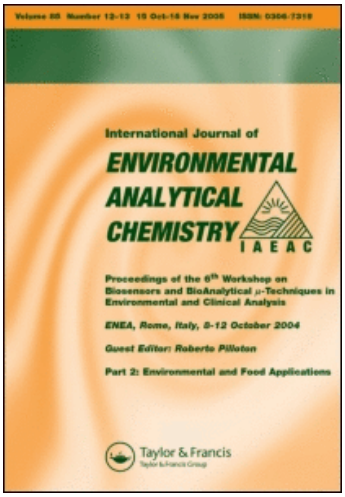

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Maria Giulia Lionetto[a]; Roberto Caricato[a]; Elisa Erroi[a]; Maria Elena Giordano[a]; Trifone Schettino[a]
[a] Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università di Lecce, 73100 Lecce, Italy

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Carbonic anhydrase-based environmental bioassay

MARIA GIULIA LIONETTO*, ROBERTO CARICATO, ELISA ERROI, MARIA ELENA GIORDANO and TRIFONE SCHETTINO

Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università di Lecce, Via prov.le Lecce-Monteroni, 73100 Lecce, Italy

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Carbonic anhydrase (CA) is a metalloenzyme catalysing the reversible idratation of CO_2 in H^+ and HCO_3^-. It is an ubiquitous enzyme in bacteria, plant and animal kingdoms, playing a fundamental role in a number of physiological processes. Previous studies demonstrated the sensitivity of CA activity to dichlorodiphenyl-dichloroethane (DDT) exposure in birds and to cadmium exposure in teleosts. The aim of the present work was to develop a new *in vitro* enzymatic bioassay for detecting toxic chemicals in environmental samples as a cost-effective tool in environmental monitoring. This bioassay uses the commercial available CA isozyme II from bovine erythrocytes whose sensitivity to the main classes of chemical pollutants of importance in water quality research was tested in this work. CA activity was determined by a modification of the electrometric method previously described by Wilbur and Anderson [K.M. Wilbur, G.N. Anderson. *J. Biol. Chem.*, **176**, 147 (1948).]: briefly, CA activity units were calculated from the rate of H^+ production in the reaction mixture (where CO_2 was present as substrate) against a blank containing the specific CA inhibitor acetazolamide. [H^+] variation was followed at 0°C in the reaction mixture using a Mettler Delta 350 pH-meter. In our experimental set-up bovine CA activity was significantly inhibited by heavy metals (Cd, Cu and Hg), by the organochlorate compound arochlor and by the carbammate pesticides carbaryl in a dose-dependent manner. CA *in vitro* bioassay can represent a novel tool for rapid and low cost understanding of the toxicity of environmental samples, for assessing bioavailability of pollutants in environmental matrices and their additive or synergistic biological effects when present in mixtures.

Keywords: Carbonic anhydrase; *In vitro* bioassay; Enzyme inhibition; Carbaryl; Heavy metals; Arochlor 1248

1. Introduction

In recent years, the increased need for improving the efficiency and effectiveness of environmental monitoring has promoted the combination of chemical analysis with biologically based techniques. This provide useful information about bioavailability of pollutants and about their toxic effect for biota, and allow us to screen large numbers of samples greatly decreasing the time and cost of analyses.

*Corresponding author. Fax: +39-0832-298626. Email: giulia.lionetto@unile.it

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The aim of the present work was to develop a new *in vitro* enzymatic bioassay for detecting toxic chemicals in environmental samples as a cost-effective tool in environmental monitoring. This bioassay uses the commercial available carbonic anhydrase (CA) isozyme II from bovine erythrocytes, whose sensitivity to the main classes of chemical pollutants of importance in water quality research was tested in this work.

Carbonic anhydrase is an ubiquitous metalloenzyme in bacteria, plant and animal kingdoms, catalysing the reversible hydration of CO_2 to produce H^+ and HCO_3^-. It plays a fundamental role in a number of physiological processes, such as respiration, ionic transport, acid–base regulation and calcification [2]. CA is a metalloenzyme using a zinc atom as cofactor in the active site. The zinc atom is liganded by three histidine residues with a hydroxide or a water molecule as a fourth ligand, giving a tetrahedral coordination geometry. For the CO_2 hydration reaction, the zinc-bound hydroxide initiates a nucleophilic attack on the substrate CO_2 to form zinc-bound HCO_3^- which is then displaced by a water molecule [3].

It is well known that CA is inhibited by sulfonamides and widely utilized in clinical application [4]. In the environmental toxicology field this enzyme has shown to be sensitive to some environmental chemical pollutants. Early studies demonstrated the sensitivity of CA activity to dichlorodiphenyl-dichloroethane (DDT) exposure in birds [5]; more recently Lionetto *et al.* [6] reported the *in vitro* inhibitory effect of cadmium on CA in the intestine and gills of the euryhaline teleost *Anguilla anguilla*, Skaggs and Henry [7] investigated the CA sensitivity to heavy metals in the gills of two euryhaline crabs and Caricato *et al.* [8, 9] found an *in vitro* and *in vivo* inhibitory effect of cadmium on CA in the sentinel organism *Mytilus galloprovincialis*.

In our *in vitro* bioassay the CAII isozyme from bovine erythrocyte was utilized as a toxicological detection tool since this is one of the most characterized CA isoforms and is commercially available in a purified form.

To our knowledge this is the first time that this enzyme has been proposed as a tool for environmental application.

2. Experimental

2.1 *Materials*

All reagent grade chemicals were purchased from Sigma (St. Louis, USA).

2.2 *Methods*

Carbonic anhydrase enzymatic activity was measured using a modification [10] of the electrometric method described earlier by Wilbur and Anderson [1].

The device for the CA based *in vitro* assay was constituted by a small beaker enclosed in a thermostated steel cup maintained at the constant temperature of 0°C by a cryostate. The reaction mixture added to the beaker was composed as follows: 0.5 μg CA isozyme II from bovine erythrocytes in 4 mL of buffer A (60 mM Mannitol, 0.2 mM Tris-Hepes pH 8.65, at 0°C) were added to 6 mL of buffer B (9.7 mM Tris, 3.5 mM Hepes pH 8.65, at 0°C). The reaction was started by the addition of 5 mL of CO_2 saturated water (0°C) and gassing the assay medium with 5% CO_2 and 95% O_2. The effect of several organic and inorganic environmental pollutants was

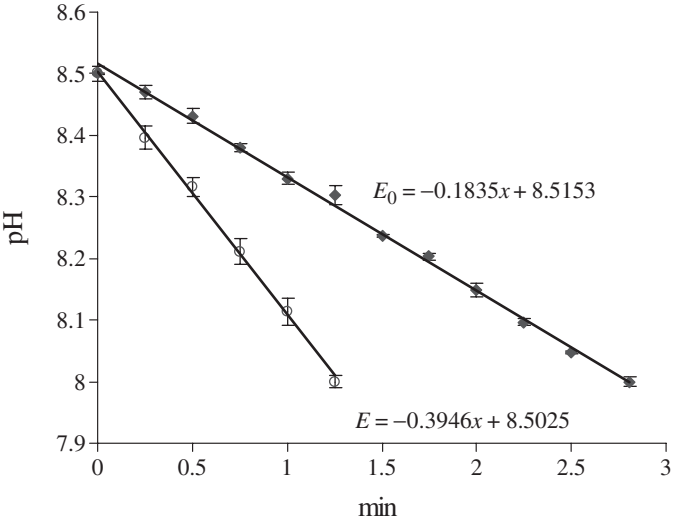

Figure 1. Time course of CA II enzymatic reaction. The slope of the E line ($-0.3946\ \Delta\text{pH min}^{-1}$) represents the rate of enzymatic production of H^+, the slope of the E_0 line ($-0.1835\ \Delta\text{pH min}^{-1}$) represents the rate of aspecific uncatalysed production of H^+. As reported in the section 2.2 the assay temperature was 0°C. 0.5 µg CA II were present in the reaction mixture.

tested by adding various concentrations of $CdCl_2$, $HgCl_2$, $CuCl_2$, arochlor 1248 and carbaryl before starting the assay.

Carbonic anhydrase activity units were calculated from the rate of H^+ production in the reaction mixture against a blank containing the specific CA inhibitor acetazolamide (1 µM). The time-course of CA II enzymatic reaction is reported in figure 1. The reaction was followed in the pH range 8.5–8.0, where the buffer capacity of the reaction mixture does not significantly change in the presence of increasing HCO_3^- concentrations developed by the hydration of CO_2, and the rate of pH decrease is linear. One unit of CA activity corresponds to 1 µmol of $H^+\,\text{min}^{-1}$ in excess of a blank sample containing the specific CA inhibitor acetazolamide (1 µM).

The enzymatic units developed in 15 mL of the reaction mixture were calculated as:

$$A = \left(\beta \times \frac{\Delta\text{pH}}{\Delta t}\right) - \left(\beta \times \frac{\Delta\text{pH}}{\Delta t'}\right) \times V$$

where A is the enzymatic activity (enzymatic units developed in 15 mL of the reaction mixture), β is the measured buffer capacity of the reaction medium in the pH range considered (it corresponds to $2.34\,\text{mmol L}^{-1}\,\text{pH}^{-1}$), $\Delta\text{pH} = 0.5$ (from 8.5 to 8.0), Δt is the time (expressed in min) required to change the pH of the reaction mixture from 8.5 to 8.0, Δt is the time (expressed in min) required to change the pH of the reaction mixture containing 1 µM acetazolamide from 8.5 to 8.0, and V is the volume of the reaction mixture, corresponding to 15 mL.

The amount of CA II utilized in the bioassay was determined on the basis of a linear relation between CA activity and CA µg in the reaction mixture (reported in figure 2).

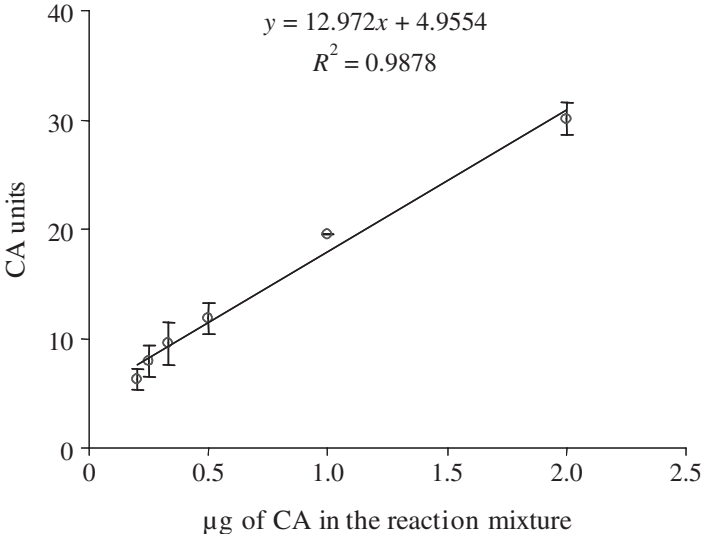

Figure 2. Relation between the enzymatic activity expressed as CA units and micrograms of CA II added in the reaction mixture (final volume 15 mL). CA units were calculated as described in section 2.2 from the rate of H^+ production in the reaction mixture against a blank containing the CA inhibitor acetazolamide (1 μM). The reaction was followed at 0°C in the pH range 8.5–8.0. It is possible to observe a linear increase of CA activity in relation to the enzyme content of the mixture.

A figure of 0.5 μg of CA II was chosen as the standard dose for the bioassay since it was included in the linear response range of CA activity *versus* CA μg (figure 2).

2.3 *Statistical analysis*

Percentage activity values of CA II for different concentrations of heavy metals, arochlor and carbaryl were drawn by using the regression analysis graph (Graph Pad Softwer Prism 2.01). Carbonic anhydrase activity without pollutants was accepted as 100% activity. The inhibitor concentrations causing up to 50% inhibition (IC_{50}) were determined from semilog plot of percentage CA activity *versus* log concentrations of pollutants in the reaction medium. Data are reported as means ± SD.

3. Results

3.1 *CA bioassay and heavy metals*

In figure 3 (a–c) the response of the CA activity based bioassay to increasing concentrations of pollutant heavy metals, such as cadmium, mercury and copper, is reported. The tested heavy metals were added as chloride salts in the assay medium. Therefore they were present in cationic form. In the concentration range tested (10^{-8}–10^{-3} M for $CdCl_2$ and 10^{-8}–10^{-4} for $CuCl_2$ and $HgCl_2$) the enzymatic activity showed a sigmoidal dose-response decrease with the logarithmic increase of heavy metal concentrations in the reaction mixture. At the low concentration of 100 nM, $CuCl_2$ was able to produce a significant inhibition of the enzymatic activity of 16.2%, while $CdCl_2$

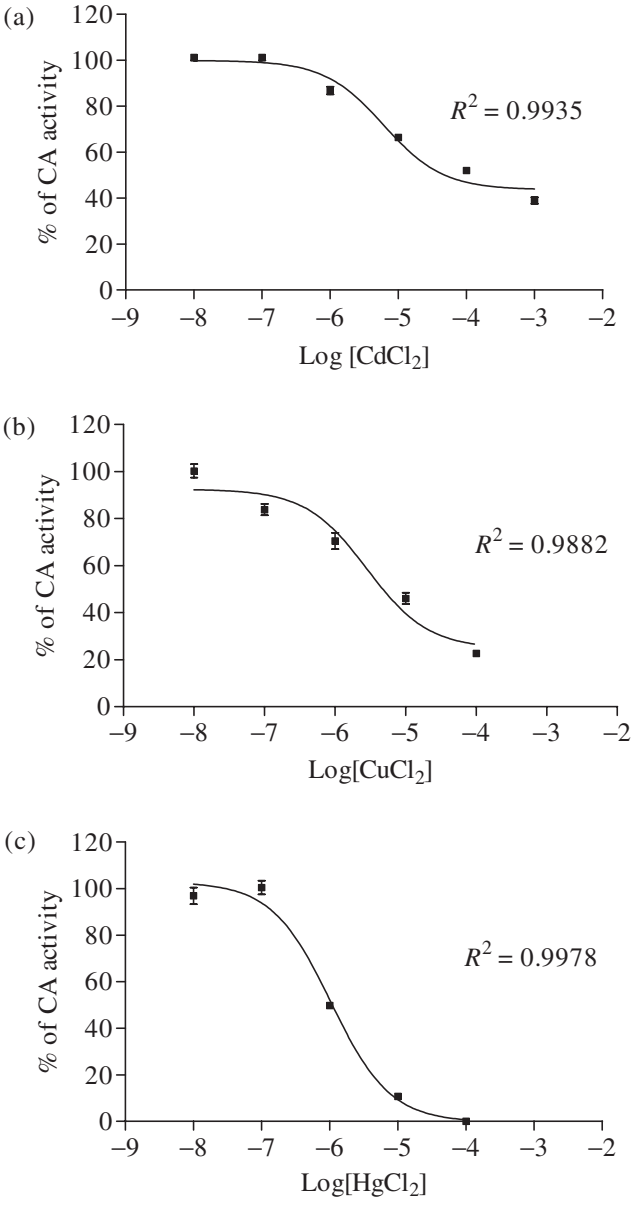

Figure 3. Dose–response curves of the effect of increasing concentrations of $CdCl_2$ (a), $CuCl_2$ (b) and $HgCl_2$ (c) on CA II activity. Ordinate: percentage of residual enzymatic activity (CA activity without pollutants was accepted as 100%); abscissa: logarithm of heavy metal concentrations. CA units were calculated from the rate of H^+ production in the reaction mixture against a blank containing the CA inhibitor acetazolamide (1 μM) (see section 2.2). The reaction was followed at 0°C in the pH range 8.5–8.0. Data are reported as mean ± SD.

and $HgCl_2$ were ineffective. $HgCl_2$ showed the lowest IC_{50} value, corresponding to 1.09 μM, followed by $CuCl_2$ (4.74 μM) and $CdCl_2$ (48.10 μM). Interestingly, when a mixture of 100 nM Hg, Cd, and Cu was added to the reaction medium the inhibition of the CA activity was significantly ($P < 0.01$) higher with respect to the effect exerted

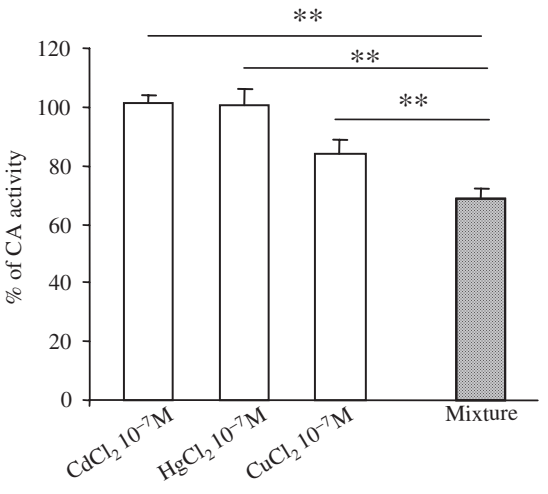

Figure 4. Effect of mixture of 10^{-7} M $HgCl_2$ plus 10^{-7} M $CdCl_2$ plus 10^{-7} M $CuCl_2$ on CA II activity compared with the effect of the single heavy metals at the same concentration. The statistical significance of data was analysed by one-way ANOVA and Newman–Keuls Multiple Comparison Test. ** = $P < 0.01$. (for details see legend to figure 3).

by each heavy metal at this concentration (figure 4) and revealed the synergic effect that several heavy metals being together can exert on biological systems.

3.2 *Carbonic anhydrase bioassay and xenobiotics*

The sensitivity of the CA based bioassay was also tested for xenobiotics such as pesticides and PCBs. The polychlorinated biphenyl (PCB) arochlor and carbamate pesticide carbaryl were used as reference toxicants.

Activity of CA II was very sensitive to arochlor, showing an inhibition of 34.4% at a concentration of 10 ng L^{-1} (figure 5). At the highest concentration tested of 1 μg L^{-1} the inhibition was of 56.5%.

The enzymatic bioassay was also very sensitive to the pesticide carbaryl. At the lowest concentration tested of 2 ng L^{-1} a significant 18% inhibition was observed (figure 6). In the concentration range tested (2 ng L^{-1}–20 mg L^{-1}) the enzymatic activity showed a sigmoidal dose–response decrease with the logarithmic increase of the pesticide concentration in the reaction mixture. The calculated IC_{50} value corresponded to 20 μg L^{-1}.

3.3 *Intra-assay precision*

The intra-assay precision of the bioassay was calculated from the coefficient of variation of four replicated determinations ((SD/mean) × 100); it was below 5% (mean value 4.63%).

4. Discussion

In the present work a new *in vitro* bioassay for detecting toxic chemicals in environmental samples is proposed. It is based on the activity of the CA enzyme, in particular

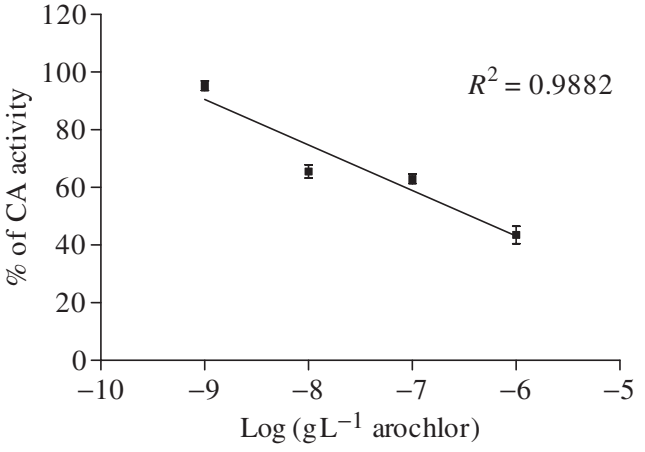

Figure 5. Dose–response curves of the effect of increasing concentration of Arochlor 1248 on CA II activity. Ordinate: percentage of residual enzymatic activity (CA activity without pollutants was accepted as 100%); abscissa: logarithm of Arochlor concentrations (for details see legend to figure 3).

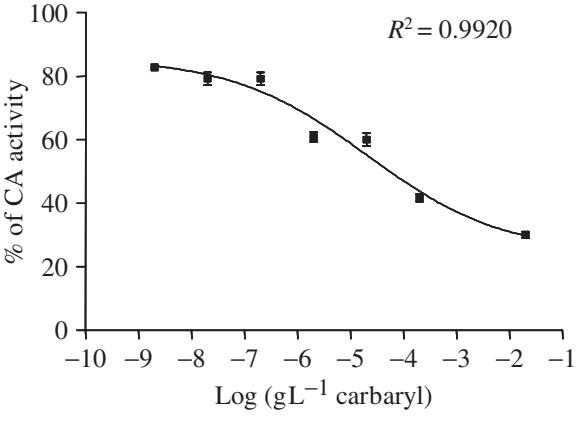

Figure 6. Dose–response curves of the effect of increasing concentration of carbaryl on CA II activity. Ordinate: percentage of residual enzymatic activity (CA activity without pollutants was accepted as 100%); abscissa: logarithm of carbaryl concentrations (for details see legend to figure 3).

the commercially available CA II isozyme, which require a simple and low cost set-up, including a pH-meter, a cryostate and a simple mixture of CO_2% and O_2%.

As indicated by the obtained results, CA based bioassay is sensitive to the main classes of environmental chemical pollutants of importance in water quality assessment, such as heavy metals, PCBs and pesticides.

Cadmium, mercury and copper, which represent the most toxic pollutant heavy metals, were tested. The natural concentration of metals in the aquatic environment, and in particular in fresh water, varies depending on the metal concentration in the soil and the underlying geological structures, the acidity of the water, its humus content and particulate matter concentration. Andersen *et al.* [11] reported high diffuse background levels of heavy metals (i.e. 0.02 ppm mercury, 0.04 ppm cadmium and

0.6 ppm copper) for surface waters, which are widely monitored for heavy metal pollution assessment. However, the total concentration of a particular heavy metal is often a poor measurement of the metal's toxicity since it does not consider the so-called 'labile metals' that represent the sum of free metal ions and metal that can easily dissociate from complexes or colloids. These are the metal species available for biological systems and responsible for toxic effects, although they are very difficult to determine analytically. The CA based bioassay was sensitive to the biological effective metal ions, showing micromolar IC_{50} values (lower for Hg followed by Cu and Cd), corresponding to fractions of ppm. The different time-course showed by the three tested metals could reflect the different sensitivity of the catalytic activity of this enzyme and corresponds to the general scale of toxicity of these three metals for biological systems, mercury being the most toxic.

Interestingly, the CA based bioassay was able not only to detect the presence of the single heavy metals but also to reveal the synergic effects that a mixture of heavy metals, each at low concentration, can exert on biological systems. In fact, it has to be pointed out that the toxicological risk for biota often comes not from the single pollutants, each in trace concentration, but from the additive and synergic effect that they can exert together.

The CA based bioassay was also sensitive to arochlor 1248 used as reference toxicant for PCBs. In arochlor chemical analysis, water samples are analysed to a detection limit of 0.1 parts per billion (ppb) (EPA Method 608/SW-846 Method 8080), while sediment samples are analysed to a detection limit of 0.1 parts per million (ppm) (EPA method 608/SW-846 Method 3550). Considering that CA II already showed an inhibition of 34.4% at a concentration of $10\,ng\,L^{-1}$ (0.01 ppb) the relevant sensitivity of this bioassay for PCBs has to be stressed. Conventional methods for the analysis of PCBs, using high resolution gas chromatography/mass spectrometry (GC/MS), are expensive and time consuming and require specialized equipment. Therefore, this bioassay offers a simple and cost-effective screening tool for these compounds; this tool is suitable for use in combination with chemical analysis.

Moreover, CA II activity also showed a high sensitivity for carbaryl, chosen as the reference toxicant for carbamate pesticides widely used in agricultural activities. Carbamates, like organophosphate pesticides, are well known for their anticholinesterase effect, but to date other possible aspects of their toxicological effects are unknown. In this work we reported on a significant carbamate induced inhibition of the enzyme carbonic anhydrase for the first time. The lowest tested carbaryl concentration effective on CA activity was $2\,ng\,L^{-1}$, showing an inhibition of 18%. This preliminary result suggests that the CA bioassay can have a higher sensitivity for carbamate pesticides than the acetylcholinesterase *in vitro* bioassay, whose detection limits are $100\,ng\,L^{-1}$ and $10\,ng\,L^{-1}$ for carbamates and organophosphate respectively [12, 13].

In the future further studies are needed to investigate the sensitivity of the CA-based bioassay for other chemical pollutants and for its application to real environmental samples. However, the results obtained already indicate clearly the potentiality of this bioassay for application in the screening of general toxicity of environmental samples. Its ability to detect different toxic chemicals on the basis of their biological negative effect, including synergic effect, makes this assay a rapid and sensitive response chemical hazard detection system. It can give useful information on the general toxicity of environmental samples without using animals, it is easy to perform, and it is cheap

and fast. Therefore, this study represent the first step for further investigations and possible applications of this bioassay in combination with chemical analysis, for the pre-screening of environmental samples that need to be analysed.

Acknowledgements

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