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Study of *Donax trunculus* as a Sentinel Species for Environmental Monitoring of Sandy Beaches on Moroccan Coasts

A. Moukrim,[1] F. El Hamidi,[1] A. Lagbouri,[1] A. Kaaya,[1] A. Zekhnini,[1] A. Bouhaimi,[1] J. F. Narbonne[2]

[1] Laboratory Water and Environment, Department of Biology, Faculty of Sciences, University of Ibn Zohr, B. P. 28/S, Agadir, Morocco
[2] Laboratory Physico and Toxicochemistry, UPRES-A-CNRS 5472, University of Bordeaux I, 33 405, Talence, France

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Biomarkers are now proposed for evaluation and control of the health of marine ecosystems (Narbonne et al. 1991; Labrot et al. 1996; Pellerin - Massicotte 1994; Mora et al. 1999). At present, they are extensively used in international programs of pollution monitoring (McCarthy and Shugart 1990; Narbonne et al. 1999). However, the ability of biological and biochemical parameters for the biomarker approach is closely related to the choice of sentinel species. Among marine organisms, mussels appeared to be one of the most appropriate candidates for use as sentinel species. These animals are suspension feeders, ubiquists, sedentary, bioaccumulators, able to survive in heavy contaminated areas and are easily handled (collection and caging). Research in this field has been carried out on these bivalves (Narbonne et al. 1991; Burgeot et al. 1996; Boucquené 1996; Najimi et al. 1997). But, these mollusks are strictly present in rocky substrate and they can't be used for pollution survey in sandy beach ecosystems.

The aim of this work was to study a mollusk, *Donax trunculus*, as a sentinel species of sandy beaches. This mollusk, living in the sediment and largely distributed in West-African and West-European Atlantic and Mediterranean costs, exhibits the same characteristics as other mussels in that they are suspension feeders as well as potential bioaccumulators (Ansell, 1983).

MATERIALS AND METHODS

Our investigations were conducted on *D. trunculus* on two sites of Agadir Bay (polluted and unpolluted). The biochemical parameters studied as biomarkers of pollution are: inhibition of acetylcholinesterase activity (involved in neurotransmission), induction of lipid peroxidation by measurement of Thiobarbituric Acid Reactifs (TBARs) production, and Gluthation S-Transferases activity perurbation (involved in metabolism of xenobiotics). These parameters were previously studied in two species of mussels of Agadir Bay, *Perna perna* and *Mytilus galloprovincialis*, living on a rocky substrate (Najimi et al. 1997; Kaaya et al. 1999).

Animals were collected monthly (February 1995 to February 1997), from two sites: i) Anza, located 10 km north of Agadir, receiving industrial and domestic wastewater of the Anza zone, and considered as the polluted area and ii) Aghroud,

Correspondence to: A. Moukrim

which is located 45 km north of Agadir, is far from any human activities, and is considered as the reference area. Mollusks with the same shell length (2-3 cm) were sampled, transferred to the laboratory, dissected and frozen immediately at -80°C until use.

The preparation of the post mitochondrial fraction was conducted on both female and male mussels. The two soft parts were entirely pooled for each sample. The resulting pool was homogenized in 1/3 w/v (weight/volume) ratio of tris buffer (0.1 M pH 7.5), for one minute in Teflon potter-Elvehjem homogenizer. The homogenate was centrifuged at 9000 g for 30 minutes to obtain the post mitochondrial fraction. All procedures were carried out at 4°C.

The AChE activity was measured at 25°C according to the spectrophotometric method of Ellman et al. (1961) with the use of acetylthiocholine (ASCh) as substrate. The activity was followed for 2 min in Perkin Elmer 550S differential spectrophotometer by measuring, at 412 nm, the formation of interaction product of the second substrate: DTNB (dithio-bis-nitrobenzoic acid) ion with free thiol groups of ASCh.

The GST activity was determined as described by Habig et al. (1974), at 25°C with the use of CDNB as substrate. The test was conducted by monitoring spectrophotometrically at 340 nm, the appearance of the conjugated complex of CDNB and GSH.

Malondialdehyde (MDA) content was measured by the thiobarbituric acid method (Sunderman, 1985). The mixture incubation, consisting of aliquot of the supernatant, phosphoric acid, thiobarbituric acid and KCl, was heated for 45 min in boiling water. The absorbency was measured at 532 nm. Then, the MDA content was determined by comparing absorbency to the samples with known concentrations of tetra-methoxy propane.

For protein (P) determination, Lowry's method (1951) was employed with bovine serum albumin used as the standard.

The study of enzymatic activity was performed according to : i) the nature of substrate (acetylthiocholine: ASch, Propionylthiocholine : PSch and Butyrylthiocholine : BuSch for AChE), ii) the variation of temperature (5-50°C), pH (5-9) and substrate concentration (0-8 mM for AChE and 0-1.2 mM for GST) and iii) the spontaneous hydrolysis and conjugation (according to increase of temperature, pH and substrate concentration) respectively for AChE and GST.

In vitro experiment consisted of adding the pollutant to the enzymatic reaction mixture (AChE and GST) prepared from animals collected at the reference site. The copper (Cu) concentrations tested were 10^{-7} and 10^{-2} M. The enzymatic activities were measured after an incubation of sample (200 µl of S9) with the metal during 30 min at 25°C.

For *in vivo* exposure to pollutants, animals sampled from the reference site were exposed for 9 days under laboratory conditions. Mollusks were placed in several 5 liter containers with sea water taken from the same site (6 individuals/l), the water, oxygenated continually and maintained at 20°C, was changed daily. The pollutant tested was Cu at 500 µg/l. A control experiment (without the contaminant) was conducted under the same conditions. Six mollusks were

sampled at 24, 48, 96, 144 and 216 h. The enzymatic parameters were then measured in these samples as described above.
For treating and expressing data, AChE and GST activities were calculated with the use of an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ at 412 nm for Acetylthiocholine and 9.6 $mM^{-1}cm^{-1}$ at 340 nm for CDNB after correcting for spontaneous hydrolysis and spontaneous conjugation respectively. Both activities are expressed in nmol/min/mg of proteins. MDA content is expressed as nmol/mg of proteins. Means ± standard deviation (SD) for each biochemical parameter were calculated for four replicates each for 10 animals per station per month. The data were analyzed by the least significant difference test (LSD) ($p<0.01$) with the use of STATISTICA.

RESULTS AND DISCUSSION

The results are presented below according to the following order : optimization of measurement conditions of AChE and GST activities, *in vitro* and *in vivo* effect of copper exposure on animals, and field study of the three biochemical parameters used (AChE and GST activities and lipid peroxidation).

Table 1. Results of kinetic parameters and optimal conditions selected for enzyme activity measurements of AChE and GST.

	AChE activity with ASCh as substrate	GST activity with CDNB as substrate
Kinetic parameters :		
-Km (µM)	44	660
-Vm (nmoles/min/mg proteins)	165	333
Values chosen for activity measurement		
- Temperature incubation (°C)	25	25
- pH	7.5	7.5
- Substrate concentration (mM)	4	1

In order to provide data on the AChE and GST activities in *D. trunculus*, and prior to investigating their use as biomarkers, the optimal conditions (pH, temperature and substrate concentrations) were determined. The study of these enzymatic parameters showed that, for AChE, the maximum activity was obtained with : i) ASch as a substrate. In fact, the comparison of Vm/Km ratio of PSch, BuSch and ASch showed that this latter substrate has the most elevated values of activity and is therefore the best substrate for this enzyme, ii) temperature between 25 and 45°C, and iii) pH between 7 and 8. Moreover, the enzyme showed a Michaelis-Menten kinetic, with increased activity up to a maximum value at 4 mM (at this concentration, the activity is constantly maximum). Similar conditions were determined for other bivalves (Najimi et al. 1997). However, the turn over of the substrate smetabolism was higher in *D. trunculus* than in other species previously studied. The maximal activity was obtained at 25 °C and pH = 7.5. This optimum temperature incubation was lower than the value reported in *M. galloprovincialis* from Arcachon Bay (37°C) and in *Corbicula fluminea* from Sanguinet Lake (42°C) (Mora et al. 1999). As for AChE, the GST activity increased

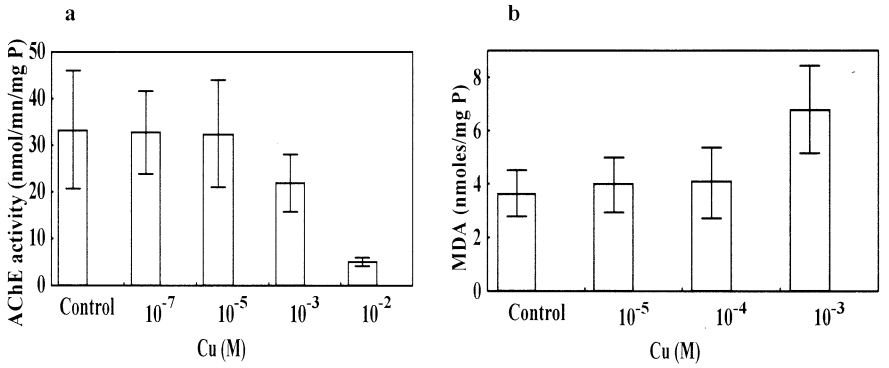

Figure 1. *In vitro* copper effect on AChE activity (a) and MDA content (b) in the *D. trunculus* post mitochondrial fraction.

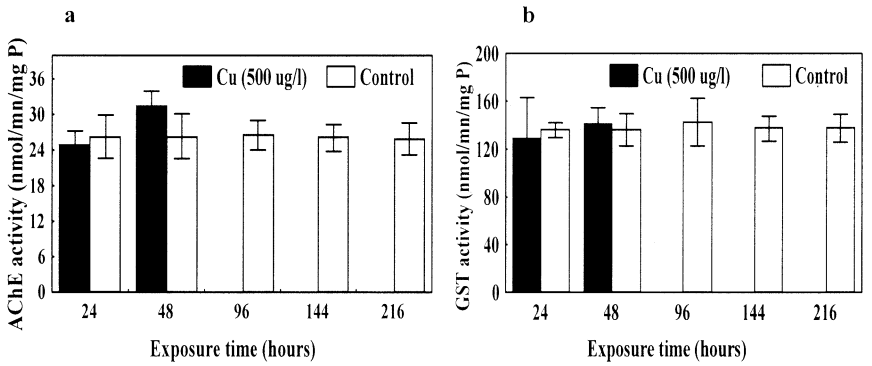

Figure 2. *In vivo* effect of copper on AChE (a) and GST (b) activities in *D. trunculus*.

proportionally to the substrate concentration, with a maximum value at 1.2 mM. Table I gives the kinetic parameters of AChE and GST and the temperature, pH and substrates concentrations selected for the two enzymes activity measurements. These results allowed us to deduce the optimal conditions for the measurements of enzymatic activities in the following experiments. The temperature, pH and substrate concentration selected for GST and AChE activities measurements took into consideration the values giving the highest activity and lowest spontaneous conjugation and spontaneous hydrolysis respectively.

The *in vitro* and *in vivo* effects of copper are presented respectively in Figure 1 and Figure 2. Figure 1a shows the direct *in vitro* effect on AChE activity by copper addition to the *D. trunculus* incubation mixture. The first significant effect was detected at 10^{-3} M. The AChE inhibition increased with the contaminant concentration. At 10^{-2} M, the inhibition reached 90%. The CI_{50} was observed at 5.37 mM. The direct inhibition of AChE activity by copper was also reported in mollusks by several authors (Boucquené, 1996; Labrot et al. 1996). The mechanism of inhibition by copper may be explained by an interaction between enzyme sulfhydryl groups and metal, leading to changes in enzyme conformation and activity.

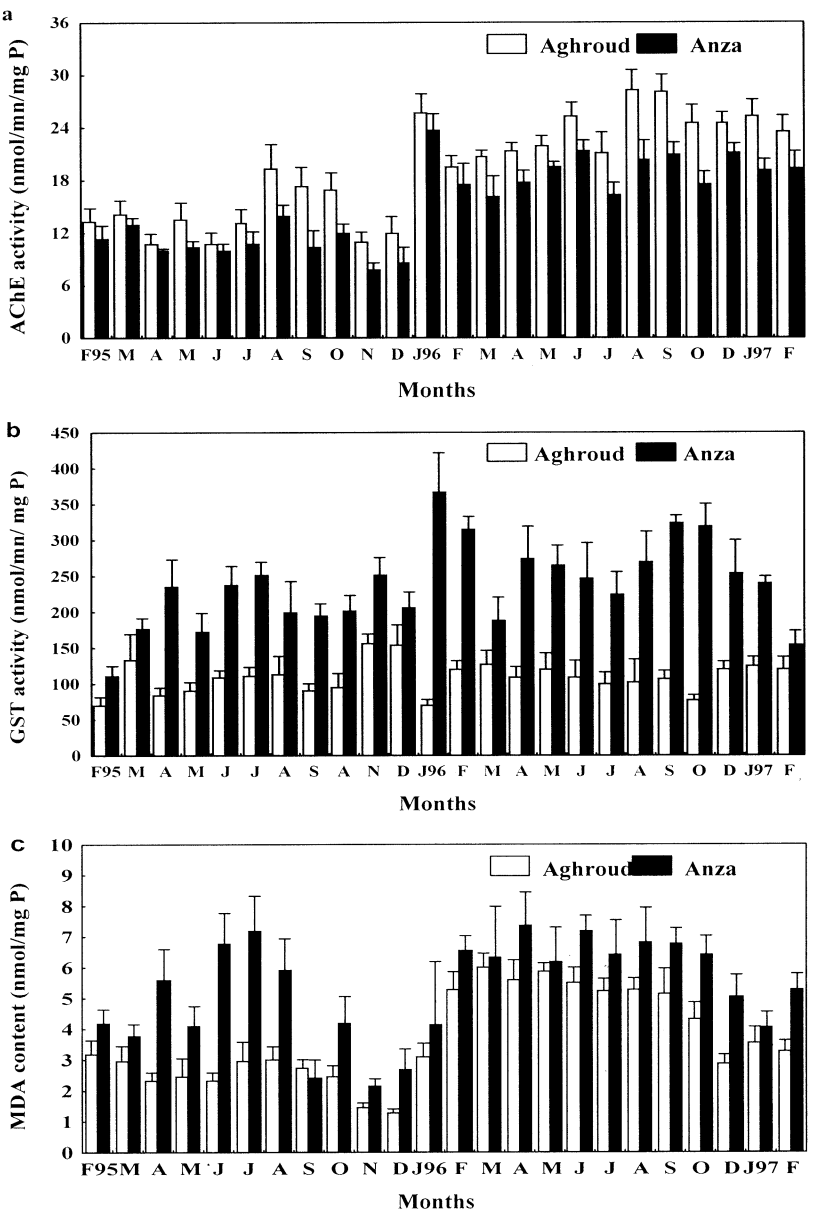

Figure 3. Seasonal variations of AChE (a), GST (b) activities, and MDA content (c) in *D. trunculus* collected from a clean site (Aghroud) and a polluted one (Anza) over two years.

As shown in Figure 1b, lipid peroxidation, evaluated by MDA content, was increased (186%) when Cu was added at the maximum concentration tested (10^{-3} M). Copper has also been reported as a potent inducer of lipid peroxidation (Gnassia - Barelli et al. 1995). It is a transition metal able to generate reactive fatty acids, in particular arachidonic acid: the main substrate for MDA formation.

In *in vivo* experiment (Figure 2), copper was found to be highly toxic at the concentration tested, which was 500 µg/l (after two days of exposure, all animals died). The shells were closed as soon as they were in contact with contaminant. This could explain the high mortality observed and consequently the absence of a biochemical response.
In reference animals, the parameters measured were constant all through the experiment in laboratory conditions. This supports the usefulness of *D. trunculus* for such studies.

Figures 3a, 3b and 3c represent the field study of the three biochemical parameters AChE activity, GST activity and lipid peroxidation respectively. Under these field conditions, all biochemical parameters measured were subject to seasonal changes over two years of study (intrasite variations). The seasonal variation of AChE activity (Figure 3a) exhibited a similar profile at the two sites: the higher values occurred in summer but were lower in spring. GST activity (Figure 3b) was highest in the autumn – winter period at the reference site and during summer at the polluted one. In contrast to AChE, GST varied seasonally at the two sites. For lipid peroxidation (Figure 3c), lower values of MDA content were recorded in winter, with higher values in the spring – summer period at both sites.

Differences were also found on intersite comparison for all biochemical parameters studied. Throughout the two years studied, AChE activity was significantly ($p<0.01$) reduced at the polluted site compared to the unpolluted one (Figure 3a). For example, in August 1995 and August 1996, the values were respectively 26% and 35% lower in Anza than in Aghroud. The GST activity (Figure 3b) was more elevated (130 to 520 %) at the polluted site compared to the reference one. The TBARs content (Figure 3c) was also significantly ($p<0,05$) higher in animals from the polluted site.
In summary, in animals from Anza, AChE activity was lower while GST activity and MDA content were higher than in molluscs collected from Aghroud. In fact, Anza was highly contaminated by domestic and industrial effluents (Touyer 1997). The correlation between AChE inhibition and pollution has been reported in field studies in fish (Galgani et al. 1992) and mussels (Narbonne et al. 1991). Moreover, similar results were previously observed in our sampling sites for *M. galloprovincialis* and *P. perna* (Najimi et al. 1997) in Agadir Bay. Lipid peroxidation was also quite sensitive to pollution in several studies (Livingstone et al. 1990; Labrot et al. 1996; Burgeot et al. 1996). Important levels have been shown to occur in response to exposure *in vivo* to heavy metals (Livingstone et al. 1990) or *in situ* to various pollutants in contaminated area (Cossu et al. 2000).
The inputs of organic and inorganic chemicals induced biochemical changes in *D. trunculus* such as inhibition of AChE (i.e. organophosphates, carbamates and heavy metals), increased lipid peroxidation (i.e. transition metals and organic pro-oxidants) and increased GST activity (i.e. organic chemicals). The results obtained from *in vivo* and *in vitro* laboratory studies with copper treatment were consistent with field study data.

In addition to investigations carried out on the biology of *D. trunculus* by our laboratory (Lagbouri and Moukrim, 1999), the data presented here suggest that *D. trunculus* is a sentinel species useful for biomonitoring studies in sandy beaches.

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