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RESEARCH ARTICLE

Comparative analysis of selected biomarkers and pesticide sensitivity in juveniles of *Solea solea* and *Solea senegalensis*

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Abstract The common sole, *Solea solea* (Linneus, 1758), and the Senegalese sole, *Solea senegalensis* (Kaup, 1858), are two important commercial species that coexist in the NW Mediterranean. In order to assess the species' ability to respond to chemical insults, a comparison of activities on enzymes involved in xenobiotic metabolism was carried out. Juveniles of both species were sampled in winter 2011 from the Ebro Delta region, and activities of selected enzymes such as acetylcholinesterase (AChE), carboxylesterase (CbE), ethoxyresorufin *O*-deethylase (EROD) and glutathione *S*-transferase (GST) were determined in several tissues. Lipid peroxidation (LP) levels in plasma were measured as a sign of oxidative stress. In vitro exposures to selected pesticides were contrasted, analysing AChE and CbE activities in several tissue homogenates. Overall, enzymatic activities were higher in *S. solea* except for gill GST and CbE and kidney GST, while plasmatic LP levels were similar. In vitro contrasts revealed lower IC50 values for CbE activities in *S. solea*, suggesting a greater buffer capacity of this enzyme to potentially reduce pesticide toxicity over AChE.

Keywords In vitro · Xenobiotic metabolism · Pollutants · Benthic fish

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Introduction

The Ebro Delta is an area of 320 km^2 located in the northwest Mediterranean Sea. In this region, the main economic interests are devoted to agricultural practices, aquaculture production and fishing activities. Also, due to its high ecological value, a significant part of its surface has been granted some legal protection by the Directive 79/409 of the European Commission (Mañosa et al. 2001). Concern on the presence of anthropogenic chemicals, mostly pesticides, and their effects over the local fauna has been addressed in a few recent studies (Damásio et al. 2010; Köck et al. 2010; Suarez-Serrano et al. 2010). Among the pesticides, organophosphates (OP) and carbamates (CB) are commonly used in agriculture, mostly during spring, and reach the bays and the surrounding marine waters by the late-spring–summer period (Gómez-Gutiérrez et al. 2006; Köck et al. 2010). The mechanism of action of these pesticides is based on the inhibition of neurotransmission acting over acetylcholinesterase (AChE), a member of the family of enzymes known as cholinesterases (ChE) (van der Oost et al. 2003), but they also inhibit other classes of B-type esterases such as carboxylesterases (CbEs) (Wheelock et al. 2008).

Among OPs, malathion is an insecticide extensively used in the region in the past. Despite its ban by the European Commission since June 2007 (ECC directive 1376/07 (07/389)), a period of grace was granted for the usage of existing stocks until December 2008. During the period 2007–2008, this pesticide was partly responsible for some fish mortality episodes in the region (Köck et al. 2010). In fish, malathion is bioactivated to the active malaoxon via oxidative desulfuration by the cytochrome P450 (CYP). Malaoxon could react with the hydroxyl group of serine in the active site of AChE and affect its activity (Aker et al. 2008). Dichlorvos

(DDVP) is another OP commonly used in the region in agriculture as well as treatments for ectoparasites in marine fish farms (Varó et al. 2008). Unlike malathion, it does not require bioactivation to become toxic as it already contains the oxon (P=O) form. Its commercialisation has also been banned recently (ECC directive 1376/07 (07/387)). Although under the present legislation these pesticides might not represent an environmental threat, they were considered in the present study as most available data on fish toxicity are based on them, and one main interest of our study was to contrast species sensitivity towards OPs.

B-type esterases are classed for their high sensitivity to OP and CB pesticides, and in fish, they are well-accepted markers of pesticide exposure (Fulton and Key 2001; Wheelock et al. 2008). In *Solea senegalensis*, AChE (EC 3.1.1.7) is the predominant form in the brain and muscle (Solé et al. 2012). On the other hand, CbE (EC 3.1.1.1) is a heterogeneous group of isoenzymes that catalyses the hydrolysis of a wide range of xenobiotic esters, amides and thioesters. CbEs are dominant in the liver and play a role in the metabolism and subsequent detoxification of many xenobiotics as well as endogenous compounds, and they are also believed to have a protective role against OP toxicity (Wheelock et al. 2008). Recently, they have also been characterised in several tissues of *S. senegalensis*, both in juveniles and adults (Solé et al. 2012).

In addition to B-type esterases, the activities of the CYP1A-dependent 7-ethoxyresorufin *O*-deethylase activity (EROD; EC 1.14.14.1), a phase I enzyme, and the phase II conjugating glutathione *S*-transferase activity (GST; EC 2.5.1.18) were considered, as they are key enzymes in the metabolism of endogenous molecules as well as xenobiotics in fish (van der Oost et al. 2003). Lipid peroxidation (LP) is a marker that integrates the negative effects caused on the lipid membranes by reactive oxygen species (ROS). Many xenobiotics undergoing metabolism can form intermediate ROS, but they can also result from incomplete oxygen reduction during the normal aerobic processes (Livingstone 2001; Valavanidis et al. 2006). LP levels in fish have been considered in pollution monitoring studies including in sole (Oliva et al. 2010) as well evaluating pesticide exposure under lab conditions (Varó et al. 2007). They are mostly measured in the muscle and liver, but they can also be reported in the plasma (Pascoli et al. 2011).

The two fish species contrasted in this study coexist in the NW Mediterranean and in the Ebro Delta region. *Solea solea* is a temperate species distributed in the Mediterranean Sea and in the Atlantic Ocean, from the Baltic Sea to Senegal. Although the natural distribution of *S. senegalensis* is in the tropical Atlantic waters (Quéro et al. 1986), it is nowadays well established in the western Mediterranean Sea according to CIESM (Atlas of Exotic Fishes in the Mediterranean Sea). Even though its presence in the Catalan Sea has been recognised since 1920, at present, there are no reliable data on its abundance compared to the common *S. solea* since they are not identified separately in the market. A contrast on their diet, growth, condition index and reproduction has been carried out in the Portuguese coast (Vinagre et al. 2008; Teixeira and Cabral 2010). In the Mediterranean, their growth and feeding ecology have also been contrasted; basically, they both feed on benthic invertebrates, such as polychaetes, bivalve molluscs and crustaceans (Garcia et al. 1991). While *S. senegalensis* has been broadly used as sentinel in field and laboratory studies in the southern Iberian Peninsula (Fonseca et al. 2011a, b; Oliva et al. 2012; Costa et al. 2012 and references within), the use of *S. solea* is more limited and has been carried out in more northern latitudes (Wessel et al. 2010; Trisciani et al. 2011).

The objective of the present study was to contrast the biotransformation capacities of two closely related species (*S. solea* and *S. senegalensis)* and relate this to potential biochemical advantages in front of a chemical challenge. To achieve this goal, in both species and selected target tissues, (1) activities of AChE, CbE, EROD, GST and LP levels were determined, and (2) in vitro exposures to selected model pesticides were used to assess AChE and CbE inhibition in tissue homogenates, the latter as an indicator of the capacity to buffer OP pesticide toxicity.

Material and methods

Chemicals

Chemical reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain), including dichlorvos (2,2-dichlorovinyl dimethyl phosphate, CAS no. 62-73-7), 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB), acethylthiocholine iodide (ASCh), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) and 1-naphthyl acetate (αNA). The solvents used in the laboratory were obtained from Merck Chemicals. Malathion (diethyl(dimethoxyphosphinothioylthio)succinate, CAS no. 121-75-5), and malaoxon (*O*-*O*-dimethyl-*S*[ethoxycarbonyl], CAS no. 1634-78-2) were obtained from Dr. Ehrenstorfer Reference Materials (Germany).

Fish sampling

Sampling was carried out in February 2011 in the platform situated in front of the Ebro Delta plain using gillnets. A total of 21 specimens were obtained from two close locations (coordinates, 40°39.429′ N 00°54.009′ E and 40°30.116′ N 00°55.213′ E). Fish were captured alive and immediately transported to a nearby lab with aerated seawater. Once in the laboratory, biological parameters were

recorded. Handling of the fish was done according to national and institutional regulations of the Spanish Council for Scientific Research and Directive 2010/63/EU. Blood was taken using heparinised syringes, and 1 % aprotinin was added to the blood extracted and thoroughly mixed. The plasma was obtained by centrifugation (1,000×*g*, 15 min, 4 °C) and stored at −80 °C until analyses. Fish were euthanized by cervical sectioning, and the target tissues (gills, liver, gonad, kidney and muscle) were dissected out, snap frozen in liquid nitrogen and kept at −80 °C until analyses. After dissection, the gonad and liver were weighted. Parameters such as condition factor (CF = body weight / (body length)^3 × 100), hepatosomatic index (HSI = (liver weight / body weight) × 100) and gonadosomatic index (GSI = (gonad weight / body weight) × 100) were also calculated.

Sample preparation

For esterase determination, a portion of tissue (between 0.1–0.5 g) was homogenised in ice-cold 50 mM phosphate buffer (pH 7.4) in a 1:5 (*w*/*v*) ratio using a Polytron® blender. The homogenate was centrifuged at 10,000×*g* for 30 min at 4 °C, and the obtained supernatant (S10) was aliquoted and stored at −80 °C until biochemical analyses. For EROD and GST determinations, the homogenisation buffer (100 mM phosphate, pH 7.4) was complemented with 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM ethylenediaminetetraacetic acid.

Biochemical determinations

Assay conditions were kept similar, and only the sample volume was changed in order to achieve linearity in the enzymatic measurements. All assays were carried out in triplicate at 25 °C, except EROD which was at 30 °C, in a 96-well plate using a Tecan Infinite M200 microplate reader.

AChE activity was measured in the undiluted S10 muscle tissue. In each microplate well, 25 μl of the sample was mixed with 150 μl of DTNB (270 μM), and after 2 min of pre-incubation, the reaction was started by adding 50 μl of the substrate ASCh (1 mM final concentration). Reading was performed in kinetic mode at 405 nm for 5 min following the Ellman et al. (1961) protocol. Activity was expressed in nanomoles per minute per milligram protein.

CbE activity was measured in S10, either fivefold diluted in the kidney, gonad and gills or 20-fold diluted in the liver. Briefly, 25 μl of the sample and 200 μl of αNA as substrate (250 μM final concentration in the well) were measured for 5 min at 235 nm as described in the Mastropaolo and Yourno (1981) protocol. Activity was expressed in nanomoles per minute per milligram protein.

EROD activity was measured using 50 μl of undiluted liver homogenate samples (S10) and incubated at 30 °C with a reaction mixture containing the following: 0.2 mM NADPH and 3.3 μM 7-ethoxyresorufin in 100 mM phosphate buffer (pH 7.4) (Burke and Mayer 1974). The reaction was followed over resorufin formation for 10 min with a transparent 96-well plate using the fluorescence mode at a 537-nm excitation and 583-nm emission. A six-point standard of resorufin (0–160 nM) was used to relate activity as picomoles per minute per milligram protein. A good agreement between S10 and microsomal EROD activity determinations was formerly confirmed ($r = 0.951$; $n = 12$).

GST activity was measured in 25 μl of the fivefold-diluted kidney, gonad and gill and 20-fold-diluted liver S10 using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione. The activity rate was measured for 5 min at 340 nm (Habig et al. 1974) and expressed as nanomoles per minute per milligram protein.

The biotransformation index (BTI) as proposed by van der Oost et al. (1998) was calculated as a ratio between EROD and GST activities in the liver.

LP was determined using 100 μl of plasma, mixed with 650 μl of 1-methyl-2-phenylindole in methanol/acetonitrile (1:3) and 150 μl of 37 % HCl. Incubation was performed at 45 °C for 40 min; the reaction was stopped in ice and further centrifuged at 13.000×*g* for 10 min to precipitate proteins. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane (MDA) treated in the same manner. LP content was expressed as nanomoles of MDA per millilitre plasma.

Total protein content of the samples for all assays was determined by the Bradford method (1976) adapted to a microplate, using Bradford Bio-Rad Protein Assay reagent and bovine serum albumin as standard. Absorbance was read at 595 nm.

In vitro exposure to pesticides

Sensitivity of AChE and CbE activities towards the pesticides dichlorvos, malathion and malaoxon was evaluated in both species. To perform the in vitro exposures, each homogenate used was obtained after centrifugation at 10.000×*g* for 30 min at 4 °C of the target tissue (S10). In vitro exposures were carried out in the undiluted S10 muscle, fivefold-diluted gonad and kidney and 20-fold-diluted liver. Stock solutions of the pesticides were dissolved in ethanol, except dichlorvos, which was dissolved in water. The final incubation concentrations for malathion and malaoxon were 0.01, 0.1, 1, 10, 100 and 1,000 μM, whereas for dichlorvos, they were 0.256, 0.512, 2.56, 12.8, 64 and 320 μM. For each incubation, 120 μl of the appropriately diluted S10 sample was mixed with 5 μl of each concentration of pesticide. Incubation with the pesticide was done at room temperature (22 °C) for 30 min. Subsequently, AChE

determinations in the muscle and CbE measurements in the gonad, kidney and liver were performed as formerly described.

Pesticide analysis residues in muscle tissue

Pesticide analysis in fish muscle was carried out to confirm that the fish had not been recently exposed to significant levels of pesticides. Analysis was carried out using liquid chromatography–tandem mass spectrometry with a previous extraction by the QuEChERS method. Four samples of pooled muscle tissue, corresponding to several individuals (7–10 g), were generated for each sex and species. A total of 45 pesticides were screened as described in Soler et al. (2007).

Statistical analysis

Data were tested for normality (Kolmogorov–Smirnov's test) and homogeneous variance (Levenes's test) and were $\log_{10}(X)$-transformed when suitable to comply with normality and homoscedasticity assumptions. A *t* test was used to compare the enzyme activities of females and males. The influence of fish size on enzyme activities was assayed using weight as covariate when performing analysis of covariance (ANCOVA) test to determine significant differences between species. Previously, the homogeneity of regression slopes was checked including the interaction factors (species × weight) in the model (Engqvist 2005). In the case of no significant effect of weight on enzyme activity, a *t* test was performed to check differences between species.

Data concerning the in vitro exposures to pesticides were analysed with one-way ANOVA, followed by Dunnett's multi-comparison test to assess which concentrations were significantly different from the control. The values expressed as percentages were arcsine-transformed to normalize the variable distribution prior to ANOVA analyses and converted back to percentages to calculate means and standard errors (Varó et al. 2007). To calculate the 50 % in vitro inhibition concentration values (IC50), the regression probit module of SPSS Systems at 95 % confidence was used. Results were presented as means ± standard error of the mean (SEM). All statistical analyses were carried out using SPSS Systems (SPSS Inc., 1989–1992), and the significance level was always set at 0.05.

Results

Biological parameters

Biological data for both sole species are presented in Table 1. All specimens were juveniles, although specimens of *S. senegalensis* were bigger than those of *S. solea* ($p < 0.05$). All fish were confirmed as immature and at a similar developmental stage as reflected in the histological analysis of gonads and sex hormone levels in the plasma (data not presented). While the CF differed significantly ($p < 0.05$) between species, no differences were observed in the indexes HSI and GSI.

Table 1 Biological parameters and morphometric indexes

	S. solea	*S. senegalensis*
N (male/female)	10 (4:6)	11 (7:4)
Length (cm)	21.75 ± 0.85	25.91 ± 0.21*
Weight (g)	79.85 ± 14.7	146.6 ± 4.96*
CF	0.72 ± 0.02	0.84 ± 0.01*
HSI	0.53 ± 0.02	0.58 ± 0.03
GSI	0.22 ± 0.06	0.34 ± 0.13

Values are mean ± SEM

CF condition factor, *HSI* hepatosomatic index, *GSI* gonadosomatic index

*$p < 0.05$ (indicates significant difference between species after *t* test)

Biomarkers

Enzyme activities in the tested tissues for both species are presented in Table 2. As no significant differences in

Table 2 Enzymatic activities (mean ± SEM) on selected tissues

		S. solea (10)	*S. senegalensis* (11)	Statistical analyses
Muscle	AChE[a]	14.7 ± 0.7	7.6 ± 0.5	ANCOVA, $F = 23.42^*$
Kidney	AChE[a]	7.3 ± 1.1	6.6 ± 0.8	*t* test, n.s.
	CbE[a]	23.9 ± 3.4	26.1 ± 1.0	*t* test, n.s.
	GST[a]	51.4 ± 4.1	64.8 ± 4.5	*t* test, $t = -2.18^*$
Gills	AChE[a]	3.2 ± 0.2	2.2 ± 0.2	ANCOVA, n.s.
	CbE[a]	6.8 ± 0.4	9.9 ± 0.4	*t* test, $t = -5.14^*$
	GST[a]	69.7 ± 5.7	87.9 ± 4.1	*t* test, $t = -2.62^*$
Liver	AChE[a]	4.3 ± 0.2	2.2 ± 0.2	ANCOVA, n.s.
	CbE[a]	84.9 ± 9.0	50.4 ± 4.5	ANCOVA, n.s.
	GST[a]	413.4 ± 16.9	364.5 ± 20.0	*t* test, n.s.
	EROD[b]	2.21 ± 0.3	0.62 ± 0.1	ANCOVA, n.s.
Plasma	LP[c]	3.1 ± 0.9	3.6 ± 0.8	*t* test, n.s.

Number of individuals indicated in brackets beside species name

AChE acetylcholinesterase, *CbE* carboxylesterase, *GST* glutathione *S*-transferase, *EROD* ethoxyresorufin *O*-deethylase, *LP* lipid peroxidation, *n.s.* not significant ($p > 0.05$)

* $p < 0.05$ (indicates significant difference between species after *t* test/ANCOVA for contrasts between species)

[a] nmol/min/mg protein

[b] pmol/min/mg protein

[c] nmol MDA/ml plasma

enzyme activities were encountered between sexes in the fish used in the present study, results were reported together regardless of sex. Both species showed the highest AChE activity in the muscle following the order muscle > kidney > liver ≈ gills. Fish size (weight) had an effect in the muscle, gill and liver AChE activity as well as in the hepatic CbE and EROD activity. Thus, this factor was considered in the contrasts, and muscular AChE activity was still confirmed as significantly higher (ANCOVA, $p < 0.05$) in *S. solea*. CbE activities in both species also followed the same trend, liver > kidney > gills, although CbE activity in the gills was significantly higher in *S. senegalensis* (*t* test, $p < 0.05$). Hepatic EROD activity was 3.6-fold higher in *S. solea* than in *S. Senegalensis*, but not significant (ANCOVA, $p > 0.05$). Hepatic GST activity was not affected by size and was similar in both species. Thus, the BTI calculated as a ratio between EROD/GST was threefold times higher in S. *solea* (5.34 ± 0.7) than in *S. senegalensis* (1.75 ± 0.4). On the contrary, extra-hepatic GST activity in the gills and kidney was significantly higher (*t* test, $p < 0.05$) in *S. senegalensis*. As per tissue, GST showed the same trend in both species: liver > gills > kidney. On the other hand, the marker of effect, LP in the plasma (measured as MDA), did not reveal significant species differences (*t* test, $p > 0.05$).

In vitro exposure to selected pesticides

The in vitro inhibitory effect of dichlorvos, malathion and malaoxon on S10 homogenates over muscle AChE and CbE activities of the gonad, kidney and liver of the Senegalese sole and common sole after 30-min exposures is contrasted (Fig. 1). No effect was due to solvent control.

In both species, dichlorvos had a significant inhibitory effect on muscular AChE and CbE activities in the gonad, kidney and liver. The IC50 values (Table 3) clearly stated a higher sensitivity of CbE to this OP pesticide in *S. solea*, while the inhibitory effect on muscle AChE was similar in both species. By contrast, in vitro exposure to malathion only caused a significant inhibition on esterase activities at the highest concentrations (100 and 1,000 μM) and had no effect on gonadal CbE in *S. senegalensis* (Fig. 1). This insensitivity is also reflected in the IC50 values (Table 3). Malaoxon, malathion's metabolite, had a significant inhibitory effect on AChE and CbE activities in all tissues examined of both species, except on CbE in the gonad of *S. senegalensis*. As seen for dichlorvos, muscle AChE was equally sensitive in both species, while CbE was more sensitive in *S. solea*, also reflected in the IC50 values for this chemical (Table 3).

Chemical analysis of pesticide residues in muscle tissue

Four pools of muscle tissue, representing each species and sex, were analysed. Out of the 45 pesticides analysed, only 6 were detected and at very low levels (0.13–0.65 ng/g wet weight (ww)) in the muscle of the pool corresponding to *S. senegalensis* males. The OP pesticides were as follows: diazinon, dimethoate and omethoate, triazines (propazine and terbuthryn) and carbamate (methiocarb). Diazinon was the only OP commonly present in the four fish groups, and its concentration ranged from 1.38 to 3.25 ng/g ww. The pesticide residues found are considered low and much below those regulated for human consumption and

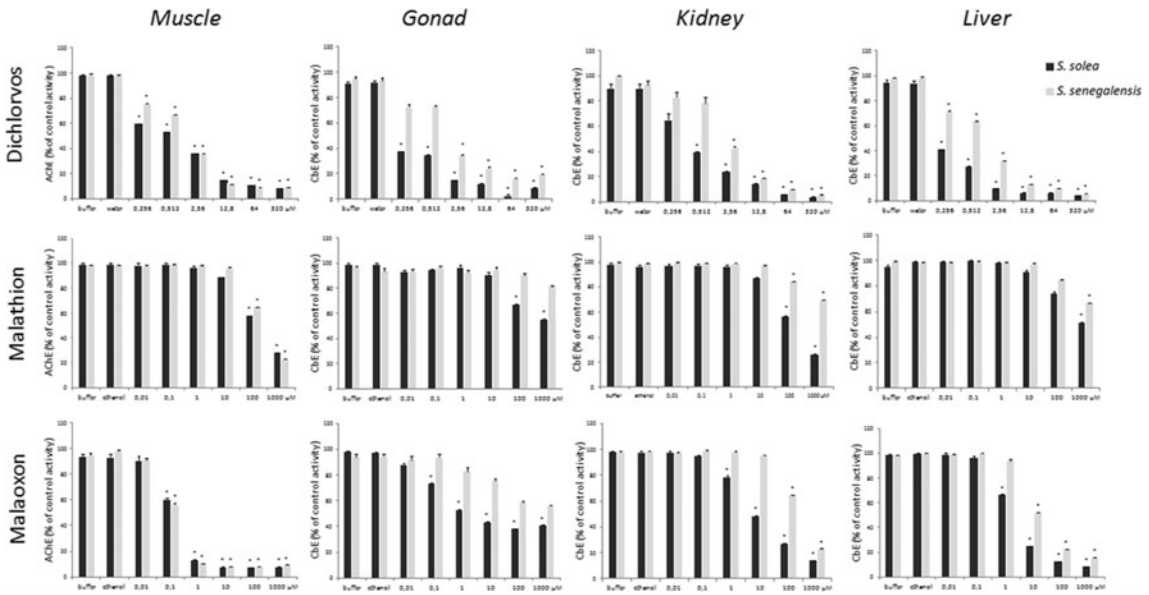

Fig. 1 Muscular AChE and gonad, kidney and liver CbE inhibition after in vitro 30-min exposures to selected pesticides. Each *bar* corresponds to $n = 4$. *Asterisk* denotes $p < 0.05$ after contrast versus the control group

Table 3 Pesticide concentration that reduce activity by half (IC_{50})

	IC50 (μM)				
	AChE	CbE			
	Muscle	Gonad	Kidney	Liver	Species
Dichlorvos	0.586	0.029	0.354	0.032	*S. solea*
	1.345	1.906	1.961	1.068	*S. senegalensis*
Malathion	218.0	4611	184.3	880.2	*S. solea*
	239.5	n.e.	5126	6126	*S. senegalensis*
Malaoxon	0.203	15.05	14.72	4.237	*S. solea*
	0.188	1311	239.5	17.87	*S. senegalensis*

AChE in the muscle or CbE in the gonad, kidney and liver in *S. solea* and *S. senegalensis*

n.e. no effect

considered as safe (<10 ng/g ww.). These results confirmed that no significant recent pesticide exposure had occurred.

Discussion

The common sole (*S. solea)* and the Senegalese sole (*S. senegalensis)* are commercially valuable fish species that, although they have different origins, presently co-exist in the Mediterranean. In this study, these two closely related sympatric soles, *S. solea* (temperate) and *S. senegalensis* (subtropical), were collected from the Ebre Delta region, a relatively clean area as far as certain organic pollutants are concerned, and during the winter period, when pesticides are found less present in this ecosystem (Köck et al. 2010). The latter was further confirmed by pesticide chemical analysis on the fish muscle. Moreover, presence of certain organic contaminants in the local sediment, collected simultaneously to the fish sampling, confirmed they correspond to low background levels reported in the Mediterranean (Gómez-Gutiérrez et al. 2007; Cardellicchio et al. 2007; Eljarrat et al. 2005), that is levels in the sediment (in ng/g dry weight) were for polycyclic aromatic hydrocarbons (PAHs), 72.50; PCBs, 15.05; and PBDEs, 1.12 (data provided by the Catalan Water Agency-ACA). Thus, under these premises of low background chemical exposure, the measure of biochemical defences of enzymes involved in xenobiotic metabolism and in vitro species sensitivity to selected pesticides was contrasted.

In general, enzyme activities in *S. solea* were higher than those in *S. senegalensis*. This supports the concept of higher enzymatic activities usually reported in species adapted to cooler environments against those from warmer climates, which is also applicable to xenobiotic-metabolising enzymes (Fitzsimmons et al. 2007). Nevertheless, the influence of factors such as sex and weight that can modulate these enzyme activities in fish (van der Oost et al. 2003), including sole (Solé et al. 2012), was considered. No sex differences were seen in this age group. However, as weight differed in both groups, this variable was considered as a cofactor in the contrast of activities and was seen relevant for esterases and EROD determinations. As in the present study, higher growth and CF for *S. senegalensis* in contrast to *S. solea* were also seen on the Portuguese coast in specimens of the same age (Vinagre et al. 2008). Thus, considering weight as a relevant factor in the contrasts of size-dependent activities, ANCOVA results confirmed that muscular AChE activities were still significantly higher in *S. solea*. Yet, AChE activity in the muscle in both sole species is low if contrasted to other teleost fish (Solé et al. 2010) although comparable to activities reported elsewhere for *S. senegalensis* (Solé et al. 2012). Despite even lower AChE activities in the gills, this tissue was considered as it was seen to be highly sensitive to exposure to antifoulants (López-Galindo et al. 2010a, b) and surfactants (Alvarez-Muñoz et al. 2007, 2009) in studies with *S. senegalensis*. Other tissues such as the kidney, gonad and liver also express AChE activity, although in these, this enzyme is likely to have roles other than neurotransmission (Karczmar 2010). They were also included in this study due to their key role in physiological processes and xenobiotic metabolism; thus, any alterations on their activities could have detrimental metabolic consequences. Other esterases, such as CbE activities, were dominant in the liver, but they were also measurable in other tissues such as the kidney, gonad and gills. The activities reported in this study fully coincide with a former study of juvenile and adult *S. senegalensis* (Solé et al. 2012).

The liver is the body's principal detoxification organ and processes the xenobiotics to which an animal is exposed in order to make them more readily excretable (van der Oost et al. 2003). An increase in EROD activity is usually used as a reliable biomarker of dioxin-like chemical exposure. In the present study, however, the pollution load of these classes of chemicals (e.g. PAHs) was low and equivalent for both species as they were simultaneously sampled from the same locations. Thus, EROD activity accurately corresponded to species differences, and although despite an apparent higher activity in the temperate *S. solea* (2.21 ± 0.3) than in the subtropical *S. senegalensis* (0.62 ± 0.3), once the fish weight was considered, these differences disappeared (ANCOVA, $p > 0.05$).

In addition to EROD, GST activity is of great importance in the detoxification of electrophilic xenobiotics

although its use in pollution monitoring is more controversial (van der Oost et al. 2003). GST activity in the liver of both species from the Ebre Delta region was similar and around 400 nmol/min/mg protein. These results are in sound agreement with the GST values (200–600 nmol/min/mg protein) found for *S. senegalensis* from the Ria de Aveiro and the Tejo estuary (Fonseca et al. 2011a, b), but they are lower than those reported for *S. solea* in a PAH-polluted site (Trisciani et al. 2011). In contrast to hepatic GST, a non-negligible but eight and 5.6 times lower GST activity was found in the kidney of *S. solea* and *S. senegalensis*, respectively, supporting its given role in the detoxification processes. Moreover, GST activity in the kidney and gills was significantly higher in the Senegalese sole than in the common sole. This fact, together with its previously reported higher CbE activity in the gills, predicts a significant extra-hepatic detoxification defence system in the Senegalese sole. Moreover, the BTI index (van der Oost et al. 1998) also confirmed species singularities.

Pesticide sensitivity was contrasted in vitro as an adequate proxy of in vivo responses (Laguerre et al. 2009), and the IC50 values for selected pesticides were calculated for muscular AChE and CbE in the gonads, kidney and liver of both Soleidae species. Both species displayed a similar response to AChE inhibition (by dichlorvos and malaoxon). However, CbE activity in *S. Solea* was more sensitive to exposure to all three pesticides (Table 3). Thanks to the protective role attributed to CbE in front of AChE inhibition (Sogorb and Vilanova 2002), a lower CbE IC50 value would indicate higher affinity for the pesticides tested in *S. solea* that could potentially imply a higher protection in front of a pesticide-polluted environment. Dichlorvos is one of the model OP pesticides more frequently used in neurotoxic sensitivity assessment. In a former study with sea bass (*Dicentrarchus labrax*) juveniles, muscle IC50 for AChE was 44.8 μM (Varó et al. 2003). This value greatly differs from muscle AChE IC50 obtained here for both sole species (0.586 μM for *S. solea* and 1.345 μM for *S. senegalensis*) and could be due to the low contribution of butyrylcholinesterase activity in the sole muscle (Solé et al. 2012) as opposed to the sea bass (Varó et al. 2003). Other OPs contrasted were malathion (parental form) and malaoxon (metabolite), and as expected for both sole species, the oxon form was more toxic as indicated by about 10^3 times lower AChE IC50 in the muscle. This chemical-form dependence on the inhibition power contrasts with studies on a freshwater fish, *Ictalurus furcatus*, in which IC50 for malathion and malaoxon only differed by twofold, 50 and 21.6 μM, respectively (Aker et al. 2008). However, more recent studies carried out with juveniles of *S. senegalensis* also revealed that the IC50 values for muscular AChE and hepatic CbE exposed to chlorpyrifos and its oxon were even lower than those of malathion/malaoxon, confirming the oxon form as the most toxic, a high species sensitivity to these pesticides and a protective role of hepatic CbE. That is, IC50 (μM) to chlorpyrifos exposure for AChE was 271.14; for CbE, it was 13.22; and for the oxon derivate, it was much lower: 0.025 in the muscle and 0.006 in the liver.

In addition to species differences, each tissue showed a particular toxicant response. In fact, CbEs represent a large family of isoenzymes that expresses species, age, tissue and chemical class specificity (Wheelock et al. 2008). In this sense, it was seen that hepatic CbE displayed a lower IC50 value than the kidney, and this value was, in turn, much lower for dichlorvos than for malaoxon in both tissues. Gonad responsiveness to pesticides also varied according to the OP pesticide and the species tested, and with the exception of dichlorvos, the gonad tissue proved to be rather insensitive to OP inhibition. Overall, the lower IC50 values of CbE after in vitro exposure to dichlorvos, chlorpyrifos and chlorpyrifos oxon support the protective role given to CbE in front of AChE inhibition, which is consistent with in vivo and in vitro studies carried out in the marine fish *Sciaenops ocellatus* (Ru et al. 2003). Conversely, a lower IC50 for CbE with respect to AChE was not confirmed for malathion and malaoxon. Nonetheless, this discrepancy in the response to two similar OP chemicals has also been reported in the Nile tilapia *Oreochromis niloticus* (Pathiratne and George 1998).

In short, considering a higher basal muscular AChE activity in *S. solea* than in *S. senegalensis*, a similar IC50 value after in vitro pesticide exposure in the muscle but a significantly lower IC50 value in the other tissues for CbE in *S. solea*, the common sole may be more resistant to AChE inhibition in front of a pesticide challenge. Further in vivo studies would be required to confirm the observation pointed out by in vitro results.

Conclusions

Enzymatic activities were, in general, higher in juveniles of *S. solea* as a species adapted to cooler waters, although *S. senegalensis* seemed to be better equipped in extra-hepatic defences as indicated by higher gill CbE activity and gill and kidney GST activities. In vitro exposure to pesticides also suggested that *S. solea* juveniles could be potentially more protected from OP exposures as expressed by higher AChE in the muscle, a similar IC50value for AChE in the muscle but a lower IC50 for CbE, the latter suggesting higher protection in front of pesticide toxicity. However, the final outcome resulting from the balance between their biochemical

differences should be confirmed under in vivo laboratory exposures to the same xenobiotic insult. In addition, the present study provides baseline data on enzymatic activities in juveniles of two closely related species of high economic value that could be of use in future monitoring studies.

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References

Aker WG, Hu X, Wang P, Hwang H-M (2008) Comparing the relative toxicity of malathion and malaoxon in blue catfish *Ictalurus furcatus*. Environ Toxicol 28:548–554

Alvarez-Muñoz D, Lara-Martín PA, Blasco J, Gomez-Parra A, Gonzalez-Mazo E (2007) Presence, biotransformation and effects of sulfocarboxylic acids in the benthic fish *Solea senegalensis*. Environ Int 33:565–570

Alvarez-Muñoz D, Gomez-Parra A, Blasco J, Sarasquete C, Gonzalez-Mazo E (2009) Oxidative stress and histopathology damage related to the metabolism of dodecylbenzene sulfonate in Senegalese sole. Chemosphere 74:1216–1223

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Burke MD, Mayer RT (1974) Ethoxyresorufin direct fluorimetric assay of microsomal *O*-dealkylation which is preferentially induced by 3-methylcholantrene. Drug Metab Dispos 2:583–588

Cardellicchio N, Buccolieri A, Giandomenico S, Lopez L, Pizzulli F, Spada L (2007) Organic pollutants (PAHs, PCBs) in sediments from the Mar Piccolo in Taranto (Ionian Sea, Southern Italy). Mar Pollut Bull 55:451–458

Costa PM, Caeiro S, Vale C, DelValls TA, Costa MH (2012) Can the integration of multiple biomarkers and sediment geochemistry aid solving the complexity of sediment risk assessment? A case study with a benthic fish. Environ Pollut 161:107–120

Damásio J, Navarro Ortega A, Tauler R, Lacorte S, Barcelo D (2010) Identifying major pesticides affecting bivalve species exposed to agricultural pollution using multi-biomarker and multivariate methods. Ecotoxicology 19:1084–1094

Eljarrat E, De La Cal A, Larrazabal D, Fabrellas B, Fernandez-Alba AR, Borrull F, Marce RM, Barcelo D (2005) Occurrence of polybrominated diphenylethers, polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in coastal sediments from Spain. Environ Pollut 136:493–501

Ellman GL, Courtney KD, Andres V Jr, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Engqvist L (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioral and evolutionary ecology studies. Anim Behav 70:967–971

Fitzsimmons PN, Lien GJ, Nichols JW (2007) A compilation of in vitro rate and affinity values for xenobiotic biotransformation in fish, measured under physiological conditions. Comp Biochem Physiol 145C:485–506

Fonseca VF, França S, Serafim A, Lopes B, Bebianno MJ, Cabral HN (2011a) Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. Aquat Toxicol 102:216–227

Fonseca VF, Franca S, Vasconcelos RP, Serafim A, Company R, Lopes B, Bebianno MJ, Cabral HN (2011b) Short-term variability of multiple biomarker response in fish from estuaries: influence of environmental dynamics. Mar Environ Res 72:172–178

Fulton M, Key P (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 20:37–45

Garcia E, Molinero A, Flos R (1991) Growth comparative study in two species of sole: *Solea solea* and *Solea senegalensis* in the Mediterranean Sea. Aquacult Environ 14:112–113

Gómez-Gutiérrez AI, Jover E, Bodineau L, Albaigés JM, Bayona JM (2006) Organic contaminant loads into the western Mediterranean Sea: estimate of Ebro River inputs. Chemosphere 65:224–236

Gómez-Gutiérrez A, Garnacho E, Bayona JM, Albaigés J (2007) Assessment of the Mediterranean sediments contamination by persistent organic pollutants. Environ Pollut 148:396–408

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Karczmar AG (2010) Cholinesterases (ChEs) and the cholinergic system in ontogenesis and phylogenesis, and non-classical roles of cholinesterases—a review. Chem-Biol Interact 187:34–43

Köck M, Farré M, Martínez E, Gadjda-Schrantz K, Ginebreda A, Navarro A, López de Alda M, Barceló D (2010) Integrated ecotoxicological and chemical approach for the assessment of pesticide pollution in the Ebro River delta (Spain). J Hydrol 383:73–82

Laguerre C, Sanchez-Hernandez JC, Kohler HR, Triebskorn R, Capowiez Y, Rault M, Mazzia C (2009) B-type esterases in the snail *Xeropicta derbentina*: an enzymological analysis to evaluate their use as biomarkers of pesticide exposure. Environ Pollut 157:199–207

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

López-Galindo C, Vargas-Chacoff L, Nebot E, Casanueva JF, Rubio D, Solé M, Mancera JM (2010a) Biomarker responses in *Solea senegalensis* exposed to sodium hypochlorite used as antifouling. Chemosphere 78:885–893

López-Galindo C, Vargas-Chacoff L, Nebot E, Casanueva JF, Rubio D, Solé M, Mancera JM (2010b) Subletal effects of the organic antifoulant Mexel 432 on osmoregulation and xenobiotic detoxification in the flatfish *Solea senegalensis*. Chemosphere 79:78–85

Mañosa S, Mateo R, Guitart R (2001) A review of the effects of agricultural and industrial contamination on the Ebro Delta biota and wildlife. Environ Monit Assess 71:187–205

Mastropaolo W, Yourno J (1981) An ultraviolet spectrophotometric assay for α-naphtyl acetate and α-naphtyl butyrate esterases. Anal Biochem 115:188–193

Oliva M, González de Canales ML, Gravato C, Guilhermino L, Perales JA (2010) Biochemical effects and polycyclic aromatic hydrocarbons (PAHs) in Senegal sole (*Solea senegalensis*) from Huelva estuary (SW Spain). Ecotox Environ Safe 73:1842–1851

Oliva M, Vicente JJ, Gravato C, Guilhermino L, Galindo-Riano MD (2012) Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): seasonal and spatial variation. Ecotox Environ Safe 75:151–162

Pascoli F, Negrato E, Di Giancamillo A, Bertotto D, Domeneghini C, Simontacchi C, Mutinelli F, Radaelli G (2011) Evaluation of oxidative stress biomarkers in *Zosterisessor ophiocephalus* from the Venice Lagoon, Italy. Aquat Toxicol 101:512–520

Pathiratne A, George SG (1998) Toxicity of malathion to Nile tilapia, *Oreochromis niloticus* and modulation by other environmental contaminants. Aquat Toxicol 43:261–271

Quéro J-C, Dessouter M, Lagardère F (1986) Soleidae. In: Whitehead P, Bauchot ML, Hureau J-C, Nielsen J, Tortonese E (eds) Fishes of north-eastern Atlantic and Mediterranean, vol 3. The Chaucer Press Ltd, Bungay

Ru S, Wei X, Jiang M, Li Y (2003) In vivo and in vitro inhibitions of red drum (*Sciaenops ocellatus*) brain acetylcholinesterase and liver carboxylesterase by monocrotophos at sublethal concentrations. Water Air Soil Pollut 149:17–25

Sogorb MA, Vilanova E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol Lett 128:215–228

Solé M, Baena M, Arnau S, Carrasson M, Maynou F, Cartes JE (2010) Muscular cholinesterase activities and lipid peroxidation levels as biomarkers in several Mediterranean marine fish species and their relationship with ecological variables. Environ Int 36:202–211

Solé M, Vega S, Varó I (2012) Characterization of type "B" esterases and hepatic CYP450 isoenzimes in Senegalese sole for their further application in monitoring studies. Ecotoxicol Environ Safe 78:72–79

Soler C, James KJ, Picó Y (2007) Capabilities of different liquid chromatography tandem mass spectrometry systems in determining pesticide residues in food: application to estimate their daily intake. J Chromatogr A 1157:73–84

Suarez-Serrano A, Ibáñez C, Lacorte S, Barata C (2010) Ecotoxicological effects of rice field waters on selected planktonic species: comparison between conventional and organic farming. Ecotoxicology 19:1523–1535

Teixeira CM, Cabral HN (2010) Comparative analysis of the diet, growth and reproduction of the soles, *Solea solea* and *Solea senegalensis*, occurring in sympatry along the Portuguese coast. J Mar Biol Assoc UK 90:995–1003

Trisciani A, Corsi I, Della Torre C, Perra G, Focardi S (2011) Hepatic biotransformation genes and enzymes and PAH metabolites in bile of common sole (*Solea solea*, Linnaeus, 1758) from an oil-contaminated site in the Mediterranean Sea: a field study. Mar Pollut Bull 62:806–814

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotox Environ Safe 64:178–189

van der Oost R, Lopes SCC, Komen H, Satumalay K, van den Bos R, Heida H, Vermeulen NPE (1998) Assessment of environmental quality and inland water pollution using biomarker responses in caged carp (*Cyprinus carpio*): use of a bioactivation: detoxication ratio as a biotransformation index (BTI). Mar Environ Res 46:315–319

van der Oost R, Beyer J, Vermeulen N (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Varó I, Navarro JC, Amat F, Guilhermino L (2003) Effect of dichlorvos on cholinesterase activity of the European sea bass (*Dicentrarchus labrax*). Pestic Biochem Phys 75:61–72

Varó I, Navarro JC, Nunes B, Guilhermino L (2007) Effects of dichlorvos aquaculture treatments on selected biomarkers of gilthead sea bream (*Sparus aurata* L.) fingerlings. Aquaculture 266:87–96

Varó I, Amat F, Navarro JC (2008) Acute toxicity of dichlorvos to *Aphanius iberus* (Cuvier & Valenciennes, 1846) and its anticholinesterase effects on this species. Aquat Toxicol 88:53–61

Vinagre C, Fonseca V, Maia R, Amara R, Cabral H (2008) Habitat specific growth rates and condition indices for the sympatric soles *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* (Kaup, 1858), in the Tagus estuary, Portugal, based on otolith daily increments and RNA–DNA ratio. J Appl Ichthyol 24:163–169

Wessel N, Santos R, Menard D, Le Menach K, Buchet V, Lebayon N, Loizeau V, Burgeot T, Budzinski H, Akcha F (2010) Relationship between PAH biotransformation as measured by biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (*Solea solea*). Mar Environ Res 69:S71–S73

Wheelock CE, Phillips BM, Anderson BS, Miller JL, Miller MJ, Hammock BD (2008) Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Rev Environ Contam Toxicol 195:117–178