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Use of Acetylcholinesterase in Perna perna and *Mytilus galloprovincialis* as a Biomarker of Pollution in Agadir Marine Bay (South of Morocco)

S. Najimi,[1] A. Bouhaimi,[1] M. Daubèze,[2] A. Zekhnini,[1] J. Pellerin,[3] J. F. Narbonne,[2] A. Moukrim[1]

[1]Laboratory of "Water and Environment," Department of Biology, Faculty of Sciences, University of Ibnou Zohr, B.P. 28/S, Agadir, Morocco
[2]Laboratory of Food Toxicology, University of Bordeaux I, 33405 Talence, France
[3]Laboratory of Marine Ecotoxicology, University of Quebec, Rimouski, Canada

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Agadir marine Bay receives domestic, industrial and agricultural discharges without previous purification. To assess the level of pollution in this ecosystem, we have established a program of research focused on the study of biomarkers of pollution in two species of mussels (African mussel, *Perna perna,* and Mediterranean mussel, *Mytilus galloprovincialis)* which cohabit along the southern coast of Morocco.

Our objective was to study the use of acetylcholinesterase (AChE) activity in mussel tissues as a biomarker of pollution. This enzyme is inhibited by some pesticides (Singh and Agarwal 1983; Zinckel *et al.* 1987) and heavy metals (Abou-donia and Mensel 1967; Galzigna *et al.* 1969; Olson and Christensen 1980; Bocquene *et al.* 1990; Narbonne *et al.* 1991). It was proposed as a biomarker of pollution by several authors (Bocquene *et al.* 1990; Narbonne *et al.* 1991).

In this work, we studied characteristics and organ distribution of AChE in the two species of mussels. The response of the enzyme to pollutants was studied in the field and in the laboratory.

MATERIALS AND METHODS

The study was conducted on adult mussels *P. perna* and *M galloprovincialis,* collected from two stations: a reference station, Cap Ghir, and a polluted station, Anza, which receives domestic and industrial waste waters of Anza city.

The animals were quickly transferred to the laboratory and frozen at -30°C. Eight to ten animals were thawed and drawn out of the shells. To each individual tissue was added 3 vol of 0.1 M phosphate buffer, pH 7.4 and homogenized for 1 min using a T25 Ultra Turrax at 9000 rpm. The extract was centrifuged at 9000 g for 25 min at 4°C. The supernatant was stored at -30°C before use.

The method of Lowry *et al.* (195 1) was used for quantitative determination of proteins using bovine serum albumin as the standard. The AChE activity was measured by the method described by Elhnan *et al.* (1961) using acetylthiocholine

Correspondence to: A. Moukrim

iodide (ATC) as substrate. Seasonal variations of AChE activity were studied in S-10 *P. perna* collected monthly from October 94 to September 95.

The effect of heavy metals on the AChE activity was studied *in vitro, in the two* species of mussels. The metals tested were: copper, cadmium, iron and zinc (in the form of $CuS0_4$, $CdCl_2$, $ZnCl_2$ and $FeCl_3$, respectively). The range of doses were: 10^{-2}-10^{-7} M for (Cd, Cu, Zn), and 10^{-2}-10^{-4} M for Fe. The assays were performed on the extract of the whole animal. In this case Tris-HCl buffer (0.1 M, pH 7.4) was used since no inhibition was found with phosphate buffer even with high concentrations of metals in preliminary experiments. Ionic solutions (prepared in distilled water) were incubated with the enzyme solution for 30 min before reaction was started by addition of substrate.

To compare the toxicity of different metals, the I_{50} was determined (I_{50} is the molarity of the tested compound in the reaction mixture which causes an inhibition of 50% of the enzyme activity).

The *in vivo* effect of heavy metals was tested for two metals, Cd and Zn in the form of $CdC1_2$ and $ZnCl_2$. The test organisms were *P. perna* of both sexes which were obtained from the reference station (Cap Ghir). They were acclimated in aerated sea water, salinity 32‰, at 21°C for 3 d prior to the experiments. Subsequently, groups of 30 individuals were held in 5-L plastic containers and exposed to a cadmium and zinc concentration, respectively, of 10 µg/l and 1 mg/l for 11 d. Control groups were maintained in clean sea water throughout the experiment. The medium was changed every two days, using natural sea water and the stock solutions of metals.

The data were analyzed by ANOVA, and the least significant difference (LSD) using STATISTICA.

RESULTS AND DISCUSSION

Effects of incubation temperature, pH and substrate concentration on AChE activity were investigated in the two species of mussels. The optimal activity occurred between 37 and 42°C for *M. galloprovincialis,* and between 28 and 40 *°C* for *P. perna* (Fig. la). AChE activity was not very sensitive to pH variations ranging between 6 and 8.5 (Fig. lb).The decreasing values were obtained above 8.5 for the two species. The same results were reported by Bocquene *et al.* (1990) in *Mytilus edulis*. The enzyme presents the same kinetic in the two species. The affinity constant (Km) of AChE for acetylthiocholine was calculated on extracts of the whole animal. Km values were 49 and 73 µM, respectively, for *P. perna* and *M. galloprovincia1i.s.* Maximal velocities were the same for the two mollusks (Vm=3.4nmoles/min).

Organ distribution of the enzyme was different between the two species (Fig. 2). The highest activity was noted in the hepatopancreas and gills in *M galloprovinci-*

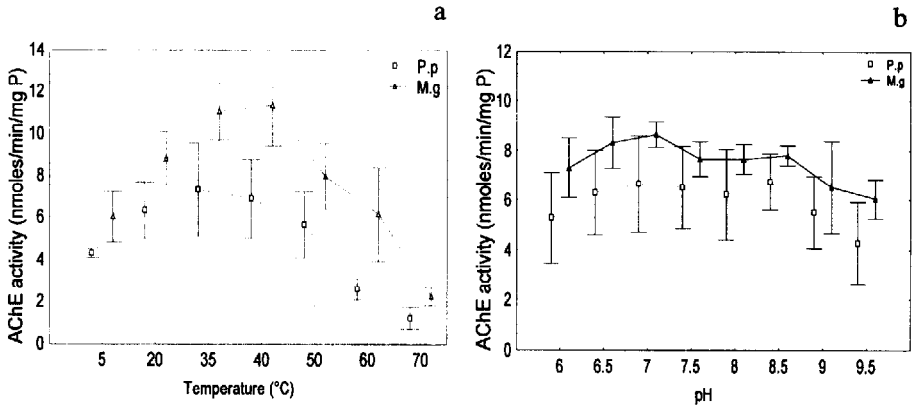

Figure 1. The effect of temperature (a) and pH (b) on the AChE activity of two species of mussel P.p. *: perna perna* and M.g. *: Mytilus galloprovincialis. The* values indicate means ± standard deviations (SD).

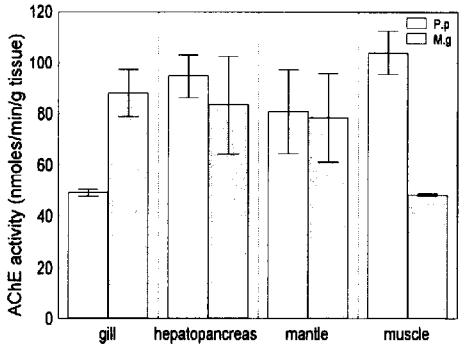

Figure 2. Organ distribution of AChE activity in *P. perna* (P.p.) and *M. galloprovincialis* (M.g.) The values indicate means ± SD.

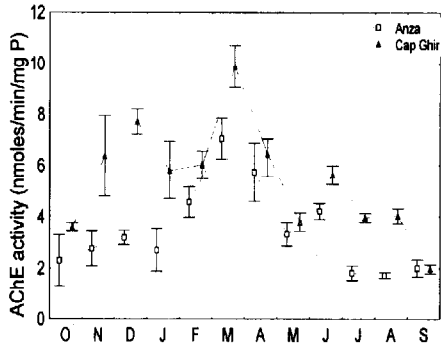

Figure 3. Evolution of AChE activity of *P. perna* in the polluted and unpolluted stations.

alis (84 and 88 nmoles/min/g tissue, respectively). *In P. perna,* high values were recorded in the hepatopancreas and muscle (95 and 101 nmoles/min/g tissue, respectively); minimal values were noted in the gills. Bocquene *et al.* (1990) reported a high AChE activity in the gills of *Mytilus edulis,* and in muscle of the prawn, the plaice and the mackerel. This enzyme activity must be related to the importance of nerve impulse transmission in tissues with high neuromuscular activity.

Figure 3 shows differences of the AChE activity related to season *in P. perna.* The curve shows three periods of high activity which alternate with three periods of low activity: the maximal values (9.88 nmoles ATC/min/mg P.) were obtained

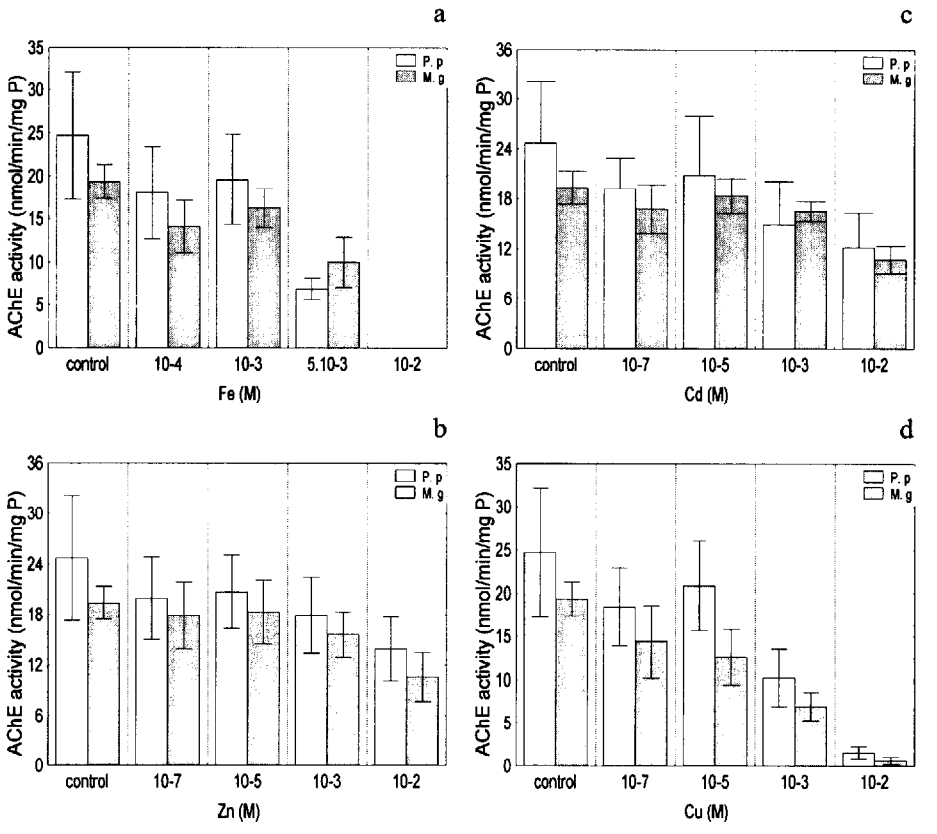

Figure 4 . The *in vitro* effect of Fe (a), Zn (b), Cd (c) and Cu (d) on the AChE activity of two species of mussel P.p. *: Perna perna*, M.g. : *Mytilus galloprovincialis.*

The values indicate means ± SD.

in March, December (7.73 nmoles ATC/min/mg P.) and June (5.62 nmoles ATC/min/mg P.), and the minimal activity (5.81 nmoles ATC/min/mg P.) was noted in January, May (3.78 nmoles ATC/min/mg P.) and September (1.99 nmoles ATC/min/mg P.). The variation of AChE activity related to the season was reported previously by Bocquene (1991) in the plaice and the prawn. The periods of maximal and minimal activity coincide respectively with spawning and sexual rest determined by Id-halla *et al.* (1994) in the two mussels of Agadir Bay. These variations are probably related to different levels of the activation of the cholinergic system during the sexual cycle. Although, these variations can also be related to variation in temperature and to changes in content of protein which increase during the sexual rest and decline in the spawning period.

The link between sea water contamination and AChE activity was investigated by measuring the activity of the enzyme in *P. perna* collected from the polluted site and the reference site. Figure 3 shows that AChE activity was significantly

Table 1. I_{50} values of metals in the mussels *Perna perna* and *Mytilus galloprovincialis*

Mollusk	Metal	I_{50}
P. perna	Cd	$9.3\ 10^{-3}$
	Cu	$3.7\ 10^{-3}$
	Fe	$4.2\ 10^{-3}$
	Zn	$1.0\ 10^{-2}$
M. galloprovincialis	Cd	$1.0\ 10^{-2}$
	Cu	$3.0\ 10^{-3}$
	Fe	$4.8\ 10^{-3}$
	Zn	1.010^{-2}

($p<0.01$) reduced in Anza compared to Cap Ghir. Correlation between inhibition of AChE activity and the gradient of pollution was reported by Galgani *et al.* (1992) in fish collected in polluted sites in the North Sea, and by Narbonne *et al.* (1991) in mussels obtained from polluted Mediterranean sites. This result indicates that AChE activity in *P. perna* is sensitive to pollutants in sea water, and validate the enzyme in this species as a tool for biological monitoring at sea. Other studies must be carried out to determine the nature and levels of inhibitors, in order to determine correlations between contamination levels of sea water and AChE activity.

Concerning the four metals tested *in vitro* on AChE activity, a significant ($p<0.0l$) inhibitory effect was detected (Figs. 4a-d). These results were noted in the two mussels. In addition, the inhibition was very important when the concentration of the metals increased. For Zn and Cd, a significant ($p<0.01$) inhibitory effect was noted at 10^{-3} and 10^{-2} M. A slight decrease of the enzyme activity was also noted at 10^{-7} and 10^{-5} M, but was not different significantly in comparison to the control (-22% and -13%, respectively, for *P. perna* and *M. galloprovincialis* for Cd at $1\ 0^{-7}$ M). For Cu and Fe a total inhibition was observed at high concentrations (10^{-3}-10^{-2} M). These metals were still effective even at low concentrations (-25% and -26% for *P. perna* and M *galloprovincialis,* respectively, for Cu at 10^{-7} M).

The determination of I_{50} allowed us to compare the toxicity of the four metals. Table 1 shows that copper and iron were more inhibitory than cadmium and zinc.

Most of the work on anticholinesterase agents has focused on carbamates and organophosphates. In contrast, few studies has been concerned with the *in vitro* effect of heavy metals. It has been demonstrated that copper, mercury, lead, iron and nickel affect the activity of cholinesterases (Abou-donia and Mensel 1967). The same results were found with alkyl lead by Galzigna *et al.* (1969). Olson and Christensen (1980) determined the order of effect of several heavy metals on AChE activity and demonstrated that copper and cadmium had the most inhibitory

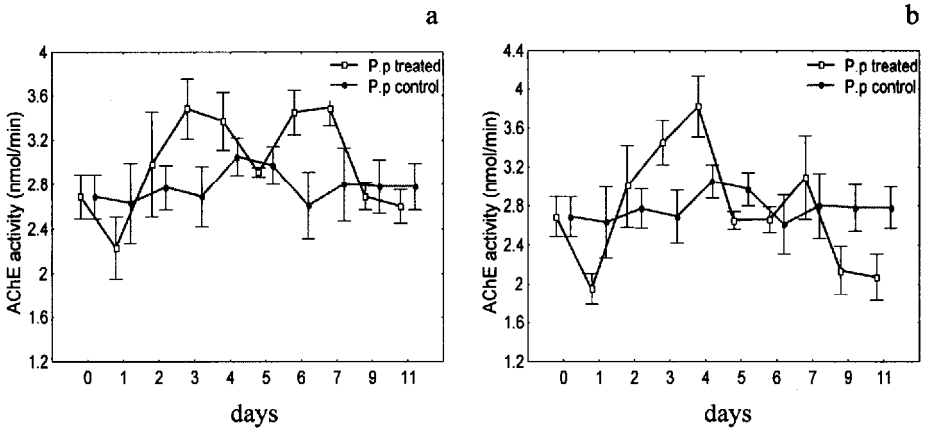

Figure 5. Evolution of AChE activity in *P. perna in vivo* exposed to Cd (10 µg/l) (a) and Zn (1mg/l) (b). The values are means ± SD.

effect on this enzyme. Recently, Bocquene *et al.* (1990) reported a strong inhibitory effect of cadmium, arsenic and zinc on AChE activity.

Figure 5 (a, b) show, respectively, the *in vivo* effect of Cd and Zn on AChE activity of *P. perna.* A significant ($p<0.01$) decrease in enzyme activity was noted for the two metals after one day of exposure. After 3 d, a significant ($p<0.01$) increase in AChE activity was noted in mussels. This high activity was also noted after 7 d for Cd and 4 d for Zn. At the end of the experiment, a significant ($p<0.01$) decrease of AChE activity was observed for Zn. For Cd, the AChE activity tended to return to control values at the end of treatment.

To our knowledge, there are few studies that tested the *in vivo* effect of heavy metals on AChE activity. Thaker and Haritos (1989) have demonstrated that, *in vivo,* mercury (0.4 mg/l) caused a significant increase in esterase activity in the shrimp. Other studies have shown different enzyme behavior depending on whether activity is assayed after *in vivo* exposure to *the* metal, or by *in vitro* addition to the incubation mixture. Viarengo (1985) demonstrated that alkaline phosphatase in fish is inhibited *in vitro* by Cu and Pb but is stimulated after *in vivo* exposure to these metals. Esterase activity in the shrimp has been found to be inhibited *in vitro* by mercury, but, *in vivo,* this metal caused a significant increase of the enzymatic activity (Thaker and Haritos 1989).

The in vitro reduction of the enzyme activity might be explained by the binding of metals to functional groups of proteins such as imidazole, sulphydryl, carboxyl and peptide groups. A possible molecular mechanism for enzyme-related metal toxicity had been identified. The toxic metal binds to a deactivating site on the molecule (Viarengo 1985). It should be noted that the binding of metallic cations to enzymes could alter their activity, not only by inhibiting, but also by stimulating the catalytic function of the enzymes Eichhorn *et al.* 1969).

The in *vivo* exposure of animals to metals may result in stimulation, no change, or depression of the enzyme activity tested, depending on the duration and the dosage used (Jackim 1974). In *in vivo* experiments other factors contribute to the changes in synthesis and/or degradation rates of the enzyme due to the metal accumulation in the tissues. In fact, a differential activation of multiple molecular forms of the esterase system was reported previously by Thaker and Haritos (1989) for the shrimp *Callianassa thyrrhena* after mercury exposure.

The inhibitory effect of AChE activity found for Zn *in vivo* can be related to the high dose used (1 mg/l), which can disturb the regulation process and, therefore, lead to the accumulation of the metal and its interaction with the enzyme. In fact, the high concentration of Zn which can be regulated by mollusks was 100 µg/l (Amiard *et al.* 1987). These authors also demonstrated that low concentrations of Zn can disturb the regulation process when the duration of exposure to this metal increases.

This preliminary study gives us basic informations on the functioning of AChE in the two mussel species of Agadir Bay and suggests that the enzyme is sensitive to the presence of pollutants in sea water. Therefore, the activity of this enzyme may possibly be used as a biomarker of pollution in Agadir Bay.

The results obtained with *P. perna* constitute a new find. They allow us to propose this species for the monitoring of aquatic ecosystems, specially in Africa where it takes the place of the Mediterranean mussel *M galloprovincialis*. Every program of research must consider the behavior of the enzyme in this species, as a result of different organ levels, and variations in AChE activity related to the sexual cycle.

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