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Multi-biomarkers approach to the assessment of the southeastern Mediterranean Sea health status: Preliminary study on *Stramonita haemastoma* used as a bioindicator for metal contamination

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HIGHLIGHTS

- A significant spatiotemporal variation in *S. haemastoma* biomarker responses from the Annaba Gulf and the El Kala coastline.
- A significant levels of trace metal elements (TME) in the digestive gland of *S. haemastoma*.
- The response of the different biomarkers studied is due to anthropogenic disturbances and to the location of the site.
- *S. haemastoma* could be used in surveillance programs as a bioindicator of contamination by TME from the Mediterranean sea.

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ABSTRACT

The present study aimed to evaluate the responses of different biochemicals parameters associated with environmental pollution in the digestive gland of the gastropod mollusc *Stramonita haemastoma*. Physiochemical parameters and trace metal elements (Copper (Cu), Zinc (Zn), Chromium (Cr), Cadmium (Cd) and Lead (Pb)) were measured in seawater. Spatiotemporal variations in reduced glutathione (GSH), malondialdehyde (MDA) and metallothionein (Mt) as well as the specific activities of glutathione S-transferase (GST) and catalase (CAT) were evaluated in digestive gland of this species during a one-year period in 2013–2014. Samples collection was conducted at three sites. The results obtained showed seasonal fluctuations in GST and CAT activities and in the rate of Mt content. In addition, intersite variations in GSH, MDA, Mt and CAT were recorded in individuals. Also, trace metal elements concentrations determined by season in the digestive gland revealed spatial and temporal variations for Cu and Zn but they are below the limit of detection for Cd and Pb. The highest values were generally recorded in spring for Cu and in winter for Zn. In this first regional study using in *S. haemastoma* as a model, the biomarkers measured were seen to be inducible parameters to evaluate the health state of the organism and the overall quality of the study sites.

1. Introduction

Marine ecosystems are highly vulnerable to pollution due to their recipient position from continents (Belabed et al., 2013b). Marine pollution has different origins. It can be of industrial (such as hydrocarbons, trace metal elements (TME) or chemicals) or agricultural origin (such as nutrients or pesticides) or simply be produced by domestic discharges following the presence of numerous wastewater outfall urban areas, wherein a wide variety of pollutants are concentrated (Valavanidis et al., 2006). The presence of these compounds in environmental media, biota and food poses nowadays a serious threat to human health and environmental integrity.

Metal pollution is one of the most abundant and dangerous forms of anthropogenic pollution threatening the littoral zone.

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Metal pollutants derive in the marine environment mainly by superficial runoff of rain, by direct atmospheric deposition and by discharges from sewage and industrial establishments. Maritime traffic is also a considerable source of metals given their presence in the antifouling paints composition of boats (Boulajfene et al., 2017). TME are micropollutants that can affect the marine environment health, as they do not undergo biological or chemical degradation. As a result, TME can accumulate in different trophic chain links at toxic concentrations in marine organisms. Due to the TME solubility, seawater analysis sample cannot be considered a reliable means of determining the pollution degree of marine environment. However, study of TME bioaccumulation in organisms exposed to them is a very important means of assessing metal pollution (Belhaouari et al., 2011). Metal contamination can have adverse effects on aquatic organisms after assimilation and accumulation. Nevertheless, not all metals have the same health impact: some (copper "Cu" and zinc "Zn") are essential at low doses and harmful at high doses, while others (cadmium "Cd" and lead "Pb") are harmful at even low doses. It is important to say that the toxic effects of Cd and Pb in marine species are multiple. Cd indirectly induces the production of reactive oxygen species (ROS) and lipid peroxidation by interference with antioxidant systems. It is also described as an inhibitor of DNA damage repair (Kamel, 2014). At the sublethal level, it can cause physiological effects (abnormalities in embryonic and larval development in bivalve molluscs) and growth inhibitions (Chiffoleau et al., 2001). Sobrino-Figueroa et al. (2007) have shown that Cd is the most toxic metal on juvenile populations of *Argopecten ventricosus*, which inhibits their growth, followed by Pb. In the mussel *Mytilus edulis*, the toxic effect of Pb can result in a competition with divalent essential metals with disruption of their metabolism: notably calcium, magnesium and Cu (Belabed, 2010). Cu is an important and essential micro-element that acts as a respiratory pigment in marine invertebrates. Its accumulation in the cell is the cause of cytotoxicity, which is manifested by enzymatic inhibition of the pyruvate oxidase system, glucose-6-phosphodehydrogenase and glutathione reductase (Kamel, 2014). High concentrations of this metal can lead to oxidative damage to lipids and proteins. It can also cause DNA deformation. Furthermore, it has been shown that, in excess, Zn becomes a prooxidant by inducing the indirect production of free radicals, and by inhibiting the enzymatic activity of certain antioxidant enzymes such as glutathione reductase and peroxidase (Sensi and Jeng, 2004).

In general, environmental or ecotoxicological monitoring in a marine ecosystem is based on two complementary approaches: biomarkers and bioindicators (Valavanidis et al., 2006).

Biomarkers can indicate links between contaminants and ecological responses and can be used to indicate the presence of harmful substances in the marine environment (Fernández et al., 2010), but data obtained are sometimes difficult to interpret due to the large amount of natural variables affecting biological processes, wich could act as confounding factors on biomarkers responses (González-Fernández et al., 2015). So, the use of a biomarkers battery is a trust approach to assess the environment health state and to increase the possibility for the detection of early biological changes (Almamoori et al., 2013).

Many species have been used as bioindicators of pollution, especially the bivalves: *M. galloprovincialis*, *Perna perna* and *Donax trunculus* (Abbes et al., 2003; Sifi et al., 2007, 2013; Amira et al., 2011; Soltani et al., 2012; Bensouda and Soltani-Mazouni, 2014).

The littoral is highly vulnerable to a wide assortment of contaminants and micropollutants directly released into the seas and oceans, to which are added those released into the air and drained by soils and rivers (Bensouda-Talbi, 2015).

The east Algerian coastline is the most important touristic and economic zone. It is continuously affected by various contaminants from urban, agricultural, harbor and industrial activities (Boucetta et al., 2016a).

The Annaba Gulf and the El Kala coastline, which represent the extreme northeastern part of the Algerian coastline, know as well as the rest of the latter, the same environmental problems. They are exposed to the risks of the different types of anthropogenic pollution that have an impact on the organisms that live there and on humans.

The Annaba Gulf is a standout amongst the most vital vacationer and financial focuses on the east coast of Algeria. It is considered as the receptacle for all residues, toxic or not, produced by the various industrial units located along the coast. This has made fishery stocks threatened by pollution linked to burgeoning economic activity. In addition, previous work has shown that the Annaba Gulf region is influenced by metal-rich effluents and is subjected to agricultural, industrial and urban activities as well as tourism development (Semadi and Deruelle, 1993; Abdenour et al., 2000; Beldi et al., 2006); with the impact of many chemicals and stressors, making the assessment of the marine ecosystem quality essential.

The El Kala coastline is minimally influenced by anthropogenic inputs, given the low urbanization of the region. Moreover, there is a notable absence of industries and consequently, little or no industrial atmospheric or continental pollution (Ounissi and Khelifi-Touhami, 1999).

Stramonita haemastoma, a gastropod mollusc commonly known as "Bakouma" in Algeria, has been the subject of several studies concerning its use as a bioindicator of tributyltin pollution (imposex phenomenon) (Chiavarini et al., 2003; Lemghich and Benajiba, 2007; El Mortaji et al., 2011). To date, however, much less attention has been paid to this species in ecotoxicology.

In this context, a multiparametric approach was implemented using more than one biomarker for the evaluation of the oxidative stress potential of *S. haemastoma* population. For this purpose, we chose to monitor seasonal variations in the rate of reduced glutathione (GSH), malondialdehyde (MDA), and metallothionein (Mt), as well as the specific activity of glutathione S-transferase (GST) and catalase (CAT) in this gastropod mollusc from three study sites: Cap de Garde (Annaba Gulf), Aouinète beach and Messida beach (El Kala coast) in the East coastal zone of Algeria.

Despite the importance and the large diversity of gastropods and their ability to bioaccumulate TME, they only interested few ecotoxicological studies (Yüzereroğlu et al., 2009; Belhaouari et al., 2011; Rabaoui et al., 2013; Boulajfene et al., 2017). This led us to conduct a seasonal study on the accumulation of Cu, Zn, Cd and Pb in the digestive gland of the same species and to analyze these same TME as well as Chromium (Cr) in the seawater to assess the environmental quality of the marine ecosystem.

2. Materials and methods

2.1. Sampling sites

The study area corresponds to the extreme northeastern part of the Algerian coastline (extreme southeast of the Mediterranean), which is bounded on the west by Cap de Garde and on the east by Cap Segleb and includes the Annaba Gulf and the El Kala coastal zone (Fig. 1). The Annaba Gulf is a bay open to the Mediterranean Sea on the north, bounded by two headlands: Rosa to the east (8°15′E, 36°58′N) and Garde to the west (7°47′E, 36°58′N), which are approximately 40 km apart with a maximum depth not exceeding 65 m (Sifi et al., 2007; Belabed et al., 2008; Belabed et al., 2013b; Amri et al., 2017a). The Annaba Gulf receives fresh water through two wadis: the Mafrag in the east and the Seybouse in the

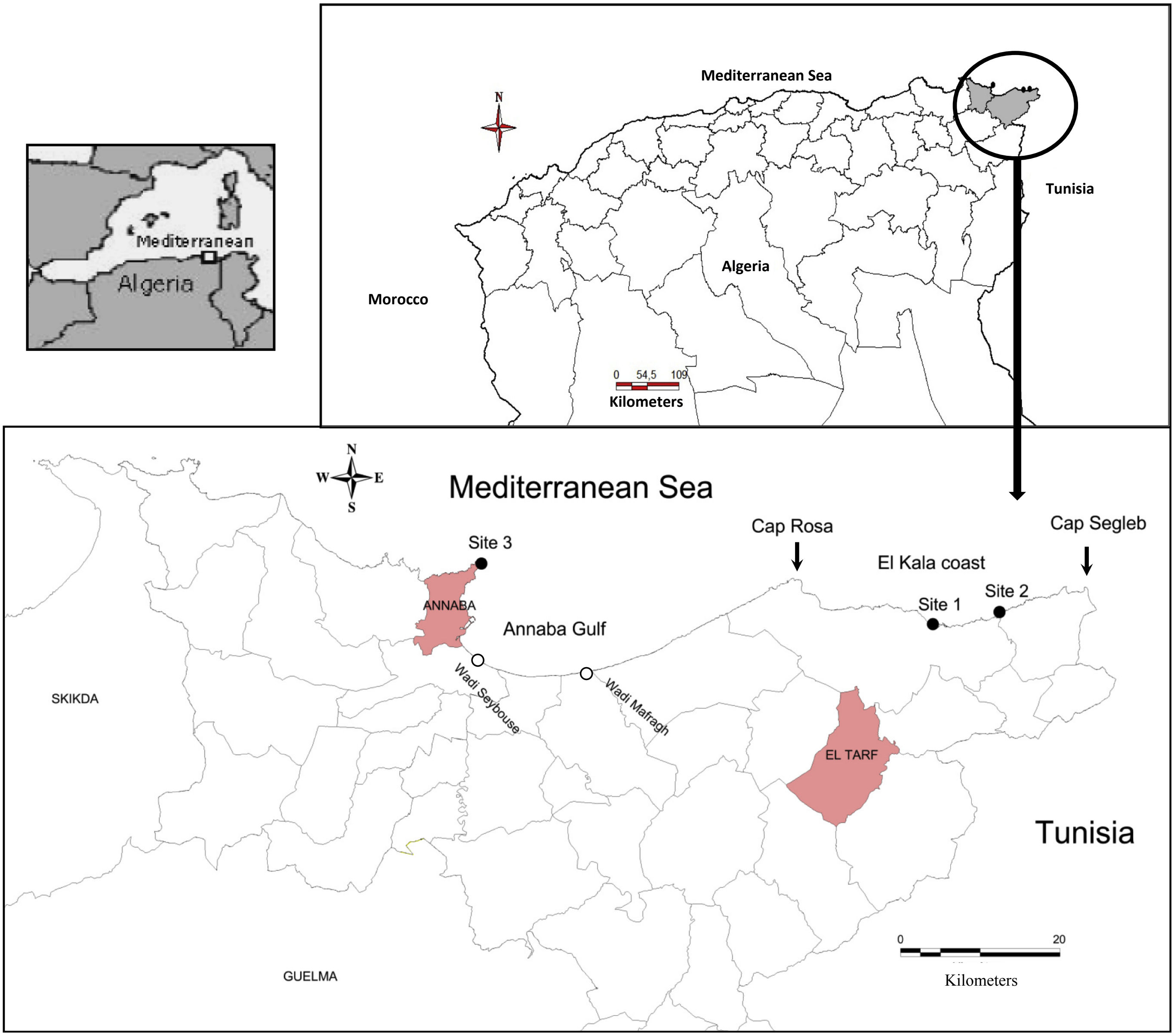

Fig. 1. Map of the three selected sampling sites.

southeast (the second longest river in Algeria), whose flow is very irregular according to the seasons. These wadis are an important source of TME in the Annaba Gulf (Belabed et al., 2013b; Boutabia-Trea et al., 2017), they receive agricultural water discharges and domestic releases from important conurbations (Khélifi-Touhami et al., 2006) and untreated sewage (Abdennour et al., 2000). Moreover, discharges from industries such as Arcelor Mittal (El Hadjar), which represents the largest integrated steel production site of the Maghreb region, and other major industrial complexes: mechanical production site, cement works, battery recycling sites and metallic equipment manufacturing plants … (Belabed et al., 2013b). The El Kala coastline is located at the extreme east of the Algerian coast; it extends from Cap Rosa to the west (8°15′E and 36°58′N) and Cap Segleb (the Tunisian border) to the east (8°13.6′E, 36°57′N). The coastline receives very few continental extrusions (Wadis Nhal, Boutribicha and Messida) because of the low freshwater inputs (Ounissi and Khelifi-Touhami, 1999).

The choice of study sites was based on the sites accessibility, the abundance of the species studied and the sampling ease. Three monitoring sites were selected, one located in the Annaba Gulf and two on the El Kala coastline:

- Aouinète beach (Site 1) is located in the eastern part of the El Kala coast (36°45′38.2″N, 8°31′21″E) and is not exposed to any source of pollution because of its location, which is quite remote from the various discharges. However, Aouinète beach is frequented by summer visitors.
- Messida beach (Site 2) is also located in the eastern part of the El Kala coast (36°54′52.3″N, 8°31′21″E) and is frequented by fishermen and summer vacationers. Messida beach receives wastewater discharges through the Wadi Messida, which is currently threatened by human activities; this constitutes a considerable threat to the fauna and flora of the study area. The Wadi Messida is a coastal canal connecting the Lake Tonga with the Mediterranean Sea. This canal is located between 36°53′60″N and 8°31′0″E, its length is approximately 1500 m

with a maximum depth of 2.5 m in the center (Benhalima et al., 2015).

- Cap de Garde (Site 3) is located in the west of the Annaba Gulf (36°58′04″N, 7°47′32″E), approximately 300 m southeast of the cap tip. It is assumed that Cap de Garde is not exposed to any source of pollution due to its location away from the various discharges, apart from the presence of a few houses not linked to the sewerage network. The Shems les Bains tourist complex is one of these and is accessible during summer.

2.2. Environmental characterization

Water temperature, pH, salinity, dissolved oxygen, and conductivity were measured *in situ* at the time of sampling using a field multi-parameter (WTW Multi 340i). Measurements of nitrates (NO_3^-), nitrites (NO_2^-), ammoniacal nitrogen (NH_4^+), orthophosphates (PO_4^{-3}) as well as suspended solids (MES) were performed in the laboratory using the manual colorimetric methods of Aminot and Kérouel (2004a). Water samples were collected in plastic bottles and transported in a cooler.

2.3. Sampling strategy

Four seasonal samplings of the *S. haemastoma* gastropod were conducted during the years 2013–2014 at the three (3) study sites. Gastropods sampling (approximately 15 per site and per season) was performed in autumn (September 2013: sampling 1), winter (January 2014: sampling 2), spring (May 2014: sampling 3) and summer (August 2014: sampling 4). Six individuals were subjected to biochemical analyses and 9 individuals to analyses of TME concentrations. The gastropods collection was carried out by hand or by diving between 0 and 2 m below the seawater surface. The collection was random and did not consider the gastropod's sex. Each harvest was placed in plastic containers containing 15 individuals bathing in their original water, depending on each site and for each season. These gastropods were then transported to the laboratory for biochemical and chemical analysis. The size of all the individuals studied was greater than 41 mm from the apex to the end of the siphonal channel.

2.4. Sample preparation

Upon return to the laboratory, the animals were dissected, and their total soft masses were frozen at −20 °C. After being thawed, the digestive gland (maintained at 4 °C throughout the duration of the assays) was ground in phosphate buffer (0.1 M, pH 7.5) using an Ultra-Turrax homogenizer; the homogenate obtained was centrifuged at 9000 × g for 20 min. The supernatant, which contained the cytosol, endoplasmic reticulum, Golgi apparatus and cytosolic proteins, was recovered for the biochemical assay and was designated S9.

2.5. Determination of related oxidative stress biomarkers

2.5.1. Reduced glutathione (GSH)

The GSH level was estimated according to the method of Weckbecker and Cory (1988), based on the absorbance of 2-nitro-5-mercapturic acid resulting from the reduction of 5-5′-dithio-bis-2-nitrobenzoic acid (DTNB) by the thiol (SH) group of the glutathione. The absorbance was measured at 412 nm. The glutathione concentration was expressed in nmol mg^{-1} protein.

2.5.2. Glutathione S-transferase (GST)

The assay of GST activity was performed according to Habig et al. (1974). This assay consists of providing the enzyme with a substrate: 1-chloro-2, 4-dinitrobenzene (CDNB), which reacts readily with reduced glutathione. The conjugation reaction of these two products results in the formation of a novel molecule, which absorbs light at a 340 nm wavelength. The optical density (OD) was measured every minute for 5 min. The specific GST activity was expressed in nmol min^{-1} mg^{-1} protein.

2.5.3. Catalase (CAT)

CAT activity was performed according to the method of Saint-Denis et al. (1998). This method is based on the measurement of the reduction of oxygenated water (H_2O_2) into an oxygen molecule (O_2) and two molecules of water (H_2O) in the presence of CAT at a UV wavelength of 240 nm. CAT activity was expressed in μmol min^{-1} mg^{-1} protein.

2.5.4. Malondialdehyde (MDA)

MDA content was assayed according to the method of Uchiyama and Mihara (1978), whose principle is based on the spectrophotometric measurement of the red colour produced during the thiobarbituric acid reaction and MDA content. The intensity of the colour increases with the MDA concentration. The absorbance was measured at 532 nm. MDA contents were determined using 1, 1, 3, 3 tetra ethoxypropane as a standard with the results expressed in nmol mg^{-1} protein.

2.5.5. Metallothionein (Mt)

Mt level was assayed according to the method of Viarengo et al. (1997). The absorbance was measured at 412 nm. The Mt concentration was determined using the equation 1 mol Mt = 20 mol GSH and was expressed in nmol g^{-1} tissue.

2.6. Extraction and measurement of trace metal elements (TME) in water and digestive gland of *S. haemastoma*

The TME extraction (Cu, Zn, Cd and Pb) in the digestive gland was performed via hot nitro-perchloric acid ($HNO_3/HClO_4$; 4 v/1 v) attack. Specifically, 50 mg of the digestive gland fine powder, previously dried in an oven at 60 °C for 72 h, was placed in the presence of 8 ml of the nitro-perchloric acid mixture in teflon bottles. After 2 h of mineralization at 110 °C, 50 ml of 0.5% nitric acid was added. The solutions thus obtained were filtered and kept cold (4 °C) until the assay. The Cu and Zn were measured directly upon mineralization or after dilution using a Perkin Elmer atomic absorption spectrophotometer (PinAAcle 900T, USA) in flame mode. The TME measurement (Cu, Zn, Cr, Cd and Pb) in seawater was directly conducted without the addition of nitric acid. For each metallic element, a standard range was prepared from a 1000 mg L^{-1} Perkin Elmer stock solution (Bankaji et al., 2016). The results were expressed in mg g^{-1} dry weight for the digestive gland and in mg L^{-1} for water.

2.7. Statistical analysis

The statistical analysis of the results was performed using software R, version 3.1.2 (R Core Team, 2014), created by Ihaka and Gentleman (1996). The condition of normal distributions was verified in advance by the application of the Shapiro-Wilk test. The distributions were mostly asymmetrical, which led us to choose non-parametric alternatives for the statistical analysis. The data are presented as the averages plus or minus standard error (m ± se). Intersite and interseason comparisons were performed using the Kruskal-Wallis nonparametric test. This last was followed by the Dunn's test application (pairwise comparisons) in the case of hypothesis rejection by the Kruskal-Wallis test. Furthermore, a principal component analysis (PCA) was performed using the FactoMineR package (Husson and Josse, 2014) on standardized data, whose objective is to characterize, by a multivariate approach, the structuration of spatiotemporal variations at the Algerian coastline. In addition, a dendrogram based on Hierarchical ascending classification “HAC” was constructed to better visualize the similarities between the study sites.

3. Results

3.1. Environmental characterization

The spatiotemporal variations of physicochemical parameters and TME (Cu, Zn, Cr, Cd and Pb) in water are represented in Table 1. The thermal water values varied between 22.73 ± 2.72 °C at site 1 and 23.65 ± 2.92 °C at site 2. However, they presented seasonal fluctuations with values ranged from 15.50 ± 0.50 °C to 27.27 ± 0.27 °C in winter and summer, respectively. Regarding pH, the analytical data revealed similar mean values in the three sites, they are between 8.51 ± 0.09 at site 3 and 8.58 ± 0.06 at site 1, which marks an alkaline pH in all the study sites. Salinity showed a variation between the sites, mean values of 31.42 ± 2.29 PSU at site 2 and 36.98 ± 0.22 PSU at site 3 were observed. The spring period is characterized by a decrease in salinity with 31.37 ± 3.25 PSU, while the highest mean values were recorded in winter with 36.47 ± 0.09 PSU. Dissolved Oxygen levels reveal similar means between the three sites, they were ranged from 9.66 ± 0.28 mg L^{-1} to 12.42 ± 1.41 mg L^{-1} at site 2 and site 3, respectively. Conductivity results showed that the mean values ranged from 47.35 ± 5.77 mS cm^{-1} at site 2 to 55.95 ± 0.18 mS cm^{-1} at site 3; the maximum seasonal mean values are registered in autumn with 56.17 ± 0.15 mS cm^{-1} and the lowest mean values are recorded in spring with 45.30 ± 7.28 mS cm^{-1}. We noted that nutritional salts concentrations are similar in all sites except for NO_3^- where they were of the order of 2.95 ± 0.77 μM at site 1 and 10.97 ± 2.05 μM at site 3; between 0.24 ± 0.06 μM at site 2 and 0.46 ± 0.29 μM at site 3 for NO_2^-; between 2.24 ± 0.54 μM at site 1 and 3.92 ± 0.75 μM at site 2 for NH_4^+; between 0.74 ± 0.13 μM at site 2 and 0.98 ± 0.05 μM at site 3 for PO_4^{-3}. Mean values of suspended solids were in the order of 28.32 ± 1.55 mg L^{-1} at site 2 and 34.00 ± 2.48 mg L^{-1} at site 1. The spring period was characterized by the lowest suspended solids means with 24.95 ± 3.13 mg L^{-1}, while the highest mean values were in the autumn period with 36.67 ± 2.15 mg L^{-1}.

With regard to TME in water, the mean Cu concentrations of the three sites ranged from 0.14 ± 0.00 mg L^{-1} at site 2 to 0.16 ± 0.00 mg L^{-1} at site 1. The mean Zn concentrations were between 0.01 ± 0.00 mg L^{-1} at site 3 and 0.02 ± 0.00 mg L^{-1} at site 1 and site 2; they reached their maximum values in winter with 0.03 ± 0.00 mg L^{-1}. The mean Cr concentrations ranged from 0.07 ± 0.01 mg L^{-1} at site 2 to 0.09 ± 0.01 mg L^{-1} at site 3. For Cd and Pb, the concentrations were below the limit of detection.

The application of the Kruskal-Wallis test for the comparison of physicochemical parameters and TME in water showed that there were significant intersite differences for Salinity, NO_3^- and Cu; as well as significant differences among seasons for water temperature and Zn. No significant spatial and seasonal variations differences were observed for others parameters.

3.2. Biomarker response

In general, by examining the box plots (Figs. 2 and 3), we found that the intersite and seasonal variations in the parameters were important. A comparison of the minimum and maximum values highlighted the extent of the range of variation in the parameters.

Based on the box plots in Figs. 2A and 3A, we observed that the GSH values at sites 1 and 2 showed less variation than those at site 3. The mean values ranged from 12.73 ± 2.45 nmol mg^{-1} protein at site 3 to 28.12 ± 1.75 nmol mg^{-1} protein at site 2, with a minimum value (3.05 nmol mg^{-1} protein) recorded at site 3. Mean values of 18.43 ± 2.05 nmol mg^{-1} protein in spring and 23.60 ± 3.74 nmol mg^{-1} protein in winter were observed.

Regarding GST activity, the box plots in Figs. 2B and 3B show that the concentrations were high but remained similar among different sites. The mean values ranged from 109.26 ± 12.82 nmol min^{-1} mg^{-1} protein at site 2 to 147.78 ± 9.79 nmol min^{-1} mg^{-1} protein at site 3, with a maximum value (264.15 nmol min^{-1} mg^{-1} protein) recorded at site 2. Mean values of 81.09 ± 13.21 nmol min^{-1} mg^{-1} protein in winter and 183.72 ± 7.46 nmol min^{-1} mg^{-1} protein in spring were observed.

CAT activity presented in Figs. 2C and 3C reveals significant variation of this parameter at site 2. The mean values ranged between 62.69 ± 5.10 μmol min^{-1} mg^{-1} protein and 106.55 ± 9.96 μmol min^{-1} mg^{-1} protein at site 3 and 2, respectively. The maximum value was recorded at site 2, with 222.97 μmol min^{-1} mg^{-1} protein. Mean values of 52.91 ± 3.93 μmol min^{-1} mg^{-1} protein in autumn and 113.70 ± 8.53 μmol min^{-1} mg^{-1} protein in spring were observed. The seasonal variation exhibited different dispersions and was much higher in winter period.

Table 1
Seasonal and spatial variations of physicochemical parameters and TME concentrations in water samples analyzed at the three sampling sites of Annaba Gulf and El Kala coastline (Mean values ± SE, n = 3).

Variable	Site 1	Site 2	Site 3	Autumn	Winter	Spring	Summer
Temperature (°C)	22.73 ± 2.72a	23.65 ± 2.92a	22.90 ± 2.26a	25.63 ± 0.47ab	15.50 ± 0.50a	23.97 ± 0.83ab	27.27 ± 0.27b
pH	8.58 ± 0.06a	8.54 ± 0.04a	8.51 ± 0.09a	8.59 ± 0.04a	8.52 ± 0.14a	8.51 ± 0.07a	8.56 ± 0.05a
Salinity (PSU)	35.23 ± 1.16ab	31.42 ± 2.29a	36.98 ± 0.22b	35.97 ± 1.43a	36.47 ± 0.09a	31.37 ± 3.25a	34.37 ± 1.87a
Dissolved oxygen (mg L^{-1})	11.27 ± 0.62a	9.66 ± 0.28a	12.42 ± 1.41a	10.00 ± 0.72a	11.74 ± 1.26a	12.41 ± 1.78a	10.32 ± 0.51a
Conductivity (mS cm^{-1})	53.40 ± 1.66a	47.35 ± 5.77a	55.95 ± 0.18a	56.17 ± 0.15a	55.77 ± 0.19a	45.30 ± 7.28a	51.70 ± 2.62a
NO_3^- (μM)	2.95 ± 0.77a	6.25 ± 1.91ab	10.97 ± 2.05b	3.56 ± 1.70a	10.48 ± 3.10a	7.96 ± 2.91a	4.89 ± 1.76a
NO_2^- (μM)	0.24 ± 0.12a	0.24 ± 0.06a	0.46 ± 0.29a	0.08 ± 0.02a	0.13 ± 0.10a	0.72 ± 0.30a	0.35 ± 0.05a
NH_4^+ (μM)	2.24 ± 0.54a	3.92 ± 0.75a	3.66 ± 1.76a	3.40 ± 1.29a	2.74 ± 0.45a	2.68 ± 0.90a	4.28 ± 2.35a
PO_4^{-3} (μM)	0.78 ± 0.09a	0.74 ± 0.13a	0.98 ± 0.05a	0.85 ± 0.10a	0.74 ± 0.13a	0.94 ± 0.09a	0.82 ± 0.17a
Suspended matter (mg L^{-1})	34.00 ± 2.48a	28.32 ± 1.55a	28.43 ± 4.17a	36.67 ± 2.15a	30.81 ± 3.16a	24.95 ± 3.13a	28.57 ± 2.30a
Cu (mg L^{-1})	0.16 ± 0.00a	0.14 ± 0.00b	0.15 ± 0.00ab	0.15 ± 0.00a	0.16 ± 0.00a	0.14 ± 0.01a	0.15 ± 0.00a
Zn (mg L^{-1})	0.02 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.03 ± 0.00b	0.01 ± 0.00a	0.01 ± 0.00c
Cr (mg L^{-1})	0.08 ± 0.01a	0.07 ± 0.01a	0.09 ± 0.01a	0.08 ± 0.01a	0.08 ± 0.01a	0.07 ± 0.02a	0.09 ± 0.01a
Cd (mg L^{-1})	< 0.028	< 0.028	< 0.028	< 0.028	< 0.028	< 0.028	< 0.028
Pb (mg L^{-1})	< 0.45	< 0.45	< 0.45	< 0.45	< 0.45	< 0.45	< 0.45

a and b indicate that inter-sites and seasonal variation is significant at $p < 0.05$, using the Dunn's test.
Limit of detection: Cu = 0.077 mg L^{-1}, Zn = 0.01 mg L^{-1}, Cr = 0.07 mg L^{-1}, Cd = 0.028 mg L^{-1}, Pb = 0.45 mg L^{-1}.

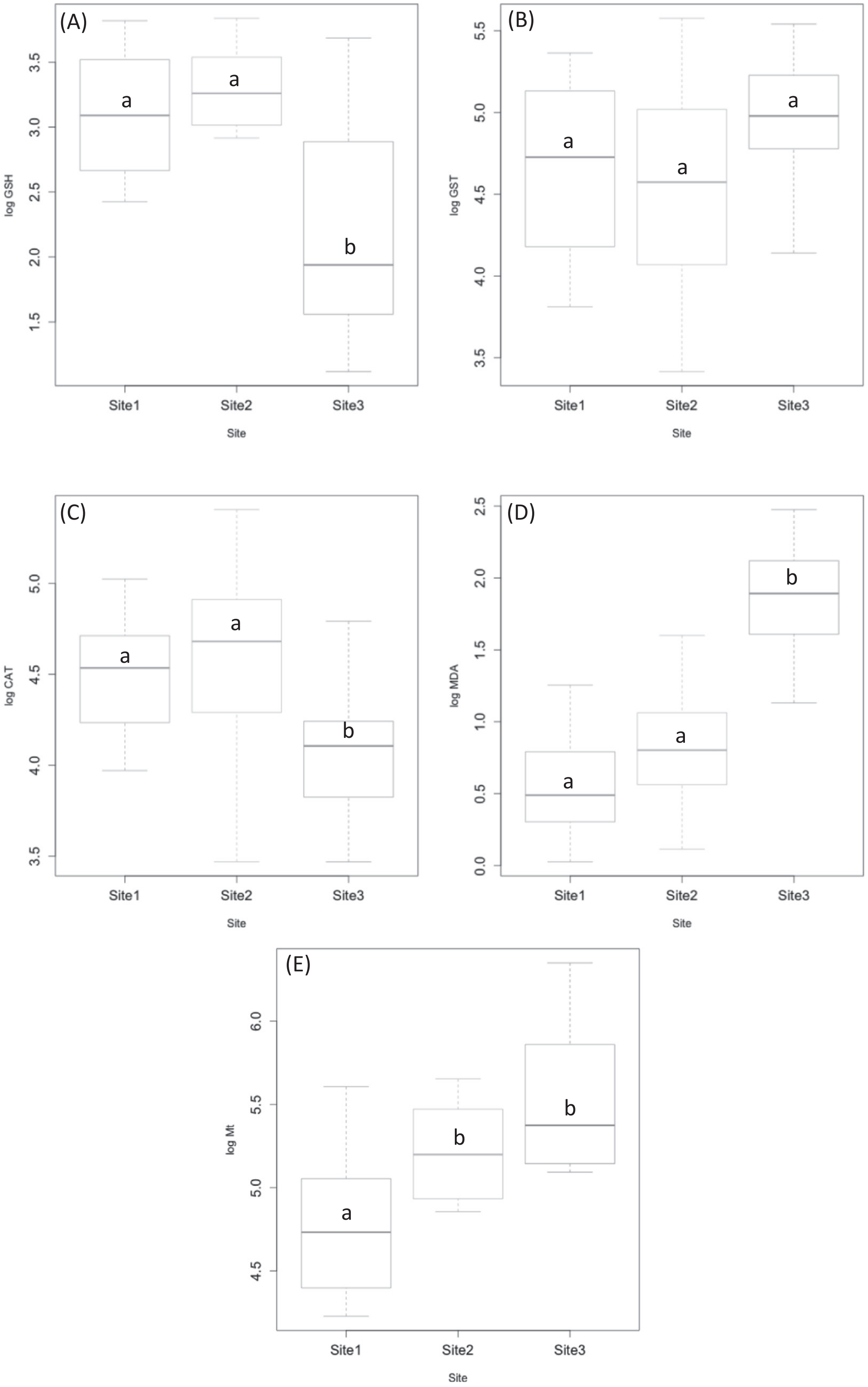

Fig. 2. Spatial variations in biomarker response in the digestive gland of *S. haemastoma* collected from Aouinète beach (Site 1), Messida beach (Site 2) and Cap de Garde (Site 3): GSH (A), GST (B), CAT (C), MDA (D) and Mt (E). a and b indicates that intersite variation is significant at $p < 0.05$, using the Dunn's test. Boxplots labeled with the same letter are not significantly different at $p > 0.05$ (Mean values ± SE, $n = 6$).

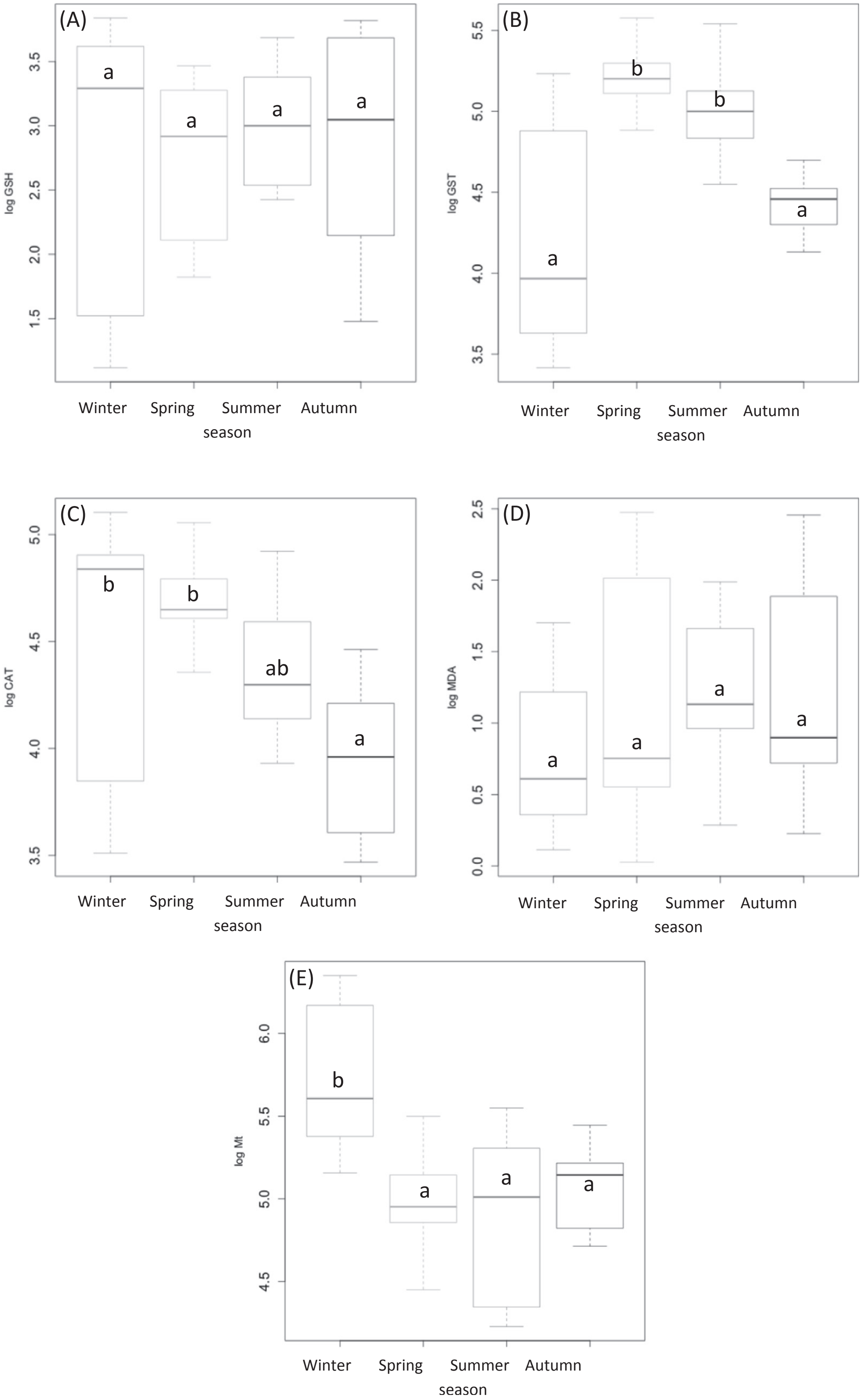

Fig. 3. Seasonal variations in biomarker response in the digestive gland of *S. haemastoma* collected from Aouinète beach (Site 1), Messida beach (Site 2) and Cap de Garde (Site 3): GSH (A), GST (B), CAT (C), MDA (D) and Mt (E). a and b indicates that seasonal variation is significant at $p < 0.05$, using the Dunn's test. Boxplots labeled with the same letter are not significantly different at $p > 0.05$ (Mean values ± SE, $n = 6$).

The MDA box plots (Figs. 2D and 3D) clearly showed that the variation in this parameter at the 3 sites was almost the same. However, the site 3 values were higher than those recorded in sites 1 and 2. The mean values ranged from 1.87 ± 0.15 nmol mg^{-1} protein at site 1 to 6.85 ± 0.54 nmol mg^{-1} protein at site 3, with a maximum of 11.88 nmol mg^{-1} protein at site 3. Mean values of 2.54 ± 0.33 nmol mg^{-1} protein in winter and 4.41 ± 0.93 nmol mg^{-1} protein in spring were observed. Thus, the box plots of the seasonal variation revealed the existence of homogeneity in the four seasons.

By analysing the Mt box plots (Figs. 2E and 3E), we noted the existence of an increasing gradient (S1<S2<S3) of Mt levels. The mean values ranged from 129.46 ± 18.09 nmol g^{-1} tissue to 280.18 ± 44.68 nmol g^{-1} tissue at site 1 and 3, respectively, with a minimum of 68.57 nmol g^{-1} tissue at site 1 and a maximum of 572.14 nmol g^{-1} tissue at site 3. Mean values of 152.86 ± 23.72 nmol g^{-1} tissue in summer and 330.24 ± 51.99 nmol g^{-1} tissue in winter were observed. An examination of the box plots revealed the existence of heterogeneity in all four seasons.

3.3. TME in the digestive gland

Cu box plots (Fig. 4A and C) and Zn (Fig. 4B and D) revealed spatiotemporal variation. The mean Cu values ranged from 0.26 ± 0.03 mg g^{-1} dry weight (dw) to 0.67 ± 0.09 mg g^{-1} dw at site 2 and 3, respectively, with a maximum of 1.80 mg g^{-1} dw at site 3 and a minimum of 0.06 mg g^{-1} dw at site 1. Mean values of 0.18 ± 0.03 mg g^{-1} dw in winter and 0.62 ± 0.11 mg g^{-1} dw in spring were observed. The average Zn values ranged from 0.25 ± 0.03 mg g^{-1} dw at site 3 to 0.54 ± 0.03 mg g^{-1} dw at site 2. The maximum value was recorded at site 2, with 0.79 mg g^{-1} dw, and the minimum value was 0.08 mg g^{-1} dw at site 3. Mean values of 0.28 ± 0.05 mg g^{-1} dw in summer and 0.48 ± 0.03 mg g^{-1} dw in winter were observed. For Cd and Pb, the concentrations were below the limit of detection.

The results of the Kruskal-Wallis test for the comparison of variables for both site and season revealed significant intersite differences ($p < 0.05$) for all variables except GST. The effect of the "site" factor appeared to be very marked, and there were significant

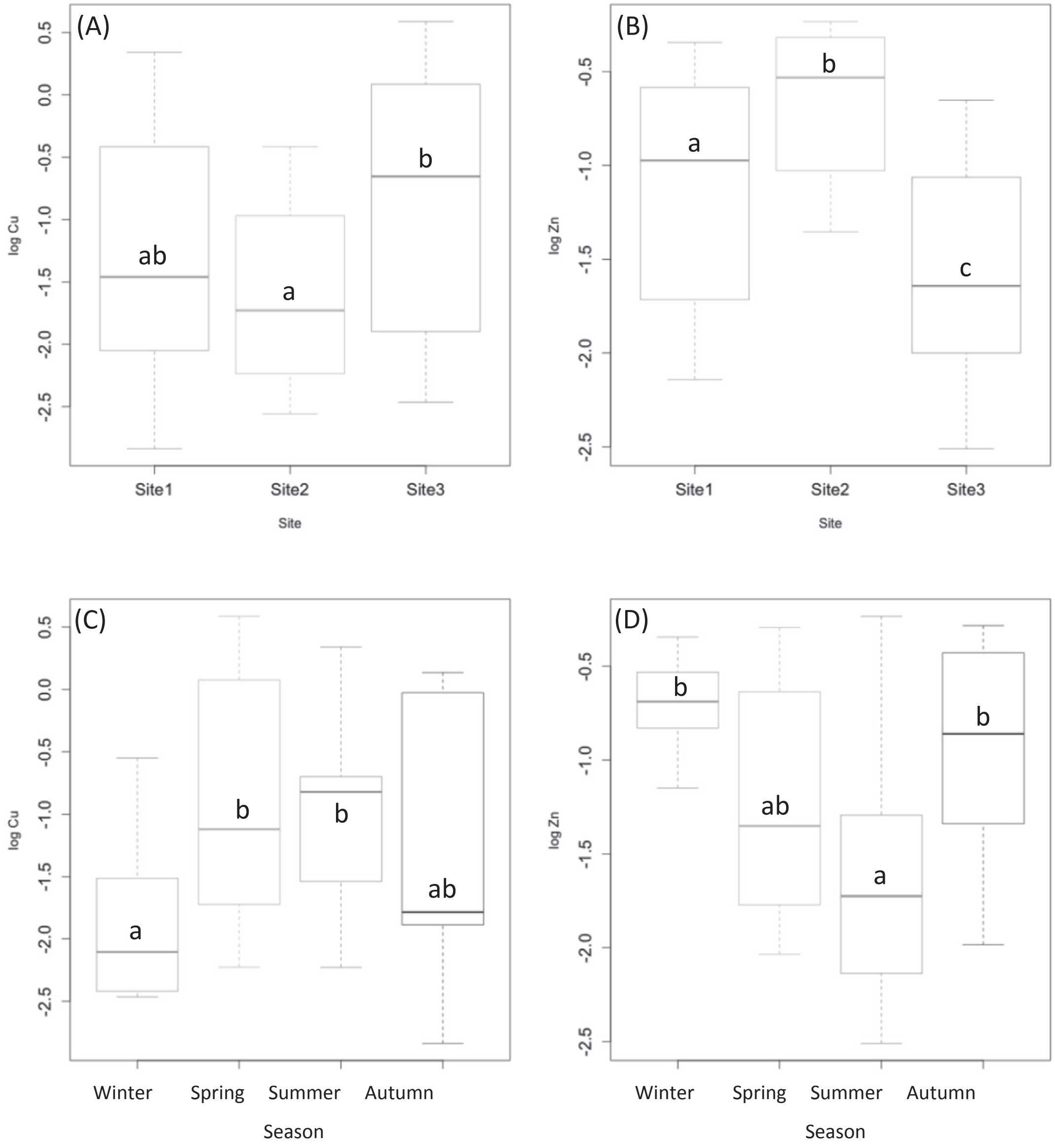

Fig. 4. Spatial (A, B) and seasonal (C, D) variation in Metal trace elements in the digestive gland of *S. haemastoma* collected from Aouinète beach (Site 1), Messida beach (Site 2) and Cap de Garde (Site 3): Cu (A, C) and Zn (B, D). a and b indicates that seasonal and intersite variations are significant at $p < 0.05$, using the Dunn's test (Mean values ± SE, n = 9).

differences among seasons ($p < 0.05$) for all parameters except GSH and MDA.

Dunn's test clearly shows that there are significant intersite differences for all of the following combinations:

- As regards GSH, CAT and MDA (Fig. 2A, C and 2D), the difference is between individuals collected from Site 3 and those of Site 1 and Site 2, which allowed us to group these sites into two groups: the Site 1 and Site 2 represent the group (a), the Site 3 corresponds to the group (b).
- For Mt (Fig. 2E), Dunn's test was permitted the classification of sites into two groups, group (a) includes Site 1 and group (b) matches Site 2 and Site 3.
- Concerning Cu (Fig. 4A), Dunn's test allows us to classify the study sites into three groups, where significance difference occurred between the group (ab) corresponding to Site 1, group (a) and (b) representing Site 2 and Site 3, respectively.
- For Zn (Fig. 4B), Dunn's test permits the classification of the sites in three groups: (a), (b) and (c). This classification clearly shows the existence of a difference between all sites.

Dunn's test also reveals significant differences between seasons for the following combinations:

- Concerning GST and Mt (Fig. 3B and E), this test allows us to classify the seasons into two groups: for GST group (a) representing winter and autumn, the group (b) corresponds to spring and summer. For Mt group (a) contains spring, summer and autumn, the group (b) includes winter.
- Eventually Dunn's test reveals that CAT, Cu and Zn levels divided seasons into three groups: for CAT (Fig. 3C) group (a) includes autumn, group (b) matches winter and spring, group (ab) corresponds to summer. For Cu (Fig. 4C) group (a) contains winter, group (b) spring and summer, group (ab) autumn. For Zn (Fig. 4D) group (a) represents summer, group (b) autumn and winter, group (ab) spring.

3.4. Seasonal and intersite variation of parameters by Principal Component Analysis (PCA)

The use of Principal Component Analysis (PCA) as a preliminary and exploratory descriptive approach made it possible to visualize the structuring of the temporal and spatial variation at the three sites according to 20 variables: water temperature (T), pH, salinity (Sal), dissolved oxygen (Oxy), conductivity (Cond), NO_3^- (Nitra), NO_2^- (Nitri), NH_4^+ (Ammo), PO_4^{-3} (Orth), suspended solids (MES), Cu Zn Cr in water (Cu.e, Zn.e, Cr.e), GSH, GST, CAT, MDA, Mt and Cu Zn in the digestive gland (CuDG, ZnDG). Our PCA (Fig. 5) clearly shows that the first two factorial axes yielded nearly 46.58% of the information. Axis 1 explains 26% of the total variation, it is positively correlated with the variables MDA ($r = 0.86$), Cr.e ($r = 0.74$), CuDG ($r = 0.71$), Nitri ($r = 0.64$), Oxy ($r = 0.64$) and Sal ($r = 0.52$) which strongly contribute to the construction of this axis ($\cos^2 = 0.74$, $\cos^2 = 0.54$, $\cos^2 = 0.50$, $\cos^2 = 0.41$, $\cos^2 = 0.40$ and $\cos^2 = 0.27$, respectively); and negatively with the variables ZnDG ($r = -0.75$), GSH ($r = -0.59$) and Zn.e ($r = -0.53$), which also contribute significantly to its construction ($\cos^2 = 0.56$, $\cos^2 = 0.35$ and $\cos^2 = 0.28$, respectively). In addition, axis 2 explains 20.58% of the total variation, it is built mainly by the strong positive correlations of the variables Cond ($r = 0.82$), Sal ($r = 0.78$), Cu.e ($r = 0.71$) and Zn.e ($r = 0.52$) and the negative correlation of variables GST ($r = -0.80$) and T ($r = 0.61$) which contribute considerably to its construction ($\cos^2 = 0.67$, $\cos^2 = 0.61$, $\cos^2 = 0.51$, $\cos^2 = 0.27$, $\cos^2 = 0.64$ and $\cos^2 = 0.37$, respectively).

3.5. Hierarchical ascending classification (HAC) of intersite variation

Fig. 6 shows the dendrogram resulting from the HAC based on the intersite variation in the measured parameters. An examination of the dendrogram indicates the existence of two clusters. The first cluster encompasses site 3, which corresponds to the Cap de Garde beach in the Annaba Gulf. The second cluster consists of sites 1 (Aouinète beach) and 2 (Messida beach), which represent the El Kala coastline.

4. Discussion

4.1. Environmental characterization

Temperature is one of the most sensitive physico-chemical parameters to natural changes, it varies according to the outside temperature (air), seasons, the geological nature of the soil and the depth of the water level relative to the sediment surface (Rodier et al., 2005); and anthropogenic changes such as wastewater discharges. In the three study sites, water thermal values did not show large variations. However, they revealed the existence of a typically mediterranean seasonal cycle. The recorded pH values demonstrated a slight water alkalinity and did not show a variation between the three sites. The dissolved oxygen of the seawater at site 2, which is very close to the northern part of the Messida canal, revealed slightly lower mean values compared to the other sites, this would probably be related to biodegradable organic matter loads. These will undergo oxidation which will result in increased consumption of oxygen leading to a decrease in its content in water. The results obtained of temperature, pH and the dissolved oxygen correspond with the work performed in the northeastern coast of Algeria (Kadri et al., 2015; Amri et al., 2017b). Salinity showed the absence of a difference between the seasons with a nonsignificant decrease in spring due to the arrival of continental freshwater, which is linked to the abundance of rains that dilute seawater. We also noted a significant difference recorded between the three study sites, where the decrease in salinity at site 2 may be due to its proximity to the Messida canal which is characterized by low salinity. Our results seem to be in agreement with the means observed in the Mediterranean Sea which are in the order of 38–39 PSU (Aminot and Kéroual, 2004b) and with those measured on the Algerian coasts, particularly on the Annaba Gulf (Hadjadji et al., 2014; Ounissi et al., 2014; Kadri et al., 2015; Amri et al., 2017b). The water conductivity showed no significant seasonal fluctuations, values were slightly high in autumn and low in spring. These variations could be explained by the dilution phenomenon during wet periods (Nassali et al., 2005) and the high evaporation and low water flow during the dry period (El Morhit et al., 2008). The monitoring of NO_3^-, NO_2^- and NH_4^+ concentrations is often systematic in water quality surveillance programs (Aminot and Kérouel, 2004a), because these elements play a crucial role in the coastal waters eutrophication (Howarth and Marini, 2006). The NO_3^- concentrations recorded in the water of site 3 were slightly higher than other two sites. According to Bremond and Perrodon (1979), concentrations above 12 $\mu g\ L^{-1}$ of NO_3^- are directly related to human activity. In addition, the NO_3^- concentrations showed insignificant seasonal fluctuations, with high values in winter period that would be the result of enrichment due to active nitrification. The results obtained from nutritional salts are more or less similar compared to the work performed at the Annaba Gulf (Ziouch, 2014; Boutabia-Trea, 2016; Amri et al., 2017b). The analysis of the results obtained showed that waters from the three sites are not overloaded with suspended solids. In the coastal environment, suspended solids are most often associated with continental inputs or solid

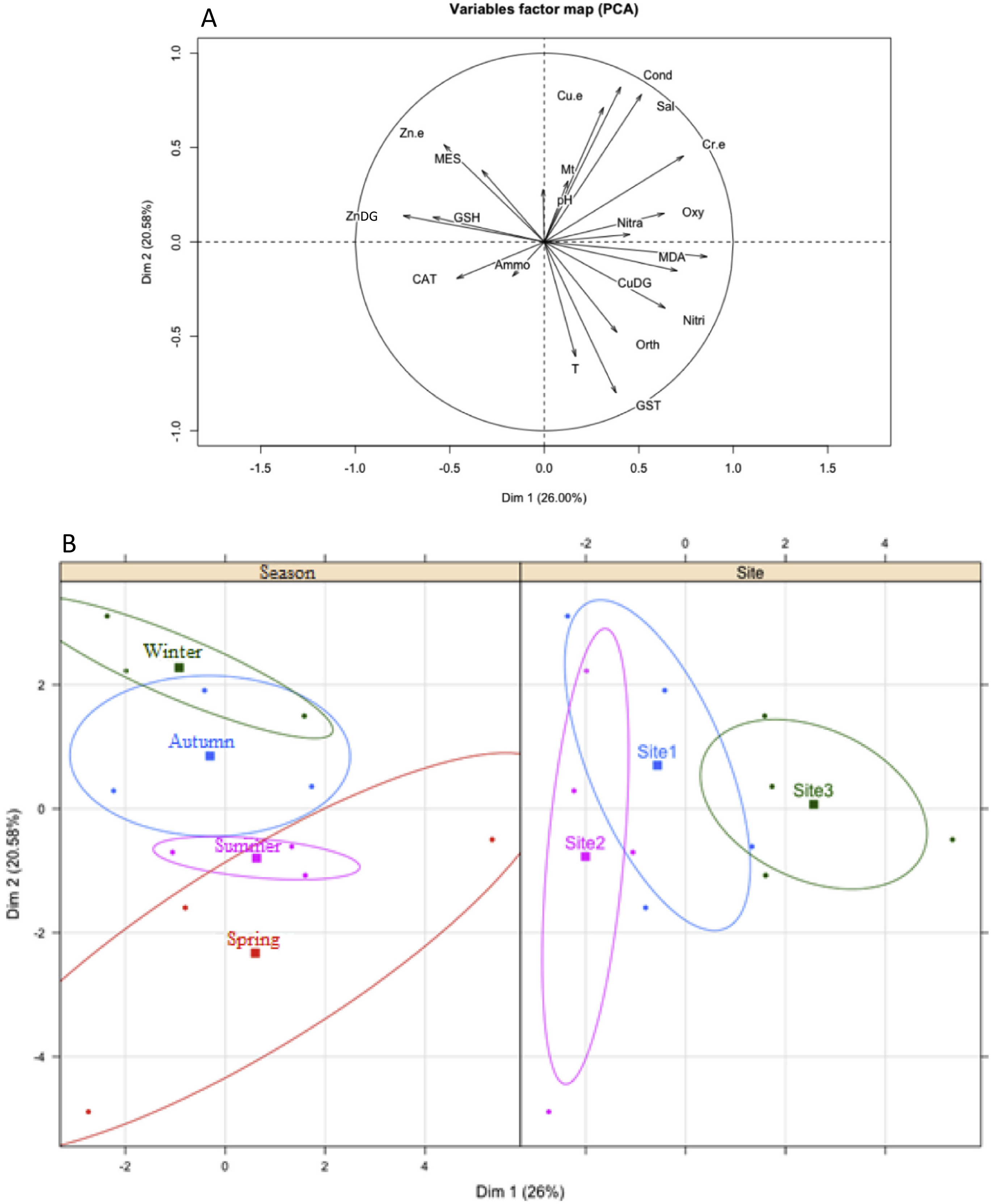

Fig. 5. Principal component analysis based on the spatiotemporal variation. Factorial plane: D1: 26%, D2: 20.58%. (A): Correlation circle of variables assayed with the first two principal axes. (B): Projection of seasons and sites on the first two principal axes.

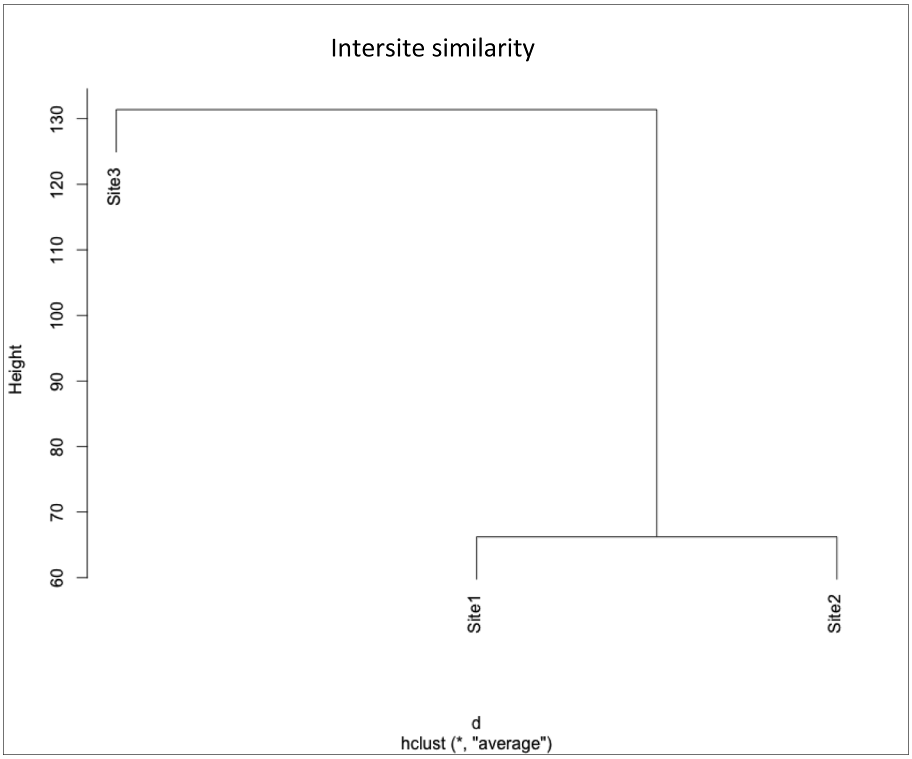

Fig. 6. Hierarchical ascending classification of sampling sites according to the variation of the measured parameters. Aouinète beach (Site 1), Messida beach (Site 2) and Cap de Garde (Site 3).

material resuspension by swell and tidal currents (Aminot and Kéroual, 2004a). Likewise, suspended solids concentrations showed no significant seasonal fluctuations; the waters appear a little less loaded in suspended solids in the spring, this would be explained by the dilution phenomenon caused by floods (Neal et al., 2000).

The measured TME in the surface seawaters of these three sampling sites revealed that the three elements (Cu, Zn and Cr) are present while the two others (Cd and Pb) are less than the limit of detection. The order of enrichment of these TME was identical for the three sites by placing the Cu in first position followed by Cr then Zn. This enrichment in TME would have for origin the numerous emissaries of wastewater from the surrounding agglomerations without any preliminary treatment, of which the El Kala littoral and especially the Annaba Gulf are the receptacles. Also, the use of motorized boats by the fishermen, all year long and those of Jet Ski in summer and autumn period could be at the origin of TME water enrichment. It is reported that fuels contain TME (Calamari and Naeve, 1994). Belabed et al. (2008) reports that Cu is strongly present in the sediment of many sites in the Annaba Gulf, suggesting that the main source in this element is telluric and would be related to the soil geology of this part of the coastline. In the mediterranean area, the importance of runoff and erosion is likely to increase transfers of pesticides based on Cu to surface waters. In the case of our study area, the main natural sources of exposure would be soil dust, plant decomposition and forest fires. In addition, these levels are probably related to industrial discharges (fertilizer manufacturing plant “FERTIAL”). Concerning Zn, it would come naturally from wind transport of soil particles and forest fires. But with regard to anthropogenic inputs, they would result from agricultural spreading (feeding of animals, slurry), urban activities (road traffic, garbage incineration), whereas Cr is used by several industries (colors and lacquers, photography films, wood, leather …). Belabed et al. (2013b) and Boutabia-Trea (2016) report the presence of these three TME in the sediment of the Annaba Gulf. Enrichment in TME during the wet and rainy season would be mainly due to the increase of metallic pollutants loads in the runoff waters of the first floods.

4.2. *Response of biomarkers to environmental stress*

The application of biomarkers to bioindicators is an important approach in the field of aquatic biomonitoring to assess the effects of and relationship between exposure to environmental pollutants and increased long-term effects on individuals and populations. The use of a battery of biomarkers in field surveillance has increased over the past decade.

Coastal waters are exposed to several disturbance mechanisms; chemical pollution associated with industrial production and high urbanization are among the most important concerns (Giarratano et al., 2010). The study of biological responses in sentinel species exposed to different contaminants has become a useful tool for assessing the quality of the environment. Many ecotoxicological biomarkers proposed over the past three decades based on molecular and cellular responses represent the first signals of environmental disturbance and are commonly used in biomonitoring programs (Moore et al., 2004; Viarengo et al., 2007). In the present work, seasonally monitored biomarker responses were measured in the digestive gland of *S. haemastoma*. On the whole, the results significantly highlight a spatiotemporal effect. The fluctuations in our biomarkers seem to reflect a disturbance of the environment.

GSH is a tripeptide (γ-L-glutamyl-L-cysteinyl glycine) that plays a central role in intracellular antioxidant defence processes due to its cysteine thiol group (Beldi, 2007). No seasonal variation in GSH levels was observed in *S. haemastoma*, but the results generally showed reduces rates compared to others such as those of Verlecar et al. (2008), Borvinskaya et al. (2016), Lavradas et al. (2016) and Amri et al. (2017b) who have demonstrated a seasonal variation in

GSH concentrations in the digestive gland of green-lipped mussel *Perna viridis*, the whitefish (*Coregonus muksun* and *Coregonus lavaretus*), *P. perna* and *Paracentrotus lividus*, respectively. To prevent the cellular damage caused by high ROS levels, GSH could be used for detoxification, resulting in its decrease (Gismondi et al., 2012). Several studies have helped elucidate the relationship between the decrease in the GSH rate and the level of environmental contamination; this was observed by Gorbi et al. (2008) in *M. galloprovincialis* transplanted in the Adriatic Sea and exposed to polluted TME sediments, and by Sifi et al. (2013) in *D. trunculus*. However, a significant effect of the sampling site was highlighted in the present work. Site 3 individuals had the lowest GSH level. This site represents the Annaba Gulf, which is subject to many permanent discharges of urban, industrial and port origin. Relatively high TME concentrations (Zn and Cu) were previously obtained in *D. trunculus* tissues in the same Gulf (Beldi et al., 2006). The decrease in GSH levels clearly indicates the stress of GSH-dependent detoxification pathways aimed at reducing peroxides to non-toxic, water-soluble primary alcohols. GSH is key in metal scavenging in the organism due to the high affinity of metals to its (−SH) group (Jozefczak et al., 2012). GSH has been proposed to complex and detoxify metal cations as soon as they enter the cells, representing a first line of defence against metal cytotoxicity. According to the literature, metal accumulation in biological tissues can result in the reduction of GSH availability (Lavradas et al., 2016). Verlecar et al. (2007) reported a decrease in GSH level in *P. viridis* exposed to TME. This decrease was observed in the digestive gland of *Crassostrea virginica* (Ringwood et al., 2004), in *P. perna* exposed to Cd and Cu (Khati et al., 2007, 2012), in the hepatopancreas of the freshwater crab *Sinopotamon yangtsekiende* (Wang et al., 2008), in *Ruditapes decussates* exposed to Cd (Khebbeb et al., 2010) and in the clam *Semele solida* exposed to anthropogenic pollution in the Chile Gulf (Srain and Rudolph, 2010).

The environment subjected to numerous pollutants imposes on certain animal species to develop mechanisms of detoxification of xenobiotics and resistance to oxidative stress. These mechanisms, including GST activity, are used as defence biomarkers (Amiard-Triquet et al., 2009). GST can be induced by certain pollutants and is therefore widely used as a stress biomarker (Cunha et al., 2007; Lam, 2009). In the present work, results related to the GST activity revealed a significant response absence of this enzyme with respect to site. Similar results were reported by Van der Oost et al. (2003), who showed similar activity between a reference site and a polluted site. However, seasonal variations were revealed. Maximal activity was noted in spring. Our results seem to be in agreement with the work of Guemouda et al. (2014) and Amri et al. (2017b), which showed differences in GST activity as a function of season, with spring peaks in the annelid polychaete *Perinereis cultrifera* and in the sea urchin *P. lividus*, respectively. Seasonal oscillations in GST activity were reported by Duarte et al. (2011), Giarratano et al. (2013), Schmidt et al. (2013), Jarque et al. (2014), Benali et al. (2015) and Balbi et al. (2017) in mussels; by Nahrgang et al. (2013) in *M. edulis* and *Chlamys islandica*; and by Fossi Tankoua et al. (2013), Barda et al. (2014), Braghirolli et al. (2016) and Louiz et al. (2016) in *Scrobicularia plana*, *Macoma balthica*, *Hyalella kaingang* and *Gobius niger*, respectively. Variations in GST activity would be due to an oxidative stress closely related to environmental factors and the environment quality such as food availability, physicochemical parameters of water (Mebarki et al., 2015), and the level of pollutants in tissues, which may vary with time. Moreover, the GST activity induction can be regarded as an adaptive response to an altered environment (Vidal-Liñán et al., 2010). This enzymatic induction catalyses the conjugation reaction between GSH and endogenous substrates and xenobiotics, allowing the formation of hydrophilic compounds which are less toxic and easy to eliminate (Cunha et al., 2007; Schmidt et al., 2012). This would be also likely due to the exposure of sites to various sources of pollution. Beldi et al. (2006) revealed an increase in GST activity as a function of water pollution in the Annaba Gulf, which is characterized by the introduction of polluants via the rivers and wadis (Chaoui et al., 2013; Bougherira et al., 2015; Keblouti et al., 2015). Similar results were obtained in the sea urchin *P. lividus*, in which GST activity was used as a biomarker of environmental contamination in a coastal area of Portugal (Cunha et al., 2005). Abbassi et al. (2015) have described a relationship between environmental pollution and GST activity in *M. galloprovincialis*. Several other studies showed higher GST activity in organisms from polluted sites when compared to those from reference sites (Sáenz et al., 2010; Bouzenda et al., 2017; Mejdoub et al., 2017). Increased GST activity has also been reported by several other authors in *D. trunculus* from Annaba Gulf (Sifi et al., 2007; Amira et al., 2011; Soltani et al., 2012; Bensouda and Soltani-Mazouni, 2014). Barhoumi et al. (2014) showed that GST was induced in *M. galloprovincialis* by organochlorine pesticides such as Dichlorodiphenyltrichloroethane (DDT). Likewise Lavarías et al. (2013) indicated that GST induced in the prawn *Macrobrachium borellii* exposed to organophosphate fenitrothion. GST activity variation may also be related to the age and the reproductive cycle (Lau et al., 2004; Giarratano et al., 2011).

Many authors have advocated using CAT as a biomarker in assessing the oxidative contaminants. CAT is the primary antioxidant defence involved in H_2O_2 detoxification, It has a very important role in the protection of aquatic invertebrates (Valavanidis et al., 2006). CAT activity in *S. haemastoma* showed relatively the same profile as GST, with a significant increase in spring. The CAT activity induction can be attributed to higher levels of exogenous hydrogen peroxide, which is the major cellular precursor of the toxic hydroxyl radical (OH^-) (Mejdoub et al.., 2017). Our results are supported by the work of Guemouda et al. (2014) and Amri et al. (2017b), which focused on the strong CAT activity in spring in *P. cultrifera* and *P. lividus*, respectively on the eastern coast of Algeria. Kamel et al. (2014) and Balbi et al. (2017) revealed seasonal variation in CAT activity in the digestive gland of *M. Galloprovincialis* and Barda et al. (2014) in *M. balthica*. These enzymatic responses, particularly those of CAT, can be modulated by seasonal changes in both environmental and biological factors, potentially affecting responsiveness and susceptibility to pollutants (Amiard-Triquet, 2009). This phenomenon has been described by Gorbi et al. (2008). Our outcomes reveal a site effect, demonstrated by an increase in CAT activity in individuals collected at site 2. The later receives discharges of wastewater and agricultural waste through the Wadi Messida, which is currently threatened by human activities (Benhalima et al., 2015). The increase in this activity at site 2 could then be explained by exposure to discharges from the Wadi Messida causing terrigenous inputs during periods of flooding. Thus, it is likely that environmental factors have an influence on CAT activity. Induction of this activity was recorded in *M. galloprovincialis* (Box et al., 2007); in the same species due to TME (Vlahogianni et al., 2007); in *P. viridis* due to organic contaminants (PAHs) and organochlorine pesticides (Richardson et al., 2008); in *Bathylomodiolus azoricus* due to metal contamination (Cu) (Company et al., 2008); in *R. decussatus* collected at several sites on the Tunisian coast following a pollution gradient (Jebali et al., 2007; Banni et al., 2009); and in *D. trunculus* (Tlili et al., 2010; Amira et al., 2011; Soltani et al., 2012; Bensouda and Soltani-Mazouni, 2014). El Jourmi et al. (2012, 2014, 2015) showed that this activity was high in *P. perna* in polluted sites compared to reference sites. Chandurvelan et al. (2015) demonstrated that CAT act to neutralise the ROS effects that can be generated by metal exposure.

Based on our results and the work described above, the CAT and GST activities are induced simultaneously and present a high values in spring period, it would be mainly caused by biological processes: This season corresponds to the period just before spawning. This later occurred between the end of spring and the beginning of the summer (Lahbib et al., 2011). The increased metabolic rates during spring period could possibly increase the rate of ROS formation and causes oxidative stress (Verlecar et al., 2007). According to Giarratano et al. (2013), the simultaneous induction of GST and CAT activities suggests a similar pattern for hydrogen peroxide elimination.

MDA is the byproduct of lipid peroxidation stimulated by ROS, which alters the cell membrane structure. The MDA content is closely related to the degradation of the cell membrane and reveals the effects of a xenobiotic penetration into the organism. MDA is therefore an early indicator of oxidative stress (Del Rio et al., 2005; Lykkesfeldt, 2007). Our results showed a site effect revealed by a slight increase in the MDA concentration at site 3 in the Annaba Gulf. In general, organisms with lowered antioxidant status could be more susceptible to lipid peroxidation, and therefore presenting higher levels of MDA (Giarratano et al., 2011). According to Soltani et al. (2012) and Sifi et al. (2013), the presence of large amounts of pollutants can overwhelm the antioxidant system, which caused lipid peroxidation of cell membranes by a high MDA level in *D. trunculus* at a site exposed to industrial pollution in the same Gulf. The increase in MDA concentrations, a potency marker of the lipid peroxidation, can be explained by environmental TME contamination, which cause overproduction of free radicals in the cell. It is known that the exposure of aquatic organisms to TME may increase ROS generation, which can lead to an imbalance in antioxidant defences, enhance oxidative stress and generate lipid peroxidation (Giarratano et al., 2013). Giguère et al. (2003) showed an increase in MDA concentrations in *Perna grandis* exposed to metal contamination in its natural environment. Tlili et al. (2010) noted the same observation in *D. trunculus* in a polluted site compared to a reference site in the Tunis Gulf. Similar results were reported by Vlahogianni et al. (2007) and Abbassi et al. (2015) in *M. galloprovincialis*; by Bergayou et al. (2009) in *S. plana* and *Cerastoderma edule* from an estuary; by Banni et al. (2009) and Kamel et al. (2014) in the digestive glands of *R. decussatus* and *M. galloprovincialis*, respectively; by El Jourmi et al. (2012, 2014, 2015) in *P. perna*; and by Khati et al. (2012) in the same species exposed to Cu and Cd. Machreki-Ajmi et al. (2008) noted an increase in MDA concentrations at a site with high Cd levels compared to a control site, as well as the oyster *C. gigas* exposed to diesel oil (Zanette et al., 2011) and *Chlamys farreri* exposed to ammonia nitrogen (Wang et al., 2012).

Mts are the proteins most susceptible to metal contamination in many marine organisms (Choi et al., 2007, 2008; Zorita et al., 2007). They are involved in homeostasis of essential metals such Cu and Zn, but also performs a major role in the detoxification of non-essential metals (Chandurvelan et al., 2015).

In this study, the Mt quantification in the digestive gland of the gastropod *S. haemastoma* revealed spatiotemporal fluctuations. The highest concentrations were recorded in individuals collected in winter and at site 3, which represents the Annaba Gulf. Kamel et al. (2014) noted seasonal variation in Mt in the digestive gland of *M. galloprovincialis*. Mt induction is considered a good biomarker of exposure to TME and is commonly used in environmental biomonitoring programs (Viarengo et al., 2007; Banni et al., 2007). Falfushynska et al. (2009) revealed that Mt was an adequate biomarker for environmental pollution in *Anodonta* sp. On the other hand, it was demonstrated that metallic elements such as Cu, Zn and Cd accumulated in the *R. decussatus* tissues exposed distinctly and/or to a mixture of these metals, causing strong induction of Mt synthesis (Serafim and Bebianno, 2010). De Montaudouin et al. (2010) and Paul-Pont et al. (2010a, 2010b) noted that the Mt concentration reflected metal contamination in *C. edule* and *Ruditapes philippinarum*. El Jourmi et al. (2012, 2014) showed high levels of Mt in *P. perna* at polluted sites compared to reference sites. The Mt induction has been reported by Ladhar-Chaabouni et al. (2009b) in *Cerastoderma glaucum* exposed to Cd, by Figueira et al. (2012b) in *R. decussatus* and *R. philippinarum* both exposed to Cd, by Figueira et al. (2012 (a)) and Freitas et al. (2012a, 2012b) in *C. edule* exposed to metals, by Khati et al. (2012) in *P. perna*, exposed to Cu and by Santoviro et al. (2015) in *Venerupis philippinarum* exposed to Cu and Zn. Ladhar-Chaabouni et al. (2009a) found that an increase in the amount of bioaccumulated Cd was followed by an increase in the Mt synthesis in the digestive gland of *C. glaucum*.

However, the Mt accumulation in the tissues could also be due to oxidative stress irrespective of the TME presence in the environment. Indeed, Banni et al. (2007) reported significant increases in Mt isoforms in terms of both protein and mRNA in *M. galloprovincialis* not exposed to TME but showing a state of oxidative imbalance. Other than metal exposure, Mt can also be induced by other factors, such as physicochemical factors (salinity, temperature, dissolved oxygen, etc.) and by the physiological state of the organism (age, sex, reproductive stage) (Boulajfene et al., 2017).

4.3. Trace metal elements (Cu and Zn) in the digestive gland

Their concentrations in the digestive gland of *S. haemastoma* revealed their accumulation, and monitoring of their concentrations showed similar seasonal fluctuations at the three study sites, with maximum bioaccumulation observed in spring and at site 3 for Cu and in winter and at site 2 for Zn. Season is an important factor, and numerous studies have shown that Cu and Zn concentrations measured in marine species vary seasonally. Our results are comparable to those observed by Beldi et al. (2006) and Belabed et al. (2008), in which a significant seasonal effect was revealed with relatively high levels of Zn in winter in the *D. trunculus* tissues at a polluted site and in *P. perna*, respectively, in the Annaba Gulf; by Boucetta et al. (2016b) in the marine gastropod *Phorcus (Osilinus) turbinatus* in the same Gulf; by Belhaouari et al. (2011) in *Osilinus turbinatus* and by Kamel et al. (2014) and Rouane-Hacene et al. (2015) in *M. galloprovincialis*. Thus, the analyzed elements (Cu and Zn) in the *S. haemastoma* gastropod presented marked seasonal fluctuations with a spring and winter maximum concentration of Cu and Zn, respectively. The increased Cu concentration in spring may be related to the release of TME in the water after the organic matters degradation of the colloidal and dissolved phases when water temperatures increase (Belabed et al., 2017). The winter Zn maximum concentration could be explained by the fact that at this time of year, storms act as a major restructuring force by changing significantly the manner of sediments granulometric distribution; this increases the sediments resuspension rate that can lead to higher remobilization rates and consequently, a significant bioavailability metals in sediments and water (Boutabia-Trea et al., 2015). Furthermore, during this cold period the increased rains led to an important inflow of fresh water to the coastal zone from the wadi system and so induce the salinity decrease. TME uptake and accumulation in body tissues is affected by element bioavailability which generally tends to increase with decreasing salinity, according to the free TME ions concentration in the surrounding seawater (Belabed et al., 2013b). Likewise, Cu and Zn concentration reached a maximum level during the cold period, which corresponding to a high storage period of energy reserves before spawning (corresponding to a period that body reserves have been depleted due to the reproduction).

It is generally accepted that the TME accumulation mainly occurs through three compartments: water, food and sediments. However, the efficiency of metals absorption from these sources may vary according to ecological needs, animal metabolism and TME concentrations in water, food and sediments, as well as other factors such as salinity and temperature. Importantly, the diet of our studied species (carnivorous) allows it to bioconcentrate and amplify TME in the digestive gland through feeding on animal prey (bivalves). It can be deduced that the accumulation of these metals in mussels may lead to an increase in metal concentrations in *S. haemastoma*. Biological factors such as food acquisition capability (Saavedra et al., 2004), sex (Sokolowski et al., 2004), age, size and spawning contribute significantly to the variation in TME bioaccumulation. Among abiotic factors, physicochemical factors (such as temperature, salinity, dissolved oxygen, and pH) of the environment play an essential role because they influence both the physicochemical form of the metals (and thus their bioavailability) and the metabolic processes of species (such as osmoregulation, respiration, reproduction, and trophic activity), which partly depend on the kinetics of metal accumulation and excretion. These environmental factors are site-specific and vary over time. The results of this study demonstrate the importance of anthropogenic inputs into the contamination of the Annaba Gulf and the El Kala coastline by TME: the highest levels of Cu and Zn were observed in the digestive gland of organisms inhabiting site 3 (Cu) located in the Annaba Gulf, which receives industrial (FERTIAL factory), urban and domestic discharges; and site 2 (Zn) located on the El Kala coast, which receives wastewater discharges and agricultural waste through the Wadi Messida. These high concentrations were primarily due to the main anthropogenic discharges from the Annaba Gulf, corresponding to the emissaries of untreated sewage due to the polluting loads carried by the Wadis Seybouse and Mafragh, Wadi Forcha, Wadi Sidi Harb, Wadi Edheb, Wadi Kouba, Wadi Bouhdid and Wadi Bedjima. These polluting loads are also influenced by port activity on the one hand and a major road axis on the other hand. In the marine environment, coastal areas close to estuaries or rivers are subject to periodic anthropogenic contamination by TME. Previous studies have highlighted the importance of the FERTIAL industrial complex in the emission of various discharges into the atmosphere and water of the Annaba Gulf (Belabed et al., 2008). In addition, Cu and Zn have been detected in sediments of the same Gulf (Abdennour et al., 2010; Belabed et al., 2013b, 2017; Boutabia-Trea et al., 2017) and in lake Tonga's surface sediments (Messida Wadi is the Tonga lake's connection with the Mediterranean Sea) (Belabed et al., 2013a). The low levels found at site 1 could be explained by the remoteness of this site from the major sources of contamination.

To authorize the consumption of these edible organisms, international legislations of regulatory agencies have fixed the maximum allowable concentrations of some TME as follows: for the United states Environmental Protection Agency (USA EPA) Cu 20 $\mu g\ g^{-1}$ wet weight and Zn 30 $\mu g\ g^{-1}$ wet weight; and for the Food and Agriculture Organisation of the United Nations (FAO) Cu 30 $\mu g\ g^{-1}$ wet weight and Zn 50 $\mu g\ g^{-1}$ wet weight (Lavradas et al., 2016). By comparing the average concentrations of TME measured in our species with the sanitary thresholds tolerated, it appears that the Cu and Zn levels constitute a danger to consumers, since they are higher than the recommended maximum allowable doses. This bioaccumulation is likely to affect the physiological processes of this gastropod, which has been demonstrated in bivalves (Merzouki et al., 2009).

4.4. PCA and HAC

Axis 1 explains a clear difference between the group consisting of two sites 1 and 2, which represent the El Kala coastline, and that of site 3 representing the Annaba Gulf, which is characterized by high rates of MDA, Cr.e, CuDG, Nitri, Oxy and Sal, and lower levels of ZnDG, GSH and Zn.e compared to other sites.

Axis 2 allowed us to identify the specificity of site 2 compared to other sites because it is characterized by lower rates in Cond, Sal, Cu.e and Zn.e, and higher rates in T and GST compared to other sites. Moreover, axis 2 highlighted a seasonal pattern that distinguishes between the spring-summer group and the autumn-winter group. It can be concluded that axis 1 differentiates the sites and axis 2 differentiates the seasons.

In general, the typology of the dendrogram obtained by the HAC agrees with our results regarding the spatiotemporal variation in the measured parameters. The induction of GSH-dependent detoxification pathways and increased lipid peroxidation were more marked during spring at site 3, which represents the Annaba Gulf. Indeed, according to several works cited above, metal pollution is relatively high in water and sediments in this Gulf, particularly during this period.

5. Conclusion

This work provides an example of antioxidant multibiomarkers potentiality, emphasizing the importance of setting how the seasonality affects the antioxidant status of the gastropod *S. haemastoma*. A global analysis of *S. haemastoma* responses of a parameters battery reflects a discrimination of sites. The interpretation of these responses in an environmental context is very complex taking into account all possible causes. Biomarkers responses could be allocated to differences in both pollution levels and seasonal variability. It could be concluded that there were direct or indirect effects of climatic conditions and/or other seasonal exogenous and endogenous factors on the variation in the measured parameters. Change of season means change of the whole complex of these factors. It has also been shown that TME founded in water and the digestive gland of *S. haemastoma* are significant and clearly reflect the pollution levels in both coasts. This first study in the area confirm that variations of antioxidant parameters could be used as prospective biomarkers of toxicity in environmental monitoring programs; and demonstrate that *S. haemastoma* is a useful tool in biomonitoring of aquatic pollution and can be employed as a sentinel species. Referring to international guideline values the concentration levels detected in our samples indicate significant contamination by TME, so *S. haemastoma* can be considered as contaminated and seems to have an ability to accumulate TME. Finally to better understand the direct effect of these pollutants, this work needs more detailed studies *in vivo* (experimental data).

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