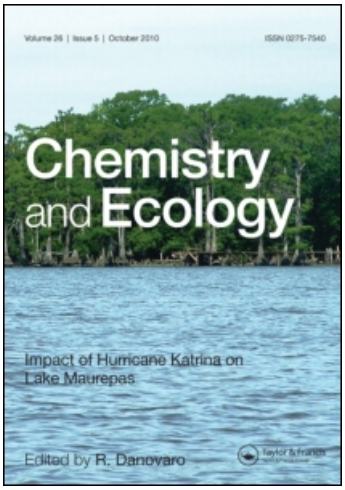

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Potential application of carbonic anhydrase activity in bioassay and biomarker studies

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Carbonic anhydrase (CA) (EC 4.2.1.1), a ubiquitous enzyme in bacteria, plant, and animal kingdoms, catalyses the reversible hydration of CO_2 to produce H^+ and HCO_3^- 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. The aim of the present work was to investigate the sensitivity of this enzyme to heavy metals with a view to possible future applications of CA activity inhibition measurement in biomonitoring as either an *in vitro* bioassay or a biomarker. CA activity was determined by modifying a previously described electrometric method: briefly, CA activity units were calculated from the rate of H^+ production in the reaction mixture (where CO_2 was present as a substrate) against a blank containing the specific CA inhibitor acetazolamide. As regards the possible application as an *in vitro* bioassay, the sensitivity to heavy metals (cadmium, mercury, and copper) of the commercially available purified carbonic anhydrase (isozyme II) from bovine erythrocyte was tested *in vitro*. In our experimental set-up, bovine CA activity was significantly inhibited by micromolar concentrations of heavy metals, showing a dose–response behaviour. As regards the possible application as biomarkers, CA was investigated in the filter-feeding *Mytilus galloprovincialis*, widely used in pollution-monitoring programmes as a sentinel organism. Following *in vitro* and *in vivo* exposure to 1.785 μM cadmium chloride as a reference toxicant, mantle CA activity was significant inhibited. In conclusion, 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 applications in the sentinel organism *M. galloprovincialis*.

Keywords: Biomarker; Bioassay; Heavy metals; Carbonic anhydrase; *Mytilus galloprovincialis*

1. Introduction

Contamination of the environment by anthropogenic chemicals has become a serious problem worldwide: pollutants can cause disturbances in ecosystems [1], loss of living resources [2], and damage to human health [3]. In recent years, increasing sensitivity to these problems has promoted the development of environmental 'diagnostic' tools for early-warning detection of pollution. It is known that the harmful effects of pollutants are typically first manifested at lower levels of biological organization before disturbances are realized at the population,

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community, and ecosystem levels [4]. This is the reason why in recent years the study of molecular and cellular effects of pollutants has yielded important advancements in developing biologically based methodologies useful for environmental biomonitoring and risk assessment. Enzymatic inhibition studies have been very useful for environmental monitoring applications. For example, the inhibition of acetylcholinesterase enzymatic activity by organophosphate and carbamate pesticides has been widely used in biomonitoring for neurotoxicant risk assessment of aquatic environments [5–12]. In the present work, carbonic anhydrase (CA) and its sensitivity to heavy metals was investigated. CA is a metalloenzyme using zinc atom as a cofactor in the active site; it is a ubiquitous enzyme present in the bacteria, plant, and animal kingdoms. CA catalyses the reversible hydration of CO_2 to produce H^+ and HCO_3^- and plays a fundamental role in a number of physiological processes. In animals, it plays a key role in respiration, ionic transport, acid–base regulation, and calcification [13]. In chloroplasts of plant cells, it is essential for photosynthetic fixation of CO_2 [14].

It is well known that CA is inhibited by most monovalent anions [15] and sulphonamides [16] widely utilized in clinical applications. On the contrary, in the area of environmental toxicology, very few investigations have been carried out on the effect of chemical pollutants on CA. Early studies demonstrated the sensitivity of CA activity to dichlorodiphenyldichloroethane (DDT) exposure in birds [17]; more recently, Lionetto *et al.* [18] reported the *in vitro* inhibitory effect of cadmium on CA in the intestine and gills of the euryhaline teleost European eel (*Anguilla anguilla* L.), and Skaggs and Henry [19] investigated the CA sensitivity to heavy metals in the gills of two euryhaline crabs.

In the present work, we investigated the *in vitro* sensitivity to heavy metals (cadmium, mercury, and copper) of the commercially available purified carbonic anhydrase (isozyme II) from bovine erythrocyte and the *in vitro* and *in vivo* sensitivity to cadmium of carbonic anhydrase present in the mantle of the sentinel organism, *Mytilus galloprovincialis.* This study was carried out in view of future applications of CA inhibition results for developing CA-based *in vitro* bioassay and biomarker.

2. Materials and methods

All chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO). CA enzymatic activity was measured using a modification of the electrometric method described by Wilbur and Anderson [20] where the time required to change the pH of the reaction medium from 8.5 to 8.00 was followed at 0 °C using a Mettler Delta 350 pH meter. Briefly, 4 ml of buffer A (60 mM mannitol, 0.2 mM Tris-Hepes pH 8.65, at 0 °C) containing the enzyme was 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. CA 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) (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 $H^+ \times \text{min}^{-1}$ in excess of a blank sample containing the specific CA inhibitor acetazolamide (1 μM).

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

$$A = \frac{\beta \Delta \text{pH}}{\Delta t} - \frac{\beta \Delta \text{pH}}{\Delta t'} V,$$

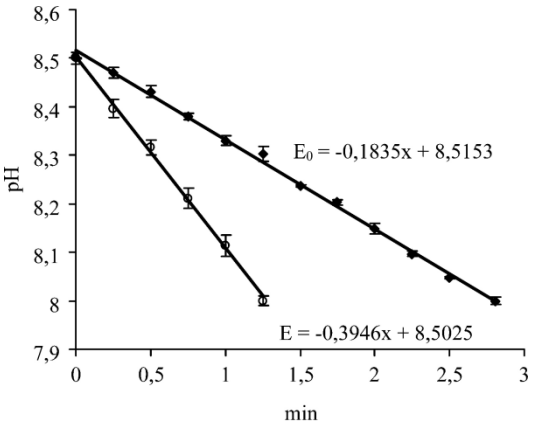

Figure 1. Time course of the CA II enzymatic reaction. The slope of the E line (-0.3946ΔpH min^{-1}) represents the rate of enzymatic production of H^+, and the slope of the E_0 line (-0.1835ΔpH min^{-1}) represents the rate of aspecific uncatalysed production of H^+.

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, corresponding to 2.34 mmol l^{-1} pH^{-1}; ΔpH is 0.5 (from 8.5 to 8.0); Δt is the time (expressed in minutes) required to change the pH of the reaction mixture from 8.5 to 8.0; $\Delta t'$ is the time (expressed in minutes) 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.

In experiments carried out on the commercially available purified carbonic anhydrase II, 0.5 μg of enzyme was added to the reaction mixture as a standardized dose. This was determined on the basis of a linear relation (not shown) between CA activity and CA μg in the reaction mixture.

In experiments carried out on the sentinel organism *Mytilus galloprovincialis*, CA was measured in the mantle crude homogenates, and its sensitivity to cadmium was evaluated in *in vitro* and *in vivo* exposure conditions. Mussels (a homogeneous stock was utilized: 66.0 ± 5.0 mm shell length) were purchased from a farm in a reference site (Mare Vivo, Castro-Lecce, Italy). Prior to their use, bivalves were acclimated for 48 h in glass tanks containing aerated sea water under the laboratory conditions: 15 ± 1 °C, 35‰ salinity and a 12 h/12 h light/dark regime. After the acclimation period, the test group, composed of 50 specimens, was exposed to 1.785 μM $CdCl_2$ for 14 d. This concentration is widely utilized for mussel chronic toxicity exposure experiments [21]. The laboratory conditions (described above) were maintained to these constants for the duration of the experiments. The control group was exposed for the same period to the same laboratory conditions of the test group except for $CdCl_2$ exposure. Test and control media were changed every third day. After 14 days of exposure, mantles were rapidly excised, frozen in liquid nitrogen, and maintained at −80 °C until analysis.

The crude homogenate was prepared as follows: the mantle was removed and homogenized in a 1:5 (tissue weight:buffer volume) ratio in ice-cold buffer (60 mM Mannitol, 0.2 mM HEPES-Tris, Leupeptin 6 mM, 0.5 mM PMSF). For the enzymatic activity determination, 200 μl of homogenates was utilized. This volume was determined on the basis of a linear relation (not shown) between mantle CA activity and homogenate protein content added in the reaction mixture.

In the *in vitro* exposure, the mantle crude homogenate of control organisms was incubated with $CdCl_2$ (100 μM) for 1 h before starting the enzymatic activity reaction. Considering the

high bioconcentration factor of heavy metals in bivalves [22], the $CdCl_2$ concentration used (100 μM) for *in vitro* experiments can resemble the real concentration of heavy metals reached in mussel tissue when the animal is chronically exposed $CdCl_2$. An incubation time of 1 h was chosen on the basis of previous results on *in vitro* CA inhibition by cadmium in teleost [18].

2.1 *Statistical analysis*

Data are reported as means ± S.D. Statistical analysis was performed using Student's *t*-test. The significance of the results was ascertained at $P < 0.05$.

3. Results

3.1 *Purified CA II from bovine erythrocyte and heavy metals*

Figure 2 shows the response of CA II activity to increasing concentrations of heavy metals, such as cadmium, mercury and copper. The tested heavy metals were added as chloride salts in the assay medium, so they were present in the cationic form. In the concentration range tested (10^{-8} M to 10^{-3} M for $CdCl_2$ and 10^{-8} M to 10^{-4} M for $CuCl_2$ and $HgCl_2$), the enzymatic activity showed a dose–response decrease with logarithmic increase in heavy-metal concentrations in the reaction mixture. At the low concentration of 100 nM, $CuCl_2$ was able to induce a significant inhibition of enzymatic activity of 16.2%, while $CdCl_2$ and $HgCl_2$ were ineffective. However, as concentrations increased, $HgCl_2$ was able to induce higher inhibitions with respect to the other two heavy metals. Comparing the inhibitory effect of the three heavy metals, cadmium was the least effective.

3.2 Mytilus galloprovincialis *CA activity and heavy metals*

In *Mytilus galloprovincialis* mantle tissue, significant CA activity was found, corresponding to 254 ± 19 U CA g^{-1} (tissue wet weight). Interestingly, *M. galloprovincialis* CA activity was sensitive to both *in vitro* and *in vivo* Cd exposure. Cadmium is recommended by the Environmental Protection Agency as a reference toxicant in tests to evaluate the long-term toxicity in effluents and outlet waters [23, 24]. As reported in figure 3, 1 h of incubation of the

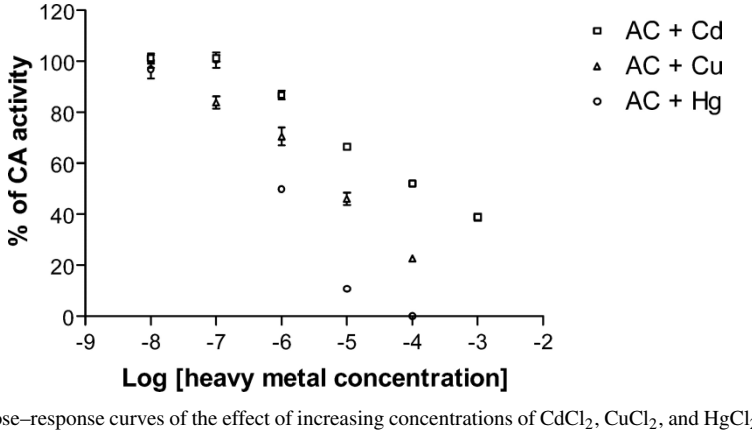

Figure 2. Dose–response curves of the effect of increasing concentrations of $CdCl_2$, $CuCl_2$, and $HgCl_2$ on purified bovine CA II activity. Ordinate: percentage of residual enzymatic activity (CA activity in the absence of heavy metals was accepted as 100% activity); abscissa: logarithm of heavy-metal concentrations. Data are reported as mean ± S.D.

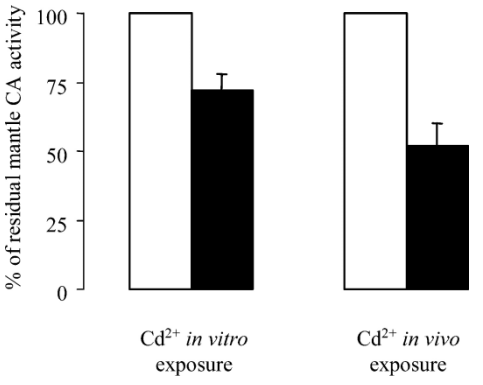

Figure 3. Effect of *in vitro* and *in vivo* $CdCl_2$ exposure of CA activity in the mantle of *M. galloprovincialis.* In the *in vitro* exposure, 100 μM $CdCl_2$ was present in the enzymatic reaction mixture; under *in vivo* conditions 1.8 μM $CdCl_2$ were added in the aquaria medium. Ordinate: percentage of residual enzymatic activity. CA activity in the absence of heavy metals was accepted as 100% activity. Data are reported as mean ± S.D.

mantle crude homogenate with 100 μM $CdCl_2$ induced a significant inhibition of CA activity of about 30%, while 14 d of exposure of the organisms to 1.785 μM $CdCl_2$ decreased the enzymatic activity by about 50%.

4. Discussion

In the present paper, the ubiquitous enzyme carbonic anhydrase has been investigated for its sensitivity to heavy metals in view of its possible future applications as an *in vitro* bioassay and biomarker.

In recent decades, pollution monitoring of aquatic environments has been increasingly concerned with the use of biological responses to pollutants at the molecular and cellular level for evaluating the biological hazards of toxic chemicals. Methods based on biological effects and their underlying mechanisms can complement, and for some applications could replace, the use of analytical chemistry in monitoring aquatic environments [25, 26]. The major advantages of such biological, mechanism-based methods are their toxicological specificity, rapidity, and low cost. Toxicological specificity refers to the relationship between the assay response and the toxic potential rather than simply the contaminant concentrations (provided by chemical analysis) of the sample being analysed. Moreover, biological assays provide rapid, sensitive, easily learned, and readily interpretable useful tools for environmental biomonitoring and risk assessment. These include biomarkers, and *in vivo* and *in vitro* bioassays.

Biomarkers are indicies of the biological effects of pollutants, occurring in organisms as a result of natural exposure to contaminants in their environment. As reported by several authors, the evaluation of biomarkers in bioindicator organisms sampled in one or more areas suspected of chemical contamination and their comparison with organisms sampled in a control area can allow potential risks of toxicological exposure in the community in question to be evaluated [27–30].

In the present work, a cadmium-sensitive CA activity was found in the mantle of *Mytilus galloprovincialis*, a sentinel organism widely utilized in marine-coastal-environment biomonitoring programmes [31, 32]. The presence of CA enzymatic activity in the mantle of *M. galloprovincialis* can be related to the role of this tissue in mussel shell formation and growth [33]. In fact, carbonic anhydrase, catalysing the reversible hydration of CO_2 to produce H^+ and HCO_3^-, plays a fundamental role in a number of physiological processes, including

calcification [13]. Mantle CA activity was sensitive to both *in vitro* and *in vivo* inhibition by cadmium. After 1 h of *in vitro* incubation with 100 μM Cd added in the reaction mixture, mantle CA was inhibited by about 30%. The high *in vitro* cadmium concentration utilized accounts for the high bioconcentration factor for heavy metals observed in bivalves [22]. In *in vivo* conditions, the organisms were exposed to 1.785 μM $CdCl_2$, a concentration found in cadmium-contaminated environments and widely utilized for chronic toxicity exposure [21]. After 14 d of exposure, CA activity showed a significant inhibition of about 50%. The inhibitory effect of cadmium on mantle CA activity can explain results previously obtained by Soto *et al.* [34], who observed a significant decreased in shell growth in *M. galloprovincialis* exposed to heavy metals. Future studies are needed for a better comprehension of the inhibitory effect of heavy-metal exposure on carbonic anhydrase activity in mussels; however, preliminary results form a good basis for future research on mussel CA activity as a potential biomarker of chemical contamination.

In the present work, the possible application of CA measuring for an *in vitro* bioassay development was also explored. Bioassays use biological systems to detect the presence of toxic chemicals in environmental matrices (water, sediment, sewage, soil, etc.). In recent years, *in vitro* bioassays, employing cultured cells or cellular extracts, are increasingly being developed and used to detect the presence of contaminants. Examples include assays that measure enzyme inhibition, receptor-binding, or changes in gene expression in *in vitro* systems. Although the *in vitro* assay is not a substitute for the biomarker approach, it can be used as an adjunct model to whole-animal *in vivo* exposure and to ecotoxicological evaluation of the potential risk of trace pollutants in aquatic environments. These are rapid, low cost, and simple tools that can be utilized in combination with chemical analysis, for pre-screening environmental samples to be analysed. In the present paper, purified commercially available CA (isozyme II) from bovine erythrocytes showed a high sensitivity to the most toxic pollutant heavy metals (cadmium, mercury, and copper). The results obtained suggest that CA activity inhibition could be used as a rapid and sensitive chemical hazard detection system for standardizing rapid, sensitive, and low-cost CA-based *in vitro* bioassays.

Further studies are needed for the application of CA to real environmental samples and to organisms in the field. However, the results obtained already clearly indicate the potential of CA inhibition measurements for field applications as a biomarker in sentinel organisms and for *in vitro* applications as a bioassay for environmental-sample toxicity assessment.

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