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Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity

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Abstract

The enzymatic activity of acetylcholinesterase (AChE) has been shown to be altered by environmental contaminants such as metals. However, the available literature illustrates a background of contradictory results regarding these effects. Therefore, the main purpose of this study was to investigate the potential of five metal ions (nickel, copper, zinc, cadmium and mercury) to inhibit AChE activity *in vitro*. First, to accomplish this objective, the possible interference of metals as test toxicants in the Ellman's assay, which is widely used to assess AChE activity, was studied. The potential influence of two different reaction buffers (phosphate and Tris) was also determined. The results suggest that the selected metals react with the products of this photometric technique. It is impossible to ascertain the artefactual contribution of the interaction of the metals with the technique when measuring AChE inhibition. This constitutes a major obstacle in obtaining accurate data. The presence of phosphate ions also makes enzymatic inhibition difficult to analyse. Attending to this evidence, an assay using the substrate o-nitrophenyl acetate and Tris buffer was used to investigate the effects of metals on AChE activity. O-nitrophenyl acetate is also a substrate for esterases other than cholinesterases. It is therefore only possible to use it for the measurement of cholinesterase activity with purified enzymes or after a previous verification of the absence of other esterases in the sample tissue. Under these conditions, the results indicate that with the exception of nickel, all tested metals significantly inhibit AChE activity.

Keywords: *Biomarker, acetylcholinesterase, metals, Ellman's assay,* in vitro *studies*

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Introduction

Acetylcholinesterase (AChE), a crucial enzyme in the nervous system of vertebrates and invertebrates, is the functional target of several xenobiotics, some of them have

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pharmacological interest, but also of others, such as pesticides used as agrochemicals or drugs used in chemical warfare (Carr & Chambers 1996, Porcelli et al. 1999). It is well established that AChE inhibition is a useful biomarker for organophosphate and carbamate pesticides both *in vivo* and *in vitro* conditions (Key & Fulton 2002). However, several studies published in the last few decades have demonstrated the effects of other classes of environmental contaminants on AChE activity of several species (Labrot et al. 1996, Payne et al. 1996, Guilhermino et al. 1998, Diamantino et al. 2003). One of these chemical classes is the group of metals that includes several common environmental contaminants (e.g. cadmium, copper, mercury, zinc).

The studies that have been published regarding the effects of metals on AChE activity revealed some contradictory results. For instance, mercury has been described as both an AChE inhibitor (Suresh et al. 1992, Devi & Fingerman 1995) and a stimulator (Sanz & Repetto 1995). Likewise, after *in vitro* exposure to copper, Romani et al. (2003) demonstrated an increase in AChE activity (V_m/K_m), while other studies showed enzyme inhibition (Olson & Christensen 1980). *In vitro* studies with aluminium chloride showed an activation of mouse brain AChE (Zatta et al. 2002), while others reported an inhibition of human serum butyrylcholinesterase (Sarkarati et al. 1999). In fish, exposure to cadmium has been shown to cause *in vitro* AChE inhibition, while no effects or an increase of enzyme activity has been found in *in vivo* conditions (Beauvais et al. 2001).

One of the factors that may introduce bias when studying AChE inhibition is the possibility of interference of metals with the methodologies used to measure AChE activity. The technique most frequently used to assess cholinesterase activity is the Ellman's reaction (Ellman et al. 1961), mainly due to its sensitivity and simplicity. The principle of this method is the following. Acetylthiocholine is used as a substrate, the enzyme hydrolyses it into acetate and thiocholine (TCh) and this last compound complexes with the Ellman's reagent (DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid)) to form a mixed disulphide and the yellow chromophore 5-thio-2-nitrobenzoic acid (TNB) (Figure 1). The colour development due to TNB may then be followed photometrically at 412 nm.

It is well known that thiol groups provide chemical targets for metals. Given the described chemical reactions, metals may interfere with Ellman's technique at least by reacting with thiol groups of TCh and/or of TNB, decreasing in both cases the final yellow colour. These interferences may be relevant when measuring the effects of metals on cholinesterase activity, since they contribute to a decrease of the produced colour, which may be erroneously interpreted as enzymatic inhibition.

An additional question when assessing the effects of metals on cholinesterase activity is the buffer choice due to possible interactions between buffer components and metals. In this respect, inorganic buffers such as phosphate, which is generally used either in the sample preparation and/or during the enzymatic assay, can be problematic since metals may be chelated by phosphate ions. In fact, removal and immobilization of aqueous divalent metal cations in the presence of phosphates have been described (Weil & Glaum 2001, Sugiyama et al. 2003), as well as the release of protons upon chelation or precipitation of metal-buffer complexes. In corroboration, some studies revealed no AChE inhibition while testing various metals (e.g. cadmium, iron) when the samples were prepared in phosphate buffer, even with high concentrations of metals, making necessary the use of an alternative buffer (Tris-HCl) (Najimi et al. 1997). Considering this, zwitterionic buffers (e.g. organic amines

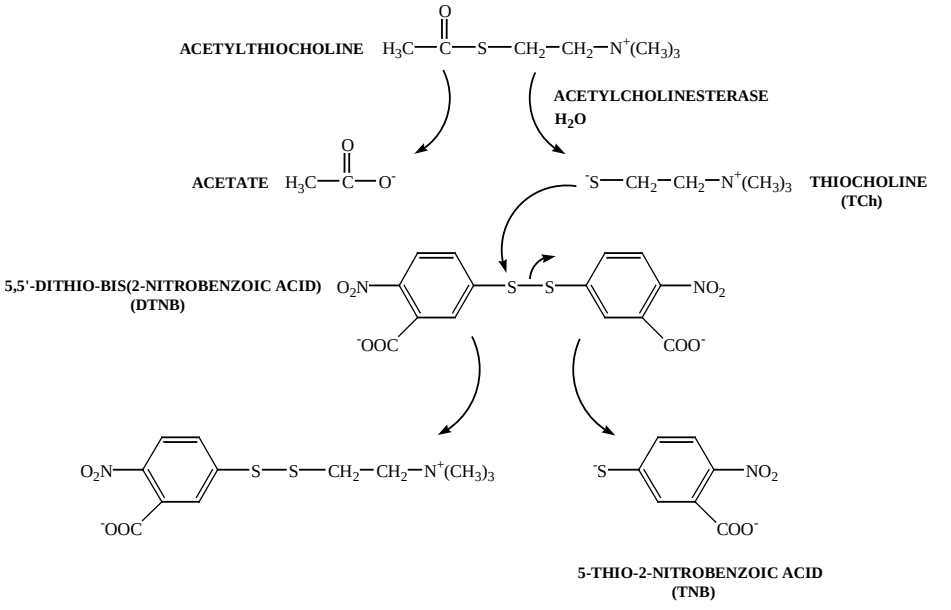

Figure 1. Ellman's chemical reaction used to assess cholinesterase activity.

derivatives) should be a better alternative since, besides having other properties they are weak metal binders (Stevens 1992).

The effect of metals on AChE activity is a major issue due to its implications in biomonitoring studies (particularly in polluted environments where several classes of chemicals may be present) and in toxicity tests with metals when using AChE as effect criteria. Considering this, the main purpose of this work was to investigate the possible interference of metals as test toxicants with the Ellman's assay used to measure AChE activity. Therefore, the *in vitro* effects of five metal ions (zinc(II), nickel(II), copper(II), cadmium(II) and mercury(II)) were independently investigated in separated steps of the Ellman's technique. Furthermore, potential buffer influence in the assessment of the effects of metals on AChE activity was also investigated in two separate stages of the experimental procedure: on the sample preparation and on the Ellman's solution. Two different buffers, phosphate and Tris, were chosen to accomplish this purpose. Finally, an assay using the substrate o-nitrophenyl acetate and Tris buffer was used to confirm the putative inhibitory effects of metals.

Materials and methods

Chemicals

The chemicals potassium dihydrogen phosphate, di-potassium hydrogen phosphate, tris(hydroxymethyl)aminomethane (Tris), zinc chloride, nickel chloride hexahydrate, copper chloride dihydrate, cadmium chloride hydrate and o-nitrophenol were purchased from Merck (Darmstadt, Germany). Mercury chloride, bovine serum albumin, reduced glutathione, dimethyl sulfoxide, acetylthiocholine iodide and DTNB were acquired from Sigma Chemical Co. (St Louis, MO, USA). The substrate o-nitrophenyl acetate was from Research Organics (Cleveland, OH, USA).

TCh preparation. TCh was prepared by enzymatic digestion of acetylthiocholine. *Drosophila melanogaster* AChE (5 units ml^{-1}) was incubated with acetylthiocholine in 25 mM Tris buffer, pH 7.0 for 2 h. The reaction was stopped by enzyme denaturation through 5 min of heating at 95°C. The final concentration of TCh in the cuvette reaction solutions was 65.70 ± 0.44 μM, assessed using reduced glutathione as standard.

TNB preparation. TNB was first prepared by incubation of a concentrated DTNB solution in 0.1 M phosphate buffer, pH 9.0 or 25 mM Tris buffer, pH 9.0. After dilution in both types of buffers at pH 7.2 for phosphate and pH 7.0 for Tris, the final concentration of TNB in the cuvette reaction solutions was 8.351 ± 0.266 μM at 25°C. This value was calculated through TNB molar absorption coefficient (ε), being considered to be the value of 14.15×10^3 M^{-1} cm^{-1}, at 25°C, as described by Eyer et al. (2003).

Enzyme

Truncated cDNA encoding soluble wild type *D. melanogaster* AChE was expressed with the baculovirus system (Chaabihi et al. 1994). Secreted AChE was purified and stabilized with 1 mg ml^{-1} bovine serum albumin as reported by Estrada-Mondaca and Fournier (1998).

Reactivity of metals with phosphate buffer

To investigate the potential reactivity of metals with phosphate buffer, the experimental procedure was divided in two phases. In the first phase, experiments were designed to choose the most suitable buffer (phosphate or Tris) for sample preparation; in the second phase, the possible interference of Ellman's solution prepared in phosphate buffer on AChE activity measurements was investigated.

Buffer choice for sample preparation. In independent experiments, several metal concentrations (up to a maximum final concentration of 10^{-3} M) were added to each of the tested buffers (0.1 M phosphate buffer, pH 7.2 or 25 mM Tris buffer, pH 7.0). Precipitation was observed for all metals after their addition to phosphate buffer. Therefore, in all the subsequent experiments, Tris buffer (25 mM, pH 7.0) was used for sample preparation. In addition, other ions that may be present in AChE samples were previously discarded by exclusion on Sephadex G25 chromatography (PD10, Amersham) to prevent possible interferences with the tested metals.

Possible interference of Ellman's solution prepared in phosphate buffer with AChE activity measurements: to investigate this hypothesis, the effects of metals on AChE activity were assayed using two Ellman's reaction mixtures. One of the mixtures was prepared in phosphate buffer and the other in Tris buffer. A similar approach was used in the fractionated colorimetric experiments of the reactivity of metals with TCh and/or TNB.

Assessment of AChE activity by using Ellman's assay

AChE was incubated for 15 min with each metal, using three different concentrations (0.1, 1 and 10 mM) at 25°C. Remaining activities were estimated after ten-fold

dilution in phosphate (0.1 M, pH 7.2) or Tris (25 mM, pH 7.0) buffers containing 472.5 μM acetylthiocholine and 315.0 μM DTNB. Activities were recorded by measurements of colour development at 412 nm.

Reactivity of metals with TCh and/or TNB

Colorimetric assays were carried out to investigate the reaction between metals and TCh and/or TNB. Metal concentrations (0.01, 0.1 or 1 mM, similar to those present in the revealing solutions of the previous Ellman's assays) were added to TCh (65.70 ± 0.44 μM) in independent experiments. Free TCh was immediately estimated at 412 nm by its reaction with DTNB (315.0 μM). To examine the possible reaction between metals and TNB, three concentrations (0.01, 0.1 or 1 mM) of each metal were added to a solution of TNB (8.351 ± 0.266 μM) in independent assays, and absorbances were immediately measured at 412 nm.

Reactivity of metals with o-nitrophenyl acetate and/or o-nitrophenol

The possible interference of metals either with the substrate o-nitrophenyl acetate (putative conversion into o-nitrophenol) or with o-nitrophenol was investigated. The final concentrations of metals in the cuvette mixture reactions were always 0.01, 0.1 and 1 mM. The substrate o-nitrophenyl acetate was tested at the concentration of 1 mM in 25 mM Tris, pH 7.0 (after dilution of a concentrated solution of 1 M in dimethyl sulfoxide). For o-nitrophenol, the molar extinction coefficient at 405 nm was determined in control conditions (in the absence of metals) ($\varepsilon = 2.014 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 7.0 and 25°C) and possible interference by the tested metals was assessed through significant changes to this value.

Assessment of AChE activity by using o-nitrophenyl acetate as substrate

The inhibition of AChE activity was assessed by incubating the enzyme during 15 min, at 25°C, with 0.1, 1 or 10 mM of each metal. Remaining activities were recorded following ten-fold dilution with 1 mM o-nitrophenyl acetate in 25 mM Tris buffer, pH 7.0. Colour developments, due to production of o-nitrophenol, were followed at 405 nm.

Data analysis

Differences among the effects of treatments of metals and buffers on AChE activity (measured by Ellman's assay), on TCh and/or TNB concentrations were analysed with two-way analysis of variance (ANOVA), followed by Dunnett comparison test when applicable (Zar 1996). For some of the tested samples, precipitation was detected, and thus those concentrations were excluded and not considered in the statistical analysis. The analysis of data comparing the effects of different buffers on the Ellman's assay (in the absence of metals) was performed using a two-tailed Student's *t*-test. Data from o-nitrophenyl acetate assays were analysed through one-way ANOVA and Dunnett comparison test. Figures present data as percentages in relation to the respective control values (in the absence of metals) and not values upon which statistics were based; standard errors (SE) were calculated after an arcsine data

transformation to normalize the variable distribution and reconverted to percentages. All statements of significant differences were based on $\alpha = 0.05$.

Results

Effect of metals and interference of buffers on AChE activity measured by Ellman's assay

The AChE activity measured after 15 min of incubation with metals showed inhibitory effects (Figure 2). These inhibitory effects were statistically significant for all tested concentrations in both reaction buffers, except for 0.1 mM Cd^{2+} in phosphate buffer. For Zn^{2+}, Cu^{2+} and Cd^{2+}, precipitation was visible at the highest tested concentration (10 mM) with phosphate buffer. Samples incubated with 1 mM Hg^{2+} precipitated in both reaction buffers.

Differences of AChE activity between the two buffers within each metal treatment were only significantly different for Cd^{2+} and Hg^{2+}. Furthermore, with the exception of Ni^{2+}, the interaction between buffers and metals were statistically significant and

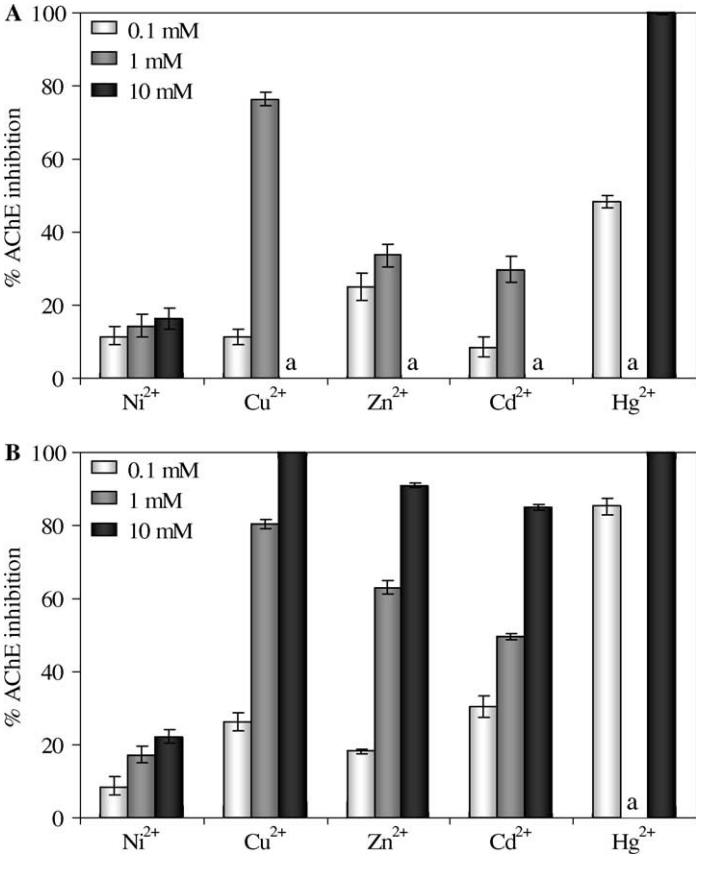

Figure 2. Inhibitory effect of metals on AChE activity (percentage of control values in the absence of metals) assessed with Ellman's assay: (A) experiments in phosphate buffer; (B) experiments in Tris buffer. Each metal was tested at three different concentrations: 0.1, 1 and 10 mM. Results are the mean of three replicates ± SE. a, Presence of precipitates in samples.

affected AChE activity for all the other metals (Table I, first column corresponding to statistical analysis of the two factor effects on AChE activity).

Also, when comparing AChE inhibition between the two buffers (Figure 2A and B), a higher inhibitory effect with Tris buffer was observed, in comparison with the effect obtained in phosphate buffer (with exception of Ni^{2+} and Zn^{2+} at the lowest concentration). These effects did not arise from an interaction between the buffer and the enzyme since the results obtained for AChE activity in control conditions (in the absence of metals) indicated no significant differences between assessments with Ellman's assay in phosphate ($517.0 \pm 6.4\ \mu M\ min^{-1}\ ml^{-1}$ enzyme) and in Tris ($543.0 \pm 23.4\ \mu M\ min^{-1}\ ml^{-1}$ enzyme) buffers ($t_{(4)} = 1.071$, $p > 0.05$).

Reactivity of metals with TCh and/or TNB

Reactivity of metals with TCh was estimated by a decrease in absorbance, upon metal addition to a solution containing TCh and DTNB. This assessment revealed high levels of precipitation with phosphate as the reaction buffer at 10 mM for all the tested metals, except for Ni^{2+}. High levels of precipitation at 1 mM Cu^{2+} and Hg^{2+} with both reaction buffers were also observed (Figure 3). All the tested metals at the assayed concentrations significantly reacted with TCh decreasing the measured absorbances. In addition, the absorbances were significantly affected by the two tested buffers for all metals, and by metal treatments × buffer interaction, but only when testing Cd^{2+} (Table I, second column corresponding to statistical analysis of the two factor effects on TCh concentration). The percentage of complexed TCh was strongly enhanced when testing Cu^{2+}, Cd^{2+} and Hg^{2+}, mainly for the higher concentration with Tris as reaction buffer (Figure 3A and B).

All metals reacted with TNB (Figure 4) but this effect was strongly influenced by the significant interaction with buffer for most of the tested metals, with the exceptions of Ni^{2+} and Hg^{2+} (Table I, third column corresponding to statistical analysis of the two factor effects on TNB concentration). No decreases in the measured absorbances (not graphically represented) were observed for Ni^{2+} at 10 mM. Attending to both buffer conditions, only 1 mM of this metal significantly affected TNB concentration. Hg^{2+} strongly reacted with TNB, leading to a significant decrease of the measured absorbances, using phosphate or Tris buffers. No observable precipitation occurred when assaying TNB at all tested concentrations of this metal. Cu^{2+} at 1 mM significantly interfered with TNB in phosphate buffer, and at 10 mM but only in Tris buffer. The measured absorbances of TNB were diminished in phosphate buffer at 0.1 and 1 mM Zn^{2+} and in Tris buffer at 10 mM. Cd^{2+} at 10 mM in phosphate buffer was the only metal that produced perceptible precipitation in the experiments with TNB. A significant depletion of TNB with 0.1 mM in phosphate buffer and with 10 mM in Tris was observed for this metal (Figure 4A and B).

Reactivity of metals with o-nitrophenyl acetate and/or o-nitrophenol

No chemical interaction was observed between any of the metals and o-nitrophenyl acetate ($F_{(6,14)} = 2.433$, $p > 0.05$). Experiments performed for each metal to investigate possible chemical degradation of the product o-nitrophenol indicated that only Cu^{2+} at 10 mM significantly reduced the measured absorbance in about

Table I. Statistical analysis results.

	AChE activity			TCh concentration			TNB concentration		
	F	d.f.	*p*	*F*	d.f.	*p*	*F*	d.f.	*p*
Ni^{2+} treatments	18.84	3,16	<0.0005	6.791	3,16	<0.005	6.670	3,16	<0.005
Buffers	2.039	1,16	n.s.	58.52	1,16	<0.0005	202.2	1,16	0.0005
Two-way Ni^{2+} treatments × buffers	1.165	3,16	n.s.	0.8083	3,16	n.s.	0.8345	3,16	n.s.
Cu^{2+} treatments	541.1	2,12	<0.0005	1657	1,8	<0.0005	25.54	3,16	<0.0005
Buffers	2.253	1,12	n.s.	9.685	1,8	<0.025	75.88	1,16	<0.0005
Two-way Cu^{2+} treatments × buffers	5.209	2,12	<0.025	0.08004	1,8	n.s.	19.74	3,16	<0.0005
Zn^{2+} treatments	149.5	2,12	<0.0005	16.60	2,12	<0.0005	13.77	3,16	<0.0005
Buffers	2.816	1,12	n.s.	21.93	1,12	<0.001	256.1	1,16	<0.0005
Two-way Zn^{2+} treatments × buffers	25.69	2,12	<0.0005	0.6590	2,12	n.s.	3.904	3,16	<0.05
Cd^{2+} treatments	96.12	2,12	<0.0005	257.0	2,12	<0.0005	10.32	2,12	<0.0025
Buffers	18.05	1,12	<0.0025	202.0	1,12	<0.0005	46.76	1,12	<0.0005
Two-way Cd^{2+} treatments × buffers	10.04	2,12	<0.005	108.2	2,12	<0.0005	6.166	2,12	<0.025
Hg^{2+} treatments	1065	2,12	<0.0005	3039	1,8	<0.0005	241.8	3,16	<0.0005
Buffers	31.85	1,12	<0.0005	17.02	1,8	<0.005	80.50	1,16	<0.0005
Two-way Hg^{2+} treatments × buffers	49.25	2,12	<0.0005	0.5626	1,8	n.s.	2.802	3,16	n.s.

Significant effects (p) and degrees of freedom (d.f. numerator degrees of freedom, denominator degrees of freedom) for AChE activity assessed with Ellman's assay, TCh and TNB concentrations due to metal treatments (each first row), reaction buffers (each second row) and the interaction of these two factors given by the two-way ANOVA metal treatments × buffer (each third row).
n.s., No statistically significant differences ($p > 0.05$).

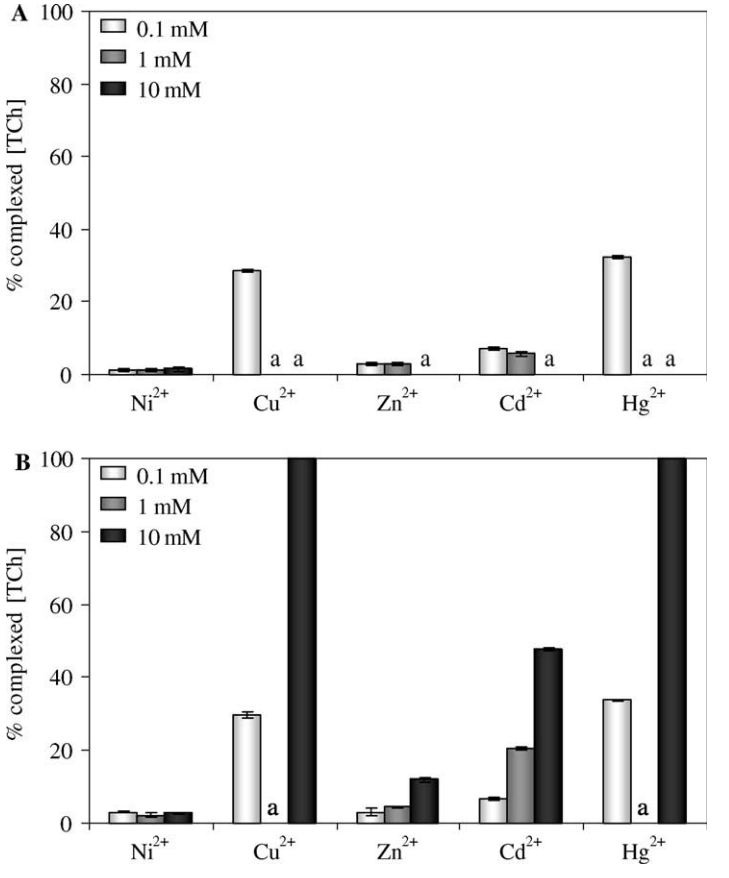

Figure 3. Reactivity of metals with thiocholine (TCh), expressed as a percentage of complexed TCh concentration: (A) experiments in phosphate buffer; (B) experiments in Tris buffer. Each metal was tested at three different concentrations: 0.1, 1 and 10 mM. Results are the mean of three replicates ± SE. a, Presence of precipitates in samples.

50% of the control value. Thus, this concentration was not included in the statistical analysis for Cu^{2+}.

Assessment of AChE activity using o-nitrophenyl acetate as substrate

When using o-nitrophenyl acetate as substrate for the assessment of AChE activity, all metals apart from Ni^{2+} ($F_{(3,11)} = 0.4336$, $p > 0.05$) significantly inhibited this enzyme (Figure 5). Statistical analysis showed that only Cd^{2+} ($F_{(3,11)} = 46.85$, $p < 0.0005$) had no significant effect at the lower concentration of 0.1 mM. Cu^{2+} ($F_{(2,9)} = 36.89$, $p < 0.0005$) at 0.1 and 1 mM significantly inhibited the enzyme. Zn^{2+} ($F_{(3,11)} = 88.29$, $p < 0.0005$) and Hg^{2+} ($F_{(3,11)} = 398.4$, $p < 0.0005$) had a significant influence on AChE activity at all tested concentrations. As indicated by the previous results, inhibition levels, measured using acetylthiocholine and Ellman's assay, were higher than those determined with o-nitrophenyl acetate. This later technique required an enzyme solution around seven times more concentrated than the former. However, this feature is not responsible for the different inhibition levels observed since

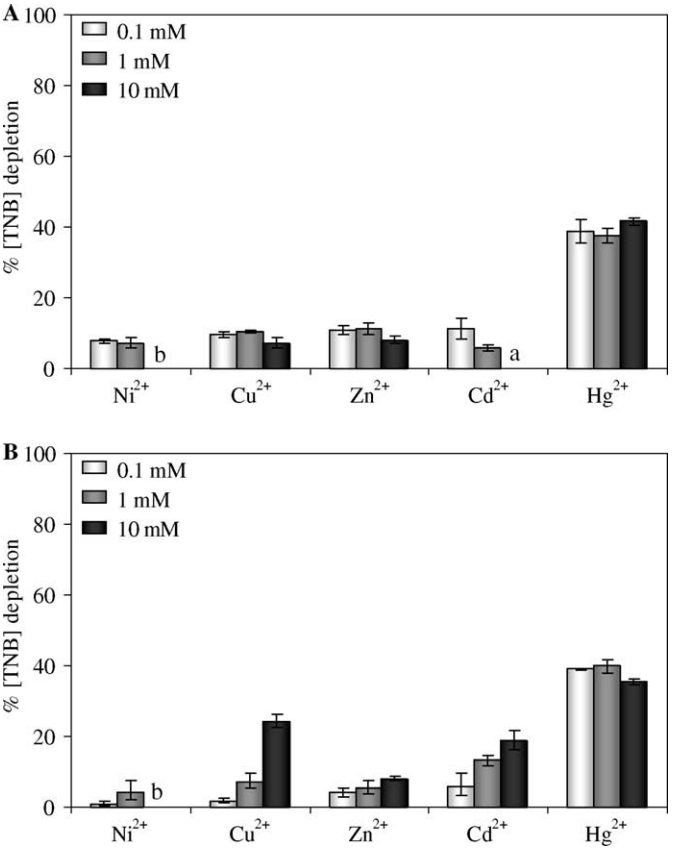

Figure 4. Reactivity of metals with TNB, expressed as a percentage of TNB concentration depletion: (A) experiments in phosphate buffer; (B) experiments in Tris buffer. Each metal was tested at three different concentrations: 0.1, 1 and 10 mM. Results are the mean of three replicates ± SE. a, Presence of precipitates in samples; b, samples where no TNB depletion occurred.

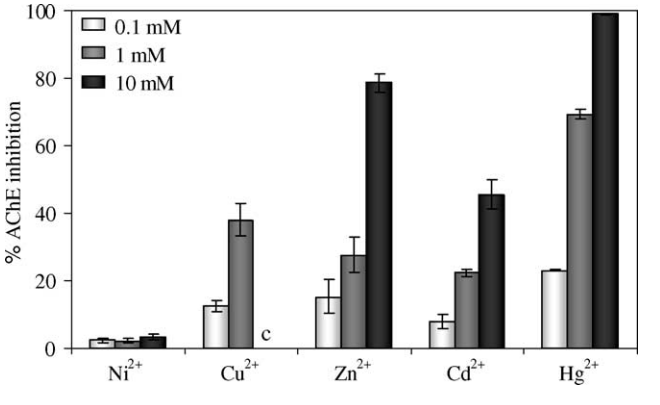

Figure 5. Inhibitory effect of metals on AChE activity (percentage of control values in the absence of metals) assessed with o-nitrophenyl acetate in Tris buffer. Each metal was tested at three different concentrations: 0.1, 1 and 10 mM. Results are the mean of three replicates ± SE. c, Samples where metal interfered with o-nitrophenol.

degradation of both substrates occurred at the same enzyme concentration range ($\mu M\ ml^{-1}$) while metals were tested at mM concentrations.

Discussion

Use of phosphate buffer should be avoided in the assessment of AChE activity in the presence of metals

The majority of the results showed higher AChE inhibition levels when Tris was used to prepare the Ellman's reaction solution, in comparison with the inhibition found in phosphate buffer. These results indicate that the measurement of AChE activity using phosphate buffer may underestimate the effects of metals when tested *in vitro*. Our findings comply with the known ability of metals to form complexes with phosphate anions (Ayati & Madsen 2000, Sugiyama et al. 2003). Furthermore, complex interactions may occur between phosphates and metals in the presence of TCh, producing high levels of precipitation, as discussed below. Therefore, the use of phosphate buffer should be avoided when studying the effects of metals in this type of *in vitro* assays.

Use of acetylthiocholine (which is metabolized into TCh) and DTNB (which is converted into TNB) should be avoided when assessing AChE activity in the presence of metals

The results of the reactivity between metals and TCh and/or TNB revealed that all tested metals tend to react (with different affinities) with thiol groups present in TCh and TNB. Cu^{2+}, Cd^{2+} and Hg^{2+} possess the highest affinities for thiol groups as shown by the elevated reactivity with TCh and TNB, while Ni^{2+} and Zn^{2+} were the metals that showed lowest affinity. The presence of all tested metals chemically decreased the respective free concentrations and the resultant yellow colour. Therefore, the real potential of metals to inhibit AChE enzyme is masked.

The large formation of complexes between metals and TCh accounted for most of the observed precipitates and hindered the measurement of activity. The lack of precipitation with 1 mM Cu^{2+} or Hg^{2+} when testing TNB, in contrast with what occurred by assaying TCh reactivity (in Tris buffer), suggests a large formation of complexes with TCh that seem to be displaced by increasing the metal concentrations. The reaction between Cu^{2+} and TCh is well supported by the histochemical method of Koelle and Friedenwald (1949), further modified by Karnovsky and Roots (1964), which is widely used for localizing cholinesterase activity in histology or in gel electrophoresis. Accordingly, the strong reducing agent TCh reacts with the cupric ion (Cu^{2+}) giving rise to a white precipitate (Koelle's cuprous (Cu^{+}) thiocholine iodide precipitate). Thus, it is possible to hypothesize a similar oxidizing action by the other tested metals, always in accordance with the different affinities for thiol groups showed by each metal. In addition, according to Tsuji and Larabi (1983), the chloride salt of cuprous thiocholine is soluble, whereas the iodide salt of cuprous thiocholine, which was the result of the reaction of Cu^{2+} and TCh used in the assays, produced precipitation.

In the case of TNB, the loss of colour may be due to reoxidation back to DTNB. The oxidation of two thiol groups can occur via two one-electron transfers with subsequent dimerization of the two cysteinyl radicals forming a disulfide (Giles et al. 2003). Thus, the tested metal cations might function as electron acceptors of the

reduced thiol group of TNB, forming reduced ions and cysteinyl radicals able to dimerize and appear as DTNB. At the highest concentration of Ni^{2+}, no significant decrease in the measured absorbance of TNB was observed. It was referred by Tomlinson et al. (1981) that this metal ion can react with DTNB. The resultant complex behaves as an activator of acetylthiocholine hydrolysis, which lead to the false initial identification of Ni^{2+} as an activator of AChE. This type of reactivity might support the results of the interaction between this metal ion and TNB obtained in the present study.

Combination of thiol groups and phosphate ions renders enzyme inhibition difficult to analyse

Depending on the different metal affinities for thiol groups, and on their ability to form stable complexes with phosphates, diverse chemical interferences arose when using Ellman's assay to assess AChE activity in the presence of metals. The high affinity of Hg^{2+} for thiol groups may explain the apparent minor formation of complexes with phosphates in relation to the other metals. In this case, the complete AChE inhibition observed was clearly affected by the interaction between Hg^{2+} and the chemicals used in the Ellman's assay. The previous mentioned precipitation at 1 mM of this cation rendered by TCh reactivity seems to have been intensified in the presence of phosphates in combination with high levels of TCh, originating precipitation also at 10 mM. Cd^{2+} clearly reacted with TCh and TNB decreasing the measured absorbances. These interferences were more obvious when Tris was used as buffer due to the large formation of complexes with phosphates (always precipitating when testing Cd^{2+} at 10 mM in phosphate buffer). The reaction between Cu^{2+} and TCh was evident by the high decrease in absorbance as well as by the occurrence of precipitation, namely at 1 mM in Tris buffer (though it also precipitated at this concentration in phosphate buffer). However, in the previous experiment of AChE inhibition, at 1 mM Cu^{2+} there was no precipitation, suggesting that TCh concentration was not enough to produce precipitated complexes with this particular metal (in contrast with what occurred with Hg^{2+}). Even so, when phosphate buffer was present, in both types of experiments, Cu^{2+} at 10 mM precipitated, which suggests more complicated interactions between this buffer, TCh and the metal. The lack of precipitation in the majority of the experiments with TNB in phosphate buffer, suggested that the presence of TCh might enhance the interactions involving these two compounds and metals and, thus, the potential formation of precipitates. The precipitation of Zn^{2+} at 10 mM only when both TCh and phosphates were present seems to support this hypothesis.

Copper reacts with acetate and o-nitrophenol

When AChE activity was measured with Ellman's method in the presence of Cu^{2+}, the reaction colour changed from yellow to orange indicating that other chemical reactions may have occurred. These results may be due to the reduction of Cu^{2+} to copper(I) (Cu^{+}). Cu^{+} cations may then react with carboxylic acid anions to form copper complexes. These complexes react with oxygen producing the red copper(I) oxide (Cu_2O). These reactions are continuously occurring and are autocatalytic (Cano et al. 1999). In these experimental conditions, the reduction of Cu^{2+} to Cu^{+} could be due to chemical interactions of the first cation with TCh and/or TNB. However, the reaction between Cu^{2+} and TCh precipitates as copper thiocholine as

described above. Using o-nitrophenyl acetate as a substrate, no changes in colour occurred despite the liberation of acetate during the enzymatic reaction. These findings suggest that TNB used in the Ellman's assay may support the reduction of Cu^{2+} to Cu^{+}. In addition, no change in colour was observed in the two experiments with TCh and TNB, supporting that the hypothesized reactions responsible for the alteration in colour require the presence and activity of the enzyme. Thus, it is possible that the reduced Cu^{+}, after reaction with TNB, reacted with the acetate produced during the enzymatic degradation of acetylthiocholine giving rise to Cu_2O. The precipitate, characteristic of this compound, was not observed probably because of its low concentration. Even so, the mixture of its red colour and the yellow from the remaining TNB may account for the final visible colour.

From all the tested metals, Cu^{2+} at the concentration of 10 mM was the only one that significantly reacted with the product o-nitrophenol. This concentration was not considered for statistical analysis, since the interference may also depend on their chemical concentrations available in the reaction medium.

Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} inhibit AChE

The results of AChE activity measured with Ellman's assay suggested high levels of inhibition for almost all of the tested metals. However, with this technique it was impossible to ascertain the artefactual contribution of the interaction of the metals with the technique, in the measurement of AChE inhibition. This constitutes a major obstacle in obtaining accurate data. In this work, an alternative method to assess the *in vitro* effects of metals on AChE activity was used. With o-nitrophenyl acetate as substrate and comparing AChE inhibition levels with those obtained by the Ellman's assay it was possible to verify the interference of metals with this last method, since AChE inhibition levels were lower and no precipitation occurred when using o-nitrophenyl acetate. These results may be explained by the inexistence of sulfhydryl reagents and thiol interchange reactions, avoiding in this way the complex reactivity of metals. Although metals do not react with the substrate acetylthiocholine, they highly interfered with the enzymatic reaction products of Ellman's assay. These interferences depend on the available metal after enzyme incubation and on TCh and TNB concentrations produced during the enzymatic reaction. The accurate and reliable establishment of reaction blanks was not possible due to the complexity of these chemical interferences. When using the substrate o-nitrophenyl acetate, AChE converts the substrate in a product that can be photometrically followed. Any possible interference is easier to detect, but may vary depending on the chemical concentrations able to react. Furthermore, o-nitrophenyl acetate is a substrate for esterases other than cholinesterases. It is, therefore, only possible to measure cholinesterase activity with purified enzymes or after a previous verification of the absence of other esterases in the sample tissue. This could constitute an added difficulty in routine AChE measurements. However, enzymatic characterization is often a prerequisite to the use of cholinesterase activity in field surveys and as effect criteria in laboratory studies (Pessah & Sokolove 1983, Sturm et al. 1999, Rodríguez-Fuentes & Gold-Bouchot 2004). The disadvantage of using o-nitrophenyl acetate as a substrate is related with the lower enzymatic affinity for it, in comparison with acetylthiocholine, requiring higher concentration of enzyme in the samples subjected to analysis. Measurements of AChE activity with o-nitrophenyl acetate made it possible to

conclude unmistakably that all the metals significantly inhibit AChE with the exception of Ni^{2+}. The fact that Ni^{2+} does not inhibit AChE activity is important since one of the standard AChE purification techniques uses histidine residues as an affinity tag, genetically engineered in recombinant proteins, which bind with high affinity Ni^{2+} ions immobilized on chelating resins (Paborsky et al. 1996). In addition, in the development of biosensors the oriented and reversible immobilization of proteins is based on the ability of Ni^{2+} to bind a histidine tail on the recombinant enzyme (Andreescu et al. 2001).

From an ecological point of view, the tested metal concentrations (minimum of 6 ppm for Ni^{2+} and maximum of 2000 ppm for Hg^{2+}) may be considered high. However, they are relevant due to three main reasons. First, in polluted environments such as estuaries and mining areas, it is possible to find concentrations of metals in the ppm range. For example, values of more than a thousand ppm of copper or zinc and more than a hundred ppm of cadmium have been found in sediments and suspended matter (Irato et al. 2003, Smolders et al. 2003, Usero et al. 2003). These environmental compartments can act as a source of metals when environmental conditions change (e.g. pH, sediment redox potential). Second, metals accumulate in the tissues of organisms, where they may reach considerably higher concentrations than those found in the surrounding medium. For instance, high concentrations of metals were found in chironomids (196 ppp cadmium, 5177 ppm zinc, 227 ppm copper) (Smolders et al. 2003), in marine bivalve molluscs (60 ppm cadmium, 3700 ppm zinc, 643 ppm copper) (Ahn et al. 1996) and in soil invertebrates (152 ppm cadmium, 488 ppm zinc, 567 ppm copper) (Cortet et al. 1999). Third, single metal concentrations were tested in this study, whereas complex mixtures of metals normally occur in the environment, therefore, toxicological interactions (e.g. additive, synergistic) are likely to occur in these conditions.

For the stated reasons, tissues for AChE determination may contain considerable amounts of metals, which may interfere with measurements of the enzymatic activity if the Ellman's method is used, as demonstrated in the present study.

In conclusion, the findings of the current study demonstrate that the metal ions of mercury, cadmium, copper and zinc inhibit AChE activity. In addition, they show that it is preferable to use the assay with o-nitrophenyl acetate as substrate to assess the effects of metals on AChE than the Ellman's method. Moreover, the effects of metals on AChE should be taken into consideration when using this enzyme as an environmental biomarker, particularly in environments polluted with several classes of chemicals.

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References

Ahn IY, Lee SH, Kim KT, Shim JH, Kim DY. 1996. Baseline heavy metal concentrations in the Antarctic Clam, *Laternula elliptica* in Maxwell Bay, King George Island, Antarctica. Marine Pollution Bulletin 32:592–598.

Andreescu S, Magearu V, Lougarre A, Fournier D, Marty J-L. 2001. Immobilization of enzymes on screen-printed sensors via an histidine tail. Application to the detection of pesticides using modified cholinesterase. Analytical Letters 34:529–540.

Ayati M, Madsen HEL. 2000. Crystallization of some heavy-metal phosphates alone and in the presence of calcium ion. Journal of Crystal Growth 208:579–591.

Beauvais SL, Jones SB, Parris JT, Brewer SK, Little EE. 2001. Cholinergic and behavioural neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). Ecotoxicology and Environmental Safety 49:84–90.

Cano E, Simancas J, Polo JL, Torres CL, Bastidas JM, Alcolea J. 1999. Early corrosion failure of copper tubing used in air-conditioning units. Materials and Corrosion 50:103–110.

Carr RL, Chambers JE. 1996. Kinetic analysis of the *in vitro* inhibition, aging, and reactivation of brain acetylcholinesterase from rat and channel catfish by paraoxon and chlorpyrifos-oxon. Toxicology and Applied Pharmacology 139:365–373.

Chaabihi H, Fournier D, Fedon Y, Bossy JP, Ravallec M, Devauchelle G, Cerruti M. 1994. Biochemical characterization of *Drosophila melanogaster* acetylcholinesterase expressed by recombinant baculoviruses. Biochemistry and Biophysics Research Communications 203:734–742.

Cortet J, Vauflery AG-D, Poinsot-Balaguera N, Gomot L, Texier C, Cluzeau D. 1999. The use of invertebrate soil fauna in monitoring pollutant effects. European Journal of Soil Biology 35:115–134.

Devi M, Fingerman M. 1995. Inhibition of acetylcholinesterase activity in the central nervous system of red swamp cray fish, *Procambarus clarkia* by mercury, cadmium and lead. Bulletin of Environmental Contamination and Toxicology 55:746–750.

Diamantino TC, Almeida E, Soares AMVM, Guilhermino L. 2003. Characterization of cholinesterases from Daphnia magna Straus and their inhibition by zinc. Bulletin of Environmental Contamination and Toxicology 71:219–225.

Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7:88–95.

Estrada-Mondaca S, Fournier D. 1998. Stabilization of recombinant Drosophila acetylcholinesterase. Protein Expression and Purification 12:166–172.

Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, Reiner E. 2003. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Analytical Biochemistry 312:224–227.

Giles NM, Watts AB, Giles GI, Fry FH, Littlechild JA, Jacob C. 2003. Metal and redox modulation of cysteine protein function. Chemistry and Biology 10:677–693.

Guilhermino L, Barros P, Silva MC, Soares AMVM. 1998. Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3:157–163.

Irato P, Santovito G, Cassini A, Piccinni E, Albergoni V. 2003. Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice. Archives of Environmental Contamination and Toxicology 44:476–484.

Karnovsky MJ, Roots L. 1964. A 'direct-coloring' thiocholine method for cholinesterases. Journal of Histochemistry and Cytochemistry 12:219–221.

Key PB, Fulton MH. 2002. Characterization of cholinesterase activity in tissues of the grass shrimp (*Palaemonetes pugio*). Pesticide Biochemistry and Physiology 72:186–192.

Koelle GB, Friedenwald JS. 1949. A histochemical method for localizing cholinesterase activity. Proceedings of the Society of Experimental Biology and Medicine 70:617–622.

Labrot F, Ribera D, Saint MD, Narbonne JF. 1996. *In vitro* and *in vivo* studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. Biomarkers 1:23–30.

Najimi S, Bouhaimi A, Daubèze M, Zekhnini A, Pellerin J, Narbonne JF, Moukrim A. 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir Marine Bay (South of Morocco). Bulletin of Environmental Contamination and Toxicology 58:901–908.

Olson DL, Christensen GM. 1980. Effects of water pollutants and other chemicals on fish acetylcholinesterase (*in vitro*). Environmental Research 21:327–335.

Paborsky LR, Dunn KE, Gibbs CS, Dougherty JP. 1996. A nickel chelate microtiter plate assay for six histidine-containing proteins. Analytical Biochemistry 234:60–65.

Payne JF, Mathieu A, Melvin W, Fancey LL. 1996. Acetlcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Marine Pollution Bulletin 32:225–231.

Pessah IN, Sokolove PG. 1983. Determination and characterization of cholinesterases in localized tissues of the giant garden slugs *Limax maximus* (Linnaeus). Comparative Biochemistry and Physiology 74C:281–289.

Porcelli F, Delfini M, Del Giudice MR. 1999. The kinetic inhibition of acetylcholinesterase from human herytrocyte by tacrine and some tacrine derivatives. Bioorganic Chemistry 27:197–205.

Rodríguez-Fuentes G, Gold-Bouchot G. 2004. Characterization of cholinesterase activity from different tissues of Nile tilapia (*Oreochromis niloticus*). Marine Environment Research 58:505–509.

Romani R, Antognelli C, Baldracchini F, De Santis A, Isani G, Giovannini E, Rosi G. 2003. Increased acetylcholinesterase activities in specimens of *Sparus auratus* exposed to sublethal copper concentrations. Chemico-Biological Interactions 145:321–329.

Sanz P, Repetto M. 1995. Implicaciones toxicologicas de las enzimas colinesterasas. In: Repetto M, editor. Toxicologia avanzada. Madrid: Diaz de Santos. p. 117–145.

Sarkarati B, Çokuğraş AN, Tezcan EF. 1999. Inhibition kinetics of human serum butyrylcholinesterase by Cd^{2+}, Zn^{2+} and Al^{3+}: comparison of the effects of metal ions on cholinesterases. Comparative Biochemistry and Physiology 122C:181–190.

Smolders AJP, Lock RAC, Van der Velde G, Hoyos RIM, Roelofs JGM. 2003. Effects of mining activities on heavy metal concentrations in water, sediment, and macroinvertebrates in different reaches of the Pilcomayo River, South America. Archives of Environmental Contamination and Toxicology 44:314–323.

Stevens L. 1992. Buffers and the determination of protein concentrations. In: Eisenthal R, Danson MJ, editors. Enzyme assays — a practical approach. New York, NY: Oxford University Press. p. 317–335.

Sturm A, Silva de Assis HC, Hansen P-D. 1999. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Marine Environmental Research 47:389–398.

Sugiyama S, Ichii T, Fujisawa M, Kawashiro K, Tomida T, Shigemoto N, Hayashi H. 2003. Heavy metal immobilization in aqueous solution using calcium hydrogen phosphates. Journal of Colloid Interface Science 259:408–410.

Suresh A, Sivaramakrishna B, Victoriamma PC, Radhakrishnaiah K. 1992. Comparative study on the inhibition of acetylcholinesterase activity in the freshwater fish *Cyprinus carpio* by mercury and zinc. Biochemistry International 26:367–375.

Tomlinson G, Mutus B, McLennan I. 1981. Activation and inactivation of acetylcholinesterase by metal ions. Canadian Journal of Biochemistry 59:728–735.

Tsuji S, Larabi Y. 1983. A modification of thiocholine-ferricyanide method of Karnovsky and Roots for localization of acetylcholinesterase activity without interference by Koelle's copper thiocholine iodide precipitate. Histochemistry 78:317–323.

Usero J, Izquierdo C, Morillo J, Gracia I. 2003. Heavy metals in fish (*Solea vulgaris*, *Anguilla anguilla* and *Liza aurata*) from salt marshes on the southern Atlantic coast of Spain. Environment International 29:949–956.

Weil M, Glaum R. 2001. Mercury phosphates with the triangular Hg_3^{4+} cluster: $(Hg_3)_3(PO_4)_4$ and $(Hg_3)_2(HgO_2)(PO_4)_2$. Journal of Solid State Chemistry 157:68–75.

Zar JH. 1996. Biostatistical analysis. 3th ed. Englewood Cliffs, NJ: Prentice-Hall.

Zatta P, Ibn-Lkhayat-Idrissi M, Zambenedetti P, Kilyen M, Kiss T. 2002. *In vivo* and *in vitro* effects of aluminium on the activity of mouse brain acetylcholinesterase. Brain Research Bulletin 59:41–45.