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Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its *in vitro* inhibition by environmental contaminants

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With a view to using the cholinesterase (ChE) activity from guppy (*Poecilia reticulata*) muscle as a biomarker, the objectives of this work were: (i) to characterize the soluble cholinesterases present in muscle homogenate using different substrates and specific inhibitors, (ii) to determine the normal range of activity in non-exposed individuals and (iii) to investigate the *in vitro* effects of two common environmental contaminants, copper sulphate and dodecylbenzene sulphonic acid sodium salt (DBS) on ChE activity. The rate of substrate hydrolysis of *P. reticulata* ChE decreased in the order acetylthiocholine, propionylthiocholine and butyrylthiocholine. Inhibition by excess of substrate was observed at concentrations higher than 1.28 mM. Furthermore, eserine sulphate and 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one (BW284C51) significantly inhibited the enzyme activity at low concentrations (mM range) and *N,N′*-diisopropylphosphorodiamic acid (iso-OMPA) had no significant effect up to 8 mM. These findings suggest that the enzyme measured in this study is acetylcholinesterase. The activity determined in non-exposed fish was 145.1 ± 44.7 SD U mg^{-1} protein. The common environmental contaminants copper and DBS significantly inhibited *P. reticulata* ChE at concentrations that can be ecologically relevant.

Keywords: biomarkers, cholinesterases, *Poecilia reticulata*, DBS, Cu^{2+}.

Introduction

Inhibition of cholinesterases (ChE) has been widely used as a biomarker for organophosphate and carbamate pesticides both in field and in laboratory conditions. A great diversity of organisms has been assayed for ChE activity including mammals, birds, fish, molluscs, crustaceans and insects (Tacha *et al*. 1994, Bocquené *et al*. 1995, Payne *et al*. 1996, Fossi *et al*. 1997, Jensen *et al*. 1997, Sturm and Hansen 1999). The results obtained in these studies confirm the sensitivity and suitability of ChE as an indicative parameter for toxic effects in ecotoxicology. However, two major difficulties must be overcome. First, the enzymes present in the species to be used as indicator in the particular conditions of

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the study should be characterized. Second, the normal range of activity in non-exposed individuals should be determined.

ChE belong to the family of enzymes designed as esterases which hydrolyse carboxylic esters. They may be distinguished from other esterases since they prefer to hydrolyse choline esters rather than other carboxylic esters and they are inhibited by physostigmine (eserine) at concentrations in the range of 10^{-5} M (Eto 1974). ChE have been divided into two types, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, 3.1.1.8). AChE, also known as true cholinesterase or specific cholinesterase, is important for the function of the nervous system (Thompson and Walker 1992). In vertebrates, it is found mainly in nervous tissue, mammalian erythrocytes, ganglia and motor end-plates (Eto 1974). BuChE, also called non-specific cholinesterase, pseudocholinesterase, serum cholinesterase and propionylcholinesterase is a less specialized enzyme whose physiological function is unknown (Witter 1963). In vertebrates it occurs principally in the serum, pancreas, liver and heart (Eto 1974, Thompson and Walker 1992). AChE and BuChE may be distinguished by distinct substrate specificity and different sensitivity toward specific inhibitors. Thus, AChE shows a high rate of hydrolysis when acetylcholine is used as substrate, a relatively lower activity with propionylcholine and a very low activity with butyrylcholine (Vellom *et al*. 1993). It is inhibited at high concentrations of substrate ($> 3 \times 10^{-3}$ M), it is very sensitive to 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) and it is relatively insensitive to *N,N´*-diisopropylphosphorodiamic acid (iso-OMPA) (Eto 1974, Xu and Bull 1994). BuChE hydrolyses butyrylcholine preferentially to acetylcholine, it is not inhibited by excess of substrate and it is inhibited at low concentrations of iso-OMPA (Eto 1974). The use of different substrates and specific inhibitors has been used to localize and to characterize the enzymes present in different species and/or tissues of both vertebrates and invertebrates (Liu *et al*. 1994, Raineri *et al*. 1995, Mionetto *et al*. 1997, Meyer *et al*. 1998). However, some recent studies indicate that ChE of some species can not be classified as AChE or BuChE since they show characteristics of both enzyme types (Liu *et al*. 1994, Bocquené *et al*. 1997).

The properties of ChE differ from species to species and also show variations in different tissues of the same species (Bocquené *et al*. 1990, Galgani and Bocquené 1990, Quadri *et al*. 1994). Distinct ChE show different sensitivities toward specific inhibitors and they also may be distinctly inhibited by environmental pollutants (Liu *et al*. 1994, Bocquené *et al*. 1997). In fact, the involvement of insensitive AChE variants has also been demonstrated in the resistance to organophosphate and carbamate insecticides (Tang *et al*. 1990, Zhu and Brindley 1990, Byrne and Devonshire 1993). Since ChE are polymorphic in most species (Xu and Bull 1994, Fontoura-da-Silva and Chautard-Freire-Maia 1996, Bocquené *et al*. 1997) and distinct forms may show different sensitivity to anticholinesterase agents, their use as biomarkers requires the biochemical characterization of the forms present in the species and in the tissue to be studied.

With a view to using the ChE activity of guppy (*Poecilia reticulata*) muscle as a biomarker, the objectives of this work were: (i) to characterize the soluble ChE present in muscle homogenate using different substrates and specific inhibitors, (ii) to determine the normal range of activity in non-exposed individuals and (iii) to investigate the *in vitro* effects of two common environmental contaminants, copper sulphate and dodecylbenzene sulphonic acid sodium salt (DBS) on ChE activity.

P. reticulata is widely used as a test organism in aquatic toxicology, being

recommended as test species in the protocol of OECD for the assessment of the potential impact of new chemicals on the aquatic environment (OECD 1993). In addition, it is relatively easy to culture and maintain in the laboratory.

Material and methods

Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide, propionylthiocholine iodide, iso-OMPA, eserine sulphate, bovine γ-globulin's, 5,5´-dithiobis(2-nitrobenzoic acid) (DTNB), BW284C51 and DBS were purchased from Sigma. Copper sulphate was from Merck. Concentrations of DBS are expressed in this study as a function of the amount of alkylbenzene sulphonate present (80% of the total product). In the case of copper, the values presented here are the concentrations of the Cu^{2+} ion.

Biological material

Fish, *Poecilia reticulata* (2—2.5 cm long), were obtained from a local supplier and acclimated in the laboratory to a temperature of 20 ± 2 °C and a photoperiod of 16h L: 8h D for at least 5 days before using them in the experiments. After the sacrifice of the animals by decapitation, dorsal muscles were immediately removed and put in ice-cold phosphate buffer (0.1 M, pH = 7.2). After removal of any remaining blood, each muscle was used to prepare homogenates in phosphate buffer using a Ystral GmbH Dottingen homogenizer and kept in ice during the homogenization. Samples were then centrifuged at 5000 *g* for 3 min, the supernatant was normalized to 0.5 mg of muscle per ml of buffer and stored at −20 °C for no more than 2 weeks. Previous experiments performed in our laboratory indicate that there is no significant decrease in the activity of ChE in frozen homogenate of *P. reticulata* during 2 weeks of storage.

Catalytic properties

The substrate preferences of *P. reticulata* muscle ChE were investigated by determining the activity of ChE at increasing concentrations of acetylthiocholine, butyrylthiocholine and propionylthiocholine (from 0.005 to 25.6 mM) in independent experiments.

Specific inhibitors and environmental pollutants

Eserine (physostigmine) is an alkaloid extracted from the calabar bean, *Physostigma venenosum,* and it is also synthesized chemically. It is a substituted phenyl ester of alkyl carbamic acids and a potent inhibitor of ChE (Volle 1971). The organophosphate iso-OMPA is a strong inhibitor of BuChE but not of AChE (Barahona and Sánchez-Fortún 1999) and BW284C51 is a selective reversible inhibitor of AChE (Xu and Bull 1994). Eserine, iso-OMPA and BW284C51 were used in this study as specific inhibitors of ChE, BuChE and AChE, respectively. For each chemical, several stock solutions were prepared in ethanol or in water.

Copper sulphate and DBS were used as representative of two groups of environmental pollutants commonly found in superficial waters: metals and surfactants. For these chemicals, stock solutions were prepared in ultrapure water. The effect of both specific inhibitors and environmental pollutants on the activity of ChE was determined after an incubation period of 30 min at room temperature as follows: for each chemical, 0.008 ml of each stock solution were added to 0.492 ml of *P. reticulata* homogenate. Controls were incubated with 0.008 ml of ultrapure water. Additional controls incubated with 0.008 ml of ethanol were included when appropriate. Three replicates per treatment were used. ChE activity was determined immediately after the end of the incubation period.

Enzyme activity

The activity of ChE was determined at 22 °C by the method of Ellman (Ellman *et al*. 1961) adapted to microplate as described in Guilhermino *et al*. (1996) using 0.05 ml of homogenate and 0.250 ml of the reaction mixture and a wavelength of 414 nm. The enzyme activity was determined in triplicate and expressed as Units (U) per mg of protein; 1 U is a nmol of substrate hydrolysed per minute. The concentration of protein was determined in triplicate by the Bradford method (Bradford 1976) adapted to microplate, using bovine γ-globulins as standard. A Labsystem Multiskan EX microplate reader was used.

Normal activity

In order to determine the normal range of ChE activity of *P. reticulata* muscle in non-exposed individuals, 30 samples prepared with different fish were assayed in the conditions described above for controls.

Data analysis

The 50% inhibition concentration (IC_{50}) was calculated by probit analysis (Finney 1971) and the values of the no-observed effect concentration (NOEC) and the low observed effect concentration (LOEC) were determined using the Analysis of Variance (nested design) and the Tukey multirange test (Zar 1996). The significance level was 0.05.

Results

In all the figures, values are the mean of three replicates (three enzymatic determinations per replicate) and the corresponding standard error (SE) bars. The activity of ChE of *P. reticulata* muscle as a function of increasing concentrations of acetylthiocholine, propionylthiocholine and butyrylthiocholine is shown in figure 1. The maximum of activity (143 ± 18 SE U mg^{-1} protein) was obtained with acetylthiocholine at 1.28 mM. Less activity was observed when propionylthiocholine was used as substrate (81 ± 27 SE U mg^{-1} protein at 1.28 mM) and even less activity was obtained with butyrylthiocholine (highest activity: 13 ± 3 SE U mg^{-1} protein at 0.32 mM).

Eserine sulphate significantly inhibited the enzymatic activity measured ($F = 53.4$, $P < 0.05$; LOEC = 6.3 μM) (figure 2). The IC_{50} value for this compound was 0.428 μM (table 1). *P. reticulata* muscle ChE is relatively insensitive to iso-OMPA since no significant inhibition was observed even at a concentration of 8 mM ($F = 0.4$, $P > 0.05$) (figure 3). BW284C51 significantly inhibited the enzyme activity ($F = 494.98$, $P < 0.05$, LOEC = 6.25 μM; IC_{50} = 0.312 μM) (figure 4) (table 2).

The normal range of ChE activity of non-exposed individuals was 145.1 ± 44.7 SD U mg^{-1} of protein. There was considerable interindividual variability.

The environmental pollutants copper and DBS significantly inhibited ChE activity (Cu^{2+}: $F = 20.0$, $P < 0.05$; DBS: $F = 9.5$, $P < 0.05$) (figures 5 and 6). LOEC values were 0.099 mM and 0.057 mM for Cu^{2+} and DBS, respectively (table 2). IC_{50} values were 0.044 mM and 0.042 mM for Cu^{2+} and DBS, respectively (table 2).

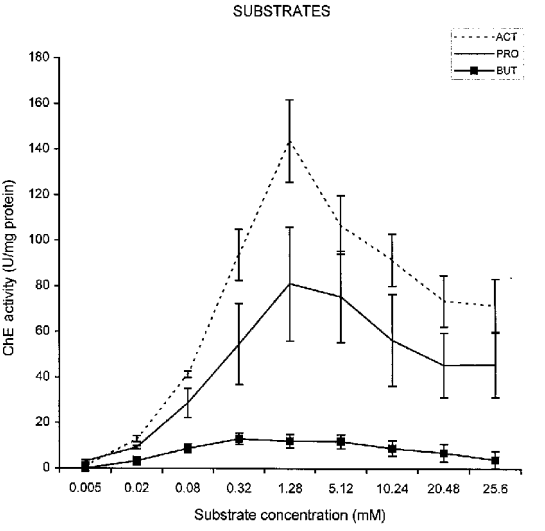

Figure 1. Activity of *P. reticulata* muscle ChE as a function of acetylthiocholine, propionylthiocholine and butyrylthiocoline concentration. Values are the mean of three replicates (three enzymatic determinations per replicate) and corresponding standard error bars.

Table 1. Effect of specific inhibitors on ChE of *P. reticulata* muscle. NOEC — no observed effect concentration, LOEC — lowest observed effect concentration, IC_{50} — 50% inhibition concentration. 95% confidence limits are shown within parentheses.

Chemical	NOEC (μM)	LOEC (μM)	IC_{50} (μM)
Eserine sulphate	< 6.3	6.3	0.428 (0.071–2.559)
Iso-OMPA	8000	–[a]	–[a]
BW284C51	< 6.3	6.3	0.312 (0.283–0.345)

[a] No significant inhibition was found up to 8 mM.

Table 2. Effect of copper (concentration of Cu^{2+}) and DBS on ChE of *P. reticulata* muscle. NOEC — no observed effect concentration, LOEC — lowest observed effect concentration, IC_{50} — 50% inhibition concentration. 95% confidence limits are shown within parentheses.

Chemical	NOEC (mM)	LOEC (mM)	IC_{50} (mM)
Cu^{2+}	< 0.099	0.099	0.044 (0.028–0.068)
DBS	0.029	0.057	0.042 (0.039–0.045)

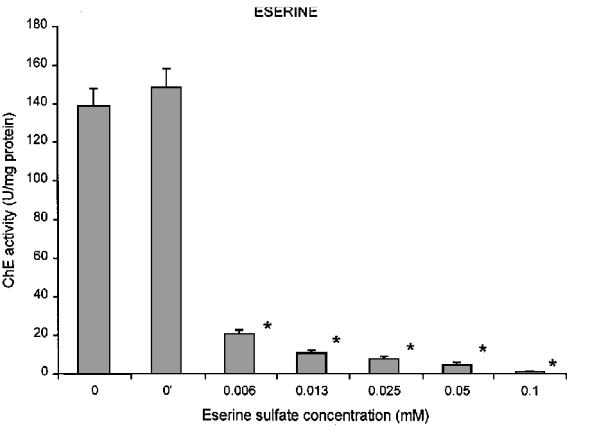

Figure 2. Effect of eserine sulphate on ChE activity of *P. reticulata* muscle. Values are the mean of three replicates (three enzymatic determinations per replicate) and corresponding standard error bars. 0'– control incubated with ethanol.

Discussion

The first objective of this study was to characterize the activity of ChE of *P. reticulata* muscle. There is a prerequisite for such characterization: the enzyme measured under the conditions of the assay must be a cholinesterase. This is important since tissues may contain significant amounts of non-specific esterases

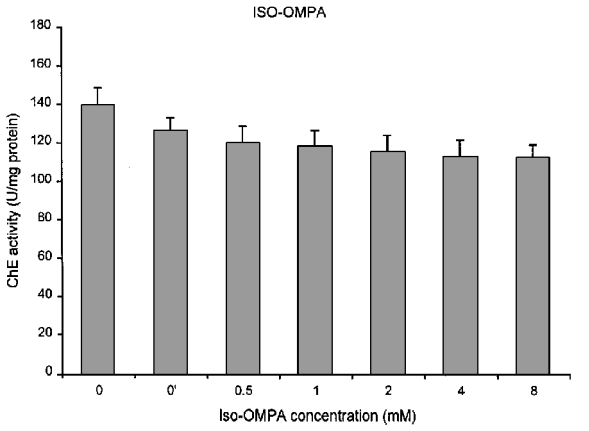

Figure 3. Effect of iso-OMPA on ChE activity of *P. reticulata* muscle. Values are the mean of three replicates (three enzymatic determinations per replicate) and corresponding standard error bars. 0'– control incubated with ethanol.

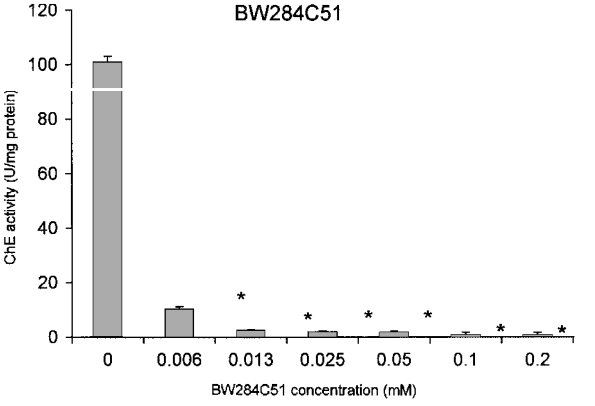

Figure 4. Effect of BW284C51 on ChE activity of *P. reticulata* muscle. Values are the mean of three replicates (three enzymatic determinations per replicate) and corresponding standard error bars. 0'– control incubated with ethanol.

which show different reaction towards anticholinesterase agents, thus, being a potential source of error in biomarker studies. The contribution of non specific esterases to the activity measured is usually determined through the inhibition of substrate hydrolysis in the presence of low concentrations (10^{-5} M range) of the carbamate eserine which is a specific inhibitor of ChE (Witter 1963, Eto 1974). In this study, the enzymatic activity of the *P. reticulata* muscle was almost completely inhibited (< 2% of control activity) at 0.1 mM, i.e. in the range considered typical for ChE. Therefore, it seems reasonable to conclude that the enzyme assayed in our experimental conditions is a ChE.

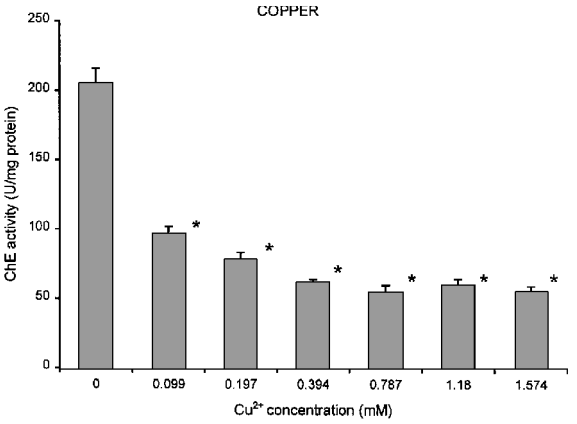

Figure 5. Effect of copper sulphate on ChE activity of *P. reticulata* muscle. Values indicate the concentrations of the Cu^{2+} ion (mM) and are the mean of three replicates (three enzymatic determinations per replicate) with associated standard error bars.

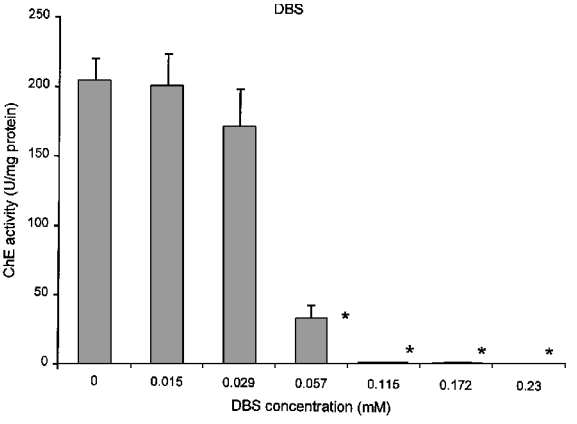

Figure 6. Effect of DBS on ChE activity of *P. reticulata* muscle. Values are the mean (mM) of three replicates (three enzymatic determinations per replicate) with associated standard error bars.

The highest activity was obtained when acetylthiocholine was used as substrate and enzymatic inhibition was observed at high concentrations of substrate (figure 1). These findings suggest that we are measuring AChE activity since this enzyme hydrolyses acetylthiocholine more rapidly than propionylthiocholine and butyrylthiocholine and it is inhibited at high concentrations of substrate (Eto 1974, Vellom *et al.* 1993). Furthermore, the activity of *P. reticulata* muscle ChE is not significantly inhibited by iso-OMPA, considered as a specific inhibitor of BuChE, at concentrations up to 8 mM and it is strongly inhibited by BW284C51, a specific inhibitor of AChE (Xu and Bull 1994), at concentrations of 0.05 mM (< 1% of control activity).

The activity of *P. reticulata* muscle ChE, determined in 30 samples prepared from different fish, was 145.1 ± 44.7 SD U mg^{-1} protein (SD = standard deviation). This value is within the range of values reported in the literature for several species of fish. For example, Labrot *et al*. (1996) indicate a mean of 59.13 ± 0.60 SD nmol min^{-1} mg^{-1} protein for the zebra fish (*Brachydanio rerio*) and Boone and Chambers (1996) refer to a value of 208.9 ± 54.9 SE nmol min^{-1} mg^{-1} protein for the mosquitofish (*Gambusia affinis*). A considerable variability of enzyme activity was found among the animals tested. Fish used in this study were obtained from a local supplier and showed colour variation and a small tail size variation. The variability of the enzyme activity may be related to genetic differences among fish. Since clients prefer to buy fish with long tails and different bright colours, suppliers tend to have a range of different colours and tail sizes. Thus, often is not possible to find a large number of similar fish and, even if it is possible, one cannot control the variability in the results due to genetic differences. Therefore, it is preferable to use fish cultured in the laboratory whenever possible. This variability among individuals is important since it can also occur in natural populations and should be considered in field studies.

The environmental contaminants copper and DBS significantly inhibited *P. reticulata* ChE at concentrations equal to or higher than 0.099 and 0.057 mM, respectively. These results agree with previous findings reported in other studies which indicate depression of ChE from several species by environmental contaminants other than pesticides, including metals and surfactants, in both *in vitro* and *in vivo* conditions (Gill *et al*. 1990, 1991, Schmidt and Ibrahim 1994, Labrot *et al*. 1996, Martínez-Tabche *et al*. 1996, Payne *et al*. 1996, Guilhermino *et al*. 2000). In this study, an IC_{50} value of 0.044 mM was determined for Cu^{2+}. This result is in agreement with the corresponding value reported by Olson and Christensen (1980) for *Pimephales promelas* (1.6×10^{-4} M), with the *in vitro* inhibition (higher than 90%) of *Scomber scomber* and *Pleuronectes platessa* AChE activity at 10^{-4} M found by Bocquené *et al*. (1990) and with the *in vivo* depression of *O. punctatus* and *C. batrachus* AChE activity reported by Mukherjee and Bhattacharya (1974) at concentrations in the mg l^{-1} range. The concentrations of metals causing a significant ChE inhibition have been considered as being irrelevant from an ecological point of view. However, the anticholinesterase effect of some metals should be a matter of concern in biomonitoring studies for three main reasons. First, several organisms accumulate metals in their bodies and the concentrations of some ions in specific tissues may be considerably higher than the concentration of these ions in the surrounding medium (Mance 1987, Borgmann *et al*. 1993, Schmidt and Ibrahim 1994). Second, frequently organisms are exposed to several pollutants simultaneously and different chemicals may have synergistic or additive anticholinesterase effects. Furthermore, some metals can intensify the inhibitory effect on ChE of some organophosphates (Bocquené *et al*. 1995). Third, these concentrations can in fact occur in contaminated sediments, polluted effluents and at certain points of water systems receiving them. For example, Whiting *et al*. (1994) report values from 3.49 to 24.79 mg l^{-1} of molybdenum in stream sites receiving drainage from a molybdenum mine and mill and Clark (1986) indicate values of 2700 ppm of zinc, 2148 ppm of copper in sediments of the river Fal estuary.

Previous studies with invertebrates found anticholinesterase effects of DBS. *In vitro* inhibition of *Moina macropa* AChE at 0.250 mg l^{-1} (IC_{50} = 6.56 mg l^{-1}) was found by Martinez-Tabche *et al*. (1996), an *in vitro* LOEC of 12.5 mg l^{-1} was

reported for AChE haemolymph of *Mytilus galloprovincialis* (Guilhermino *et al.* 1998), an IC_{50} of 6.6 mg l^{-1} and an EC_{50} of 11.4 mg l^{-1} were found for AChE of *Daphnia magna* exposed to *in vitro* and *in vivo* conditions, respectively (Guilhermino *et al.* 2000). *In vitro* LOEC and IC_{50} values determined for *P. reticulata* muscle ChE in the present study were 0.057 mM (20 mg l^{-1}) and 0.042 mM (14.5 mg l^{-1}), respectively. Thus, they are in agreement with the values previously reported for other aquatic organisms (Martinez-Tabche *et al.* 1996, Guilhermino *et al.* 1998, 2000). Linear alkylbenzene sulphonates (LAS), including DBS, are considered to be rapidly degraded in the environment (WHO 1996). However, due to a continuous imput, they may be present at relatively high concentrations in some polluted areas. Usually, the concentrations of surfactants reported in the literature for surface waters are considerably lower (μg l^{-1} range) than those causing significant AChE inhibition (mg l^{-1} range). However, LAS concentrations near 10 mg l^{-1} have been measured in effluents (WHO 1996). Furthermore, single agent concentrations are given in these studies while in the environment several compounds are in general present simultaneously. If several anticolinesterase agents coexist, the effect on the enzyme will be the result of their interactive effects. Thus, a significant inhibition may be induced at concentrations considerably lower than those required for inducing a similar effect if only one individual chemical was considered.

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