



POTENTIAL APPLICATION OF CARBONIC ANHYDRASE ACTIVITY IN BIOASSAY AND BIOMARKER STUDY

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BACKGROUND Carbonic anhydrase (CA) (EC 4.2.1.1), ubiquitous enzyme in bacteria, plant, and animals, catalyzes the reversible hydration of CO₂ to produce H⁺ and HCO₃⁻ using zinc as cofactor. CA plays a fundamental role in a number of physiological processes, such as respiration, ionic transport, acid-base regulation and calcification.

Sulfonamides and heavy metals are considered strongest CA inhibitors (Vitale et al., 1999). Moreover, dichlorodiphenyl-dichloroethane (DDT) has previously shown inhibitory effect on CA in birds (Pocker et al., 1971). However, to date there are lacking information about toxicological effects of other pollutants on this enzyme.

The **AIM** of the present work was to investigate the *in vitro* and *in vivo* sensitivity of Carbonic Anhydrase (CA) to several chemical contaminants in view of a possible future application of CA activity inhibition measurement in biomonitoring field as either (1) *in vitro* bioassay or (2) biomarker in the sentinel organism *Mytilus galloprovincialis*. To our knowledge this is the first time that this enzyme is proposed as tool for environmental application

METHODS CA enzymatic activity was measured using a modification of the electrometric method early described by Wilbur and Anderson (1948) where the time required to change the pH from 8.5 to 8.0 was followed at 0°C using a Mettler Delta 350 pH meter. Briefly CA activity units were calculated from the rate of H⁺ production in the reaction mixture (where CO₂ as substrate was present) against a blank containing the specific CA inhibitor acetazolamide.

$$A = \left(\frac{B \times \Delta pH}{\Delta t} - \frac{B \times \Delta pH}{\Delta t} \right) \times V$$

A = enzymatic activity (Units developed in the 15 ml of reaction mixture)
 B = buffer capacity of the reaction medium
 ΔpH = 0.5 (from pH 8.5 to pH 8.0)
 Δt = the time (min) required to change the pH of the reaction mixture from 8.5 to 8.0
 Δt' = the time (min) required to change the pH of the reaction mixture containing 1μM ACZ from 8.5 to 8.0
 V = volume of the reaction mixture (15 ml)

1) **CA based *in vitro* assay:** The *in vitro* CA bioassay utilizes the commercial available CA isozyme II from bovine erythrocytes. The device for the CA assay was constituted by a small beaker enclosed in a thermostated steel cup connected to a criostrate. The reaction mixture added in the beaker was maintained at the constant temperature of 0°C and contained 75 μg Carbonic Anhydrase Isozyme II from bovine erythrocytes. The reaction was started by the addition of 5 ml of CO₂ saturated water (0°C) and gassing the assay medium with 5% CO₂ and 95% O₂. The effect of several organic and inorganic environmental pollutants important in water quality research was tested by adding various concentrations of CdCl₂, HgCl₂, CuCl₂, Arochlor 1248, carbaryl, before starting the assay.

Statistical analysis. % activity values of CA II for different concentrations of heavy metals, Arochlor and carbaryl were drawn by using regression analysis graph (Graph Pad Software Prism 2.01). CA activity without pollutants was accepted as 100% activity. The inhibitor concentrations causing up to 50% inhibition (IC50) were determined from semilog plot of percentage CA activity versus log concentrations of pollutants in the reaction medium. Data are reported as means ± S.D.

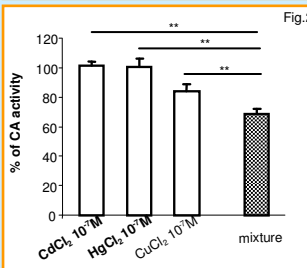
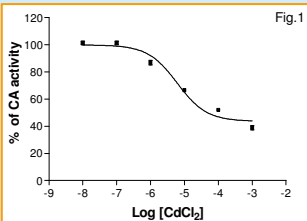
2) **CA as biomarker in the sentinel organisms *M. galloprovincialis*:** 280 specimen of *M. galloprovincialis* with a uniform shell length (66.0 ± 5.0 x 35.0 ± 1.0 mm; %male/female 60/40) were collect from the same reference sites. Prior to their contamination, the bivalves were acclimated for 48 h in glass tank containing aerated seawater at the laboratory condition: 15±1°C, 35‰ salinity and 12/12h light/dark regime.

140 mussels were exposed for 14 days to cadmium chloride (1.785 μM) and 140 were not, representing the control group. Constant Cd levels and constant feeding conditions were ensured by changing experimental and control media every third day. At time 0, 3, 7 and 14 days gills, mantle and digestive gland were collected and stored at -80°C until the CA assay. Control and exposed samples were also assayed for evaluation of standardized biomarkers such as metallothionein (MT) digestive gland levels (Viarengo et al., 1997) and granulocyte lysosomal membrane stability (Lowe et al., 1992).

Statistical analysis The statistical significance of data was tested by Two Way ANOVA. Values are given as means ± SE.

CA BIOASSAY AND HEAVY METALS

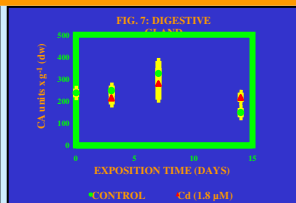
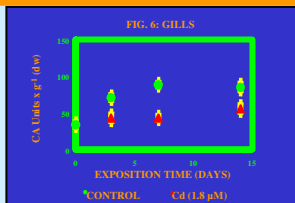
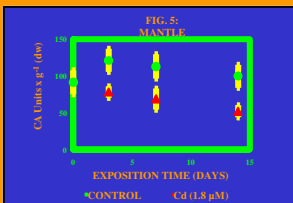
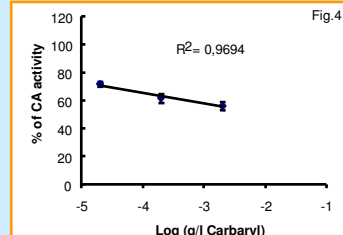
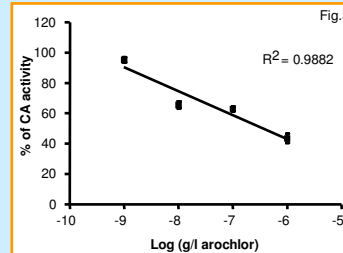
The response of CA activity based bioassay to increasing concentrations of cadmium (reported in Fig.1), mercury and copper (not reported here) (in the concentration range tested 10⁻⁸ + 10⁻³ M for CdCl₂ and 10⁻⁸ + 10⁻⁴ for CuCl₂ and HgCl₂) showed a sigmoidal dose-response decrease with the logarithmic increase of heavy metal in the reaction mixture. HgCl₂ showed the lowest IC₅₀ value, corresponding to 1.09 μM, followed by CuCl₂ (4.74 μM) and CdCl₂ (48.1 μM). Interestingly, when a mixture of 10⁻⁷M 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 by each heavy metal at this concentration (Fig.2) and revealed the synergic effect that several heavy metals being together can exert on biological systems.



CA BIOASSAY AND XENOBIOTICS

The sensitivity of the CA based bioassay was also tested for xenobiotics such as pesticides and PCBs. As reference toxicants the polychlorinated biphenyl (PCB) Arochlor 1248 and the carbamate pesticide carbaryl were used.

CA II activity was very sensitive to Arochlor, showing an inhibition of 34.4 % already at the concentration of 10 ng/l. At the highest concentration tested of 1 μg/l the inhibition was of 56.5 % (Fig.3) Also the pesticide carbamate was effective on CA II showing a 29% inhibition at the lowest concentration tested of 20 μg/l (Fig.4).



CA AS BIOMARKER IN THE SENTINEL ORGANISMS *M. galloprovincialis*

These results showed that *M. galloprovincialis* CA activity was significantly affected by *in vivo* cadmium exposure and its sensitivity was tissue-specific. **Mantle** CA activity showed a 40% maximal inhibition during the entire time-course of the experiment (Fig. 5). This result could be related to the decrease in shell growing observed in mussels chronically exposed to environmental pollutants (Manu et al., 2000). **Gill** CA activity was significantly inhibited in a time dependent manner: it showed a maximal inhibition by 50% within the first week of exposure, followed by a partial recovery (Fig. 6).

Digestive gland CA activity showed an interesting biphasic behaviour surprisingly different with respect to the other tissues. In the first week of the experiment, it was slight inhibited in the exposed mussel with respect to the control group. In the second week the enzymatic activity showed an inversion of this trend and a significant increase with respect to control group at time 14 (fig. 7). In order to explain this last response it has to be considered that CA could play a key role in the acidification of the lysosomal compartment (by catalyzing the H⁺ production from metabolic CO₂) and that pollutant induces the activation of the lysosomal system in exposed organisms (Köhler, 1991). Therefore, the observed biphasic changes of CA activity in mussels could rise from a balance between the inhibitory effect of Cd on the enzymatic catalytic activity and the induction of new CA molecules synthesis required for the activation of the lysosomal system.

CONCLUSIONS In the present work we successfully explored the possible application of carbonic anhydrase enzyme as either *in vitro* bioassay or potential biomarker in sentinel organism.

As regards the possible application as *in vitro* bioassay we standardized a CA based bioanalytical method available for monitoring environmental samples, utilizing commercial available CA isozyme II from bovine erythrocytes. In our experimental set up bovine CA activity was significantly inhibited by submicromolar concentrations of heavy metals (Cd, Cu and Hg), and ppb concentrations of arochlor (the lowest effective concentration: 0.01 ppb) and carbaryl (the lowest effective concentration: 20 ppb) showing a dose-response behaviour.

As regards the possible application as biomarkers, CA was investigated in the filter feeding *M. galloprovincialis*, widely used in pollution monitoring programs as sentinel organism. Following *in vivo* exposure of *M. galloprovincialis*, to cadmium chloride as reference toxicant, CA activity present in mantle and gills was significant inhibited.

The sensitivity to chemical pollutants and low cost and simplicity of the assay method make CA activity measurement suitable for *in vitro* bioassay of the toxicity of environmental samples and for field biomarker application in the sentinel organism *M. galloprovincialis*.

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