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Effects of xenobiotic compounds on the cell activities of *Euplotes crassus*, a single-cell eukaryotic test organism for the study of the pollution of marine sediments

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Abstract

It is now widely accepted that assays with protists are relevant to be exploited for the study of environmental modifications due to the presence of xenobiotic compounds. In this work, the possibility of utilizing *Euplotes crassus*, an interstitial marine ciliate, for the pre-chemical screening of estuarine and coastal sediments was evaluated. For this purpose, the effects of exposure to pollutants were tested on the cell viability, fission rate and lysosomal membrane stability of *E. crassus*. The following toxicants were used: an organophosphate (OP) pesticide, basudin, an organochlorine hydrocarbon, AFD25, both employed especially for pest control in agricultural sites, a toxic heavy metal, mercury ($HgCl_2$) and different mixtures of the above-mentioned compounds, as they might occur in polluted sites. Exposure to these toxicants affected cell viability at concentrations ranging from 96.6 to 966 $\times 10^3$ mg/l for basudin, from 3.3 to 33 $\times 10^3$ mg/l for AFD25 and from 0.1 to 1 mg/l for $HgCl_2$. A significant decrease in the mean fission rate ($P < 0.001$) was found after 24- or 48-h exposures to 9.66 mg/l basudin, 3.3 mg/l AFD25 and 7 $\times 10^{-2}$ mg/l $HgCl_2$. Furthermore, the Neutral Red Retention Assay showed a significant decrease in lysosomal membrane stability after 60- and 120-min exposures to AFD25 (33 mg/l) and $HgCl_2$ (0.33 mg/l). In addition, as it is well-known that the inhibition of acetylcholinesterase activity represents a specific biomarker of exposure to OP and carbamate pesticides in higher organisms, initially the presence of cholinesterase (ChE) activity was detected in *E. crassus*, using cytochemical, spectrophotometric and electrophoretic methods. Afterwards, this enzyme activity was characterized spectrophotometrically by its sensitivity to specific ChE inhibitors and to variations in pH and temperature. The ChE activity was inhibited significantly by basudin- (9.66 and 96.6 mg/l) or AFD25-exposure (3.3 mg/l). Conversely, exposure to AFD25 (33 mg/l) or $HgCl_2$ (0.1 and 0.3 mg/l) caused a significant increase in this enzyme activity. Moreover, exposure to mixtures containing basudin, AFD25 and $HgCl_2$ was found to affect the cell viability, the mean fission rate and the ChE activity differently, in an unpredictable manner. Our results indicate that *E. crassus* seems to be a suitable test organism to evaluate the toxicity of marine sediments.

Keywords: Cholinesterase activity; Toxicity tests; Xenobiotic compounds; Pollution of marine sediments; Ciliated protista; *Euplotes crassus*

1. Introduction

The notable increase that has occurred in the world's population during the last century and its concentration in urban areas and the concomitant development of agricultural and industrial activities have caused a considerable increment in wastes of anthropogenic origin and, consequently, the presence of pollutants in marine sediments. It has been observed that the pollutant concentration in the interstitial sediment water and sediments is more than 10 times higher than that present in the overlying water column (McMahon, 1989). For mercury, concentrations of 117 and of 10 ng/l were detected in the interstitial waters and water column, respectively, of a lagoon (Ramalhosa et al., 2006).

Interest in the toxicity of pesticides, such as organophosphate (OP) and organochlorine (OC) compounds, has increased during the last two decades as they enter waterways from agricultural and urban runoff and may be transported to estuaries and coastal sites. Thus, pesticides contribute greatly to marine environment pollution, potentially causing harm to a large variety of non-target wildlife organisms (Walker et al., 2001). In addi-

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tion, the presence of toxic metals, such as mercury, cadmium, nickel and chromium has been found in industrial wastewaters often in high concentrations: 1.6–7.6 mg/l for mercury (Canstein von et al., 1999), 0.01–0.02 mg/l for cadmium, 0.07–0.11 mg/l for nickel and 0.09 mg/l for chromium (Kirk and Lester, 1984).

It is generally agreed that in the field of environmental biomonitoring, toxicological bioassays can provide useful information for identifying those situations requiring a close investigation at an early stage (Luoma and Ho, 1993; Hall and Giddings, 2000). From this point of view, it is of increasing interest to identify a panel of organisms displaying direct and sensitive responses to environmental perturbations. Assays with protists are regarded as valuable bioassays to be exploited in standardized laboratory procedures for evaluating the toxicity of chemical compounds or polluted waters (Apostol, 1973; Persoone and Dive, 1978; Lynn and Gilron, 1992; Ricci, 1995; Sauvant et al., 1999; Miyoshi et al., 2003).

Many studies of the toxic effects of pollutants on protists have taken into account heavy metals, such as mercury, cadmium, zinc, lead and copper, because their concentrations have increased to toxic levels in various ecosystems in recent years due to anthropogenic activity (Dini, 1981; Piccinni et al., 1987; Nilsson, 1989; Madoni et al., 1994; Viarengo et al., 1996; Ricci et al., 1997; Coppellotti, 1998; Gutiérrez et al., 2003). Due to their nature as a eukaryotic cell/organism, protists exhibit a relatively simple organization and a high degree of specialization. Protists respond directly to environmental stimuli, such as mechanical, chemical, heat and light stimuli (Anderson, 1987), thus behaving like animals as selection units towards environmental challenges. On the other hand, as single cells directly exposed to the external environment, protists are more sensitive to environmental modifications (Gutiérrez et al., 2003) than the cells of higher organisms that have differentiated and organized complex structures, organs, so that the cell responses vary depending on the site and function of the cell. However, protist responses can be related to those of higher organisms, because of their nature as the eukaryotic ancestors of all metazoa. Due to their small size, protists generally multiply through short cell-cycles, thus making it possible to study the effects of pollutants on a large and genetically homogeneous cell population over a short time period as well as on subsequent cell generations. In addition, the absence of a cell wall in the vegetative stage allows protists to respond faster to stimuli than bacteria and yeasts. Moreover, several species of protists can be cultured in the laboratory under seemingly natural conditions, so that their biological responses are more reliable.

Not only are protists a very conspicuous component of the planktonic and benthic microecosystems inhabiting marine and freshwater environments, but they also play a key role in the trophic chain (Fenchel, 1987). Numerous species feed on bacteria and are prey of higher organisms. As a consequence, contaminants can be potentially transferred along food chains and affect organisms at higher trophic levels, eventually also leading to adverse effects on human health (Patrick and Loutit, 1976; Swartz and Lee, 1980). On the other hand, an alteration in the protist-component of microbial communities caused by the lethal effects of toxicants can alter the trophic chain and significantly affect the environmental balance. On the basis of these considerations, protist responses in toxicity tests performed in the laboratory under strictly controlled conditions may be utilized as a predictive tool for risk assessment in natural ecosystems (Delmonte Corrado et al., 2005; Trielli et al., 2006).

In this work, the possibility of utilizing *Euplotes crassus*, an interstitial marine ciliate, for the pre-chemical screening of estuarine and coastal sites under anthropic pressure was examined. For this purpose, the following xenobiotic contaminants were considered: basudin, an OP pesticide, AFD25, an OC hydrocarbon, both extensively used on agricultural sites as insecticides, mercury ($HgCl_2$), a toxic heavy metal and different mixtures of these compounds. The effect of the exposure to low concentrations of these toxicants has been evaluated on the cell viability, fission rate and lysosomal membrane stability of *E. crassus*. Lysosomal membrane integrity is considered as a general cytological biomarker of exposure to a wide range of inorganic and organic toxicants. Toxicants can destabilize the membranes of lysosomes, the cell organelles involved in compartmentalization of xenobiotics, causing the release of hydrolytic enzymes (Nicholson, 2003), as reported in eukaryotes (Moore et al., 2006) including protists (Dondero et al., 2006).

Our previous studies have shown the presence of cholinesterase (ChE) enzyme activities in protists, such as *Paramecium primaurelia* (Trielli et al., 1997; Delmonte Corrado et al., 1999, 2001) and *Dictyostelium discoideum* (Falugi et al., 2002; Amaroli et al., 2003). These activities have been found to be sensitive to exposure to classical anticholinergic agents and OP compounds (Falugi et al., 2002; Delmonte Corrado et al., 2005). In this work, the ChE activities have been detected in *E. crassus*, using cytochemical, spectrophotometric and electrophoretic methods, and characterized spectrophotometrically by their sensitivity to specific ChE inhibitors and to variations in chemical–physical parameters, such as pH and temperature. Several studies of invertebrates and vertebrates have shown that inhibition of acetylcholinesterase (AChE) activity represents a specific biomarker of exposure to OP and carbamate pesticides (Hassal, 1990). OPs phosphorylate the serine of the enzyme active site, making AChE unable to hydrolyze the choline esters (Sultatos, 1994). Therefore, the effect of basudin-exposure has been evaluated on the ChE activity of *E. crassus*, as well as the sensitivity of this enzyme activity to exposure to AFD25, $HgCl_2$ or mixtures of these compounds.

2. Material and methods

2.1. Cell culturing

E. crassus cells, strain SSt_{22}, kindly supplied by Prof. Fernando Dini, University of Pisa, were grown in artificial seawater (16 mM $MgCl_2$, 5 mM $CaCl_2$, 473 mM NaCl, 6.2 mM KCl, 1.5 mM $NaHCO_3$, 18 mM Na_2SO_4, 0.045 mM NaF, 0.56 mM KBr, 0.32 mM H_3BO_3, 0.048 mM $SrCl_2{\cdot}6H_2O$, pH 8.0), containing 0.5 g/l of proteose-peptone, and inoculated with *Enterobacter aerogenes*. The cells, cultured under dark con-

ditions, were analyzed during their logarithmic growth phase. All the experiments were carried out at 24 °C.

2.2. Chemicals

The following compounds were tested: basudin (Cyba-Geigy, I), a formulate insecticide, containing 20% diazinon, an active thionophosphate principle, diluted in artificial seawater at the final concentrations of 9.66, 96.6, 9.66×10^2, 9.66×10^3, 96.6×10^3 and 966×10^3 mg/l (the basudin concentration of 9.66 mg/l contains 1.93 mg/l of diazinon that corresponds to 6.35×10^{-6} M); AFD25 (Cifo, I), an OC hydrocarbon, containing 24% dicofol, an OC active principle, diluted once in chloroform to obtain the stock solution and then in artificial seawater at the final concentrations of 0.33, 3.3, 33, 3.3×10^2, 3.3×10^3 and 33×10^3 mg/l (the AFD25 concentration of 0.33 mg/l contains 0.08 mg/l of dicofol that corresponds to 2.13×10^{-7} M); $HgCl_2$ (Sigma, I) diluted in artificial seawater at the final concentrations of 3×10^{-2}, 7×10^{-2}, 0.1, 0.3, 0.5 and 1 mg/l (the $HgCl_2$ concentration of 3×10^{-2} mg/l contains 2.2×10^{-2} mg/l of mercury that corresponds to 1.1×10^{-7} M). Three mixtures (MIXs) of the above-mentioned compounds were prepared to obtain the following final concentrations: basudin 96.6 mg/l, AFD25 3.3 mg/l and $HgCl_2$ 7×10^{-2} mg/l (MIX1); basudin 9.66 mg/l, AFD25 0.33 mg/l and $HgCl_2$ 3×10^{-2} mg/l (MIX2); basudin 96.6 mg/l and AFD25 3.3 mg/l (MIX3).

The half-life of diazinon, the active principle of basudin, in water is of the order of 5–15 days (International Programme on Chemical Safety). Dicofol, the active principle of AFD25, has a half-life in solution at pH 5.0 of 47–85 days and is expected to adsorb to sediment when released into open waters, because of its very high absorption coefficient (Howard, 1991).

2.3. Toxicity tests

To evaluate the effect on cell viability, ten groups of five logarithmically growing cells were transferred into depression slides containing bacterized artificial seawater (i.e. inoculated with *E. aerogenes*) and basudin (9.66, 96.6, 9.66×10^2, 9.66×10^3, 96.6×10^3 and 966×10^3 mg/l) or AFD25 (0.33, 3.3, 33, 3.3×10^2, 3.3×10^3 and 33×10^3 mg/l) or $HgCl_2$ (3×10^{-2}, 7×10^{-2}, 0.1, 0.3, 0.5 and 1 mg/l) or MIX1 or MIX2 or MIX3. Ten groups of five cells were transferred into depression slides containing only bacterized artificial seawater and used as controls. The cultures were kept under observation for 60 min or 24 h to check for the number of viable cells. For each toxicant concentration, the experiments were carried out at least in triplicate. For each toxicant, the median lethal concentration (LC_{50}) after a 60-min or 24-h exposure (60-min LC_{50} or 24-h LC_{50}, respectively) was computed on the mean values, using the Trimmed Spearman-Karber method. Statistical analysis has been performed, using a two-way ANOVA followed by the Student-Newman-Keuls multicomparison test to discriminate statistically significant treatments.

To evaluate the effect on the mean fission rate, 20 logarithmically growing cells were individually isolated in depression slides containing bacterized artificial seawater and basudin (9.66 mg/l) or AFD25 (0.33 or 3.3 mg/l) or $HgCl_2$ (3×10^{-2} or 7×10^{-2} mg/l) or MIX1 or MIX2 or MIX3. Twenty cells were individually isolated in depression slides containing only bacterized seawater and used as controls. The mean fission rate was measured as follows: $n = \log_2 n_x$, where n is the number of fissions performed after 24 or 48 h, n_x is the number of cells found after 24 or 48 h and derived from a single cell isolated in a depression slide. The daily mean fission rate of a logarithmically growing cell culture was approximately 2.6 fissions. Each experiment was carried out at least in triplicate. Statistical analysis was performed using a two-way ANOVA followed by the Student-Newman-Keuls multicomparison test to discriminate statistically significant treatments.

Moreover, to evaluate the possible effect of the chloroform used to prepare the AFD25 stock solution, three groups of five logarithmically growing cells were transferred into depression slides containing bacterized artificial seawater and chloroform at the final concentrations of 0.001, 0.01, 0.1, 1 or 10 μl/ml, which corresponded to the chloroform concentrations present in the 0.33, 3.3, 33, 3.3×10^2 or 3.3×10^3 mg/l AFD25 solutions used for the cell viability tests. Likewise, six logarithmically growing cells were isolated in depression slides containing bacterized artificial seawater and chloroform at the final concentrations of 0.001 or 0.01 μl/ml, corresponding to the chloroform concentrations present in the 0.33 or 3.3 mg/l AFD25 solutions used for the fission rate tests.

2.4. Lysosomal membrane stability tests

To evaluate the lysosomal membrane stability, the Neutral Red Retention (NRR) assay was used (Moore, 1985; Lowe et al., 1992). As healthy cell lysosomes retain the vital NR dye for some length of time, dye retention times represent a marker of membrane permeability.

From a stock solution (2×10^4 mg/l in DMSO) of NR in artificial seawater, 0.5 μl were added to aliquots of 500 μl of logarithmically growing cell cultures of *E. crassus*. After 15 min, the cells were washed and resuspended in artificial seawater. Then, aliquots of 30 μl were transferred onto microscope slides and exposed to basudin (final concentrations of 9.66 or 96.6 mg/l) or AFD25 (final concentrations of 3.3 or 33 mg/l) or $HgCl_2$ (final concentrations of 0.1 or 0.3 mg/l) or MIX1 or MIX2 or MIX3. Likewise, aliquots of 30 μl were transferred onto microscope slides and used as controls. At various times up to 120 min, the slides were observed under an Olympus IMT-2 inverted microscope equipped with a charge-coupled device CUE video camera, and the images were recorded with a Dage MTI camera and digitized with the CUE2 imaging system (Galai Production, Israel). The digitized images allowed the evaluation of the NRR time within the lysosomes. The lysosomal membrane stability was expressed as the optical density (OD) percentage of the control value, computed after 60- or 120-min exposures to the toxicants.

Each experiment was carried out at least in triplicate. Statistical analysis was performed using a two-way ANOVA followed

by the Student-Newman-Keuls multicomparison test to discriminate statistically significant treatments.

2.5. Detection and characterization of ChE activities

Depending on the species, different ChEs were detected and discriminated on the basis of their catalytic properties, according both to the substrate cleaving their activity and to their sensitivity to specific inhibitors (Mendel and Rudney, 1943; Talesa et al., 1990). The AChE, also referred to as 'true' AChE (E.C. 3.1.1.7), present in higher organisms with specialized synapses (Massoulié et al., 1993), hydrolyzes acetylcholine (ACh) with high affinity, and the acetyl-β-methyl thiocholine iodide (AcTChI) substrate. The AChE enzyme activity is inhibited by eserine, a carbamate compound and, more specifically, by BW284c51, a phenol ester. Other choline esters, also referred to as 'pseudocholinesterases', are butyrylcholinesterase (BChE, E.C. 3.1.1.8) and propionylcholinesterase (PrChE). The BChE activity preferentially hydrolyzes butyrylcholine and the butyryl thiocholine iodide (BTChI) substrate (Talesa et al., 1990), whereas the PrChE activity cleaves propionyl thiocholine iodide (PrTChI) as a preferential substrate. Both BChE and PrChE enzyme activities are inhibited by eserine and, more specifically, by iso-OMPA, a phosphoramide compound.

The ChE activities of *E. crassus* were checked for with cytochemical, spectrophotometric and electrophoretic methods and characterized spectrophotometrically by their sensitivity to specific ChE inhibitors and to variations in chemico-physical parameters, such as pH and temperature.

2.5.1. Cytochemical method for detecting ChE activities

The cells were fixed with 2% paraformaldehyde, for 30 min at 24 °C, washed in maleate buffer and transferred onto microscope slides. The samples were incubated overnight at 4 °C with the reaction medium, according to Karnovsky and Roots (1964). The substrates employed were AcTChI, PrTChI or BTChI (Sigma, I), at a concentration of 0.002 M. After treatment, the samples were washed in 0.1 M phosphate-buffered saline, pH 7.4, dehydrated, transferred onto slides, mounted in Eukitt and examined under an Olympus optical microscope.

2.5.2. Spectrophotometric evaluation of ChE activities

The cells were centrifuged at 1660 × *g* for 10 min and 8 μl of pellet were resuspended in 400 μl of 0.2 M phosphate buffer, pH 8.0. Proteins were extracted in 0.5% Triton X-100. The ChE activities were recorded at λ = 412 nm with a Uvikon 930 spectrophotometer (Kontron Instruments I), for the first 10 min after exposure to AcTChI, PrTChI or BTChI, used as substrates at a concentration of 0.025 M, according to the modified method of Ellman et al. (1961). The total protein content was evaluated spectrophotometrically using the Biorad System (Biorad Microscience, Cambridge, MA) and bovine serum albumin was used as a standard, according to the manufacturer's instructions. The ChE activities were expressed as ChE units. One ChE unit hydrolyzes 1 μmol of acetyl/ or propionyl/ or butyryl group/min at pH 8.0 at 24 °C. Each experiment was carried out at least in triplicate.

2.5.3. Non-denaturing electrophoretic detection of ChE activities

The cells were centrifuged at 1660 × *g* for 10 min, and 30 μl of pellet were lysed by freezing at −80 °C for 10 min. The membrane fraction was obtained by centrifuging at 15,000 × *g* for 10 min. Proteins were extracted in 1% Triton X-100. The samples were diluted in 10% glycerol with the addition of 0.05 M bromophenol. ChE molecules were separated on an 8% polyacrylamide running gel, under non-denaturing conditions. Electrophoresis was carried out at 12 mA, for 16 h at 5 °C, according to Ornstein and Davis' procedure (1962). The ChE activities were detected with Karnovsky and Roots' method (1964), using AcTChI, PrTChI or BTChI, as substrates at a concentration of 0.002 M. One unit of *Electrophorus electricus* AChE (Sigma, I) was employed as a control.

2.5.4. Characterization of ChE activities

To characterize the ChE activities of *E. crassus*, the sensitivity to specific ChE inhibitors was evaluated spectrophotometrically (Ellman et al., 1961). After centrifuging the cells, 8 μl of pellet were incubated with 10^{-5} M iso-OMPA (Sigma, I) or 10^{-5} M BW284c51 (Burrow-Welcome, NC) or 10^{-5} M eserine (Sigma, I), for 15 min at 24 °C. Likewise, the sensitivity of ChE activities to 10^{-4} M diazinon (Ciba, I), an active thionophosphate principle present in several OP insecticides, such as basudin, was evaluated. Afterwards, the samples were exposed to the AcTChI, or PrTChI substrates at a concentration of 0.025 M. Each experiment was carried out at least in triplicate. Student's *t*-test was used to compare the mean values of the experimental samples and that of the controls.

The effects of pH and temperature variations on the ChE activity able to cleave preferentially the AcTChI substrate (0.025 M), were assessed by incubating cell homogenates with this substrate, at increasing values of pH from 5.0 to 10.0 at 24 °C, or at increasing values of temperature from 5 to 37 °C at pH 8.0. Each experiment was carried out at least in triplicate.

2.6. Evaluation of ChE activity after toxicant-exposure

The cells were cultured in test-tubes and transferred into 50 ml Falcon tubes when the cell density reached about 1000 cells/ml. The effect of xenobiotic compounds on ChE activity able to cleave preferentially the AcTChI substrate was evaluated *in vivo* by adding the following toxicants to cell cultures growing in Falcon tubes: basudin (final concentrations of 9.66 or 96.6 mg/l) or AFD25 (final concentrations of 3.3 or 33 mg/l) or $HgCl_2$ (final concentrations of 7×10^{-2}, 0.1 or 0.3 mg/l) or MIX1 or MIX2 or MIX3. After a 60-min exposure, the cells were centrifuged and lysed by freezing at −80 °C for 10 min. The homogenates were exposed to the AcTChI substrate at a concentration of 0.025 M and the ChE activity was measured spectrophotometrically (Ellman et al., 1961).

Each experiment was carried out at least in triplicate. Student's *t*-test was used to compare the mean values of the experimental samples and that of the controls.

3. Results

3.1. Effects of toxicant-exposure on cell viability

The results related to the effects of toxicant-exposure on cell viability are reported in Table 1. Each toxicant used in this work appeared to affect cell viability after a 60-min or 24-h exposure. Basudin-exposure caused 100% mortality within 60 min at concentrations ranging from 9.66×10^2 to 966×10^3 mg/l, whereas at a basudin-concentration of 96.6 mg/l the cell viability decreased significantly ($P < 0.001$) compared to that of the controls after a 60-min or 24-h exposure. No effect on cell viability was found after a 60-min or 24-h exposure to a basudin concentration of 9.66 mg/l. The 60-min LC_{50} and 24-h LC_{50} were 96.6 mg/l (70–130, 95% confidence limits) and 40 mg/l (30–50, 95% confidence limits), respectively. A 60-min exposure to AFD25 at concentrations ranging from 3.3×10^2 to 33×10^3 mg/l was found to be lethal for the whole cell population. Exposure to AFD25 concentrations of 33 or 3.3 mg/l did not affect cell viability after 60 min, but such exposures resulted in 100% or 24% mortality, respectively, after 24 h. An AFD25 concentration of 0.33 mg/l did not caused any lethal effect on the cell population. The 60-min LC_{50} and 24-h LC_{50} were 100 mg/l (95% confidence limits not computable) and 6 mg/l (4.5–7.9, 95% confidence limits), respectively. Exposure to chloroform at the concentrations of 0.001, 0.01, 0.1, 1 or 10 μl/ml (see Section 2.3) did not affect cell viability after 60 min and 24 h (data not shown). Exposure to 0.5 or 1 mg/l $HgCl_2$ caused 100% mortality within 60 min. Cell viability decreased significantly ($P < 0.001$) after a 60-min exposure and was totally suppressed after a 24-h exposure to 0.3 mg/l $HgCl_2$. No significant effect on cell viability was detected after a 60-min exposure to 0.1 mg/l $HgCl_2$, whereas 100% mortality appeared after a 24-h exposure to this concentration. Both 7×10^{-2} and 3×10^{-2} mg/l $HgCl_2$ concentrations did not affect cell viability significantly. The 60-min LC_{50} and 24-h LC_{50} were 0.24 mg/l (0.22–0.27, 95% confidence limits) and 7.9×10^{-2} mg/l (7.5×10^{-2} to 8.3×10^{-2}, 95% confidence limits), respectively. A 60-min exposure to MIX1, MIX2 or MIX3 did not significantly affect cell viability. However, a significant decrease ($P < 0.001$) was detected after a 24-h exposure to MIX2 or MIX3.

A first statistical analysis of the data, reported in Table 1, was carried out using a two-way ANOVA (Table 2). Significant differences ($P < 0.001$) appeared among the cell viability mean values after a 60-min or 24-h exposure to toxicants at different concentrations. The results of the Student-Newman-Keuls multicomparison test pointed out the following order of *E. crassus* sensitivity to the tested toxicants: $HgCl_2$ > basudin > AFD25, for a 60-min exposure; $HgCl_2$ > AFD25 > basudin, for a 24-h exposure.

Table 1
Effects of 60-min and 24-h exposures to toxicants on cell viability

Toxicants	Concentrations (mg/l)	Mean of viable cells ± S.D.	
		60 min	24 h
Basudin	966×10^3	$0 \pm 0^{***}$	–
	96.6×10^3	$0 \pm 0^{***}$	–
	9.66×10^3	$0 \pm 0^{***}$	–
	9.66×10^2	$0 \pm 0^{***}$	–
	96.6	$25 \pm 5.7^{***}$	$10 \pm 6.1^{***}$
	9.66	50 ± 0	45 ± 4.3
AFD25	33×10^3	$0 \pm 0^{***}$	–
	3.3×10^3	$0 \pm 0^{***}$	–
	3.3×10^2	$0 \pm 0^{***}$	–
	33	50 ± 0	$0 \pm 0^{***}$
	3.3	50 ± 0	$38 \pm 3.3^{***}$
	0.33	50 ± 0	50 ± 0
$HgCl_2$	1	$0 \pm 0^{***}$	–
	0.5	$0 \pm 0^{***}$	–
	0.3	$21 \pm 4.9^{***}$	$0 \pm 0^{***}$
	0.1	50 ± 0	$0 \pm 0^{***}$
	7×10^{-2}	50 ± 0	45 ± 3.8
	3×10^{-2}	50 ± 0	50 ± 0
MIX1		50 ± 0	44 ± 4.8
MIX2		47 ± 2.5	$35 \pm 6.4^{***}$
MIX3		48 ± 2.6	$37 \pm 4.9^{***}$
Control		50 ± 0	50 ± 0

Each experiment was carried out in triplicate. Sample size, 50 cells; S.D., standard deviation. For statistical analysis a two-way ANOVA was used (see Table 2), followed by the Student-Newman-Keuls multicomparison test. The means differing significantly from that of the controls are marked by the symbols *** ($P < 0.001$).

3.2. Effects of toxicant-exposure on the mean fission rate

Table 3 shows the effects of a 24- or 48-h toxicant-exposure on the mean fission rate. The toxicant concentrations that caused less than 25% mortality were used for fission rate assays. Exposure to 9.66 mg/l basudin-, 3.3 mg/l AFD25- or 7×10^{-2} mg/l $HgCl_2$-concentrations caused a significant decrease ($P < 0.001$) in the mean fission rate after 24 and 48 h. In contrast, exposure to 0.33 mg/l AFD25 or 3×10^{-2} mg/l $HgCl_2$ did not affect the mean fission rate after 24 and 48 h. A 24- or 48-h exposure to chloroform at the concentrations of 0.001 or 0.01 μl/ml (see Section 2.3) did not influence the mean fission rate (data not shown). A significant decrease ($P < 0.001$) in the mean fission rate was detected after a 24- or 48-h exposure to MIX1, MIX2 or MIX3.

A first statistical analysis of the data, reported in Table 3, was carried out using a two-way ANOVA (Table 4). Significant differences ($P < 0.001$) appeared among the mean fission

Table 2
Statistical analysis (two-way ANOVA) on data related to the effects of 60-min and 24-h exposures to toxicants on cell viability, reported in Table 1

Source of variation	SS	d.f.	MS	*F*
Treatments	60356.31	21	2874.11	541.97
Time	2367.28	1	2367.28	446.40
Interaction	3858.55	21	326.60	61.59
Error	466.67	88	5.30	
Total	70048.81	131		

Table 3
Effects of 24- and 48-h exposures to toxicants on mean fission rate

Toxicants	Concentrations (mg/l)	Mean fission rate ± S.D.	
		24 h	48 h
Basudin	9.66	$1.65 \pm 0.31^{***}$	$1.05 \pm 0.28^{***}$
AFD25	3.3	$1.86 \pm 0.24^{***}$	$1.59 \pm 0.27^{***}$
	0.33	2.73 ± 0.32	2.51 ± 0.33
$HgCl_2$	7×10^{-2}	$1.44 \pm 0.25^{***}$	$1.70 \pm 0.38^{***}$
	3×10^{-2}	2.61 ± 0.39	2.62 ± 0.30
MIX1		$2.00 \pm 0.24^{***}$	$1.87 \pm 0.20^{***}$
MIX2		$1.41 \pm 0.22^{***}$	$1.33 \pm 0.15^{***}$
MIX3		$1.63 \pm 0.17^{***}$	$1.42 \pm 0.16^{***}$
Control		2.68 ± 0.33	2.43 ± 0.59

Each experiment was carried out in triplicate. Sample size, 20 cells; S.D., standard deviation. For statistical analysis a two-way ANOVA was used (see Table 4), followed by the Student-Newman-Keuls multicomparison test. The means differing significantly from that of the controls are marked by the symbols *** ($P < 0.001$).

rate values after a 24- or 48-h exposure to toxicant different concentrations. The results of the Student-Newman-Keuls multicomparison test pointed out the following order of *E. crassus* sensitivity to the tested toxicants: $HgCl_2$ > basudin > AFD25, for both 24- and 48-h exposures.

3.3. Effects of toxicant-exposure on lysosomal membrane stability

The effects on lysosomal membrane stability were evaluated by the Neutral Red Retention (NRR) assay after 60- and 120-min exposures of cell populations to single toxicants or MIX1 or MIX2 or MIX3. Exposure to 9.66 or 96.6 mg/l basudin did not affect lysosomal membrane stability, expressed as an OD percentage compared to the control value (Fig. 1A). Likewise, a 3.3 mg/l AFD25 concentration did not affect lysosomal membrane stability after a 60-min exposure, whereas significant decreases ($P < 0.001$) in the OD percentage were found after a 120-min exposure and after 60- and 120-min exposures to 33 mg/l (Fig. 1B). Similar results were obtained for $HgCl_2$-exposure. An exposure to a concentration of 0.1 mg/l did not affect the lysosomal membrane stability after 60 min. In contrast, significant decreases ($P < 0.001$) were detected in the OD percentage after a 120-min exposure and after 60- and 120-min exposures to 0.3 mg/l (Fig. 1C). No effect on the OD was detected after 60- and 120-min exposures to MIX1, MIX2 or MIX3 (Fig. 1D).

Table 4
Statistical analysis (two-way ANOVA) on data related to the effects of 24- and 48-h exposures to toxicants on mean fission rate, reported in Table 3

Source of variation	SS	d.f.	MS	*F*
Treatments	137.55	8	17.19	152.50
Time	4.26	1	4.26	37.76
Interaction	6.01	8	0.75	6.66
Error	58.85	522	0.11	
Total	206.67	539		

Table 5
Statistical analysis (two-way ANOVA) on data related to the effects of 60- and 120-min exposures to toxicants on lysosomal membrane stability (see Fig. 1)

Source of variation	SS	d.f.	MS	*F*
Treatments	60.09	9	6.68	246.97
Time	10.78	1	10.78	398.67
Interaction	28.36	9	3.15	116.54
Error	1.08	40	0.03	
Total	100.31	59		

A first statistical analysis of the data, represented in Fig. 1, was carried out using a two-way ANOVA (Table 5). Significant differences ($P < 0.001$) appeared among the mean OD values after 60- and 120-min exposures to different concentrations of toxicants. The results of the Student-Newman-Keuls multicomparison test pointed out the following order of *E. crassus* sensitivity to the tested toxicants: $HgCl_2$ > AFD25, for both 60- and 120-min exposures.

3.4. Cytochemical, spectrophotometric and electrophoretic detection of ChE activities

The cytochemical analysis revealed the presence of three ChE activities, depending on the substrate used. The enzyme reaction product was found as a dark precipitate at the active sites. The enzyme activity was revealed by the cleavage of AcTChI (Fig. 2A), used as a substrate. The reaction product was much fainter when the PrTChI or BTChI substrates were employed (data not shown), and absent when no substrate was employed (Fig. 2B).

The spectrophotometric analysis confirmed the presence of three different ChE activities. When the AcTChI substrate was used, the enzyme activity able to hydrolyze this substrate was the most represented ChE activity compared to those able to cleave the PrTChI and BTChI substrates (Fig. 3).

The electrophoretic pattern of the ChE molecules showed the presence of two enzyme activities able to cleave the AcTChI substrate (Fig. 4, lane B), and a single enzyme activity able to cleave the PrTChI substrate (Fig. 4, lane C). In contrast, no enzyme activity was detected when employing BTChI as a substrate (Fig. 4, lane D). It is worth noting that the AcTChI substrate was hydrolyzed by both an enzyme activity migrating like the 260 kDa molecular form of the *E. electricus* AChE (Fig. 4, lane A) and by an enzyme activity corresponding to the active molecular form revealed by the PrTChI substrate.

3.5. Characterization of ChE activities

The *E. crassus* ChE activities were characterized by exposure to classical ChE inhibitors at a concentration of 10^{-5} M and to diazinon at a concentration of 10^{-4} M. The ChE activity able to cleave preferentially the AcTChI substrate was sensitive to BW284c51- or diazinon-exposure ($P < 0.001$) (Fig. 5A), whereas the ChE activity able to cleave preferentially the PrTChI

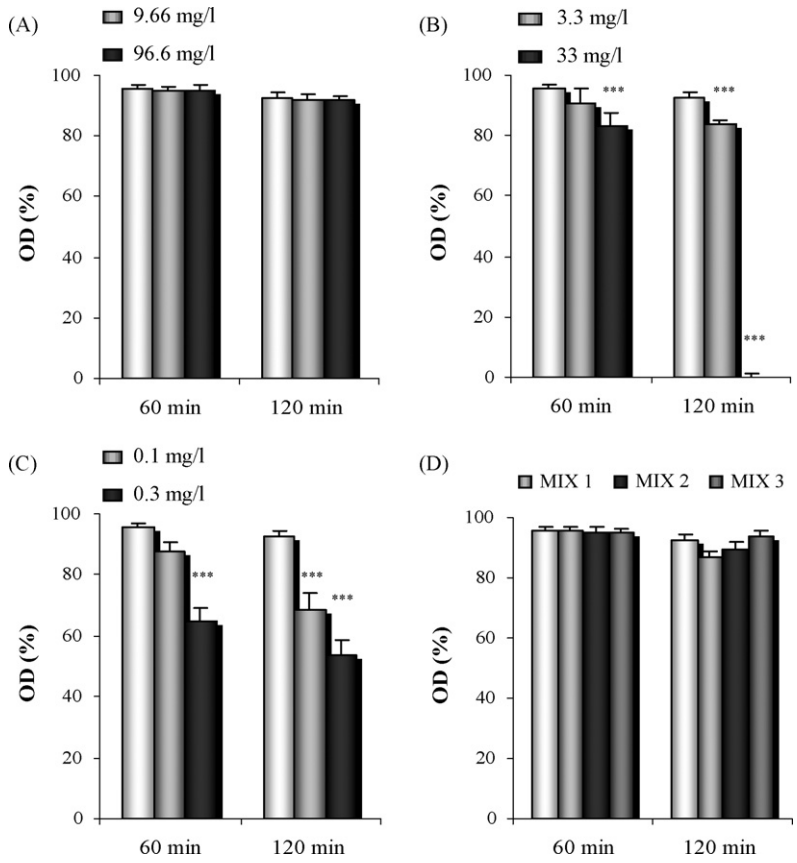

Fig. 1. Evaluation of lysosomal membrane stability, expressed as percentage of optical density (OD), after 60- and 120-min exposures to basudin (A), AFD25 (B), $HgCl_2$ (C) and MIX1, MIX2 or MIX3 (D). Control cells (white bars). The toxicant concentrations used are reported on the upper side of the relative histograms. Means of three experiments and standard deviation are shown. For statistical analysis a two-way ANOVA (see Table 5), followed by the Student-Newman-Keuls multicomparison test was used. The means differing significantly from that of the controls are marked by the symbol *** ($P < 0.001$).

substrate was inhibited by exposure to iso-OMPA, BW284c51 or diazinon ($P < 0.001$) (Fig. 5B).

The effects of pH and temperature variations on ChE activity able to cleave preferentially the AcTChI substrate were assessed. As the pH increased from 5.0 to 10.0 at 24 °C, this enzyme activity showed gradually increasing values, the lowest one corresponding to pH 5.0 and the highest one to pH 10.0 (Fig. 6A). In a temperature interval ranging from 5 to 37 °C, the ChE activity evaluated at pH 8.0 showed its maxima at 5 and 37 °C, and stable values between 15 and 25 °C (Fig. 6B). This range seems to be the best condition for the enzyme activity and corresponds to the optimal culture temperatures for growing of *E. crassus* in the laboratory.

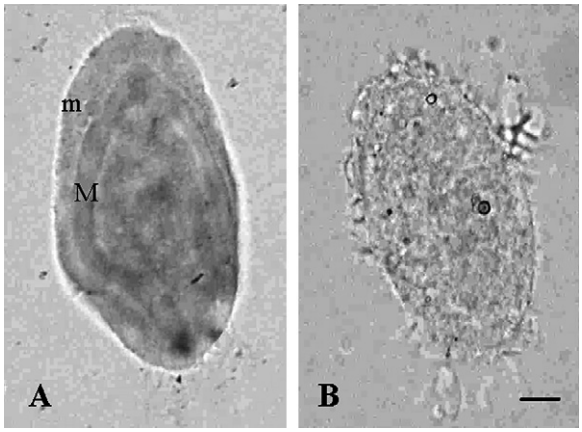

Fig. 2. Cytochemical detection of ChE activity able to cleave the AcTChI substrate. The enzyme reaction product appears as a dark precipitate (A). Control cell (B). M, macronucleus; m, micronucleus. Bar = 10 μm.

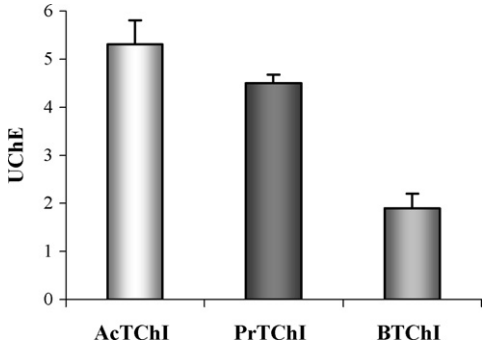

Fig. 3. Spectrophotometric evaluation of ChE activities. On the abscissa, the substrates used at a concentration of 0.025 M. On the ordinate, the ChE activities expressed as ChE Units (UChE). One UChE hydrolyzes 1 μmol of acetyl/min or propionyl/min or butyryl/min at pH 8.0 at 24 °C. Means of three experiments and standard deviation are shown.

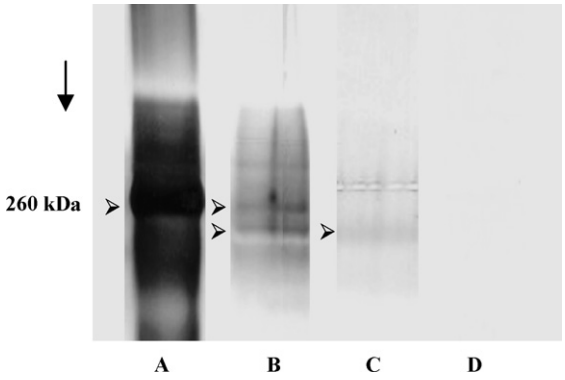

Fig. 4. Non-denaturing electrophoretic pattern of ChE activities. The substrates used were: AcTChI (lane B), PrTChI (lane C) and BTChI (lane D) at a concentration of 0.002 M. One unit of *Electrophorus electricus* AChE activity (lane A) is given as positive control.

3.6. Effects of toxicant-exposure on ChE activity

The effects of a 60-min exposure of cell populations to single toxicants, MIX1, MIX2 or MIX3 were evaluated by spectrophotometrically measuring the ChE activity able to cleave

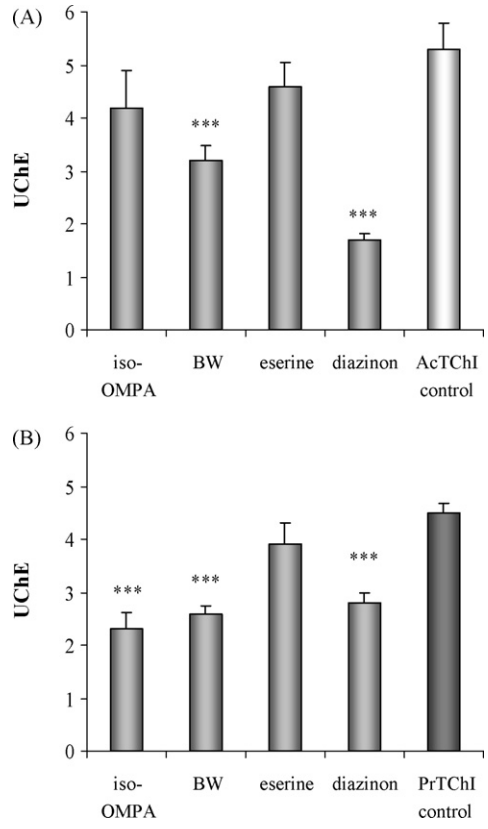

Fig. 5. Spectrophotometric evaluation of ChE activities, able to cleave the AcTChI (A) or PrTChI (B) substrates, after exposure to ChE inhibitors (10^{-5} M iso-OMPA, BW284c51 or eserine; 10^{-4} M diazinon). Both substrates were used at a concentration of 0.025 M. On the ordinate, the ChE activities expressed as ChE Units (UChE). One UChE hydrolyzes 1 μmol of acetyl/min or propionyl/min at pH 8.0 at 24 °C. Means of three experiments and standard deviation are shown. Student's *t*-test was used to compare the mean values of the experimental samples and that of the controls. The means differing significantly from that of the controls are marked by the symbol *** ($P < 0.001$).

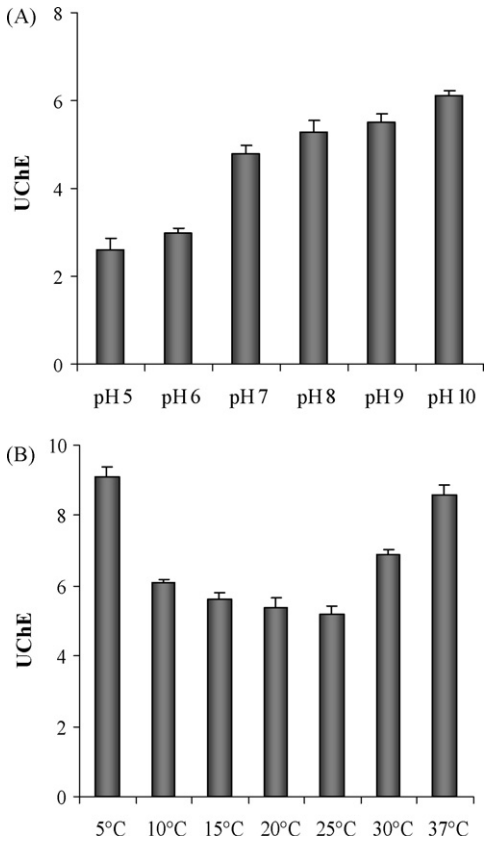

Fig. 6. Spectrophotometric evaluation of ChE activity able to cleave the AcTChI substrate, surveyed at increasing pH values at 24 °C (A) and at increasing temperature values at pH 8.0 (B). On the ordinate, the ChE activity expressed as ChE Units (UChE). One UChE hydrolyzes 1 μmol of acetyl/min at pH 8.0 at 24 °C. Means of three experiments and standard deviation are shown.

preferentially the AcTChI substrate, as this enzyme activity was inhibited significantly (more than 60%) by exposure to diazinon, an OP compound (see Fig. 5A). In cells exposed to basudin (9.66 or 96.6 mg/l) or AFD25 (3.3 mg/l), the ChE activity was significantly inhibited ($P < 0.05$) as this enzyme activity showed a 30% decrease compared to the control value (Fig. 7A and B). Conversely, exposure to AFD25 at a concentration of 33 mg/l caused a significant increase ($P < 0.05$) in ChE activity (Fig. 7B). Similarly, exposure of cells to 0.1 mg/l $HgCl_2$, 0.3 mg/l $HgCl_2$ or MIX1 caused a significant increase in ChE activity ($P < 0.05$) (Fig. 7C and D). Finally, exposure to MIX2 did not affect this enzyme activity, whereas MIX3 caused a significant decrease ($P < 0.05$) (Fig. 7D).

4. Discussion

The results indicate that *E. crassus* is sensitive to exposure to insecticides, such as basudin, an OP pesticide, and AFD25, an OC hydrocarbon, affecting significantly both cell viability and fission rate of this ciliate. Although our 60-min or 24-h LC_{50} for basudin and AFD25 were computed on only a few insecticide concentrations, it is worth noting that these values are as much as 3000 times lower than the recommended ones given by the manufacturers (1.5–3 ml/l for basudin and 3–4 mg/ml for AFD25). In the last years, assays with sea urchin have been

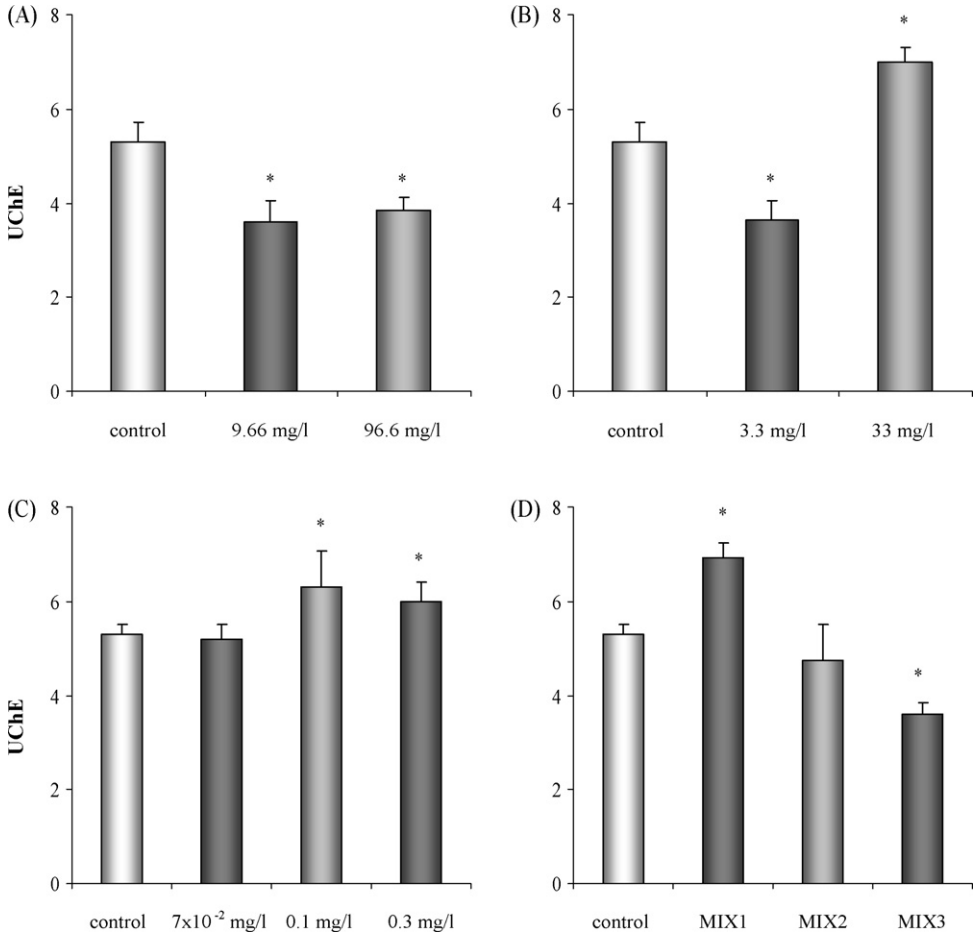

Fig. 7. Spectrophotometric evaluation of ChE activity able to cleave the AcTChI substrate, detected after a 60-min exposure to basudin (A), AFD25 (B), $HgCl_2$ (C) and MIX1, MIX2 or MIX3 (D). On the abscissa of A, B and C, the toxicant concentrations used. On the ordinate, the ChE activity expressed as ChE Units (UChE). One UChE hydrolyzes 1 μmol of acetyl/min at pH 8.0 at 24 °C. Means of three experiments and standard deviation are shown. Student's *t*-test was used to compare the mean values of the experimental samples and that of the controls. The means differing significantly from that of the controls are marked by the symbol * ($P < 0.05$).

extensively applied in environmental monitoring to test different matrices, as the first stages of development of this organism are a very sensitive model to a variety of pollutants (Pagano et al., 1986). A 9.66 mg/l basudin concentration, affecting the fission rate of *E. crassus*, corresponds to a 10^{-7} M basudin concentration that was found to alter sea urchin *Paracentrotus lividus* early development (Morale et al., 1998). Moreover, exposure to low mercury concentrations caused a serious damage to *P. lividus* developmental events (Trielli et al., 1995), as well as induced 50% mortality in populations of the rotifer *Brachionus plicatilis* (Moffat and Snell, 1995). The $HgCl_2$ concentrations used in this work appeared to be included in the mercury tolerance range shown for several *E. crassus* stocks (Dini, 1981). In addition, our results of statistical analysis indicated that mercury-exposure affected both cell viability and fission rate of *E. crassus* more than basudin- or AFD25-exposure.

The lysosomal membrane stability, a general biomarker of stress-induced by xenobiotic compounds, was altered by exposure to $HgCl_2$ or AFD25, as a significant lysosomal release of neutral red into cytoplasm was detected in toxicant-exposed cells. This result agrees with those reported in the literature on the lysosomal membrane destabilization due to heavy metal- or OC compound-exposure (Lowe and Pipe, 1994; Domouhtsidou and Dimitriadis, 2001; Marchi et al., 2004; Fernandez Freire et al., 2005).

The presence of three ChE activities able to cleave the AcTChI, PrTChI and BTChI substrates, respectively, was detected in *E. crassus* using the cytochemical and spectrophotometrical approaches. However, the BChE activity was not revealed by non-denaturing electrophoresis, due to its scanty presence (see Figs. 3 and 4). The ChE activities found in *E. crassus* showed atypical properties for their substrate affinity and sensitivity to classical ChE inhibitors, as often reported for ChE activity in lower organisms, such as *Daphnia magna* (Diamantino et al., 2003). As in higher organisms (Hassal, 1990), the inhibition of both ChEs found in *E. crassus* after diazinon-exposure, the active principle of basudin, can be exploited as a potential specific biomarker of exposure to OP compounds. However, significant variations in ChE activity were detected after exposure to the OC pesticide AFD25 and $HgCl_2$. It is interesting that AFD25 inhibited ChE activity of *E. crassus* at a concentration of 3.3 mg/l, whereas a concentration ten times higher caused a significant increase in this enzyme activity. This is not an uncommon result, as it is well known that

some drugs, such as sarin, can cause a biphasic response in rat ACh receptors, depending on the exposure doses (Khan et al., 2000). The sensitivity of *E. crassus* ChE activity to OC pesticide and $HgCl_2$ agrees with recent studies reporting that invertebrate and vertebrate ChE activity is sensitive to a variety of xenobiotic compounds other than OP and carbamate pesticides (Payne et al., 1996), and to a combination of different chemical classes, such as OPs and heavy metals (Bocquené et al., 1995). A significant increase in ChE activity after mercury-exposure was found in *E. crassus*, as well as in human peripheral blood erythrocytes (Zabinski et al., 2000). Conversely, a decrease in ChE activity was detected after mercury-exposure in rat (Lakshmana et al., 1993; El-Demerdash, 2001; Cheng et al., 2005), in the fish *Cyprinus carpio* (Suresh et al., 1992) and in the crayfish *Procambarus clarkia* (Devi and Fingerman, 1995). A significant decrease followed by an increase in the esterase activity was found after an *in vivo* mercury exposure of the shrimp *Callianassa thyrrena*, where the enhancement of ChE activity was thought to be related to the activation of multiple molecular forms (Thaker and Haritos, 1989). Another possible explanation could be that the increase in the ChE activity depends on a polymerization process of monomeric and dimeric forms into heavy forms with high activity due, in turn, to an increase in cytosolic Ca^{2+}, as reported in mammalian cell cultures (De La Porte et al., 1984; Day and Greenfield, 2002). Actually, an increase in cytosolic Ca^{2+} was observed in *E. crassus* after mercury exposure (Viarengo et al., 1996). However, the metal-induced stimulation of the ChE activity could also depend on the allosteric interactions between the cation and peripheral anionic sites of the enzyme (Gulya et al., 1990).

The results related to the effects of exposure to low concentrations of mixtures of the three xenobiotic compounds used in this work support the conclusion that the toxicity of mixtures containing different pollutants cannot generally be correlated to the toxicity of their single components, particularly when their action mechanisms are different (Fernández-Alba et al., 2001).

In conclusion, our results indicate that: (i) *E. crassus* can be exploited as a test organism for the pre-chemical screening of sediment from coastal and estuarine sites and that (ii) the use of ChE activity could be extended to biomonitoring programs investigating the biological effects of contaminants present in complex mixtures in the environment (Guilhermino et al., 1998; Lionetto et al., 2005). The sensitivity of *E. crassus* to the toxicants used in this work could have important environmental consequences. In fact, the decrease in the ciliate community of marine sediments could alter the balance of the food chain, as several ciliates are bacteria consumers and all of them, in turn, represent a food source for higher organisms (Fenchel, 1987).

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