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Biomarker responses to pollution in the Mediterranean green crab *Carcinus eastuarii* living in the Gulf of Gabès (Tunisia)

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Abstract

The central part of the Gulf of Gabès, in south-eastern Tunisia, is considered as one of the most sever pollution hotspots in the Mediterranean. However, because detailed studies are lacking little is known on the impact of pollution on the coastal organisms living in this area. We investigated this issue by using the Mediterranean green crab *Carcinus aestuarii* (Nardo, 1847) as a model species, and by considering a combination of morphological biomarkers (body condition and fluctuating asymmetry level) and biochemical ones (the activities of lactate dehydrogenase, alkaline phosphatase, as well as alanine and aspartate aminotransferase in the hemolymph and hepatopancreas). Our general approach was based on the comparison between crabs collected close to Gabès-Ghannouche factory complex of phosphate treatment, which is the source of coastal and marine pollution in this area, and crabs collected in one less contaminated site situated twenty-five kilometres faraway. We found that crabs living close to the factory complex were characterized by poorer body condition, higher level of fluctuating asymmetry and overall higher enzyme activities. These results show that the proximity to Gabès-Ghannouche factory complex was associated with remarkable perturbations in crab physiology, condition and development, revealing possible dangerous impacts of pollution on the whole coastal biodiversity in the Gulf of Gabès. Our results also stress once again, the usefulness of the Mediterranean green crab as a reliable species for biomonitoring in the polluted coastal habitats of the Mediterranean.

1. Introduction

Pollution of aquatic ecosystems results **g**enerally in dramatic effects on the inhabiting organisms at different levels [1]. This issue has received much attention during the last years, especially because of the negative repercussions of water pollution in terms of human health and environment quality [2]. Because xenobiotics can be stored in various forms, such as insoluble precipitates and concretions, pollutant levels do not always give reliable information about their toxicological significance [3]. Thus, the potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting polluted aquatic ecosystems has received increasing attention during the recent years [1-4]. Biomarkers can be defined as functional measures of exposure to pollutants at the sub-organismal, physiological and behavioural levels [5]. Because biological responses to water pollution vary according to a complex mixture of environmental and biological factors, biomonitoring works should be based on a multiparametric approach, using different and complementary biomarkers in order to assess, in a broad and synthetic manner, the effects of pollution on living organisms [6-7].

At the organism level, body condition provides a particularly useful biomarker as the most simple and easily measurable parameters.

Pollution is generally assumed to result in a remarkable decrease in body condition [8-9]. One further and more largely popular organism-level biomarker is the degree of fluctuating asymmetry (FA) in bilateral morphological and anatomical traits [10-11]. FA, which refers to the random deviation from perfect symmetry in normally symmetrical characters, reflects the disruption of developmental processes and provides one

measurable descriptor of environmentally induced developmental instability [12]. Such a biomarker is increasingly recognised as a reliable tool in assessing pollution effects and has been proposed to be used as a first step of an environmental monitoring program to identify stressed populations, regardless of the pollutants involved [13]. At the molecular level, some enzymes, especially those involved in biotransformation systems, are commonly used as key biomarkers of environmental pollution [14-15]. These molecular biomarkers have proven to provide a strong tool for specific early warning signs for aquatic pollution in a wide variety of animal species [16]. For instance, changes in the activities of Lactate dehydrogenase (LDH), alkaline and acid phosphatases (ALP and ACP), and alanine and aspartate aminotransferases (ALAT and ASAT) are commonly used as molecular bioindicators of the response of aquatic organisms to chronic exposition to various contaminants, such as metals and other xenobiotics [17-18].

With regard to the Mediterranean, studies using the biomarkers approach to assess the impact of water and sediment pollution on the living organisms have predominantly been concerned with polluted marine and coastal areas in Mediterranean Europe [19]. Detailed data on this issue from polluted areas along the southern border of the Mediterranean are relatively poor. For instance, the Gulf of Gabès, in south-eastern Tunisia, is nowadays listed as one of the most remarkable pollution hotspot in the Mediterranean, mainly due to the development of intense industrial activities in Sfax and Gabès cities [20]. In particular, the installation in the early 1970s of intense phosphate treatment industries for acid and fertilizer production in the Gabès-Ghannouche industrial complex (Figure 1), has resulted in the discharge of huge quantities of phosphogypsum, containing heavy metals such as cadmium, copper, zinc arsenic, chromium and lead in the sea [21-22]. Although detailed information are lacking, it has been proposed that during the first 20 years following the installation of Gabès-Ghannouche phosphate industries, 50 millions tonnes of phosphogypsum have been discharged into the sea [23]. This has resulted in the pollution of the nearby marine and coastal environments, leading notably to the rapid degradation of the sea grass *Posidonia oceanica* meadows and a remarkable loss of the associated biodiversity [24].

Studies dealing with pollution effects in the marine and coastal areas close to this industrial complex have mainly been concerned with quantifying the levels of pollutants, especially those of heavy metals in water [25], sediments [24-25-26] and more rarely in animal tissues [27-28]. However, biomarker-based works specifically conducted to assess the impact of pollution on the organisms living in this particular area are lacking. Such investigations are nonetheless essential for drawing a more complete picture on the environmental health in this gulf and for assessing the associated risks against humans.

In this study, we investigated this issue by using the Mediterranean green crab (*Carcinus aestuarii*) as a model species, and by considering different morphological and biochemical biomarkers. In doing so, we also tested the relevance of different common biomarkers in assessing the effect of pollution in the particular case of the Gulf of Gabès. The biomarkers used were the following: body condition and fluctuating asymmetry level in the legs as morphological biomarkers, and the activities of LDH, ALP, ALAT and ASAT in the hemolymph and hepatopancreas as biochemical biomarkers. Our general approach was based on the comparison between crabs exposed to two different pollution levels by sampling crabs from one contaminated site situated in the vicinity of the Gabès-Ghannouche industrial complex, and one less contaminated site situated faraway. Our specific objective was to answer the following question: Do crabs living in the contaminated site present lower body condition such as higher levels of fluctuating asymmetry and enzyme activities compared to those living in the less contaminated site?

2. Experimental details

2.1. Study area

This study was conducted in the coastal area south to Gabès City in the central part of the Gulf of Gabès in south-eastern Tunisia (Figure 1). This area is predominantly composed of mudflats with gentle declivity and that are largely emerged during low tides. Tide movements in this area are among the highest in the Mediterranean. In the spring tides can attain locally 2.1m (National Geospatial-Intelligence Agency 2002). As mentioned above, the coastal and marine habitats in this area have been polluted by considerable amounts of phosphogypsum discharged by the industries of phosphate treatment installed since 1971 in the Gabès-Ghannouche industrial complex.

Our sampling work was carried out in two coastal sites situated at different distances from Gabès-Ghannouche industrial complex (Figure 1). The first site is located in front of the industrial complex where the phosphogypsum is discharged and is likely to be the most polluted site. The second sampling site, namely Kettana Bay, is situated at 25 km south to Gabès-Ghannouche Industrial Complex (Figure 1) and is less affected by pollution.

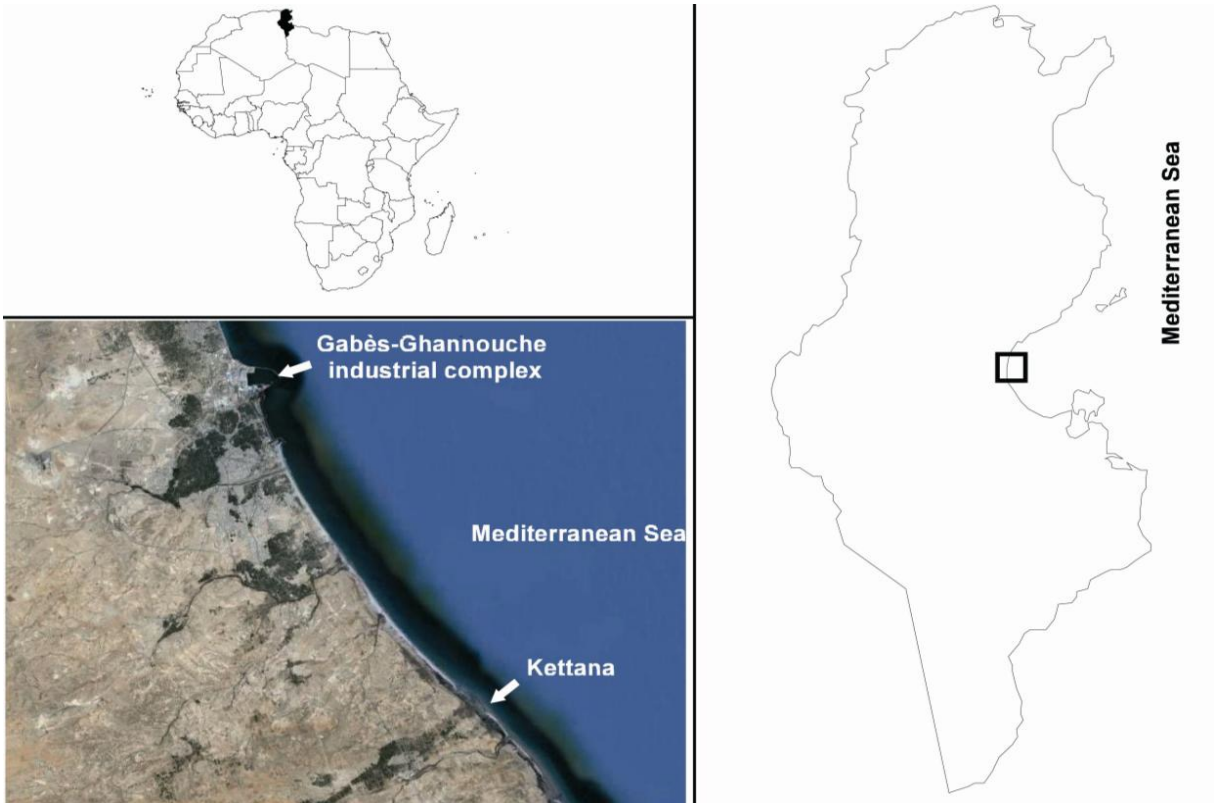

Figure 1: Map of the Gulf of Gabès showing the locations of the two study sites.

1.2. Study species

The Mediterranean green crab *Carcinus aestuarii* is a Mediterranean-native crab species that inhabits a great variety of coastal habitats, ranging from estuaries and lagoons to muddy bays, sandy banks and rocky shores [29]. It can be distinguished from the closely related Atlantic green crab (*Carcinus maenas*) by the form of the gonopods, the shape of the frontal area between the eyes and the length-width ratio [30]. *Carcinus aestuarii* is a generalist species that shows a great tolerance to environmental changes [30] and can subsist on a large spectrum of food items, including marsh vegetation, algae, crustaceans, molluscs and fishes [32].

Crabs have previously been proven to provide valuable biomonitors [33-34].Their ubiquitous habits and their capacity to accumulate contaminants from water and other food origins make them particularly useful in biomonitoring programmes [34-35]. The Mediterranean green crab was used as a model species in this study because it abundantly occurs in the coastal areas of the Gulf of Gabès and is a non-protected species that can readily be taken without restrictions.

1.3. Data collection

1.3.1. Crab sampling

During summer 2012 (June – July), 30 *C. aestuarii* male crabs (carapace width between 30 and 40 mm) were sampled in each of the two sites of study. All crabs were sampled during their inter-moult period to avoid possible influences of moult-related physiological events on biomarker responses [34]. Crabs were captured using a 15 mm-mesh fishing net at a depth less than 5 m. We avoided sampling crabs living in the intertidal zone because routine emersion during low tide is likely to induce physiological changes that may bias our results. The collected crabs were immediately placed into one plastic pail with seawater and rapidly transported to the lab for tissue sampling and morphological measurements. Time between crab capture and laboratory measurements and analyses did not generally exceed 2 hours.

Upon arrival at the laboratory, each crab was dried with a filter paper and then weighed to the nearest 0.01 g with an electronic digital scale. The crab was then chilled on ice for five minutes before hemolymph and hepatopancreas collection. This chilling step was necessary to prevent the coagulation of hemolymph and clumping of hemocytes [17]. Using a 1-ml plastic syringe, at least 500 µl of hemolymph were collected from the base of the third walking leg. The hemolymph sample was then transferred to an Eppendorf tube and centrifuged at 3,000 rpm for 10 min, and the supernatant was used for enzymatic analyses. The carapace of the crab was

also opened on the ventral face and the hepatopancreas was collected. The hepatopancreas was rinsed with distilled water to remove the adhering hemolymph and then blotted with a filter paper before it was homogenised (1:4 w/v) in cold phosphate buffer (0.1 M, pH 6.5). The homogenates were centrifuged for 10 min at 5,000 rpm and the supernatants were collected for enzymatic analyses [17]. The carcass of the crab was also preserved in 70° Ethanol for later morphological measurements.

1.3.2. Biochemical analyses

Enzyme activities were performed according to automated spectrophotometric methods. Final temperature of measurements was 37 °C. Alanin aminotransferase activity (ALAT, E.C. 2.6.1.2) and Aspartate aminotransferase activity (ASAT, EC 2.6.1.1) were assayed by the method of Bergmeyer et al. [36], using commercial Biomaghreb kits (Ref, 20046 and 20042, respectively). The rate of NADH depletion was determined photometrically (340 nm) as the catalytic rate concentration of ALAT or ASAT in the analyzed sample. The method of Wenger [37] was applied to measure the alkaline phosphatase activity (ALP, EC 3.1.3.1.) by using Biomaghreb kits (Ref, 20015). The rate of p-Nitrofenilphosphate hydrolysed to form p-Nitrofenol was monitored photometrically at 405 nm. Finally, lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed according to Vassault [38] method using Biomaghreb kits (Ref, 20011). The depletion rate of NADH as substrate was performed at 340 nm. The hepatopancreas samples were also used to estimate total protein content by applying the kit protein assay from Biomaghreb (Ref, 20161) based on the Biuret Method [39] using crystalline bovine serum albumin as standard. Enzyme activities in hepatopancreas samples were expressed as nmol of substrate degraded per min per mg of protein, while those in Hemolymph samples were expressed as nmol of substrate degraded per min per ml.

1.3.3. Morphological measurements

The preserved carcasses of the 60 sampled crabs were first used to measure carapace width as the distance (in mm) between the two lateral spines. We then randomly selected 30 carcasses (15 from Gabès-Ghannouche and 15 from Kettana) to measure the length (in mm) of the three pairs of walking legs (pairs 2, 3 and 4) and the pair of swimming legs (pair 5). The latter data were used to investigate the degree of asymmetry between right and left legs. All these measurements were carried out by the same observer (N. Hayder) using a digital calliper to the nearest 0.01 mm and were repeated three times in a random order. Following the procedure described by Lessells and Boag (1987) [40], we found that all morphological measurements were highly repeatable ($r > 97\%$ and $P < 0.0001$ for all traits).

1.3.4. Data analyses

For each sampled crab we calculated the average weight and carapace width. These data were used to derive an index of body condition as the residuals of the linear regression on weight (in g) as a function of carapace width (in mm). We then used ANOVA to assess the significance of the relationship between body condition and sampling site (Gabès-Ghannouche *vs* Kettana). We also calculated the average length (in mm) of each measured leg based on the three repeated measurements we carried out. These data were used to calculate, for each pair of legs, the difference between the right leg and the left one (R-L). In order to ensure that asymmetry distributions did not show either antisymmetry or directional asymmetry we verified for each pair of legs if (R-L) was normally distributed around a mean of zero using Shapiro-Wilk and one-sample *t* tests. We then calculated a size-corrected index of fluctuating asymmetry for each pair of legs i: $FA_i = |R_i - L_i| / (0.5 * (R_i + L_i))$. The four fluctuating asymmetry indices obtained for each crab were summed together to obtain one composite measure of fluctuating asymmetry that should provide a more reliable indicator of developmental competence [41]: $FA = \Sigma FA_i$. Finally, the obtained FA data were used to asses if the degree of fluctuating asymmetry in crab legs differed significantly between the two studied sites by means of ANOVA.

With regard to enzyme data, given that our variables were interrelated, the whole significance of enzyme activity variation as a function of site (Gabès-Ghannouche *vs* Kettana) was assessed by means of a multivariate ANOVA (MANOVA). Complementary separate ANOVAs were also carried out to more profoundly explore the direction of the relationship between the activity of each enzyme, as a response variable, and site as an explanatory variable.

All conducted analyses and tests were carried out using SAS package (SAS 1998). The linear regressions, ANOVA and MANOVA were carried out using the GLM procedure. The normal distribution of variables used in these linear models was previously verified by means of Shapiro-Wilk tests using the UNIVARIATE procedure (Shapiro-Wilk test: $P > 0.05$ for all analyses). The same procedure was also used to verify the normal distribution of (R-L) variables used in FA analyses, as well as to verify if means (R-L) departed significantly from zero. All estimated means and parameter effects (betas) are reported ± 1SE.

3. Results and Discussion

To our knowledge, this is the first biomarker-based investigation of pollution-induced stress in the polluted coastal area close to Gabès City in southern Tunisia. Using a combination of morphological and biochemical biomarkers, we found that the proximity to Gabès-Ghannouche factory complex of phosphate treatment, which is the main source of coastal and sea water pollution in this area, was associated with acute perturbations in the development, health and physiology of the Mediterranean green crab *Carcinus aestuarii*.

3.1. Body condition and fluctuating asymmetry

Crab weights ranged from 7.16 to 12.44 g (mean±SE = 9.63±0.27) in Gabès-Ghannouch sample and from 8.23 to 13.55 g (mean±SE = 10.96±0.28) in Kettana sample. The results of the regression on crab weight as a function of carapace width show a strong positive relationship between the two parameters (r^2 = 64%, β = 0.54±0.05, $F_{1,58}$ = 102.66, $P < 0.0001$). Using the residuals of this regression model as a measure of crab body condition, we found that crabs from Gabès-Ghannouch have significantly lower body condition index compared to those from Kettana bay (ANOVA: r^2 = 48%, $F_{1,58}$ = 52.69, $P < 0.0001$; Figure 2).

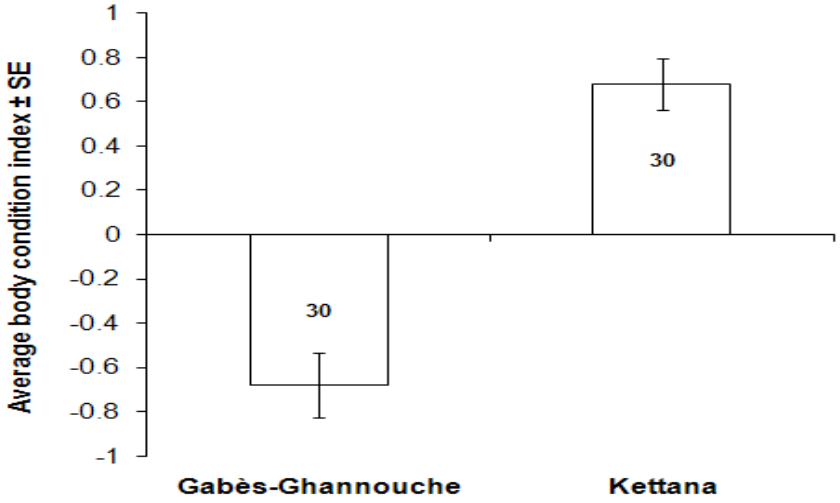

Figure 2: Comparison between crabs from the two sampled sites regarding body condition. Numbers on the bars indicate sample sizes.

With regard to fluctuating asymmetry, we first found that the (R-L) metrics relative to the four measured pairs of legs followed normal distributions around a mean of zero (Table 1). These results suggest that the observed asymmetry was fluctuating and could not be attributed to antisymmetry or directional symmetry. Furthermore, we found that the composite FA score, obtained by considering the overall fluctuating asymmetry in the walking and swimming legs, varied significantly between the two sampled sites (ANOVA: r^2 = 26%, $F_{1,28}$ = 9.69, $P < 0.0042$). Crabs from the polluted site exhibited an overall higher level of leg fluctuating asymmetry than those living in the less contaminated site (Figure 3).

Table 1: Statistical properties of signed (R-L) for the four measured pairs of legs (pair 2 to 5) and results of tests of normality and mean difference with zero. N = sample size.

Leg pair	N	Mean±SE	Skewness	Kurtosis	Student t-test for "mean = 0"		Shapiro-Wilk normality test	
					t	*P*	W	*P*
P2	30	-0.33±0.23	0.57	1.91	-1.44	0.1615	0.93	0.0598
P3	30	0.13±0.41	0.65	1.38	0.30	0.7636	0.96	0.2450
P4	30	0.21±0.23	0.86	2.33	0.88	0.3886	0.94	0.1193
P5	30	-0.16±0.25	-0.41	-0.12	-0.62	0.5374	0.95	0.1308

As expected, we found that crabs living near the factory complex were characterized by poorer body condition than those living in the control area and, hence, less exposed to pollutants. For comparable carapace width, crabs living near the factory complex were overall lighter than those living in the control area. We also found that the proximity to Gabès-Ghannouche factory complex of phosphate treatment was associated with a remarkable increase in the level of fluctuating asymmetry in crab legs. This result would suggest that the

stressful conditions suffered by crabs living in the polluted area negatively affected the harmony of their development, causing abnormal asymmetries in traits that should normally grow symmetrically.

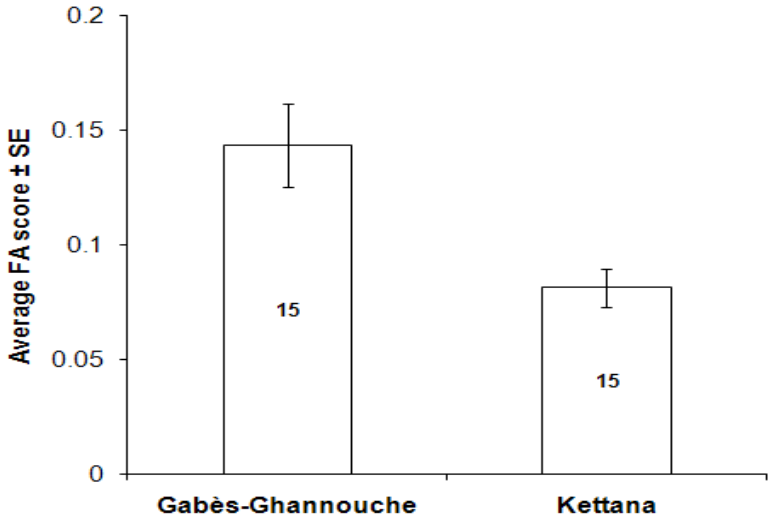

Figure 3: Comparison between crabs from the two sampled sites regarding fluctuating asymmetry score. Numbers on the bars indicate sample sizes.

Overall, these results stress once more that in spite of their simplicity, body condition and fluctuating asymmetry provide reliable tools for assessing the effects of pollution on the living organisms. It is, nonetheless, to be underlined that the mechanisms linking the observed degradation in crab body condition and developmental stability to habitat pollution remain unknown. It could be that under stressful conditions, energy reserves are channelled into energy-consuming detoxification processes, which reduces energy allocation to tissue production resulting in abnormal development and growth [8]. Alternatively, it could be that pollution decreased the availability of suitable food items, which may had caused food shortage and reduced energy intake. Further experimental investigation of crab body condition and fluctuating asymmetry in relation to water origin (polluted *vs* control area), and using a standard feeding protocol, would permit us disentangling the effect of food shortage and that of detoxification processes on crab body condition and development.

3.2. Enzyme activities

The results of MANOVA on enzyme variables as functions of site show an overall significant relationship (Wilks $\lambda = 0.11$, $F_{8,51} = 52.55$, $P < 0.0001$). All considered enzymes presented higher activities in the hemolymph samples from the polluted site compared to those from the less contaminated site (Table 2, Figure 4). However, hepatopancreas data show that the activities of ALAT and ASAT were higher in Gabès-Ghannouche samples while non-significant differences were found for LDH and ALP (Table 2, Figure 4).

Table 2: Results of the ANOVAs on enzyme activities in the crab hemolymph and hepatopancreas as functions of site (Gabès-Ghannouche *vs* Kettana). N = sample size.

Enzyme	Tissue	N	Model r^2 (%)	F	*P*
LDH	Hemolymph	60	32	26.68	< 0.0001
	Hepatopancreas	60	1	0.50	0.4821
ALP	Hemolymph	60	13	8.48	0.0051
	Hepatopancreas	60	3	1.68	0.2001
ALAT	Hemolymph	60	57	76.93	< 0.0001
	Hepatopancreas	60	60	85.91	< 0.0001
ASAT	Hemolymph	60	57	78.20	< 0.0001
	Hepatopancreas	60	63	100.14	< 0.0001

With regard to biochemical biomarkers, our results show an overall increase in enzyme activities in the tissues of crabs living in the polluted site compared to those living in the less contaminated site. However, for two enzymes out of the four measured, namely Lactate dehydrogenase (LDH) and Alkaline phosphatase (ALP), the activity changes between the two studied sites were more perceptible in hemolymph samples compared to hepatopancreas ones. This suggests that in the studied crabs, the biochemical composition of the hemolymph

was more sensitive to pollution-induced stress than that of the hepatopancreas, which would indicate that hemolymph provides more appropriate samples for detecting crab biochemical responses to habitat pollution than do the hepatopancreas ones.

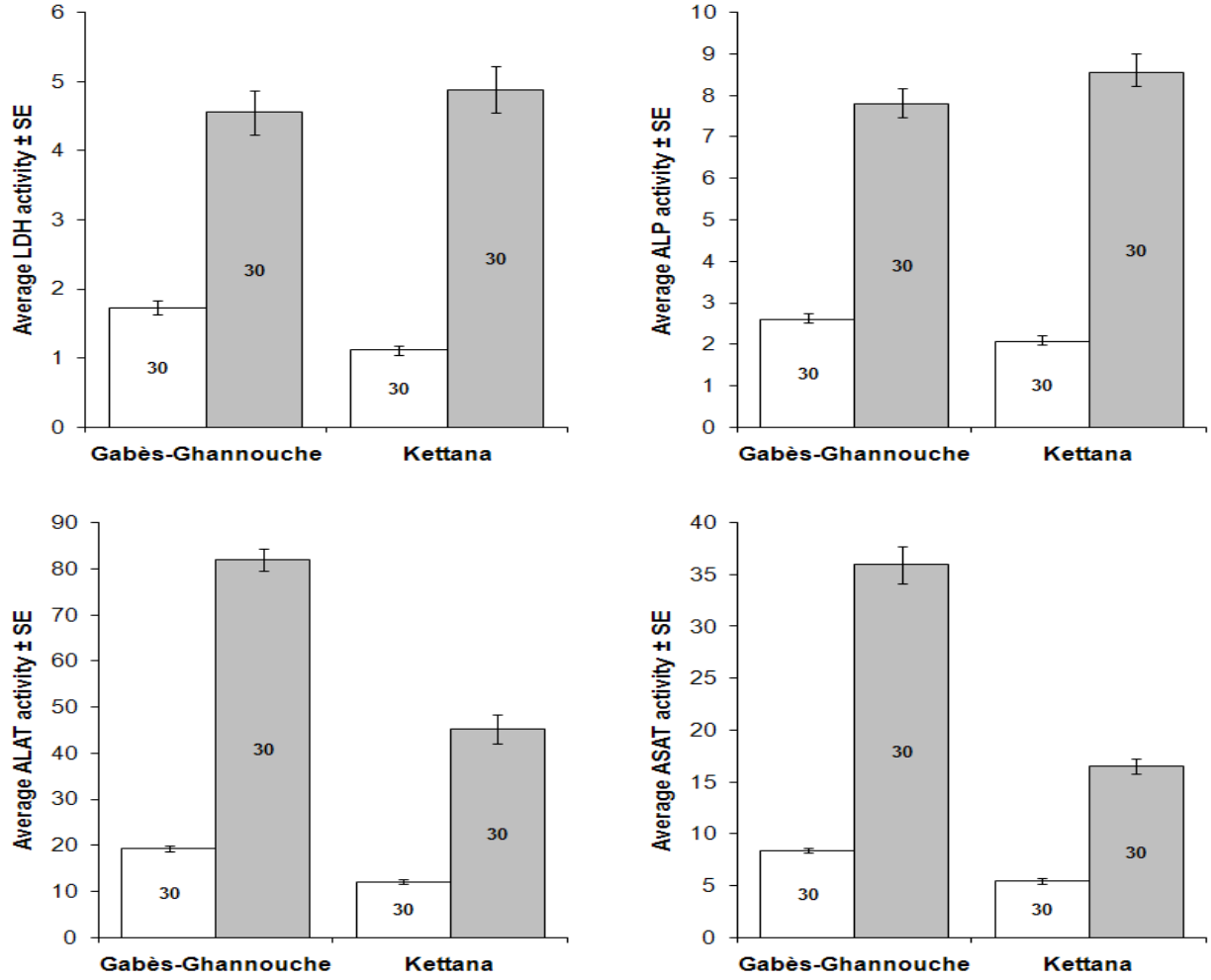

Figure 4: Variation in enzyme activity in crab hemolymph (white) and hepatopancreas (grey) between the two sampled sites. Enzyme activity is expressed as nmol/min/ml for hemolymph and as nmol/min/mg proteins for hepatopancreas. Numbers on the bars indicate sample sizes.

On the other hand, given that Alanine and aspartate transaminanses (ALAT and ASAT) showed remarkably higher activities in both the hemolymph and hepatopancreas samples from the polluted area compared to those from the control site, they are likely to be more reliable biomarkers of pollution stress than LDH and ALP in our study system. The LDH activity is generally used as an indicator of the anaerobic capacity of a tissue [42]. Any change in protein and carbohydrate metabolism may cause change in LDH activity [43-44]. It has been used as a biomarker of hypoxia [45] and in situations of chemical stress [46], when organisms require additional energy. In fact, a situation of pollution stress is usually followed by hypoxic conditions, which increases the anaerobic pathway resulting in the enhancement of LDH. The observed increase in LDH activity level in the hemolymph of crabs from the polluted site could thus be due to the interference of pollutants with energy production pathways by the induction of anaerobic glycolysis to meet the required energy demands.

This enzyme also plays major roles in the molting physiology of many crustaceans, as it is involved in calcification and mineralization processes during molting cycle [47]. Because the administration of pollutants alters the activity of ALP, this enzyme is increasingly recognised as a good biomarker in ecotoxicological studies [48]. However, different trends have been reported regarding the sense of the relationship between pollution and ALP activity, depending on the type and concentration of pollutants involved and the organs sampled. The result of our study is rather in agreement with increasing ALP activity with pollution level. However, this result remains difficult to explain unless the pollutants contents of seawater and sediment in the contaminated site were known and the factors responsible of the observed change were identified. We believe

that in our studied polluted site, ALP is likely to be affected by a mixture of metals, which complicates the interpretation of the observed trend.

Changes in the activities of these aminotransaminases (ALAT and ASAT) reflect stress-induced variations in protein metabolism [49]. ALAT is known to function as a link between carbohydrate and protein metabolism under altered physiological, pathological, and induced environmental conditions through its role in mobilizing L-amino acids for gluconeogenesis (GNG) [50-51]. This enzyme is frequently used in the diagnosis of damage caused by pollutants in various tissues [52]. The elevation of ASAT activity provides the oxaloacetate required for the gluconeogenesis pathway to meet the additional supply of glucose for the production of energy under reduced phase of oxidative metabolism [53]. The abnormally elevated activities of ALAT and ASAT that we observed in crabs living in the polluted site is likely to express the need to enhance energy production and/or gluconeogenesis processes by generating ketoacid-like ketoglutarate and oxaloacetate, in order to meet the excessive energy demands that occur under pollution stress. Similar findings have been reported in related crab species [54].

Conclusions

In conclusion, our work provides evidence that the huge quantities of phosphogypsum and related heavy metals that have been released by the Gabès-Ghannouche factory complex of phosphate treatment since the 1970s had negative effects on the Mediterranena green crab *Carcinus aestuarii* living in the neighbouring coastal area at both the organismal and molecular levels. They also show the usefulness of this species as a reliable monitor of this particularly polluted, but poorly known, area.

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