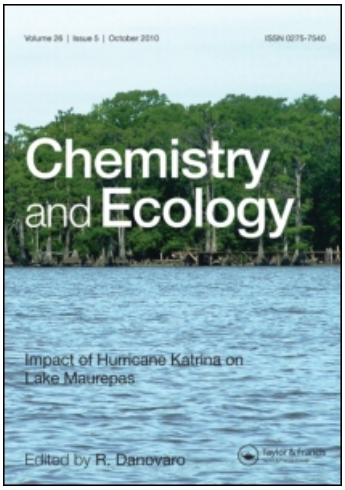

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Use of *Mytilus galloprovincialis* and *Tapes philippinarum* as sentinel organisms for the development of a biosurveillance program in the Pialassa Baiona coastal lagoon (Ravenna, Italy)

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The Pialassa Baiona is an intertidal lagoon located north from the town of Ravenna (Italy). It shows a certain degree of contamination caused by past uncontrolled discharges, and we aim at developing a bioassessment program to be used for future monitoring and conservation plans. A battery of six biomarkers (lysosome membrane stability, lipofuscin and neutral lipid accumulation, hsp70 expression, metallothionein levels and acetylcholinesterase (AChE) activity) was investigated in *Mytilus galloprovincialis* transplanted for 30 days in different sites. *Tapes philippinarum* are widely distributed within the lagoon, and we explored the possibility to use resident clams for future biosurveillance programs. Adenylyl cyclase (ACase) activity was also assessed in tissues of both bivalves as a future possible biomarker. Taken together, our results indicate that mussels are suitable sentinel organisms also within such a peculiar environment; alteration of four biomarkers was observed in tissues of mussels transplanted in two sites out of the five analyzed, indicating their low water quality. *T. philippinarum* showed a barely detectable AChE activity and absence of neutral lipids in digestive glands, therefore appeared unsuitable for our purpose, at least using the selected battery of biomarkers. ACase activity was not different among animals collected from the different sites.

Keywords: Pialassa Baiona; Biomarker; Lysosome activity; Adenylyl cyclase; Acetylcholinesterase; hsp70

1. Introduction

The Pialassa Baiona is an intertidal lagoon of $\sim$10 km^2 located north from the town of Ravenna (Italy), whose hydrodynamics is regulated by tidal fluxes and salinity varies in the range between 25 and 35 ppt. It is considered a site of relevance for the European Community (SIC IT4070004) and it is also a protected area by the intergovernmental treaty of Ramsar, recently included into the list of vulnerable areas, according to the Italian Law on body water protection (D. Lgs. 152/99, modified in 258/00).

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The basin receives fresh waters from five main channels draining agricultural run-off and effluents from industrial and municipal treatment plants. Marine water reaches the lagoon through the harbor of Ravenna (Canale Candiano). According to recent law dispositions, the Pialassa Baiona is partially exploited by professional fishermen, mainly collecting the Manila clams, *Tapes philippinarum*. As requested by law, water and resident clams are systematically collected in seven sites of the lagoon for routine analysis concerning temperature, salinity, oxygen content, heavy metals and batteriological charge [1].

Although the environmental control of the fresh water inputs is now adequately addressed, the lagoon shows a certain degree of contamination caused by uncontrolled discharges carried out in the past; as a consequence, and because of the natural importance of the area, the assessment of the health status of the Pialassa Baiona is of primary importance. Therefore, the aim of our work is the development of a bioassessment program which, together with ecotoxicological and geochemical analyses, could be a useful tool for future monitoring and conservation plans of the lagoon environment.

Harmful chemicals trigger alterations of several physiological processes in animals, potentially useful as stress indices or 'biomarkers'. The use of biomarkers has been recently included into several international environmental biomonitoring programs [2–4] for various reasons, including their unique contribution to determine the toxicity of a mixture of pollutants, although each contaminant is below the threshold prescribed by law. Moreover, changes induced by pollutants at the biochemical and molecular levels anticipate those provoked at higher levels of biological organization, so that biomarkers act as early warning signals of potential toxicological endpoints. A relevant number of biomarkers have been developed with increasing specificity and sensitivity to contaminants [5], but it has been well established that no individual biomarker can offer a complete diagnosis of overall harmful effects of contaminants on living organisms. For this reason, we have chosen a battery of six biomarkers to be analyzed on the Mediterranean mussel *Mytilus galloprovincialis*, used as sentinel organism. Mussels have been employed for biomonitoring purposes for many years [6] and we wanted to evaluate their suitability also within the peculiar environment of the Pialassa Baiona. Moreover, as the Manila clams *T. philippinarum* are widely distributed within the lagoon, and partially exploited for commercial reasons, we explored the possibility that these clams can be used as sentinel organisms for future biosurveillance programs.

2. Materials and methods

2.1 *Animals*

M. galloprovincialis were collected by fishermen of the 'Cooperativa Adriatica' (Gorino, Italy) along the coast of the north-western Adriatic sea. They were selected on size basis, between 5 and 6 cm in length, and the digestive glands and gills immediately excised from 50 individuals (referred to as controls at zero time, CT0 in figures), then stored at −80 °C until analyzed. Selected individuals were meanwhile transferred to five appropriate cages in numbers of 50 each and transplanted for 30 days at five different sites along four different channels: Baccarini (BAC), Baiona A (BAI-A), Baiona B (BAI-B), Magni (MAG) and Taglio della Baiona/Fossatone (TBF) (figure 1). The study was carried out during October 2002. On the basis of preliminary results (not shown), TBF was chosen as possible reference site. *T. philippinarum* resident in the lagoon were collected from the same four channels, in proximity of the positions where mussels were transplanted. *T. philippinarum* were not present at the site BAI-A. As no clams were available in areas where sediments could have been considered uncontaminated, about 100 individuals were collected from the four sampling sites

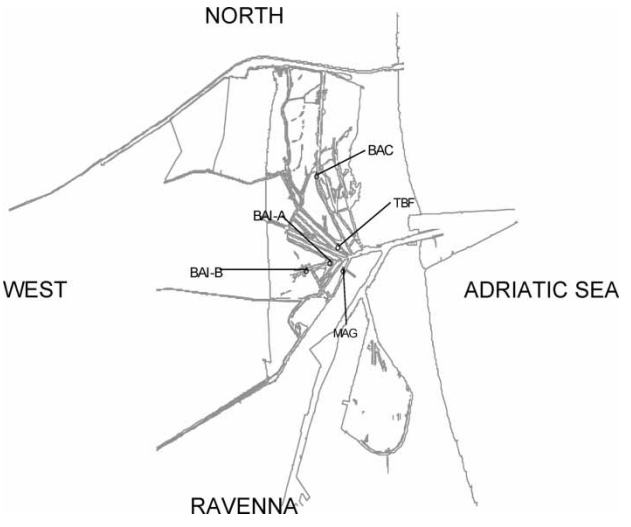

Figure 1. Sampling sites distributed along four main channels of Pialassa Baiona: BAC, BAI-A, BAI-B, MAG and TBF.

and kept for 30 days in two aquaria containing 60 l of aerated artificial 34 ppt sea water at 16 °C under natural photoperiod; these individuals were fed once a day with appropriate algal slurry (Liquifry marine, Interpet Ltd, Dorking, England) and used as controls.

2.2 *Battery of biomarkers*

The battery of biomarker was composed of three biomarkers selected according to specific problems affecting the lagoon: metallothionein (MT), induced by heavy metals; acetylcholinesterase (AChE) activity, inhibited by pesticides; hsp70 expression, mainly induced by thermal stress; three further biomarkers were chosen among the most sensitive response to generic stress factors (lysosomal membrane stability, lipofuscin and neutral lipid accumulation) *i.e.* not induced by a specific class of contaminants. Moreover, adenylyl cyclase (ACase) activity was measured to assess its possible role as a biomarker.

2.3 *Metallothionein content*

MTs were evaluated in digestive glands of bivalves according to Viarengo *et al.* [7] using the Ellman reagent DNTB [8]. Final absorbance was measured at 410 nm, using reduced glutathione as reference standard.

2.4 *AChE activity*

After thawing, pooled samples of gills were homogenized in ice-cold 0.1 M phosphate buffer, pH 7.4. Experimental procedures were as recently reported [9]. Briefly, the homogenate was centrifuged at 9000 $\times$ *g* at 4 °C for 30 min and the supernatants stored at −80 °C until analyzed. The sample protein concentration was estimated according to Lowry *et al.* [10], and the enzyme activity was then measured following the method of Ellman [8]. Gill homogenates were incubated at 25 °C in a final volume of 1.2 ml containing 100 mM phosphate buffer, pH 7.4, 0.5 mM acetylthiocholine iodide (ASCh) and 0.33 mM DTNB. The enzymatic reaction rate was quantified spectrophotometrically at 405 nm.

2.5 *Hsp70 expression*

After thawing, gills were homogenized as previously described [11] and centrifuged for 5 min at 500 × *g*; the supernatants were diluted with Laemmli buffer [12] and boiled for 5 min. Thirty micrograms of homogenate protein were loaded onto 12% polyacrylamide gels and electrophoresis carried out at 28 mA for 2 h at 4 °C. Proteins were then transferred onto a nitrocellulose membrane (300 mA, 1 h at 4 °C) and probed with anti-HSP70 rat monoclonal antibody (Affinity Bioreagents, 1:2000). A rabbit anti-rat IgG polyclonal antibody conjugated with horseradish peroxidase was used as secondary antibody (Sigma, 1:500). Immunoblots were developed by enhanced chemiluminescence reagent (ECL, Amersham Biosciences) and a densitometric analysis of the films was performed by Image Master (Amersham-Pharmacia) equipped with TotalLab software.

2.6 *Cytochemical determinations*

Digestive glands were removed from 15 randomly selected mussels per site. Five digestive glands (approximate weight 250 mg) were placed on each aluminum cryostat chuck. The chucks were immersed in *N*-hexane precooled to −70 °C using liquid nitrogen and immediately stored at −80 °C. Ten micron-thick serial sections were obtained at −30 °C using a cryostat; each group of five gland sections were then transferred to a microscope slide maintained at room temperature. To ensure impartial results, all slides were randomly coded prior to analysis and decoded only after all measurements were made. Further treatments were carried out accordingly to the different experimental protocols.

2.7 *Neutral lipid accumulation*

Sections were prepared in triplicate from each aluminum chuck, for a total of 15 individual samples per site, and treated according to Bancroft [13]. Briefly, the 10 μm sections were rinsed in distilled water, placed in 60% triethyl phosphate for 3 min and stained in a 1% solution of Oil Red O (Sigma, St Louis, MO, USA) for 15 min. Sections were then washed, rinsed and mounted with glycerol gelatin (Sigma) under a coverslip for microscope observation. Pink lipid droplets were then quantified by image analysis.

2.8 *Lipofuscin accumulation*

Lipofuscin accumulation was determined using the Schmorl reaction [14]. Triplicate cryostat sections (10 μm thick) of digestive glands were fixed in calcium-formol for 15 min at 4 °C, rinsed in distilled water and immersed in the reaction medium containing an aqueous solution of 1% ferric chloride and 1% potassium ferricyanide in a ratio 3:1 (v/v). Sections were stained for 5 min, rinsed in 1% acetic acid for 1 min and washed in distilled water before mounting with glycerol gelatin (Sigma) under a coverslip for microscope observation. Dark green granule products of peroxidation were then quantified by image analysis.

2.9 *Lysosomal membrane stability*

The procedure was based on the measurement of the activity of the marker enzyme *N*-acetyl-b-hexosaminidase [15, 16]. Multiple sections for each tissue sample were incubated at 37 °C in a 0.1 M Na–citrate buffer (pH 4.5) for different times (0, 3, 5, 10, 15, 20, 30 and 40 min). Slides were then incubated for 20 min at 37 °C in a reaction medium containing napthol

AS-BI *N*-acetyl-b-D-glucosamide (Sigma) dissolved in 2-methoxy ethanol and citrate buffer with NaCl and low-viscosity polypeptides (Sigma). Sections were rinsed, placed in phosphate buffer, stained with fast violet B (Sigma) and mounted under coverslips using glycerol gelatin. The reaction product of the *N*-acetyl-b-hexosaminidase activity was determined by using automatic image analysis to examine the different levels of reaction product on the slides, recorded as pixel density, from the various incubation times. The labilization period was calculated as the incubation time in the citrate buffer producing maximal staining reactivity.

2.9.1 Image analysis. Tissue sections were quantitatively assessed for each of the cytochemical biomarkers using a computer-enhanced automatic image analysis with Scion Image v Beta 4.0.2 software (Scion Corporation 2000).

2.10 *ACase activity*

The assay has been carried out as previously reported by Fabbri *et al.* [17] in crude membrane preparation. The cAMP produced was assessed by radiochemical determination (3H-cAMP Kit Amersham Biosciences, Amersham, UK).

2.11 *Data analysis*

Data were subjected to one-way ANOVA (SigmaStat, SPSS) using Newman–Keuls as *post hoc* test; statistical difference was accepted when $p < 0.05$.

3. Results

3.1 *MT levels*

As shown in figure 2, digestive glands of *M. galloprovincialis* transplanted for 30 days in different sites of the Pialassa Baiona displayed similar content of MT; only for mussels collected from BAC, the MT content was slightly but significantly higher than that observed in mussels from the reference site. The average content of MT in digestive glands of *T. philippinarum*

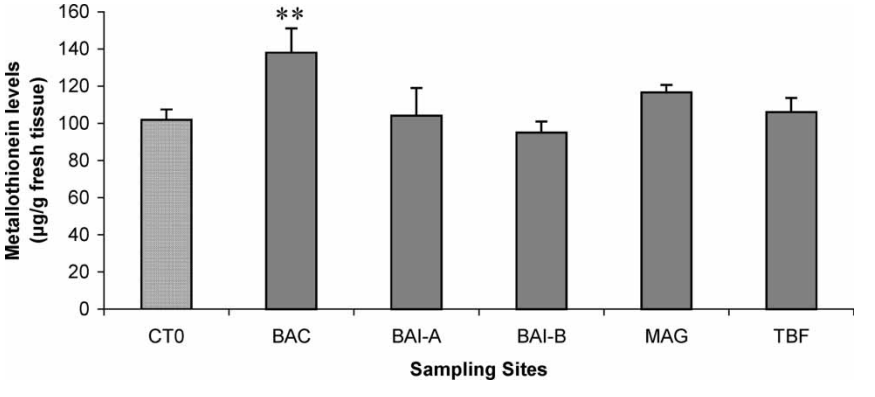

Figure 2. MT levels in mussels transplanted in different sites of the Pialassa Baiona for 30 days. For each sampling site, data are the mean ± SEM of four different evaluations on pools of four to six digestive glands. ** $p < 0.05$ *vs.* TBF. Control at zero time (CT0) has not been considered for statistical evaluations.

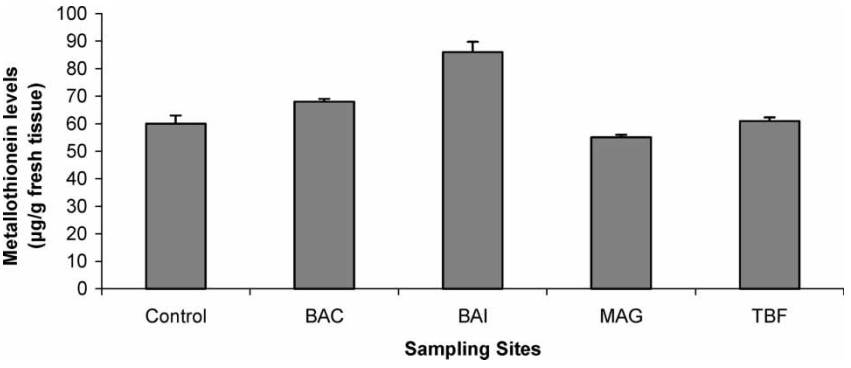

Figure 3. MT levels in clams collected at different sites of the Pialassa Baiona. For each sampling site, data are the mean ± SEM of four different evaluations on pools of 16–18 digestive glands.

was almost 2-fold lower than that of mussels. No difference was observed among individuals at the different sites or controls in the aquaria (figure 3).

3.2 *AChE activity*

The AChE activity was measured in gills from mussels and clams in the presence of ASCh as substrate. The enzyme activity calculated in mussels at zero time (CT0) and those transplanted in TBF was about 11 and 10.2 nmol min^{-1} mg^{-1}, respectively; it was slightly, although significantly, reduced in individuals collected from MAG and BAI-A (figure 4). Strange as it may seem, the AChE activity in gill clams was barely detectable, ranging from 0.3 to 0.8 nmol min^{-1} mg^{-1} in the presence of 1 mM ASCh (data not shown). Such values did not allow to establish any significant difference among samples.

3.3 *Hsp70 expression*

The monoclonal antibody clearly detected the expression of hsp70 in the gills of both mussels and clams. As typical for bivalves, two bands were observed in control conditions, which

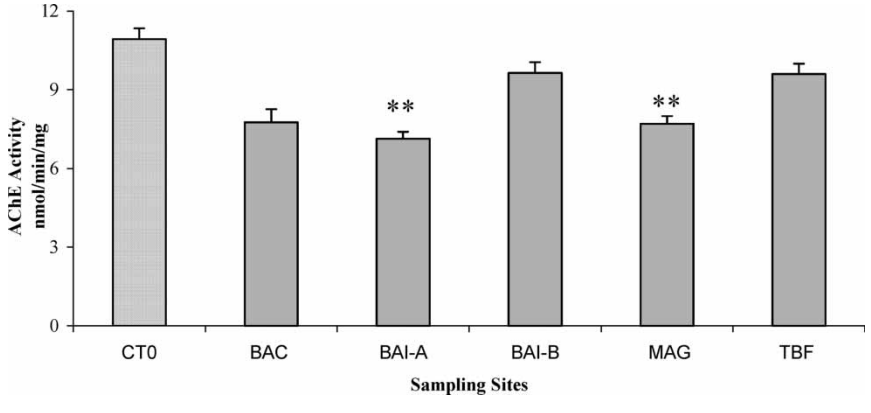

Figure 4. Acetylcholinesterase activity in the gills of *M. galloprovincialis* kept at different sites of the lagoon. For each sampling site, data represent the mean ± SEM of three different evaluations carried out in quintuplicate using pools of gills from eight individuals. $^{**}p < 0.05$ *vs.* TBF. CT0 has not been considered for statistical evaluations.

appeared significantly over-expressed in mussels from BAI-A and MAG (figure 5) and clams from TBF (figure 6) with respect to the relative controls.

3.4 *Unsaturated neutral lipid accumulation*

Mussels caged for 30 days and exposed to the water of the Pialassa Baiona did not show any significant increase in unsaturated neutral lipid accumulation when compared with those of the reference site (data not shown). As shown in figure 7, neutral lipids were easily detectable in mussels (left panel), but appeared absent in the digestive glands of clams (right panel).

3.5 *Lipofuscin accumulation*

Mussels caged in BAI-A, BAI-B and MAG showed significant accumulation of lipofuscine with respect to those from the reference site (figure 8), with the highest levels found in mussels from BAI-B. Regarding clams, accumulation of lipofuscine was not significantly different among the samples analyzed (data not shown).

3.6 *Lysosomal membrane stability*

According to the methodology used and previous data [18], a short time (2–10 min) indicates that lysosome membrane is easily labilized, therefore, indicating a critical health status; longer times (20–30 min) indicate that the lysosome membrane of sampled organisms is resistant to labilization and is an index of a good health status. The average time of labilization was estimated around 10 min in mussels from BAC and about 5 min in mussels from MAG1, BAI-A and BAI-B; it was about 15 min at the reference site TBF, whereas control animals

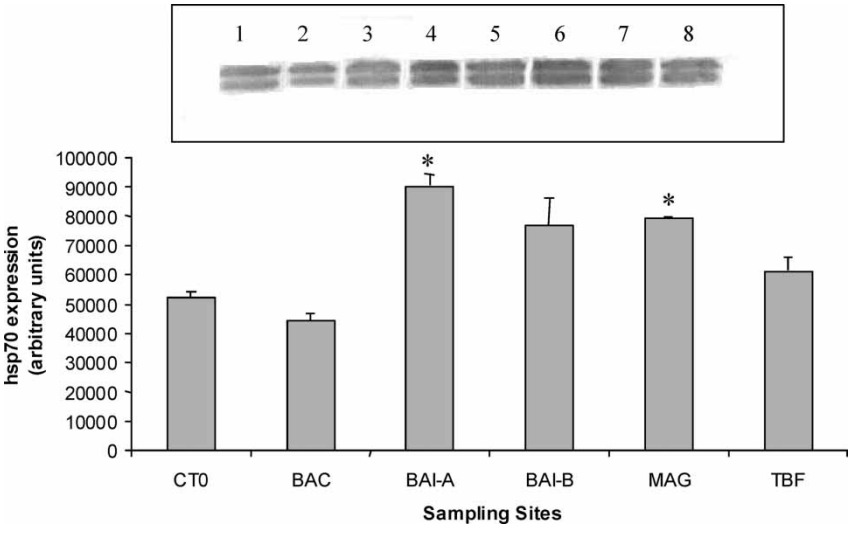

Figure 5. Representative western blotting (upper panel) and densitometry (lower panel) of hsp70 immunodetection in gills of *M. galloprovincialis*. Lanes represent samples from different sites: 1 and 2, CT0; 3 and 4, TBF; 5, MAG; 6, BAI-A; 7, BAI-B; 8, BAC. Sample order in the upper panel is different than in the lower panel because each western blotting was carried out changing the lane position of the different samples to avoid methodological influence on results. Data are expressed as pixel number and normalized with respect to an internal standard. Data represent the average ± SEM of three different experiments. $^{*}p < 0.01$ *vs.* TBF. CT0 has not been considered for statistical evaluations.

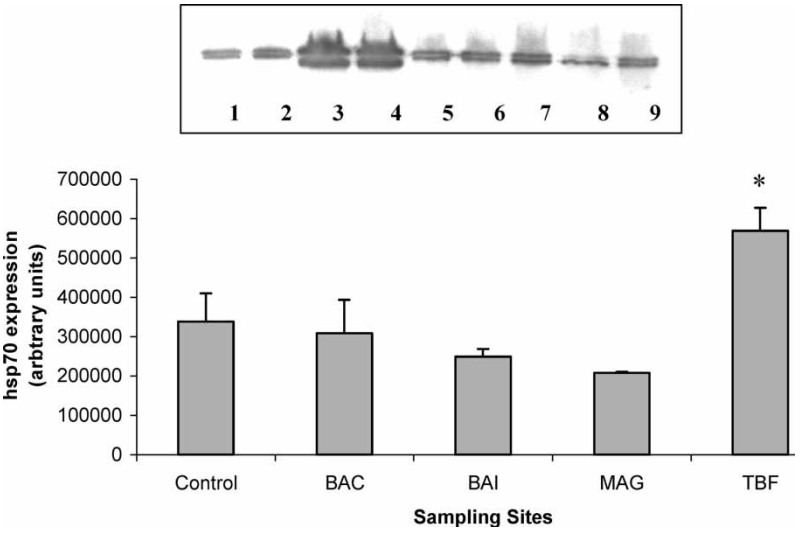

Figure 6. Representative western blotting (upper panel) and densitometry (lower panel) of hsp70 immunodetection in gills of *T. philippinarum*. Lanes represent duplicates of the same preparation obtained from different sites: 1 and 2, MAG;3 and 4, TBF, 5 and 6, BAC; 7 and 8, BAI; 9, control. Sample order in the upper panel is different than in the lower panel because each western blotting was carried out changing the lane position of the different samples to avoid methodological influence on results. Data are expressed as pixel and normalized with respect to an internal standard. Data represent the average ± SEM of three different experiments. $^{*}p < 0.01$ *vs.* all other evaluations.

at zero time showed an average time of labilization of about 25 min (figure 9). Lysosome membrane stability evaluated in the digestive glands of *T. philippinarum* was rather different among the four sites, with the highest value found in clams from MAG (25 min) and the lowest was found at TBF (4 min) indicating a good and compromised health status of the organisms, respectively. Average values of 15 and 11 min were found in BAC and BAI-A, respectively (data not shown).

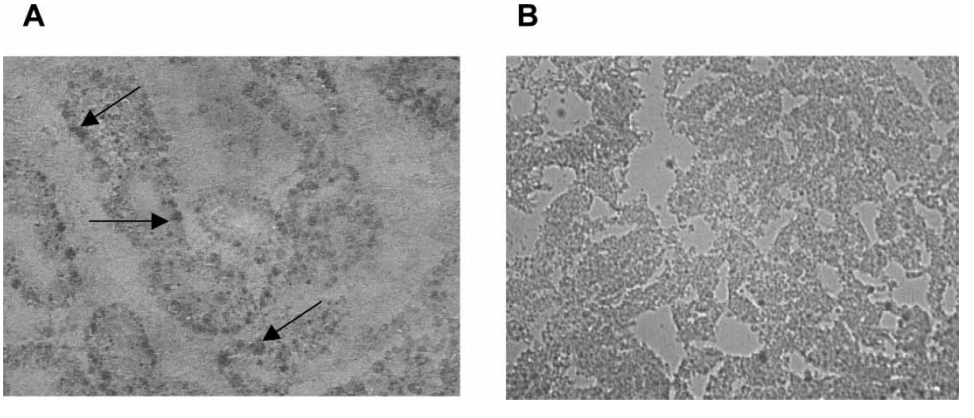

Figure 7. Light microscope images (20×) of 10 μm cryostat sections. (A) The localization of unsaturated neutral lipid accumulation in a mussel digestive gland; examples are indicated by the arrows. (B) The lack of stained structures in a clam digestive gland.

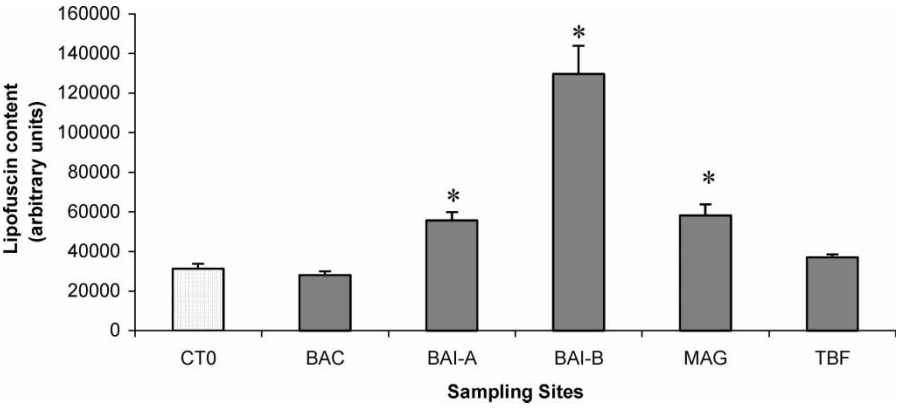

Figure 8. Lipofuscin content in mussels transplanted at different sites of the Pialassa Baiona. Data are the mean ± SEM of lipofuscin content in 15 digestive glands for each sampling site, obtained through the analysis of four different pictures from each gland. $^{*}p < 0.01$ *vs.* TBF. CT0 has not been considered for statistical evaluations.

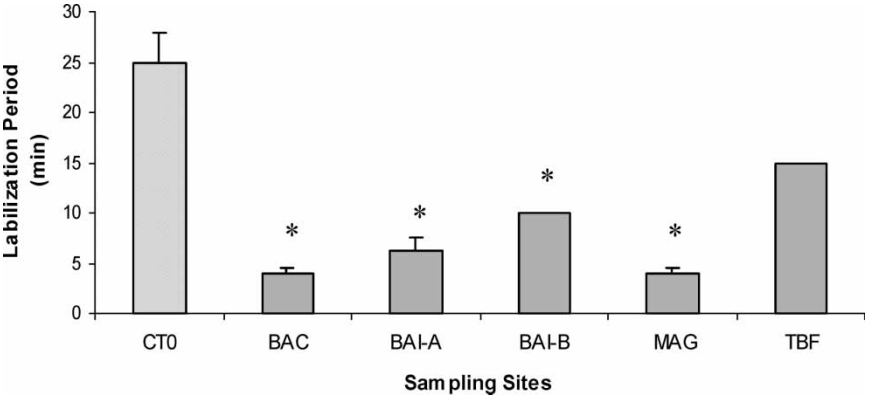

Figure 9. Evaluation of lysosome membrane stability in mussels transplanted at different sites of the Pialassa Baiona. Data represent the average ± SEM of 15 digestive glands, each analyzed in four quarters and for seven times of exposure. $^{*}p < 0.01$ *vs.* TBF. CT0 has not been considered for statistical evaluations.

3.7 *ACase activity*

Basal levels of the ACase activity were measured in the gills and digestive glands of the mussels caged at different sites of the Pialassa Baiona. Table 1 indicates that enzyme activity was higher in digestive glands than in gills, but there was no significant difference between individuals from different sites or between control and transplanted samples. ACase activity

Table 1. ACase activity in tissues of *M. galloprovincialis* (mussels) and *T. philippinarum* (clams).

	ACase activity	CTR	BAC	BAI1	BAI3	MAG	TBF
Mussels	Gills	5.3 ± 0.8	4.8 ± 0.3	5.1 ± 0.4	5.3 ± 0.8	4.2 ± 0.3	4.0 ± 0.5
Mussels	Digestive glands	10.5 ± 2	9.6 ± 1.5	8.8 ± 2.5	9.5 ± 2.9	12.1 ± 1.8	11.3 ± 1.5
Clams	Gills	8.6 ± 0.6	9.4 ± 1.2	10.5 ± 0.9	–	9.1 ± 1.5	8.8 ± 0.7

Note: Data are the mean ± SEM of four pools of tissues obtained from a total of 20 individuals and are expressed as pmol cAMP produced per 10 min/mg protein.

was also measured in the gills of clams collected in the lagoon (table 1). As with the mussel, enzyme activity was similar among individuals from the different sites.

4. Discussion

Previous studies reported that the Pialassa Baiona was exposed to harmful discharges that are now documented by the high contamination evaluated in sediments from different sampling sites [19]. Accordingly to Miserocchi *et al.* [20, 21], contamination by mercury is widely distributed within the lagoon, with maximum levels of about 160 mg/kg of superficial sediments and up to about 1100 mg/kg at 80 cm of depth, in the area of via Cupa/MAG channel. A north–south pollution gradient was described by Fabbri *et al.* [19]. Other studies reported the sediment distribution of other heavy metals such as As, Pb, Cd, Cr, Cu and Zn [22] and the presence of polycyclic aromatic hydrocarbons [23].

The battery of biomarkers analyzed in mussels transplanted for 30 days at different sites of the Pialassa Baiona provided evidence for a critical health status in two of the five selected sites. In particular, several stress indices were modified in mussels collected from MAG and BAI-A. Lysosome membrane stability examined in the digestive glands of these mussels was rather low. Lysosomes are cellular organelles with important physiological functions including a role in detoxification [24]; the alteration of their membrane permeability causes the harmful leaking of digestive enzymes into the cytoplasm. Such a phenomenon is quickly induced by environmental stress conditions, and therefore this biomarker is a highly sensitive early warning signal of compromised health conditions [25]. The mussels from TBF, chosen as the reference site, had a lysosome membrane stability 2-fold lower than the controls at zero time (CT0, figure 9). It is well accepted that 30 day exposure to a 'clean' site does not induce *per se* a stress syndrome in transplanted mussels, we can speculate that TBF site is not as clean as we had hoped. However, the modification of one single biomarker might not represent a significant indication of stress.

In the digestive glands of mussels from BAI-A and MAG, a significant rise in lipofuscin content was also observed; however, the highest lipofuscin levels were found in mussels transplanted at BAI-B. Lipofuscins are accumulated in granules constituted by oxidatively modified protein and lipid degradation products; also, carbohydrates and metals may be included [26]. These pigments are an endpoint of lipid peroxidation, easily detectable in cells of stressed organisms, in comparison with the minimum level present in cells of organisms living in unpolluted waters (Moore *et al.*, 1988).

The effects of pollutants have been associated with alteration of fatty acid metabolism and with the accumulation of high levels of unsaturated neutral lipids in lysosomes [27], and the increase of lysosomal content of neutral lipids in the digestive gland of mussels is considered as a useful indicator of alterations of cell physiology [16]. In most circumstances, mussel digestive glands showing reduced lysosome stability and high lipofuscin content also show an increase in unsaturated neutral lipid accumulation as symptom of altered cellular metabolism; this was not the case in the mussels analyzed, where the content was not significantly different among individuals. Unexpectedly, neutral lipid accumulation was not detectable in clams. We are not aware of reports showing and/or explaining this feature, and such finding deserves further studies.

Mussels from MAG and BAI-A displayed also a significant over-expression of hsp70 in their gills. Hsps of the 70 kDa family are used in environmental biomonitoring [28], although their employment is criticized because of the variability of the basal levels between organisms and the variety of stress stimuli responsible for enhancing their expression [29]. Heat, but also

changes in salinity, hypoxia and heavy metals are among these factors [28], and we recently reported that Cd exposure evokes hsp70 over-expression and MT synthesis in tissues of *Ostrea edulis* [30]. In the present work, however, we did not find any significant change in the content of MT in digestive glands of mussels transplanted for 30 days into the Pialassa Baiona, except for BAC.

We can hypothesize that, in general, the water to which the sentinel organisms were exposed was not contaminated in a significant manner by those heavy metals able to induce MT [7]. Several factors could have also contributed to induce the over-expression of hsp70, including the fact that MAG channel flows through the most contaminated southern part of the lagoon. We are now planning further studies on some chemical and physical parameters of this area aimed at clarifying whether natural (*e.g.* salinity, oxygen content) or anthropogenic (*e.g.* thermal stress) factors are responsible for the observed hsp70 and/or other biomarker alterations. The Pialassa Baiona is the site of discharge of industrial cooling water (500–600 million of cubic meters per year) from two power plants, and temperatures as high as 35 °C have previously been reported in the southern part of the lagoon [31] along the MAG channel. As the MAG channel is closely connected to the Baiona channel, this possibly explains the similar stressful condition experimented by the animals in the two sites.

AChE activity was not modified in mussels during the period of exposure to the water of the Pialassa Baiona, except for a reduction at MAG and BAI-A, small but significant with respect to the reference site TBF. More data need to be collected in order to clarify whether this reduction is consistent with biological variations or is a true sign of pesticide contamination.

We also assessed the ACase activity in the gills and digestive glands of mussel and clams. In a recent report, Dailianis *et al.* [32] reported higher cAMP levels in mussels from contaminated coastal sites of northern Greece and suggested the possibility that the nucleotide content could be used as biomarker. Our data show that the activity of the enzyme responsible for the cAMP production is not modified in tissues of bivalves exposed to the Pialassa Baiona. Although a significant reduction of ACase activity was reported by us in fish hepatocytes exposed to heavy metals [33], we did not observe any significant modifications of the ACase activity in gills of clams exposed *in vitro* to Hg and tributyltin [34]. However, no conclusion can be inferred at the moment comparing the results obtained from Greece to those from the Pialassa Baiona for numerous reasons. First, the environmental characteristics of the two areas examined are quite different. Secondly, cAMP levels are the results of the activity of both ACase, the synthesizing enzyme, and phosphodiesterase, the degrading enzyme, and the latter has not been evaluated in either of the two field assessments. However, as cAMP is a second messenger with a pivotal role in modulating cellular functions and also gene expression, its alteration by environmental contamination is a phenomenon of great importance and needs further evaluation.

The results discussed so far indicate that mussels from MAG and BAI-A have been exposed to stressful environmental conditions, although no relationship with a particular factor can be identified. In fact, the most significant variations have been found for the biomarkers classified as general stress indices.

As the present work is aimed at developing an integrated bio/geo/chemical monitoring strategy based on sentinel organisms and the assessment of a number of sediment and water parameters of the lagoon, the information collected confirms that mussels can be transplanted for at least 1 month along the different channels of the Pialassa Baiona and employed as sentinel organisms in transition environments. The use of ACase activity and/or cAMP levels as biomarkers appears rather premature; however, the battery of six biomarkers employed here can be further implemented with other validated stress indices such as the micronuclei test [35] and TOSC activity [36]; moreover, mussel tissues can be used for bioaccumulation tests.

Because of its wide distribution, *T. philippinarum* would be rather useful for environmental biomonitoring, and some attempts in this regards are reported [37]. The data presented

here are in full agreement with previous observations we made in a pilot assessment of the lagoon health status [38] and definitively confirm that this clam species is not useful as a sentinel organism, at least according to the battery of biomarkers used. In particular, the responses of the specific biomarkers for pesticides (inhibition of AChE activity) and that for heavy metal accumulation (MT) seemed rather doubtful. In the first case, we recently carried out a characterization of AChE activity in bivalves and reported that the enzyme activity of *T. philippinarum* is extremely low also in the presence of substrates different from ASCh, *e.g.* butyrylthiocholine or proprionylthiocholine, and at different incubation conditions [9]. As to MT, no difference was noted among clams exposed to different sites of the Pialassa Baiona, but we have also observed in the laboratory that weekly treatments with $CdCl_2$ (100 μg per liter per day) did not cause a significant increase of MT in gills and digestive glands of *T. philippinarum* (unpublished results). Further reducing the usefulness of the battery of biomarker employed, *T. philippinarum* did not accumulate unsaturated neutral lipids in their digestive glands. This clam is an allochtonous bivalve species that rapidly expanded along the North Adriatic sea (Italy). Among other reasons, its rapid development has been ascribed to a high tolerance to environmental changes, resistance to parasites and to pollution [39, 40]. We can hypothesize that some adaptive mechanisms may reduce the environmental effects on biochemical and cytological responses of this organism. However, only scarce attention has been addressed to understand the regulation of physiological functions possibly related to the ecological success of the clam *T. philippinarum*, and future studies are needed in this regard.

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