Use of oxidative stress biomarkers in Carcinus maenas to assess littoral zone contamination in Tunisia

Jihene Ghedira1, Jamel Jebali1, Mohamed Banni1, Lassaad Chouba2, Hamadi Boussetta1, Juan López-Barea3, José Alhama3,*

1Laboratory of Biochemical and Environmental Toxicology, Higher Institute of Agriculture, Chott-Mariem, 4042 Sousse, Tunisia
2Chemical Laboratory, Higher Institute of Marine Sciences and Technology, La Goulette Center, 2060 Tunis, Tunisia
3Department of Biochemistry and Molecular Biology, University of Córdoba, Severo Ochoa Building, Rabanales Campus, Highway A4, Km 396a, 14071 Córdoba, Spain

ABSTRACT: Biological effects of pollutants were studied in Carcinus maenas crabs from 3 polluted sites (Bizerte, Teboulba, Gargour) along the Tunisian littoral zone using biochemical biomarkers. A metal contamination gradient was found, Bizerte standing out as the most metal-polluted area. Gargour animals nonetheless showed higher oxidative stress responses, such as glutathione reductase and 6-phosphogluconate dehydrogenase activities, as well as malondialdehyde (MDA) levels in gills. The gills showed higher lipid peroxidation than did the digestive gland, in keeping with their respiratory role. Animals were also exposed for different periods to 2 model pollutants, cadmium and chlorpyriphos-ethyl. Although cadmium induces oxidative stress, mainly in gills, thus increasing lipid peroxidation, principal-component analysis indicated that metal content in sediments and crabs from in-field monitoring does not fully correlate with oxidative stress biomarker responses. Catalase and MDA were the most sensitive biomarkers, and gills the most responsive organ. A lower catalase content in gills was linked to higher MDA levels.

KEY WORDS: Biomonitoring · Biochemical effects · Antioxidant enzymes · Lipid peroxidation · Multivariate analysis · Digestive gland · Gills · Metals · Organophosphates · Sediment pollution

INTRODUCTION

The marine environment is threatened by increasing levels of pollutants from many anthropogenic activities, including urban, agricultural and industrial discharges. This endangers the health of organisms and affects the marine ecosystem and humans consuming food of marine origin. Levels of metals, pesticides and hydrocarbons are increasing along the Tunisian littoral zone, especially at Bizerte Lagoon (Dellali et al. 2001, Khessiba et al. 2001, Louiz et al. 2009, Bouraoui et al. 2010) and in the area of Sfax/ Gabès gulf (Louati et al. 2001, Hamza-Chaffai et al. 2003, Zaghdan et al. 2005, Machreki-Ajmi et al. 2008, Bouraoui et al. 2010). Although both areas are subject to many ecological pressures, they are, due to the variety of fishing and aquaculture activities, key aquatic resources.

The diversity of chemicals dumped into the marine environment, the costs of chemical measurement of water and sediment quality, and the difficulty of evaluating the toxicity of pollutant mixtures has led to increasing use of biomarkers in marine organisms (Dellali et al. 2001, Banni et al. 2009a, Bouraoui et al. 2010). Biomarkers are useful for assessing chemical exposure and for predicting the detrimental effects of contaminants before damage reaches higher organizational levels (Monserrat et al. 2003). Many contaminants, including organic compounds and metals, convert O2 into reactive oxygen species (ROS) that...
are highly toxic and mutagenic. ROS damage various biomolecules, such as fatty acids that generate oxidized by-products, including malondialdehyde (MDA) (Gutteridge 1984, Brown & Kelly 1996).

Aerobic organisms have several lines of defence against oxidative stress (Sies 1986). Primary antioxidant enzymes such as catalase (CAT) detoxify ROS. Glutathione peroxidase (GPX) reduces reactive lipid hydroperoxides to prevent MDA formation (Flohé 1989). Ancillary enzymes such as 6-phosphogluconate dehydrogenase (6PGDH) recycle NADP⁺ into NADPH (Pickett 1989). Glutathione-S-transferases (GSTs) conjugate electrophilic compounds to reduced glutathione (GSH), and some GSTs play significant roles in the detoxification of lipid peroxidation by-products (Pickett 1989). Allant enzymes such as 6-phosphogluconate dehydrogenase (6PGDH) recycle NADP⁺ into its reduced form (NADPH). Then glutathione reductase (GR) uses NADPH to turn oxidized glutathione (GSSG) into GSH, which keeps cytosol reduced (Sies 1986). Biochemical biomarkers, including antioxidant enzymes and evidence of oxidative damage to biomolecules, are powerful tools for detecting the exposure and biological effects of pollutants, allowing early detection of environmental problems (López-Barea 1995, Monserrat et al. 2003, Martín-Díaz et al. 2004, Vioque-Fernández et al. 2009, Bouraoui et al. 2010). Chemical analysis and biomarker assessment, when combined, can offer more complete and biologically relevant information on the impact of pollutants on organism health (Martín-Díaz et al. 2005, Allan et al. 2006).

The shore crab Carcinus maenas was selected as a ‘model organism’. C. maenas, a eurythermal and euryhaline species (Hebel et al. 1997), is a common littoral crab and an important invasive species, widespread along the Mediterranean coast (Carlton & Cohen 2003). Its long life cycle, wide distribution, and sedentary lifestyle make it a good bioindicator for assessment of contaminant effects in situ (Martín-Díaz et al. 2009, Montes Nieto et al. 2010) or under controlled conditions (Martín-Díaz et al. 2005, 2007, 2008). It can be exposed to relatively high levels of a broad range of contaminants, yet remain common and abundant, without apparent detrimental effects (Pedersen et al. 1998). This suggests that compensatory mechanisms which allow the shore crab to survive natural environmental fluctuations may also confer some degree of tolerance to contaminant exposure (Hebel et al. 1997, Brown et al. 2004).

Exposure to toxic metals has become an increasing source of disease. Due to its widespread industrial use, Cd, a non-essential metal, is a ubiquitous and increasingly common contaminant of great ecological and human concern. Due to its wide range of organ toxicity and to its 10 to 30 yr half-life, Cd is one of the most hazardous substances known (Patrick 2003). In crustaceans, Cd is accumulated in key organs, including digestive glands and gills (Ray 1986), where it becomes toxic through any of various mechanisms (Simpson 1981, Rainbow 1998): (1) alteration of sulfhydryl homeostasis by GSH depletion and binding to protein thio (-SH) groups, which decreases its antioxidant capacity by inhibiting antioxidant enzymes (Casalino et al. 2002, Patrick 2003, Valko et al. 2005, Pan & Zhang 2006, Silverste et al. 2006); (2) displacement of Zn and Se in metallo-enzymes, decreasing their activity (Patrick 2003); (3) displacement of Fe²⁺, which induces ROS via a Fenton reaction (Casalino et al. 1997); or (4) generation of lipid peroxidation and DNA damage (Casalino et al. 1997, 2002, Patrick 2003, Pan & Zhang, 2006). Organophosphates (OPs) are highly neurotoxic synthetic insecticides used to control agricultural pests (Banni et al. 2005, Vioque-Fernández et al. 2007, Gbedira et al. 2009). They are widely used in the north of Tunisia to treat cereal pathologies. They often end up in aquatic habitats, carried there by wind, runoff, or uncontrolled waste disposal, thus threatening freshwater and marine ecosystems, especially during the rainy season (Dellali et al. 2001, Jebali et al. 2007, Gbedira et al. 2009).

In the present study, biochemical biomarkers were used to assess the exposure and biological effects of pollutants in Carcinus maenas collected from relatively polluted sites along the Tunisian littoral zone. Parallel to this, crabs were exposed to Cd and chlorpyrifos-ethyl (cPFe), to evaluate their biochemical responses to 2 representative models of metals and pesticides.

**MATERIALS AND METHODS**

**Sampling sites, animals and handling**

In October 2006, intermoult Carcinus maenas (n = 8; length: 45 to 55 mm) were manually collected at 3 different sites from the north and eastern coast of Tunisia (Fig. 1). Many industries (e.g. cement, textile, electronic, metallurgical, tyre manufacturing, oil refineries) are located in Bizerte, an area of high growth and intense urbanization, with a busy shipyard and commercial harbour (Dellali et al. 2001, Khessiba et al. 2001, Louiz et al. 2009, Bouraoui et al. 2010). Agricultural waste reaches Bizerte Lagoon (area: 150 km²) through runoff from nearby cereal fields, where fertilizers are used at the rate of 20.5 t
yr⁻¹ (ANPE 1989). The Gabès Gulf is a major aquatic resource, contributing 65% of national aquaculture production (CGP 1996). Sfax is the second largest city in Tunisia, the fourth busiest commercial harbour, and the major industrial area in the south. Crude phosphate treatments, chemical industries, tanneries, and plastics plants release pollution-loaded effluents into the marine environment; these pollutants include toxic metals, which are washed back by tides to the littoral area (Louati et al. 2001, Banni et al. 2005, Zaghden et al. 2005, Machrek-Ajmi et al. 2008, Smaoui-Damak et al. 2009). Cd from phosphate plants is one of the main contaminants in the Gabès area (Hamza-Chaffai et al. 2003, Smaoui-Damak et al. 2003, Machrek-Ajmi et al. 2008). Samples were collected from: (1) Bizerte (BIZ) Lagoon, and (2) Gargour (GAR), in Gabès Gulf, both of which areas receive urban, industrial and agricultural pollution, and (3) Teboulba (TEB), with no main contamination sources and considered a reference site in many field studies (Banni et al. 2007, Jebali et al. 2007, Bouraoui et al. 2010).

Crabs from these areas were transported alive to the laboratory immediately upon collection in bucketed with aerated seawater. Once there, the cephalothorax was opened on ice and the digestive gland and gills were quickly removed. For metals analysis, organs were freeze-dried and ground; for biochemical studies, they were frozen at −80°C, ground in a mortar with liquid N₂, and stored at −80°C until use.

Three samples of surface sediments (up to 10 cm depth), collected in the same place where the crabs were sampled, were mixed, transported to the laboratory and freeze-dried for 12 h. After removal of large plant fragments, they were sieved to obtain the fine fraction (<60 µm), which was stored at 4°C until metals analysis.

Metals analysis

Metals were analysed in the sediment samples and crab organs by atomic absorption spectrophotometry (AAS). Both sediment samples and freeze-dried organs (0.2 g) were digested for 35 min in a mixture of 5 ml concentrated HNO₃ and 2 ml HF in a microwave oven (Ethos) set at 100% power and 120 psi. After digestion, samples were diluted with Milli-Q water, and 9.8 g of H₃BO₃ was added (PNUE/COI/AIEA/FAO 1994). Concentrations of Cd, Pb, and Cu were determined by AAS with graphite furnace using a Vectra 220 Z spectrophotometer (Varian) with ZEEMAN correction. Zn concentration was determined by an AA 10 flame AAS (Varian). Total Hg was determined by cold vapour technique, employing a VGA system coupled to a spectrophotometer using a reducing dissolution of SnCl₂ in HCl. Quality assurance was assessed using dogfish liver International Atomic Energy Agency (IAEA) 405 as reference material. Standards were treated and analysed under the same conditions as the sediment and organ samples. All metal concentrations are reported in µg g⁻¹ of dry weight of sample. Values represent the mean of 2 independent extractions from the sediment and organ samples.

Exposure of crabs to Cd and OP₈₉

Crabs from TEB were transported alive to the laboratory as described above. There were divided into 4 groups of 8, placed in plastic tanks with 10 l of seawater (37 ± 1% salinity) and maintained at 17.2°C. Animals were fed regularly with diced fish every 3 d. After 7 d of acclimation, Group 1 was exposed to 500 µg l⁻¹ CdCl₂ (Merck), Group 2 to 3.12 µg l⁻¹ OP₈₉ (Dursban®), and Group 3 to a mixture of Cd and OP...
at the concentrations indicated above (all nominal values). Group 4, the control group, received no treatment. After 2 d exposure, all crabs were sacrificed. For each of the 4 groups, the digestive gland and gills were carefully removed and frozen at −80°C until analysis. The concentrations of Cd and cPFe, chosen as suggested in the literature (Schuwerack et al. 2001, Schuwerack & Lewis 2003), were well below their respective LC$_{50}$. (The LC$_{50}$ of cPFe for the adult crab *Eriocheir sinensis*, e.g. is 460.9 µg l$^{-1}$; Li et al. 2006).

**Cell-free extract preparations and biomarker assays**

All steps for cell-free extract preparation were carried out at 4°C. Organs were ground up and disrupted in a T 25 Ultra-Turrax® (Janke & Kunkel) in 10 mM Tris HCl (pH 7.5) containing 1 mM EDTA, 1 mM GSH and 1 mM phenylmethylsulfonyl fluoride at a concentration of 3 ml g$^{-1}$. For the MDA assay, 300 µl of homogenate was set apart and the rest centrifuged for 20 min on a Beckman J2-21 centrifuge at 10 000 × $g$. Supernatants were stored at −20°C for no longer than 1 wk until biomarker determinations.

The cytosolic activities of primary and ancillary antioxidant enzymes, and phase II biotransforming enzymes were assayed (as published) as biomarkers (Rodríguez-Ariza et al. 1992). CAT was measured at 240 nm with 20 mM H$_2$O$_2$. GPX followed at 340 nm with 1 mM GSH, 2 mM cumene hydroperoxide, 0.24 U GR and 0.15 mM NADPH. 6PGDH was analyzed at 340 nm with 1 mM 6-phosphogluconate and 0.25 mM NADP$^+$. GR was assayed at 340 nm with 2.5 mM GSSG and 0.12 mM NADPH. GST was determined at 340 nm with 2 mM GSH and 2 mM 1-chloro-2,4-dinitrobenzene. Data were expressed as mU mg$^{-1}$ protein, one unit being defined as µmol of substrate consumed, or of product formed, per min. Thiobarbituric-acid-reactive substances were fluorometrically assayed using 515 nm excitation and 550 nm emission to assess the MDA level, a known biomarker of lipid oxidative damage (Brown & Kelly 1996). MDA was expressed as nmol MDA mg$^{-1}$ protein. Total protein content was measured at 595 nm following the Bradford method (Bradford 1976), using bovine serum albumin as the standard.

**Statistical analysis**

The numbers represent the mean ± SD of 3 independent extractions with 3 assays per extract. Statistical significance of the results compared to the reference was determined by Student’s *t*-test using InStat™ software, v. 2.01/93 (GraphPad), after checking normality of data and homogeneity of variance. Factorial analysis was also performed to determine the major responses with respect to metal levels. Factor analysis using principal component analysis (PCA) was applied to the original sets of variables using the SPSS Statistics 17 package. Using this same software, a correlation between metal concentrations and biomarker responses was undertaken using a Pearson correlation analysis. Results were considered significant at $p < 0.05$.

**RESULTS AND DISCUSSION**

**Metal concentrations in sediments and crab organs**

To evaluate metal pollution levels in the Tunisian littoral zone, concentrations of 5 elements were determined in sediments (Table 1). BIZ stood out as the most metal-polluted area. The levels of all metals in the sediments were much higher at BIZ than at GAR and at the reference site TEB. Pb levels were >11 times higher, Cd and Cu >9 times higher, Zn >6.8 times higher and Hg >2.5 times higher at BIZ than at TEB. Cu levels at BIZ were 56 times higher than at GAR, which in turn had higher levels of Cd, Pb and Hg than TEB. Cu levels, however, were 6 times

<table>
<thead>
<tr>
<th>Metal</th>
<th>Sediment</th>
<th>Digestive gland</th>
<th>Gills</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEB</td>
<td>BIZ</td>
<td>GAR</td>
</tr>
<tr>
<td>Cd</td>
<td>0.08</td>
<td>0.77</td>
<td>0.15</td>
</tr>
<tr>
<td>Pb</td>
<td>9.34</td>
<td>106.5</td>
<td>25.9</td>
</tr>
<tr>
<td>Cu</td>
<td>12.49</td>
<td>116.6</td>
<td>2.08</td>
</tr>
<tr>
<td>Zn</td>
<td>176.9</td>
<td>1208.9</td>
<td>173.5</td>
</tr>
<tr>
<td>Hg</td>
<td>0.19</td>
<td>0.49</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 1. Metal content (µg g$^{-1}$ dry wt) in sediments, and digestive gland and gills of *Carcinus maenas* from 3 sampling sites along the Tunisian coast. TEB: Teboulba; BIZ: Bizerte Lagoon; GAR: Gargour. Values represent mean of 2 independent extractions. Numbers in parentheses represent ratio of metal content at BIZ and GAR to TEB. nd = not detected.
higher at TEB than at GAR. Notably, except for Cd, values at BIZ were above effects range-low (ERL) levels of the sediment quality guidelines proposed by Long et al. (1995; Cd: 1.2, Pb: 47, Cu: 34, Zn: 150 µg g⁻¹). At GAR and TEB, only Zn showed values higher than ERL levels. Recently, evidence of reproduction alterations and gonadal lesions related to pollution stress has appeared in BIZ fish, causing an upsetting drop in fish productivity during the last few years (Louiz et al. 2009).

Carcinus maenas is widely distributed, has many sediment-burrowing features and exhibits a wandering lifestyle, roaming over muddy substrates in coastal areas. When these animals are exposed to metal pollution within their habitats, toxic metal loads accumulate and subsequently have a biological effect on the crabs. Bioaccumulation of the 5 selected metals was evaluated in the digestive gland and gills of C. maenas from the 3 study sites (Table 1). In agreement with the results obtained in the sediments, the levels of metals were higher in organs from BIZ animals, except for Hg, which was not detected in the studied organs. In the digestive gland, Cd at BIZ was 34.7 times the levels found in C. maenas at TEB; Pb and Zn levels in BIZ animals were, respectively, 6.72 and 9.62 times higher than in TEB animals. Cd was 4.9 times higher in GAR crabs than in TEB crabs, while Pb and Cu levels were higher in TEB animals. In gills, importantly, Zn levels were 7.52 times higher in BIZ animals than in TEB animals. Crude phosphate treatment industries located at Sfax are suggested as the main source of the Cd found in the sediments (1.9 times higher than TEB levels) and in the digestive gland of the GAR crabs (4.9 times higher than in TEB animals). On the other hand, it is relevant to highlight the significantly high levels of all analyzed metals in BIZ sediments as compared to TEB sediments (Cd, Pb, and Zn ~10 times higher in BIZ sediments than TEB). This is probably due to the intensive industrial and agricultural activities carried out in this area. Our results agree with those previously described for the nereid worm Nereis (Heiste) diversicolor, which showed the highest content of heavy metals at BIZ (Cd levels 19.4 times, Cu levels 4.6 times, and Zn levels 3.4 times those measured in animals at TEB), lower levels at GAR (Cd levels 4.7 times those measured in animals at TEB) and the lowest levels at TEB (Bouraoui et al. 2010).

Differences in the distribution of metals in the organs were also apparent. Thus, although a fairly similar accumulation of the 2 essential metals, Cu and Zn, was found in both organs, Cd was higher in the digestive gland than in the gills, while the gills showed much higher Pb levels. Cu bioaccumulation was detected in both organs, Cu being ~30 times higher in Carcinus maenas at TEB, ~7.5 times higher in animals at BIZ, and ~100 times higher in animals at GAR than in the corresponding sediments. Significantly, Cd bioaccumulation was also found in the digestive gland, with levels in TEB animals 7.8 times higher, levels in BIZ animals 27.9 times higher and levels in GAR animals 20.3 times higher than in the corresponding sediments. Unlike C. maenas, the river crab Potamonautes warreni showed higher Cd and Cu contents in gills than in the digestive gland, although Zn levels were similar in organs from both species (Schuwerack et al. 2001). When crabs are exposed to Cd without other metals, the highest content is found in gills. When they are exposed to Cd in company with other metals, Cd values are highest in the digestive gland (Martín-Díaz et al. 2005). Cd accumulation was also found to be higher in the digestive gland of the cockle Cerastoderma glaucum from Gabès Gulf than in the gills (Machrek-Ajmi et al. 2008).

**In-field biomarker responses**

Chemical monitoring to assess environmental quality has its limitations, and therefore biological monitoring, including the use of biomarkers, is strongly recommended in monitoring programmes (Allan et al. 2006). Application of biochemical biomarkers under field conditions has been proposed by many investigators to assess chronic responses and to address the integrated effects of anthropogenic and environmental stressors. ROS formation has been proposed as the basis of the toxicity of many contaminants, including metals and organic compounds. For this reason, biochemical biomarkers responsive to oxidative stress were used to determine pollution levels along the Tunisian littoral zone (Fig. 2). Compared to TEB, CAT activity was lower in the digestive gland of BIZ animals (1.5 times less, p < 0.01), and even lower in animals from GAR (3.7 times less, p < 0.001). The gills from these 2 metal-polluted sites showed more drastic CAT decreases (from 3.9- to 4.8-fold, p < 0.001) than what was observed in control animals from TEB. Antioxidant enzymes, including CAT, decrease with time or with exposure to high metal levels (Pan & Zhang 2006). CAT activity has also shown as a sensitive biomarker in other studies, since Procambarus clarkii crayfish from polluted areas showed lower activity than the reference animals (Vioque-Fernández et al. 2009). Decreased CAT
activity has also been reported in mice dwelling at sites contaminated by toxic metals (Montes-Nieto et al. 2007). It is noteworthy, however, that other investigators have found higher CAT activity in clams and worms from BIZ and from sites near Sfax than at control sites (Jebali et al. 2007, Banni et al. 2009b, Bouraoui et al. 2010).

Although BIZ was the most metal-polluted area, GAR crabs had more extensive symptoms of oxidative stress compared to control animals from TEB, as indicated by the increased activity of the 2 antioxidant auxiliary enzymes, GR and 6PGDH, and mostly by a higher MDA content, showing increased damage to lipids. Thus, GR and 6PGDH were higher in both organs of GAR crabs but not in those from BIZ animals, with the highest increases in GAR animals being found in GR rather than in 6PGDH (GR 3.8 times higher in the digestive gland, p < 0.001; 6.4 times higher in gills, p < 0.05; 6PGDH ~1.9 times higher in both organs). Similarly, MDA was higher in the digestive gland of GAR crabs (p < 0.05), but even higher in gills (p < 0.001) from the 2 most polluted areas, with the highest levels in GAR crabs (6.6 times levels measured in control animals) than in BIZ (3.5 times levels measured in control animals). Greater DNA damage (8-oxodG) has also been found in clams at BIZ and Gabès Gulf sites (Jebali et al. 2007). Oxida-

Fig. 2. Carcinus maenas. Concentrations of biomarkers analyzed in the digestive gland and gills of crabs from 3 sites (see Fig. 1). One unit (U) is defined as μmol of substrate consumed, or of product formed, per min. CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; 6PGDH: 6-phosphogluconate dehydrogenase; GST: glutathione-S-transferase; MDA: malondialdehyde. Data represent mean values ± SD of 3 independent extractions with 3 assays per extract. Statistical significance of results based on reference site TEB: ***p < 0.001, **p < 0.01, *p < 0.05
Ghedira et al.: Using oxidative stress biomarkers in Carcinus maenas

damaging damage to biomolecules, including lipid peroxidation, DNA damage and enzyme inactivation, occurs when antioxidant systems are overcome by an excessive ROS production (Winston & Di Giulio 1991). Finally, GPX and GST did not change significantly in any of the studied organs.

**Relationships between metals and in-field biomarkers**

A multivariate analysis was performed to compare biomarker responses and metal levels in sediments and in crab organs. According to PCA, 2 principal components described the 25 original variables, explaining 100% of the total variance (Fig. 3). Each component is described according to the dominant group of variables.

The first principal component, accounting for 65.1% of the variance, was associated with metal content, in sediments (s), in crab digestive glands (dg) and in gills (g), as well as with an increase in GPX and GST in the digestive gland and a decrease in GPX in the gills. Clearly, the concentrations of all metals analysed were highly correlated with each other (results not shown), both in sediments and in crab organs. The significant positive correlation between GPX (dg), and Pb (dg) and Cu (g) (p < 0.05), and the significant negative correlation between GPX (g), and Cd (s, dg) and Zn (s, dg, g) (p < 0.05) should also be highlighted.

The second component, accounting for 34.9% of the variance, was associated with CAT, GR, 6PGDH, GST and MDA in both organs (except for GST in the digestive gland), although these 5 biomarkers were not associated with metals. It should be emphasized that CAT activity was negatively correlated with all other biomarkers, especially in the case of CAT (dg) and MDA (g) (p < 0.01). Reduced CAT enzymatic activity, along with increased levels of damaged biomolecules (MDA, oxidized glutathione), evidenced by the oxidative lesions promoted by toxic metals, have also been described in crabs and mice from sites contaminated by toxic metals (Huelva Estuary, SW Spain; Montes-Nieto et al. 2007, Montes Nieto et al. 2010). In contrast, a positive correlation was found between GR (dg), GR (g) and 6PGDH (dg) (p < 0.05), and between GST (g) and MDA (dg) (p < 0.01).

**Biochemical responses to Cd and cPFe**

Cd, discharged from crude phosphate plants near Sfax, is one of the most significant contaminants at Gabès Gulf. Metal analysis showed enormous Cd bioaccumulation in the digestive gland of crabs from BIZ, and a lesser but still significant load in that of GAR animals. Both areas also have important agric不同意 damage to biomolecules, including lipid peroxidation, DNA damage and enzyme inactivation, occurs when antioxidant systems are overcome by an excessive ROS production (Winston & Di Giulio 1991). Finally, GPX and GST did not change significantly in any of the studied organs.

**Relationships between metals and in-field biomarkers**

A multivariate analysis was performed to compare biomarker responses and metal levels in sediments and in crab organs. According to PCA, 2 principal components described the 25 original variables, explaining 100% of the total variance (Fig. 3). Each component is described according to the dominant group of variables.

The first principal component, accounting for 65.1% of the variance, was associated with metal content, in sediments (s), in crab digestive glands (dg) and in gills (g), as well as with an increase in GPX and GST in the digestive gland and a decrease in GPX in the gills. Clearly, the concentrations of all metals analysed were highly correlated with each other (results not shown), both in sediments and in crab organs. The significant positive correlation between GPX (dg), and Pb (dg) and Cu (g) (p < 0.05), and the significant negative correlation between GPX (g), and Cd (s, dg) and Zn (s, dg, g) (p < 0.05) should also be highlighted.

The second component, accounting for 34.9% of the variance, was associated with CAT, GR, 6PGDH, GST and MDA in both organs (except for GST in the digestive gland), although these 5 biomarkers were not associated with metals. It should be emphasized that CAT activity was negatively correlated with all other biomarkers, especially in the case of CAT (dg) and MDA (g) (p < 0.01). Reduced CAT enzymatic activity, along with increased levels of damaged biomolecules (MDA, oxidized glutathione), evidenced by the oxidative lesions promoted by toxic metals, have also been described in crabs and mice from sites contaminated by toxic metals (Huelva Estuary, SW Spain; Montes-Nieto et al. 2007, Montes Nieto et al. 2010). In contrast, a positive correlation was found between GR (dg), GR (g) and 6PGDH (dg) (p < 0.05), and between GST (g) and MDA (dg) (p < 0.01).

**Biochemical responses to Cd and cPFe**

Cd, discharged from crude phosphate plants near Sfax, is one of the most significant contaminants at Gabès Gulf. Metal analysis showed enormous Cd bioaccumulation in the digestive gland of crabs from BIZ, and a lesser but still significant load in that of GAR animals. Both areas also have important agric...
cultural activity, with increasing levels of pesticides such as OPs (Jebali et al. 2007, Ghedira et al. 2009). Thus, Cd and cPFe were chosen as model pollutants in exposure experiments to evaluate their biochemical responses. Fig. 4 shows the effect of 2 d exposure to Cd or cPFe on 8 biomarkers, both in digestive gland and gills. Since crabs inhabiting polluted water bodies are exposed to complex pollutant mixtures, including heavy metals and organic pollutants, exposure to a Cd-cPFe mixture was also monitored to evaluate their combined effect (Fig. 4). CAT activity decreased extensively \((p < 0.001)\) after exposure to Cd, cPFe and the mixture, both in the digestive gland and, to a lesser extent, in gills. Lower GPX activity was also found in the digestive gland, this being significant \((p < 0.05)\) only in the mixture, where the effect was synergistic, but not in the gills. An important GR increase was found after exposure to Cd, both in the digestive gland \((10.8\text{-fold increase}, p < 0.001)\) and gills \((6.5\text{-fold increase}, p < 0.01)\). GR activity increase was not observed with cPFe in any organ, but a huge rise \((20\text{-fold increase}, p < 0.001)\) was observed in gills upon exposure to the Cd-cPFe mixture. On the other hand, a higher 6PGDH activity was found after exposure to cPFe and to the mixture in both organs, but only in the digestive gland after Cd exposure \((p < 0.05)\). While no combined effect of the mixture was observed on 6PGDH activity, a complex effect was ob-

**Fig. 4. Carcinus maenas. Biomarkers (see Fig. 2) analysed in the digestive gland and gills of animals exposed for 2 d to Cd, chlorpyrifos-ethyl (cPFe) and to a mixture of Cd and cPFe. One unit (U) is defined as µmol of substrate consumed, or of product formed, per min. Data represent mean values ± SD of 3 independent extractions with 3 assays per extract. Statistical significance of results based on corresponding groups of crabs receiving no treatment, used as control: ***\(p < 0.001\), **\(p < 0.01\), *\(p < 0.05\).**
tained with GR, with cPFe exerting an antagonistic effect on the action of Cd in the digestive gland, and a synergistic effect in gills. A slight but significant (p < 0.05), increase in GST activity was found after exposure to cPFe (gills) or the mixture (both organs), but not to Cd. Although GST is highly induced in insects, being involved in insect resistance to OP pesticides, the response of this enzyme in freshwater invertebrates is low and variable (Hyne & Maher 2003, Domingues et al. 2009). Parallel to the drastic changes in so many activities related to oxidative stress, MDA increases were detected in gills after exposure to Cd (2.4-fold increase, p < 0.01), but especially to cPFe (6.1-fold increase, p < 0.001) and the mixture (7- to 9-fold increase, p < 0.001), in which an additive effect was observed. In the digestive gland, MDA increased slightly (p < 0.05) after exposure to cPFe and the mixture, but not to Cd. Acute effects of cPFe on acetyl and butyrylcholinesterases have been reported in digestive gland and gills (Ghedira et al. 2009).

Biomarker responses from crabs subject to chronic exposure (in-field) (Fig. 2) are similar to those obtained after 2 d of acute exposure (Fig. 4), since decreased CAT and higher GR and 6PGDH activities and MDA levels are found in both cases, while no important change is found in GPX or GST activities. The highly significant decrease in CAT could be due to heme oxygenase, a metal-inducible enzyme that would destroy free heme groups, thus lowering the activity of heme-containing enzymes such as CAT (Montes Nieto et al. 2010). The increased proteolytic susceptibility of this enzyme following exposure to various oxidants has also been offered as a plausible explanation (Grune et al. 2003, Montes-Nieto et al. 2007). We also observed a significant CAT decrease after cPFe exposure, both in digestive gland and gills (Fig. 4). GR showed an increase (both organs) after exposure to Cd and to the mixture (gills), but not after OP exposure alone (Fig. 4). However, a significantly higher GR was observed at GAR than at BIZ (Fig. 2), even though higher Cd levels were measured at the last sampling site, both in sediments and organs (Table 1). 6PGDH was also found to be significantly higher only at GAR (Fig. 2), coinciding with the cPFe and Cd-cPFe acute-exposure (Fig. 4).

We wish to highlight the much higher lipid oxidative damage in gills than in the digestive gland, although both organs accumulated metals to a similar extent, Cd levels being even higher in the digestive gland. MDA in GAR crab gills was 1.37 ± 0.24 nmol mg⁻¹ of protein (Fig. 2), rising to 1.64 ± 0.33 nmol mg⁻¹ in animals exposed to the Cd-cPFe mixture (Fig. 4). This is 2 orders of magnitude higher than in the digestive gland, where MDA ranged from 0.012 to 0.036 nmol mg⁻¹. Gills are much more sensitive than the digestive gland to pollutant exposure, and a high proportion of DNA strand breaks and a positive dose-response relationship between DNA damage and Cd levels has been shown in gills, but not in the digestive gland (Pan & Zhang 2006). Oxidative damage and mitochondrial alterations have also been reported in gills, but not in the digestive gland, of the crab Potamonautes warreni exposed to 0.2 mg Cd²⁺ l⁻¹ (Schuwerack & Lewis 2003). Digestive glands have cumulative defence and detoxification mechanisms, not sufficiently developed in gills, to better protect from oxidative stress. Further, higher O₂ levels are found in gills than in the digestive gland, also explaining the more intense lipid oxidative damage of gills, a key organ in gas exchange. Since the similar activities of the antioxidant enzymes measured here cannot explain lipid peroxidation differences between these two organs, other mechanisms must be involved.

Although metals such as Cd induce oxidative stress, especially in gills, thus increasing lipid peroxidation (Fig. 4), the metal content we measured in sediments and crab organs from the Tunisian littoral area (Table 1) does not correlate clearly with oxidative biomarker responses (Fig. 2), as shown by PCA analysis (Fig. 3). Metal levels were much higher at BIZ, both in sediments and in Carcinus maenas organs, than at the other 2 sites, following the order BIZ>GAR>TEB. In contrast, GAR crabs showed higher oxidative stress responses, the order being GAR>BIZ>TEB. In C. maenas from another metal-polluted coastal system (Óbidos Lagoon, Portugal), oxidative stress and biotransformation responses were not directly attributable to metal concentrations in the digestive gland; additive effects of metals, nutrients, alterations in dissolved oxygen and other abiotic variables, and their interactions (synergism, potentiation) could be crucial in inducing biochemical responses (Pereira et al. 2009). How the variability in physico-chemical parameters in estuarine regions can affect biochemical and physiological responses, by altering the bioavailability and the toxicity of pollutants, has been reviewed by Monserrat et al. (2007). The use of more holistic and unbiased proteomic approaches could help to clarify the complexity of responses and interactions (López-Barea & Gómez-Ariza 2006, Monserrat et al. 2007). Recent nereid monitoring studies of Tunisian coasts, using nereid worms have shown similar contamination values, corresponding to a ‘critically contaminated envi-
ronment’ in BIZ and GAR (Bouraoui et al. 2010). However, in the clam *Ruditapes decussatus*, greater contamination has been found at GAR than at BIZ (Banni et al. 2005). High levels of organic contaminants from agricultural, industrial and domestic-urban sources, capable of inducing ROS production, may be present at GAR, thus contributing to its higher pollution level and elevated oxidative stress condition. High levels of petroleum hydrocarbons (from oil transportation, shipping and industrial activities, urban runoff and waste discharge) have been found along the coasts of Sfax and Gabès Gulf (Louati et al. 2001, Zaghdén et al. 2005). It should be noted that pesticides (cPFe), alone or in company with metals, increase lipid peroxidation well above levels induced by Cd exposure (Fig. 4).

**CONCLUSIONS**

The present study shows that, given the increasing number and diversity of pollutants dumped into the marine environment and the high cost of chemical measurements of water and sediment quality, biomarker assessment in sentinel organisms is an effective method for predicting detrimental effects and evaluating toxicity of pollutant mixtures. In our study, a set of early-warning exposure biomarkers (several antioxidant enzymes) and an effect biomarker (MDA) were analyzed in crab tissues (liver and gills). CAT and MDA were the most sensitive biomarkers and gills is the most responsive organ. A lower CAT activity in gills is associated with higher MDA levels. Nevertheless, although the response of conventional biomarkers has been well established, they can be biased and may give only partial information (Vioque-Fernández et al. 2009, Montes Nieto et al. 2010). For this reason, we are presently carrying out a more general and comprehensive proteomic study using 2-dimensional electrophoresis and mass spectrometry analysis in the same areas along the Tunisian littoral zone, to elucidate new and unbiased pollution biomarkers.

**Acknowledgements.** This work was funded by a mobility grant (A/016113/08) from the Spanish Ministry of Foreign Affairs, Spanish Agency of International Cooperation, within the Spain-Tunisia program, and a grant (CTM2009-12858-C02) from the Spanish Ministry of Science and Education. The authors also thank the Ministry of Higher Education, Scientific Research and Technology, Tunisia (Unité de Recherche en Biochimie et Toxicologie Environnementale grant no. UR (4AGR03)), and the Institution de la Recherche et de l’Enseignement Supérieur Agricole, Tunisia.

**LITERATURE CITED**

Ghedira et al.: Using oxidative stress biomarkers in *Carcinus maenas*


Submitted: May 13, 2011; Accepted: September 8, 2011
Proofs received from author(s): November 8, 2011

Editorial responsibility: Matthias Seaman, Oldendorf/Luhe, Germany