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Monitoring of oxidative stress and immunotoxic responses in clams (*Ruditapes decussatus*) reared in the Tunisian north coast

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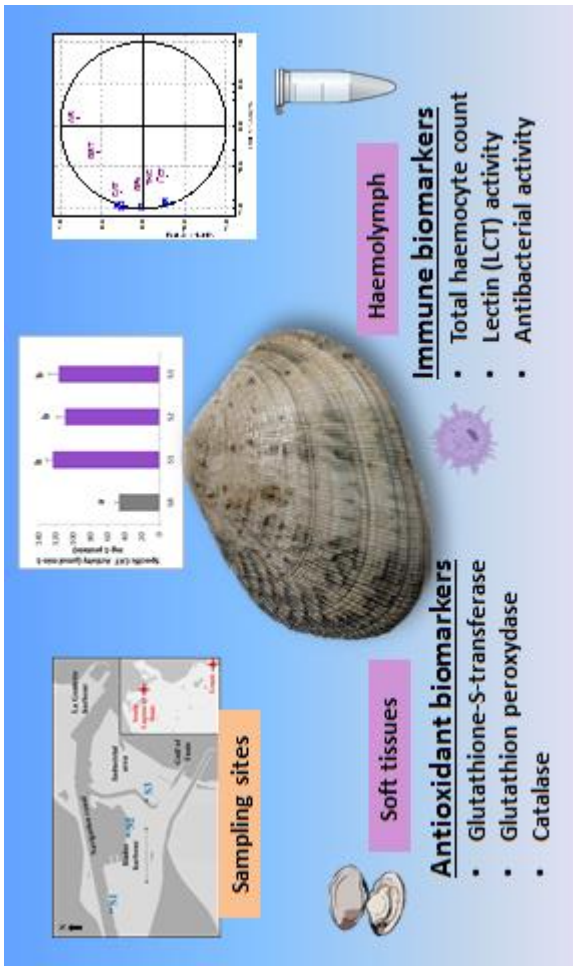
Graphical Abstract



Abstract

The aim of this study was to validate immunotoxic and oxidative stress responses as ecotoxicological biomarkers in the carpet shell clams (*Ruditapes decussatus*) to detect and monitor biological effects of anthropogenic pollution in the South Lagoon of Tunis (Tunisia). Clams were collected from four sites: three of them were located within the polluted lagoon of Tunis (S1, S2 and S3) and another one was allocated in a clean site on the Mediterranean coast (SR). Oxidative and immune status of clams was evaluated through the analysis of glutathione S-transferases, glutathione-peroxidase, catalase, lectin and antibacterial activities and total haemocyte count. Our results revealed activation of antioxidant enzymes and immune alteration in clams sampled from contaminated sites. Overall, the current study clearly showed that affected biomarkers could be useful tools for biomonitoring in the study area.

Keywords: *Ruditapes decussatu*; Biomarkers; Biomonitoring; Tunisian north coast

1. Introduction

Biomarker is a biochemical, cellular, physiological or behavioral change which can be measured in tissue, body fluid or at the level of the whole organism and that reveals the exposure at/or the effects of one or more chemical pollutants (Depledge 1994). Biomarkers have been suggested as practical tools for environmental management for a number of decades. Biomarkers, particularly those detected at low levels of biological organization, are generally early and sensitive indices of chemical stress. Biomarkers are subdivided into biomarkers of defense that allow organisms to cope with the presence of contaminants and biomarkers of damage that reveal deleterious effects (de Lafontaine *et al*. 2000).

Among benthic invertebrates, several species of bivalve molluscs such as clams, mussels and oysters have been introduced as biological models for research in ecotoxicology. As sedentary filter-feeders, bivalves are able to accumulate many chemicals in their tissues (Fournier *et al*. 2002, Bebianno *et al*. 2003, Sandrini-Neto *et al*. 2016).

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. Exposure of aquatic organisms to pollutants can induce an increase in the production of reactive oxygen species (ROS) (Chakraborty *et al*. 2013, Aguirre-Martínez, and Martín-Díaz 2020).

Aerobic organisms have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants that are usually effective in blocking harmful effects of ROS. The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione transferase (GST) and glutathione reductase (GR). The antioxidant systems can be induced or inhibited under stress conditions. Thereby, the assessment of antioxidant enzymes can provide information on the organism's health status and could be used as a biomarker of pollutant-induced oxidative stress in aquatic organisms (Borković *et al.* 2008). In recent years, the antioxidant system response has been widely studied and employed as a defense biomarker in aquatic organisms (Regoli *et al.* 2011).

Under normal conditions the immune system of molluscs maintains efficient protection against most microbial or parasitic attacks. However, many chemical contaminants may induce immunological disorders, even at low concentration (Ahmad *et al.* 2011, Chakraborty *et al*. 2013, Ray *et al.* 2013, Hannam *et al.* 2010). In this context, immune-biomarkers have been proposed to be sensitive tools in eco-immunology studies to detect signs of impaired bivalve health (Matozzo *et al.* 2013, Auffret *et al.* 2006, Cotou *et al.* 2013). However, the use of immune biomarkers is not widespread in monitoring studies.

The main goal of this study was to assess the impact of pollution in the Southern Lagoon of Tunis on the health state of the carpet shell clam *Ruditapes decussatus*. For this reason, clams collected from three sites of the Lagoon: the navigation canal, the Rades harbor and the chemical industrial area. Control samples were collected from Louza beach. Oxidative and immune status of clams was evaluated through the analysis of glutathione S- transferases, glutathione-peroxidase, catalase, lectin and antibacterial activities and total haemocyte count.

2. Material and methods

2.1. Indicator organism and selected areas

The Mediterranean clam *Ruditapes decussatus* (Family: Veneridae), *is* a common species, indigeneous for the Mediterranean. It is an important component of marine infaunal communities (Velez *et al.* 2017). The harvest of this species mainly occurs in the Atlantic coasts of France, Spain, Portugal and Ireland, and in the Mediterranean basin, where it is considered as an economically important bivalve (Velez *et al.* 2017). Due to its ecological and economic interest, this species is commonly used as sentinel in ecotoxicological investigations. In order to minimize the effects of the reproductive cycle which can influence the biochemical and immunological biomarkers responses, clams were sampled after their spawning period (February) which occurs irregularly from June to December (Hamida *et al.* 2004).

Clams were collected during February from a reference site (SR) and three polluted sites (S1-S3) geographically located near contamination sources and differently influenced by anthropogenic impact **(Fig. 1)**. The reference site is located in Louza beach (rural area) which has been considered as a reference site in monitoring programs along the Tunisian coasts (Banni *et al.* 2009) with low concentration of trace metals (Mansour *et al.* 2020a, Chalghmi *et al.* 2016a, b) and hydrocarbons (Mansour *et al.* 2020b) in bivalves and surface sediments. The thee polluted sites are located in the Lagoon of Tunis that is adversely affected by industrial contaminants from the industrial area, the important harbor activities (La Goulette, Tunis and Rades harbors) and the urban untreated sewage from the city of Tunis and its southern suburbs (Jouini *et al.* 2005). This lagoon is considered as a mesotrophic ecosystem due to the richness of organic matter (Charrada 1992, Jouini *et al.* 2005). Moreover, it has been reported a low water mass renewal/turnover rates (i.e. long residence times) in the lagoon (Jouini *et al.* 2005) not observed in the reference site. High levels of polycyclic aromatic hydrocarbons (Chalghmi *et al.* 2020) and trace metals such as cadmium, lead, mercury, zinc and nickel, among others, have been found in the lagoon sediments (Hellal *et al.* 2011, Chalghmi *et al.* 2016b).

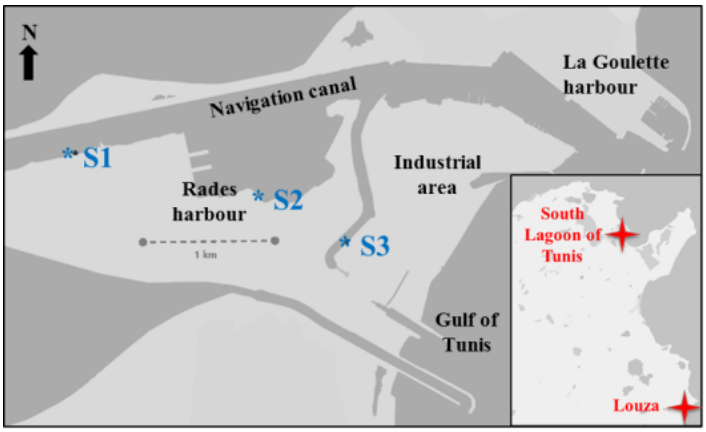


Fig. 1. The location of the stations in Tunis lagoon and Louza beach (Tunisia).

2.2. Samples preparation

After sampling, clams were immediately transported to the laboratory and maintained in aquaria filled with aerated sea water from each sampling sites for 24 h. Temperature was kept at 13±1 °C in order to coincide with the temperature at the sampling sites (Reference and polluted sites). The next day, the whole soft body of 20 clams for each site was separated and kept at -20 °C until biochemical

biomarkers analysis. For immune biomarker analysis, 1 mL of haemolymph were drawn from the anterior adductor muscle of 20 clams with sterile syringes and kept on ice. A volume of 25 µL of haemolymph was kept at 4 °C in order to determine the total haemocyte count (THC). To measure enzymatic activities, the haemolymph samples were centrifuged (780 x g, 10 min at 4 °C) and the supernatants, corresponding to cell-free haemolymph, were collected and stored at -20 °C until analysis.

2.3. Antioxidant biomarkers

Frozen soft tissues were thawed and homogenized in a motor-driven glass-teflon homogenizer at 500 rpm with TRIS buffer (TRIS 50 mM, NaCl 150 mM, DTT 1mM, protease inhibitor cocktail pH 7.4). Homogenates were centrifuged for 25 min at 9000g at 4°C and supernatants (called S9 fractions) were immediately collected for enzymatic activity determinations. All measurements were performed at 4°C to prevent enzyme or tissue degradation. Each measurement was carried out on 20 individual clams and each measurement was performed in triplicate. Total protein concentration in S9 fractions and haemolymph samples was measured spectrophotometrically by the Bradford method (Bradford 1976), adapted for microplate reader at 595 nm using bovine serum albumin (BSA) as a standard. Glutathione-S-transferase (GST) activity assay was determined as described by Habig *et al.* (Habig *et al.* 1974). The conjugation of reduced glutathione (GSH) with 1-Chloro-2,4-dinitrobenzene (CDNB) was followed spectrophotometrically at 340 nm. The enzymatic activity was expressed in nmol of substrate conjugated min^{-1} mg^{-1} protein. Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (Buege and Aust 1978). Glutathion peroxydase (GPx) activity assay was performed according to Lawrence and Burk (Lawrence and Burk 1985) adapted for microplate reader. GPx activity was determined by measuring the decrease of absorbance at 340 nm. The reaction consists in the reduction of oxidized glutathione linked to the oxidation of NADPH in the presence of excess glutathione reductase. Cumene hydroperoxide was used as substrates. The enzymatic activities were expressed in $nmol.min^{-1}$ mg^{-1} protein. Catalase (CAT) activity was measured based on the method adapted from Clairbone (Clairbone 1985). CAT activity was determined by measuring the decrease of absorbance at 240 nm due to the presence of H_2O_2 concentration using a microplate reader. The enzymatic activity was expressed in µmol of H_2O_2 min^{-1} mg^{-1} protein.

2.4. Immune biomarkers

The cellular innate immunity was assessed by calculating the number of free haemocytes in the hemolymph. Briefly, a volume of 25 mL of haemolymph was mixed with the same volume of formol in order to prevent cell clotting and the total haemocyte count (THC) was performed using a Malassez cell counter. Then, THC was expressed as the number of haemocytes (x 10^6) mL^{-1} in the haemolymph. The humoral innate immunity was assessed with lectin and antibacterial activities. Lectin (LCT) activity in haemolymph samples was determined by the reaction of hemagglutination (HA) following the method described by Ordás *et al.* (Ordás *et al.* 2000). This activity was measured by adding 25 µL of 3% human blood to 25 µL of serially diluted haemolymph in 96-well plates. After mixing, plates were kept at room temperature for 2 h. Agglutination was determined by the presence or the absence of a button of blood on the bottom of the well. The agglutination title (inverse of the highest haemolymph dilution factor) was recorded and expressed as its $\log_2$. All samples were run in duplicate. Antibacterial activity was determined according to the method described by Ordás *et al.* (Ordás *et al.* 2000) modified by Mansour *et al.* (Mansour *et al.* 2017). Aliquots of 25 µL of haemolymph were mixed with75 µL of a suspension of *Escherichia coli* (ATCC35218) (10^8 cell mL^{-1} in Tryptone soy broth (TSB, Sigma)) in a 96 well plate. In the control, the haemolymph was substituted by TSB. After

incubation for 3 h at 18 °C, 100 µL of 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (0.5 mg mL^{-1} in TSB) were added to each well. After 15 min in the dark at 18 °C, the absorbance at 600 nm was measured. All samples were run in triplicate. The antibacterial activity index (BI) was calculated as follows:

BI=sample ABS_{600}/control ABS_{600}

2.5. Statistical analysis

All our results were expressed as mean ± standard error. Data were statistically analysed using a one-way analysis of variance (ANOVA). Significant differences were determined at the $p < 0.05$ level using Tukey test. Principal component analysis (PCA) was used to find correlations between the different biomarkers. Statistical analysis was performed using the software STATISTICA (Statsoft STATISTICA version 6.1.478.0).

3. Results and discussion

3.1. Antioxidant biomarkers

Biochemical biomarker responses are shown in **Figure 2**. Glutathione S- transferases (GST) represent a major group of the phase II of biotransformation playing an important role as indirect antioxidants usually used as a biomarker of pollution by persistent organic pollutants (POPs), mainly organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), trace metals (Marques *et al.* 2018, Jiang *et al.* 2019). Overall GST activity **(Fig. 2A)** was significantly higher (ANOVA, p=0.006) in clams collected at S1 (537.69 ± 37.16 nmol min^{-1} mg^{-1} protein) compared to clams sampled from SR (355.24 ± 48.98 nmol min^{-1} mg^{-1} protein). No significant difference was found between clams sampled from the reference site and S2 or S3. In previous studies, Chalghmi *et al*. (Chalghmi *et al*. 2016a, b) reported high levels of GST activity in clams living in the sites of the southern lagoon of Tunis, with the highest level of GST in organisms sampled in the Canal (or S1). These results are in line with ours. The results of the present study confirm contamination of the Tunis lagoon.

GPx activity **(Fig. 2B)** was significantly higher in clams sampled from the three polluted sites (S1, S2 and S3) compared to clams sampled from SR (ANOVA, p=0.004, p=0.016 and p=0.037, respectively). The highest GPx levels were observed in clams from the site S1 compared to the site SR (6.63 ± 0.71 nmol min^{-1} mg^{-1} protein). These results are in agreement with previous studies in clams *Ruditapes decussatus*, in which the GPx activity was higher in the gills of clams collected from Bizert lagoon compared to those collected from Louza (Bejaoui *et al*. 2020). In a recent study, no effect by local pollution was observed on GPx activities measured in Green-lipped mussels *Perna viridis* and Manila clams, *Ruditapes philippinarum*, transplanted in different polluted sites at Hong Kong (De Luca-Abbott *et al*. 2005). According to these studies, antioxidant responses may differ between species.

Overall CAT activity **(Fig. 2C)** was significantly higher (ANOVA, $p < 0.05$) in clams sampled at the three sites of Tunis lagoon (S1, S2 and S3) compared to clams sampled from the reference site (SR). The highest CAT levels were observed in clams collected at site S1 (123.9 ± 7.71 µmol min^{-1} mg^{-1} protein) compared to site SR (46.71 ± 3.73 µmol min^{-1} mg^{-1} protein). A similar pattern of induced CAT activity was observed in previous studies carried out on the same species collected from Tunis (Banni *et al*. 2003, Chalghmi *et al*. 2016a, b) and Bizert (Bejaoui *et al*. 2020) lagoons. Increase in CAT activity means oxidative stress, often related to excessive ROS production during the catabolism of various organic compounds (Clairbone 1985). Indeed, an increase of ROS generation was recorded

aquatic organisms exposed to several contaminants (Coles *et al.* 1994, Dyrynda *et al*. 1998, Camus *et al*. 2002, Chakraborty *et al*. 2013). Thus, our results can be explained by an increase in ROS production owing to the presence of organic contaminants in the lagoon.

3.2. Immune biomarkers

Immunological biomarkers responses are shown in **Figure 3**. Haemocytes are circulating cells involved in bivalve immune defense such as haemocytosis (increases in circulating haemocyte numbers), phagocytosis of small particles, and encapsulation of large particles and production of reactive oxygen species. The change in total haemocyte count (THC) measured in the haemolymph of clams *Ruditapes decussatus* collected from three sites in the South lagoon of Tunis and the control site is illustrated in the **Figure 3A**. Overall total haemocyte count (THC) was higher in clams collected at the three contaminated sites of Tunis lagoon (S1, S2 and S3) compared to the values found in the clams from the reference site (SR) with significant differences at S1 and S2 (ANOVA, $p=0.005$ and $p=0.047$, respectively). A wide variety of studies have demonstrated a similar increase in the number of haemocytes in bivalves exposed to various pollutants. Indeed, *in vivo* exposure to phenanthrene resulted in an increase in THC in the scallop *Pecten maximus* (Hannam *et al.* 2010). In addition, dietary PAH exposure resulted in an increase in THC in the oyster *Crassostrea virginica* (Croxton *et al.* 2012). An increase in the haemocytes density was recorded in the blue mussel, *Mytilus edulis*, exposed *in tubo* to 10^{-4} M and 10^{-3} M of Hg for 24 h (Duchemin *et al.* 2008). Similar increase has also been reported in the bivalve *Scrobicularia plana* inhabiting a mercury contaminated area (Laranjo basin, Ria de Aveiro, Portugal) (Ahmad *et al.* 2011). Moreover, an increase of haemocyte concentration was recorded in the Pacific oyster, *C. gigas* exposed to a mix of Cd and Cu for 4 day (Haberkorn *et al.* 2014). Thus, the present study demonstrates strong effects of pollution level in the South Lagoon of Tunis on the total haemocyte count. Therefore, THC seems ideal tools for biomonitoring.

Lectin activity plays a crucial role in eliminating potential pathogens in marine invertebrates such as bacteria and parasites (Chu 1988). In the current study, lectin activity (LCT) was higher in clams collected at the three contaminated sites of Tunis lagoon S1, S2 and S3 (1.11 ± 0.27, 1.93 ± 0.36 and 1.13 ± 0.12, respectively) compared to the values found in the clams from the reference site (0.60 ± 0.17) with significant differences at site S2 (ANOVA, $p<0.001$). Study carried out on the bivalve *Scrobicularia plana* reported a reduction in plasma agglutination in animals environmentally exposed to mercury (Ahmad *et al.* 2011). Moreover, Chikalovets *et al.* (Chikalovets *et al.* 2010) revealed significant changes in agglutination in the mussel *Mytilus trossulus* after exposure to cadmium. In the current study, lectin activity was affected by pollution level in the South Lagoon of Tunis.

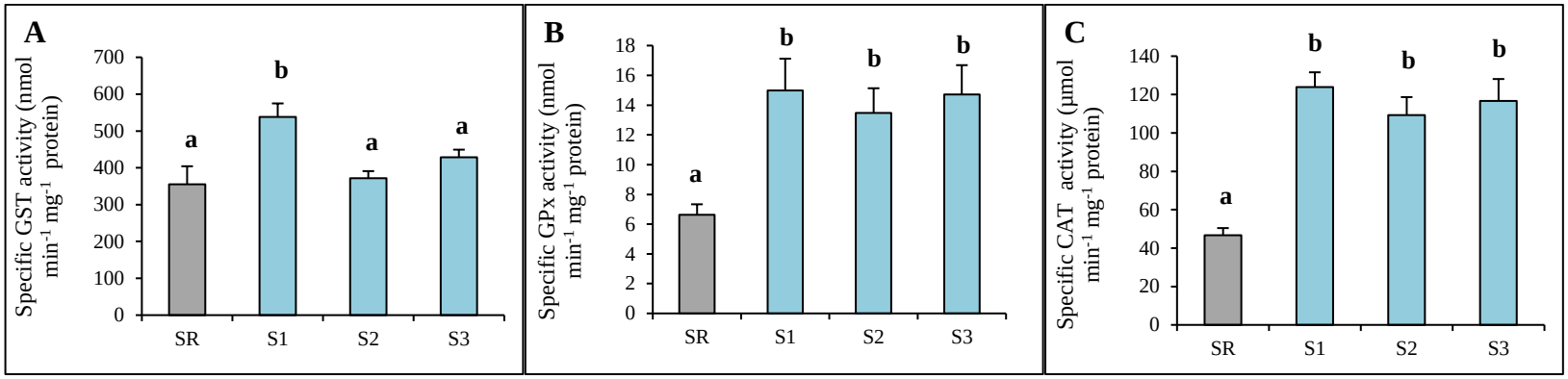


Fig. 2. Glutathione S- transferases (A), Glutathione peroxidase (B) and Catalase (C) activities in clam *Ruditapes decussatus* collected from three sites of the lagoon (S1, S2 and S3) and the control site (SR). The bars represent the mean ± SE. Small letters denote significant differences between sites (ANOVA, $p < 0.05$).

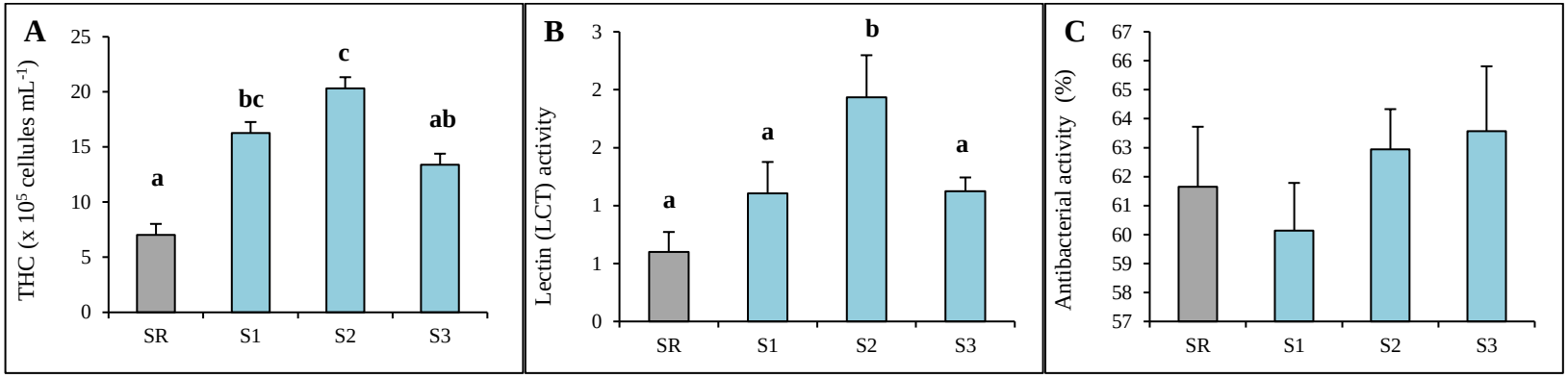


Fig. 3. Total haemocyte count (A), Lectin activity (B) and Antibacterial activity (C) in clam *Ruditapes decussatus* collected from three sites of the lagoon (S1, S2 and S3) and the control site (SR). The bars represent the mean ± SE. Small letters denote significant differences between sites (ANOVA, $p < 0.05$).

Regarding the antibacterial activity against *E. coli*, no significant difference (ANOVA, $p > 0.05$) was observed between the four sampling sites while the antibacterial activity was slightly higher at the polluted sites compared to the reference site. A wide variety of studies have demonstrated the antimicrobial action of several classes of organic hydrocarbons on different microorganisms (Heipieper and Martínez 2018). However, our results not revealed variations between the sampling sites. Thus, the bactericidal activity does not provide a suitable spatial discrimination for practical purposes.

3.3. Principal component analysis

Principal component analysis (PCA) was performed to obtain an overview of the spatial distribution of the biochemical and immunological biomarker data. Two principal components were extracted which accounted for 51.15 % of the total variance (**Fig. 4**). PC1 explained 33.07 % of the total variance and was positively loaded by biochemical and immunological parameters in clams. PC2 explained 18.08% of the total variance. According to PC2 axis, the site S1 and S2 were clearly discriminated from the sites S3 and SR.

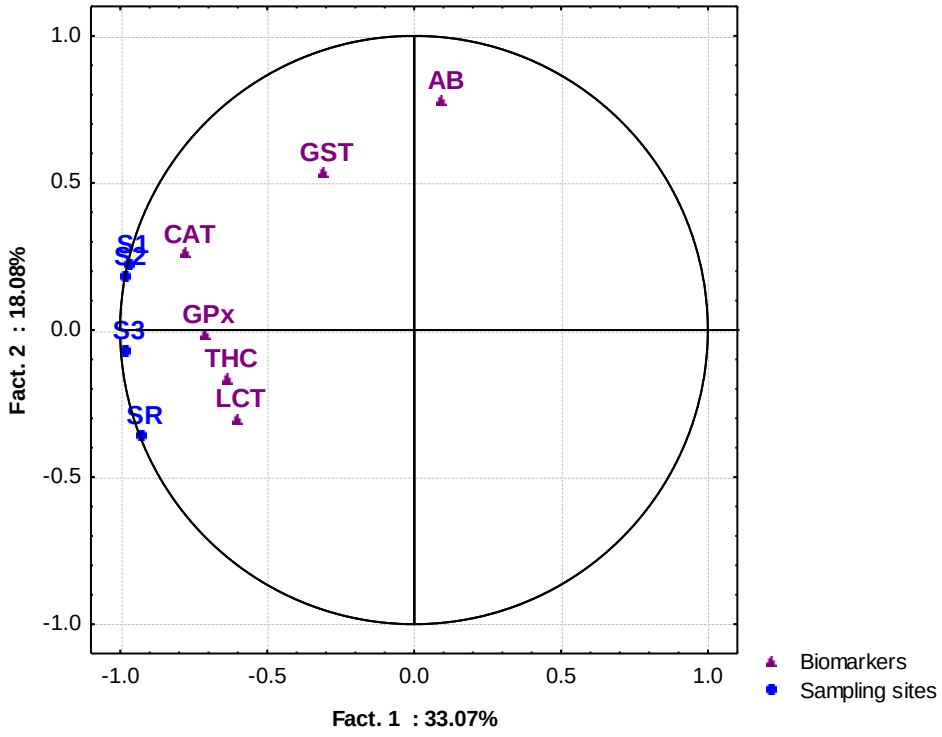


Fig.4. Results of PCA of the two main factors produced by biochemical (GST, GPx and CAT) and immunological (THC, LCT and AB) biomarkers in clams, *Ruditapes decussatus*, collected from the three sites of the southern lagoon of Tunis (S1, S2 and S3) and the control site (SR).

Pearson's correlation coefficients between the immunological and the biochemical biomarkers studied are shown in **Table 1**. A correlation coefficient higher than 0.5 was considered as significant at $P<0.05$. In our experimental conditions, no correlation was recorded between the different parameters.

Table 1. Pearson's correlation coefficients (r) of the immunological and the biochemical parameters studied.

	AB	LCT	THC	CAT	GST	GPx
AB	1					
LCT	-0.128	1				
THC	0.044	0.215	1			
CAT	0.021	0.353	0.367	1		
GST	-0.077	0.053	-0.008	0.225	1	
GPx	-0.110	0.268	0.293	0.378	0.180	1

4. Conclusion

In conclusion, the present study reveal activation of antioxidant enzymes in clams *Ruditapes decussatus* sampled from the polluted area as well as immune activation. The antioxidant enzymes GST, GPx and CAT were sensitive, responding to different pollution scenarios in the south lagoon of Tunis. Moreover, total haemocyte count and lectin activity were affected by pollution level in the Lagoon. Overall, the current study reveals the efficiency of this methodological approach to evaluate physiological responses in *Ruditapes decussatus* to environmental disruption caused by anthropogenic pollution.

Acknowledgements

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