Science of the Total Environment 584–585 (2017) 813–827

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# 2D-DIGE as a proteomic biomarker discovery tool in environmental studies with *Procambarus clarkii*

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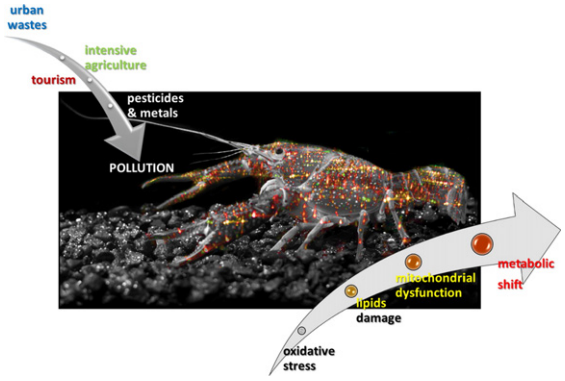
## HIGHLIGHTS

- Biological responses were analyzed in *P. clarkii* captured at sites of Doñana National Park and its surroundings (SW Spain).
- Mn, Zn and Cu contents highly correlated in *P. clarkii* tissues and sediments, while Cd and As were less correlated.
- The higher metal levels promoted oxidative stress, increasing lipid peroxidation and reversibly oxidized protein thiols.
- Protein expression profiles were traced by 2D-DIGE and 68/82 spots showed $>\pm$ 2-fold changes.
- MS/MS analysis identified 30 non-redundant proteins that were confirmed by Western blotting.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 15 July 2016
Received in revised form 17 January 2017
Accepted 18 January 2017
Available online 31 January 2017

Editor: D. Barcelo

## ABSTRACT

A 2D-DIGE/MS approach was used to assess protein abundance differences in the red swamp crayfish *Procambarus clarkii* from polluted aquatic ecosystems of Doñana National Park and surrounding areas with different pollution loads. *Procambarus clarkii* accumulated metals in the digestive glands and gills reflecting sediment concentrations. We first stated that, probably related to elements accumulation, pollution increased oxidative damage in *P. clarkii* tissues, as shown by the thiol oxidation status of proteins and MDA levels. In these animals, the altered redox status might be responsible for the deregulated abundance of proteins involved in cellular responses to oxidative stress including protein folding, mitochondrial imbalance and inflammatory processes. Interestingly, polluted *P. clarkii* crayfish also displayed a metabolic shift to enhanced aerobic glycolysis, most

*Abbreviations:* ACN, acetonitrile; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; 2-DE, two-dimensional gel electrophoresis; DBR, Doñana Biological Reserve; 2D-DIGE, two-dimensional differential in gel electrophoresis; DNP, Doñana National Park; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; 5-IAF, 5-iodoacetamidofluorescein; IAM, iodoacetamide; ICP-MS, inductively-coupled plasma-mass spectrometry; IEF, isoelectro-focusing; IPG, immobilized pH gradient; LC-MS, liquid chromatography-mass spectrometry; LDP, Lucio del Palacio; LOD, limit of detection; LPO, lipid peroxidation; MAT, Matochal stream; MALDI, matrix-assisted laser desorption ionization; MDA, malondialdehyde; PAR, Partido stream; PCA, principal component analysis; PMSF, phenyl-methyl-sulphonyl fluoride; PVDF, Polyvinylidene fluoride; PTFE, Polytetrafluoroethylene; PTM, post-translational modifications; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; RNS, reactive nitrogen species; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBP, tributylphosphine; TFA, trifluoroacetic acid; TOF, time-of-flight.

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http://dx.doi.org/10.1016/j.scitotenv.2017.01.125

*Keywords:*
2D-DIGE proteomics
*Procambarus clarkii*
Biomarkers
Environmental pollution
Metals
Oxidative stress

likely aimed at generating ATP and reduction equivalents in an oxidative stress situation that alters mitochondrial integrity. The deregulated proteins define the physiological processes affected by pollutants in DNP and its surrounding areas and may help us to unravel the molecular mechanisms underlying the toxicity of environmental pollutants. In addition, these proteins might be used as exposure biomarkers in environmental risk assessment. The results obtained might be extrapolated to many other locations all over the world and have the added value of providing information about the molecular responses of this environmentally and economically interesting animal.

*Significance:* Metal content in digestive gland and gills of *P. clarkii* crayfish reflects their contents in sediments at sites of Doñana National Park and its surroundings. Accumulation of essential and toxic transition metals is paralleled by clear signs of oxidative stress to lipids and proteins and by significant deregulation of many proteins involved in protein folding, mitochondrial respiratory imbalance and inflammatory response. These results indicate that *P. clarkii* is an excellent bioindicator to be used in aquatic ecosystems quality monitoring. Additionally, results evidence that the anthropogenic activities carried out around Doñana National Park represent an extremely serious threat to this unique Biosphere Reserve and pose a risk to the environment and their inhabitants health.

The identified deregulated proteins provide information about the metabolic pathways and/or physiological processes affected by pollutant-elicited oxidative stress, may also be useful as biomarkers of environmental pollution and have the added value of providing information about the molecular responses of this environmentally and economically interesting animal.



## 1. Introduction

Thousands of chemicals released into the environment stress both organisms and ecosystems, and many governmental efforts have been made to evaluate the associated risks. This is a challenging task due to diversities in the chemical nature and toxicity mechanisms of the pollutants and due to the different sensitivities of the exposed organisms. Environmental risk assessment is usually based on toxicity tests, from which a presumed safe concentration is established. However, substantial difficulties are involved in extrapolating results from short laboratory experiments to long-term environmental effects because even low (possibly harmless) concentrations of pollutants often produce deleterious effects on organisms that are expressed only after prolonged exposure. Thus, it is crucial to develop early warning signals (biomarkers) to expose the adverse biological effects of environmental toxins even at small concentrations.

Proteomic analysis provides global information on protein abundance, thus allowing for better insight into the molecular responses to pollutant-induced toxicity. As with all omics, Environmental Proteomics (EP) provides a holistic assessment of toxic and defensive mechanisms triggered by pollutants without any previous knowledge of their toxic pathways. This makes it possible to identify proteins that are significantly altered in an organism after pollution exposure, helping to reveal toxic mechanisms and leading to the identification of new biomarkers useful in biomonitoring. During the last decade, EP gained popularity in environmental risk assessment, mostly relying on two-dimensional gel electrophoresis (2-DE). An advanced version of 2-DE is two-dimensional difference gel electrophoresis (2D-DIGE), which advanced this field by allowing the accurate and reproducible quantification of multiple samples. While LC-MS has recently become more popular for proteome quantification, 2D-DIGE remains relevant, as it can be used to directly visualize hundreds to thousands of protein species, providing key information of pI/MW changes caused by truncation, degradation, genetic code variation, alternative splicing, post-translational processing, and PTMs (Arentz et al., 2015). 2-DE followed by protein identification by mass spectrometry has been used extensively in ecotoxicology (i.e., Lopez-Barea and Gomez-Ariza, 2006)

We have used 2D-DIGE/MS to quantify protein abundance differences in the red swamp crayfish *Procambarus clarkii*, which dwell in polluted aquatic ecosystems, to identify these proteins for use as biomarkers in environmental risk assessment. We compared sites at Doñana National Park (DNP, SW Spain) and surrounding areas with different pollution loads. DNP is an area of marshes, shallow streams and sand dunes located at the mouth of the Guadalquivir River. DNP was declared a World Heritage Site in 1981, sheltering millions of migratory birds and endangered species. Its marshes are fed by the Rocina, Partido, and Guadiamar streams and the Guadalquivir River, which carry pollutants from areas of intense urbanization, industry and agriculture located nearby. Therefore, the presence of contaminants at the DNP core is of great concern (Bonilla-Valverde et al., 2004; Fernández-Cisnal et al., 2014; Gago-Tinoco et al., 2014; Ruiz-Laguna et al., 2001; Vioque-Fernandez et al., 2009a; Zorrilla-Miras et al., 2014). We used *P. clarkii* as a bioindicator. This highly abundant decapod, native to southeastern USA, was introduced to the Doñana marshes in 1973, becoming an invasive species because of its high adaptability, tolerance, and fecundity. It is a crustacean model organism used in many research fields, including animal behavior, viral infection, and environmental stress and toxicity, particularly those focused on the effects of metals and pesticides near DNP (Dorr et al., 2008; Gago-Tinoco et al., 2014; Martín-Díaz et al., 2006; Osuna-Jiménez et al., 2014; Pueyo et al., 2011; Suarez-Serrano et al., 2010; Vioque-Fernandez et al., 2009a; Vioque-Fernandez et al., 2009b).

## 2. Material and methods

### 2.1. Sampling areas and animals

Samples were collected in April/June 2009 at three sites of DNP and the surrounding areas, shown in Fig. 1, including the UTM coordinates and site codes. Lucio del Palacio (LDP), located at the DNP core (Doñana Biological Reserve-CSIC, DBR), is a reference site as shown in previous studies (Ruiz-Laguna et al., 2006; Ruiz-Laguna et al., 2001; Vioque-Fernandez et al., 2007a; Vioque-Fernandez et al., 2009b). The other two sites are close to areas of intensive agricultural use. Partido (PAR), located upstream of the El Partido stream, is under the influence of citrus fruit and grape fields and "El Matochal" (MAT), next to the Guadiamar stream, is affected by rice-growing fields and suffered the input of metals transported by the Guadiamar river during the rupture of the Aznalcollar mine tailing pond in 1998 (Grimalt et al., 1999).

Crayfish were collected with tubular plastic mesh traps, baited with raw chicken parts, that were placed in the evenings and checked the next morning (Vioque-Fernandez et al., 2009a; Vioque-Fernandez et al., 2007a). Adults were taken alive to DBR and dissected after recording the site of capture, date of capture, sex and weight. The digestive glands and gills from fourteen healthy male crayfish per site (weight/length $25.8 \pm 2.6$ g/$9.3 \pm 0.18$ cm, respectively) were individually frozen in liquid $N_2$. The organs were taken to the laboratory at Córdoba University (UCO), individually homogenized in a SPEX SamplePrep cryogenic