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Doñana National Park survey using crayfish (*Procambarus clarkii*) as bioindicator: Esterase inhibition and pollutant levels

A. Vioque-Fernández [a], E. Alves de Almeida [a], J. Ballesteros [a], T. García-Barrera [b], J.-L. Gómez-Ariza [b], J. López-Barea [a,*]

[a] *Department of Biochemistry and Molecular Biology, University of Córdoba, Spain*
[b] *Department of Chemistry and Materials Science, University of Huelva, Spain*

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Abstract

Utility of carboxylesterase and acetylcholinesterase inhibition as pesticide exposure biomarker was studied at Doñana National Park (SW Spain) in crayfish (*Procambarus clarkii*). Activities were measured in animals from reference sites or potentially exposed to pesticides, and their reactivation studied after dilution or 2-PAM treatment. Crayfish from affected sites had significantly less carboxylesterase and acetylcholinesterase activity than reference ones. No significant differences were found after dilution or 2-PAM treatment, showing that inhibition was irreversible. High pesticide levels were found in water and/or soil at rice growing sites, and lower levels at other affected places. High metal levels existed at rice growing sites and lower at other affected and at both reference sites. A combined effect on esterase inhibition of pesticides and metals is proposed. This field study suggest that the rice growing areas near Guadiamar stream are most polluted, followed by strawberry and citrics growing zones near Partido and Rocina streams. However, no correlation exist between the pesticide concentration at different sites and the extent of esterase inhibition, indicating that other factors could affect esterase response of animals from polluted sites.

Keywords: Carboxylesterase; Acetylcholinesterase; Inhibition and recovery; Carbamates; Organophosphorous pesticides; Metals

1. Introduction

Doñana National Park (DNP) is a 550 km^2 wildlife reserve North of Guadalquivir River Estuary in Huelva SW Spain (Fig. 1A and B). Due to its variety of ecosystems, plant and animal species, it is a UNESCO Reserve of the Biosphere, a Ramsar Site and a World Heritage Site. This sanctuary receives millions of migrating birds, mainly at its core, Doñana Biological Reserve (Grimalt et al., 1999). Yet it is not risk-free, being located 60 km S of Iberian Pyrite Belt that generates acid mine drainage and 40 km SE of industrial areas at Huelva Estuary. To monitor the biological effects of metals released when a tailings dam of Aznalcóllar pyrite mine collapsed in 1998 (Grimalt et al., 1999) biochemical biomarkers were measured in wild mice (*Mus spretus*), including the levels of biotransforming/antioxidative enzymes and oxidative damage to biomolecules (Ruiz-Laguna et al., 2001; Bonilla-Valverde et al., 2004). This accident did not cause serious impacts to DNP, but the effects of pesticides used in agriculture in its surroundings now arouse increasing concern (Bonilla-Valverde et al., 2004).

* Corresponding author. Tel.: +34 957 218 687; fax: +34 957 218 688.
E-mail address: bb1lobaj@uco.es (J. López-Barea).

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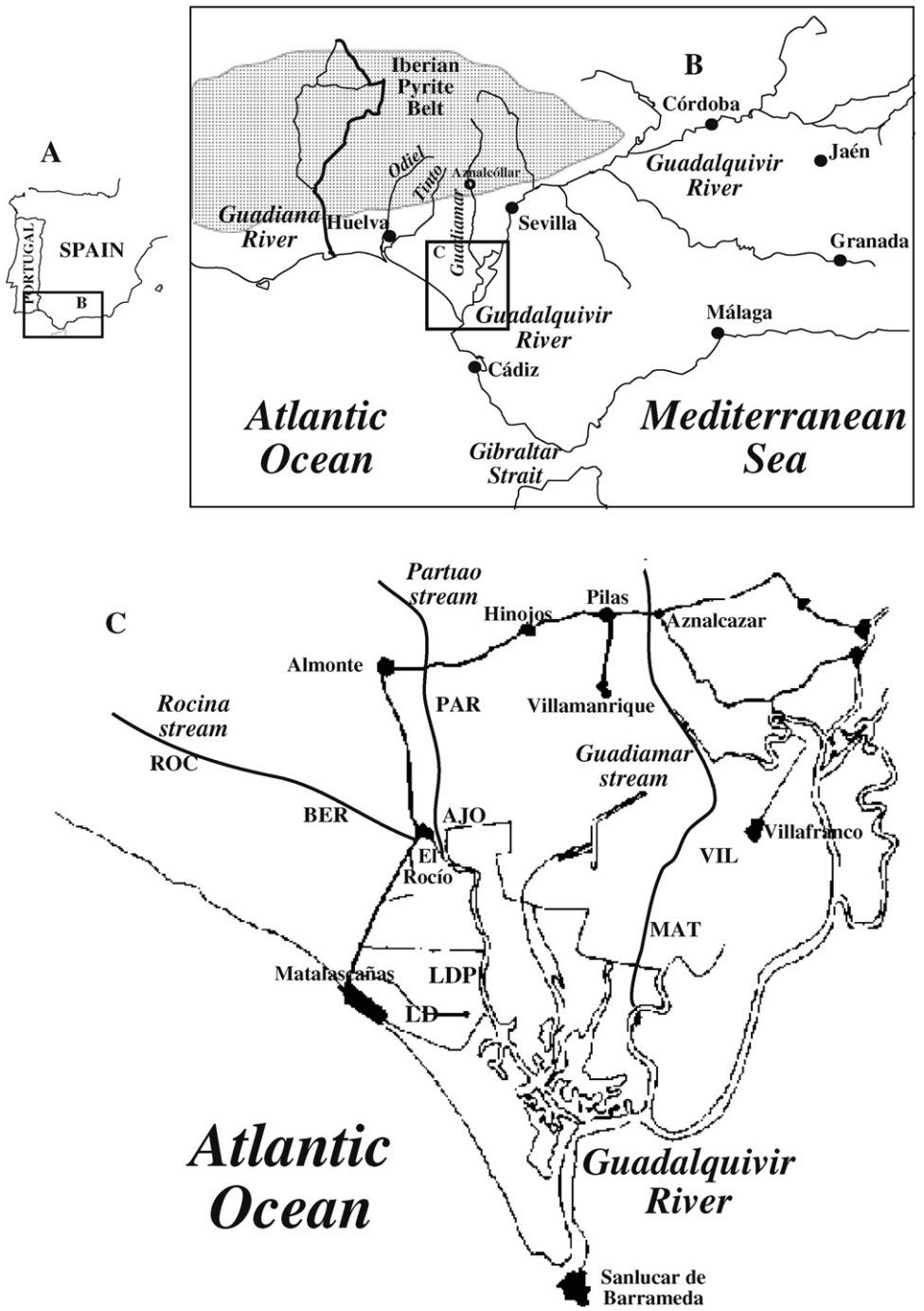

Fig. 1. Location of areas studied. (A) The Iberian Peninsula with Portugal and Spain; rectangle B enlarged on the right. (B) Eastern Portugal and Andalusia with the courses of Guadalquivir and Guadiana Rivers, the Portuguese border, the Gibraltar Strait, and the locations of seven Andalusian cities; square C enlarged below. (C) Doñana National Park and its surrounding areas; positions of two problem sites along the course of "Rocina" stream (ROC, BER), two along the course of "Partido" stream (PAR, AJO), two near the course of Guadiamar stream (VIL, MAT), and two reference sites within the Doñana Biological Reserve (LD, LDP).

Table 1
Areas studied, UTM coordinates, and main ecological characteristics

Area (code) (location)	UTM coordinates	Ecological characteristics
Rocina (ROC) (upstream Rocina stream)	$X = 178{,}653$, $Y = 4{,}119{,}937$	Problem, under influence of strawberry crops
Bernabe (BER) (Rocina stream, near "El Rocío")	$X = 187{,}036$, $Y = 4{,}116{,}086$	Problem, under influence of citrus and grape crops
Partido (PAR) (upstream Partido stream)	$X = 191{,}173$, $Y = 4{,}124{,}977$	Problem, under influence of citrus and grape crops
Ajolí (AJO) (Partido stream, near "El Rocío")	$X = 192{,}352$, $Y = 4{,}115{,}565$	Problem, under influence of citrus and grape crops
Villafranco Guadalquivir (VIL) (Guadiamar stream, near "Villafranco")	$X = 212{,}496$, $Y = 4{,}112{,}103$	Problem, under the influence of rice crops
El Matochal (MAT) (Guadiamar stream, near DNP)	$X = 208{,}681$, $Y = 4{,}102{,}207$	Problem, under the influence of rice crops
Laguna Dulce (LD) (Inside Biological Reserve of Doñana)	$X = 189{,}947$, $Y = 4{,}098{,}578$	Reference
Lucio del Palacio (LDP) (Inside Biological Reserve of Doñana)	$X = 193{,}800$, $Y = 4{,}099{,}515$	Reference

Organophosphates (OP) and carbamates (CM) replaced organochlorine insecticides as main defence line from agricultural pests. Although less persistent, they still pose risks to non-target organisms triggering increasing disquiet regarding effects on vulnerable species (Scholz and Hopkins, 2006). Pesticides elicit toxic effects by inhibiting esterases, hydrolases with a Ser active-site, of three types. Acetylcholinesterase (AChE) hydrolyses ACh at synapses in nerve transmission. Butyrylcholinesterase (BChE), of uncertain physiological role, releases fatty acids from triacylglycerols, phospho- and sphingolipids. Carboxylesterases (CbE) hydrolyze short-chain fatty acids from xenobiotics and participate in their detoxication (Maxwell, 1992; Hyne and Maher, 2003). Farmland-related bird species have declined due to extensive use of insecticides, although use of less-toxic products has reduced bird fatalities, except for products used in small fruit crops (Mineau and Whiteside, 2006).

Peakall (1994) included AChE inhibition among gold standard biomarkers, useful to diagnose a problem without any need for chemical analysis. AChE and BChE are used as exposure biomarkers for CMs/OPs pesticides (Payne et al., 1996). Though few reports exist on CbE inhibition, its greater activity and sensitivity make it an outstanding biomarker (Galloway et al., 2002; Bonacci et al., 2004). Aquatic invertebrates are excellent indicators due to their great sensitivity to OPs/CMs, but AChE and CbE are neglected as biomarkers (Hyne and Maher, 2003). The first esterase study in crayfish (*Procambarus clarkii*) reported triclorfon inhibition of muscle AChE (Repetto et al., 1988), followed by sensitivity of AChE and BChE to fenitrothion (Escartin and Porte, 1996) and use of AChE in field studies (Escartin and Porte, 1998). OPs and CMs differ in their effects on esterases: while CM-exposed enzymes reactivate after pesticide elimination, OP effects are more persistent and activity is only recovered when the inactive enzyme is treated with oximes, such as 2-pyridinealdoxime iodide (2-PAM) (Hyne and Maher, 2003). Thus, recovery from esterase inhibition can differentiate CM and OP exposure.

AChE, BChE and CbE activities have been established in several *P. clarkii* tissues and their sensitivities to model OP and CM pesticides evaluated; *in vitro* conditions for recovery from inactivation by dilution or oxime treatment have been also optimized to differentiate dilution reversible effects (CMs) from dilution-irreversible (OPs) (Vioque-Fernández et al., unpublished results[1]). We now evaluate AChE and CbE inhibition and recovery as pesticide exposure biomarkers in a field study carried out at DNP. These two esterase activities were measured in crayfish from reference and potentially affected sites of aquatic DNP environments, and their possible reactivation was studied after dilution or 2-PAM treatment. Possible relationships between biomarker responses and levels of pesticides and metals were also studied.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, phenylthioacetate, 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB), pesticides and PCBs standards, sodium sulphate, Florisil and basic alumina were from Sigma Chemical Co (St. Louis, MO, USA). Bradford reagent was from Bio-Rad (Hercules, CA, USA) and solvents from Teknokroma (Barcelona, Spain).

[1] Vioque-Fernández, A., Alves de Almeida, E., López-Barea, J. *Procambarus clarkii* esterases as pesticide biomarkers: tissue distribution, sensitivity to model compounds and recovery from inactivation (unpublished results).

2.2. Animals and sampling sites

Twenty animals (aprox. 8 cm and 15–25 g) were caught in May 2003, in each of two reference sites of Doñana Biological Reserve, at DNP core, lucio del Palacio (LDP) and laguna Dulce (LD), and in each of the six potentially polluted sites near DNP: upstream Rocina stream (ROC) and close to Bernabé house (BER), upstream Partido stream (PAR) and close to Ajoli bridge (AJO), and close to Guadiamar stream, near Villafranco (VIL) and Matochal (MAT) rice growing areas (Fig. 1C). Table 1 shows the areas studied and their ecological characteristics.

2.3. Sample preparation, enzyme assays and protein quantification

Animals were taken to the laboratory, their digestive gland and nervous tissues excised, weighed and frozen in liquid N_2 until used for extract preparation. Tissues were homogenized in 4 vol 0.1 M Tris–HCl buffer, pH 8.0, centrifuged 30 min at 30,000 × *g* (Beckman J2-21), and the supernatant frozen at −80 °C until used for biochemical analysis.

Esterases were assayed following release of thiol-derivatives (412 nm, Beckman DU650) (Ellman et al., 1961), in 0.1 M Tris–HCl buffer, pH 8.0. Then, 1 mM DTNB was added and buffer and sample blanks were incubated at 25 °C for 5 min to assess endogenous cross-reaction with DTNB. Assays started by adding 10 mM acetylthiocholine or 4.5 mM phenylthioacetate as substrates for AChE or CbE, respectively, and were measured for 2 min. Triplicate assays were made in each of two pools of five digestive glands and in each of two pools of 10 neural tissues. For reactivation, extracts were diluted 50-/30-fold with 0.1 M Tris–HCl buffer, pH 8.0, buffer plus 1 mg/ml ovoalbumin for CbE and AChE, respectively, or with 1/0.5 mM 2-PAM for CbE and AChE, respectively, for 2 h (Vioque-Fernández et al., see footnote 1). Specific activities are shown as U mg protein^{-1}. Protein concentration was determined by Bradford method (1976).

2.4. Pesticide extraction and analysis

EPA methods 3550/3620B and 3500/3610B were used for extraction/clean-up from soils and water. A 3800 GC apparatus with CPSIL-8 column (30 m, 0.25 mm, 0.25 μm film thickness) with a Saturn 2000 ion-trap MS detector (Varian, Sunnyvale, CA, USA) were used. Extract was injected (1 μl, splitless time 60 s) using an 8200 autosampler. Oven was kept at 60 °C (2 min), raised to 180 °C (25 °C min^{-1}) and maintained for10 min, raised to 300 °C (4 °C min^{-1}) and maintained for 5 min. Full scan electron impact ionization data were acquired at 7 min solvent delay, 70 eV impact energy, 30 μA emission current, 1 s scan^{-1} scan time and 50/200 °C manifold/trap temperature. Gain was switched on with a 20,000 counts target. After 7 min, scanning at 90–400 *m/z* was made from 7 to 51.8 min.

The MS–MS option was used, emission current was 80 μA and scan time 0.6 s scan^{-1}. To isolate precursor ions, the run time was split into nine segments. Due to the variety of pesticides, multiple reaction monitoring mode was used. Ions were isolated using 3 amu window and subjected to collision-induced dissociation after optimising for maximum sensitivity with automated method development software. Excitation storage level was set at the minimum value that allowed the dissociation of the precursor ion.

Due to the greater sensitivity of the electron capture detector (ECD) for some pesticides, extracts were also analysed in a 6890N GC-μECD (Hewlett Packard, Wilmington, USA) using a 7683 autosampler. A Chrompack CP-SIL8 column was used (30 m × 0.25 mm × 0.25 μm). Oven and injector temperature and He flow-rate were as described above. Make up gas was N_2 at 60 ml min^{-1} and detector temperature was set at 330 °C.

2.5. Metal analysis and estimation of sand percentage

Metals were analysed in a 4500 ICP-MS (Hewlett-Packard, Palo Alto, CA) at 1301 W RF power, 1.76 V RF matching, carrier gas (Ar) 1.19 l/min, blend gas (Ar) 0.08 l/min (Bonilla-Valverde et al., 2004). Detection limits (ppb) were 7.85 (Fe), 3.58 (Zn), 0.88 (Cu), 0.56 (Mn), 0.39 (Pb), 0.36 (Ni), 0.24 (Hg), 0.17 (Cr) and 0.08 (As, Cd). Particle size was analysed on 50 g sample using Na hexametaphosphate and 10 min sonication. Slurry was sieved through <63 μm to separate the sand fraction, evaluated by gravimetry after drying.

2.6. Statistical analysis

Statistical significance was assessed with the Instat™ software (Graphpad, San Diego, CA, USA). The Dunnett's test was used to compare all affected sites to the reference one (LDP) and the Bonferroni test to compare each site without treatment and after dilution or 2-PAM addition. Statistical significances are shown as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Normality of data and homogeneity of variance were checked.

3. Results

Vioque-Fernández et al. (see footnote 1) have adapted the assay of crayfish digestive gland CbE and neural tissue AChE as pesticide exposure biomarkers; reactivation was also proposed to distinguish CM and OP exposure. Table 2 shows CbE without treatment and after 50-fold dilution or 2-PAM addition. Crayfish of five potentially polluted sites of Doñana, ROC, BER, PAR, AJO and VIL, had a significantly lower activity, (−43 to −23%) than those from the LDP reference site. Conversely, no significant differences were found at the other potentially polluted site, MAT. Since CbE decrease in five of the six in potentially polluted locations suggested that animals living there could be exposed to OPs or CMs, possible reactivation was studied to distinguish among

Table 2
Carboxyl esterase activity in *P. clarkii* collected during spring 2003 from several sites of DNP area, without treatment, after 50-fold dilution or after 2-PAM treatment

Site	No treatment U/mg ± S.E. (significance[a])	Change vs LDP (%)	+50-fold dilution U/mg ± SE (significance[b])	% of untreated	+1 mM 2-PAM U/mg ± S.E. (significance [b])	% of untreated
ROC	8.1 ± 1.3 (**)	−34	6.5 ± 0.8 (ns)	81	6.9 ± 0.8 (ns)	86
BER	8.7 ± 0.5 (**)	−29	9.1 ± 0.6 (ns)	105	7.5 ± 1.4 (ns)	87
PAR	9.4 ± 0.2 (*)	−23	9.5 ± 0.2 (ns)	100	9.8 ± 0.1 (ns)	104
AJO	7.0 ± 0.6 (**)	−43	6.8 ± 0.5 (ns)	98	7.2 ± 0.6 (ns)	102
VIL	7.0 ± 0.5 (**)	−43	7.4 ± 0.5 (ns)	106	7.1 ± 0.6 (ns)	102
MAT	10.1 ± 0.7 (ns)	−18	9.2 ± 0.3 (ns)	91	9.8 ± 0.4 (ns)	97
LD	9.9 ± 0.1 (ns)	−19	9.5 ± 0.1 (ns)	96	9.9 ± 0.2 (ns)	100
LDP	12.3 ± 1.1 (–)	0	14.6 ± 0.8 (ns)	118	13,4 ± 0.2 (ns)	109

[a] Statistical significance vs. the result obtained at LDP, according to the Dunnett's test.
[b] Statistical significance vs. the result obtained at the same site but without treatment, using Bonferroni test.

them. No significant differences with untreated extracts were found after 50-fold dilution or 2-PAM treatment in any of the sites, indicating that CbE was irreversibly inhibited.

Table 3 shows that AChE of animals from four sites potentially polluted by pesticides, ROC, PAR, AJO and MAT, was significantly lower (−34 to −20%) than that of those from the LDP reference site, while no significant differences were found at another two affected sites, BER and VIL. Animals from LD reference site had 11% higher activity than those from the other reference, LDP, the difference being not statistically significant. Although AChE inhibition was less extensive than that of CbE, the lower AChE activity of crayfish from four of the six problem sites confirmed that they could also be exposed to pesticides. Again, AChE did not significantly increase by 30-fold dilution or 2-PAM treatment.

To assess pollution of several DNP sites, the levels of different types of chemicals were assessed in water and soil from the sites studied (Table 4). No pesticides were detected in water of LD site and only pendimethalin was found in LDP water. While pesticides were not detected in LDP soil, LD soil had high trifluraline and α-cipermetrin contents, and lesser levels of DDT, bromopropylate and acrynatrin. Different pesticide types were present in water and/or soil from rice growing areas near Guadiamar, at VIL and, especially, MAT sites. They included high malathion content, moderate levels of diclofuanid, bupirimate, bromacyl and dimethoate, and lower of DDT, chlorpyryfos, methidathion, bromopropylate, trifluraline, captafol, acrynatrin and penconazole. Some chemicals were also found at moderate levels in water (but not in soil) from the lower course of Rocina stream, BER, and the upper course of Partido stream, PAR, and at lower levels at AJO site but not at the upper

Table 3
Acetylcholinesterase activity in *P. clarkii* collected during spring 2003 from several sites of DNP area, without treatment, after 30-fold dilution or after 2-PAM treatment

Site	No treatment U/mg ± S.E. (significance[a])	Change LDP (%)	+30-fold dilution U/mg ± S.E. (significance[b])	% of un-treated	+0.5 mM 2-PAM U/mg ± S.E. (significance[b])	% of un-treated
ROC	1.3 ± 0.1 (*)	–20	1.4 ± 0.05 (ns)	105	1.5 ± 0.05 (ns)	114
BER	1.80 ± 0.11 (ns)	+9	2.03 ± 0.13 (ns)	113	1.99 ± 0.10 (ns)	110
PAR	1.27 ± 0.01 (**)	−23	1.26 ± 0.01 (ns)	99	1.39 ± 0.01 (ns)	110
AJO	1.10 ± 0.01 (**)	−34	1.19 ± 0.01 (ns)	108	1.29 ± 0.01 (ns)	118
VIL	1.49 ± 0.01 (ns)	−10	1.67 ± 0.01 (ns)	112	1.63 ± 0.01 (ns)	109
MAT	1.30 ± 0.05 (**)	−22	1.40 ± 0.09 (ns)	108	1.42 ± 0.05 (ns)	109
LD	1.85 ± 0.14 (ns)	+11	1.94 ± 0.12 (ns)	105	2.00 ± 0.08 (ns)	108
LDP	1.66 ± 0.01 (–)	0	1.80 ± 0.07 (ns)	109	1.80 ± 0.01 (ns)	109

[a] Statistical significance vs. the result obtained at LDP, according to the Dunnett's test.
[b] Statistical significance vs. the result obtained at the same site but without treatment, using Bonferroni test.

Table 4
Pesticide and total metal concentrations in water/soil from DNP sites in spring 2004

Site	Pesticide (type)[a]	Water $x \pm \sigma$ (ng l^{-1})	Soil $x \pm \sigma$ (pg g^{-1})	$\sum$Metals (μg g^{-1})[b]	Sand (%)
ROC	PCB138 (OCl)	1.8 ± 0.2	nd[c]	389.3	82.3
BER	Dimethoate (CB)	101.0 ± 14.0	nd	491.4	91.1
	Bromacyl (OBr)	210.0 ± 27.0	nd		
PAR	Molinate (OP)	11.4 ± 1.3	nd	635.3	32.5
	4,4′-DDD (OClD)	24.5 ± 2.9	nd		
	Dichlofuanid (SFM)	15.2 ± 2.3	nd		
	Folpet (PHT)	14.3 ± 1.6	nd		
AJO	Molinate (OP)	7.3 ± 1.1	nd	567.4	22.4
VIL	4,4′-DDT (OCl)	nd	19.0 ± 3.0	1243.5	12.3
	Dichlofuanid (SFM)	nd	410.0 ± 40.0		
	Bupirimate (Pyr)	nd	298.1 ± 36.0		
MAT	Malathion (OP)	1099.0 ± 27.0	nd	1188.2	28.5
	Chlorpyriphos (OP)	57.0 ± 8.0	nd		
	Me-chlorpyriphos (OPD)	nd	4.5 ± 0.5		
	Methidathion (OP)	15.0 ± 2.0	nd		
	Dimethoate (CB)	196.0 ± 31.0	nd		
	4,4′-DDT (OCl)	nd	30.0 ± 4.0		
	Bromacyl (OBr)	210.0 ± 25.0	nd		
	Bromopropylate (OBr)	10.6 ± 1.2	nd		
	Trifluraline (OFl)	25.0 ± 4.0	nd		
	Captafol (PHT)	10.6 ± 14.0	nd		
	Acrynathrin (PTR)	76.0 ± 13.0	nd		
	Penconazole (TRZ)	16.0 ± 2.0	nd		
LD	PCB52 (OCl)	nd	1.0 ± 0.1	454.2	76.2
	4,4′-DDT (OCl)	nd	4.5 ± 0.6		
	Bromopropylate (OBr)	nd	49.0 ± 5.0		
	Trifluraline (OFl)	nd	2094.0 ± 53.0		
	Acrynathrin (PTR)	nd	42.0 ± 5.0		
	α-Cipermethrin (PTR)	nd	1688.0 ± 47.0		
LDP	Pendimethalin (TRZ)	226.0 ± 31	nd	378.3	79.2

[a] Pesticide types shown as follows: OCl (D), organochlorine (derivative); CB, carbamate; OBr, organo-bromine; OP (D), organophosphate (derivative); SFM, sulfamide; PHT, phtalamide; PTR, pyrethroid; TRZ, triazole.
[b] Total concentration of Mo, Zn, Pb, Cd, Co, Ni, Mn, Cr, Cu and As.
[c] Not detected.

course of Rocina stream, ROC, where only one PCB isomer was detected.

Metals (Mn, As, Zn, Cr, Ni, Cu, Pb, Co, Cd and Mo) were also analysed in the same soil samples in which agrochemicals were assessed. Table 4 shows the total metal concentrations. High levels were detected at the rice growing areas near Guadiamar, at VIL and MAT sites, including high Mn, Zn, Cr, Ni and Co contents (not shown). Lower metal levels were found at other problem sites along the Rocina and Partido streams, ROC, BER, PAR and AJO, and at both reference sites, LD and LDP. High As levels were found in soils from MAT and LD, 221 and 154 μg/g, respectively, although it was below detection levels at the other sites studied. The sand percentage of soil samples is included in Table 4. A significant correlation was found between sand proportion and total metal content of soil: high metal contents were detected in low sand soils, such as VIL or MAT, and low metal levels being present in high sand soils, as ROC, BER, LD and LDP. This inverse relationship had a correlation coefficient of −0.765 and a *P*-value of 0.0269.

4. Discussion

Esterase inhibition is used as a biomarker of pesticide exposure in aquatic organisms, although the general response, sensitivity and tissular distribution of esterases vary significantly with the species. *P. clarkii* esterases were recently characterized, including their inhibition

by OPs/CMs and their reactivation, and inhibition and reactivation of CbE/AChE was proposed as pesticide exposure biomarkers (Vioque-Fernandez et al., see footnote 1). Until now, few studies have assessed esterase inhibition and pesticide levels in real environments (De la Vega Salazar et al., 1997; Robillard et al., 2003; Hamers et al., 2003; Abdel-Halim et al., 2006). We have studied the possible correlation of esterase inhibition and chemicals levels to validate esterases as biomarkers in aquatic DNP areas. While CbE and AChE were fully active in crayfish from reference sites of Doñana Biological Reserve, LD and LDP, they were significantly lower in potentially polluted sites, such as PAR/AJO, along Partido stream, ROC/BER, along Rocina stream, with extensive citrus and strawberry crops, and VIL/MAT, in the rice growing areas near Guadiamar stream. Inhibition of CbE was more extensive than that of AChE, suggesting that CbE is a more sensitive biomarker of OP/CM exposure.

Yet, relation of esterase inhibition and pollutant levels is complex. (1) AJO animals had extensively inhibited esterases (43/34% for CbE/AChE, respectively) but no pesticides were found in AJO soil, and water had only low molinate levels. (2) ROC crayfish had moderately inhibited esterases (34/20%) although ROC water had only low concentration of one PCB isomer. (3) MAT animals had low esterase inhibition (18/22%) while many pesticides existed at high levels in water and soil from this site. The lack of correlation of pesticide levels in water and soil and esterase inhibition led us to study the percentage of sand in soils, that should be negatively related to their binding capacity. Considering the low pesticide content in soils with high/low sand content (BER versus PAR) and the variety and high level of pesticides in high/low sand soils (LD versus VIL) this idea was excluded. However, an inverse relationship was found between sand proportion and total metal content of soil, although no significant correlations were found between sand or metal content and AChE or CbE inhibitions. The high metal load of VIL/MAT soils, added to the variety and high pesticide content in water and/or soil from these two sites, could explain esterase inhibitions of VIL/MAT animals. Nevertheless, the higher esterase inhibitions of AJO, ROC and PAR crayfish contrast with the moderate levels of pesticides and metals in water and/or soil from these sites. We think that, in addition to pesticides and metals, other pollutants not considered in our study could affect esterase activity.

Previous field studies have illustrated a complex relationship between AChE inhibition and pesticide levels, that can be altered by pesticide transformation and induction by their metabolites (De la Vega Salazar et al., 1997), or by natural variation of many abiotic factors (Robillard et al., 2003), necessitating powerful statistical methods to show such correlations (Hamers et al., 2003), that could even not being recognized (Robillard et al., 2003). In addition to pesticides, esterases are inhibited by toxic metals (Martínez-Tabche et al., 2001; Diamantino et al., 2003), although some metals activate AChE under acute exposure (Zatta et al., 2002; Bainy et al., 2006). Actually, Cu, Hg, Pb, Fe, Cd, Ni and As affect esterase activity (Abou-Donia and Menzel, 1967; Olson and Christensen, 1980; Bocquene et al., 1990).

The lack of AChE inhibition in LD crayfish is puzzling, considering the variety and high content of pesticides and of As content in soil from this putative reference site. Heavy As levels strongly inhibit AChE in aquatic organisms (Bocquene et al., 1990), but those present in LD could not be enough to affect *P. clarkii* enzyme. In addition, none of the pesticides detected at LD were OPs or CMs, and they were only found in soil but not in water, where the crayfish dwell. Despite the popular use of esterase inhibition as a biomarker, it is sensitive to only few pesticide types, mainly OPs and CMs, while other agrochemical types (pyrethroids, organochlorines, etc.) can be present in polluted sites without inhibiting esterases. The pesticide source in LD soil is unknown: this lagoon only receives water from rain, not being connected to any stream. In fact, trifluraline has been detected in rainfall water (Trevisan et al., 1993). In addition, since LD receives each year thousands of birds, they might transfer to the soil chemicals adhering to their limbs. The presence of moderate pendimethalin levels at the LDP reference site can be due to its volatilization from nearby areas, not included in our study, considering the vapour pressure and Henry constant values of this pesticide.

Esterase reactivation differentiates OP and CA exposure (Rotenberg et al., 1995). OP effect is reverted only by oxime treatment, while esterases inhibited by CAs are reactivated by dilution. *P. clarkii* esterases inhibited *in vitro* by CAs and OPs strictly follow this pattern (Vioque-Fernandez et al., see footnote 1), but reactivation is complex in organisms exposed *in vivo* to OPs and CAs (Almeida et al., unpublished results[2]). Other factors affect reactivation in animals exposed to OPs or CAs, including "aging" and/or degradation of inhibited enzymes, requiring the synthesis of new enzyme molecules to recover from inhibition. Aquatic species

[2] Almeida, E.A., Vioque-Fernández, A., López-Barea, J. Proteomic alterations and biomarker responses in *Procambarus clarkii* after controlled exposure to clorpyrifos and carbaryl (unpublished results).

need weeks to recover esterases after OPs exposure, but only hours after CAs exposure (Ferrari et al., 2004). In our study, enzymes with a lower activity than reference were not reactivated by 2-PAM treatment or dilution. Despite the wide use of reactivation to distinguish CA and OP exposure, in field studies this approach could be concealed by factors that can alter esterase activities. The simultaneous presence of CA and OPs, and the synergistic effect of organic and inorganic contaminants and pesticides (and their decay products) could alter the response to established *in vitro* reactivation protocols (Vioque-Fernandez et al., unpublished results, see footnote 1). Reactivation could also depend on time after exposure and on concentration of pesticides present in water, contributing to an overall decline of activity, hindering its reactivation by 2-PAM treatment or dilution.

5. Conclusions

This field study in Doñana suggests a combined effect on esterases of pesticides and metals, derived from agricultural practices or from the Aznalcóllar accident. For crayfish, LDP is a better reference than LD, although both are within the Biological Reserve. Esterase inhibition and assessment of pesticides and metals in water/soil suggest that the rice growing fields near Guadiamar stream are the most polluted areas, followed by strawberry and citrics growing zones near Partido and Rocina streams. Esterases are sensitive to pollution, since crayfish from sites with historical contamination had lower esterase activity than those from reference sites. However, no correlation exist between the pesticide concentration at different sites and the extent of esterase inhibition, indicating that other factors could affect esterase response of crayfish from polluted sites, which requires further studies.

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