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New Methodologies for Assessing the Presence and Ecological Effects of Pesticides in Doñana National Park (SW Spain)

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1. Introduction

Environmental quality can be assessed by a variety of methods (chemical, biological, and ecological) that, when used individually, have multiple limitations. In fact, it is convenient to use different approaches to understand the status of any ecosystem. Actually, in addition to show the presence of pollutants in the environment, their appearance in the organisms and their biological effects on them has to be demonstrated before their possible risks can be evaluated. The chemical analysis of pollutants requires sophisticated tools due to the growing number of compounds, the subsequent transformation of their parent compounds, the need to distinguish between their different chemical forms, and the interest in characterizing the metabolites derived from them. In addition to conventional pollutants (e.g. linear hydrocarbons, PAHs, PCBs, metals, pesticides) there are many others like metal species and persistent organic pollutants (such as aldrin, chlordane, DDT, dieldrin, endrin). Recently further concern has been shown about input of new emerging pollutants. These include pharmaceuticals, alkylphenols, linear alkylsulphonates, new metal species, etc., which, although found in the ecosystems at very low concentrations, are ecologically highly active, even acting as endocrine disruptors with deleterious effects for the reproduction of different species (Strauch et al., 2008). Antiinflamatory drugs (diclophenac, ketoprofen, naproxen, and ibuprofen), antibiotics (penicillins, tetracyclins, sulfonamides, and quinolones), anticonceptives, etc., are used in human and animal health care. Their growing use and increasing presence in the ecosystems has become an important issue, the focus of research being shifted from conventional to emerging pollutants that are highly dangerous since are designed to have biological activity. The amount of pharmaceuticals reaching the environment depends on human consumption and excretion via feces/urine that reach the sewage system. Effluents of wastewater plants are the main source in the aquatic environment, followed by the release of outdated medicines down household (Ruhoy et al., 2007) and pharmaceutical industry waste (Kümmerer, 2004; Larsson et al., 2007).

Homeostasis describes the constant state of the internal environment of living beings. Homeostatic regulation allows an organism to function effectively in a broad range of environmental conditions, and pollution seriously endangers this situation. Biomarkers are used in sentinel organisms (bioindicators) to provide evidence of exposure/effect to one or more pollutants (Livingstone, 1993; Peakall, 1994). Environmental studies usually rely on the assessment of classic bioindicators (bivalve mollusks or fish), but there is a lack of studies on free-living animals, such as *Procambarus clarkii* (red swamp crayfish) or *Mus spretus* (Algerian mouse), from barely polluted ecosystems (Fig. 1).

Fig. 1. *Procambarus clarkii* and *Mus spretus,* and the model laboratory mouse, *Mus musculus*.

P. clarkii is native to Southeastern USA. In Southern Europe, this crustacean is actively colonizing new territory, at the expense of the native crayfish. This organism, of relatively small size (10 cm; 25-30 g) reaches sexual maturity in the marshes of the Guadalquivir River at 6 cm total length. This omnivorous and detritivorous species feeds on plants, rhizomes and small animals like aquatic larvae, insects and cladocerans (Gutiérrez-Yurita & Montes, 1998). *P. clarkii* is an excellent bioindicator of contamination in fresh water ecosystems (Martín Díaz et al., 2006). The mouse is the favorite model organism for generation of knowledge useful to humans. *Mus musculus* is the source of most classical inbred laboratory mouse strains and its genome has been sequenced (Mouse Genome Sequencing Consortium, 2002). *M. spretus* is the best characterized of the aboriginal species. This small animal attains high population densities, typically inhabits marshlands, and feeds on seeds, insects and small invertebrates around its burrow. This free-living rodent has proved to be an excellent bioindicator of contamination in terrestrial ecosystems (Nunes et al., 2001; Ruiz-Laguna et al., 2001; Bonilla-Valverde et al., 2004). The two murine species separated more than one million years ago (Gao & Zhang, 2003) and exhibit a relatively high gene sequence homology. Consequently, molecular studies with the aboriginal species can take advantage of the laboratory species databases (Prieto-Álamo, et al., 2003; Ruiz-Laguna et al., 2005,

2006). Both bioindicators are non-protected species and highly prolific, therefore they are very suitable in environmental studies.

Doñana National Park (DNP, SW Spain, Fig. 2) was settled in 1969 and declared a World Heritage Site in 1981. This area of marsh, shallow streams and sand dunes, in the mouth of the Guadalquivir River, has a biodiversity unique in Europe, contains many ecosystems and shelters wildlife including millions of migratory birds, and endangered species such as the Imperial Eagle and Iberian Lynx (Grimalt et al., 1999). Doñana is watered by the Rocina, Partido and Guadiamar streams and by Guadalquivir River, and is surrounded by areas of intense agricultural activities (citric, strawberry, rice). It is also threatened by industries located at Huelva estuary, 40 Km West, formed by Odiel and Tinto rivers, carrying metals from mining activity, and where the Domingo Rubio stream discharges petrochemical refuses, and pesticides from strawberry crops (Montes-Nieto et al., 2007, 2010). Finally, in 1998 metals released from Aznacóllar pyrite mine, 60 Km north, threatened Doñana through Guadiamar stream (Grimalt et al., 1999).

Fig. 2. South-Atlantic Spanish littoral, between Huelva and Guadalquivir estuaries. The limits of DNP are shown in white, the Odiel, Tinto and Guadalquivir Rivers and the Domingo Rubio (DRS), Rocina (ROC), Partido (PAR) and Guadiamar streams are shown in blue and the Lucio del Palacio (LDP) reference site is indicated in green. The numbers included in the figure show a variety of polluted sites located at: 1, phosphogypsum stacks; 2-4, low, medium and high DRS course, respectively; 5-6, ROC; 7-8, PAR; 9, Matochal; 10, Isla Mayor; 11, Cangrejo Grande; 12, Brazo de la Torre; 13-15 Doñana bank at Guadalquivir Estuary, at the mouth of Guadiamar stream, San Rafael saltworks and across Bonanza harbor, respectively.

While there are many studies in polluted environments there are few in natural ecosystems, and in particular in National Parks around the World. Due to the variety of pollutants, these studies focuse on metals and organic pollutants, in relatively polluted areas (Livingstone et al., 2000; Hobbelen et al., 2004; Hamers et al., 2006; Schwindt et al., 2008). Initial assessment of

contaminants at Doñana area was centered on Guadiamar stream. The high metal levels and low pH detected in its upper course were related to acid-mine draining from Aznalcóllar pyrite mine; metals and herbicides were also found at the rice growing fields East of DNP (Cabrera et al., 1984). A global evaluation of DNP contamination was made by the Scientific Research Council (Albaiges et al., 1987). Streams feeding Doñana marshes were the main source of contaminants. In addition to metals and pesticides, Guadiamar stream contributed organic matter from urban/agro-industrial sources, and petroleum hydrocarbons entered from Guadalquivir River at the South. In addition to water origin, the uniform distribution of PAHs and PCBs at stream-isolated lagoons suggested aeolian transport from industries at Huelva Estuary (Albaiges et al., 1987). After the tailings dam of Aznalcóllar pyrite mine, the effect of this spill in DNP was studied (López-Pamo et al., 1999; Manzano et al., 1999). Although the spill did not have a dramatic effect within Doñana, increasing concern raised about the presence of contaminants at DNP core, not directly related to the spill but possibly derived from the agrochemicals used in nearby areas (Ruiz-Laguna et al., 2001; Bonilla-Valverde et al., 2004, 2006). Actually, the effect of agricultural activity is the most serious gap in the knowledge of Doñana environmental quality, since this type of contamination is more diffuse, difficult to assess and less relevant in the media than a mining spill.

Biomarkers are used in sentinel organisms to provide evidence of exposure, effect or risk to pollutants in biomonitoring studies (Livingstone, 1993; Peakall, 1994; López-Barea, 1995). The so-called "classic" biomarkers are suggested *a priori* by their biological roles but they are somewhat biased in pollution assessment since they concentrate in a small number of proteins but exclude other also altered but whose relationship with pollution is still unknown (López-Barea & Gómez-Ariza, 2006). Table 1 summarizes a number of biomarkers that indicate the responses of organisms to pollutants. They include: 1) Induction (inhibition) of phase I, and 2) phase II biotransformation enzymes (Livingstone, 1993; George, 1994; Goksøyr, 1995; Van der Oost et al., 2003), responsive to PAHs, PCBs, PCDFs, dioxins (inhibited by organotins), that are coordinately regulated by the Ah receptor (Stegeman & Han, 1994). 3) Levels of reduced and oxidized glutathione or non-enzymatic antioxidants, and, 4) activity of primary/ancillary antioxidant enzymes and oxidative damages to biomolecules, responsive to metals and prooxidants. 5) Esterase activities inhibited by organophosphate or carbamate pesticides (Peakall, 1994). 6) Stress proteins, responsive to many organic compounds, such as natural products and anthropogenic contaminants, and transition metals. 7) Reproductive and endocrine effects, sensitive to organotins and endocrine disruptors. 8) Genotoxicity, responsive to genotoxins and carcinogens (López-Barea, 1995). 9) Physiological and morphological parameters.

The cellular genome is in a dynamic equilibrium between processes that damage it and those that maintain its integrity. While genes exert their functions at protein level, the genetic responses to stress are often regulated at the transcriptional level. After completion of the Human Genome Project, an increasing range of post-genomic techniques are available to asses the complete content of genes, proteins or metabolites in a cell under a particular situation. In contrast to the conventional biomarker strategy, these *omic* approaches, supplemented with *in vitro* and *in silico* methods, are becoming a powerful multidisciplinary strategy in environmental studies. Nevertheless, their application is still at an early developmental stage, mainly since most popular bioindicators are poorly represented in gene/protein sequence databases (Ruiz-Laguna et al., 2006; González-Fernández et al., 2008a). The *omic* sciences include *genomics* for study of DNA variations, *transcriptomics* for genome-wide characterization of gene expression via the measurement of mRNAs,

proteomics for measuring cell and tissue-wide expression of proteins, and *metabolomics* for global assessment of metabolite concentrations. These technologies give detailed molecular information that help to identify toxicity pathways and to define pollutant mechanisms and modes of action without requiring previous knowledge.

Category	Biomarker/abbreviation	Reference
Phase I biotransform	CYP 1A protein/mRNA	Goksøyr, 1995
	EROD/AHH activity	Livingstone, 1993
Phase II biotransform	Glut. S-transfer., GSTc,m	Van der Oost et al., 2003
	UDP-glucuronyl transfer	George, 1994
Antioxidants	GSSG/GSH redox status	López-Barea, 1995
	Vitamins C or E	Van der Oost et al., 2003
Primary antiox enzym	Superoxide dismutase, SOD	López-Barea, 1995
	Catalase, CAT	López-Barea, 1995
	Glut. peroxidase, GSHPx	Van der Oost et al., 2003
Auxilary antiox enzym	Glut. reductase, GSSGRase	Van der Oost et al., 2003
	Glucose-6P dHase, G6PDH	López-Barea, 1995
	6Pgluconat. dHase, 6PGDH	López-Barea, 1995
Oxidative damages	Lipid peroxidation, MDA	López-Barea, 1995
	DNA oxidation	López-Barea, 1995
Esterase inhibition	Acetyl cholinesterase, AChE	Peakall, 1994
	Carboxyl esterase, CbE	Galloway et al., 2002
Stress proteins	Heat shock proteins, HSPs	López-Barea, 1995
	Metallothioneins, MTs	López-Barea, 1995
Reproduction	Vitellogenin, VTG	Van der Oost et al., 2003
Genotoxicity	DNA adduct/strand breaks	Van der Oost et al., 2003
	Sister-chromatid exchanges	Van der Oost et al., 2003
	Micronucleous assay	Van der Oost et al., 2003
Physiol/Morphology	Histopathology	Nunes et al., 2001
Other	Promutagens activation	López-Barea, 1995

Table 1. Biochemical pollution biomarkers used in biomonitoring studies

Several technologies are available to analyze the transcriptome, the entire complement of transcripts in a cell, tissue or organism under certain conditions. *Microarrays technology* allows thousands of gene transcripts to be monitored simultaneously, but they are based in the complementarity of sequences between probes and tested samples. Commercial microarrays are not usually applicable for environmental studies, since most bioindicators are poorly represented in databases. Custom cDNA microarrays can be developed for any species of interest but it is labor intensive, time consuming and costly since it requires to previously obtaining expressed sequence tags, cDNA libraries or genome sequence data. Different strategies allow overcoming these inconvenients. One is to choose a bioindicator close to a model organism; in this case, the similitude between their genic sequences allows the use of commercially available microarrays by means of heterologous hybridization. Application of heterologous microarray has gained wide interest to study the response to pollutants and environmental stressors (Bar-Or et al., 2007; Buckley, 2007; Osuna-Jiménez et al., 2009).

When heterologous microarrays cannot be used due to sequence divergence between the studied species and a possible reference, an alternative is *suppressive subtractive hybridization* (SSH) (Williams et al., 2003; Prieto-Álamo et al., 2009). This is a PCR-based technique for generating cDNAs enriched for differentially expressed genes, useful for large-scale gene identification in non-model organisms (Diatchenko et al., 1996). The identification of differentially expressed genes by SSH is shown in a number of papers that analyze the responses to model contaminants under controlled exposure conditions. In comparison, few studies examine individuals from ecosystems with different degrees of pollution, mainly centered in aquatic ecosystems. It is worth mentioning the studies in the black tiger shrimp (*Penaeus monodon*), the grass shrimp (*Palaemonetes pugio*) and the American winter flounder (*Pseudopleuronectes americanus*) (Straub et al., 2004; Griffitt et al., 2006; De la Vega et al., 2007). Hybridization-based approaches provide data that must be confirmed by more refined quantitative methods like real-time reverse transcription (RT) followed by polymerase chain reaction (PCR), the most powerful tool for transcript quantification. Most RT-PCR studies are taken semiquantitatively (fold-variation) and assume that housekeeping genes are stably expressed, or that any changes that might occur are balanced. Absolute quantification (real quantification) gives the molecule copy number of each transcript in each of the samples under study using a calibration curve. Although relative quantification is much easier to perform because a calibration curve is not necessary, relative data are much less informative than absolute data (Jurado et al., 2003; Prieto-Álamo et al., 2003; Jiménez et al., 2005; Ruiz-Laguna et al., 2005, 2006). The commercialization of real-time PCR equipments allows for absolute expression data with precision levels unattainable for those generated by conventional end-time PCR. Real-time RT-PCR is adequate for studies focused in a few number of transcripts, because it is expensive and time-consuming.

Proteomics addresses the post-genomic challenge of examining the entire complement of proteins (proteome) expressed by a genome in a cell, tissue or organ at a given time under defined conditions (James, 1997; Anderson & Anderson, 1998; Blackstock & Weir, 1999). Protein expression is modulated at different levels from transcription to maturation of the polypeptides produced by translation of mature mRNAs. Post-translational modifications are of key importance as they give rise to multiple protein products from a single gene, each of which may have different functions. Proteins were initially separated by *two-dimensional electrophoresis* (2-DE, Wilkins et al., 1996), their expression being analyzed by 2-D softwares (Melanie, etc.). Proteins were identified by mass spectrometry analysis of their peptide mass fingerprint (MALDI-TOF-PMF) or *de novo* sequencing of some peptides (nESI-MS/MS), then contrasting the results with public databases (Simpson, 2003). 2-DE, labor intensive and of a low reproducibility, requires a large amount of sample, and its narrow dynamic range is problematic with proteins of extreme Mr/pI. Fluorescent labeling gave rise to *difference gel electrophoresis* (DIGE), which analyzes two samples in one single gel, facilitating a quantitative assessment of expression (Ünlü et al., 1997). Alternatively, shotgun methods allow the analysis of complex protein mixtures after full digestion, by multidimensional separation coupling tandem liquid chromatography (LC/LC) and MS/MS (Washburn et al., 2001). *Isobaric reagents* (iTRAQ) revolutionized proteomics by allowing the simultaneous quantification and identification of proteins in complex mixtures in one single experiment (Ross et al., 2004). A set of multiplexed isobaric reagents are used to label peptides in their amino groups (Lys and N-terminal). Although the labeled peptides are undistinguishable in MS, they generate low-mass MS/MS signature ions (113-121), supporting their relative and absolute quantification. The application of proteomic technology in environmental studies

(Environmental Proteomics, EP) faces the problem of the lack of genomic information on most non-model sentinel organisms. This makes it difficult to identify differentially expressed proteins by high-throughput methods like MALDI-TOF-PMF analysis (Barrett et al., 2005; López-Barea & Gómez-Ariza, 2006; González-Fernández et al., 2008a).

Within the growing body of Proteomics, issues addressing environmental problems are on the rise. Ecotoxicology uses expression changes of proteins known to be involved in toxicological responses. Unlike directed approaches, proteomics examines how multiple expression changes are associated with a contamination suspected to be harmful. Thus, proteins involved in toxicological responses that have not been described before may be revealed. Following identification of key proteins indicating exposure or effect, Proteomics can be used in risk assessment. To this end, bioinformatics may unveil protein patterns specific to an environmental stress that would constitute a classifier able to distinguish an exposure from a control state. Two main trends are used in expression proteomics: 1) *Pattern-only approaches*, to recognize proteomic patterns and not at protein identification in the first place, initially named PES approach (Shepard & Bradley, 2000; Shepard et al., 2000). 2) *Identity-based approaches*, relying on the identification of proteins with differential expression (Monsinjon & Knigge, 2007). In EP studies the difficulty of protein identification in non-model organisms was initially circumvented by the PES approach. These sets of proteins observed in 2-DE, useful as state markers, signal early pathological stages or stress exposure. Initial studies showed that PES is specific for the type and extent of stress. Since altered PES are used as multimarkers, to diagnose adverse effects there is no need to identify the proteins with altered expression levels (Shepard et al., 2000; Baker 2005), being enough to show the appearance of altered PES patterns to demonstrate pollution effects.

Metal ions play a key role in life chemistry, so that it is essential to identify and quantify the chemical species containing metals in living beings. The corpus of research dealing with metals in biological systems has been named "metallomics" (Haraguchi, 2004). The function of a third of the proteins depends on their interaction with transition metals like Cu, Fe, Zn, or Mo (Hasnain, 2004). Thus, it is vital to find out how an organism generates biomolecules containing metals in response to signals and environmental stress. Therefore, cell chemistry implies knowing, in addition to its genome and proteome, its metallome, i.e. the distribution of metals/metalloids among its different biomolecules and compartments (Szpunar, 2004, 2005; López-Barea & Gómez-Ariza, 2006). There are not analytical procedures to directly determine the chemical forms of the elements in biological matrices at the existing concentrations. To avoid any changes in its original form, it is necessary to study the species in which the element is strongly bound to the biomolecules. As electrospray mass spectrometry does not permit their analysis due to matrix effects, high resolution on-line separations with HPLC or CE have to be combined with sensitive and specific atomic detectors (ICP-MS). These hybrid techniques allow the measurement of metal species in cytosolic extracts using the heteroelement as a marker (Sanz-Medel, 2003). The couplings based on size exclusion (SEC-ICP-MS) permit a rapid screening through the molecular mass of metal-molecules (Szpunar, 2004, 2005; Sanz-Medel et al., 2003; González-Fernández et al., 2008b), although purification of the peaks requires a second chromatographic separation by ionic exchange, reverse phase or hydrophobic interaction, to identify the molecule (Arias-Borrego et al., 2008). Other alternatives use CE-ICP-MS couplings, gel electrophoresis with laser ablation and ICP-MS detection, or detection of the metal by spot digestion and ICP-MS analysis (Sussulini et al., 2007; Monicou & Lobinski, 2008). The potential of isotopic dilution and of the MS in MALDI mode and in the diverse forms of ESI-MS is also used. The basic

work scheme, used with variants in metallomics, incorporates three elements (Fig. 3): i) a separation system; ii) a detector for the element (metal or metalloid) (ICP-MS); and iii) a system for the identification of the molecule (MS) (Gómez-Ariza et al., 2004).

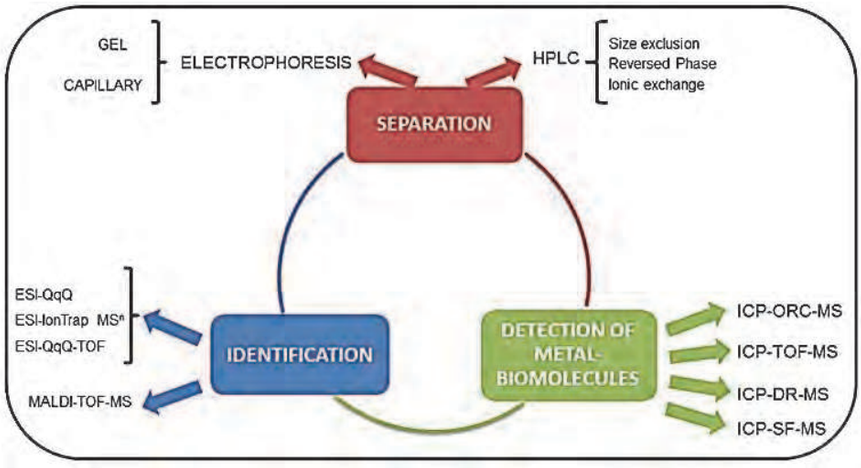

Fig. 3. The basic work scheme of the metallomics approach (Gómez-Ariza et al., 2004).

2. Our previous studies

We will review previous studies of the groups that monitor the pollution status of Doñana and surrounding areas, whose recent results will be summarized in section 3. We only refer to the development of analytical methods for contaminants present at DNP, and the application of conventional biomarkers and new *omic* methods to assess the responses of organisms living in the area. This section is centered in the following aspects: 2.1) New analytical approaches for emerging pollutants determination. 2.2) Developments in metallomics studies for environmental assessment. 2.3) Conventional biomarkers and proteomics in environmental studies. 2. 4) Transcriptomics in environmental studies.

2.1 New analytical approaches for emerging pollutants determination

Procedures for determination of pharmaceuticals and other emerging pollutants in animal tissues have been reviewed, though few exist in fish or bivalves. Most methods imply solid-liquid extraction and further clean up by solid-phase extraction prior to chromatographic analysis, but there is interest in improving detection sensitivity not paying much attention to sample treatment procedures, the most crucial and time consuming step for analyzing the compounds of interest in complex matrices. Additionally, few reports have been published on analysis of bioactive compounds and drugs, in animal tissues.

One of the main problems for quantitative analysis of pharmaceuticals in biological samples is that the analyte is usually bound to proteins or peptides, thus requiring the previous cleavage of these structures. Although enzymatic digestion has been traditionally used, these methods are time consuming and usually require further clean-up (Fernández-Torres et al., 2010a). The use of enzymes combined with ultrasonic energy has been reported as an alternative and since ultrasonic probes provide 100 times more energy than ultrasonic baths, these last are preferred for enzymatic sonication extraction (Fernández-Torres et al., 2010b) in the so called ultrasound probe sonication-assisted extraction. To improve extraction

efficiencies, enzymatic digestion combined to subsequent inhibition of enzyme activity by microwaves (microwave-assisted extraction, MAE) was used finding that at low intensity the enzymatic activity remained stable and recoveries of almost all analytes increased. Thus, a procedure combining enzymes and microwaves was studied further. The parameters to optimize for the enzymatic treatment were: type and quantity of enzyme, and time and power applied. In a bibliography search three enzymes were selected: protease, proteinase-K and a combination protease-lipase. Proteinase-K gave the best recoveries (Fernández-Torres et al., 2011). It is cheap, reliable and environmentally friendly, and shows equal or better extraction efficiency than more conventional techniques. MAE has widespread use in several areas, including environmental analysis, food and clinical determinations.

The presence in carps (*Cyprinus carpio*) of antibiotics was studied (Fernández-Torres et al., 2011) to evaluate MAE approach in fish exposed to these pharmaceutical compounds. Carp was selected since this species live for a long period in aquaria and is obtained in pet shops. Before the assay, fish were maintained in a 1000 L tank (with filtration and aeration systems) for two months to eliminate the antibiotics possibly used in the pet shop for prophylaxis. Four assays were made with different antibiotic mixes to assure that doses and mixes were not lethal: *assay 1*, tianphenicol, trimethoprim and chloramphenicol; *assay 2*, sulfadiazine, sulfamerazine, sulfadiazine and sulfamethoxazole; *assay 3*, sulfadiazine, sulfamethoxazole, oxytetracycline and amoxicillin, and *assay 4*, oxytetracycline, chlortetracycline, amoxicillin and ampicillin. Three animals (~30 g each) were isolated into a 20 L aquarium before antibiotic administration. Following recommended doses (Durborow et al., 1996), 100 mg of each antibiotic were mixed in a small bread ball to assure that animals ingested it fully; a second dose was administered 24 h later. After 1 h, fish were sacrificed and muscle tissue was processed by MAE. Most of the analytes administered were detected, although in some cases their levels were not quantifiable. Several of their metabolites were also detected and/or quantified. In all quantified samples the levels were above maximum recommended limit (MRL, Council Directive 37/2010 EC), highlighting that an adequate time has to elapse from antibiotic administration to commercialization. The viscera of exposed carps were also analyzed by the same procedure, as the presence of the analytes was also expectable. Data obtained confirmed this, although levels in fish viscera are not submitted to legislation, these are higher than the obtained in muscle samples.

To verify whether the presence of pharmaceuticals is significant in aquatic ecosystems, a variety of marine specimens were captured near Aguilas, a town of some importance SW of Murcia (Spain), with an area of 252 km^2 and 28 km coastline at the Mediterranean Sea. It has a stable population of 28,800 inhabitants that reaches 150,000 in summer. The specimens were captured through artisan fishing by net placed 100-200 m from the beach. Specimens of different fish species, *Thrachynotus ovatus, Salpa salpa, Oblada melanura* and *Liza ramada*, were analyzed according to the HPLC-MAE method previously developed. In general, the results obtained show that some specimens had been exposed to antibiotics in some way but levels found were below the MRL fixed by the Council Directive 37/2010 EC and subsequent amendments. To notice the results obtained for oxytetracycline in samples of *T. ovatus* which are really surprising and unexpected, with contents about 3-5 times the corresponding MRL, which could imply a long exposure of the animal to the compound.

Methods for the determination of PAHs and linear alkyl benzene sulfonates (LAS) were also optimized. DNP includes a mosaic of unique ecosystems which are particularly affected by the quality of the incoming flowing water. Characterization of wastewater and sludge from water treatment plants is a key issue due to their high contaminant power if depuration has

not been effective. An analytical procedure was developed using MAE prior to liquid chromatography coupled to diode array (DAD) or fluorescence (FLD) detectors for the determination of PAHs in sewage sludge (Villar et al., 2004). The new method has shown to be suitable for determination of the 16 PAHs recommended by EPA in sewage sludge. The extraction method requires only 0.5 g of sample and no additional clean-up is required prior to the final determination by LC using a FLD or DAD detector. A new method for the extraction and determination of LAS from sewage sludge based on MAE and HPLC coupled to DAD and FLD has been proposed (Villar et al., 2007). The extraction of C_{10}-C_{13} homologues of LAS was carried out by using an extraction time of 10 min and 5 mL of methanol and the determination of LAS by HPLC lasted less than 6 min. The method did not require clean-up or preconcentration steps.

2.2 Developments in metallomics studies for environmental assessment

The concept of metallome and metallomics has evolved to encompass a more complete picture of the role of metals in the living organisms, considering not only the different metal species in a cell but also their identity, quantity and localization, e.g. their presence in a particular tissue or compartment, or a set of metal complexes with a given class of ligands, e.g. metalloproteome (all metalloproteins) or metallometabolome (all metallometabolites) (Monicou et al., 2009). Metallomics is becoming a very multidisciplinary area with impact in the study of biological catalysis, because an important number of enzymes exert their activity through the metal included in the prosthetic groups, such as *heme* for Fe, or covalently bound to the active site, as Se-Cys in GSH peroxidase. Transition metals play an essential role in the metabolism of living organisms and, unlike metabolites, metals are neither produced nor consumed during cell reactions. Thiele & Gitlin (2008) assert that metals and their chemistries act as driving force for the evolution of life, and even more, metals metabolism depend upon their oceanic and atmospheric cycles, so that some living organisms exist due to the presence of metals in an area (Thiele & Gitlin, 2008). This allows to correlate the proteome of sentinel organisms with the metal contamination, the rationale for their use in environmental proteomics and metallomics (Lopez-Barea & Gomez-Ariza, 2006). In addition, the metabolism of plants involves the mobilization of low solubility metals from soils and translocation into the plant and sequestering metal ions in cytosol or in cellular compartments. Therefore, metallomics is a useful tool for the study of plants hyperaccumulation for environmental bioremediation (Shah & Nongkynrih, 2007) and plant defense mechanisms against heavy metal stress (Clemens, 2001).

Several analytical approaches have been proposed in environmental metallomics, mainly focused on detection, identification and analysis of metal-containing biomolecules in the sample under study. The trace levels of metallobiomolecules (proteins and metabolites) require the use of high resolution separation techniques with very sensitive elemental and/or molecular MS detectors. The separation component role is to avoid co-elution of another species of the same element (when ICP-MS detection is used) or that of any easily ionizable species able to suppress the ionization of the analyte (if molecular MS is used). One key point in analytical techniques hyphenation is to consider metal species kinetically inert which are not involved in reactions that provoke replacement of one or more ligands in the coordination sphere by others on the timescale of the analytical procedure.

The specificity of metalloproteomics studies requires the description of metal-binding sites, metal stoichiometry and metal-dependent structure or conformation changes. This is not compatible with many sample treatments used in conventional proteomic methods that

produce proteins denaturation and, thus, loss of non-covalently bound metals (Gomez-Ariza et al., submitted). A valid metallomic protocol requires the preservation of the binding between protein and metal, a suitable system for purification and/or preconcentration of the metal-binding protein of concern, and an instrumental platform for specific detection and identification of metalloproteins from the sample (generally the combined use of HPLC-ICP-MS and HPLC-MS).

Use of size exclusion chromatography hyphenated to inductively coupled plasma mass spectrometry (SEC-ICP-MS) has been recently proposed for pollution assessment (González-Fernández et al., 2011). The approach is based on the use of *M. spretus* as bioindicator in contaminated and non-contaminated DNP areas. Metal SEC-ICP-MS profiles of liver and brain extracts reveal Cu, Mn and Zn-containing proteins and biomolecule-bound non-essential elements, such as Cd, Pb and As. Clear differences existed between the chromatograms corresponding to polluted areas compared to non-polluted. An apparent upregulation of a Cu and Zn-peak at ~7 kDa, which may correspond to metallothionein (induced by pollution), and a downregulation of a ~32 kDa Cu-peak, possibly related to superoxide dismutase (Cu-SOD), are observed in liver extracts from contaminated areas, while opposite results were observed in unpolluted areas. However, in brain extracts the differences between the SEC-ICP-MS chromatograms from polluted and unpolluted areas for Cu and Zn-peaks, related to metallothioneins and superoxide dismutase, are very small, possibly due to the protection of blood–brain barrier.

2.3 Conventional biomarkers and proteomics in environmental studies

2.3.1 Studies in bivalve molluscs

Bivalve mollusks have gained a worldwide importance as bioindicators of marine/estuarine pollution. Different species were used as sentinels at the Huelva and Guadalquivir Estuaries from 1999 to 2003. Since metals and organics that promote oxidative damages are the main contaminants in the area, biomarkers responsive to oxidative stress were selected to monitor their biological effects, paying special attention to antioxidant defenses and oxidative damage to biomolecules. Differences existed in pollution responses of clams (*Chamaelea gallina*) and oysters (*Crassostrea angulata*) in comparison to mussels (*Mytilus galloprovincialis*) (Rodríguez-Ortega et al., 2002; Funes et al., 2006). Clams and oysters were very sensitive bioindicators, since animals from contaminated Huelva sites accumulated high pollutant loads and displayed higher antioxidant defenses, thus containing less oxidative damages. Actually, bivalves chronically exposed to pollutants released by Huelva and Guadalquivir Estuaries had less lipid peroxidation and 8-oxodG level in DNA, and a less oxidized GSSG/GSH status. In contrast, mussels were less adapted to pollution and showed clear increases in oxidized biomolecules, in agreement to their small increases in antioxidant defense mechanisms, in spite of their much lower metal accumulation (Funes et al., 2006).

Its high metal affinity/inducibility and free radical scavenging ability makes of metallothionein (MT) one of the most sensitive biomarkers of pollution in several bivalve species, such as clams (Rodríguez-Ortega et al., 2002), mussels and oysters (Funes et al., 2006), in which MT levels correlated positively with metal load. In search of a more specific, sensitive and reliable assay, a total MT quantification method was developed using reversed-phase HPLC coupled to fluorescence detection (RP-HPLC-FD) after monobromobimane (mBBr) derivatization. The RP-HPLC-FD method was described and successfully applied to assess the MT content in heat-denatured digestive gland extracts of *C. gallina* from different South-Spanish coastal sites (Alhama et al., 2006). The method was later improved, by optimizing mBBr derivatization and

heating treatment, in *Scrobicularia plana* clams (Fig. 4) to assess the pollution status of the Guadalquivir Estuary after the Aznalcóllar spill (Romero-Ruiz et al., 2008). MT content was higher in unheated samples than in heated extracts, and correlated better to metal (Zn, Pb, Ni, Mn and Fe) contents and antioxidant activities. MT correlated negatively to 6PGDH and glyoxalase II activities, inhibited/inactivated at metal polluted sites, and positively to CAT activity and MDA level, increased by metal-promoted oxidative stress. Protein expression profiles were studied also by 2-DE in *S. plana* gills (Romero-Ruiz el al, 2006). The results showed a higher number of upregulated protein spots in animals from sites of Guadalquivir Estuary near its mouth in the Atlantic Ocean, that contained higher content of several redox-active elements (Fig. 4). Taken together, our results suggest that elements detected at Guadalquivir Estuary did not originate from Aznalcóllar spill, but were carried out by Guadalquivir River and deposited at its concave bank at high tide conditions.

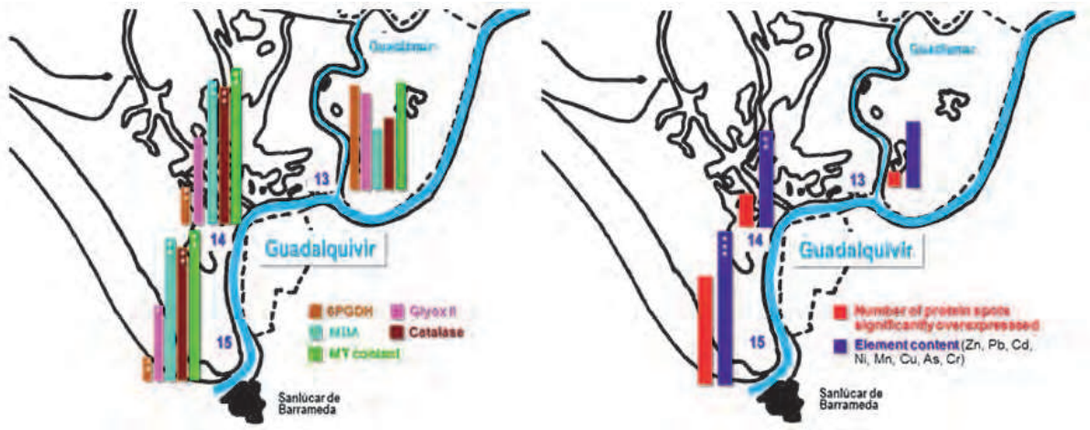

Fig. 4. Pollution of Guadalquivir Estuary studied in *S. plana* in October 2003. A) Responses of "classic" biomarkers, and B) total number of protein spots showing after 2-DE significant upregulated expression levels, compared to content of a number of elements.

2.3.2 Effects of Aznalcóllar spill assessed in *M. spretus*

On April 25, 1998, a tailings dam of the Aznalcóllar pyrite mine partially collapsed releasing to Guadiamar stream acidic water and mud with toxic metals (Fe, Zn, Pb, As, Cu, Sb, Tl, Bi, Cd, Ag, and Sr) that threatened DNP. To assess their possible biological effects, biochemical biomarkers were assessed in *M. spretus* from several Doñana areas and along the Guadiamar course (Ruiz-Laguna et al., 2001). Biomarkers assayed responded to different contaminant types: 1) Metals and prooxidant compounds (CAT, SOD, GSSGRase, GSHPx, G6PDH, MDA, GSSG/GSH status). 2) Aromatic compounds (EROD). 3) Chemicals with both characteristics (cytosolic and microsomal GST). Before the spill mice from the "Brazo de la Torre" (Fig. 2) had GSHPx and EROD activities close to animals of Huelva Industrial Park, suggesting similar levels of oxidants and aromatic contaminants at both sites (Ruiz-Laguna et al., 2001). Six months after the spill, mice from the lower Guadiamar areas, also showed significantly higher GSTc and GSTm activities, altered levels of several antioxidant enzymes, and a highly oxidized GSSG/GSH status. Thus, chemicals spilled from Aznalcóllar dam induced further biological effects in mice from exposed areas.

Monitorization of terrestrial ecosystems of DNP and Guadiamar course continued till 2002 using *M. spretus*. Kidney Pb, Cd and As levels correlated with antioxidative enzymes.

Oxidative stress biomarkers indicated the presence of metals in the upper/medium Guadiamar course, and their response decreased with the distance to the mine. From 1997 to 2002 biomarkers evolved as follows: 1) Antioxidative (CAT, SOD, GSSGRase, G6PDH) and biotransforming (GSTc, GSTm) enzymes and MDA and GSSG levels were lower in LDP mice than in those along Guadiamar course, and decreased in 2001-2002, indicating a clear recovery after dilution of the metals released. 2) Other biomarkers (G6PDH, GSHPx and especially EROD) were also high in LDP mice and increased in 2001-2002, suggesting the presence in the medium/low Guadiamar course, and even in Doñana, of organic pollutants, such as the pesticides used in intensive agriculture of nearby areas, strawberry and citric crops West of DNP, and the rice fields East of Guadiamar (Bonilla-Valverde et al., 2004).

In 2002, the effects of Aznalcóllar spill and those of agrochemicals used around DNP were compared in *M. spretus* from sites along Guadiamar, Rocina ($ROC_{5/6}$) and Partido ($PAR_{7/8}$) streams, within Doñana Biological Reserve (LDP) and Matochal rice fields. In addition to the biomarkers used previously, a PES proteomic approach was undertaken, detecting up-/down-regulation of proteins after 2-DE gels (Bonilla Valverde, 2006). Figure 5A summaryzes the responses of "classic" biomarkers: compared to LDP, mice from areas of intensive agricultural practices, ROC, PAR, Matochal, had increased EROD and GSHPx activities and GSSG levels, indicating that these sites were polluted, even more extensively than sites from Guadiamar course. Near 3,000 proteins were resolved in 2-DE gels (24 cm, pH 4-7), of which 30 showed significant intensity differences when compared to gels of LDP mice. Figure 5B summarizes the conclusions of this PES approach: the intensities of 18 spots were signifycantly altered in mice from intense agriculture ($ROC_{5/6}$, $PAR_{7/8}$, Matochal), and 20 spots in animals collected along Guadiamar course. It should be noticed that the intensity of 8 of the total 30 proteins were simultaneously altered in animals from both sites, suggesting the presence of similar contaminants, probably metals, in areas exposed to Aznalcóllar spill and of intense agricultural practices (Bonilla Valverde, 2006).

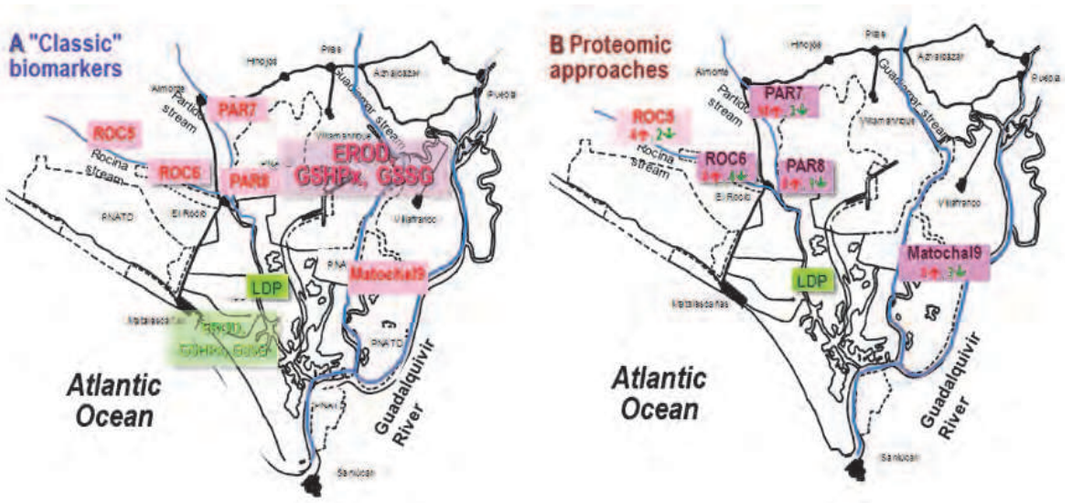

Fig. 5. Pollution of DNP and Guadiamar sites compared to LDP site studied in *M. spretus* liver. A) Responses of "classic" biomarkers. B) Number of proteins with significantly altered expression levels (red up-, green down-regulated).

2.3.3 Conventional biomarkers and PES-proteomic studies in *P. clarkii*

Organophosphate (OP) and carbamate (CM) pesticides replaced organochlorines as the main defense line against agricultural insects. Although less persistent, they affect nontarget organisms and increasing concern exists about their impacts (Scholz & Hopkins, 2006). OPs and CMs are toxic by inhibiting esterases, hydrolases of three types: (i) Acetylcholinesterase (AChE) active at synapses during nerve transmission. (ii) Butyrylcholinesterase (BChE) of uncertain physiological role. (iii) Carboxylesterases (CbE) that hydrolyze fatty acids from xenobiotics (Maxwell, 1992) and play a role in pesticide detoxification and tolerance (Hyne & Maher, 2003). Inhibition of AChE and BChE has been used as an exposure biomarker for CMs and OPs: Peakall (1994) included AChE inhibition in the gold standard biomarkers, useful to diagnose a problem without need for chemical analysis.

AChE, BChE and CbE activities were characterized in different *P. clarkii* tissues, and their sensitivity to inhibitors and model OP and CM pesticides was studied (Vioque-Fernández et al., 2007a). Nervous tissue AChE and digestive gland CbE were proposed as biomarkers; low BChE was found in all tissues studied. Use of TritonX-100 was avoided due to its diverging effects in esterase assays. CMs inhibited AChE 100-fold more strongly than OPs. *In vitro* conditions to assess recovery from inactivation were established for AChE and CbE. The new protocol was proposed as a biomarker of pesticide exposure to differentiate between dilution-reversible inhibitions, indicating CM exposure, from irreversible effects, treating with 2-pyridinealdoxime (2-PAM), attributed to OPs.

Utility of AChE and CbE inhibition and reactivation was assessed in a field study carried out at different freshwater sites of DNP. Esterases were measured in *P. clarkii* from reference or potentially exposed sites, and their possible reactivation was studied after dilution or 2-PAM treatment (Vioque-Fernández et al., 2007b). As summarized in Table 2, crayfish of five potentially polluted sites, $ROC_{5/6}$, $PAR_{7/8}$ and Isla $Mayor_{10}$ (Fig. 2), had lower CbE activity (from -43 to –23%) than those from LDP. Yet, no differences with untreated extracts were found after 50-fold dilution or 2-PAM treatment, indicating that CbE was irreversibly inhibited. Several pesticides were detected in water and/or soil at Matochal and Isla Mayor rice growing sites, and lower levels at agricultural areas near the Rocina and Partido streams. Since no correlation was found between pesticide content and esterase inhibition, other factors, such as the concomitant effects of metals, should affect pesticide response.

Site	CbE activity			Organic compound detected
	% LDP	Dilution	2-PAM	
LDP	**100**	118	109	
ROC 5	**66****	81	86	PCB138
ROC 6	**71****	105	87	Bromacyl, Dimethoate
PAR 7	**77***	100	104	4,4´-DDD, Dichlofluamid, Folpet, Molinate
PAR 8	**57****	98	102	Molinate
Matochal 9	**82**(ns)**	91	97	Malathion, Bromacyl, Dimethoate, Acrynathrin, Chlorpyrifos, Trifluraline, Penconazole, Methidathion
Isla Mayor 10	**57****	106	102	4,4´-DDT, Dichlofluanid, Bupirimate

Table 2. BChE activity in *P. clarkii* and pesticides detected in water/soils from DNP sites. CbE is shown as % of LDP animals, and after 50-fold dilution or 2-PAM treatment, and compared to untreated values. Pesticides are shown in decreasing order of concentration.

Freshwater aquatic DNP ecosystems were monitored using *P. clarkii* in four campaigns carried out from 2003 to 2004 (Vioque-Fernández et al., 2009a). In the "classic" approach, twelve biomarkers, responsive to pesticides, organics and prooxidants were used. Low CAT, G6PDH, CbE and AChE activities existed at $ROC_{5/6}$, $PAR_{7/8}$, Matochal and Isla Mayor, plus high MDA, MT and GSSG levels, suggesting that metals and prooxidants were probably present at sites potentially polluted by agrochemicals. In contrast, high CAT, CbE and AChE activities and GSH levels, and low MDA, MT and GSSG contents existed at LDP (Fig. 6A). The superiority of proteomics was clearly established. 2-DE resolved over 2,500 gill spots, and 35 proteins had significant intensity differences (Vioque-Fernández et al., 2009a). The fold-number of up-/down regulation separated different PES (Fig. 6B). Animals captured within or close to Doñana Biological Reserve (LDP, LD, ROC_6, and PAR_8) showed clean/low polluted PES, with 32 proteins of unaltered intensity compared to LDP. Site PAR_7 had a moderately polluted PES, with 7 proteins up- and other 6 downregulated. Crayfish from the upper "Rocina" course (ROC_5) showed a polluted PES, with 13 proteins up- and other 13 downregulated compared to LDP. The higher proteomic responses at the upper Rocina and Partido courses suggest that non-persistent agrochemicals are used in Doñana surroundings. The highest responses correspond to crayfish from the rice growing areas at Matochal and Isla Mayor, with 30 of the 35 proteins being significantly altered, according to the extended and intensive use of agrochemicals in such areas. Both the "classic" biomarker and the environmental proteomic approach indicate that sites within Doñana Biological Reserve are scarcely polluted, while the agricultural areas around DNP are highly polluted.

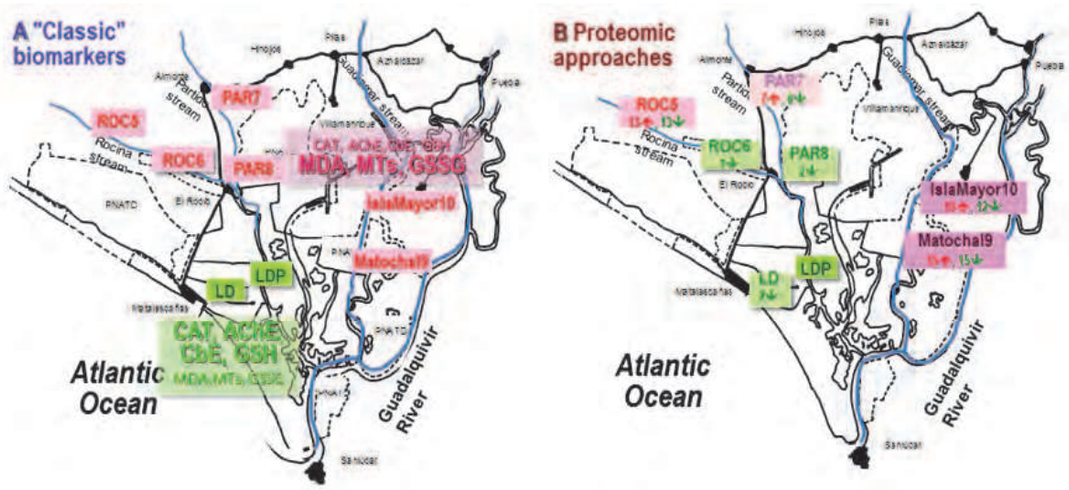

Fig. 6. Pollution of DNP sites compared to LDP assessed in *P. clarkii*. A) Responses of "classic" biomarkers. B) Number of proteins with significantly altered expression levels (red up-, green down-regulated) in gill extracts of crayfish from the different sites studied.

DNP monitoring using *M. spretus* (Bonilla Valverde, 2006) and *P. clarkii* (Vioque-Fernández et al., 2009a) differed to some extent. While the "classic" approach yielded similar results, with higher biomarkers levels being detected at the agricultural areas surrounding Doñana, along the Rocina and Partido streams and the rice growing fields east, the PES approach differed significantly. Actually, while in the *M. spretus* proteomic study the areas close to DNP, ROC_6/PAR_8, were as polluted as Matochal, in the *P. clarkii* PES study these two areas were classified as low or faintly polluted, in view of the small number of proteins whose

intensity was significantly altered when compared to LDP animals. It is worth noticing that mice evaluate the status of terrestrial ecosystems and crayfish that of fresh water aquatic environments, whose pollution could be quite different.

In vivo effects of two model pesticides, chlorpyrifos and carbaryl, were studied under controlled exposure conditions in *P. clarkii* (Vioque-Fernández et al., 2009b). The organophosphate chlorpyrifos inhibited CbE in a concentration-dependent mode, AChE being less sensitive; in contrast, the effects of carbaryl, a carbamate, were less clear. Chlorpyrifos lowered EROD, CAT and GSSG levels but raised GST activity, while carbaryl raised EROD, CAT and GST, but lowered GSHPx and GSH levels. The effects on protein expression were studied also by 2-DE (Vioque-Fernández et al., 2009b). In gill and nervous tissue about 2,000 spots were resolved, with quite different expression patterns. As shown in Figure 7, chlorpyrifos altered 72 proteins, mostly in nervous tissue, and carbaryl 35, distributed evenly between organs. Several spots were selected as specific protein expression signatures for chlorpyrifos or carbaryl exposure in gills and nervous tissue, respectively.

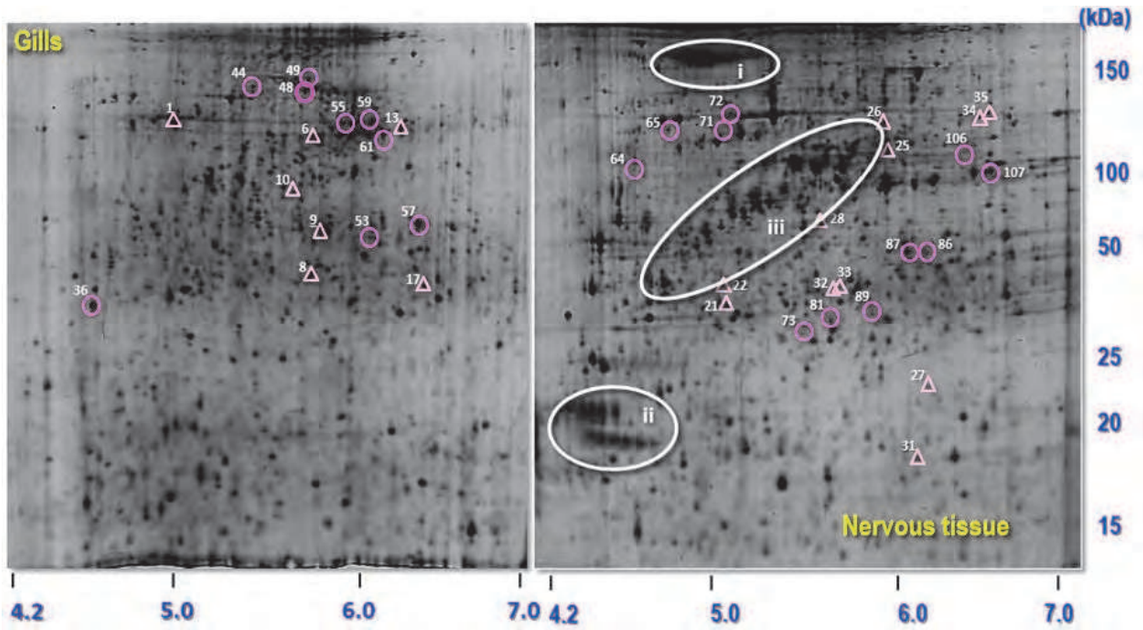

Fig. 7. Representative 2-DE gels of proteins from *P. clarkii* gills and nervous tissue. Symbols show spots with significantly altered intensities after 7 days exposure to chlorpyrifos (O) or carbaryl (Δ) concentrations. Areas i-iii mark clearly different patterns in nervous tissue.

2.3.4 Use of fisrt-generation methods in the environmental proteomic approach

We will now review several studies made by our groups using first-generation proteomic methods for protein separation and identification. These studies were carried out comparing animals from sites within DNP and Doñana surroundings, the Domingo Rubio Stream, and the Guadalquivir Estuary. Bivalves, including *Chamaelea gallina*, *Scrobicularia plana*, the crustacean *Carcinus maenas*, and *M. spretus* mice were used as bioindicators. Proteins showing significantly altered levels were identified by *de novo* nESI-MS/MS sequencing in the non-model species *C. maenas* and *S. plana*, and by MALDI-TOF-PMF in *M. spretus*, due to its homology to model mouse species *Mus musculus*.

Proteomics was used in *C. gallina* clams as a preliminary screening of changes in protein expression caused by pollutants, potentially useful as new biomarkers (Rodríguez-Ortega et al., 2003). Clams were exposed for seven days to four model contaminants, Aroclor 1254, copper (II), tributyltin (TBT), and arsenic (III), and cytosolic fractions were initially analyzed by 2-DE (7 cm, pH 4–7). About 1,000 spots were resolved and altered expression was qualitatively detected in 9–26 spots per treatment. Aroclor 1254, Cu (II) and As (III) had a mainly upregulating effect, in contrast to TBT. Altered protein expression was confirmed in 18 cm gels. The 15 spots more drastically altered were excised and analyzed by MS. Aroclor 1254 and Cu(II) upregulated putative isoforms of tropomyosin and light myosin chain. Actin was downregulated by Aroclor and Cu(II) but upregulated by TBT and As(III), while the opposite behavior was shown by a truncated actin form. The exclusive identification of cytoskeletal proteins could reflect their relative abundance, their prevalence in databases in mollusks, or their role as major targets of pollutant-related oxidative stress.

The utility of proteomics to assess pollutant response of *S. plana* clams from three sites of Guadalquivir Estuary at the southern end of DNP was studied (Romero-Ruiz et al., 2006). Protein expression profiles were analyzed by 2-DE in soluble fractions of *S. plana* gills. Nearly 2,000 well-resolved spots were detected in silver-stained gels, with focused areas in the 4–6.5 pH range. Different protein expression signatures were found at each site, with the highest number of more intense spots in animals with the highest metal content. Nineteen more intense protein spots were analyzed out by nanospray-ion trap tandem MS/MS, *de novo* sequencing and a bioinformatics search for their possible identification. While sequence tags of 16 more intense protein spots were obtained, including several proteins induced by pollutant exposure of model organisms, only 2 proteins were unambiguously identified: hypoxanthine guanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Both enzymes were significantly higher in animals with the highest metal contents.

The suitability of high-throughput proteomic methods to monitor terrestrial ecosystems was evaluated in *M. spretus* from three sites in the DRS and from LDP, using specimens from an industrial settlement (phosphogypsum stacks) and Matochal rice fields as positive controls (Montes-Nieto et al., 2007). The 2-DE analysis showed 36 spots with significantly altered expression. Sixteen were identified by MALDI-TOF-PMF and peptide matching with *M. musculus* databases. Identified proteins play different roles: cytoskeletal dynamics, proteolysis, biotransformation, oxidative-stress adaptation, and metabolism. Animals from different polluted environments showed contrasting differences in their proteomes, with specific increases and decreases in selected groups of proteins that seem to be coordinately regulated. Proteomic data were consistent with metal biomonitoring and conventional biomarker responses, indicating that DRS animals sustained a heavier pollutant burden than LDP mice and suffered a chronic oxidative stress. Whereas some protein expression differences may protect mice from pollutant toxicity, others should make them more susceptible. Transcript expression signatures agree with the documented lack of correlation between mRNA and protein levels. Nonetheless, there is a positive significant correlation between the gpx1 mRNA molecules and the intensity of one of the two identified GPX1 isospots. It was underlined the usefulness of additional information (element content, biomarker responses and absolute mRNA expression signatures) to assist the biological interpretation of environmental proteomic data.

Element load, "classic" biomarkers and altered protein expression were studied in *C. maenas* crabs, to assess DRS contamination (Montes-Nieto et al., 2010). Lower antioxidative

activities (G6PDH, 6PGDH, CAT) were found in parallel to higher levels of damaged biomolecules (MDA, GSSG), due to oxidative lesions promoted by contaminants, as the increased levels of essential (Zn, Cu, Co) and nonessential (Cr, Ni, Cd) elements. Utility of Proteomics to assess environmental quality was confirmed, especially after considering the six proteins identified by *de novo* sequencing. They include tripartite motif-containing protein 11 and ATF7 transcription factor (upregulated), plus CBR-NHR-218 nuclear hormone receptor, two components of the ABC transporters and aldehyde dehydrogenase (downregulated). These proteins could be used as novel potential biomarkers of the deleterious effects of pollutants present in the area.

Table 3 summarizes a number of proteins with significantly altered expression identified in previous studies of Environmental Proteomics using fisrt-generation methodologies. The Table includes the cellular functions in which these proteins are involved, including: cytoskeleton, axonal transport and cell division, membrane transport, proteolysis and autophagy, biotransformation, adaptation to oxidative stress, central pathways of glucid, fatty acid, aminoacid, methyl and urea metabolism, and transcriptional regulation.

Cellular function	**Protein identified** (reference)
Cytoskeletal dynamics	Tropomyosin[1], light Myosin chain[1], Actin[1], Tubulin beta-3 chain[2]
Membrane transport	Peptide ABC transporter[3], ABC transporter G-family[3]
Proteolysis	Cys-protease ATG4B[2], tripartite motif-containing protein 11[3]
Redox functions	GST ω-1[2], GSHPX 1[2], Peroxiredoxin 1, 2 and 6[2]
Intermediary metabolism	Triose-P isomerase[2], Glyceraldehyde 3-P, DHase[4], Fructose-1,6-bisphosphatase[2], Hypoxanthine P-ribosyl transferase[4], Aldehyde DHase[3], EnoylCoA hidratase[2], HMG-CoA synthase 2[2], L-Asp DHase[2], Met adenosyl transferase[2], Gly-N-methyl transferase[2], Ornithine transcarbamylase[2],
Transcriptional regulation	ATF-7, cAMP-dependent transcription factor, CBR-NHR218 nuclear hormone receptor[3].

Table 3. Proteins with significantly altered expression identified in: [1]Rodriguez-Ortega et al., 2003; [2]Nontes-Nieto et al., 2007; [3]Nontes-Nieto et al., 2010; [4]Romero-Ruiz et al., 2006.

2.4 Transcriptomics in environmental studies

Studies reporting absolute transcript abundance of genes are infrequent, even though it is the only adequate procedure to accurately assess the expression of a gene (Prieto-Alamo et al., 2003). We used this rigorous approach to provide the first comprehensive and absolute quantification analysis of the overall expression patterns of transcript coding for several ecotoxicological interesting protective and detoxificant enzymes in *M. spretus*. A total of 20 transcripts involved in oxidative stress response (heme oxygenase, HO) and biotransformation of electrophilic compounds (CYPs, GSTs) was examined (Prieto-Alamo et al., 2003; Ruiz-Laguna et al., 2005, 2006). Different sites located inside DNP and in Huelva province were chosen due to their proximity to industrial or agricultural areas. Metal biomonitoring indicated that animals from contaminated areas had heavier pollutant burden than those from LDP. Interesting organ-associated differences were found in the expression levels of the transcripts analyzed, being in general the liver the organ with the highest levels, according to its detoxificant role.

The hepatic expression levels of transcripts encoding different CYPs and GSTs showed up-regulation of Cyp1a2, Cyp2a5, Cyp2e1, Cyp4a10, Gsta1, Gsta2, Gstm1, and Gstm2 mRNAs in liver of male mice dwelling polluted areas (Table 4). Hepatic level of *Cyp* and *Gst* mRNAs are collectively activated by many structurally unrelated compounds through a variety of mechanisms. Additionally, CYP proteins usually generate reactive molecules that must be detoxified by GSTs. The concomitant up-regulation of Cyp and Gst genes, that respond to many different classes of chemicals and regulatory mechanisms, seems the logical results of exposure to a complex profile of pollutants, like those associated with the intensive agricultural activity developed close to our studied areas. Important gender-related differences were found also in the expression of most Cyp and Gst genes, suggesting that absolute amounts of transcripts of biotransformation enzymes are more potent biomarkers in males than in females. Individual quantitation's is also mandatory to prevent biased interpretations by specimens with abnormal expression levels.

Up-regulated genes	**Known inducer mechanisms**
HO1	Induced by oxidants
Cyp1a2	Induced by PAHs/dioxins through the xenobiotic response element (Noda et al., 2003)
Cyp2a5	Induced by N heterocycles non P450 inducers (Su & Ding, 2004)
Cyp2e1	Act on solvents and industrial monomers (González & Kimura, 2003)
Cyp4a10	Induced by peroxisome proliferators via PPAR (cited Kroetz et al., 1998)
Gsta1, Gsta2 Gstm1, Gstm2	Induced by PAHs, phenolic antioxidants, ROS, isothiocyanates, arsenicals, barbiturates, etc., by mechanisms usually involving the antioxidant response element (Chanas et al., 2002).

Table 4. Genes upregulated identified in: Prieto-Álamo et al., 2003; Ruiz-Laguna et al., 2005

3. Ongoing research

The global aim of our current project focused in Doñana and its surroundings is the development and multidisciplinary integration of innovatory analytical tools, that would subsequently be applicable to different types of ecosystems to contribute to a technological updating of environmental evaluation. The following goals are being pursued during the development of our project: 1) Information is being obtained on pollutant presence in Doñana and their biological effects. 2) Analysis methods for the characterization of metals and elements and new species with a toxic potential are being optimized. 3) The presence of pesticides and other pollutants, including some emerging ones, is being evaluated by developing screening and fast analysis methods. 4) Finally, the chemical analyses and the responses of some established biomarkers will be integrated with the expression profiles detected with high-throughput methods at genomic, proteomic and metallomic levels, permitting a global evaluation of environmental quality.

3.1 Novel analytical approaches for emerging pollutants

Analysis of complex samples and detection or quantitation of analytes at very low levels are two of the key analytical problems, whose complexity increases when both problems are

present. The use of clean-up procedures is a traditional tool that has recently undergone very important developments. Liquid-liquid extraction (LLE) used to be a key sample-preparation technique prior to carry out the chemical analysis. Recently, solid phase extraction (SPE), using several sorbent types, has been preferred to extract pharmaceuticals from environmental and biological matrices. Yet, in the last years, high interest exists in developing new extraction and clean-up procedures. Liquid phase microextraction (LPME) is an attractive alternative to the widely used solid phase extraction (SPE). Single, low-cost, disposable, porous, hollow fibers made of polypropylene are used as support for liquid membranes (HF-LPME) as an efficient clean-up procedure that yields also high degree of pre-concentration. Additionally, the low organic solvent consumption makes HF-LPME an interesting and environmental friendly analytical procedure. This extraction procedure has been used for the determination of some pharmaceuticals in environmental and biological samples (Ramos-Payan et al., 2009a,b, 2010, 2011a,b,c) that also decreases matrix effects and ionic suppression when mass spectrometry detection is used.

An electric potential can promote efficient analytical extraction through a supported liquid membrane (SLM). This system, known as electromembrane extraction (EME) produces the analytes to be extracted from an aqueous sample through an organic solvent immobilized as SLM in the wall of a polypropylene porous hollow fiber to an aqueous acceptor solution placed within the lumen of the hollow fiber. Essentially, it is similar to a HF-LPME where the migration through the SLM is forced by the electrical field generated by two electrodes placed out of the fiber and in its lumen, respectively (electromigration, EMI). This extraction procedure has been used to determine six widely used non-steroidal anti-inflammatory drugs. The method was applied to their determination in such a highly complex matrix as urban wastewaters. The EME procedure provides very clean extracts that can be directly injected into the chromatographic system thus producing excellent baselines.

Currently, the efforts are directed to combine enzymatic extraction assisted by ultrasounds probe sonication or microwaves and a subsequent HF-LPME to reach higher selectivity and sensitivities. Extraction procedures are being developed for determination of substituted (chloro-, nitro- and alkyl-) phenols, well known as endocrine disruptors. Procedures that combine microwave and ultrasound energy with use of enzymes followed by a HF-LPME, will be applied to environmental samples and to fish and crustaceans tissues. Work is also being carried out in the design and optimization of exposure experiences to emerging contaminants for *P. clarkii*, an excellent bioindicator in fresh water ecosystems.

3.2 Novel analytical approaches for pesticides

Conventional analytical approaches for pesticides based on gas chromatography with ECD or MS detectors have been improved to enhance sensitivity and selectivity. A coupling based on a two-dimensional detector, electron capture/inductively coupled plasma MS, combines the high sensitivity (ECD) and the selectivity (ICP-MS) for halogens, avoiding interferences from other organic compounds present in organism samples (Gómez-Ariza et al., 2006). Pretreatment is critical to get quantitative recovery and preconcentration from samples in a short time. Polymeric membranes are being used for this purpose since it overcomes well established approaches such as solid phase microextraction with lower cost and no cross contamination. A method based on hollow fiber liquid-phase microextraction has been optimized for extraction of forty COP's, including PCB's, PBDE's and pesticides. Preliminary univariated optimization allowed the selection of more significant variables, subsequently subjected to a central composite rotable design (CCRD) for multivariate

optimization of extraction time, membrane length and temperature. Efficiency and preconceentration factors and recoveries are very high (Manso-Sayago et al., 2010).

3.3 Metallomic analysis in animals from Doñana National Park

The approach based on SEC-ICP-MS coupling is being used in exposure experiments of the laboratory mice, *M. musculus*. Preliminary results show that exposure to a toxic element, Cd, yield clear biological responses, namely the upregulation of Cd-metallothionein in liver and the concomitant downregulation of Cu-superoxide dismutase (González-Fernández et al., 2011). 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) is being applied to extracts of Cd-exposed *M. musculus* to identify all proteins differentially expressed after Cd treatment. Comparison of proteins identified by the massive 2D-DIGE approach and by SEC-ICP-MS will allow to understand the mechanisms involved in Cd response.

The SEC(HPLC)-ICP-MS approach is being optimized and applied to *M. spretus* liver to identify differentially expressed metalloproteins/metallobiomolecules (González-Fernández et al., 2011). Future studies will be focused on experimental exposure to other toxic elements or metal species, such as Hg^{2+}, methyl-Hg, As(V), and organic pollutants (pesticides, pharmaceutical drugs, PCB), involving both mice species, *M. musculus* and *M. spretus*.

3.4 Transcript expression patterns in animals from Doñana National Park

3.4.1 Heterologous microarrays for analysing pollution effects on *M. spretus*

The Domingo Rubio stream (DRS) is a contaminated place near Huelva industrial area that receives elements of pyritic origin from the Tinto River. Tidal changes affect mainly its lower course (Fig. 2, site 2), but also reach its medium course (site 3) that is also affected by nearby chemical plants and a petrochemical complex. The intensive agriculture carried out at adjoining strawberry fields affect the upper DRS course (site 4) and also reach site 3. Previous studies reported that DRS mice accumulate several elements, the metal loads being particularly high in animals from the medium DRS course (Montes-Nieto et al., 2007).

The transcriptomes of *M. spretus* from DRS sites 2, 3 and 4 were studied using heterologous microarrays available for the laboratory mouse, *M. musculus*, and compared to those of LDP reference mice. Although both species diverged ~1-3 million years ago, they conserve a great sequence similitude (one sequence variant every 50 bp) what permit the heterologous hybridization and identification of genes differentially expressed between reference and problem mRNA populations. Microarrays analysis (Fig. 8) identified 1,872 spots as differentially expressed in mice living in at least one DRS site. Expression of 242 genes was significantly altered in mice from the three DRS sites, and 39 genes showed ≥10-fold changes (34 up-/5 down-regulated) in animals from at least one DRS site compared with LDP site. DRS3 animals showed the maximal amplitude and the highest number of changes compared to those from the DRS2 and DRS4 sites (Abril et al., 2011) (Fig. 9).

Many pollutants, including heavy metals and pesticides, modulate the immune function (Galloway & Depledge, 2001). Chemicals in pesticides, mitocides, herbicides and fumigants stimulate, suppress or deregulate the immune system depending on dose and its duration (Rea & Lian, 1991); pesticides generate also reactive oxygen species (Agraval & Sharma, 2010). Genes of the "immune response" and "stress response/DNA repair" categories, such as Chi3l3 or A2m, Gpx3 or Sgk1 genes, are highly induced in DRS mice. CHI3l3 (chitinase 3-like 3 protein), produced by macrophages upon inflammation (Welch et al., 2002), mediates asthma inflammatory response (Shuhui et al., 2009). A2M (α2-macroglobulin), a plasma proteinase inhibitor, binds many biologically important molecules (LaMarre et al., 1991), inhibits the

degradation of matrix proteins and reduce inflammation in liver (Ho et al., 2010). Thus, A2M overexpression in DRS mice might help to the resolution of the inflammatory response and to recover homeostasis. GPX3 (GSH peroxidase 3), a key enzyme in the defense from oxidative stress, induces the Sgk1 gene expression (Leong et al., 2003; Loffing et al., 2006). SGK1 (serum- and glucocorticoid-regulated protein kinase 1) is a protein kinase activated via PI3-kinase to counteract apoptosis (Lutz et al., 2010). Most genes of the "cell cycle/cell differentiation" categories (e. g., Btc) are strongly up-regulated in DRS mice.

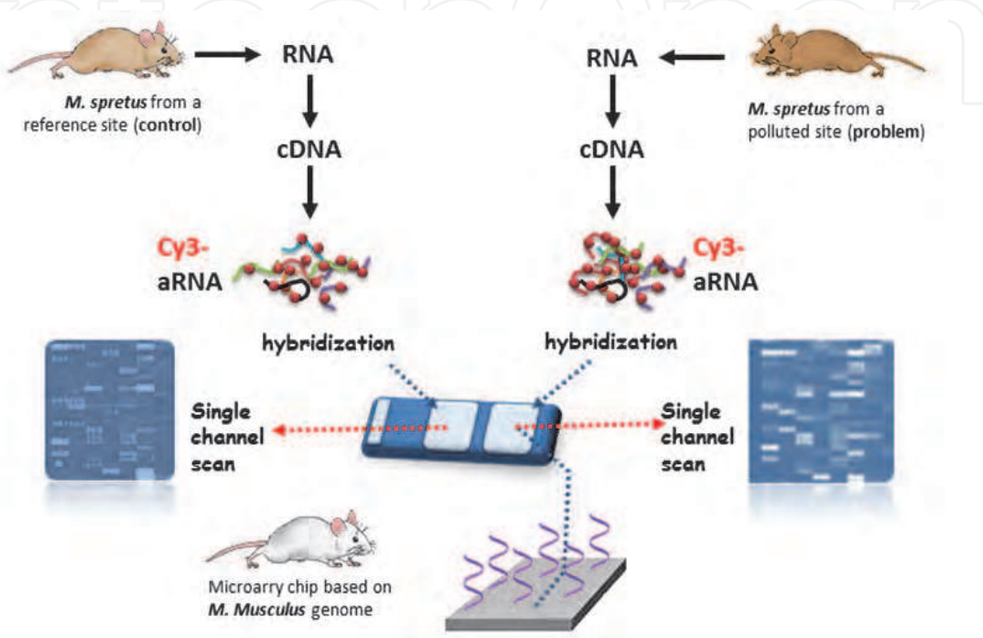

Fig. 8. Workflow for one-color heterologous microarray experiments. RNA is extracted, reverse transcribed into cDNA and labelled with Cy3 while synthetizing aRNA. Each sample is hybridized to a single microarray chip, that is washed and scanned. The relative signal intensity is used to estimate differentially expressed genes in the problem sample. The species for which the microarray was developed, here *M. musculus*, differs from that is hybridized to the microarray, here *M. spretus*.

Pesticide exposure may alter lipid, protein and carbohydrate metabolism (Karami-Mohajeri & Abdollahi, 2010). Interestingly, repression of Inhbe gene, negative regulator of cell growth (Chabicovsky et al., 2003) and inducer of gluconeogenesis (Hashimoto et al., 2009), might be linked to co-induction of genes coding MUP proteins, of the "cell signalling" category. MUPs (major urinary proteins) transport small hydrophobic ligands (pheromones), and inhibit the expression of gluconeogenic and lipogenic genes (Hui et al., 2009; Zhou et al., 2009). The Fgf21 gene was down-regulated in DRS animals (>11 fold), probably explaining their lower gluconeogenesis and lipid metabolism (Tong et al., 2010). Hence, the induction of Mup genes and the repression of Inheb and Fgf21 genes might be considered as a coordinated response to drive energy towards the inflammatory process. As a whole, data from the heterologous microarray study in *M. spretus*, and previous proteomic results (Montes-Nieto et al., 2007), indicate that DRS mice sustained a heavier pollutant burden than LDP animals and, therefore, suffer a chronic stress situation that elicit and maintain immune and an oxidative stress responses.

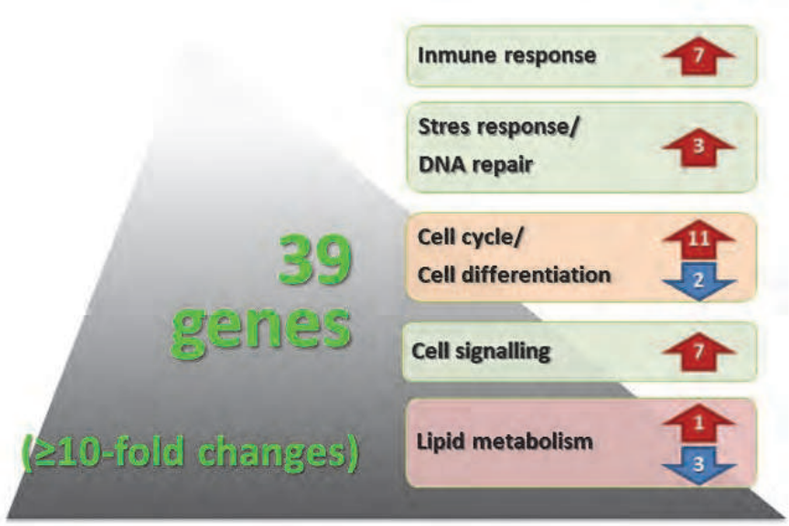

Fig. 9. Summary of genes significantly altered (≥10-fold, 34 up-, 5 down-regulated) in animals from the three DRS sites as compared with the LDP site (Abril et al., 2011).

3.4.2 Substractive suppressive hybridization to analyze pollution effects on *P. clarkii*

The reproductive success of *P. clarkii*, its ability to tolerate environmental changes and to feed on near anything contribute to its huge potential to colonise new areas and exploit natural resources. SSH is used to analyze the pollution response of *P. clarkii*, identifying genes differentially expressed in two populations without previous knowledge of their sequence. Animals from Matochal rice fields were considered as problem, and those from LDP as reference. The analysis was carried out in the digestive gland, main site of xenobiotic biotransformation involved also in key physiological processes (Reddy et al., 1997). Two libraries, one forward (genes up-regulated in MAT) and one reverse (down-regulated in MAT), were obtained. Most of the identified genes are related to the immune response (Abril et al., 2011). One of the up-regulated genes codes for hemocyanin, a Cu-containing protein linked to invertebrate response to PAHs and metals, after activation by Ser-proteases (Lee et al., 2004). The parallel up-regulation of the Ser-proteinase inhibitor-3 gene and the down-regulation of cathepsin-L gene in Matochal crayfish, might decrease the proteolytic generation of immune effectors, seeking for homeostasis recovery and resolution of the inflammatory situation (Jiang et al., 2009). Though the results obtained by SSH have to be accompanied by clone analysis and qRT-PCR validation, the data show that environmental stresses are causing a strong response in crayfish collected at polluted site.

3.5 Protein expression patterns in animals from Doñana National Park

Second generation proteomic methodologies, 2D fluorescence difference gel electrophoresis (2D-DIGE) and quantitative determination after labeling with isobaric reagents (iTRAQ) are being used to identify altered protein expression patterns in animals from sites of DNP with different pollution levels. In *M. spretus*, labeling with isobaric iTRAQ reagents is used to identify hundreds of mouse proteins. To diminish the complexity of mouse liver proteome, after trypsin digestion and iTRAQ labeling the peptides are prefractionated by isoelectrofocusing in IPG strips of 24 cm and 3-10 pH range, that are cut into 1 cm fractions before being extracted and analysed by tandem mass spectrometry in an Orbitrap system.

In *P. clarkii*, 2D-DIGE is being used to identify, by nESI-MS/MS, a high number of proteins with differential expression. Thus, from 2,400 proteins resolved in crabs from a polluted site, BER, 6 proteins are overexpressed in males and 3 in females. The DeCyder software has detected 50 proteins with significantly altered levels of expression when comparing male crabs from two polluted, PAR, MAT, and one reference, LDP, sites in Doñana surroundings. The highest changes have been found in animals from the Partido stream, the changes being lower in crabs from the Matochal rice fields. Pollutant-promoted modifications of proteins, including carbonylation and oxidation of –SH groups, are being studied by electrophoretic methods. Higher modification is being found in extracts from crayfish living in sites potentially affected by pollutants.

4. Conclusion

The difficulty in applying omic methods in field studies with free-living animals is being solved by: (i) a precise experimental design based on a profound knowledge of the area and the bioindicators, (ii) the continuous validation of omic results with more conventional and sensitive methods, (iii) the absolute quantification at the individual level of selected mRNAs, and (iv) the analysis of selected conventional biochemical biomarkers. The integration of results from contaminant analysis and metallomic results with the biological response will allow defining a non-biased set of novel biomarkers for a global biomonitorization of any ecosystem, thus contributing to the technological renewal.

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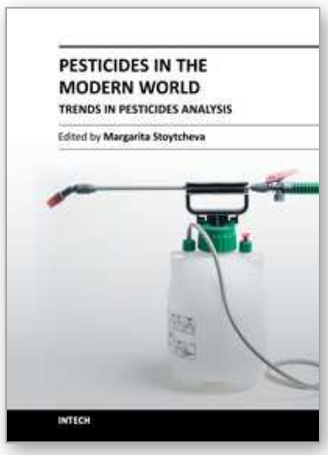

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The book offers a professional look on the recent achievements and emerging trends in pesticides analysis, including pesticides identification and characterization. The 20 chapters are organized in three sections. The first book section addresses issues associated with pesticides classification, pesticides properties and environmental risks, and pesticides safe management, and provides a general overview on the advanced chromatographic and sensors- and biosensors-based methods for pesticides determination. The second book section is specially devoted to the chromatographic pesticides quantification, including sample preparation. The basic principles of the modern extraction techniques, such as: accelerated solvent extraction, supercritical fluid extraction, microwave assisted extraction, solid phase extraction, solid phase microextraction, matrix solid phase dispersion extraction, cloud point extraction, and QuEChERS are comprehensively described and critically evaluated. The third book section describes some alternative analytical approaches to the conventional methods of pesticides determination. These include voltammetric techniques making use of electrochemical sensors and biosensors, and solid-phase spectrometry combined with flow-injection analysis applying flow-based optosensors.

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