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**RESEARCH ARTICLE**

# Biochemical and proteomic effects in *Procambarus clarkii* after chlorpyrifos or carbaryl exposure under sublethal conditions

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**Abstract**

*In vivo* effects of two sublethal doses of chlorpyrifos and carbaryl were studied in *Procambarus clarkii* after 2 and 7 days of exposure, and after pesticide removal. Chlorpyrifos inhibited carboxylesterase activity in a concentration-dependent manner, but acetylcholinesterase was less sensitive. Compared with chlorpyrifos, carbaryl had a less marked effect on esterase activity. The effects of selected pesticides on biotransformation or oxidative stress biomarkers were contradictory. Chlorpyrifos lowered ethoxyresorufin-*O*-deethylase (EROD), catalase and oxidized glutathione (GSSG) levels but raised glutathione-*S*-transferase activity, while carbaryl raised EROD, catalase and glutathione-*S*-transferase, but lowered glutathione peroxidase and reduced glutathione (GSH) levels. The effects on protein expression patterns depending on pesticide type and the tissue used for analysis were studied in parallel by 2-DE. In gill and nervous tissue about 2000 spots (pI 4–7) were resolved, with quite different expression patterns. Chlorpyrifos altered 72 proteins, mostly in nervous tissue, and carbaryl 35, distributed evenly between organs. Several specific spots were selected as specific protein expression signatures for chlorpyrifos or carbaryl exposure in gills and nervous tissue, respectively.

**Keywords:** *Pesticide; carboxylesterase; acetylcholinesterase; biomarkers; proteomic; two-dimensional gel electrophoresis*

## Introduction

Organophosphate (OP) and carbamate (CM) pesticides inhibit different esterases, which are useful as biomarkers of pesticide exposure/effect (Hyne & Maher 2003). Recently, we showed in *Procambarus clarkii* that the OP chlorpyrifos and the CM carbaryl inhibit *in vitro* acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxylesterase (CbE) and optimize conditions for reactivation of these three enzymes (Vioque-Fernandez et al. 2007b). This crayfish species was used in Doñana National Park (South Spain) to monitor water contamination by agricultural pesticides and metals released from the Aznalcóllar tailings dam through the evaluation of CbE and AChE inhibition (Vioque-Fernandez et al. 2007a, 2008). However, there are no data on *P. clarkii* about the *in vivo* responses of BChE, CbE and other biomarkers for pesticide exposure under controlled conditions.

OP and CM toxicity go well beyond brain AChE inhibition, including generation of reactive oxygen species (ROS) (Bagchi et al. 1995, Gultekin et al. 2000, Gultekin et al. 2001), but further information on factors regulating their effects is needed (Akturk et al. 2006). In rats, chlorpyrifos increases lipid peroxidation and affects antioxidant defences (Bebe & Panemangalore 2003), including catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH), parameters that are well known to respond to contaminants in different organisms (Lopez-Barea & Pueyo 1998). As OPs are biotransformed by cytochrome P450 and subsequently bound to GSH by glutathione *S*-transferases (GST) (Fujioka &

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Casida 2007) exposure to pesticides could alter the levels of these systems. Although these parameters have been used to assess pesticide exposure in invertebrates (Lionetto et al. 2003), their use as biomarkers is far from well established.

Because of its potential for discovering new/unbiased pollutant biomarkers and establishing toxicity mechanisms, the use of proteomics in ecotoxicology is becoming a new exciting approach for exposure and effect assessment of contamination (Snape et al. 2004, Lopez-Barea & Gomez-Ariza 2006, Dowling & Sheehan 2006, Monsinjon & Knigge 2007). Altered protein expression signatures (PESs) show early pathological stages or exposure to stress. As they are specific for the type and extent of stress, they are useful as exposure/effect biomarkers (Shepard & Bradley 2000, Shepard et al. 2000). The proteomic approach allows the study of exposure to different chemicals/doses or to complex mixtures, and also the assessment of the status of organisms from natural ecosystems and even the development of new biomarkers, from traditional assays to proteomic panels (Clarke et al. 2003, Oberemm et al. 2005). As altered PES patterns are used as multimarkers, to diagnose adverse effects there is no need to identify any altered proteins (Shepard & Bradley 2000, Petricoin et al. 2002, Baker 2005), a challenging task in ecotoxicological studies, in which species of unsequenced genomes/proteomes are used as bioindicators (Barrett et al. 2005, Lopez-Barea & Gomez-Ariza 2006, Monsinjon et al. 2006) thus seriously hindering their identification.

Here we report the *in vivo* effects on AChE and CbE activities of two sublethal concentrations of chlorpyrifos and carbaryl after 2 and 7 days of exposure, and 7 days after pesticide removal. Other well-established biomarkers related to biotransformation or oxidative stress were also assessed with conflicting results. Effects of pesticide exposure on protein expression signatures were also studied by two-dimensional gel electrophoresis (2-DE). In addition to further expanding the study of the *in vivo* *P. clarkii* response to OPs and CMs, our study opens up new perspectives in the discovery of new, sensitive, specific and unbiased biomarkers of pesticide exposure and effect.

## Material and methods

### *Chemicals*

Acetonitrile, acetylthiocholine, carbaryl (Pestanal®, 99.5%), 1-chloro-2,4-dinitrobenzene (CDNB), chlorpyrifos (Pestanal®, 99.8%), 5,5-dithiobis-2-dinitrobenzoic acid (DTNB), dithiothreitol (DTT), 7-ethoxy-resorufin, ethylenediaminetetraacetic acid (EDTA), GSH, oxidized glutathione (GSSG), hydrogen peroxide, iodoacetamide, monobromobimane (mBBr), phenylmethanesulfonylfluoride (PMSF), phenylthioacetate, bovine seralbumin, reduced β-nicotinamide-adenine-dinucleotide phosphate (NADPH), resorufin and 2- thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, glycine, Immobiline DryStrips®, Pharmalyte 3–10, sodium dodecyl sulfate (SDS), thiourea, Tris aminomethane (Tris) and urea were from Amersham GE Healthcare (Amersham, UK). Duracryl was from Genomic Solutions® Ltd. (Huntingdon, UK). Bradford reagent and mineral oil were from Bio-Rad (Hercules, CA, USA). Analytical quality chemicals and Milli-Q water (Millipore®) were used throughout.

### *Exposure experiments*

*P. clarkii* crayfish (from Seafood S.L., Villafranco, Seville, Spain), were treated in three 100-l tanks, divided into 21 compartments with plastic mesh walls, using 20 animals per tank, 1 per partition to avoid cannibalism. Exposures were performed in an isothermal room kept at 15°C with a 12 h light/12 h dark photoperiod. Membrane pumps immersed in the extra tank partition assured water circulation, oxygenation and pesticide distribution. Water (controls and contaminated tanks) was changed every 2 days, and Tetra® fish feed was provided daily *ad libitum*. Tanks were filled with 30 l water, so animals were partly immersed in keeping with their aquatic–terrestrial habits. Chlorpyrifos exposure was carried out in August 2004 and that of carbaryl in January 2005.

In each experiment, one tank was used as a vehicle-exposed control and the other two received chlorpyrifos or carbaryl at low (12 μg $l^{-1}$ or 1 mg $l^{-1}$, respectively) or moderate (24 μg $l^{-1}$ or 2 mg $l^{-1}$) concentrations, based on the published $LC_{50}$ (72–96 h) for *P. clarkii*, 24 μg $l^{-1}$ and 2 mg $l^{-1}$, respectively (Cebrian et al. 1992, Muncy & Oliver 1963). The weight (g) of animals in each tank (media ± SD) was: 22.4 + 4.7 (control animals; OP experiment), 22.7 ± 4.8 (12 μg $l^{-1}$ OP), 21.2 ± 4.6 (24 μg $l^{-1}$ OP), 26.5 ± 3.3 (control animals, CM experiment), 26.1 ± 3.0 (1 mg $l^{-1}$ CM) and 25.9 ± 3.2 (2 mg $l^{-1}$ CM). The effect of temperature on exposure was first optimized in search of sublethal conditions. At 25°C, there was high mortality (± 5 crayfish/day) and a rising water pH. At 10°C although there was no mortality the animals had a low metabolic rate and slow movements. The optimal temperature was 15°C, as no mortality or pH change, and normal motility, were found at 7 days. After 2 and 7 days of exposure to pesticides, five crayfish were collected per tank. According to the EXTOXNET website (http://extoxnet.orst.edu) the half-life of carbaryl at neutral water pH and 25°C is 10 days, while the half-life

for chlorpyrifos can vary from 35 to 78 days. Because the treatments were changed every 2 days, we consider that there was no substantial pesticide degradation during the exposure time. After pesticide exposure, residual pesticides were cleaned from the tank and the remaining animals were maintained for 7 additional days to assess their possible recovery. At each time and condition, digestive gland, gills and nervous tissue were excised and frozen in liquid $N_2$.

### *Sample preparation, enzyme assays and protein quantification*

AChE and CbE were assessed in the nervous tissue and digestive glands, respectively. Tissues were disrupted in 4 vol 0.1 M Tris–HCl, pH 8.0, centrifuged (Beckman J2-21) for 30 min at 30 000*g* and the supernatant frozen at -80°C. Activity was assayed in the above described buffer following (412 nm, Beckman DU650) the release of thiol-derivatives from 1 mM DTNB (Ellman et al. 1961). Assays started by adding 10 mM acetylthiocholine/4.5 mM phenylthioacetate for AChE/CbE, respectively, conditions in which no substrate inhibition was detected, as previously reported (Vioque-Fernandez et al. 2007b). Triplicate assays were made in pools of the five nervous tissues and in each of the five digestive glands per condition. To assay for possible reactivation, extracts were diluted 50-/30-fold in disruption buffer and 1 mg $ml^{-1}$ ovoalbumin, or 1/0.5 mM 2-pyridinealdoxime iodide (2-PAM) for CbE and AChE, respectively, for 2 h (Vioque-Fernandez et al. 2007b). Specific activities are shown as U mg $protein^{-1}$.

For CAT, GPx, GST and ethoxyresorufin-*O*-deethylase (EROD) assays, digestive glands were individually disrupted in 4 vol of 50 mM Tris–HCl, pH 7.4, containing 1 mM EDTA and 1 mM PMSF. Aliquots of 250 µl were taken for malondialdehyde (MDA) analysis, and the remaining extract was spun at 9000*g* for 20 min. Supernatants were re-centrifuged for 1 h at 105 000*g* (Beckman Optima TLX) to obtain cytosolic (CAT, GPx and GST) and microsomal fractions (EROD, resuspended in 0.1 M phosphate, pH 7.5, 20% glycerol, 0.1 mM EDTA), that were stored at –80°C. CAT was assayed following $A_{240}$ decrease of $H_2O_2$ (Beutler 1975). GPx was assayed following $A_{340}$ decrease during GSSG-linked NADPH oxidation using cumene hydroperoxide (Sies et al. 1979). GST was assayed following $A_{340}$ increase using GSH and CDNB as substrates (Keen et al. 1976). EROD was assayed following fluorimetrically ($\lambda_{ex}$ 537, $\lambda_{em}$ 583) resorufin release from 7-ethoxyresorufin (Fernandes et al. 2002). Thus, 10 µl microsomes were incubated at 30°C in 0. 5 ml 80 mM phosphate, pH 7.4, 3.3 µM 7-ethoxyresorufin and 0.2 mM NADPH and the fluorescence measured after 1 h. Enzymatic data are expressed as U $mg^{-1}$ or protein; one enzyme unit is defined by µmol of substrate consumed or product formed per min (pmol $min^{-1}$ for EROD activity). MDA was measured by high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) radiation detection (de Almeida et al. 2004), GSSG and GSH by HPLC coupled to electrochemical detection (Rodriguez-Ariza et al. 1994), metallothionein (MT) by HPLC coupled to fluorescent detection (Alhama et al. 2006) and protein by dye-binding (Bradford 1976).

### *Protein expression analysis*

Extracts were prepared in vehicle-exposed crayfish and in those exposed for 7 days to low and moderate concentrations of each pesticide. Proteins (100 µg) were incubated for 30 min in 350 µl final volume of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 0.5% Pharmalyte 3–10) and loaded on Immobiline DryStrips (pH 4–7, 18 cm). After 6 h passive/6 h active (50 V) rehydration, isoelectric focusing (IEF) was carried out (20°C, 50 mA/strip) in a BioRad IEF cell in six steps: 500 V (90 min), 1000 V (90 min), 2000 V (90 min), 4000 V (90 min), 8000 V (120 min) and 8000 V, until reaching 60 000 Vh (~4 h). IPG-strips were soaked for 15 min in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS containing 50 mM DTT, and again in this buffer with 250 mM iodoacetamide. Strips were loaded on 12.5% SDS-PAGE gels and separated simultaneously at 20°C in a BioRad Dodecacell at 2.5 W/gel (10 min) and 15 W/gel until separation end (~5 h). After silver staining (Mortz et al. 2001), images of four replicate gels/condition were obtained in a GS-800 densitometer (BioRad) and analysed with PDQuest software (V7.1; BioRad), once normalized by the total intensity of all the matched spots in the experiment. The intensity (in arbitrary U) of each spot is shown as mean ± SE of the four gels per group of animals. Protein spots were individually analysed in all gels simultaneously in order to verify their intensities, so allowing the identification of specific spots with significant differential expression. Significantly altered proteins were chosen only if their profiles were repeated in at least three of the four replicates.

### *Statistical analysis*

Statistical significance compared with controls, was determined by the Dunnett test using the Instat™ software (Graphpad, San Diego, CA, USA), and was shown as $p < 0.05$. Spots with significant differences ($p < 0.001$) in the Student's *t*-test and with at least threefold over/underexpression among conditions were included in the altered PESs. Normality of data and homogeneity of variance had been previously checked

## Results

### In vivo *effects of chlorpyrifos and carbaryl on* P. clarkii esterases

Figure 1 shows esterase inhibition by chlorpyrifos. CbE activity was inhibited at both times in a concentration-dependent mode, near totally at 7 days with moderate level. Although AChE increased at 2 days with low exposure, it was also inhibited at 7 days in a concentration-dependent mode. One week after OP removal was not enough to recover the initial CbE or AChE activities, which remained below the control and $T_0$ values. Carbaryl (CM) had less inhibitory effects than chlorpyrifos against both esterases (Figure 2): CbE was inhibited at 7 days with both concentrations, but not at 2 days, and AChE was unaffected. In contrast to the lack of recovery from OP, significant CbE and AChE increases were found after carbaryl removal, but it should be mentioned that this significant increase would be due to a decrease in esterase activities in the control animals.

Extracts of crayfish exposed to chlorpyrifos or carbaryl were diluted or treated with 2-PAM to test for possible differences in the reactivation process, although significant reactivation of phosphorylated or carbamylated esterase activities was not observed in either case (data not shown).

### In vivo *effects of chlorpyrifos and carbaryl on conventional pollution biomarkers*

Eight biomarkers, related to biotransformation or oxidative stress, were also assessed in pesticide-exposed crayfish. Figure 3 shows the effect of chlorpyrifos on CYP1A and antioxidant enzyme activities. EROD activity decreased at 2 days with low exposure, remaining below $T_0$ level at 7 days and after pesticide removal. In contrast, GST (phase II) increased significantly at 2 days with the higher concentration, recovering near-initial values after pesticide removal. CAT activity (antioxidant) had a puzzling behaviour: it decreased at 2 and 7 days with the low concentration and remained low after pesticide

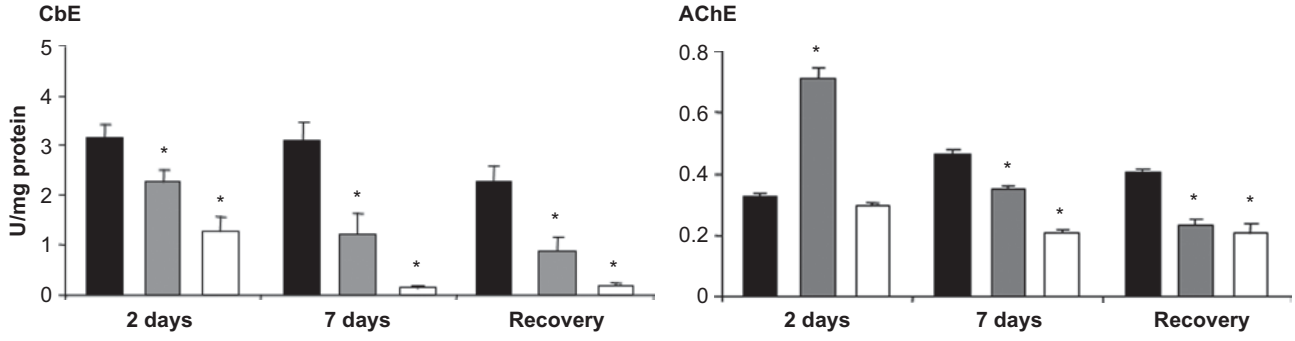



**Figure 1.** Digestive gland carboxylesterase (CbE) and nervous tissue acetylcholinesterase (AChE) levels in *Procambarus clarkii* exposed to low (▒) and moderate (□) chlorpyrifos concentrations for 2 and 7 days, and 7 days after pesticide removal, and compared with controls at each time point (■). Initial CbE and AChE levels were 3.84 ± 0.73 and 0.30 ± 0.01 U $mg^{-1}$ protein, respectively. Data are means ± SEM from five crayfish (CbE) and three replicates of five pooled crayfish (AChE). Statistical significance vs the respective control group is shown as $^*p < 0.05$.

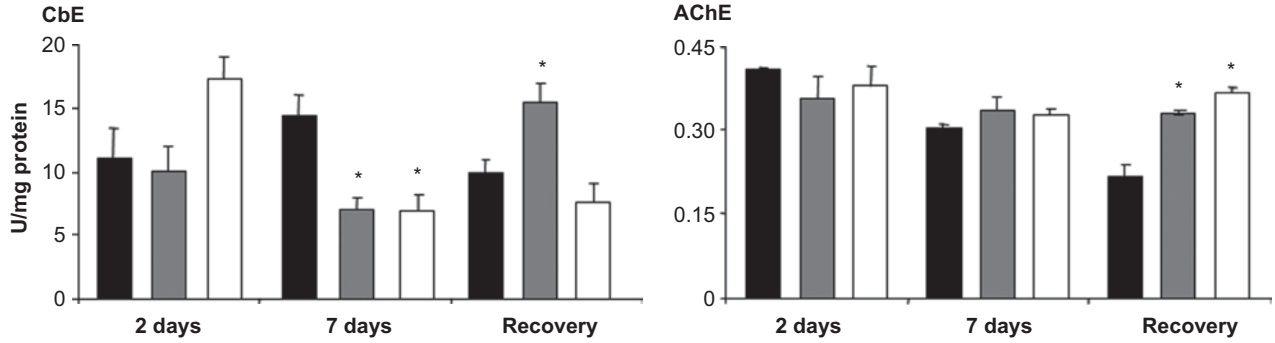
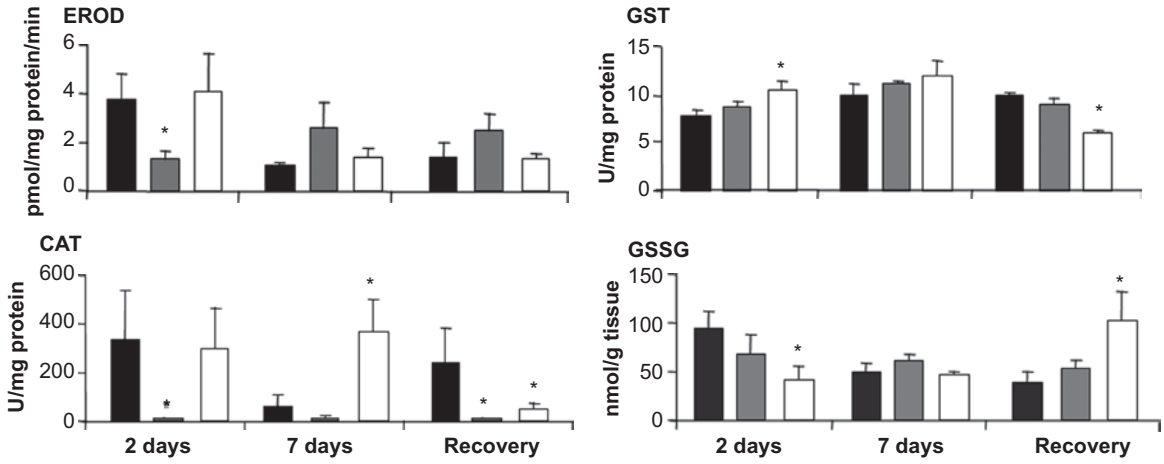



**Figure 2.** Digestive gland carboxylesterase (CbE) and nervous tissue acetylcholinesterase (AChE) levels in *Procambarus clarkii* exposed to low (▒) and moderate (□) carbaryl concentrations for 2 and 7 days, and 7 days after pesticide removal, and compared with controls at each time point (■). Initial CbE and AChE activities were 16.2 ± 1.73 and 0.41 ± 0.01 U $mg^{-1}$ protein, respectively. Data are means ± SEM from five crayfish (CbE) and three replicates of five pooled crayfish (AChE). Statistical significance vs the respective control group is shown as $^*p < 0.05$.




| | 2 DAYS | | | 7 DAYS | | | RECOVERY | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 1/2 LC50 | LC50 | Control | 1/2 LC50 | LC50 | Control | 1/2 LC50 | LC50 |
| **GSH** nmol/gr tissue | 1624 ±120 | 706 ±188 | 1375 ±239 | 1543 ±71.4 | 1790 ±294 | 1193 ±240 | 1155 ±258 | 1588 ±186 | 1380 ±194 |
| **MDA** nmol/gr tissue | 300 ±17.0 | 269 ±37.2 | 276 ±239 | 310 ±38.7 | 318 ±22.4 | 329 ±100 | 304,68 ±27.64 | 344 ±9.58 | 291 ±26.3 |
| **GPx** U/mg protein | 0.90 ±0.13 | 1.04 ±0.14 | 0.98 ±0.20 | 1.02 ±0.10 | 1.14 ±0.09 | 1.07 ±0.06 | 1.17 ±0.12 | 1.13 ±0.11 | 0.91 ±0.07 |
| **MTx** nmol/mg protein | 19.9 ±2.23 | 19.6 ±1.34 | 20.4 ±3.31 | 23.5 ±3.89 | 22.4 ±1.07 | 21.7 ±2.03 | 22.4 ±3.19 | 20.8 ±1.02 | 23.0 ±1.51 |

**Figure 3.** Response of conventional pollution biomarkers in digestive glands of *Procambarus clarkii* exposed to low (▒) and moderate (□) chlorpyrifos concentrations for 2 and 7 days, and 7 days after pesticide removal, and compared with controls at each time point (■). Initial ethoxyresorufin-*O*-deethylase (EROD), glutathione *S*-transferase (GST) and catalase (CAT) activities and oxidized glutathione (GSSG) levels were 3.1 ± 1.1 pmol $mg^{-1}$, 7.08 ± 0.53 U $mg^{-1}$ protein, 420 ± 192 U $mg^{-1}$ protein, 50.6 ± 24.3 nmol $g^{-1}$, respectively. The table inserted below shows glutathione peroxidase (GPx) activity and reduced glutathione (GSH), malondialdehyde (MDA) and metallothionein (MT) levels in the same experiment. Data are means ± SEM from five crayfish. Statistical significance vs the respective control group is shown as $^*p < 0.05$.

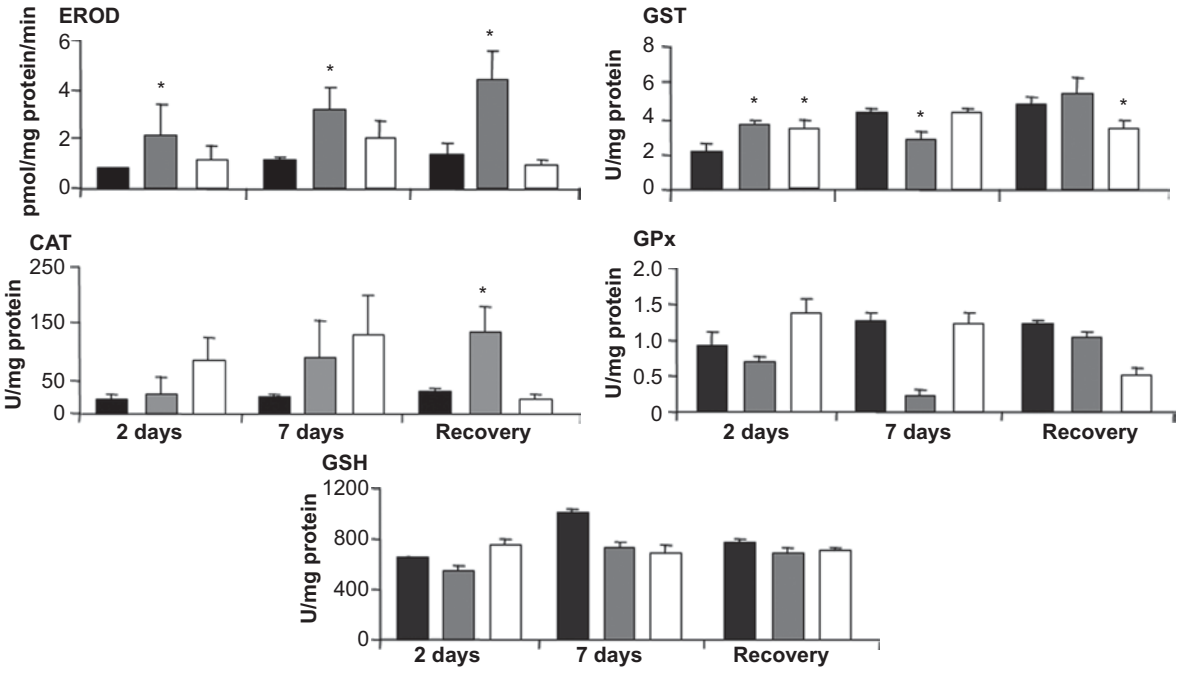


removal, but had near-initial values at the moderate concentration. CAT activity decreased significantly after 7 days in control animals compared with values observed after 2 days, and increased again after 7 additional days (pesticide removal), and this should be also be accounted for in the differences observed between the controls and treatments. Similar control variation was observed for EROD activity. GSSG decreased in a concentration-response mode at 2 days, significantly at the moderate exposure, and after pesticide removal rose again to a moderate concentration. Lower, but not significant, GSH values were observed at the moderate exposure, and higher MDA was usually found, although again the increase was not significant. GPx and MTs levels remained unaltered.

Figure 4 shows carbaryl responses. Both biotransformation activities increased significantly, EROD only at the low exposure at 2 and 7 days of exposure and also after pesticide removal, while GST increased at both concentrations after 2 days only. After 7 days of exposure to lower the carbaryl concentration and 7 recovery days from the higher concentration, GST decreased. CAT activity had a concentration-dependent increase, although only significant at low exposure after carbaryl removal. GPx decreased at low exposure, significantly at 7 days. GSH diminished at both concentrations at 7 days compared with $T_0$ and to the vehicle-exposed control in that time. GSSG, MDA and MTS levels remained mainly unaltered.

### *Alterations of protein expression profiles*

Protein expression signatures were studied in gills and in nervous tissue, after optimizing conditions for protein extraction and 2-DE analysis. Alteration of PES was analysed in four replicate gels after 7 days of pesticide exposure in three conditions: vehicle-exposed control, and crabs exposed to low and moderate concentrations. Figure 5 shows representative 2-DE gels of soluble proteins from gills (A) and nervous tissue (B). These two proteomes displayed 2000 and 2100 well-resolved spots, respectively, of 4–7 pI and 14–150 kDa Mr. Both organs had clearly different patterns, especially in the areas marked with roman numbers in the figures. After pesticide exposure, a total of 107 spots had significant intensity differences ($p < 0.001$). Table 1 summarizes the 35 spots with major intensity changes selected as specific PES for each treatment and tissue. Nine spots (36,




| | 2 DAYS | | | 7 DAYS | | | RECOVERY | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 1/2 LC50 | LC50 | Control | 1/2 LC50 | LC50 | Control | 1/2 LC50 | LC50 |
| **GSSG** nmol/gr tissue | 59.3 ±10.9 | 70.5 ±10.9 | 85.0 ±18.8 | 98.5 ±18.7 | 69.3 ±4.05 | 141 ±27.7 | 82.9 ±25.0 | 82.1 ±10.8 | 106 ±15.6 |
| **MDA** nmol/gr tissue | 666 ±176 | 498 ±127 | 1468 ±844 | 1296 ±378 | 672 ±402 | 1237 ±759 | 767 ±363 | 411 ±30.9 | 579 ±151 |
| **MTs** nmol/mg protein | 21.4 ±1.63 | 22.0 ±1.23 | 27.2 ±2.15 | 24.9 ±1.72 | 24.5 ±2.34 | 20.1 ±4.93 | 21.3 ±3.06 | 25.5 ±3.71 | 21.1 ±276 |

**Figure 4.** Response of conventional pollution biomarkers in digestive glands of *Procambarus clarkii* exposed to low (▬) and moderate (□) carbaryl concentrations for 2 and 7 days, and 7 days after pesticide removal, and compared with controls at each time point (■). Initial ethoxyresorufin-*O*-deethylase (EROD), glutathione *S*-transferase (GST) and catalase (CAT) and glutathione peroxidase (GPx) activities and oxidized glutathione (GSH) levels were 0.47 ± 0.18 pmol $mg^{-1}$, 1.93 ± 0.49 U $mg^{-1}$ protein, 12.2 ± 4.13 U $mg^{-1}$ protein, 0.87 ± 0.18 U $mg^{-1}$ protein, 806 ± 86.6 nmol g protein, respectively. The table inserted below shows the GSSG, malondialdehyde (MDA) and metallothionein (MT) levels in the same experiment. Data are means ± SEM from five crayfish. Statistical significance vs the respective control group is shown as $^*p < 0.05$.

**Table 1.** Spots selected in *P. clarkii* as specific PES for chlorpyrifos or carbaryl exposure in gills and nervous tissue, respectively. Numbers correspond to those indicated in Fig. 5 A/B.

| Pesticide | Gills | Nervous tissue |
|---|---|---|
| **Chlorpyrifos** | 36, 44, 48, 49, 53, 55, 57, 59, 61 (***N* = 9**) | 64, 65, 71, 72, 73, 81, 86, 87, 89, 106, 107 (***N* = 11**) |
| **Carbaryl** | 1, 6, 8, 9, 10, 13, 17 (***N* = 7**) | 21, 22, 25, 26, 27, 28, 31, 32, 33, 34, 35 (***N* = 11**) |

**Table 2.** Number of spots showing significantly altered expression in *P. clarkii* after 7 days exposure to chlorpyrifos or carbaryl.

| | | Organ/Tissue | | | | | |
|---|---|---|---|---|---|---|---|
| Pesticide | Effect on expression | Gill | | Nervous tissue | | Partial nº | Total nº |
| **Chlorpyrifos** | Over | 22 | (27) | 24 | (45) | 46 | 72 |
| | Under | 5 | | 21 | | 26 | |
| **Carbaryl** | Over | 8 | (18) | 13 | (17) | 21 | 35 |
| | Under | 10 | | 4 | | 14 | |
| | | | | | | | 107 |

44, 48, 49, 53, 55, 57, 59, 61) were chosen as chlorpyrifos-specific PES in gills and eleven (64, 65, 71, 72, 73, 81, 86, 87, 89, 106, 107) in the nervous tissue. Seven totally different spots were included in the carbaryl-specific PES in gills (1, 6, 8, 9, 10, 13, 17) and another eleven spots (21, 22, 25, 26, 27, 28, 31, 32, 33, 34, 35) in the nervous tissue.

Table 2 shows the number of over- and underexpressed proteins detected in each organ after 7 days of exposure to each pesticide. Two-thirds of the proteins, 72 of 107, were significantly altered by chlorpyrifos, whose effects dominated in the nervous tissue, where 45 proteins were altered versus only 27 in gills. The remaining third of the proteins (35) were affected by carbaryl, although they were evenly distributed between both tissues, 17 altered in the nervous tissue and 18 in gills. Most of the proteins with an altered intensity, 67, were upregulated,

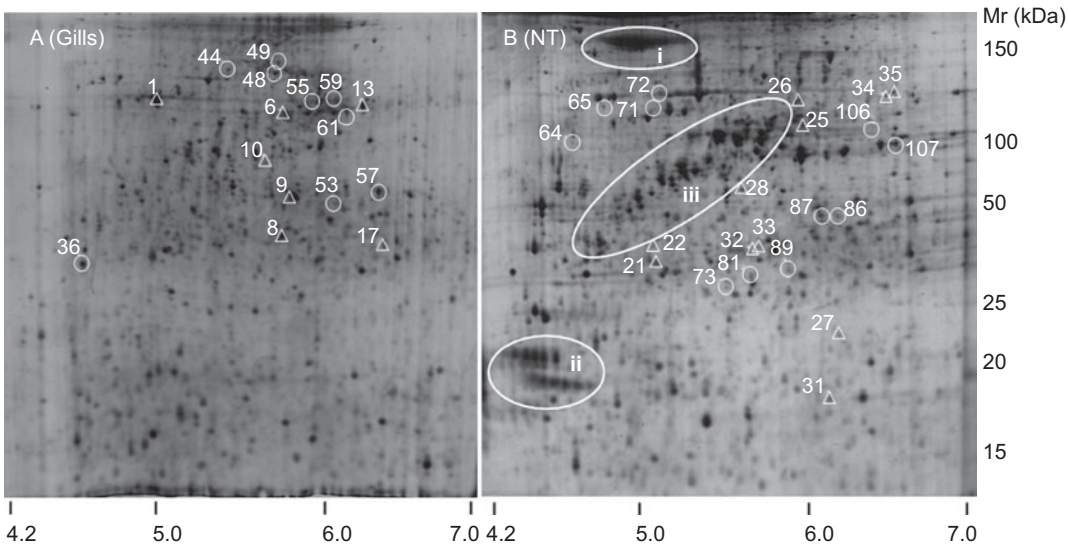



**Figure 5.** Representative two-dimensional gel electrophoresis (2-DE) of soluble proteins from gills (A) and nervous tissue (NT) (B) of *Procambarus clarkii*. Symbols indicate the spots with significant intensity differences ($p < 0.001$) after exposure for 7 days to low and moderate chlorpyrifos (○) or carbaryl (Δ) concentrations. Areas (i), (ii) and (iii) mark clearly different patterns in nervous tissue.

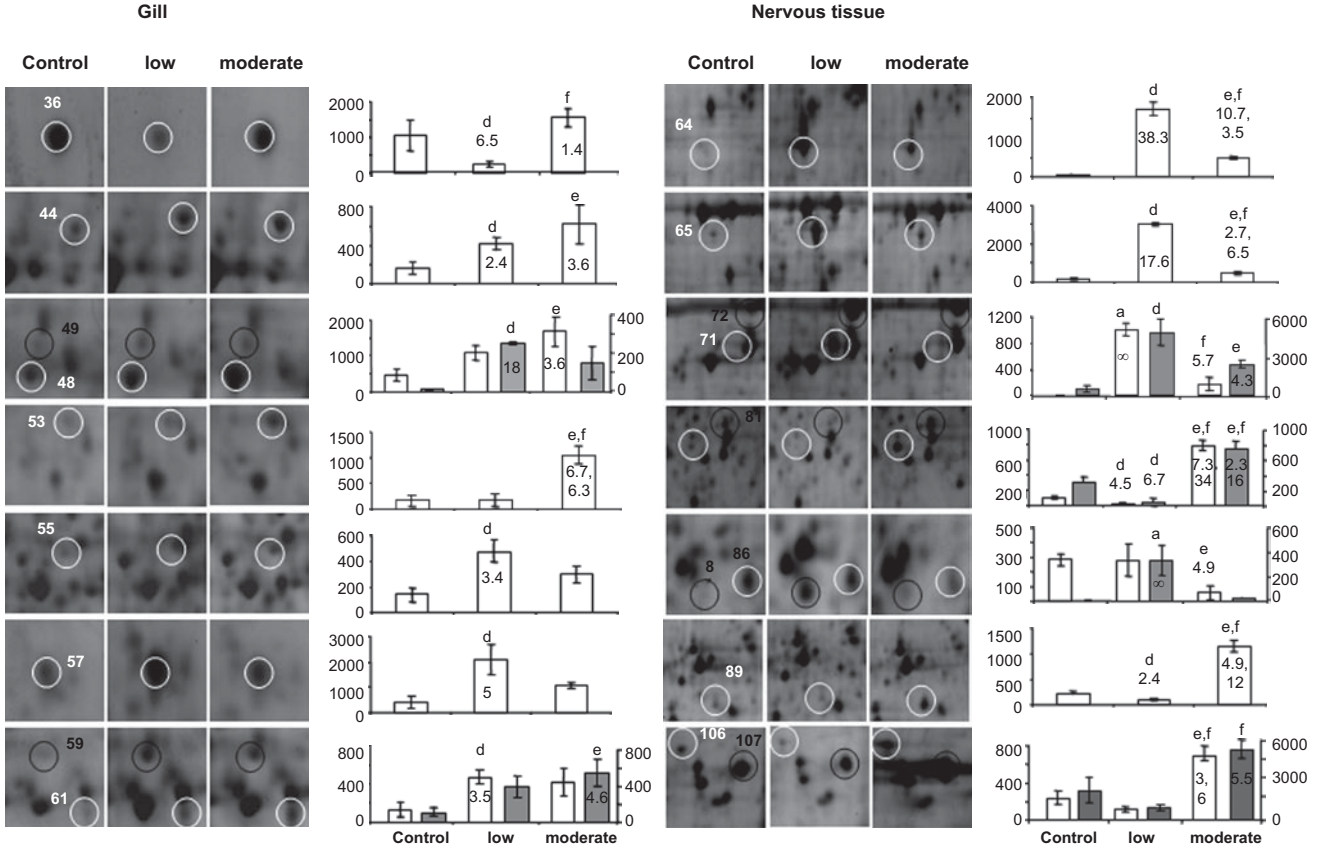



**Figure 6.** Proteins with major intensity alterations in gills/nervous tissues of *Procambarus clarkii* after 7 days of exposure to chlorpyrifos. Significant statistical differences ($p < 0.001$) are shown as: *Qualitative*, ***a*** control vs low concentration, and ***c*** low vs moderate concentration. *Quantitative*, ***d*** control vs low concentration, ***e*** control vs moderate concentration, and ***f*** low vs moderate concentration.

46 by chlorpyrifos and 21 by carbaryl. The remaining 40 proteins were downregulated, 26 by chlorpyrifos and 14 by carbaryl. Of the 107 spots altered, only 23 had qualitative changes, i.e. they were present in one condition (vehicle-exposed control, or animals exposed to low or moderate concentration) but absent in another or in

both the other conditions. Most changes, 84 out of 107, were quantitative, i.e. spots with over threefold intensity deviations; the vast majority of them were upregulated compared with the unexposed controls.

Figure 6 shows close-up views of proteins with the most intense alterations in gills and nervous tissue at 7 days without or with low or moderate chlorpyrifos levels. The histograms show the spot intensity in each condition, its variation pattern and fold-number change, and its sorting into six types of qualitative or quantitative classes. Nine spots were altered in gills and 11 in nervous tissue. Nineteen of the 20 spots were overexpressed: three (44, 48, 59) in a dose-dependent mode; nine (49, 55, 57, 61, 64, 65, 71, 72, 87) only at a low chlorpyrifos dose and seven (36, 53, 73, 82, 89, 106, 107) only at the moderate dose. Only three proteins were underexpressed, two (73, 82) at low, and one (86) at moderate dose.

The analysis was also made in gills and nervous tissue after 7 days of exposure to carbaryl (Figure 7). Seven of the 18 proteins were altered in gills and 11 in nervous tissue. Compared with the controls, 12 spots (9, 10, 13, 21, 25, 26, 28, 31, 32, 33, 34, 35) were overexpressed and six (1, 6, 8, 17, 22, 27) underexpressed. Six spots (13, 25, 26, 31, 32, 33) were over- and one underexpressed (27) in a dose-dependent mode. Another ten were over- (9, 10, 28, 34, 35) or underexpressed (1, 6, 8, 17, 22) after exposure to a low carbaryl concentration, and another five spots were up- (6, 10) or downregulated (8, 21, 34) following exposure to moderate carbaryl concentrations.

## Discussion

### *Effects on esterase activities*

Assessment of esterase inhibition is the classical approach to monitoring environments probably polluted by pesticides (Hyne & Maher 2003). However, there are few studies reporting the pesticide effects on *P. clarkii* biomarkers (Escartín & Porte 1996a, b, Porte & Escartín 1998, Vioque-Fernandez et al. 2007). In the present work, chlorpyrifos and carbaryl effects were assessed under sublethal conditions in three steps: (1) esterase inhibition, (2) changes in biomarkers involved in biotransformation or antioxidant defences, and (3) variation in protein expression profiles.

Chlorpyrifos extensively inhibited CbE, usually in a dose-response mode, but not AChE, which remained at an increased level after 2 days of exposure. A higher AChE synthesis to remove the acetylcholine accumulated during its previous inhibition could explain this increase,

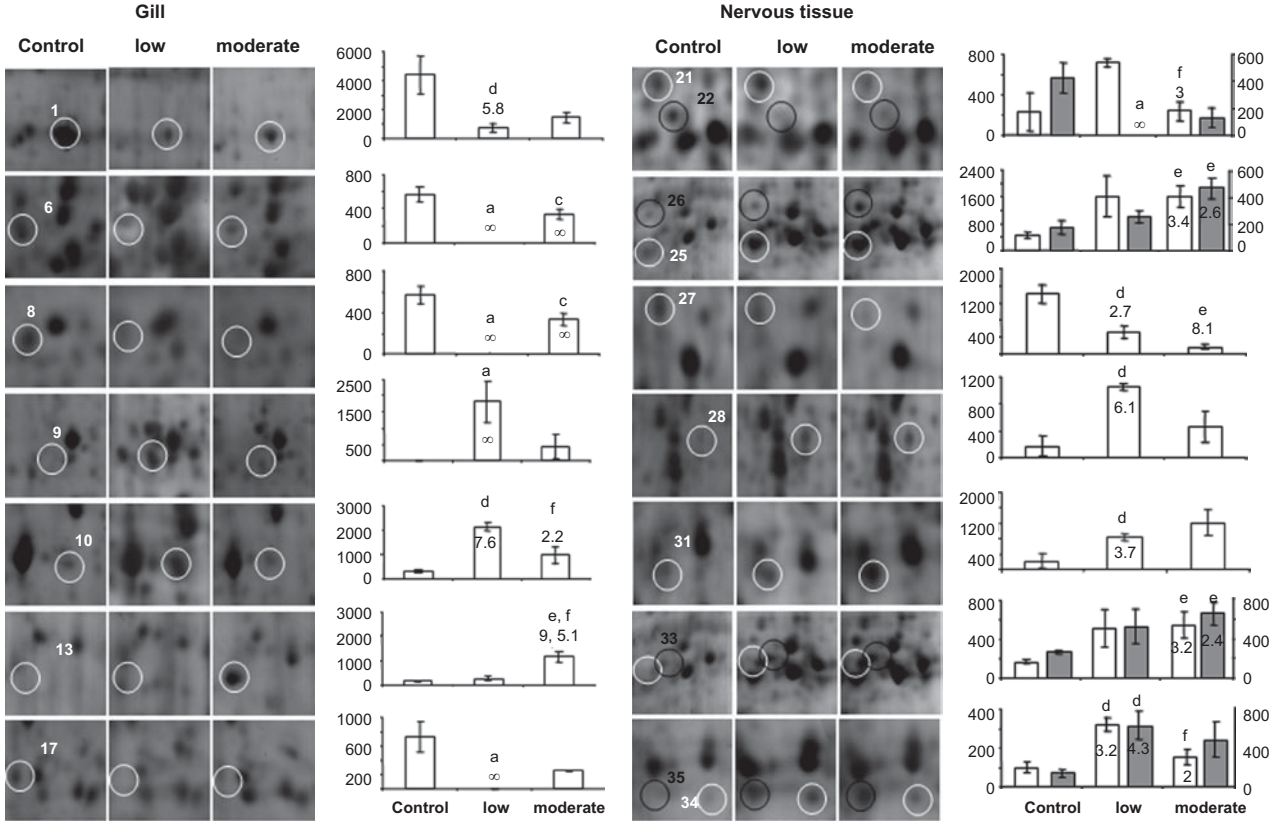



**Figure 7.** Proteins with major intensity differences in gills/nervous tissues of *Procambarus clarkii* after 7 days of exposure to carbaryl. Significant statistical differences ($p < 0.001$) are shown as: *Qualitative*, ***a*** control vs low concentration, ***b*** control vs moderate concentration, and ***c*** low vs moderate concentration. *Quantitative*, ***d*** control vs low concentration, ***e*** control vs moderate concentration, and ***f*** low vs moderate concentration.

as shown by the effects of metals on this enzyme (Bainy et al. 2006). Carbaryl was less effective than chlorpyrifos in causing esterase inhibition, as only CbE was inhibited after 7 days, and agrees with the higher doses tested for carbaryl (1 and 2 mg $l^{-1}$) compared with chlorpyrifos concentrations (12 and 24 µg $l^{-1}$).

Our work also confirms CbE as the preferred biomarker for OP exposure compared with AChE, as shown in *P. clarkii* in real ecosystems (Vioque-Fernandez et al. 2007a, 2008). Some other studies also demonstrated that CbE activity is often a more sensitive biomarker to OP exposure than ChE activity. The cladoceran *Daphnia magna* shows a higher CbE activity and a greater sensitivity to inhibition by OPs than AChE activity (Barata et al. 2004). The same response for CbE activity was also reported in mussels, *Mytilus galloprovincialis* (Escartin & Porte 1997) and *M. edulis* (Galloway et al. 2002). In a field survey, Fourcy et al. (2002) also found that CbE activity of the polychaete *Nereis diversicolor* sampled in an area treated with the pesticide Abate 500E (containing temephos as the active ingredient) was more inhibited than the AChE activity. Indeed, Wogram et al. (2001) found that *in vitro* inhibition of *Gasterosteus aculeatus* liver CbE activity was more pronounced ($IC_{50} = 5.6 \times 10^{-7}$ M) than brain ($IC_{50} = 7.5 \times 10^{-6}$ M) or muscle ($IC_{50} = 9.4 \times 10^{-6}$ M) AChE inhibition. An *in vivo* experiment carried out by Küster (2005) and using whole embryo homogenates of zebrafish (*Danio rerio*) also showed a higher sensitivity of CbE activity to OP exposure.

In contrast to the CbE and AChE reactivation by dilution or 2-PAM treatment after *in vitro* exposure of *P. clarkii* extracts to OPs and CMs (Vioque-Fernandez et al. 2007b), the lack of reactivation detected in the present study after *in vivo* exposure agrees with a similar absence observed in crayfish from pesticide-exposed sites near Doñana (Vioque-Fernandez et al. 2007a, 2008). Nevertheless, Escartín & Porte (1996a) reported a slow esterase recovery in muscle of *P. clarkii* exposed to fenitrothion after oxime treatment. The increase of AChE and CbE activities after carbaryl removal, but not of chlorpyrifos, agrees with the studies in aquatic crustacean esterases that took from 2 to > 4 weeks to recover to control levels after OP removal (Barata et al. 2004). Other factors might affect esterase reactivation or recovery in exposed crayfish, including 'aging' and/or degradation of inhibited enzymes, which would require a synthesis of new enzyme molecules.

Also, the presence of free active pesticides in the crayfish tissues and fluids could account for a slow recovery of OP-inhibited esterase activity or an absence of oxime-induced reactivation, because re-inhibition could take place when the inhibited esterase is spontaneously or chemically (2-PAM) released from the inhibitor. The study by Escartin & Porte (1996a, b) shows that residues of fenitrothion still persist in the muscle of *P. clarkii* during the first 7 days of transferring the animals to clean water.

### *Effects on biotransformation and antioxidant enzymes*

As OPs and CMs cause severe physiological alterations to other biochemical systems (Akturk et al. 2006), effects on other biomarkers were also studied in pesticide-exposed *P. clarkii*. Several biomarkers involved in phase I or II biotransformation, antioxidative defence and oxidative damage were altered, although chlorpyrifos and carbaryl elicited differential responses. Biotransformation enzymes, including cytochrome P450-linked EROD (phase I) and GST (phase II) activities, increased in general during exposure, in agreement to previous reports showing that OPs and CMs are biotransformed by cytochrome P450 and later conjugated by GSTs (Jokanovic 2002, Tang et al. 2002, Usmani et al. 2004, Fujioka & Casida 2007). Alterations in antioxidative defences and oxidative damage to lipids and glutathione were difficult to explain as they differed from our expectations or were contradictory: chlorpyrifos exposure paralleled low CAT, low (not high) GSSG and unaltered MDA levels, and carbaryl exposure with high CAT and unaffected GSSG or MDA levels. Thus, while significant alterations were visible in pesticide-exposed *P. clarkii*, agreeing with variations in antioxidant defences in rat liver, kidney and spleen (Bebe & Panemangalore 2003), no cause–effect relationships could be established. A poor fit between responses of these general biomarkers, pesticide doses and exposure times indicates that, contrary to esterase inhibition, these systems are not adequate biomarkers for OP and CM exposure under the conditions tested.

### *Proteomic responses*

Conventional biomarkers are suggested *a priori* by their known biological roles and are somewhat biased in pollution assessment. In contrast, proteomics is a powerful tool in ecotoxicology, with a high potential for identifying novel biomarkers and for gaining insight into pollutant toxicity mechanisms (Dowling & Sheehan 2006, Lopez-Barea & Gomez-Ariza 2006, Monsinjon & Knigge 2007). However, a proteomic disadvantage in ecotoxicological studies nowadays is to know what this overexpression or underexpression of unspecific proteins means for individual health. At the moment, changes in the protein expression would be considered as biomarkers of exposure only.

Environmental proteomic studies started when unique protein expression signatures were described after 2-DE analysis of pollutant-exposed mussels and fish (Shepard & Bradley 2000, Shepard et al. 2000, Bradley et al. 2002).

Later, we showed that 15 proteins were altered in clams exposed to model pollutants, and four were unambiguously identified by *de novo* sequencing and related to cytoskeletal structure or function (Rodriguez-Ortega et al. 2003). In contrast to the early studies on non-model organisms, and the ensuing studies in model organisms, there is still little research on animals from natural ecosystems. In polycyclic aromatic hydrocarbon (PAH)- and metal-polluted mussels several induced peptides were identified by surface-enhanced laser desorption/ionization–time of flight–mass spectrometry (SELDI-TOF-MS) (Knigge et al. 2004). Contamination of Doñana National Park (SW Spain) by pesticides, or by metals released in the Aznalcollar tailings dam spill, has been monitored with the PES approach using mice and crayfish as bioindicators (Lopez-Barea & Gomez-Ariza 2006) and a bivalve at the nearby Guadalquivir estuary (Romero-Ruiz et al. 2006). However, the application of proteomic approaches to ecotoxicology is still at an early stage, due to the under-representation of the usual bioindicators in protein/gene sequence databases, hindering identification of differentially expressed proteins via high-throughput matrix-assisted laser desorption/ionization–time of flight–peptide mass fingerprinting (MALDI-TOF-PMF) analysis (Dowling & Sheehan 2006, Lopez-Barea & Gomez-Ariza 2006, Monsinjon & Knigge 2007). Although this can be circumvented by *de novo* sequencing (Rodriguez-Ortega et al. 2003, Romero-Ruiz et al. 2006), this is an expensive, time-consuming, and highly complex approach for routine biomonitoring, especially when using as a bioindicator non-model species such as *P. clarkii*, totally absent from current sequence databases.

For this reason, in the present study we avoided identifying proteins significantly altered after pesticide exposure, relying on the alteration of PESs specific for each pesticide in two different tissues. We agree that further identification of these PESs will represent an inestimable contribution for the identification of new proteins affected by OPs and CMs, so contributing to a better understanding of the toxicity mechanism elicited by these compounds. However, due to the absence of a protein database on *P. clarkii* and other crustaceans as well, in this work we resolved to focus on establishing PESs that respond specifically to carbaryl and chlorpyrifos, in the concentration studied, after 7 days of exposure.

Protein expression was studied in *P. clarkii* gills and nervous tissue, not in digestive gland, as the high protease activity found in the latter (Teschke & Saborowski 2005), interfered with 2-DE analysis of its extracts, as confirmed by the lack of high Mr proteins observed in this proteome. In contrast, gill and nervous tissue proteomes had over 2000 proteins with quite different patterns, in agreement with their dissimilar structure and functions. The high quality of both proteomes and the puzzling responses of conventional biomarkers prompted us to embark on an unbiased and global search for proteins altered by pesticides. A total of 107 spots had significant intensity differences after chlorpyrifos or carbaryl exposure. Thirty-five spots were sorted into four pesticide- and organ-specific PES, which should be useful for future studies. In fact, the utility of the PES approach was established in seminal studies carried out in bivalves and fish in 2000 (Shepard & Bradley 2000, Shepard et al. 2000, Bradley et al. 2002).

The quantitative analysis of altered proteins shows that chlorpyrifos alters the expression of a two-fold higher number of spots than carbaryl (72 vs 35), in agreement with the greater sensitivity of CbE and AChE to the OP than to the CM. However, it should be mentioned that it is possible that other proteins not present in the gels (not focused on a pH range of 4–7, or with very low expression and then low abundance) would produce a different panorama. So, affirmations on higher or lesser sensitivity to OP or CM are based on the experimental procedure adopted in this work.

Chlorpyrifos affected more extensively nervous tissue than gills (45 spots vs 27). Most altered proteins were upregulated. The time-course of variation of several altered spots was quite similar to the variations observed in some conventional biomarkers assessed, with drastic alterations at low but not at moderate exposure. The similarity between the patterns of conventional biomarkers and of protein alterations might indicate that they are real and not artefactual.

Many proteins with a significantly altered expression in organisms from polluted sites have been related to biotransformation or contaminant-promoted oxidative stress. Thus, in pollutant-exposed bivalves, four cytoskeletal proteins were identified that were oxidized or proteolysed due to contaminant exposure (Rodriguez-Ortega et al. 2003). Two oxidative stress-related enzymes were also identified among the 16 proteins overexpressed in bivalves from metal-polluted sites of Guadalquivir estuary (Romero-Ruiz et al. 2006). An increased number of carbonylated and glutathionylated proteins was also shown in bivalves from contaminated sites or exposed to oxidizing conditions (McDonagh et al. 2005). Recently, in *Mus spretus* from pesticide- and/or metal-polluted sites from the Huelva estuary, we identified several overexpressed proteins related to biotransformation and oxidative stress and showed the emergence of modified forms of 2 pH units, lower pI or truncated, due to oxidative modifications (Montes-Nieto et al. 2007).

We realize that the present study represents some initial steps on future advances that should be developed in the field of ecotoxicology, considering sentinel organisms with no available data on protein databases.

The identification of proteins by current MS procedures can better indicate the involvement of proteins that respond specifically to particular xenobiotics, so increasing the knowledge on toxicity mechanisms, and this remains to be further investigated in *P. clarkii*. In fact, the present work contributes to indicate that there are several proteins specifically and significantly altered in response to carbaryl and chlorpyrifos, which could *per se* indicate a toxic effect and be useful as a PES to assess the effects of these compounds.

In conclusion, under our experimental conditions, *P. clarkii* was more sensitive to chlorpyrifos than to carbaryl, as shown by the higher esterase inhibition, usually in a dose- and time-dependent mode, and a twofold higher number of proteins with drastic intensity differences. Carboxylesterase was the most specific biomarker for pesticide exposure, as it responded to chlorpyrifos and carbaryl. The response of general conventional biomarkers was puzzling and requires further studies to disclose the underlying mechanisms. The usefulness of proteomic approaches for studying the general effects of pesticide exposure has been clearly established, especially by using the alteration of protein expression signatures. Some of these differentially expressed proteins could be used to develop new pesticide biomarkers and to gain insight into their toxicity mechanisms.



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