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Integrated assessment of biochemical responses in Mediterranean crab (*Carcinus maenas*) collected from Monastir Bay, Tunisia

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Abstract

The biochemical response of Mediteranean Crab (*Carcinus maenas*) collected at five stations of Monastir Bay and from Kuriat station as control was studied using a set of complementary biomarkers. The catalase, glutathione S-transferase, lactate dehydrogenase, acetycholinesterase activities; and metallothionein and malonediladehyde levels in gills were evaluated. Results revealed differences among sites in relation to each specific biomarker. Hence, a suite of biomarkers can be used to discriminate sampling sites according to types of pollution, reflecting differing conditions of anthropogenic impact. Based on Integrated Biomarker Response, the highest values and critical biochemical alteration were observed at Khniss and Ksibat in response to urban and industrial discharges and the lowest IBR value was found at reference site. The current study has shown clearly that a biomarker-based index is usefulness tool in the monitoring Tunisian coast using *C. maenas* as sentinel specie. Further studies in progress to investigate the seasonal variations of IBR levels and its relationship to pollutants concentrations in the sediment, gills and digestive gland of *Carcinus maenas* from Monastir Bay.

Key words: *Carcinus maenas*; acetycholinesterase; metallothionein; glutathione S-transferase; lactate dehydrogenase; catalase; malonedialdehyde

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Introduction

Organisms in polluted ecosystem, develop various physiological and biochemical strategies facing the toxicities of chemicals pollutants. Many biochemical techniques have been developed in the last decade to examine and monitor environmental pollution. The biochemical endpoints (biomarkers) can provide valuable information regarding the working mechanism of toxic compounds and be used as early reporters related to endpoints at higher levels of biological organization. Various biomarkers in crustacean species have been used as a tool for ecotoxicological assessments (Martín-Díaz et al., 2007, 2008; Ghedira et al., 2009; Pereira et al., 2009).

The catalase activity and malonediladehyde (MDA) levels are sensitive, widely used biomarkers of bivalve exposure to chemicals pollutants in the Tunisian coast area (Banni et al., 2005; Jebali et al., 2007); whereas few in-field studies interested in the validation of theses biomarkers in crustacean species. Among other enzyme biomarkers, measurements of crustacean acetylcholinesterase (AChE) and glutatione S-transferase (GST) activities have become tools used in the biomonitoring of marine waters (Astley et al., 1999; Martín-Díaz et al., 2008; Morales-Caselles et al., 2009). GST conjugate electrophilic compounds to glutathione (Martinèz-Lara et al., 1997; Cunha et al., 2007). The AChE is inhibited principally by exposure to pesticides (Lundbaye et al., 1997), heavy metals and polycyclic aromatic hydrocarbons (PAHs) at higher concentrations (Jebali et al., 2006; Bonacci et al., 2008). Metallothioneins (MT) are low molecular weight (about 7 kDa), cysteine-rich and metal-binding proteins (Roesijadi, 1992; Viarengo et al., 2007). The induction of MT as a measure of response to metal exposure in aquatic organisms has been widely investigated in laboratory and field conditions (Alhama et al., 2006; Jebali et al., 2008; Ghedira et al., 2010). Lactate dehydrogenase (LDH) is a cytoplasmatic enzyme which catalyses the interconversion of pyruvate to lactate in glycolysis. Its activity has been used as indicative of potential effects on energy production mechanisms induced by chemical (Guilhermino et al., 1994) and may be inducible by oxygen stress in marine organisms (Wu and Lam, 1997). The battery of biomarkers (CAT, GST, AChE, LDH activities and MDA and MT levels) has been selected to detect a wide range of possible

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effects of anthropogenic activities on crab health in the Lagoon of Monastir (Tunisia) base on Integrated Biomarker Response (IBR) developed by Beliaeff and Burgeot (2002). Recent research (Banni et al., 2005; Bouraoui et al., 2010) has highlighted the successful use of a suite of biochemical biomarkers response to provide an integrated relative measure of the general health status of coastal marine invertebrates (Banni et al., 2005).

In invertebrates, digestive gland is the most frequently used tissue for biochemical biomarkers determinations. Gills are recommended by several authors to test and validate it's usefulness in in-field study (Maria et al., 2009; Ricciardi et al., 2010). Gills are considered an important route for uptake, bioconcentration and excretion of toxicants and a prime target to contaminants, due to the wide surface area in contact with the external medium and reduced distance between internal and external media (Montes Nieto et al., 2010).

The goals of this study were: (1) to evaluate the responses of six biomarkers, acetylcholinesterase, catalase, gluthation S-transferase, lactate dehdrogenase activities; and metallthioneine and malonedialdehyde levels in Mediterranean crab (*Carcinus maenas*) collected from different sites of Monastir bay (Tunisia); and (2) to develop an IBR index and star plot for interpretation of those biomarker responses to assess the biological health status of crabs.

1 Materials and methods

1.1 Studied areas

Mediterranean crab (*C. maenas*) were collected from five locations of Monastir Bay and one site from Kuriat Island as reference site. Monastir Bay (latitudes 35°50′S–35°40′S) is widely opened north eastward; it covers more than 70 km^2 and does not exceed 3 m depth up to 2 km off the shoreline. It's represents an economically important body of water due to a variety of fishing and aquaculture activities. Sampling sites were chosen because of their geographical locations near urban, industrial areas (Fig. 1); they are constantly threatened by contamination due to their proximity to human settlements and high economic and industrial activities (Sahnoun, 2000).

Khniss and Ksibat sites are highly influenced by the industrial discharges from the industrial zone of Monastir and obvious sources of urban discharges at Khniss site (Dahmane, 2000). The Lamta, Téboulba and Bekalta sites influenced by the treated and none treated municipal wastewater from limitroph agglomerations and fishing harbour. The control crabs were collected from Kuriat Island, which is characterized with no apparent contamination sources.

1.2 Crabs collection and handling

Intermoult *C. maenas* (45–55 mm) were collected with hands on January 2010 at five different selected sites from the Monastir Bay and Kuriat Island (Fig. 1). Six adult crabs were caught from each site. Immediately, crabs were dissected *in situ* and gills were removed and washed briefly in ice-cold homogenizing buffer and transported into the laboratory. Samples were stored at –40°C until biochemical analysis.

1.3 Biomarkers assays

1.3.1 Cell-free extract preparations

All steps were carried out at 4°C. Crab gills were homogenised in ice-cold phosphate buffer (100 mmol/L; pH 7.5; 1 mmol/L EDTA; 1 mmol/L reduced glutathione; GSH) with an T25 Ultraturrax (Biolock, Germany) at

Fig. 1 Map of Tunisia indicting the location of sampling sites along Monastir Bay: Khniss, Ksibat, Lamta, Téboulba, Bekalta and Kuriat as reference site.

24,000 r/min for 50 sec and at a ratio of 3 mL/g (buffer volume/tissue weight). The homogenate was centrifuged at 9000 ×*g* at 4°C for 15 min. The supernatant of each sample was stored at –20°C, for no longer than a week, until enzymatic activities and malonediladehyde (MDA) levels determinations.

Total protein content in the homogenate was measured following the Bradford method (Bradford, 1976), at 595 nm, using bovine serum albumin as standard protein.

1.3.2 Catalase (CAT) determination

Catalase activity was determined by the method of Claiborne (1985). The rate of enzymatic decomposition of hydrogen peroxide (H_2O_2) determined as absorbance decrements at 240 nm. The assay mixture consisted of 780 μL of sodium phosphate buffer (0.1 mol/L, pH 7.5 and 25°C, 200 μL solution of 0.5 mmol/L (H_2O_2) and 20 μL of cytosolic fraction. Results were expressed as μmol H_2O_2 consumed per minute per milligram protein.

1.3.3 Glutathione S-transferase (GST) determination

GST activity was assayed by the method described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as substrate and glutathione (1 and 4 mmol/L final concentration, respectively) in 100 mmol/L sodium phosphate buffer at pH 7.5. All GST activity assays were realised in conditions of linearity with respect to incubation time. The results were expressed as micromoles produced per minute per milligram protein.

1.3.4 Acethylcholinesterase (AChE) determination

AChE activity was determined according to the method of Ellman et al. (1961). Reaction mixture contained 0.1 mol/L sodium phosphate buffer (pH 7.5), 8 mmol/L 2,4-dinitrothiocyanatebenzene and the stock cytosolic solution containing acetylcholinesterase fractions. After pre-incubation, the reaction was started by the addition of 8.25 mmol/L acetylthiocholine (AtCh) as substrate. AChE activity was determined by kinetic measurement at 412 nm. Results were expressed as nmol AtCh hydrolyzed per minute per milligram protein.

1.3.5 Lactate dehydrogenase (LDH) determination

The enzymatic activity was determined following the changes of absorbance at 340 nm by UV-spectrophotometric method (Laganà et al., 2007). LDH was assayed in a reaction mixture of 1 mmol/L pyruvate and 0.15 mmol/L NADH in 0.1 mol/L sodium phosphate buffer at pH 7.5. One enzyme unit was defined as the amount catalyzing the production of 1.0 μmol per litter NAD^+per minute at 25°C.

1.3.6 Malondialdehyde (MDA) accumulation

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species with use of 1,1,3,3-treaethyloxypropane as a standard. The reaction was determined at 532 nm using thiobarbituric acid reagent as per the method of Buege and Aust (1978). MDA content was expressed as nmol equivalent MDA per milligram protein.

1.3.7 Metallothionein (MT) determination

MT content was evaluated in gills according to the spectrophotometric method described by Viarengo et al. (1997), based on cysteine residue titration of a partially purified MT extract. MT protein levels were determined using a spectrophotometric assay for MT using Ellman's reagent (0.4 mmol/L DTNB in 100 mmol/L KH_2PO_4) at pH 8.5 in a solution containing 2 mol/L NaCl and 1 mmol/L EDTA. Reduced GSH standard solutions were used for calibration (2–100 μmol/L) and data are expressed as micrograms MT per milligram protein taking, into consideration mussel MT molecular weight (8600 Da) and number of cysteine residues (21 residues) (Viarengo et al., 1997). In brief, gills samples were homogenized with 3 mL/g of 0.5 mol/L sucrose, 20 mmol/L Tris-HCl buffer at pH 8.6, with added 0.006 mmol/L leupeptine, 0.5 mmol/L PMSF (phenylmethylsulfonyl fluoride) as antiproteolitic, and 0.01% β-mercaptoethanol as reducing agent. The homogenate was then centrifuged at 15,000 ×*g* for 30 min at 4°C. The supernatant obtained was treated with ethanol/chloroform. Cold (–20°C) absolute ethanol (1.05 mL) and chloroform (80 μL) were added to aliquots of 1 mL supernatant. The mixture was acidified with 6 mol/L HCl (10 μL) and RNA was added to coprecipitate MT and improve recovery. The sample was maintained at –20°C for 1 hr and centrifuged at 10,000 ×*g* for 10 min. The MT pellet obtained was resuspended in HCl/EDTA to remove metal cations still bound to the MT. Finally, 2 mol/L NaCl was added to the solution to facilitate thiol interactions with DTNB by reducing the interaction of divalent metals with the apothionein.

1.4 Statistical analysis

Data from different stations were compared by a one-way analysis of variance (ANOVA) and statistically different treatments were identified by Duncan's test. All differences were considered significant at $P < 0.05$. Statistical analyses were performed using the SPSS statistical package (version 10.0). Different letters a, b, c, d and e indicated significant differences between groups. The star plots were derived using Microsoft Office Excel 2003 program.

1.5 Integrated Biomarker Response (IBR) determination

A method for combining all the measured biomarker responses into one general "stress index" termed "Integrated Biomarker Response" (Beliaeff and Burgeot, 2002) was applied to evaluate an integrated impact of toxicants from different monitoring sites.

For each biomarker: (1) calculation of mean and SD for each station. (2) Standardization of data for each station: $X_i' = (X_i - \bar{x})/S$, where X_i' is the standardized value of the biomarker, X_i is the mean value of a biomarker from each station, $\bar{x}$ is the mean of the biomarker calculated for all the stations, and S is the standard deviation calculated for the station-specific values of each biomarker. (3) Using standardized data, addition of the value obtained for each station (X_i') to the absolute (non-negative) value of the min-

imum value $B = X_i' + |X_{\min}|$ in the data: $B = X_i' + |X_{\min}|$, where $B = X_i' + |X_{\min}|$ the minimum value for all stations and for each biomarker; and B is the score of each biomarker and for each station. Result: adjusts the lowest value in the set to zero. For all the biomarkers treated this way. (4) Calculation of star plot areas by multiplication of the obtained value of each biomarker (B_i) with the value of the next biomarker, arranged as a set. (5) Summing-up of all values. The corresponding IBR value is: $\{[(B_1 \times B_2)/2] + [(B_2 \times B_3)/2] + ... + [(B_{n-1} \times B_n)/2]]\}$ and B_n are two scores of two successive biomarkers. Result: IBR (average of different arrangements of biomarkers in the set).

2 Results

2.1 Biomarker responses

The responses of the biochemical parameters determinate in *Carcinus maenas* specimen collected along the Monastir Bay coast and from the Kuriat site as reference site are presented in Fig. 2.

The measurement of CAT and MDA levels as oxidative stress biomarkers in Mediterranean crab *C. maenas* differ among the sampling sites. The activity of CAT, in crabs from Bekalta (Bek), Lamta (Lam), Ksibat (Ksi) did not differ from controls but it was significantly enhanced in those from Téboulba (Teb) and Khniss (Khn). The CAT activity levels were 1.41 and 1.48 fold higher in crab from Teb and Khe, respectively, when compared to Kuriat (Kur). Concerning MDA, and comparing to Kur, its levels were found 2.02 and 2.14 fold higher in crab from Bek and Khn respectively.

MT levels were significantly enhanced in crab from all the sampling sites of Monastir Bay except Bek, with respect to the reference site. Morever, at Teb and Ksi, crab had a maximum MT level induction when compared to Kur (4.45 and 4.55 fold respectively). GST activity showed a similar pattern of response along the different sampling sites as MT levels. Its levels in crab from Lam, Ksi, Khn and Teb were respectively 2.35, 2.15, 1.96 and 1.67 fold

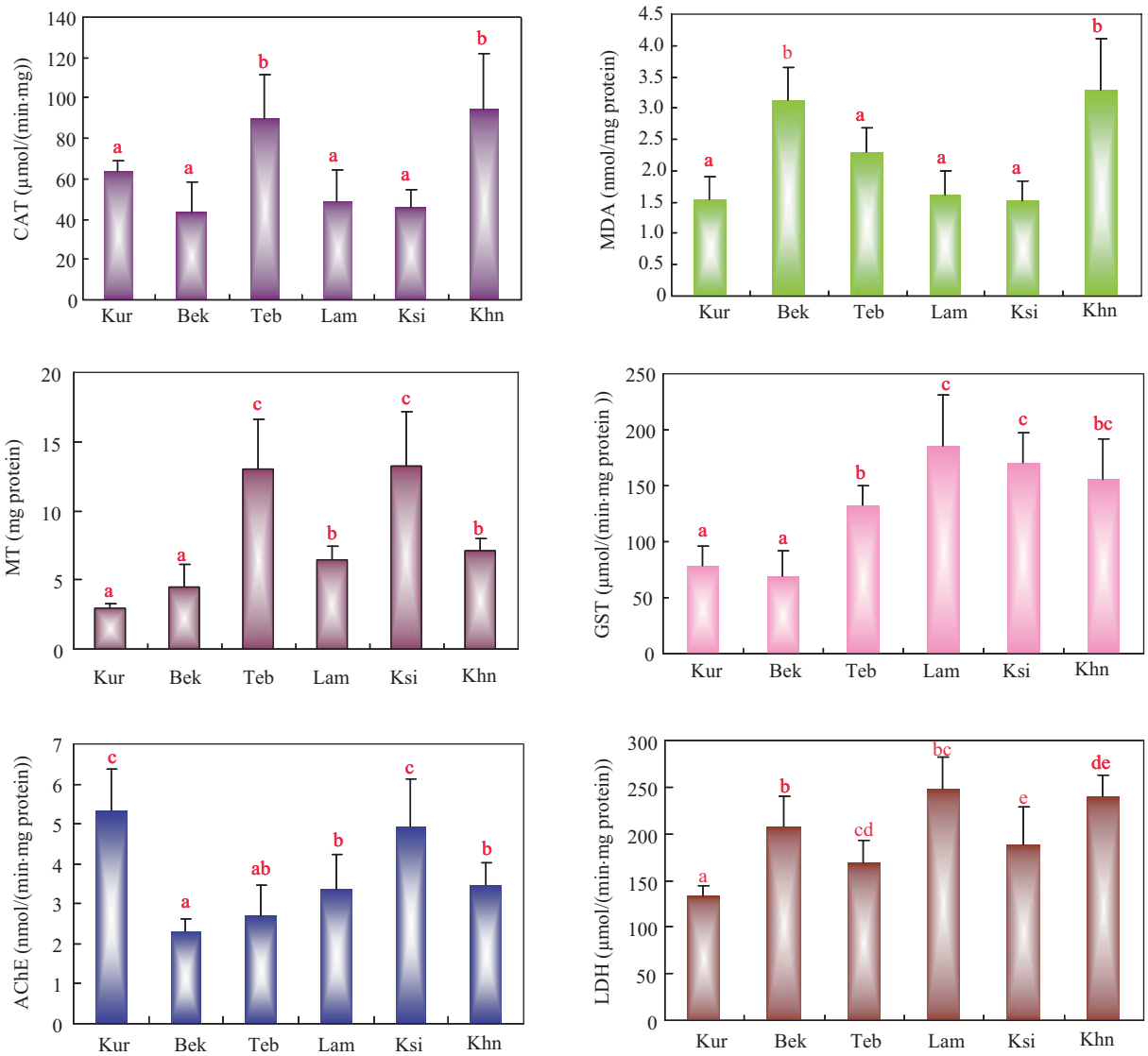

Fig. 2 Responses of catalase (CAT), gluthation S-transferase (GST), acetylcholinesterase (AChE), lactate dehydrogenase (LDH) activities; and metallothionein (MT) and malonedialdehyde (MDA) levels measured in gills of *Carcinus maenas* collected from six stations: Kuriat (Kur), Bekalta (Bek) Téboulba (Teb) Lamta (Lam), Ksibat (Ksi) and Khniss (Khn). All results are expressed as mean ± SD; n = 6. Letters a, b, c, d and e indicate a significant differences between groups.

higher than Kur.

AChE activity levels were significantly lower in crab from all the sampling sites except Ksi, when compared to Kur. Furthermore, the AChE activity levels of Teb, Lam and Khn were found decreased respectively 49.07%, 36.94%, 34.92% lower than Kur. The lowest AChE activity was found in crab collected at Bek representing a 56.78% decrease when compared to Kur.

2.2 Integrated biomarker response (IBR)

To diagnose and compare the overall physiological state of crabs studied at the Monastir Bay and Kuriat sites, the IBR index was applied to the six biomarkers measured. IBR in crab collected from different sites were evaluated and are represented as star plot (Fig. 3). Star plots using IBR values instead of biomarker data make it possible to visualize differences between studied sites. In general, IBR values show a large range of variation at different monitoring sites. A higher IBR values were measured at Khn and Ksi (95.78 and 91.13) as well as at Lam, Teb and Bek with respectively values: 67.68, 65.58 and 35.62. The lowest IBR value was found at Kur, reference site (0.00). Thus, and according to the obtained index values, the studied sites were ranked from the higher pollution level and critical biological responses as: Khn and Ksi > Lam and Teb > Bek > Kur.

3 Discussion

The potential use of multi-marker approach for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years since, a single biomarker may not reflect the health status of a sentinel species

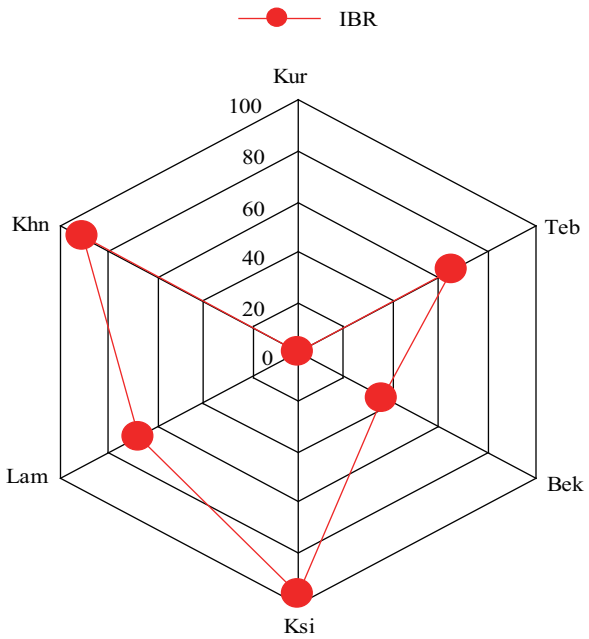

Fig. 3 Star plots of integrated biomarker response (IBR) from different monitoring sites: Kuriat (Kur), Bekalta (Bek), Téboulba (Teb), Lamta (Lam), Ksibat (Ksi) and Khniss (Khn).

(Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Rodríguez-Ortega et al., 2009; Tlili et al., 2010). Due to lack of information about the use of biochemical systems as pollution biomarkers in Mediterranean crab *C. maenas* on the Tunisian coastline, the in-field study of some biochemical responses and BRI were evaluated in *C. maenas* collected from Monastir Bay and Kuriat site as reference site.

The shore crab *C. maenas* was selected as a "model organism" since the single-system approach has been applied frequently to this common littoral species and has provided a wealth of background information. *C. maenas*, an extremely eurythermal and euryhaline species, is widely distributed along Mediterranean coast including Tunisian coast area (Astley et al., 1999; Pereira et al., 2009; Ghedira et al., 2009). Consequently, shore crabs may be exposed to a broad range of anthropogenic contaminants, yet remain common and abundant (Pedersen et al., 1998). In addition, it has the capability of accumulating various pollutants including heavy metals, PAHs and PCBs (Hagger et al., 2009) and, thus, it seems to be a suitable bioindicator of environmental contamination by these agents. It has been also found to be a suitable organism for use in "*in situ*" assays (Morales-Caselles et al., 2008; Martín-Díaz et al., 2009).

Our previous biomonitoring studies using fish or invertebrate organisms focused their attention on liver or digestive gland due to its importance on the metabolism and storage of xenobiotics (Jebali et al., 2007; Tlili et al., 2010; Banni et al., 2009).

In the present, six biomarkers were measured in gills of *C. maenas* collected from Monastir Bay and the obtained results show differences in individual biomarker responses. Multivariate analysis techniques are a useful tool for interpreting biomarker data. In this study, we used IBR developed by Beliaeff and Burgeot (2002), to evaluate the crab physiological disturbances at sampling sites. Among the studied sites where crab were characterised by critical responses to biomarkers, and maximum IBR values, we found at Khniss and Ksibat. The chronic of none treated wastewater discharges were apparent at Khniss and Ksibat sites increases the chemicals concentrations in different components of marine ecosystem of theses sites (El Asmi-Djellouli et al., 2001). In deed, the marine currents north-south can disperse the chemical pollutants by geochemical process from sources discharges to the other sites, and thus may aggrieved the health status of crab and their environment in Monastir Bay (Sahnoun, 2000). Moreover, the geochemical study of Sassi et al. (1998) demonstrate a higher degradation of sediment littoral Khniss-Ksiabt due to the higher none treated domestics and industrials discharges and thus, may explain the higher pollution levels, critical biochemical alterations and higher IBR scores of crab from these sites.

Although the anthropogenic discharges were chronic and continuous at the Monastir Bay area, a very few chemical and ecological data were available. The interesting study of Dahmane (2000) shows higher metallic concentrations in sea water in front of domestic discharge

point at Khniss site. The plumb (Pb) and copper (Cu) were particularly found more than 150 ng/g sediment. The lack of information about the metallic and organic pollutants and as well as it's potential effects in Mediterranean crab collected from Monastir Bay, further studies will be carried out to investigate the seasonal variations of IBR levels's relationship to pollutants concentrations in sediment, gills and digestive gland.

For both Lamta and Téboulba sites, BRI scores corresponded to the higher pollution level and higher alterations in biological response and Bekalta has moderate alterations in biological responses. In contrast to the urban discharges at Lamta and Téboulba sites, Bekalta havenot any apparent pollution sources and it could be influenced by contaminants transported by the marine currents from the higher contaminated sites (Khniss and Ksibat). Similarly, Banni et al. (2005) illustrated how a biomarker index could distinguish both spatial and temporal variations in biomarker responses in clam (*Ruditapes decussatus*) populations from anthropogenic-contaminated sites by using discriminant analysis.

To our knowledge, there are no reports of the use of crab species in biomonitoring programs along the Tunisian coasts. The present work as the first report on the usage of IBR based on biochemical markers in crab *C. maenas* from a local area of Tunisian coast (Monastir Bay).

4 Conclusions

In this study, we have shown that a biomarker-based index (IBR) has the potential to be used in monitoring in Tunisian coast using *C. maenas* sentinel specie. We report the integration of many biomarkers indicating the presence of various stressors based on IBR score, thus allowing a better comprehension of the real toxicological risk of an investigated site. Further studies are still needed and are in progress to investigate the seasonal variations of IBR levels its relationship to pollutants concentrations in Mediterranean crab *C. maenas*.

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