Environ Sci Pollut Res (2014) 21:13315–13323
DOI 10.1007/s11356-014-2741-y

Metabolic signatures associated with environmental pollution by metals in Doñana National Park using *P. clarkii* as bioindicator

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Received: 17 July 2013 / Accepted: 4 March 2014 / Published online: 24 April 2014

Abstract Bioindicators can reflect the effects of pollutants on their metabolism, being widely used to assess environmental stress. In this sense, the crab *Procambarus clarkii* has been previously proposed to monitor the contamination in Doñana National Park (southwest Spain) using conventional biomarkers. In this work, a metabolomic approach based on direct infusion mass spectrometry, which allows an easy and quick study of a large number of metabolites in a single run, was used for pollution assessment of this area, considering the biological response of this organism to contamination. In addition, metal accumulation in crab tissues was determined. Thus, the integrated analysis of metabolomic and metallomic data enabled the study of metabolic response of the organism against pollution. Several metabolites were discovered as potential biomarkers of pollution, such as decreased levels of carnosine, alanine, niacinamide, acetoacetate, pantothenic acid, ascorbate, glucose-6-phosphate, arginine, glucose, lactate, phospholipids, and tryglicerides, as well as elevated levels of acetyl carnitine, phosphocholine, choline, and uric acid. In this way, metal-induced toxicity could be related to metabolic impairments, principally oxidative stress, metabolic dysfunction, and dyslipidemia.

Keywords *Procambarus clarkii* · Metabolomics · Doñana National Park · Pollution · Metals · Biological response

Responsible editor: Markus Hecker

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Introduction

The study of environmental stressors is generally performed by the use of bioindicators or sentinel organisms, which reflect the biological response to the presence of contaminants (Beeby 2001). In this sense, invertebrates have been frequently proposed for the study of aquatic environments since they can indicate changes in the environment through their responses at different levels of organization, ranging from the individual animal to the total invertebrate community (Phillips and Rainbow 1993; Rosenberg and Resh 1993). The advantage of biological monitoring using invertebrates relies on their demographic characteristics, such as wide distribution in the environment, abundance, and population turnover times (Hodkinson and Jackson 2005). Thus, a multitude of different aquatic and terrestrial invertebrates from protozoa, nematodes, bivalves, crustaceans, and insects have all been suggested for this purpose.

The crab *Procambarus clarkii* has a long life cycle, wide distribution, and sedentary lifestyle, and it can live in a wide range of environmental conditions, even in highly polluted waters, which render this species a good bioindicator to assess

the effects of contaminants (Alcorlo et al. 2006; Schilderman et al. 1999). This organism was introduced into the marshes of the lower Guadalquivir River (SW, Spain) catchment in 1974, colonizing quickly all the nearby aquatic systems in the absence of natural predators, reaching Doñana National Park (DNP) (Cruz and Rebelo 2006). The National Park involves the inner core of the whole reserve and it is the area with highest protection, declared Reserve of the Biosphere by the United Nations Organization for Education, Science and Culture in 1981. Environmental monitoring of this area is necessary due to intense activity in its surroundings, namely agriculture, mining, tourism, and industrial activities (Oñate et al. 2003). In addition, the biological reserve was affected by the Aznalcóllar mining spill on the Guadalquivir estuary in 1998, which damaged about 4,328 ha situated at both shores of the Guadiamar river (Grimalt et al. 1999). The crab *P. clarkii* has been frequently used to monitor contamination of water in DNP, and numerous authors proposed the determination of metals concentrations in this organism to assess metal contamination in Doñana, as well as their bioavailability and bioaccumulation processes (Sánchez-López et al. 2003, 2004; Alcorlo et al. 2006). On the other hand, environmental stress induced in *P. clarkii* has been also evaluated by the use of several molecular biomarkers reflecting the effects of pollutants on cellular metabolism (Vioque-Fernández et al. 2007, 2009).

To characterize biological responses induced by metal pollutants in bioindicators, conventional ecotoxicological studies normally include a battery of biomarkers, such as proteins for detoxification of metal contamination like metallothioneins, mixed function oxidase (EROD), and glutathione *S*-transferase (GST) for detoxification of organic compounds, as well as glutathione peroxidase (GPX) and glutathione reductase (GR) antioxidant enzymes, among others (Martín-Díaz et al. 2008). However, the use of conventional biomarkers requires a deep knowledge about the toxicological mechanisms involved in the metabolism, and they do not consider other changes that can be induced by the presence of metals or other contaminants which would not be represented by the conventional list of biomarkers. For this reason, −omic sciences are emerging as a new tool to evaluate the global biological response to contamination. Metabolomics is based on the study of the complete set of endogenous low-molecular-weight compounds in living organisms at a specified time under specific environmental conditions. This approach is a good tool to understand biological processes occurring in complex systems since metabolites may be considered as the final product of interactions between genes and proteins expression with the cellular environment. Thus, environmental metabolomics is gaining importance for the characterization of interactions of living organisms with their environment (Bundy et al. 2009). On the other hand, metallomics, which is focused on the study of metal-species related to cells, organs, and tissues, can provide complementary information about the biochemical changes associated with metal pollution due to the importance of metals in biological systems (Lobinski et al. 2006).

The aim of this work was to determine the response induced by contaminants in *P. clarkii* specimen from Doñana National Park and surroundings in order to assess the effects of environmental pollution in this species. For this purpose, total metal levels were evaluated in different tissues as a primary source of contamination in the studied area. In addition, metabolomic analysis was carried out by direct infusion into a triple quadrupole time-of-flight mass spectrometer with electrospray ionization source (DI-ESI-QqQ-TOF) to investigate the relationships between metal levels and the metabolic response of the organism.

Experimental

Reagents and samples

Methanol and chloroform (high-performance liquid chromatography grade) were purchased from Fisher Scientific (Leicestershire, UK), while formic acid and ammonium acetate were supplied by Aldrich (Steinheim, Germany). Nitric acid and hydrogen peroxide (Suprapur quality) were obtained from Merck (Darmstadt, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

Three specimens of crab *P. clarkii* were collected per sample point during the spring 2009 from DNP, southwest Spain. Animals were collected form five different sampling areas with differential contamination (Fig. 1). The control point was “Lucio del Palacio” (LDP, green spot), a low contaminated area located in the center of the park. The other sampling points were “Matochal” (MAT, yellow rhombus), next to Guadiamar river that is affected by rice-growing fields and suffered the input of metals during the rupture of Aznalcollar mine tailing pond in 1998 (Grimalt et al. 1999); “Partido” (PAR, blue triangle) and “Ajolí” (AJO, white triangle), up- and downstream of El Partido stream, respectively, which are under the influence of citrus fruit and grape fields; and “Bernabé” (BER, orange square), in La Rocina stream, with strawberry, citrus fruit, and grape fields in the surroundings, and in addition affected by diffused pollution from industrial and metallurgical activities.

Crabs were caught with tubular plastic mesh traps, baited with raw chicken parts. Adult animals were taken alive to the laboratory, and site/date of capture, sex, weight, and size were recorded. Crabs were dissected for excision of individual organs (digestive gland, muscle, gills, and antennal glad), which were cleaned with 0.9 % NaCl solution, weighed in Eppendorf tubes and frozen in liquid nitrogen. Finally, tissues were cryohomogenized in a cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770), during 1 min at rate of 15 strokes per second, and stored at −80 °C until analysis.

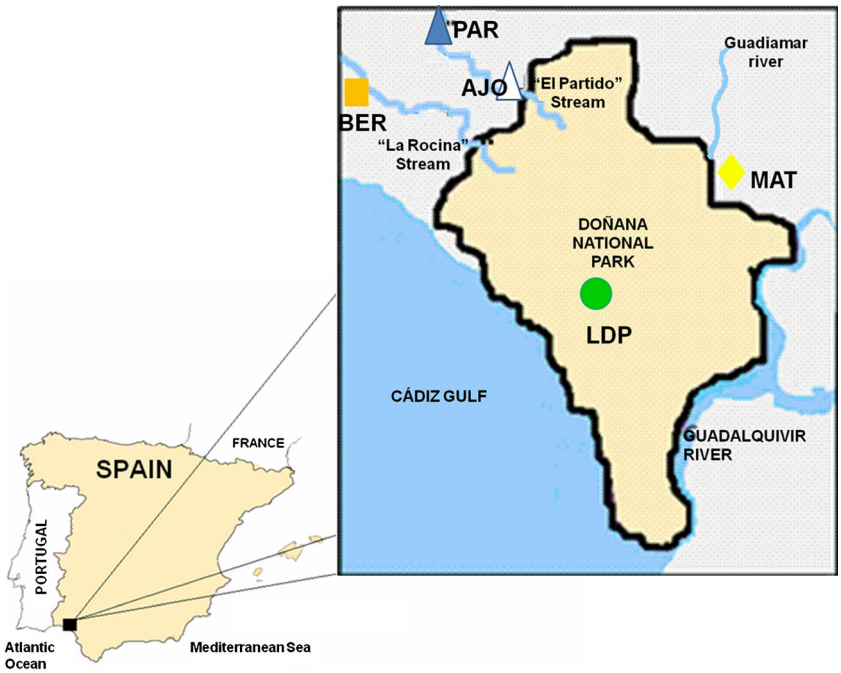

Fig. 1 Sampling areas in Doñana National Park and surrounding—control point, "Lucio del Palacio" (LDP, *green spot*); contaminated areas, "El Matochal" (MAT, *yellow rhombus*), "Ajolí" (AJO, *white triangle*), "Partido" (PAR, *blue triangle*), "Bernabé" (BER, *orange square*)

Total metals content analysis

Total metals content in main crab tissues (digestive gland, muscle, and gills) was analyzed in order to evaluate the pollution in the five areas considered in this study. For this purpose, homogenized tissue samples from three different specimens in each area were pooled and subjected to microwave-assisted acid digestion. For this purpose, 0.200 g of sample was exactly weighed in 5-ml microwave vessels, and 800 μL of a mixture containing nitric acid and hydrogen peroxide (4:1 *v*/*v*) was added. After 10 min, the PTFE vessels were closed and introduced into a microwave-accelerated reaction system (MARS, CEM, Matthews, NC, USA). The mineralization was carried out at 400 W, from room temperature to 160 °C in 15 min and held for other 40 min at this temperature. Then, solutions were made up to 2 ml with ultrapure water, adding rhodium as internal standard (1 μg L^{-1}). All these analysis were performed in triplicate.

Concentrations of heavy metals were determined using an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan), equipped with an octopole collision/reaction cell and a micromist nebulizer. Helium of high-purity grade (>99.999 %) was used as collision gas, and a tuning solution containing Li, Co, Y, and Tl at 1 μg L^{-1} was employed for instrumental conditions optimization. The forward power was set at 1,500 W, and the gas flow rates were fixed at 15 L min^{-1} for plasma gas, 1 L min^{-1} for both auxiliary and nebulizer gases, 0.15 L min^{-1} for makeup gas, and 3.5 mL min^{-1} for helium. Isotopes monitored were ^{63}Cu, ^{64}Zn, ^{65}Cu, ^{66}Zn, ^{75}As, and ^{112}Cd, with a dwell time of 0.3 s per isotope.

Metabolomics

Tissue samples were treated in a two-step procedure for subsequent mass spectrometry-based metabolomic analysis. In a first step, homogenized tissues were extracted with an 80:20 (*v*/*v*) methanol/water mixture, using a pellet mixer (VWR International, UK) to obtain a polar extract of metabolites. To this end, 30 mg of digestive gland, muscle, or gills were stirred with 500 μL of methanolic extractant during 1 min, followed by vortexing for 5 min at room temperature and centrifugation at 10,000 rpm for 10 min at 4 °C. In the case of antennal glands, the extraction was performed by adding 100 μL of methanol/water mixture to 10 mg of sample, and following the same procedure as explained for other tissues. Then, the pellet isolated during the first step was again extracted in order to recover the lipophilic fraction of metabolites. Thus, the precipitate was mixed with 500 μL of chloroform/methanol in a proportion 2:1 (*v*/*v*) (or 100 μL for antennal glands), and extracted in the same way as the first step. Finally, to improve sensitivity of the analysis by electrospray mass spectrometry, different additives were employed. For positive ion mode analysis, 0.1 % (*v*/*v*) formic acid was added to polar extracts, while addition of ammonium ions (10 mM ammonium acetate) was selected for lipophilic extracts, since neutral lipids are not readily ionized by electrospray ionization source (ESI). In the case of negative ionization, it was checked that addition of additives to samples was not necessary to increase sensitivity. Therefore, further experiences were performed by injection of extracts.

For metabolomic analysis by direct infusion mass spectrometry (DIMS), a QSTAR XL Hybrid system was employed

(Applied Biosystems, Foster City, CA, USA) using an ESI source according to the method previously optimized (González-Domínguez et al. 2012). The samples were introduced into the mass spectrometer at 5 μL min^{-1} flow rate using an integrated apparatus syringe pump and a 1,000-μL volume Hamilton syringe. Data were obtained both in positive and negative ion modes acquiring averaged full-scan spectra for 0.2 min in the *m*/*z* range of 50–1,100 with 1.005 s scan time. In positive mode, the ion spray voltage (IS) was set at 3,300 V, and high-purity nitrogen was used as curtain and nebulizer gases at flow rates about 1.13 and 1.56 L min^{-1}, respectively. The source temperature was fixed at 60 °C, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. For ESI(−), only a few parameters were modified with respect to ESI(+) method, which were ion spray voltage at −4,000 V, DP of −100 V, and FP of −250 V. To acquire tandem mass spectra (MS/MS), nitrogen was used as collision gas.

Data analysis

Markerview™ software (Applied Biosystems) was employed to preprocess the mass spectrometry results and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1 Da, and a minimum response of 10 counts was considered for filtering. Finally, data were normalized according to the total area sum. Then, data were submitted to Pareto scaling and processed by partial least squares discriminant analysis (PLS-DA) in order to discriminate between samples from different sites, using SIMCA-P™ software (version 11.5, UMetrics AB, Umeå, Sweden). Quality of the PLS-DA models was assessed by their class separation (R^2) and predictive power (Q^2) values, which are indicative of goodness-of-fit and goodness-of-prediction, respectively. In addition, these models were validated using permutation tests (Y-scrambling) of the Y-predicted values. In Y-scrambling, class labels are randomly permuted for refitting a new model with the same number of components as the original one, and then these new models are compared with the original models to test the possibility that the original model arose by chance. Thus, an overfitted model will have similar R^2 and Q^2 to that of the randomly permuted data, while well fitted and meaningful models will have R^2 and Q^2 values that are always higher than that of the permuted data. Finally, potential biomarkers of environmental pollution were selected according to the variable importance in the projection or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 2, indicative of significant differences among groups. These metabolites were identified matching the experimental accurate mass and MS/MS, with those available in open-access databases, such as the METLIN (http://metlin.scripps.edu) or Mass Bank (http://www.massbank.jp).

Results

Environmental metal pollution

Morphometric data of crab specimen sampled in the areas under study are collected in Table 1. Analysis of total metals content in the main tissues of *P. clarkii* (digestive gland, muscle, and gills) allowed assessing the different levels of contamination in each site of study. Concentrations of the most representative metals (zinc, copper, arsenic, and cadmium) show that digestive glands present the highest levels of metals, followed by gills and muscles (Table 2). These results show important pollution in "Matochal" area, probably due to the spill of mining waste in 1998 from Aznalcóllar mine (Grimalt et al. 1999), followed by "Ajolí" and, to a lesser extent, "Partido" and "Bernabé" areas. On the other hand, specimens captured in "Lucio del Palacio", a reference site inside Doñana National Park, present the lowest metal levels. In addition, these results are in agreement with previous data published about total metals levels content in soils, sediments, and water collected in the same points under study (García-Sevillano et al. 2012).

Comparison of metabolomic profiles

The metabolomic approach based on direct infusion electrospray mass spectrometry, together with the use of complementary analytical methodologies combining a two-step extraction of polar and lipophilic metabolites with analysis by ESI(+)/ESI(−), gave comprehensive metabolic profiles of crab samples. Moreover, the study of both digestive and antennal glands, muscle, and gills provides an overview of the biochemical differences between the tissue types. Using the intensity of the *m*/*z* peaks in ESI full-scan mass spectra as

Table 1 Morphometric data of crab specimen under study

Sampling site	Sample	Sex	Weight (g)	Size (cm)
Lucio del Palacio (LDP)	1	Male	57.4	11.5
	2	Male	57.9	12.0
	3	Male	60.4	12.0
Partido (PAR)	1	Male	31.3	10.5
	2	Male	28.3	9.5
	3	Male	28.0	10.0
Ajoli (AJO)	1	Male	37.0	10.0
	2	Male	35.0	10.0
	3	Male	39.0	9.5
Bernabe (BER)	1	Male	36.0	10.5
	2	Male	27.0	9.5
	3	Male	34.5	10.5
Matochal (MAT)	1	Male	28.0	9.5
	2	Male	27.5	9.5
	3	Male	62.0	13.5

Table 2 Metal concentrations in *Procambarus clarkii* tissues (means±SD, μg g^{-1} dry weight) in the five studied sites

Tissue	Sampling point	Cu (μg g^{-1})	Zn (μg g^{-1})	As (μg·g^{-1})	Cd (μg·g^{-1})
Digestive gland	LDP	2.946 (±1.128)	27.410 (±7.707)	1.102 (±0.012)	0.040 (±0.001)
	BER	73.425 (±1.419)	67.097 (±0.946)	0.477 (±0.005)	0.094 (±0.002)
	PAR	84.056 (±4.421)	29.825 (±2.570)	1.281 (±0.024)	0.088 (±0.010)
	AJO	156.169 (±4.541)	52.254 (±1.702)	1.002 (±0.022)	0.161 (±0.002)
	MAT	340.344 (±7.312)	135.388 (±5.325)	2.027 (±0.023)	3.838 (±0.001)
Muscle	LDP	1.414 (±1.320)	9.506 (±2.320)	0.111 (±0.007)	<LOD
	BER	2.662 (±1.420)	8.815 (±1.850)	0.145 (±0.003)	<LOD
	PAR	4.459 (±1.171)	9.707 (±2.456)	0.281 (±0.100)	<LOD
	AJO	8.710 (±1.322)	10.398 (±2.450)	0.154 (±0.002)	<LOD
	MAT	3.637 (±1.178)	11.292 (±2.671)	0.137 (±0.003)	<LOD
Gill	LDP	10.281 (±0.429)	4.331 (±0.281)	0.403 (±0.020)	<LOD
	BER	27.589 (±6.814)	6.136 (±3.121)	0.321 (±0.066)	<LOD
	PAR	46.591 (±2.640)	5.540 (±0.877)	0.396 (±0.088)	<LOD
	AJO	39.678 (±1.949)	4.596 (±0.481)	0.229 (±0.011)	<LOD
	MAT	29.992 (±0.693)	23.846 (±5.605)	2.767 (±0.809)	0.082 (±0.026)

LOD limit of detection (calculated as the concentration that corresponds to a signal equal to three times the standard deviation of a blank), *LDP* Lucio del Palacio, *BER* Bernabe, *PAR* Partido, *AJO* Ajoli, *MAT* Matochal

chemical descriptors, PLS-DA was applied to establish whether there were defined groups between different sampling points. One statistical model was built with each data batch, considering analysis in positive and negative modes of polar and lipophilic extracts for the four different tissues, so a total of 16 discriminant analyses were performed employing the metabolomic data obtained. Figure 2 shows a representative scores plot of one of these models in which polar extracts from digestive glands (n=3), analyzed in positive ionization mode, are classified according to their provenance. As can be observed, samples are classified in different clusters of points, but separation between groups is not perfect, occurring overlapping among several of them. However, the uncontaminated area of "Lucio del Palacio" was clearly classified in a support cluster with respect to the samples from polluted sites.

These metabolic differences could be attributed to pollution effects, considering LDP as control point (inside the National Park) versus the other contaminated sites affected by metal input from mine spillage or agriculture pollution. In this way, metabolomic data were reprocessed considering only two groups of study: the reference site (LDP) and the polluted sites (MAT, AJO, PAR, and BER). Scores plots from these statistical analyses show a complete separation between the two cases considered (Fig. 3).

In total, four latent components were calculated producing the best predictive modeling capabilities in terms of R^2 and Q^2 values (Table 3). As can be observed, models provided good classification regarding the R^2 value and Q^2. In addition, permutation tests demonstrated the validity of this discrimination since R^2 and Q^2 values of permuted models were lower than those for the original PLS-DA models indicating that the models were not overfitted.

Finally, a considerable number of metabolites could be identified as potential biomarkers of environmental pollution

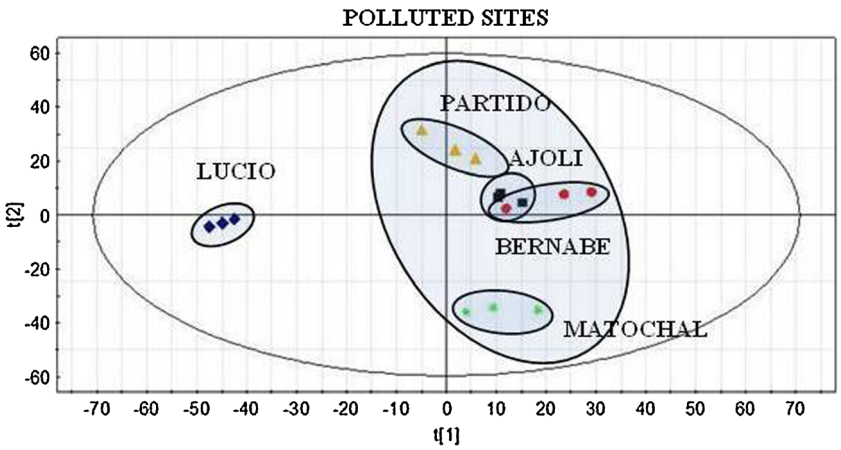

Fig. 2 Scores plot of the PLS-DA considering five areas of study. *Blue diamonds* Lucio del Palacio (LDP), *red circles* Bernabe (BER), *yellow triangles* Partido (PAR), *green stars* Matochal (MAT), *black squares* Ajoli (AJO)

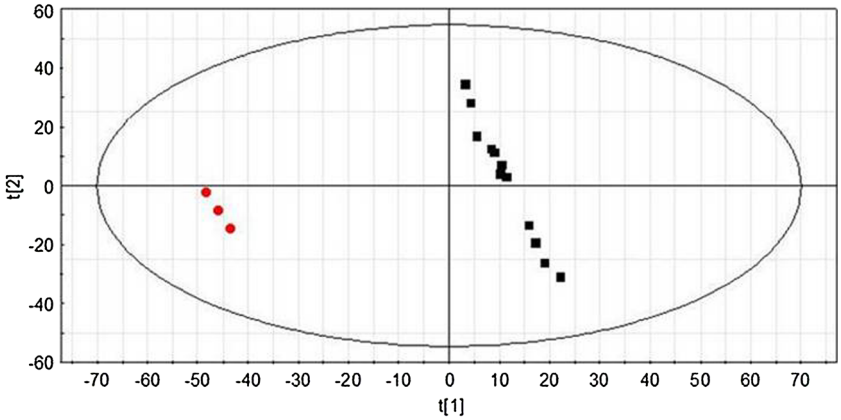

Fig. 3 Scores plot of the PLS-DA considering polluted and clean points. *Red circles* LDP, *black squares* polluted sites (MAT, AJO, PAR, and BER)

using the variable importance in the projection as explained in the "Experimental" section. Thus, these most important signals in the discrimination were identified with the help of metabolomic databases (Table 4). The most important markers were related to decreased levels of carnosine, alanine, niacinamide, acetoacetate, pantothenic acid, ascorbate, glucose-6-phosphate, arginine, glucose, lactate, and increased levels of acetyl carnitine, phosphocholine, choline, and uric acid. On the other hand, besides these individual compounds, some signals in the *m*/*z* ranges of 650–850 and 850–900, which can be assigned to phospholipids (PLs) and triglycerides (TGs), respectively, decreased considerably in polluted samples. However, specific PLs and TGs were not individually identified since overexpression was found in the complete group of lipids. Finally, it must be remarked that some of these biomarkers were tissue-specific, since altered levels were found only in one of them, while others were common to all samples studied from the different sampling points.

Discussion

Discriminant analysis of metabolomic fingerprints from crab tissues of Doñana National Park showed clear differences between specimens captured in contaminated and noncontaminated areas. Thus, several metabolites were identified as potential biomarkers of pollution that could be used as indicators of environmental status, but in addition, this information is of great value in the study of the mode of action of toxicants.

Downregulation of phospholipids, ascorbate, and carnosine and overexpression of phosphocholine, choline, and uric acid were indicative of oxidative impairment due to an imbalance between the physiologic rate of oxidants production and response time of antioxidant defense system. In aquatic toxicology, oxidative stress has become an important subject in environmental exposure to both anthropogenic stressors (toxicants from pollution) and natural stressors (variations in salinity, temperature, and oxygen and viral and bacterial infections). In particular, there is a great interest in the contaminant-stimulated production of reactive oxygen species (ROS), and the resulting oxidative damage in proteins, lipids, and DNA as a mechanism caused by toxicants in aquatic organisms (Livingstone 2001). Among them, membrane phospholipids are important targets of free radicals action, since they are rich in unsaturated fatty acids, resulting in lipid peroxidation that leads to a loss of membrane integrity and function and as a result, to structural and functional damage of cells, tissues, and whole organs (Winston and Di Giulio 1991). Thus, decreased levels of total phospholipids were consistent with this oxidative injury, as well as with the increase of degradation products of phosphatidylcholines, free choline, and phosphocholine, which belong to one of the most important phospholipid families (Table 3). Other important metabolic changes related to oxidative stress in metabolomic profiles of *P. clarkii* exposed to pollutants are deficiencies of ascorbate and carnosine. While ascorbic acid is one of the most studied antioxidants that represent a scavenger of free radicals, carnosine is a dipeptide that also presents protective effects, preventing peroxidation of lipids, proteins, and carbohydrates by reactive oxygen radicals, as a quencher of 4-

Table 3 Statistical parameters of the PLS-DA models

		Digestive gland		Gills		Muscle		Antennal gland	
		R^2	Q^2	R^2	Q^2	R^2	Q^2	R^2	Q^2
ESI+	Polar extract	1	0.882	0.996	0.621	0.994	0.856	0.999	0.862
	Lipophilic extract	0.99	0.90	0.994	0.322	0.998	0.831	1	0.875
ESI-	Polar extract	1	0.927	0.989	0.567	0.999	0.866	0.997	0.858
	Lipophilic extract	1	0.941	1	0.814	1	0.903	1	0.892

Table 4 Potential biomarker of pollution, up- (↑) or downregulated (↓) in polluted sites

Metabolite	Metlin ID	Tissue	Change in polluted sites
Phospholipids	–	Digestive gland, muscle, gills, antennal gland	↓
Choline	56	Gills	↑
Phosphocholine	3,318	Antennal gland	↑
Carnosine	38	Digestive gland	↓
Ascorbate (vitamin C)	249	Digestive gland	↓
Niacinamide (vitamin B3)	1,497	Digestive gland	↓
Pantothenic acid (vitamin B5)	241	Digestive gland	↓
Acetoacetate	276	Digestive gland	↓
Acetyl carnitine	956	Digestive gland	↑
Uric acid	88	Gills	↑
Glucose	133	Muscle, antennal gland	↓
Glucose-6-phosphate	145	Muscle	↓
Alanine	11	Digestive gland, muscle	↓
Arginine	13	Muscle	↓
Triglycerides	–	Digestive gland, gills	↓

hydroxy-2-nonenal and malonaldehyde, and preventing glycation of proteins (Kohen et al. 1988). It has also been demonstrated that carnosine acts as neutralizing agent for copper overload-induced damages in cultured human cells, and it is also an effective antioxidant that can dismutate superoxide radicals, scavenge hydroxyl radicals, and neutralize the lipid peroxidation (Arnal et al. 2011). In this sense, depletion of cellular antioxidant defense systems has been previously reported in toxicant exposure, causing decreases in both primary antioxidant protection by the enzymes superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic antioxidant levels (Limón-Pacheco and Gonsebatt 2009). Finally, there is also evidence for the role of hyperuricemia in the development of oxidative stress, since excess of uric acid can be associated with overexpression of xanthine oxidase (XO), an enzyme involved in catabolism of purines that generates endogenous reactive oxygen species. Induction of XO activity has been previously related to pollutant exposure, mainly cadmium, causing conversion of xanthine dehydrogenase into xanthine oxidase and abnormalities in urate transporters (Wang et al. 2012). Taking into account that cadmium is an important metal toxicant in the polluted areas considered in this study, but is practically absent in the reference site (Table 2), an overactivation of XO is expected, leading to uric acid accumulation in these samples (Table 4).

Changes in pathways of energy production, induced by pollutants, represent also an important harmful effect of contamination. One of the most important factors related to impaired energy metabolism in crustaceans is the decrease in oxygen uptake associated with a significant reduction in oxygenation due to gill damage by pollutants, including metals. This hypoxia generated by long-term exposure produces a hypoglycemic response and disturbance in the anaerobic metabolism (Bonvillain et al. 2012) leading to decreased levels of carbohydrates and related compounds involved in glycolysis in tissues of exposed organisms, while elevated lactate is normally observed as a consequence of enhanced anaerobiosis (Reddy and Bhagyalakshmi 1994). In this sense, decreased glucose and glucose-6-phosphate have been observed in *P. clarkii* specimens from polluted areas, demonstrating impairment in glycolysis. In addition, alanine was also reduced in these same samples. Alanine is an important end product of

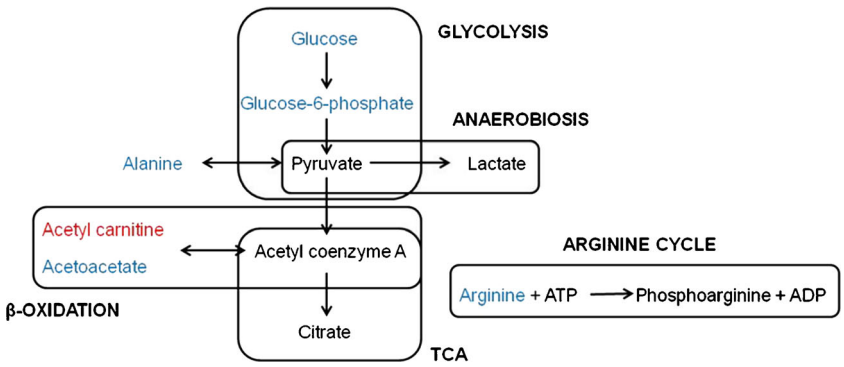

Fig. 4 Schematic energy metabolism dysregulation in *P. clarkii*. *Red* metabolites overexpressed in polluted sites; *blue* metabolites downexpressed in polluted sites; *black* unchanged metabolites

anaerobic breakdown of glucose, and its dysregulation has been previously associated with disturbances in the energetic metabolism, being decreased in numerous exposure experiences to metallic pollutants principally in bivalves (Zhang et al. 2011a, b; Liu et al. 2011a; Blasco and Puppo 1999). On the other hand, this hypoxia is also responsible for liberating ROS, increasing oxidative damage that can interfere directly in energy metabolism through mitochondrial failure, and impaired oxidative phosphorylation (OP) (Halliwell and Gutteridge 1999). This has been previously related to depletion of tricarboxylic acid cycle (TCA) in other species including a decrease in lactate dehydrogenase activity and an increase in lactate content, reduced mobilization of pyruvate into the cycle, and decreased activity of related enzymes such as succinate dehydrogenase and malate dehydrogenase (Reddy and Bhagyalakshmi 1994). In our case, deficiencies of B vitamins (niacinamide and pantothenic acid), which are required as coenzymes for maintaining mitochondrial function, could indicate a deteriorated OP. Niacinamide (B3) is required to supply protons for oxidative phosphorylation, while pantothenic acid (B5) is involved in coenzyme A formation and is also essential for α-ketoglutarate and pyruvate dehydrogenase complexes as well as fatty acid oxidation (Depeint et al. 2006). Thus, this abnormal status of B vitamins content could potentially be employed as an index of proper mitochondrial function. Arginine levels are also closely related to this slowed energetic metabolism since the equilibrium arginine/phosphoarginine is responsible for energy buffer in high-energy demand situations in invertebrates. It has been demonstrated that ATP levels are protected by this cycle in crabs subjected to hypoxia by lead exposure, causing decreased ATP/ADP ratio due to increased ADP levels (Morris et al. 2005). Additionally, depletion of arginine and ATP/ADP has been proposed as biomarker of disorder of energy metabolism in clams exposed to mercury (Liu et al. 2011a, b). In this way, lowered levels of arginine in muscles of exposed *P. clarkii* could be considered as a reliable marker of energy dysfunction. Finally, altered levels of acetyl-carnitine and acetoacetate, which are involved in lipid metabolism, could also be associated with this impaired energetic status. Acetyl-carnitine is in equilibrium with carnitine and acetyl-coenzyme A, controlling the transport of fatty acids into the mitochondria for oxidation. On the other hand, acetoacetate is a ketone body derived from acetyl-coenzyme A during oxidation of free fatty acids, whose down expression is known in bivalves exposed to metals (Liu et al. 2011a, b). In this sense, the observed increase of acetyl-carnitine and decrease of acetoacetate reflected the effects of pollution in crab, suggesting a shift in energy metabolism by β oxidation of fatty acids. Therefore, a global imbalance in the energy production pathways can be concluded from experimental metabolomic profiles of *P. clarkii* (Fig. 4) in which the most important metabolic changes were impact on glycolysis, anaerobiosis, beta-oxidation, and arginine cycle as indicated by the values from VIP plots.

Finally, besides alterations related to oxidative stress and shifts in energy metabolism, reduced levels of triglycerides denoted a dyslipidemic situation, which could be related to nephrotoxicity and hepatic failure. Thus, changes in lipid content have been demonstrated that is a common stress-induced response in crustaceans, and which have been demonstrated for the exposure to both inorganic (Torreblanca et al. 1991; Chinni and Yallapragada 2002) and organic pollutants (Wang and Stickle 1988).

Conclusions

The integration of metabolomic analysis in crab tissues from Doñana National Park and surroundings and total metal concentrations allowed the assessment of environmental pollution in this important ecological reserve. This study illustrates that high-throughput metabolomics based on tandem mass spectrometry is an efficient method for the characterization of biological response to heavy metal and possibly other contamination, and important metabolites related to stress induced by pollutants can be qualitatively evaluated using metabolite profiling. This combination of metabolomics and metal profiling allows a more efficient discrimination of the level of contamination (mainly associated with metals in this study but extensible to other pollutants) and its effect on free-living organisms in the five areas under study, ranging from noncontaminated areas such as "Lucio del Palacio", the reference site in the center of DNP, to "Matochal", highly contaminated by agriculture activities and mining spillage. In addition, several biomarkers were identified, which could be used as indicators of environmental health. In this way, oxidative stress and energy impairments were found to be the principal metabolic alterations associated to toxicological response, regarding changes in phospholipids, glucose, alanine, arginine, or different vitamins, among others. In conclusion, it has been demonstrated that the strong impact of pollutants, principally metals, in the metabolism *P. clarkii* used as bioindicator. However, further studies are required to understand the responses of crabs exposed to single metal or other contaminants, as well as the quantification of the metabolites identified as biomarkers.

Acknowledgments This work was supported by the projects CTM2012-38720-C03-01 from the Ministerio de Economia y Competitividad and P008-FQM-3554 and P009-FQM-4659 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía). Raúl González Domínguez thanks the Ministerio de Educación for a predoctoral scholarship.

References

Alcorlo P, Otero M, Crehuet M, Baltanas A, Montes C (2006) The use of the red swamp crayfish (*Procambarus clarkii*, Girard) as indicator of the bioavailability of heavy metals in environmental monitoring in the River Guadiamar (SW, Spain). Sci Total Environ 366:380–390

Arnal N, de Alaniz MJT, Marra CA (2011) Carnosine and neocuproine as neutralizing agents for copper overload-induced damages in cultured human cells. Chem-Biol Interact 192(3):257–263. doi:10.1016/j.cbi.2011.03.017

Beeby A (2001) What do sentinels stand for? Environ Poll 112:285–298

Blasco J, Puppo J (1999) Effect of heavy metals (Cu, Cd and Pb) on aspartate and alanine aminotransferase in *Ruditapes philippinarum* (Mollusca: Bivalvia). Comp Biochem Physiol C-Toxicol Pharmacol 122(2):253–263

Bonvillain CP, Rutherford CA, Kelso WE, Green CC (2012) Physiological biomarkers of hypoxic stress in red swamp crayfish *Procambarus clarkii* from field and laboratory experiments. Comp Biochem Physiol A-Mol Integr Physiol 163:15–21

Bundy JG, Davey ÆMP, Viant MR (2009) Environmental metabolomics: a critical review and future perspectives. PubliCat 5(1):3–21. doi:10.1007/s11306-008-0152-0

Chinni S, Yallapragada PR (2002) Energy levels of *Penaeus indicus* postlarvae on exposure to lead. Ecotox Environ Safe 52:173–179

Cruz MJ, Rebelo R (2006) Colonization of freshwater habitats by an introduced crayfish, *Procambarus clarkii*, in Southwest Iberian Peninsula. Hydrobiologia 575(1):191–201. doi:10.1007/s10750-006-0376-9

Depeint F, Bruce WR, Shangari N, Mehta R, O'Brien PJ (2006) Mitochondrial function and toxicity: role of the B vitamin family on mitochondrial energy metabolism. Chem-Biol Interact 163:94–112

González-Domínguez R, García-Barrera T, Gómez-Ariza JL (2012) Metabolomic approach to Alzheimer's disease diagnosis based on mass spectrometry. Chem Pap 66(9):829–835

Grimalt JO, Ferrer M, Macpherson E (1999) The mine tailing accident in Aznalcollar. Sci Total Environ 242(1–3):3–11

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. Oxford University Press, Oxford

Hodkinson ID, Jackson JK (2005) Terrestrial and aquatic invertebrates as bioindicators for environmental monitoring, with particular reference to mountain ecosystems. Environ Manage 35(5):649–666

Kohen R, Yamamoto Y, Cundy KC, Ames BN (1988) Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proc Natl Acad Sci U S A 85:3175–3179

Limón-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutation Res 674:137–147

Liu X, Zhang L, You L, Cong M, Zhao J, Wu H, Li C, Liu D, Yu J (2011a) Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. Environ Toxicol Phar 31:323–332

Liu X, Zhang L, You L, Yu J, Cong M, Wang Q, Li F, Li L, Zhao J, Li C, Wu H (2011b) Assessment of clam *Ruditapes philippinarum* as heavy metal bioindicators using NMR-based metabolomics. Clean Soil Air Water 39:759–766

Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42:656–666

Lobinski R, Moulin C, Ortega R (2006) Imaging and speciation of trace elements in biological environment. Biochimie 88:1591–1604

Martín-Díaz ML, Blasco J, Sales D, Delvalls T (2008) Field validation of a battery of biomarkers to assess sediment quality in Spanish ports. Environ Poll (Barking Essex 1987) 151(3):631–640. doi:10.1016/j.envpol.2007.03.019

Morris S, van Aardt WJ, Ahern MD (2005) The effect of lead on the metabolic and energetic status of the Yabby, Cherax destructor, during environmental hypoxia. Aquat Toxicol 75:16–31

Oñate JJ, Pereira D, Suarez F (2003) Strategic environmental assessment of the effects of European Union's regional development plans in Doñana National Park (Spain). Environ Manage 31:642–655

Phillips DJH, Rainbow PS (1993) Biomonitoring of trace aquatic contaminants. Elsevier, London

Reddy PS, Bhagyalakshmi A (1994) Changes in oxidative metabolism in selected tissues of the crab (*Scylla serrata*) in response to cadmium toxicity. Ecotox Environ Safe 29:255–264

Rosenberg DM, Resh VH (1993) Freshwater biomonitoring and benthic macroinvertebrates. Chapman and Hall, London

Sánchez-López FJ, Gil-García MD, Sanchez-Morito NP, Martínez-Vidal JL (2003) Determination of heavy metals in crayfish by ICP-MS with a microwave-assisted digestion treatment. Ecotox Environ Safe 54:223–228

Sánchez-López FJ, Gil-García MD, Martínez-Vidal JL, Aguilera PA, Garrido-Frenich A (2004) Assessment of metal contamination in Doñana National Park (Spain) using crayfish (*Procamburus clarkii*). Environ Monit Assess 93(1–3):17–29

Schilderman PA, Moonen EJ, Maas LM, Welle I, Kleinjans JC (1999) Use of crayfish in biomonitoring studies of environmental pollution of the river Meuse. Ecotoxicol Environ Saf 44:241–252

Sevillano M, González-Fernández M, Jara-Biedma R, García-Barrera T, López-Barea J, Pueyo C, Gómez-Ariza JL (2012) Biological response of free-living mouse *Mus spretus* from Doñana National Park under environmental stress based on assessment of metal-binding biomolecules by SEC-ICP-MS. Anal Bioanal Chem 404(6–7):1967–1981. doi:10.1007/s00216-012-6274-2

Torreblanca A, Del Ramo J, Diaz-Mayans J (1991) Effects of cadmium on the biochemical composition of the freshwater crayfish *Procambarus clarkii* (Girard, 1852). Bull Environ Cont Toxicol 47:933–938

Vioque-Fernández A, Alves de Almeida E, Ballesteros J, Garcia-Barrera T, Gomez-Ariza JL, Lopez-Barea J (2007) Doñana National Park survey using crayfish (*Procambarus clarkii*) as bioindicator: esterase inhibition and pollutant levels. Toxicol Let 168:260–268

Vioque-Fernández A, Alves de Almeida E, López-Barea J (2009) Assessment of Doñana National Park contamination in *Procambarus clarkii*: integration of conventional biomarkers and proteomic approaches. Sci Total Environ 407(5):1784–1797

Wang SY, Stickle WB (1988) Biochemical composition of the blue crab *Callinectes sapidus* exposed to the water-soluble fraction of crude oil. Mar Biol 98:23–30

Wang J, Pan Y, Hong Y, Zhang QY, Wang XN, Kong LD (2012) Quercetin protects against cadmium-induced renal uric acid transport system alteration and lipid metabolism disorder in rats. Evid Based Complement Alternat Med 2012:548430. doi:10.1155/2012/548430

Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol 19:137–161

Zhang L, Liu X, You L, Zhou D, Yu J, Zhao J, Feng J, Wu H (2011a) Toxicological effects induced by cadmium in gills of Manila clam *Ruditapes philippinarum* using NMR-based metabolomics. Clean Soil Air Water 39:989–995

Zhang L, Liu X, You L, Zhou D, Wu H, Li L, Zhao J, Feng J, Yu J (2011b) Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics. Mar Environ Res 72:33–39