Interdisciplinary Studies on Environmental Chemistry — Biological Responses to Contaminants, Eds., N. Hamamura, S. Suzuki, S. Mendo, C. M. Barroso, H. Iwata and S. Tanabe, pp. 227–236.

Acetylcholinesterase Characterization in the Terrestrial Isopod *Porcellionides pruinosus*

Nuno G. C. FERREIRA, Fernanda ROSÁRIO, Inês DOMINGUES, Carla F. CALHÔA, Amadeu M. V. M. SOARES and Susana LOUREIRO

CESAM & Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

(Received 25 January 2010; accepted 21 February 2010)

Abstract—In the last decades biomarkers have been widely used for the assessment of effects and/or exposure to environmental contaminants, but to our knowledge few data has been disseminated for isopods. Here, the cholinesterase of the isopod *Porcellionides pruinosus* was characterized using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and *S*-butyrylthiocholine iodide) and three ChE inhibitors (eserine hemisulfate, BW284C51, and iso-OMPA). The results showed that this organism has only one cholinesterase form, the acethylcolinesterase with a mean basal level of 113.6 ± 4.7 U/mg protein. The present study highlights the relevance of ChE characterization before its use as a biomarker in ecotoxicology and biomonitoring studies.

Keywords: biomarkers, acetylcholinesterase, acetylcholinesterase characterization, kinetic curves

INTRODUCTION

Biomarkers can be described as any biological response to an environmental stressor below the individual level, measured as biochemical, molecular, genetic, immunologic, physiologic signals or even organism products (e.g., urine, faeces, hair, feathers, etc.) or events in biological systems (vanGestel and vanBrummelen, 1996). These events indicate a shift from the normal status or fitness and most of the times can not be detected by phenotypic or life trait changes, but will be possibly linked to them in a near future. The definition of biomarkers also comes associated to two predominant features: (1) their sensitivity and quick response may act as early alarms to toxicant impacts on organisms, before ecological disturbances can be observed, (2) they may also provide a more accurate relationship between toxicant exposure and biological response before causing irreversible effects (Morgan *et al*., 1999).

The knowledge of biomarkers basal levels can build a threshold from where changes can be indicative of the health status of organisms and how stressors may act upon exposure.

In ecotoxicological studies or biomonitorization procedures, enzymatic

biomarkers are usually used to screen specific chemical groups. Among these, acetylcholinesterase is usually related to carbamate and organophosphate exposures which have a direct mode of action inducing an overstimulation of the central nervous system and causing neurotoxic effects (Barata *et al.*, 2004).

In terrestrial ecosystems edaphic organisms are often exposed to xenobiotics that may jeopardize all decomposition and fragmentation processes, causing a decrease in soil quality and soil services (MEA, 2005). As macrodecomposers, isopods play an important role in decomposition processes by the fragmentation of litter material and in the re-cycling of nutrients (Zimmer, 2002; Zimmer *et al.*, 2003; Loureiro *et al.*, 2006). Terrestrial isopods and particularly the species *Porcellionides pruinosus* have been described as good sensors for soil contamination or changes in their habitat (Takeda, 1980; Vink *et al.*, 1995; Jansch *et al.*, 2005; Loureiro *et al.*, 2005, 2009; etc.).

The main goal of this study was divided into two parts: i) to determine the best homogenization methodology for isopod test-species: the use of homogenizer vs. sonicator; ii) to characterize the cholinesterase in this isopod species.

This information and methodology will be crucial as a foundation for future studies where the effects of contaminants or other stressors will be assessed in the terrestrial isopod *Porcellionides pruinosus*.

MATERIALS AND METHODS

Test organism and culture procedure

The organisms used in this study belong to the species *Porcellionides pruinosus* (Brandt, 1833), and were previously collected from a horse manure pill and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 ± 2°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water sprayed and food provided. Only adult animals (15–25 mg wet weight) were used in the experiments and no distinction between sexes was made, although pregnant females were excluded.

Experimental procedure

To optimize the methodology for the enzymatic measurements two procedures were applied to our sampling animals. Ten organisms were processed using a homogenizer (*Ystral GmbH D-7801*, Dottingen, Germany) and the other ten using a sonicator (*Kika Labortechnik*, V200Scontrol, Germany). Total protein and AChE activity were measured for each homogenization method.

Test organisms were collected from culture boxes, weighted and cautiously observed: animals with abnormalities, moulting and pregnant females were discarded.

Cholinesterase characterization

Cholinesterase characterization was performed by the determination of

substrate preferences and selective inhibitor effects. A pool of twelve heads from culture organisms were homogenized using a sonicator in 6 ml of K-Phosphate buffer (0.1 M, pH 7.2) and centrifuged (1700 g, 3 min, 4°C) for cholinesterase activity determination, which was performed with six replicates, according to the Ellman method (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al*., 1996).

In independent experiments, acetylthiocholine iodide (AcSCh), *S*-butyrylthiocholine iodide (BuSCh), and propionylthiocholine iodide (PrSCh) within a dose range (0.005 to 20.48 mM) were used as substrates. Eserine hemisulfate was used as selective inhibitor of the activity of all the ChE, tetraisopropyl pyrophosphoramide (iso-OMPA) as selective inhibitor of pseudocholinesterase (PChE) and 1,5-bis(4-allyldimethyl-ammonimphenyl) pentan-3-one dibromide (BW284C51) as selective inhibitor of AChE. In the selective inhibitor experiments, all enzymatic activities were determined using AcSCh as substrate after an incubation period of 30 min at 25 ± 1°C. For each inhibitor, 5 μl of a stock solution was incubated with 495 μl of homogenate sample extract. Inhibitor concentrations ranged from 6.25 to 200 mM (eserine and BW284C51) and from 0.25 to 8.0 mM (iso-OMPA). Ultrapure water was added to controls and an additional control with ethanol was used in the experiments with iso-OMPA.

Acetylcholinesterase

One isopod head per sample was homogenized using a homogenizer or sonicator in 500 μl of potassium phosphate buffer (0.1 M, pH 7.2), and the supernatants obtained after centrifugation of the homogenates (4°C, 1700 g, 3 min) were removed and stored at –80°C until enzymatic analysis.

In a 96 well microplate 250 μl of the reaction solution was added to 50 μl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20 min. The reaction solution had 1 ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM solution, 1.280 ml of 0.075 M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36×10^{-3} $M^{-1}cm^{-1}$.

Protein quantification

The protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

Chemical compounds

All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany).

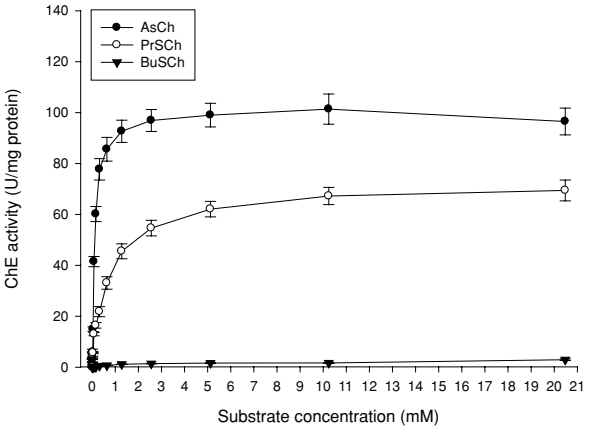

Fig. 1. ChE activity measured in *Porcellionides pruinosus* as a function of acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and *S*-butyrylthiocholine iodide (BuSCh) concentrations. Values are means of 6 isopods' heads with 4 enzymatic determinations per isopod and the corresponding standard error bars.

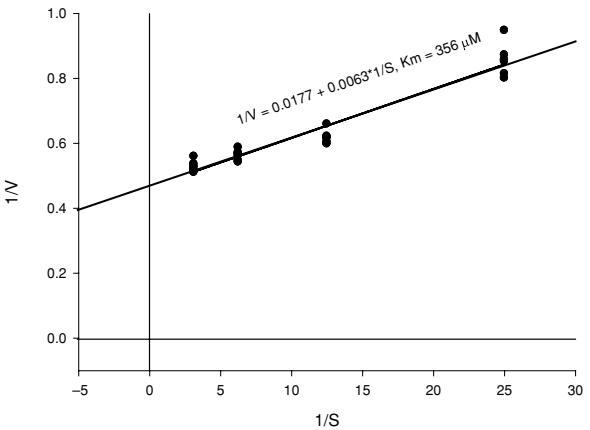

Fig. 2. Apparent K_m value for acetylthiocholine iodide (ASCh) substrate presented in a Lineweaver and Burk graph.

Statistics

For the cholinesterase characterization, values for *in vitro* inhibition concentration (IC_{50}) were calculated using a nonlinear four parameter logistic curve for eserine hemisulfate and a nonlinear 2 parameters exponential decay curve for BW284C51 (SPSS, 1999). An analysis of variance (ANOVA) was performed to compare differences between inhibitor's concentrations after data

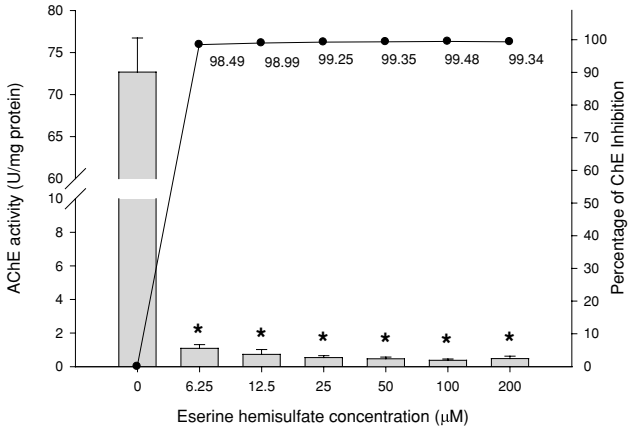

Fig. 3. ChE activity measured in *Porcellionides pruinosus* using as substrate acetylthiocholine iodide (AcSCh) as a function of the inhibitor eserine hemisulfate concentrations. Values are means of 6 isopods' head with 4 enzymatic determinations per isopod and corresponding standard error bars. Bars correspond to AChE activity and the line to the percentage of ChE inhibition. * = Dunnett's test, $p < 0.05$.

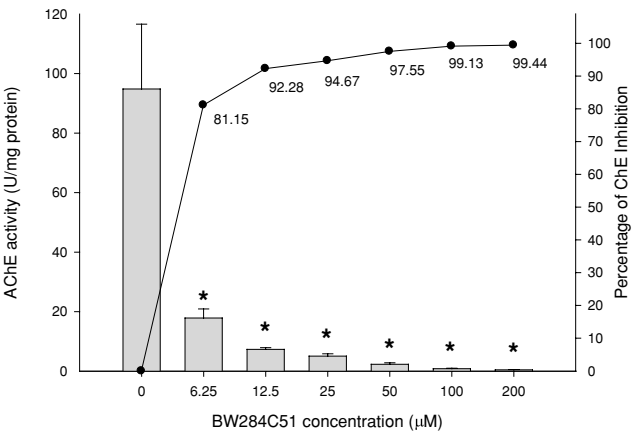

Fig. 4. Effect of BW284C51 on ChE activity of *Porcellionides pruinosus*. Values are mean of 6 isopods' head, with 4 enzymatic determinations per isopod and corresponding error bars. Bars correspond to AChE activity and the line to the percentage of ChE inhibition. * = Dunnett's test, $p < 0.05$.

transformation using natural logarithm (ln). Dunnett's comparison test was carried out to discriminate statistical different treatments (SPSS, 1999).

The comparison between the two types of sampling processing (homogenize vs. sonicator) was made using the Students *t*-test (SPSS, 1999).

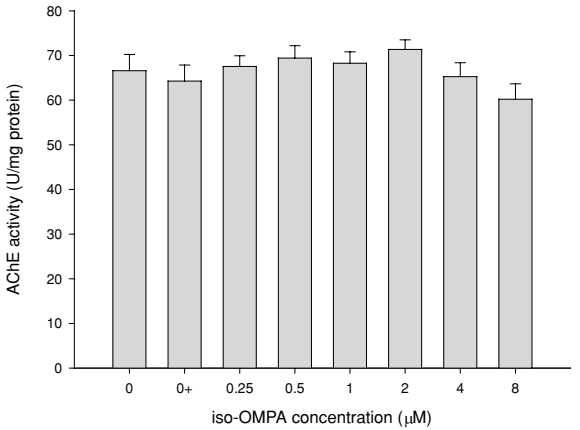

Fig. 5. ChE activity measured in *Porcellionides pruinosus* using as substrate acetylthiocholine iodide (AcSCh) as a function of a concentration range of the inhibitor iso-OMPA. Values are means of 6 isopods' head with 4 enzymatic determinations per isopod and corresponding standard error bars. 0 is the control and 0+ is the control solvent.

RESULTS

Homogenization methodology

The two homogenization procedures were compared and significant differences were found for the amount of protein extracted ($t_{18} = 5.959$; $p < 0.001$), and AChE activity ($t_{18} = 7.872$; $p < 0.001$). These biomarker activity, measured by the sonicator procedure, showed higher activities than when using an homogenizer.

Therefore, all samples were processed with a sonicator.

Cholinesterase characterization

To investigate the substrate preferences of ChE in the head tissues of *P. pruinosus*, three substrates were assayed: AcSCh, PrSCh, and BuSCh (Fig. 1). Although the maximum activity of protein was obtained with AcSCh at 10.24 mM (201.94 ± 5.38 SE U/mg), in the stable zone of the graph, we have considered the value of 2.56 mM (99.55 ± 3.24 SE U/mg protein) at the end of the exponential phase of the enzymatic activity as the concentration of AcSCh to be used in future studies. Lower ChE activities were observed when using PrSCh and BuSCh as substrates (e.g., 70.69 ± 3.16 SE and 2.80 ± 0.87 SE for PrSCh and BuSCh, respectively, at 20.48 mM). Therefore, ChE activity measurements hereafter were performed using AsSCh as substrate at the concentration of 2.56 mM.

The apparent K_m value for the AcSCh substrate calculated by the Lineweaver and Burk method was 356 μM (Fig. 2).

Table 1. Examples of Michaelis-Menton constant (K_m) for the AcSCh substrate in species used as test-organisms in ecotoxicological approaches. Values for this study on *Porcellionides pruinosus* are expressed as mean value of 6 replicates. Values for other species were reported in previous works.

Species	K_m (μM)
Porcellionides pruinosus	356
Spodoptera frugiperda (Yu, 2006)	33.5
Mytilus galloprovincialis (Mora *et al*., 1999)	34
Nucella lapillus (Cunha *et al*., 2007)	91
Octopus vulgaris (Talesa *et al*., 1995)	70
Crassostrea gigas (Bocquene *et al*., 1997)	30
Pecten jacobaeus gills (Stefano *et al*., 2008)	275
Pecten jacobaeus adductor muscle (Stefano *et al*., 2008)	234
Monodonta lineate (Cunha *et al*., 2007)	157
Cathorops spixii (Tortelli *et al*., 2006)	196
Cnesterodon decemmaculatus (de la Torre *et al*., 2002)	170
Cyprinus carpio (de la Torre *et al*., 2002)	230
Haemulon plumieri (Leticia and Gerardo, 2008)	310
Micropogonias furnieri (Tortelli *et al*., 2006)	179
Odonthestes bonaeriensis (Monserrat *et al*., 2001)	40
Odonthestes argentinensis (Monserrat and Bianchini, 2001)	50
Oreochromis niloticus (Rodriguez-Fuentes and Gold-Bouchot, 2002)	102

Eserine hemisulfate significantly inhibited ChE activity ($p < 0.001$) (Fig. 3), and similar results were obtained with the selective inhibitor of AChE, BW284C51 ($p < 0.001$), although data did not show a normal distribution (Fig. 4). Inhibition by eserine hemisulfate and BW284C51 was almost complete (>99%) at the highest concentrations tested. The effect of the selective inhibitor of BChE iso-OMPA did not affect *P. pruinosus* ChE activity ($p > 0.005$) at concentrations up to 8 mM (Fig. 5). IC_{50} values for eserine hemisulfate and BW284C51 are 0.12 ± 3.22 SE U/mg protein and 0.26 ± 0.06 SE U/mg protein, respectively; IC_{50} values for iso-OMPA could not be determined since no significant inhibition was found up to the maximum concentration.

DISCUSSION

Sample's preparation showed to be a very important step in the measurement of biomakers activity. When applying two different methodologies for the homogenization procedure (homogenizer and sonicator), there were significant differences in the analyses. The amount of protein extracted and AChE activity was higher when using the sonicator.

The objective of this study, the characterization of the ChE activity in *P. pruinosus*, included a first step to distinguish ChE from nonspecific esterases. This procedure is important because tissues may contain several nonspecific esterases, which contribute to the measured activity and may show different sensitivities towards anticholinesterase agents (Garcia *et al*., 2000). Nonspecific

esterases contribution was estimated using the compound eserine hemisulfate, which is considered a specific inhibitor of ChE at low concentrations, in the 10^{-6}–10^{-5} M range (Eto, 1974). In the present study the measured enzymatic activity was almost fully inhibited by eserine hemisulfate at the lowest concentration tested, 6.25 μM, (98.49%), which indicates the predominant presence of ChE and not of other esterases.

The highest ChE activity in *P. pruinosus* was obtained with AcSCh, showing a distinct preference over the other substrates. Furthermore, there was an almost complete inhibition when BW284C51 was used, while no significant inhibition was observed with iso-OMPA. Thus, it seems that only one ChE form is present in this species, with typical characteristics of an AChE. Cholinesterase forms in terrestrial invertebrate species have been less studied. To our knowledge there are no other studies where this characterization have been carried out for isopod species, therefore no comparisons could be made within isopoda. Considering other crustaceans, these results are in agreement with those obtained for several marine and freshwater species, since, in general, only AChE is present, such as in decapods (e.g., Key and Fulton, 2002; Quintaneiro *et al*., 2006), amphipods (Xuereb *et al*., 2007) and copepods (Forget and Bocquene, 1999). However, some crustacean species have shown different results, displaying a ChE form with atypical characteristics, as is the case of the cladoceran *Daphnia magna* (Diamantino *et al*., 2003).

The K_m value obtained for AcSCh was higher than the ones published for other invertebrates (Table 1) such as the fall armyworm *Spodoptera frugiperda*, with 33.5 μM (Yu, 2006), the mollusc bivalve *Mytilus galloprovincialis*, 34 μM (Mora *et al*., 1999), *Nucella lapillus*, 90.83 μM (Cunha *et al*., 2007), *Octopus vulgaris*, 70 μM (Talesa *et al*., 1995) or *Crassostrea gigas*, 30 μM (Bocquene *et al*., 1997), but similar to the earthworm *Eisenia andrei*, 160 μM (Gambi *et al*., 2007), the mollusc bivalve *Pecten jacobaeus*, 274.8 μM in gills and 233.9 μM in the adductor muscle (Stefano *et al*., 2008) and *Monodonta lineate* 157.04 μM (Cunha *et al*., 2007). Values obtained were also very similar to others found for several fish species (Monserrat and Bianchini, 2001; Monserrat *et al*., 2001; de la Torre *et al*., 2002; Rodriguez-Fuentes and Gold-Bouchot, 2002; Tortelli *et al*., 2006; Leticia and Gerardo, 2008).

Results reported here will be used as a basis for future studies on the evaluation of biomarkers in the species *Porcellionides pruinosus* upon xenobiotic exposures in the laboratory, but also as a possible biomonitorization tool for *in situ* testing.

We also believe that these results and the approach carried out can be used as a control tool to evaluate isopods status in laboratory cultures.

Acknowledgments—The study was supported by the Portuguese project Agromix-Mixture Toxicity evaluation in agricultural soils (PTDC/AGR-AAM/68676/2006), financed by Fundação para a Ciência e a Tecnologia. The authors would like to thank the laboratorial support given by Dr. Abel Ferreira.

REFERENCES

Barata, C., A. Solayan and C. Porte (2004): Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aqua. Toxicol*., **66**, 125–139.

Bocquene, G., A. Roig and D. Fournier (1997): Cbolinesterases from the common oyster (*Crassostrea gigas*)—Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. *Febs Lett*., **407**, 261–266.

Bradford, M. M., (1976): Arapid and sensitive method for the quantification of microgram quantities of protein, utilising the principle of protein-dye landing. *Anal. Biochem*., **72**, 248–254.

Cunha, I., E. Mangas-Ramirez and L. Guilhermino (2007): Effects of copper and cadmium on cholinesterase and glutathione *S*-transferase activities of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*). *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol*., **145**, 648–657.

de la Torre, F. R., L. Ferrari and A. Salibian (2002): Freshwater pollution biomarker: response of brain acetylcholinesterase activity in two fish species. p. 271–280. In *21st International Congress of the European-Society-of-Comparative-Physiology-and-Biochemistry*, Elsevier Science Inc., Liege, Belgium.

Diamantino, T. C., E. Almeida, A. Soares and L. Guilhermino (2003): Characterization of cholinesterases from *Daphnia magna* straus and their inhibition by zinc. *Bull. Environ. Contam. Toxicol*., **71**, 219–225.

Ellman, G. L., K. D. Courtney, V. Andres and R. M. Featherstone (1961): A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*., **7**, 88–95.

Eto, M. (1974): *Organophosphorus Pesticides*. CRC Press, Ohio.

Forget, J. and G. Bocquene (1999): Partial purification and enzymatic characterization of acetylcholinesterase from the intertidal marine copepod *Tigriopus brevicornis*. *Comp. Biochem. Physiol.—Part B: Biochem. Mol. Biol*., **123**, 345–350.

Gambi, N., A. Pasteris and E. Fabbri (2007): Acetylcholinesterase activity in the earthworm *Eisenia andrei* at different conditions of carbaryl exposure. *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol*., **145**, 678–685.

Garcia, L. M., B. Castro and L. Guilhermino (2000): Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its *in vitro* inhibition by environmental contaminants. *Biomarkers*, **5**, 274–284.

Guilhermino, L., M. C. Lopes, A. P. Carvalho and A. Soares (1996): Acetylcholinesterase activity in juveniles of *Daphnia magna* straus. *Bull. Environ. Contam. Toxicol*., **57**, 979–985.

Jansch, S., M. Garcia and J. Rombke (2005): Acute and chronic isopod testing using tropical *Porcellionides pruinosus* and three model pesticides. *Eur. J. Soil Biol*., **41**, 143–152.

Key, P. B. and M. H. Fulton (2002): Characterization of cholinesterase activity in tissues of the grass shrimp (*Palaemonetes pugio*). *Pesticide Biochem. Physiol*., **72**, 186–192.

Leticia, A. G. and G. B. Gerardo (2008): Determination of esterase activity and characterization of cholinesterases in the reef fish *Haemulon plumieri*. *Ecotoxicol. Environ. Safe*., **71**, 787–797.

Loureiro, S., A. Soares and A. J. A. Nogueira (2005): Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. *Environ. Pollut*., **138**, 121–131.

Loureiro, S., A. Sampaio, A. Brandao, A. J. A. Nogueira and A. Soares (2006): Feeding behaviour of the terrestrial isopod *Porcellionides pruinosus* Brandt, 1833 (Crustacea, Isopoda) in response to changes in food quality and contamination. *Sci. Total Environ*., **369**, 119–128.

Loureiro, S., M. J. B. Amorim, B. Campos, S. M. G. Rodrigues and A. Soares (2009): Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. *Environ. Pollut*., **157**, 625–636.

Millennium Ecosystem Assessment (MEA) (2005): *Ecosystems and Human Well-Being: Synthesis*. Island Press, Washington, D.C.

Monserrat, J. M. and A. Bianchini (2001): Anticholinesterase effect of eserine (physostigmine) in fish and crustacean species. *Brazil. Arch. Biol. Technol*., **44**, 63–68.

Monserrat, J. M., A. Bianchini and A. C. D. Bainy (2001): Kinetic and toxicological characteristics of acetylcholinesterase from the gills of oysters (*Crassostrea rhizophorae*) and other aquatic species. p. 781–785. In *11th International Symposium on Pollutant Responses in Marine Organisms (PRIMO 11)*, Elsevier Sci. Ltd., Plymouth, England.

Mora, P., X. Michel and J. F. Narbonne (1999): Cholinesterase activity as potential biomarker in two bivalves. *Environ. Toxicol. Pharmacol.*, **7**, 253–260.

Morgan, A. J., S. R. Sturzenbaum and P. Kille (1999): A short overview of molecular biomarker strategies with particular regard to recent developments in earthworms. *Pedobiologia*, **43**, 574–584.

Quintaneiro, C., M. Monteiro, R. Pastorinho, A. Soares, A. J. A. Nogueira, F. Morgado and L. Guilhermino (2006): Environmental pollution and natural populations: A biomarkers case study from the Iberian Atlantic coast. *Mar. Pollut. Bull.*, **52**, 1406–1413.

Rodriguez-Fuentes, G. and G. Gold-Bouchot (2002): Characterization of cholinesterase activity from different tissues of *Nile tilapia* (*Oreochromis niloticus*). p. 505–509. In *12th International Symposium on Pollutant Responses in Marine Organisms (PRIMO 12)*, Elsevier Sci. Ltd., Safety Harbor, FL.

SPSS (1999): Statistical package of the social sciences vol. 10.0. SPSS Inc., Chicago, Illinois.

Stefano, B., C. Ilaria and F. Silvano (2008): Cholinesterase activities in the scallop *Pecten jacobaeus*: Characterization and effects of exposure to aquatic contaminants. *Sci. Total Environ.*, **392**, 99–109.

Takeda, N. (1980): The aggregation pheromone of some terrestrial isopod crustaceans. *Experientia*, **36**, 1296–1297.

Talesa, V., M. Grauso, E. Giovannini, G. Rosi and J. P. Toutant (1995): Acetylcholinesterase in tentacles of octopus-vulgaris (cephalopoda)-histochemical-localization and characterization of a specific high salt-soluble and heparin-soluble fraction of globular forms. *Neurochem. Int.*, **27**, 201–211.

Tortelli, V., E. P. Colares, R. B. Robaldo, L. E. M. Nery, G. L. L. Pinho, A. Bianchini and J. M. Monserrat (2006): Importance of cholinesterase kinetic parameters in environmental monitoring using estuarine fish. *Chemosphere*, **65**, 560–566.

vanGestel, C. A. M. and T. C. vanBrummelen (1996): Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology*, **5**, 217–225.

Vink, K., L. Dewi, J. Bedaux, A. Tompot, M. Hermans and N. M. Vanstraalen (1995): The importance of the exposure route when testing the toxicity of pesticides to saprotrophic isopods. *Environ. Toxicol. Chem.*, **14**, 1225–1232.

Xuereb, B., P. Noury, V. Felten, J. Garric and O. Geffard (2007): Cholinesterase activity in *Gammarus pulex* (Crustacea Amphipoda): Characterization and effects of chlorpyrifos. *Toxicology*, **236**, 178–189.

Yu, S. J. (2006): Insensitivity of acetylcholinesterase in a field strain of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith). *Pesticide Biochem. Physiol.*, **84**, 135–142.

Zimmer, M. (2002): Is decomposition of woodland leaf litter influenced by its species richness? *Soil Biol. Biochem.*, **34**, 277–284.

Zimmer, M., G. Kautz and W. Topp (2003): Leaf litter-colonizing microbiota: supplementary food source or indicator of food quality for *Porcellio scaber* (Isopoda:Oniscidea)? *Eur. J. Soil Biol.*, **39**, 209–216.

N. G. C. Ferreira (e-mail: nunoferreira@ua.pt)