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Integrated cellular stress indices in trace metal contamination: critical evaluation in a field study

A. Viarengo[1], G. Mancinelli[1], G. Martino[2], M. Pertica[1], L. Canesi[1], A. Mazzucotelli[3]

[1] Institute of General Physiology, Corso Europa 26, I-16132 Genova, Italy
[2] Institute of Marine Environmental Science, Via Balbi 5, I-16132 Genova, Italy
[3] Institute of General Chemistry, V. le Benedetto XV, I-16132 Genova, Italy

ABSTRACT: Metal bound to metallothioneins and cytosolic proteins significantly increased in the digestive gland of mussels *Mytilus edulis* exposed in the GEEP Workshop mesocosm experiment to different levels of a copper and diesel oil mixture. Analysis performed on mussels sampled at 4 field sites in Langesundfjord, Norway, showed that the mussels were contaminated by metals, although to a limited degree. The concentration of metal bound to thioneins was essentially the same in the digestive gland extracts from mussels sampled at reference and polluted sites, but the values of copper bound to cytosolic proteins significantly increased at the field sites characterized by heavy metal pollution. These results indicate that the parameters studied (metals bound to cytosolic proteins and to metallothioneins) can be considered as satisfactory integrated stress indices in evaluating the biological impact of heavy metal contamination. Calcium concentration significantly increased in the digestive gland of mussels exposed to pollutants in the field as well as in the highest dosed mesocosm basins. The possibility of further application of this finding as a general stress index is discussed.

INTRODUCTION

Lamellibranch molluscs, mussels in particular, can accumulate high metal concentrations in their tissues, albeit with variability between species and tissues (Coombs 1980, George 1982). Metal accumulation may also vary with the physiological status of the animal, the effects of environmental factors on metal chemistry in sea water, cellular homeostasis mechanisms, the period of metal exposure and the route of metal uptake (Harrison 1979, Bouquegneau & Gilles 1979, George & Viarengo 1985, Viarengo 1985, Viarengo et al. 1986). In the last few years, emphasis has been on studies of important biological cations such as zinc and copper, which are toxic at cellular concentrations above normal physiological levels, and of cadmium and mercury, which are among the most toxic environmental contaminants. Heavy metals can be stored in non-toxic forms in mollusc tissues as inorganic precipitates (Coombs 1980), in membrane limited vesicles (George et al. 1978) and in lysosomes (Viarengo et al. 1981, George 1983a, b). Metals can also be trapped by cytosolic cystein rich proteins called metallothioneins (Kägi & Nordberg 1979), the synthesis of which rapidly increases in response to the accumulation of heavy metals in the cells (Noel-Lambot 1976, Viarengo et al. 1980, Roesijadi et al. 1982, Viarengo 1985, Viarengo et al. 1985). Therefore, metal toxicity appears to be related not to its total tissue concentration, but to the amount of metal free to interact with cell structures and/or enzymes, in this way affecting metabolic pathways (Webb 1979). Numerous authors have suggested tissue metallothionein concentrations to be an index of the biological response of marine organisms to specific effects of heavy metal environmental contamination at the cellular level (Brown et al. 1977, Viarengo et al. 1982). Such data could be usefully integrated by determining the amount of metal bound to cytosolic protein, in order to gain information about the possible interference that abnormal metal interactions with soluble enzymes may cause in cell metabolism.

At the GEEP Workshop we studied the applicability of these integrated measurements specific to heavy metal contamination (metal bound to thioneins and to cytosolic proteins), on *Mytilus edulis* sampled from 4 sites in Langesundfjord, designated 1 to 4 in order of anticipated pollution gradient. They were also tested in a mesocosm experiment in which mussels were

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exposed to controlled copper-containing contaminant mixtures at different concentrations. Furthermore, since cellular damage of different sources frequently enhances peroxidation processes in the cells (Bus & Gibson 1979), thus affecting calcium homeostasis (Di Monte et al. 1984), the concentration of this cation in the mussel digestive gland was evaluated and the possible use of this parameter as a general stress index examined.

Finally, since large numbers of samples must be analyzed in field studies in order to determine the toxic effects of metals on marine organisms, a rapid procedure is presented for routinely assaying different metals in biological samples, using the Inductively Coupled Plasma (ICP) system. In addition, a rapid separation of the metallothionein fraction by HPLC gel filtration analysis is proposed, and the possibility of determining metal concentrations in the eluate by AAS and/or ICP is compared.

MATERIAL AND METHODS

Animals. *Mytilus edulis*, of shell length 4 to 6 cm, were collected at 4 sites in Langesundfjord, Norway, as described by Follum & Moe (1988). The conditions of exposure of the mussels sampled from the 4 experimental basins at the Solbergstrand mesocosm facility are described by Bakke et al. (1988).

Materials. Phenylmethylsulphonyl fluoride (PMSF) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, USA); Protein-Pak 125 gel filtration column was obtained from Millipore Waters (Milford, Massachussets, USA). All other reagents used were of analytical grade.

Preparation of the soluble fraction and cytosolic proteins. Digestive glands of *Mytilus edulis* were rapidly removed and homogenized with 2 volumes of 0.5 *M* sucrose, 100 m*M* NaCl, 20 m*M* Tris, pH 8.6, 0.006 m*M* leupeptine, 0.5 m*M* PMSF, 1 m*M* DTT. The homogenate was then centrifuged at 100 000 × *g* for 60 min to obtain the cytosolic fraction. Metallothioneins were isolated by heating the cytosol at 60 °C for 15 min and centrifuging at 10 000 × *g* for 15 min; the supernatant contained metallothionein. The pellet was resuspended in 3 ml of the homogenization buffer and centrifuged again at 10 000 × *g* for 10 min. The washed pellet was used for the determination of metal bound to cytosolic proteins.

HPLC gel filtration and metallothionein determination. Metallothioneins were partially purified by HPLC on a TSK gel filtration column, using 0.05 *M* phosphate buffer, pH 7.0, at a 0.8 ml min^{-1} flow. The elution of the protein was monitored at 280 nm and the copper content evaluated by directly connecting the chromatographic column to the torch of the ICP system. Copper was monitored at 327.396 nm. The wavelength was routinely checked to control the correct position of the emission maximum. Alternatively, the gel filtration HPLC eluate was collected, and copper present in the metallothionein fractions was evaluated by furnace Atomic Absorption as described previously (Mazzucotelli et al. 1976).

Determinations of cadmium, zinc, copper and calcium by plasma ICP analysis. Determinations of the metal content of homogenates and cytosolic fraction proteins were performed by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) utilizing Jobin Yvon (Model JY38) apparatus. To prepare the samples, homogenates or cytosolic proteins were hydrolyzed with 2 volumes of HNO_3. (65 %) at 70 °C for 2 h in a closed Pyrex vessel. The solutions obtained were analyzed by ICP-AES at wavelengths of 226.506 for cadmium, 213.876 nm for zinc, 327.396 nm for copper and 393.360 nm for calcium.

Statistical analysis. Mean values were compared by the Mann-Whitney *U*-test.

RESULTS

Total metal concentrations (copper, zinc, cadmium, calcium) in the digestive gland of *Mytilus edulis* from 4 field sites in Langesundfjord are given in Table 1. Mussels from the reference area (Site 1) had minimal amounts of all 4 metals analyzed. In samples from polluted sites (2, 3 and 4), the concentrations of copper and cadmium were slightly (no more than 40 %) but significantly higher than control values. These data indicate that only a limited degree of heavy metal pollution is present in the fjord, reaching the highest values at Site 3 (copper 4.48 μg g^{-1} wet weight, 39 % over reference site values). The values for copper concentrations are in reasonable agreement with those of Abdullah & Steffenak (1988); confirming the limited metal contamination of these Langesundfjord sites. Table 1 also shows that the concentration of calcium in the mussel tissues increased by 90 % at Sites 3 and 4 in comparison with Site 1.

Table 2 shows the concentrations of metals (copper and cadmium) bound to metallothioneins in digestive gland extracts of mussels. There were no significant differences between field sites. Table 2 also shows the concentrations of copper and zinc present in the cytosolic, heat-denaturated, protein fraction. There is a net and significant enhancement of the concentration of copper bound to cytosolic proteins. The soluble protein metal content in the digestive gland of mussels sampled at Site 3 was about 70 % higher than at the reference site. Cadmium concentrations in the cytosolic

Table 1. *Mytilus edulis*. Metal concentrations (μg g^{-1} wet wt, measured by ICP) in digestive glands of mussels from 4 sites in Langesundfjord and 4 exposure levels of a copper-containing pollutant in mesocosm basins (mean ± SD, n = 4 pools each of 10 to 12 individuals, all determinations in triplicate)

Source	Zn	Cd	Cu	Ca
Site				
1	35.16 ± 2.76	0.93 ± 0.04	3.23 ± 0.45	290.9 ± 26.7
2	39.47 ± 3.09	1.26 ± 0.07**	3.92 ± 0.41*	426.5 ± 64.7**
3	37.11 ± 4.63	1.22 ± 0.12**	4.48 ± 0.37**	520.8 ± 37.7**
4	33.30 ± 1.99	1.19 ± 0.03**	4.01 ± 0.48*	544.0 ± 102.4**
Basin				
C	38.46 ± 8.00	1.69 ± 0.20	4.18 ± 0.87	343.4 ± 56.6
L	44.71 ± 1.52	1.79 ± 0.07	7.98 ± 1.13**	366.5 ± 21.8
M	43.78 ± 6.94	1.57 ± 0.12	11.58 ± 1.77**	378.2 ± 51.4
H	26.73 ± 5.49*	1.51 ± 0.15	13.23 ± 2.74**	425.3 ± 71.7*

Significant differences of field site means from the reference site (1) and mesocosm basins from controls (C), indicated by: * $p<0.05$, ** $p<0.01$

protein fraction were always below the limit of detection of the method, whereas the zinc content of the protein soluble fraction did not differ significantly between field sites.

In the mesocosm experiment at Solbergstrand, differing concentrations of a copper and diesel oil mixture were dosed to three basins (L: low, M: medium, H: high dose) with a 4th basin acting as a control (C). Nominal doses of Cu were L: 0.8 μg l^{-1}, M: 5 μg l^{-1}, H: 20 μg l^{-1}. Exposure was for 16 wk for L and M mussels but only 3 wk for H mussels, due to high mortality (after 7 wk) of mussels originally stocked in the H basin.

Table 1 shows the total metal content (zinc, copper, cadmium, calcium) in the digestive gland of *Mytilus edulis* from the 4 basins. In control mussels, the copper concentration was 4.18 μg g^{-1} wet weight; it significantly increased (90 %) for L mussels and was correspondingly higher for M and H mussels (180 % and 220 % of controls, respectively). The cadmium concentration was essentially constant in all mussels tested. Zinc levels in the digestive gland of control mussels (38.5 μg g^{-1} wet weight) were similar to those in L and M mussels but decreased in H mussels (26.7 μg g^{-1}). Table 1 further shows that there was a steady increase in the calcium content in the digestive gland of mussels in line with the increasing contaminant dose, although significant differences from the control were only established for H mussels.

Table 2. *Mytilus edulis*. Concentrations (μg g^{-1} wet wt) of copper bound to thionein (MT-Cu, measured by AAS) and copper and zinc bound to cytosolic proteins (measured by ICP) for 4 field sites and 4 mesocosm basins. Zinc bound to thioneins in reference/control individuals: 0.10/0.11 μg g^{-1}. Other details as for Table 1

Source	MT-Cu	Cytosolic-Cu	Cytosolic-Zn
Site			
1	0.27 ± 0.08	0.07 ± 0.01	4.35 ± 0.85
2	0.29 ± 0.07	0.11 ± 0.01*	3.85 ± 0.75
3	0.31 ± 0.06	0.15 ± 0.02**	4.87 ± 0.74
4	0.30 ± 0.06	0.12 ± 0.01*	4.10 ± 0.31
Basin			
C	0.35 ± 0.08	0.12 ± 0.03	4.79 ± 0.14
L	0.51 ± 0.16	0.36 ± 0.06*	5.43 ± 0.21
M	0.61 ± 0.15**	0.94 ± 0.22**	5.41 ± 0.32
H	0.94 ± 0.19**	1.39 ± 0.23**	3.24 ± 0.19**

Statistical significance as for Table 1

Table 2 lists copper content (analyzed by absorption spectrophotometry) in the metallothioneins in the digestive gland of mussels in the 4 experimental exposures. Metallothionein concentration was around 0.35 μg g^{-1} for controls, increasing by about 50 % in L mussels and by 75 and 170 % for M and H mussels. Similar results were obtained when the values of copper in metallothioneins were evaluated by direct ICP analysis of the eluate of the gel filtration chromatography (Fig. 1), in this way reducing analysis time to the 30 min necessary for HPLC separation. Standard deviations of metallothionein determinations by ICP were high, especially when control samples were analyzed (coefficient of variation = 0.37). Rigorous control of the monochromator temperature is required to avoid variations in maximum peak position of metal emission.

In the mesocosm basins subject to Cu dosing, there was a dramatic increase in the copper bound to cytosolic proteins of the mussel digestive glands. Concentrations were in the same order as exposure levels, with the copper content of the soluble protein fraction being 11 times higher in H mussels than in control mussels; in the same samples the concentration of zinc was significantly lower (−22 %).

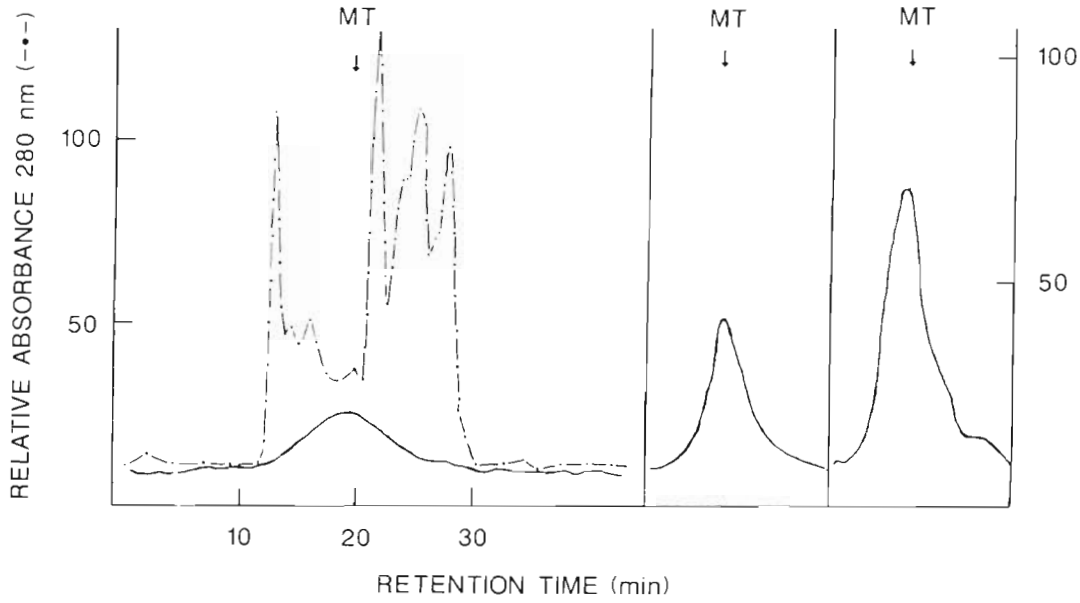

Fig. 1. *Mytilus edulis*. Gel-filtration elution profile of the soluble extracts from digestive gland of C, M and H mussels. Protein elution was monitored at 280 nm (-·-) and copper content (—) evaluated by direct analysis of the HPLC eluate by the ICP-AES system at 327.396 nm. MT = metallothionein peak

DISCUSSION

Data on copper, cadmium and zinc content of the digestive gland of *Mytilus edulis* from the Langesundfjord sites demonstrate that there is limited heavy metal contamination. At the more contaminated sites, there was no significant increase in concentrations of thionein-bound metals in digestive gland cells, whereas copper binding to cytosolic proteins (which may interact with soluble enzymes probably affecting digestive gland metabolism) significantly increased. However, interpretation of these data is complicated by the presence of other pollutants, specifically a clear hydrocarbon gradient in mussel tissues across Sites 1 to 4. Such hydrocarbon contamination could alter the normal pathways of heavy metal homeostasis in mussel tissues, also affecting the protective role of metallothionein. It is interesting that the concentration of a cation such as calcium, often thought to be involved in cellular toxicity induced by oxidative stress (Sies 1985), increased along the field pollution gradient.

In the mesocosm experiment, copper exposures led to an accumulation of copper in the digestive gland of mussels from dosed basins, but in no case did this affect the cadmium content. A significant decrease in zinc was observed only in mussels with high levels of copper accumulation.

The increase in calcium concentration in mesocosm mussels, in line with contaminant dosing levels, was consistent with results from other laboratory experiments (Viarengo unpubl.) where exposure of mussels to Cu^{++} significantly altered the redox balance in the gills and digestive gland, increasing malondialdehyde production and lipofuscin (insoluble peroxidation product) accumulation in the lysosomes. In the same experiments, an enhancement of the total Ca concentration in the cells, as well as the free calcium levels in the soluble fraction, was always observed. These results are be interpreted as indicating calcium cytotoxicity induced by oxidative stress which, in mesocosm mussels, is probably due partly to copper contamination. Such an interpretation appears to be in agreement with the data of Moore (1988), which demonstrates that there is an increase in lipofuscin accumulation in digestive gland lysosomes of mussels from the polluted field sites, as well as in the dosed mesocosm basins. Moreover, the possibility that an abnormal Ca homeostasis may play a role in cytotoxicity seems to be in agreement with cytochemical and physiological data reported by other authors in this Special MEPS Volume; this indicates that, for some fjord sites and mesocosm basins, mussels are subjected to stress.

Our results indicate the need for more research on the possibility of adopting calcium levels as a general stress parameter. In fact, although the level of total Ca in tissues seems to vary in relation to oxidative stress due to pollution, only the value of free calcium in the cytosol is usually considered related to cytotoxicity; for this reason direct estimation of this variable may be a more useful stress index. However, hypoxic hypercapnic conditions may also cause Ca increase in mussel

haemolymph and tissues, probably due in part to $CaCO_3$ mobilization from the shell (caused by a decrease of pH in fluids and tissues). In this study only H mussels showed a significant decrease in O_2 consumption; there were no significant respiration differences between any of the field sites (Widdows & Johnson 1988). Therefore, this possibility cannot on its own explain the general Ca increase observed in the current samples, though the role of hypoxic hypercapnic conditions needs to be taken into account in future studies as a possible additional means of Ca enhancement in mussel tissues.

Metallothionein determinations from mesocosm mussels confirm that the evaluation of metal bound to this protein often gives a good indication of the biological response to heavy metals. The ratio between metal bound to thioneins and total metal in the tissue was lower in the samples analysed in this study (especially in the Cu-exposed mussels in the mesocosm experiment) than in previous laboratory experiments (Viarengo et al. 1985). A possible explanation is that high concentrations of hydrocarbons in the water may have altered copper homeostasis mechanisms, affecting the rate of metallothionein synthesis or catabolism or the capacity of lysosomal metallothionein compartmentation (Viarengo 1985). In addition, a high accumulation of lipofuscin was found in digestive gland lysosomes of impacted mussels (Moore 1988), and lipofuscin is a peroxidative product able to complex heavy metals in an insoluble form (George 1983a).

Estimation of metal bound to thioneins by gel filtration HPLC gave quite similar results, whether interfaced with ICP or followed by the usual estimation of metal present in eluate fractions by furnace AAS analysis. In view of its greater versatility, the ICP system may be suggested as a future improvement in multi-element analysis, thus saving time in an analysis which often presents matrix interference problems. However, it requires more detailed research to reduce the high variability which was observed here in the analysis of mesocosm control samples, with a low copper content (about 0.02 $\mu g\ ml^{-1}$ in the HPLC eluate).

The results reported strongly suggest that the levels of copper bound to cytosolic proteins are useful as an indicator of potential toxicity of metal present in the cell. In fact, marked increases in copper bound to soluble proteins in mussel digestive gland were found not only for the clear-cut tissue Cu gradient in mesocosm mussels but also for the extremely limited field gradient. The heat treatment used to prepare the cytosolic proteins probably causes a redistribution of metals between high and low molecular weight compounds; hence it is difficult to quantify the biological significance of interactions of excess copper with the enzymic cytosolic pool. However, there is good agreement between the variation of this specific stress index and the cytochemical and physiological data collected on the same mussel populations by other workshop participants. This index can also be applied to toxic metals, such as lead and chromium, etc. which do not stimulate metallothionein synthesis.

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