2017. Assessment of trace metal concentrations and human health risk in clam (*Tapes decussatus*) and mussel (*Mytilus galloprovincialis*) from the Homa Lagoon (Eastern Aegean Sea). Environmental Science and Pollution Research International 24, 4174-4184.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Cabiscol, E., Ros, J., 2006. Oxidative damage to proteins: structural modifications and consequences in cell function, in: Dalle-Donne, I., Scaloni, A., Butterfield, D.A. (Eds.), Redox Proteomics: From Protein Modifications to Cellular Dysfunction and Diseases. John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp. 399-471.

Cabral, H., Fonseca, V., Sousa, T., Costa Leal, M., 2019. Synergistic Effects of Climate Change and Marine Pollution: An Overlooked Interaction in Coastal and Estuarine Areas. International Journal of Environmental Research and Public Health 16, 2737.

Carreira, S., Costa, P.M., Martins, M., Lobo, J., Costa, M.H., Caeiro, S., 2013. Ecotoxicological heterogeneity in transitional coastal habitats assessed through the integration of biomarkers and sediment-contamination profiles: a case study using a commercial clam. Archives of Environmental Contamination and Toxicology 64, 97-109.

Costa, P.M., Carreira, S., Costa, M.H., Caeiro, S., 2013. Development of histopathological indices in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality. Aquatic Toxicology (Amsterdam, Netherlands) 126, 442-454.

Cravo, A., Pereira, C., Gomes, T., Cardoso, C., Serafim, A., Almeida, C., Rocha, T., Lopes, B., Company, R., Medeiros, A., Norberto, R., Pereira, R., Araujo, O., Bebianno, M.J., 2012. A multibiomarker approach in the clam *Ruditapes decussatus* to assess the impact of pollution in the Ria Formosa Lagoon, South Coast of Portugal. Marine Environmental Research 75, 23-34.

Chalghmi, H., Bourdineaud, J.P., Chbani, I., Haouas, Z., Bouzid, S., Er-Raioui, H., Saidane-Mosbahi, D., 2019. Occurrence, sources and effects of polycyclic aromatic hydrocarbons in the Tunis Lagoon, Tunisia: an integrated approach using multi-level biological responses in *Ruditapes decussatus*. Environmental Science and Pollution Research International. https://doi.org/10.1007/s11356-019-04220-3.

Chalghmi, H., Bourdineaud, J.P., Haouas, Z., Gourves, P.Y., Zrafi, I., Saidane-Mosbahi, D., 2016a. Transcriptomic, biochemical, and histopathological responses of the clam *Ruditapes decussatus* from a metal-contaminated Tunis Lagoon. Archives of Environmental Contamination and Toxicology 70, 241-256.

Chalghmi, H., Zrafi, I., Gourves, P.Y., Bourdineaud, J.P., Saidane-Mosbahi, D., 2016b. Combined effects of metal contamination and abiotic parameters on biomarker responses in clam *Ruditapes decussatus* gills: an integrated approach in biomonitoring of Tunis Lagoon. Environmental Science 18, 895-907.

Cherkasov, A.S., Biswas, P.K., Ridings, D.M., Ringwood, A.H., Sokolova, I.M., 2006a. Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. The Journal of Experimental Biology 209, 1274-1284.

Cherkasov, A.S., Ringwood, A.H., Sokolova, I.M., 2006b. Combined effects of temperature acclimation and cadmium exposure on mitochondrial function in eastern oysters

728 *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). Environmental Toxicology and
729 Chemistry 25, 2461-2469.

730 Cherkasov, A.S., Taylor, C., Sokolova, I.M., 2010. Seasonal variation in mitochondrial
731 responses to cadmium and temperature in eastern oysters *Crassostrea virginica* (Gmelin)
732 from different latitudes. Aquatic Toxicology (Amsterdam, Netherlands) 97, 68-78.

733 Davies, M.J., 2005. The oxidative environment and protein damage. Biochimica et Biophysica
734 Acta 1703, 93-109.

735 Draper, H.H., Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation.
736 Methods in Enzymology 186, 421-431.

737 Eaton, P., 2006. Protein thiol oxidation in health and disease: techniques for measuring
738 disulfides and related modifications in complex protein mixtures. Free Radical Biology &
739 Medicine 40, 1889-1899.

740 Ellman, G.L., 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82, 70-
741 77.

742 Filgueiras, A.V., Lavilla, I., Bendicho, C., 2004. Evaluation of distribution, mobility and
743 binding behaviour of heavy metals in surficial sediments of Louro River (Galicia, Spain)
744 using chemometric analysis: a case study. The Science of the Total Environment 330, 115-
745 129.

746 Flohe, L., Gunzler, W.A., 1984. Assays of glutathione peroxidase. Methods in Enzymology
747 105, 114-121.

748 Funes, V., Alhama, J., Navas, J.I., López-Barea, J., Peinado, J., 2006. Ecotoxicological effects
749 of metal pollution in two mollusc species from the Spanish South Atlantic littoral.
750 Environmental Pollution 139, 214-223.

751 Gebicki, J.M., 2016. Oxidative stress, free radicals and protein peroxides. Archives of
752 Biochemistry and Biophysics 595, 33-39.

753 Ghedira, J., Chicano-Gálvez, E., Fernández-Cisnal, R., Jebali, J., Banni, M., Chouba, L.,
754 Boussetta, H., López-Barea, J., Alhama, J., 2016. Using environmental proteomics to
755 assess pollutant response of *Carcinus maenas* along the Tunisian coast. The Science of the
756 Total Environment 541, 109-118.

757 Ghedira, J., Jebali, J., Banni, M., Chouba, L., Boussetta, H., López-Barea, J., Alhama, J., 2011.
758 Use of oxidative stress biomarkers in *Carcinus maenas* to assess littoral zone
759 contamination in Tunisia. Aquatic Toxicology 14, 87-98.

760 Hellal Mel, A., Hellal, F., El Khemissi, Z., Jebali, R., Dachraoui, M., 2011. Trace metals in
761 algae and sediments from the North-Eastern Tunisian lagoons. Bulletin of Environmental
762 Contamination and Toxicology 86, 194-198.

763 Holmstrup, M., Bindesbol, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Kohler, H.R., Loureiro,
764 S., Soares, A.M., Ferreira, A.L., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E.,
765 Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of
766 environmental chemicals and natural stressors: a review. The Science of the Total
767 Environment 408, 3746-3762.

768 Ihaka, R., Gentleman, R., 1996. R: A language for data analysis and graphics. Journal of
769 Computational and Graphical Statistics 299-314.

770 Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Flores, E.M., Rocha, J.B., 2001. Diphenyl
771 diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and
772 brain of mice. Pharmacology & Toxicology 88, 119-125.

773 Kägi, J.H., 1991. Overview of metallothionein. Methods in Enzymology 205, 613-626.

774 Kalousova, M., Zima, T., Tesar, V., Dusilova-Sulkova, S., Skrha, J., 2005. Advanced
775 glycoxidation end products in chronic diseases-clinical chemistry and genetic background.
776 Mutation Research 579, 37-46.

777 Kayali, R., Cakatay, U., Akcay, T., Altug, T., 2006. Effect of alpha-lipoic acid supplementation
778 on markers of protein oxidation in post-mitotic tissues of ageing rat. Cell Biochemistry and
779 Function 24, 79-85.

780 Kessabi, K., Annabi, A., Navarro, A., Casado, M., Hwas, Z., Said, K., Messaoudi, I., Pina, B.,
781 2012. Structural and molecular analysis of pollution-linked deformities in a natural
782 *Aphanius fasciatus* (Valenciennes, 1821) population from the Tunisian coast. Journal of
783 Environmental Monitoring 14, 2254-2260.

784 Kharroubi, A., Gargouri, D., Baati, H., Azri, C., 2012. Assessment of sediment quality in the
785 Mediterranean Sea-Boughrara Lagoon exchange areas (southeastern Tunisia): GIS
786 approach-based chemometric methods. Environmental Monitoring and Assessment 184,
787 4001-4014.

788 Khedhri, I., Afli, A., Aleya, L., 2017. Structuring factors of the spatio-temporal variability of
789 macrozoobenthos assemblages in a southern Mediterranean lagoon: How useful for
790 bioindication is a multi-biotic indices approach? Marine Pollution Bulletin 114, 515-527.

791 Klomsiri, C., Karplus, P.A., Poole, L.B., 2011. Cysteine-based redox switches in enzymes.
792 Antioxidants & Redox Signaling 14, 1065-1077.

793 Lahbib, Y., Abidli, S., Trigui-El Menif, N., 2018. First assessment of the effectiveness of the
794 international convention on the control of harmful anti-fouling systems on ships in Tunisia
795 using imposex in *Hexaplex trunculus* as biomarker. Marine Pollution Bulletin 128, 17-23.

796 Lannig, G., Flores, J.F., Sokolova, I.M., 2006. Temperature-dependent stress response in
797 oysters, *Crassostrea virginica*: pollution reduces temperature tolerance in oysters. Aquatic
798 Toxicology (Amsterdam, Netherlands) 79, 278-287.

799 López-Barea, J., 1995. Biomarkers in ecotoxicology: an overview, in: Degen, G.H., Seiler, J.P.,
800 Bentely, P. (Eds.), Toxicology in Transition. Springer, Berlin, pp. 57-79.

801 Louiz, I., Palluel, O., Ben-Attia, M., Ait-Aissa, S., Hassine, O.K.B., 2018. Liver histopathology
802 and biochemical biomarkers in *Gobius niger* and *Zosterisessor ophiocephalus* from
803 polluted and non-polluted Tunisian lagoons (Southern Mediterranean Sea). Marine
804 Pollution Bulletin 128, 248-258.

805 Martoja, R., Martoja-Pierson, M., 1967. Initiation aux techniques de l'histologie animale.
806 Masson, Paris.

807 Mhadhbi, T., Pringault, O., Nouri, H., Spinelli, S., Beyrem, H., Gonzalez, C., 2019. Evaluating
808 polar pesticide pollution with a combined approach: a survey of agricultural practices and
809 POCIS passive samplers in a Tunisian lagoon watershed. Environmental Science and
810 Pollution Research International 26, 342-361.

811 Mubiana, V.K., Blust, R., 2007. Effects of temperature on scope for growth and accumulation
812 of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. Marine Environmental
813 Research 63, 219-235.

814 Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F., 2017. Indirect
815 effects of climate changes on cadmium bioavailability and biological effects in the
816 Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere 169, 493-502.

817 Nardi, A., Benedetti, M., d'Errico, G., Fattorini, D., Regoli, F., 2018. Effects of ocean warming
818 and acidification on accumulation and cellular responsiveness to cadmium in mussels
819 *Mytilus galloprovincialis*: Importance of the seasonal status. Aquatic Toxicology 204, 171-
820 179.

Ou, P., Wolff, S.P., 1996. A discontinuous method for catalase determination at 'near physiological' concentrations of H_2O_2 and its application to the study of H_2O_2 fluxes within cells. Journal of Biochemical and Biophysical Methods 31, 59-67.

Oueslati, W., Helali, M.A., Mensi, I., Bayaoui, M., Touati, H., Khadraoui, A., Zaabooub, N., Added, A., Aleya, L., 2018. How useful are geochemical and mineralogical indicators in assessing trace metal contamination and bioavailability in a post-restoration Mediterranean lagoon? Environmental Science and Pollution Research International 25, 25045-25059.

Parache, A., 1982. La Peche Maritime. La Palourde.

Park, H.R., Oh, R., Wagner, P., Panganiban, R., Lu, Q., 2017. New insights into cellular stress responses to environmental metal toxicants. International Review of Cell and Molecular Biology 331, 55-82.

Perceval, O., Couillard, Y., Pinel-Alloul, B., Giguère, A., Campbell, P.G.C., 2004. Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses. Aquatic Toxicology 69, 327-345.

Perceval, O., Couillard, Y., Pinel-Alloul, B., Campbell, P.G.C., 2006. Linking changes in subcellular cadmium distribution to growth and mortality rates in transplanted freshwater bivalves (*Pyganodon grandis*). Aquatic Toxicology 79, 87-98.

Petrovic, S., Ozretic, B., Krajnovic-Ozretic, M., Bobinac, D., 2001. Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. Marine Pollution Bulletin 42, 1373-1378.

Pompella, A., Visvikis, A., Paolicchi, A., De Tata, V., Casini, A.F., 2003. The changing faces of glutathione, a cellular protagonist. Biochemical Pharmacology 66, 1499-1503.

Rekik, A., Drira, Z., Guermazi, W., Elloumi, J., Maalej, S., Aleya, L., Ayadi, H., 2012. Impacts of an uncontrolled phosphogypsum dumpsite on summer distribution of phytoplankton, copepods and ciliates in relation to abiotic variables along the near-shore of the southwestern Mediterranean coast. Marine Pollution Bulletin 64, 336-346.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods in Enzymology 233, 357-363.

Richards, R.G., Chaloupka, M., 2009. Temperature-dependent bioaccumulation of copper in an estuarine oyster. The Science of the Total Environment 407, 5901-5906.

Roberts, R.A., Laskin, D.L., Smith, C.V., Robertson, F.M., Allen, E.M., Doorn, J.A., Slikker, W., 2009. Nitrative and oxidative stress in toxicology and disease. Toxicological Sciences 112, 4-16.

Rodríguez-Ortega, M.J., Alhama, J., Funes, V., Romero-Ruiz, A., Rodríguez-Ariza, A., López-Barea, J., 2002. Biochemical biomarkers of pollution in the clam *Chamaelea gallina* from south-Spanish littoral. Environmental Toxicology and Chemistry 21, 542-549.

Romero-Ruiz, A., Alhama, J., Blasco, J., Gómez-Ariza, J.L., López-Barea, J., 2008. New metallothionein assay in *Scrobicularia plana*: heating effect and correlation with other biomarkers. Environmental Pollution 156, 1340-1347.

Serafim, A., Bebianno, M.J., 2007. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. Environmental Toxicology and Chemistry 26, 960-969.

Sheehan, D., McDonagh, B., Bárcena, J.A., 2010. Redox proteomics. Expert Review of Proteomics 7, 1-4.

Sies, H., 1986. Biochemistry of oxidative stress. Angewandte Chemie International Edition in English 25, 1058-1071.

867 Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on
868 metabolism in aquatic ectotherms: implications of global climate change. Climate Research
869 37, 181-201.

870 Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical
871 Biology & Medicine 18, 321-336.

872 Triki, H.Z., Laabir, M., Lafabrie, C., Malouche, D., Bancon-Montigny, C., Gonzalez, C.,
873 Deidun, A., Pringault, O., Daly-Yahia, O.K., 2017. Do the levels of industrial pollutants
874 influence the distribution and abundance of dinoflagellate cysts in the recently-deposited
875 sediment of a Mediterranean coastal ecosystem? The Science of the Total Environment
876 595, 380-392.

877 Valko, M., Morris, H., Cronin, M.T., 2005. Metals, toxicity and oxidative stress. Current
878 Medicinal Chemistry 12, 1161-1208.

879 Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of
880 metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. The
881 American Journal of Physiology 277, R1612-1619.

882 Viarengo, A., Burlando, B., Ceratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneins:
883 a comparative overview. Cellular and Molecular Biology (Noisy-le-Grand, France) 46,
884 407-417.

885 Yan, L.J., Forster, M.J., 2011. Chemical probes for analysis of carbonylated proteins: a review.
886 Journal of Chromatography 879, 1308-1315.

887 Zaaboub, N., Helali, M.A., Martins, M.V., Ennouri, R., Bejaoui, B., da Silva, E.F., El Bour, M.,
888 Aleya, L., 2016. Assessing pollution in a Mediterranean lagoon using acid volatile sulfides
889 and estimations of simultaneously extracted metals. Environmental Science and Pollution
890 Research International 23, 21908-21919.

891 Zakhama-Sraieb, R., Zribi, I., Mnasri, I., Charfi-Cheikhrouha, F., 2019. A comparative study of
892 trace elements in *Cymodocea nodosa* from three semi-enclosed coastal areas in Tunisia.
893 Environmental Science and Pollution Research International 26, 10000-10012.

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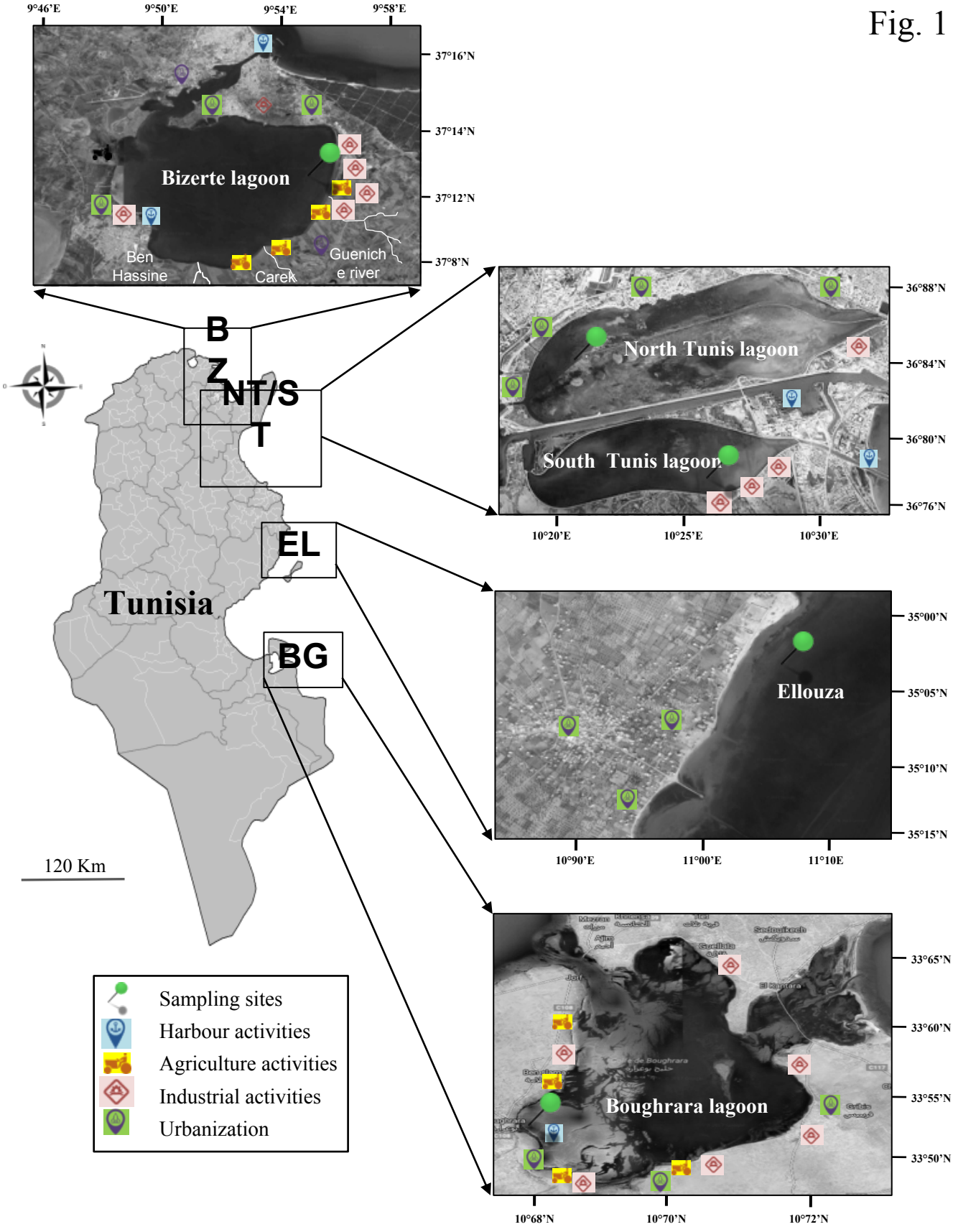
895

Fig. 1


9°46'E
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Bizerte lagoon
Ben Hassine
Carek
Guenich e river
B
Z
NT/S
T
EL
BG
Tunisia
120 Km
North Tunis lagoon
South Tunis lagoon
36°88'N
36°84'N
36°80'N
36°76'N
10°20'E
10°25'E
10°30'E
Ellouza
35°00'N
35°05'N
35°10'N
35°15'N
10°90'E
11°00'E
11°10E
Boughrara lagoon
33°65'N
33°60'N
33°55'N
33°50'N
10°68'N
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10°72'N
Sampling sites
Harbour activities
Agriculture activities
Industrial activities
Urbanization

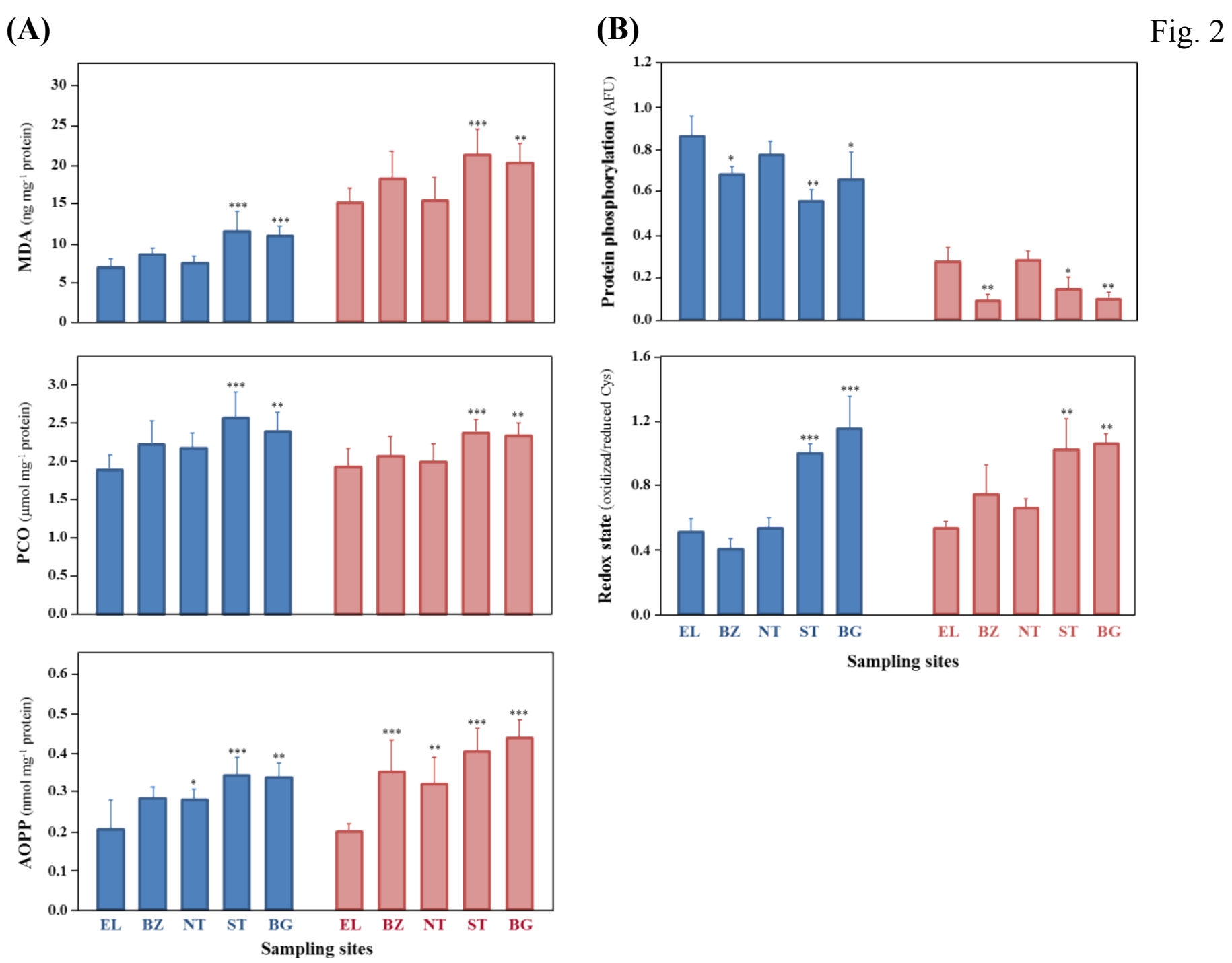
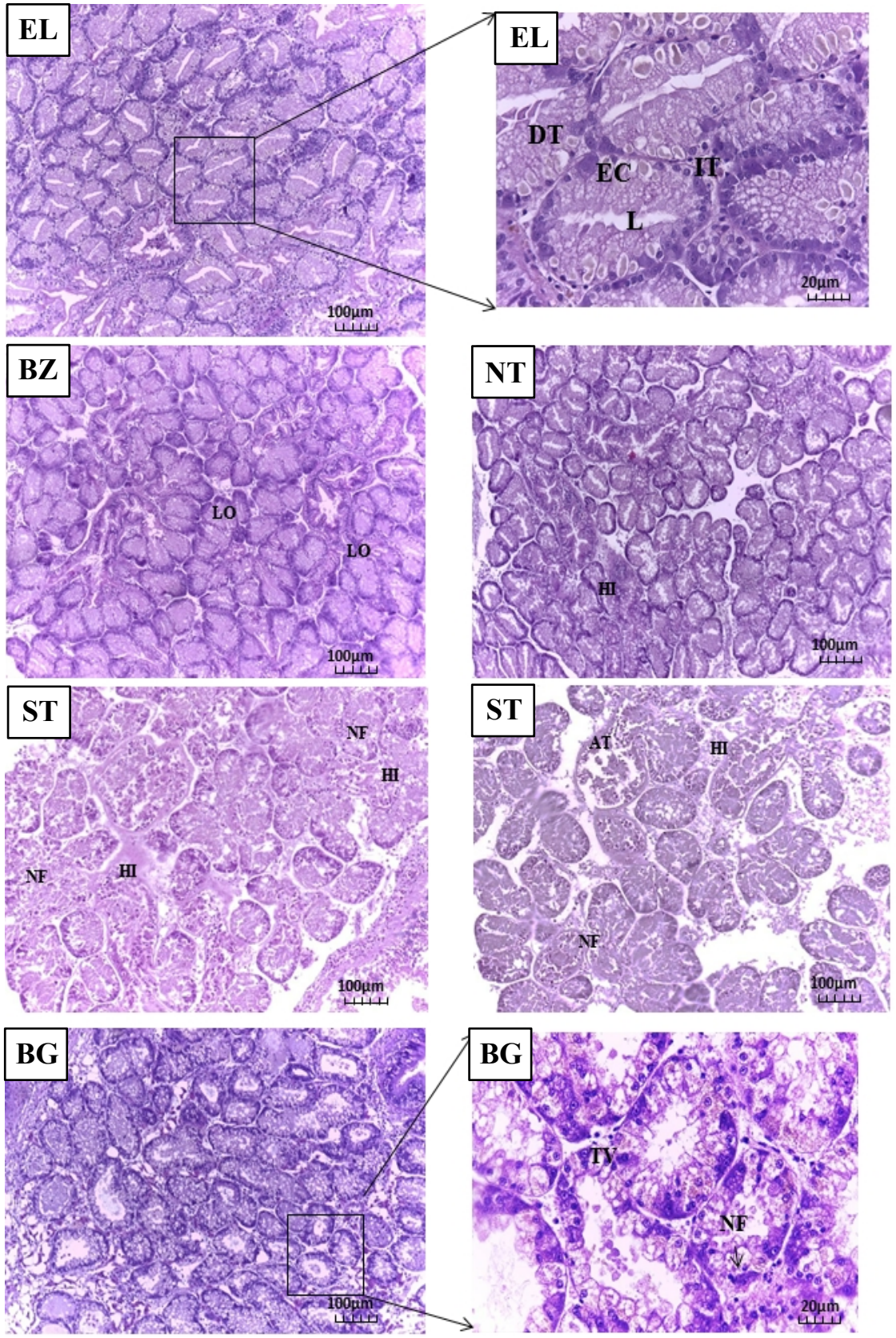

(A)
(B)
Fig. 2
MDA (ng mg-1 protein)
PCO (μmol mg-1 protein)
AOPP (nmol mg-1 protein)
Protein phosphorylation (AFU)
Redox state (oxidized/reduced Cys)
EL BZ NT ST BG EL BZ NT ST BG
Sampling sites

Fig. 3

(A)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(B)

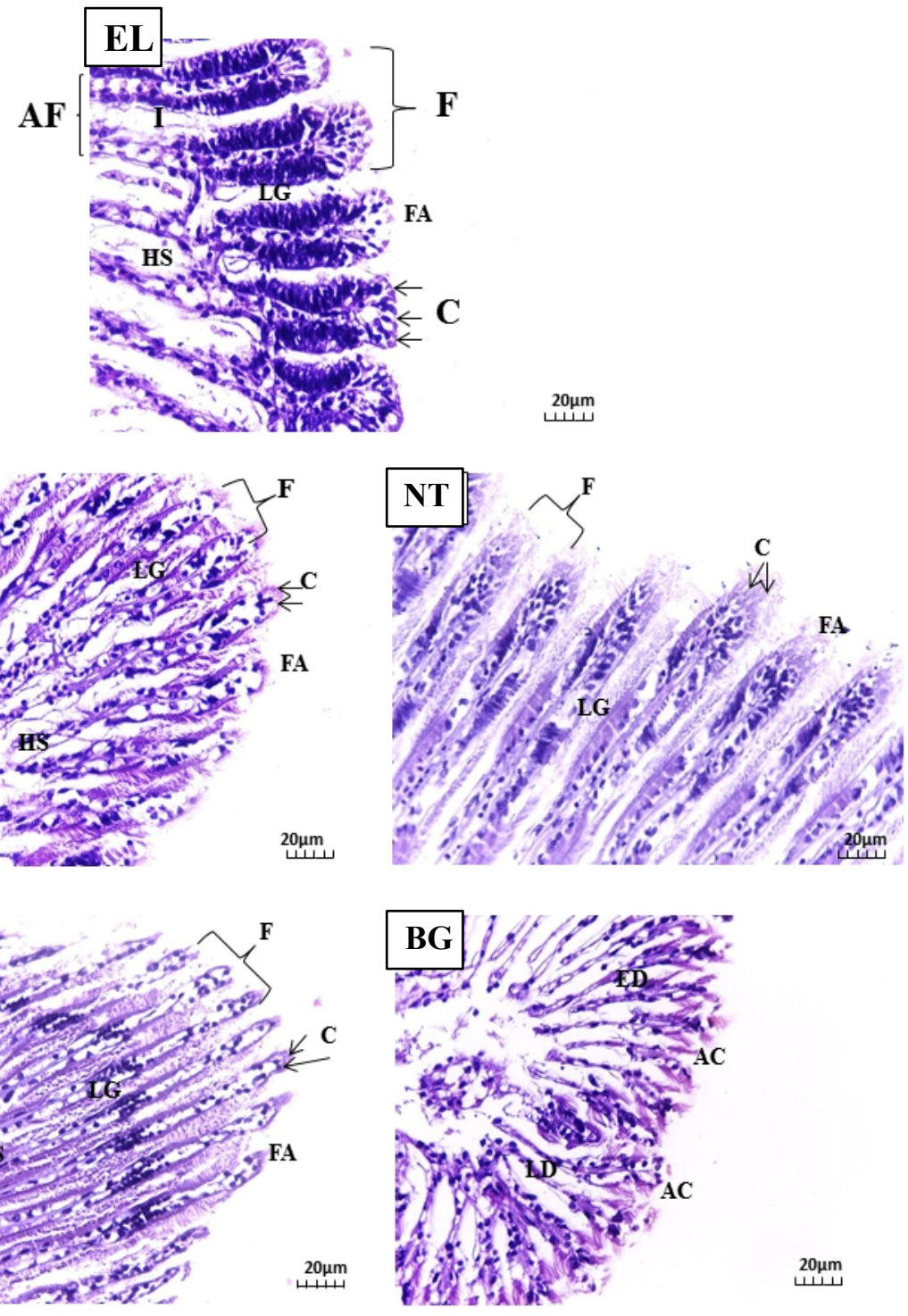
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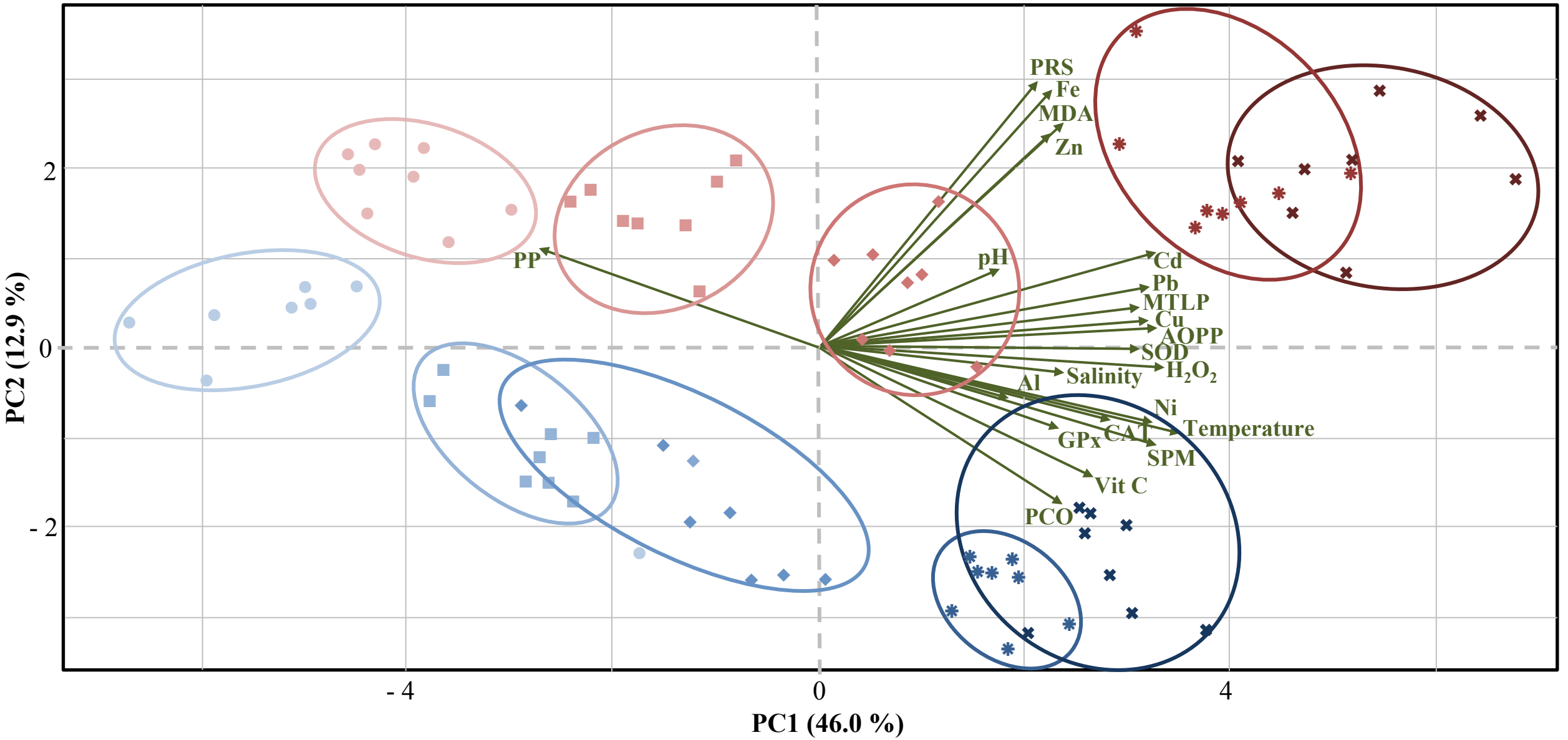
Fig. 4


PRS
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SPM
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- 2
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0
4
PC2 (12.9 %)
PC1 (46.0 %)

Table 1. Physico-chemical parameters determined in water at the different studied locations along the Tunisian coast.

Sites[a]	Temperature (°C)	Salinity (psu)	pH	Suspended Particulate Matter (mg L^{-1})
EL	23.36 ± 2.82[b]	37.91 ± 2.10	8.15 ± 0.02	112.51 ± 6.78
BZ	28.06 ± 2.12	37.33 ± 1.63	8.00 ± 0.03	**200.45 ± 14.36*****[c]
NT	26.41 ± 1.53	37.22 ± 2.62	8.01 ± 0.01	**163.22 ± 10.32*****
ST	**29.17 ± 2.05***	38.01 ± 1.11	8.10 ± 0.02	**198.66 ± 11.75*****
BG	**31.47 ± 1.99****	39.11 ± 1.39	8.00 ± 0.11	**231.22 ± 9.47*****

[a] Sampling sites along the Tunisian coast are as follows: Ellouze (EL) and Bizerte (BZ), North (NT) and South Tunis (ST), and Boughrara (BG) lagoons.

[b] Results represent the mean ± SD from the analysis of three independent samples. Determinations were recorded during February 2017.

[c] Statistical significance was compared with the values at EL, the reference site: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant results are highlighted in bold.

Table 2. Trace metals concentrations in the gills and the digestive gland of *Venerupis decussata* clams from different locations along the Tunisian coast.

Tissues	Sites[a]	Metals (µg g^{-1} dry weight)						
		Cd	Pb	Cu	Zn	Fe	Al	Ni
Gills	EL	0.85 ± 0.09[b]	0.99 ± 0.17	4.10 ± 1.11	25.73 ± 5.35	367.39 ± 7.41	19.84 ± 0.50	0.32 ± 0.05
	BZ	0.92 ± 0.12	**1.29 ± 0.10***	4.89 ± 0.83	30.51 ± 2.52	369.35 ± 6.22	21.47 ± 2.88	**0.44 ± 0.03***
	NT	0.90 ± 0.18	1.38 ± 0.27	4.69 ± 1.04	30.83 ± 3.95	345.27 ± 6.94	21.32 ± 2.76	**0.59 ± 0.03*****
	ST	**1.26 ± 0.18****[c]	**1.68 ± 0.17*****	**6.87 ± 1.02*****	32.02 ± 6.88	373.74 ± 10.97	**23.83 ± 1.43*****	**0.77 ± 0.10*****
	BG	**1.42 ± 0.25*****	**1.91 ± 0.21*****	**8.77 ± 1.13*****	34.19 ± 6.95	377.37 ± 7.32	**22.49 ± 0.51***	**1.06 ± 0.10*****
Digestive gland	EL	0.87 ± 0.10	1.13 ± 0.33	5.39 ± 0.97	36.08 ± 7.49	395.92 ± 15.03	16.90 ± 4.25	0.42 ± 0.07
	BZ	1.03 ± 0.10	**1.66 ± 0.46***	5.54 ± 0.95	36.52 ± 7.02	406.36 ± 12.66	20.96 ± 4.16	0.54 ± 0.09
	NT	1.01 ± 0.07	**1.82 ± 0.31****	6.31 ± 0.65	37.17 ± 7.33	402.92 ± 8 .97	20.25 ± 3.98	0.58 ± 0.12
	ST	**1.32 ± 0.10****	**2.33 ± 0.31*****	**8.42 ± 1.06*****	**50.44 ± 6.36****	**433.83 ± 13.51*****	21.87 ± 4.25	**0.82 ± 0 .18*****
	BG	**1.87 ± 0.38*****	**2.31 ± 0.32*****	**8.13 ± 1.17*****	**50.08 ± 6.23****	**435.22 ± 13.07*****	22.99 ± 4.32	**0.84 ± 0.09*****

[a] Sampling site along the Tunisian coast are as follows: Ellouze (EL) and Bizerte (BZ), North (NT) and South Tunis (ST), and Boughrara (BG) lagoons.

[b] Results represent the mean ± SD of six independent replicates of twenty pooled clams.

[c] Statistical significance was compared with the values at EL, the reference site: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant results are highlighted in bold.

Table 3. Hydrogen peroxide levels and antioxidant responses in the gills and in the digestive gland of *V. decussata* clams from different locations along the Tunisian coast.

Tissues	Sites[a]	H_2O_2 (µmol mg^{-1})	Enzymatic antioxidants			Non-enzymatic antioxidants		
			CAT[b] (U mg^{-1})	GPx (U mg^{-1})	SOD (U mg^{-1})	MTLPs (µmol g^{-1})	NPSH (µmol mg^{-1})	Vit C (nmol mg^{-1})
Gills	EL	1.08 ± 0.37[c]	10.68 ± 1.82	33.57 ± 5.71	14.08 ± 4.52	0.23 ± 0.08	0.23 ± 0.06	11.27 ± 2.95
	BZ	1.54 ± 0.18**[d]	13.13 ± 2.21	**38.58 ± 1.72***	16.43 ± 2.10	0.34 ± 0.10	0.30 ± 0.06	13.42 ± 1.54
	NT	1.15 ± 0.15	10.95 ± 2.83	37.77 ± 1.90	14.07 ± 1.27	0.28 ± 0.026	0.26 ± 0.07	12.37 ± 1.96
	ST	**1.73 ± 0.18***	**18.05 ± 3.58****	**44.75 ± 2.14*****	**19.21 ± 2.47****	**0.40 ± 0.07****	**0.35 ± 0.08***	**15.69 ± 1.77*****
	BG	**1.74 ± 0.10***	**17.74 ± 3.20****	**43.26 ± 2.26*****	**18.82 ± 0.88****	**0.44 ± 0.09****	0.32 ± 0.07	**14.44 ± 1.72***
Digestive gland	EL	1.30 ± 0.08	10.30 ± 1.65	28.35 ± 2.76	15.74 ± 3.34	0.31 ± 0.05	0.15 ± 0.02	11.39 ± 2.06
	BZ	**1.69 ± 0.14***	**17.03 ± 4.18****	28.17 ± 3.53	18.60 ± 1.81	**0.44 ± 0.07****	0.17 ± 0.01	13.05 ± 0.98
	NT	1.46 ± 0.17	11.15 ± 2.85	27.27 ± 4.95	15.80 ± 2.88	0.36 ± 0.06	0.15 ± 0.01	11.39 ± 1.51
	ST	**1.84 ± 0.27*****	**17.18 ± 1.83****	**41.46 ± 8,99*****	**21.22 ± 1.07*****	**0.48 ± 0.08*****	**0.24 ± 0.03*****	**14.88 ± 1.26*****
	BG	**1.92 ± 0.08*****	**17.73 ± 2.53*****	**52.07 ± 4.95*****	**20.11 ± 1.66****	**0.47 ± 0.06*****	**0.21 ± 0.01***	**14.27 ± 1.10****

[a] Sampling site along the Tunisian coast are as follows: Ellouze (EL) and Bizerte (BZ), North (NT) and South Tunis (ST), and Boughrara (BG) lagoons.

[b] Abbreviations of the different antioxidants are as follows: Catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), metallothionein-like proteins (MTLPs), non-protein sulfhydryls (NPSH), and ascorbic acid (Vitamin C, Vit C).

[c] Results are given as the mean ± SD of six (H_2O_2) or eight (antioxidant responses) replicates of twenty pooled clams.

[d] Statistical differences between sites, compared to EL, are represented as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant results are highlighted in bold.

Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author statement:

Safa Bejaoui: Conceptualization, Investigation, Resources, Writing, Funding acquisition; **Carmen Michán:** Conceptualization, Formal analysis, Resources, Writing, Visualization, Supervision; **Khaoula Telahigue:** Investigation; **Salwa Nechi:** Investigation; **Mhamed el Cafsi:** Supervision, Funding acquisition; **Nejla Soudani:** Investigation; **Julián Blasco:** Writing, Funding acquisition; **Pedro M. Costa:** Writing, supervision, Funding acquisition; **José Alhama:** Conceptualization, Formal analysis, Resources, Writing, Visualization, Supervision, Funding acquisition

Supplementary Table 1. Biometric parameters determined in *Venerupis decussata* clams collected at different locations along the Tunisian littoral zone.

Sites[a]	Shell length (mm)	Shell width (mm)	Weight[b] (g)
EL	39.5 ± 2.5	16.3 ± 2.0	13.3 ± 2.9
BZ	40.2 ± 1.5	17.9 ± 0.5	13.6 ± 1.7
NT	42.2 ± 1.0	16.6 ± 1.2	12.9 ± 2.4
ST	41.2 ± 2.0	18.3 ± 0.4	12.7 ± 0.5
BG	43.3 ± 1.3	19.3 ± 1.1	14.2 ± 1.8

[a] Sampling sites along the Tunisian coast are as follows: Ellouze (EL) and Bizerte (BZ), North (NT) and South Tunis (ST), and Boughrara (BG) lagoons.

[b] Weight values of the whole clams before dissection.

Forty clams were analyzed for each location. Statistical differences among clams from the different locations and those at EL reference site were not significant.

Supplementary Table 2. Spearman correlation coefficients between the physico-chemical parameters determined in water, and metal concentrations and biochemical determinations measured in clam tissues (digestive gland and gills) along the Tunisian littoral zone.

	Temperature	Salinity	pH	SPM	Cd	Pb	Cu	Zn	Fe	Al	Ni	H_2O_2	CAT	GPx	SOD	MTLP	NPSH	Vit C	MDA	PCO	AOPP	PP	PRS
Temperature	1.000																						
Salinity	**0.700 ***	1.000																					
pH	-0.616	0.051	1.000																				
SPM	**0.900 ****	0.500	**-0.872 ****	1.000																			
Cd	**0.960 *****	**0.665 ***	-0.568	**0.837 ****	1.000																		
Pb	**0.812 ****	0.492	-0.391	0.615	**0.915 *****	1.000																	
Cu	**0.837 ****	**0.689 ***	-0.328	0.640	**0.915 *****	**0.903 *****	1.000																
Zn	0.419	0.271	-0.164	0.295	0.636	**0.782 ***	**0.697 ***	1.000															
Fe	0.468	0.394	-0.253	0.419	**0.661 ***	**0.697 ***	**0.673 ***	**0.939 *****	1.000														
Al	**0.911 *****	0.615	-0.442	**0.739 ***	**0.818 ****	**0.673 ***	**0.661 ***	0.188	0.200	1.000													
Ni	**0.886 ****	0.591	-0.455	**0.689 ***	**0.903 *****	**0.891 ****	**0.855 ****	0.503	0.406	**0.818 ****	1.000												
H_2O_2	**0.935 *****	**0.714 ***	-0.492	**0.812 ****	**0.964 ******	**0.867 ****	**0.891 ****	**0.661 ***	**0.721 ***	**0.794 ****	**0.806 ****	1.000											
CAT	**0.935 *****	0.640	-0.480	**0.788 ***	**0.891 ****	**0.733 ***	**0.794 ****	0.309	0.358	**0.927 *****	**0.806 ****	**0.855 ****	1.000										
GPx	**0.788 ***	**0.788 ***	-0.227	0.591	**0.661 ***	0.491	0.527	0.055	0.103	**0.891 ****	**0.697 ***	**0.684 ***	**0.733 ***	1.000									
SOD	**0.862 ****	**0.714 ***	-0.316	**0.689 ***	**0.891 ****	**0.818 ****	**0.855 ****	0.636	**0.709 ***	**0.758 ***	**0.697 ***	**0.964 ******	**0.842 ****	**0.648 ***	1.000								
MTLP	**0.840 ****	0.580	-0.456	**0.728 ***	**0.924 *****	**0.900 *****	**0.888 ****	**0.796 ****	**0.827 ****	0.638	**0.748 ***	**0.954 ******	**0.766 ***	0.468	**0.936 *****	1.000							
NPSH	0.506	0.346	-0.177	0.383	0.279	0.134	0.209	-0.460	-0.474	**0.711 ***	0.426	0.255	0.596	**0.687 ***	0.267	0.079	1.000						
Vit C	**0.877 ****	0.617	-0.329	**0.679 ***	**0.802 ****	**0.693 ***	**0.748 ***	0.310	0.304	**0.918 *****	**0.766 ***	**0.839 ****	**0.912 *****	**0.784 ****	**0.851 ****	**0.744 ***	**0.671 ***	1.000					
MDA	0.443	0.295	-0.177	0.345	0.636	**0.721 ***	**0.661 ***	**0.964 ******	**0.964 ******	0.236	0.418	**0.709 ***	0.370	0.091	**0.721 ***	**0.833 ****	-0.413	0.389	1.000				
PCO	**0.886 ****	0.591	-0.379	**0.689 ***	**0.794 ****	**0.685 ***	**0.733 ***	0.212	0.176	**0.952 *****	**0.830 ****	**0.782 ***	**0.915 *****	**0.818 ****	**0.758 ***	**0.657 ***	**0.754 ***	**0.973 ******	0.248	1.000			
AOPP	**0.837 ****	0.492	-0.505	**0.739 ***	**0.903 *****	**0.855 ****	**0.745 ***	**0.685 ***	**0.733 ***	**0.721 ***	**0.721 ***	**0.903 *****	**0.806 ****	0.515	**0.891 ****	**0.930 *****	0.140	**0.723 ***	**0.745 ***	**0.648 ***	1.000		
PP	-0.369	-0.246	0.253	-0.369	-0.552	-0.539	-0.539	**-0.867 ****	**-0.927 *****	-0.139	-0.285	-0.624	-0.309	0.006	-0.612	**-0.748 ***	0.486	-0.292	**-0.939 *****	-0.139	**-0.673 ***	1.000	
PRS	**0.812 ****	**0.739 ***	-0.303	0.615	**0.903 *****	**0.855 ****	**0.939 *****	**0.673 ***	**0.661 ***	0.636	**0.867 ****	**0.842 ****	**0.770 ***	0.539	**0.782 ***	**0.845 ****	0.146	**0.669 ***	0.624	**0.661 ***	**0.758 ***	-0.567	1.000

Number in bold indicate significant correlations (red, positive; green, negative). Asterisks indicate the signification of the correlations: *, $p < 0.0332$; **, $p < 0.0021$; ***, $p < 0.0002$; ****, $p < 0.0001$.

Abbreviations are as follows: Suspended particulate matter (SPM), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), metallothionein-like proteins (MTLP), non-protein sulfhydryls (NPSH), vitamin C (Vit C), malondialdehyde (MDA), protein carbonyl levels (PCO), advanced oxidation protein products (AOPP), protein phosphorylation (PP) and protein redox state (PRS).