Effects of Cadmium in the Sea Mussel, *Mytilus Edulis* L.

A Stress Approach

Margarita B. Veldhuizen - Tsoerkan

Proefschrift

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Limnologisch Instituut
3631 AC Nieuwersluis (U)

Effects of Cadmium in the Sea Mussel, *Mytilus Edulis* L.

A Stress Approach

Effecten van cadmium op de zeemossel, *Mytilus edulis* L.
Een stress benadering
(met een samenvatting in het Nederlands)

Proefschrift

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Margarita Borisovna Veldhuizen-Tsoerkan

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FLANDERS MARINE INSTITUTE
Oostende - Belgium

Promotor:

Prof. Dr. D.I. Zandee
(verbonden aan de Faculteit Biologie, Rijksuniversiteit Utrecht)

Co-promotores:

Dr. D.A. Holwerda
(verbonden aan de Faculteit Biologie, Rijksuniversiteit Utrecht)

Dr. C.A. van der Mast
(verbonden aan de Faculteit Biologie, Rijksuniversiteit Utrecht)

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Посвящаю моей маме
Ирине Владимировне Семенченко

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C H A P T E R 1

Introduction

Introduction

The continually increasing pollution of the marine environment from expanding human activities associated with industries and agriculture constitutes a serious threat to the inshore marine ecosystem. Elevated levels of numerous toxic chemicals denote the deteriorating quality of the Dutch coastal and estuarine waters (Akkerman *et al.*, 1989).

Sea mussels, *Mytilus edulis*, are widely used as bioindicators of water quality in the marine environment. As sessile filter-feeders, mussels are forced to absorb high amounts of pollutants in areas with anthropogenic stress (Simkiss *et al.*, 1982). Due to their accumulation capacity, sea mussels are employed in "International Mussel Watch" programs to estimate the bioavailability of contaminants (Stephenson *et al.*, 1980, 1982). These programs are mainly based on measuring of contaminant content in whole-body soft tissues. Less known are the harmful effects of toxic chemicals as induced changes are not always easy to determine in such animals like mussels with low metabolic activity. Sea mussels are also a source of human food and this makes it important to understand the effects of pollutants within this organism in order to assess the possible hazards for human consumers.

The direct effects of pollutants are manifested foremost at the molecular/biochemical level as important macromolecules and cell membranes are damaged, and enzyme activities and metabolic pathways are changed. These alterations may often constitute sensitive and specific indicators of particular toxicants. The effects at this level can result in changes at higher levels of biological complexity and may eventually be expressed at population, community or ecosystem levels. So, studies on the biological impact of pollutants must integrate the effects at different levels, thus providing more information on the nature and extent of environmental damage than direct measurements of pollutant concentration in tissues and/or pollutant-induced alterations at one particular level. Cadmium (Cd) is a widespread environmental pollutant with a broad range of toxic effects. Cadmium intoxication has been extensively studied in mammals and is characterized by kidney damage, gonadal atrophy, bone demineralization, muscle and connective tissue damage (Webb, 1979).

Cadmium is believed to exert its toxic effects via two major mechanisms. First, the metal ion can bind directly to the sulfhydryl groups of proteins and to bases of nucleic acids, thus affecting structure and function of these important macromolecules (Vallee

and Ulmer, 1972; Koizumi and Waalkes, 1990). Secondly, due to physical and electrochemical similarities, cadmium can also interfere with the metabolism of essential divalent cations such as calcium and zinc which are involved in the regulation of a large number of cellular processes (Vallee and Falchuk, 1981; Suzuki *et al.*, 1985; Sunderman and Barber, 1988; Brostrom and Brostrom, 1990; Sutoo *et al.*, 1990). The toxicity of cadmium is thought to be counteracted by the induction of metallothioneins (MT) which are cysteine-rich proteins of low molecular weight (LMW) and with high Cd-binding capacity (Hamer, 1986; Kägi and Schäffer, 1988).

In sea mussels, the uptake, accumulation and excretion of cadmium have been studied in detail. The rate of uptake and accumulation was found to depend on the external Cd concentration, exposure time, food availability, salinity and temperature (Coombs, 1979; Jansen and Scholz, 1979; Köhler and Rijsgard, 1982; Borchardt, 1983; Everaarts, 1990). The final tissue accumulation of cadmium decreases in the order: kidney > viscera > gills > mantle > muscle > foot (George and Coombs, 1977). Accumulation of cadmium occurs to that extent as the metal uptake exceeds the elimination. George and Coombs (1977) have reported that the rate of metal excretion is 18 times slower than the rate of uptake. Elimination of cadmium was not observed in mussels transplanted to an unpolluted area for 70 days (Luten *et al.*, 1986).

Retention and detoxication of cadmium within the molluscan body is attributed to the binding of cadmium to MT-like proteins. Noël-Lambot (1975) found that cadmium is associated with a LMW protein in the homogenate of Cd-treated *M. edulis*. George *et al.* (1979) isolated Cd-binding proteins with molecular weight of 10 kDa from mussels. Amino acid analysis of metal-binding proteins from the tissues of Cd-exposed mussels has indicated that these proteins belong to the MT protein family (George *et al.*, 1979; Frazier, 1986). The amount of MT-like proteins is greatest in kidney and digestive gland of *M. edulis* (Nolan and Duke, 1983). This correlates with the uptake order mentioned earlier. The amount of these proteins was found to increase when Cd body burden was augmented (Köhler and Rijsgard, 1982). Experiments with purified lysosomes of mussel kidney have demonstrated the presence of MT in secondary lysosomes, but not in tertiary lysosomes, although the latter contain significant amounts of cadmium (George, 1983).

Sea mussels appear to be extremely tolerant to cadmium. Body burdens up to 150 µg Cd/g caused no measurable effects on physiological parameters such as clearance, ingestion, assimilation or growth of *M. edulis* (Poulsen *et al.*, 1982). It has been shown that only acute intoxication with 500 µg Cd/l resulted in severe histological changes in the gills and mid-gut of *M. edulis* (Jansen and Jansen, 1983). Studies of Sunila (1986, 1988) have confirmed that exposure to high Cd concentrations (1-10 mg/l) induces pathological alterations in gill tissue of mussels. Cadmium at low concentration (50 µg/l) has been shown to suppress the gametogenesis only in the initial stages of gonad development in *M. edulis* (Myint and Tyler, 1982) while exposure to higher concentrations (2 and 8 mg Cd/l) resulted in disorganization of male follicles (Sunila, 1984). These morphological changes are linked to biochemical/molecular perturbations, but the molecular effects of cadmium are poorly studied in *M. edulis*. Investigation of the Cd effects at this level

could not only help to elucidate the molecular mechanism(s) of Cd toxicity, but could also lead to manageable parameters for measuring the impact of this pollutant on the individual animal and on the ecosystem.

With regard to other pollutants, mussels have been shown to accumulate organic xenobiotics such as polyaromatic hydrocarbons, polychlorinated biphenyls (PCBs) and pesticides (Martin *et al.*, 1984; De Kock, 1986; Akkerman *et al.*, 1989). The response of mussels to organic pollution is induction of the cytochrome P-450 monooxygenase system that has a key position in biotransformation of lipophilic xenobiotics (Stegeman, 1985). Elevated activity of this system has been reported for the digestive gland of mussels and other bivalves exposed to PCBs, polybrominated biphenyls and petroleum hydrocarbons (Payne *et al.*, 1983). PCBs belong to the most serious and persistent organic pollutants in the aquatic ecosystem, but their toxicity has been studied mainly in marine vertebrates, *e.g.*, seals (Reijnders, 1987; Brouwer *et al.*, 1989) and fishes (Thomas, 1989). Data on the effects of PCBs in marine invertebrates are scarce. Recent studies of Den Besten *et al.* (1990a,b) have shown that PCBs display adverse effects on the reproduction of the sea star, *Asterias rubens*. In *M. edulis*, short-term exposure to 5 mg PCBs/l caused loosening of intercellular connections in epithelia and shrinkage of the cells in the gills (Sunila, 1988). Investigation into effects of PCBs was not a main object of this thesis, but PCBs were rather employed as an organic pollutant in order to compare its effects with those of cadmium.

In the unstable environment of the tidal zone, sea mussels evolved special mechanisms for sensing and responding to natural environmental stresses such as aerial exposure, fluctuations in temperature and salinity. Survival and ecological fitness of mussel populations depend on proper functioning of these mechanisms. Anoxic tolerance and the underlying biochemical mechanism have been the object of study in *M. edulis* (De Zwaan, 1977; Zandee *et al.*, 1986). As the majority of organisms, mussels also respond to elevated temperature by the synthesis of a limited set of heat shock proteins (hsps) (Lindquist, 1986; Sanders, 1988; Steinert and Pickwell, 1988). The specific hsp response is associated with the acquisition of thermotolerance (Tomasovic, 1989).

De Zwaan and De Kock (1988) have suggested that pollution would make mussels more vulnerable to natural stress. Pollution stress superimposed on already existing natural stress(es) may have more harmful effects. For example, diesel oil stress in *M. edulis* was promoted by lowered salinity (Tedengren and Kautsky, 1987). It is conceivable that the naturally-stressed organism constitutes a more sensitive system for the expression of pollutant effects which could otherwise remain concealed. Moreover, the toxic influence of contaminants may modify the response of animals to a natural environmental stress and, thus, would cause a deterioration of the ecological fitness of a mussel population.

The major aim of the present study was to investigate the effects of cadmium in sea mussels at the molecular and higher levels of organization, in order to develop new sensitive indices. These indices should help to create an early warning system to predict the damage by the contamination in the marine environment. The basic concept of this thesis involved the "stress approach", or the use of the response of mussels to a natural

stress as a tool to disclose the effects of pollutants. This approach was elaborated under laboratory conditions, checked in long-term, semi-field experiments and applied in a field study in the strongly polluted estuary of the Western Scheldt.

In Chapter 2, the effects of short-term exposure to cadmium and long-term exposure to cadmium or PCBs are described. Both pollutants were found to reduce the ability of mussels to survive the natural stress of anoxia. Based on these results, research was carried out into the toxic effects of cadmium at the molecular level (Chapters 3-7). Short-term exposure of mussels to cadmium inhibited the synthesis of proteins and RNA while inducing the expression of specific proteins, *viz.*, MT-like proteins and hsps, in the excised gills (Chapters 3-5). In Chapter 3, the effects of metal- and heat stress are compared. It is shown there that pre-exposure to cadmium alters the hsp response of mussels. In Chapter 6, Cd-induced alterations in the phosphorylation of several gill proteins are described. The effects of chronic exposure to cadmium were studied in Chapter 7. These effects include an induction of MT-like proteins and an altered hsp response although now the inhibitory effect of cadmium on the overall protein synthesis in the gills could not be detected. Chapter 8 summarizes the results of the field study in which the "stress approach" appeared to be a promising method to pinpoint early alterations in animals exposed at the contaminated sites. Concluding remarks are presented in Chapter 9.

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C H A P T E R 2

Anoxic survival time and metabolic parameters as stress indices in sea mussels exposed to cadmium or polychlorinated biphenyls

M.B. Veldhuizen-Tsoerkan, D.A. Holwerda, and D.I. Zandee

Department of Experimental Zoology, University of Utrecht, 8 Padualaan, 3584 CH Utrecht, The Netherlands

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Anoxic survival time and metabolic parameters as stress indices in sea mussels exposed to cadmium or polychlorinated biphenyls

57311

Abstract

Sea mussels, *MYtilus edulis*, were exposed to cadmium under laboratory and semi-field conditions and to polychlorinated biphenyls (PCBs) under semi-field conditions. After various exposure times, the animals were subjected to environmental anoxia by aerial exposure. The anoxic survival time was significantly reduced by short-time laboratory or semi-field exposure to cadmium. The effect of PCBs on the anoxic survival time was apparent after six months. In addition, during long-term semi-field exposure to cadmium or PCBs, also other potential stress indices, such as the adenylate energy charge, condition indices, glycogen and metabolite contents, and cadmium-binding proteins were examined.

Introduction

Sea mussels, *Mytilus edulis*, are typical inhabitants of the Dutch coastal and estuarine waters. By their sessile mode of life and high capacity to accumulate heavy metals and organic micropollutants (Martin *et al.* 1984; De Kock 1986), mussels are considered to be suitable biomonitors for the registration of pollution. During the last decade mussels have been employed in "Mussel Watch" programs to evaluate the bioavailability of contaminants and the quality of marine waters (Stephenson *et al.* 1980, 1982). It could be advantageous if, at the same time, *M. edulis* could constitute the model organism for revealing the deleterious effects of toxicants. Therefore, an inquiry has been started into sensitive signals of pollution-induced stress. Mussels were exposed, both acutely and chronically, to cadmium and polychlorinated biphenyls, which are among the most noxious pollutants delivered by the rivers Rhine and Western Scheldt, and especially threatening the Wadden Sea ecosystem.

Sea mussels are often subjected to unfavorable environmental conditions, such as fluctuations in salinity, temperature, food and oxygen availability. Sustained valve closure, accompanied by oxygen depletion, is a response to a stress-generating condition. *M. edulis* can tolerate hypoxic or anoxic conditions for days or even weeks due to the evolved biochemical strategy: a strong reduction of energy demand and activation of highly efficient pathways of anaerobic energy metabolism (Zandee *et al.* 1986). Recently, it was suggested that pollution and natural stress in general will cause the energy expenditure to increase and, therefore, the rate of anaerobic energy metabolism to become elevated, thus making the mussel more vulnerable to anoxia (De Zwaan and De Kock 1988).

The experimental approach was to limit the life span of mussels to the survival time under anoxia, *i.e.*, exposure to air. A possible reduction in survival time of pollutant-exposed mussels could then be considered as an indication of toxicity or as a stress index of pollution. Important criteria of stress are measurable changes in a biological (physiological or other) process that have a detrimental effect on the survival of the organism, or its reproduction, or its capacity to resist any further environmental change (Bayne 1980; Livingstone 1982). The majority of these alterations, measured at various levels of organization (biochemical, physiological, cytological), form 'general' indices which respond to several unrelated environmental stressors, but some are 'specific' indices which respond to only one stressor (Livingstone 1982).

The anoxic survival time of mussels was examined after short-term exposure to cadmium under laboratory conditions. The observed changes were checked in the system of long-term exposure to cadmium or PCBs under semi-field conditions. In addition, several established and general indices of sublethal stress, *e.g.*, adenylate energy charge (AEC) and condition indices, were measured after long-term exposure to either pollutant. Binding of cadmium to cellular proteins was employed as a specific index of metal pollution. Substrate (glycogen) and end product (succinate) were determined both in the normoxic and anoxic state to assess possible changes in the pathways of anaerobic energy metabolism.

Materials and methods

Animals and exposure system

Sea mussels, *M. edulis* L., were collected in the Eastern Scheldt (a relatively unpolluted area) in January 1987. Mean shell length was 4.9 ± 0.3 cm. The animals were kept in aquaria with recirculating sea water at 12^{0}C under a natural light-dark regime. Sea water salinity was 2.8%. The mussels were not fed.

Animals were exposed to cadmium at approximately 50 µg/L (0.5 µM) in 40-L glass aquaria to which sea water and metal solution were supplied with a pump at rates of 1 L/h and 10 mL/h, respectively. Cadmium was added as $CdCl_2$ (Merck, no. 2011). Cadmium concentration in the water was measured twice a week by atomic absorption spectrophotometry. The actual concentration amounted to 47 ± 3 µg/L.

Mussels were collected in the Eastern Scheldt in August 1988 for semi-field experiments. Mean shell length was 5.5 ± 0.4 cm. The animals were exposed to cadmium or PCBs in the field laboratory of Tidal Waters Division (Ministry of Transport and Public Works). Mussels were kept in 1,000- L tanks to which sea water from the Eastern Scheldt (salinity 2.8%, ambient temperature) was pumped at a flow rate of 41 L/h. The mussels were fed *Phaeodactylum tricornutum* at a concentration of 50 x 10 cells/L. A group of 250 mussels was exposed to cadmium from August 1988 till June 1989. Cad-

mium was added as $CdCl_2$ (BHD Chemicals Ltd) to give an actual Cd concentration of 16.5 ± 2.9µg/L (0.15 µM). Mussels were exposed to PCBs by feeding with *P. tricornutum*, cultured in sea water to which PCBs (technical mixture Clophen A50, Bayer, Leverkussen, FRG) were added continuously to result in a concentration of approximately 0.5 µg PCBs/L. Exposure to PCBs lasted from August 1988 till February 1989. Mussels were kept in unspiked sea water for 24 h until analyzed.

Metal and PCB analysis

Mussels were examined individually for the Cd content. Whole soft tissues were lyophilized for 48 h and decomposed in 65% (w/v) nitric acid (Merck, no. 456) at 90^{0}C for 2 h, using Teflon (PTFE) bombs placed in sandbath. Cadmium concentration in the nitric acid was determined by atomic absorption spectrophotometry (AAS) with a Varian AA-10, equipped with a deuterium lamp for background correction.

Polychlorinated biphenyls in 2 samples, composed of three mussels, were analyzed by the Netherlands Organization for Applied Scientific Research (TNO). The method comprised enzymatic destruction of wet tissue and extractive steam distillation with n-hexane. The hexane extract was cleaned up with a benzene sulfonic acid column and analyzed with a Hewlett Packard 5880A gas-chromatograph with electron capture detector (column 25 m x 0.22 mm, fused silica DB-1). Total amount of PCBs was calculated on the basis of measurements of 8 individual PCB-components (PCB-52, 87, 101, 105, 118, 138, 153 and 180).

Anoxic survival test

Groups of 20 (laboratory experiments) or 30 mussels (semi-field experiments) were subjected to anoxia. Mussels were exposed to air at 18^{0}C in closed humid boxes. Survival was assessed daily. Death symptoms were considered to be a specific smell and open valves.

Condition indices

Two indices of condition were determined as formulated by Bayne and Widdows (1978) and Fischer (1988), respectively: CI_1 = soft tissue dry wt (mg)/shell length (mm); and CI_2 = (soft tissue dry wt x 100)/(soft tissue dry wt + shell wt).

Biochemical analysis

Groups of 6 to 7 mussels were subjected to anoxia by keeping them just above the water surface for 6 h at 12^{0}C. Anoxic mussels and 'normoxic' controls (animals taken directly from the aquarium) were examined individually for glycogen, succinate and adenosine nucleotide contents. Soft tissues were frozen in liquid nitrogen immediately after dissection and lyophilized for 48 h. In order to obtain representative values, the lyophilized tissues of individual animals were pulverized prior to preparation of the samples for metabolite analysis. Preparation of tissue extracts for glycogen and adenylate assay was as follows: 100 mg dry tissue was homogenized (w/v = 1:25) in 5% ice-cold trichloroacetic acid. Homogenates (0.2 ml) were kept on ice until used for glycogen determination. Glycogen concentration was measured enzymatically (Keppler and Decker 1971) after enzymatic hydrolysis with amylo-α-1,6-glucosidase. The homogenate remainder was centrifuged at 40,000 g for 15 min. The supernatant was adjusted to pH 7-8 with 5 M K_2CO_3 and immediately assayed for the adenylates ATP (Lamprecht and Trautschold 1971), ADP and AMP (Jaworek *et al.* 1971). Preparation of tissue extracts for succinate analysis was as follows: 100 mg dry tissue was homogenized (w/v = 1:25) in 70% ethanol, 10 mM $KHCO_3$, pH 7.0. The homogenate was centrifuged at 40,000 g for 15 min and in the supernatant succinate was determined according to Kmetec (1966).

Analysis of metal-binding proteins

Soft tissue of 6 mussels was pooled and lyophilized. A sample of 300 µg was gently homogenized under a nitrogen stream in 4.5 mL 25 mM TRIS-HCl + 50 mM NaCl, pH 8.0. After centrifugation for 1 h at 100,000 g, 2 mL of the supernatant were chromatographed on a Sephadex G-75 superfine column (Pharmacia, 1.4 x 54 cm). The column was eluted at 4.5 mL/h at room temperature with the above-mentioned TRIS-buffer. Fractions of 1.12 mL were eluted. Cd content of the column fractions was determined by AAS.

SDS-polyacrylamide electrophoresis (SDS-PAGE) was applied to analyze the protein fractions (Laemmli 1970). An aliquot of each column fraction was mixed 2:1 with loading buffer (0.125 M TRIS-HCl, pH 6.8, 5% SDS, 25% glycerol, 12.5% 2-mercaptoethanol and 0.02% bromophenol blue), heated to 95^{0}C for 10 min and subjected to SDS-PAGE analysis on 15% gels. The gels were run at 200 V for 6 h at room temperature in a BioRad Proteon II slab cell. Gels were stained in 40% methanol and 10% acetic acid with 0.25% Coomassie Brilliant Blue R-250 and photographed.

Statistical analysis

The Kaplan-Meier survival curve estimate was applied in the statistical treatment of the anoxic survival time. Data from other experiments were analyzed for significance of difference with Student's t-test, taking a probability limit $P < 0.05$ as significant.

Results

Anoxic survival time

Preliminary experiments were carried out to examine the effect of temperature on the anoxic survival time of *M. edulis*. At 12°C a three time higher LT_{50} was found that at 22°C (Figure 1A). For practical reasons, a temperature of 18°C was chosen for the further experiments. Two weeks of Cd exposure under laboratory conditions caused a significant decrease in anoxic survival time. LT_{50}'s of the exposed and control groups were 9.5 and 10.7 days, respectively (Figure 1B). A further decrease, to 7.6 days, was found after 4 weeks of exposure (Figure 1C). Cadmium concentration in the soft tissue of mussels increased from 0.59 to 21.1± 5.9 (mean of 20 animals ± SD) µg/g dry wt after 2 weeks, and 40.3 ± 11.4 (ibid.) µg/g dry wt after 4 weeks.

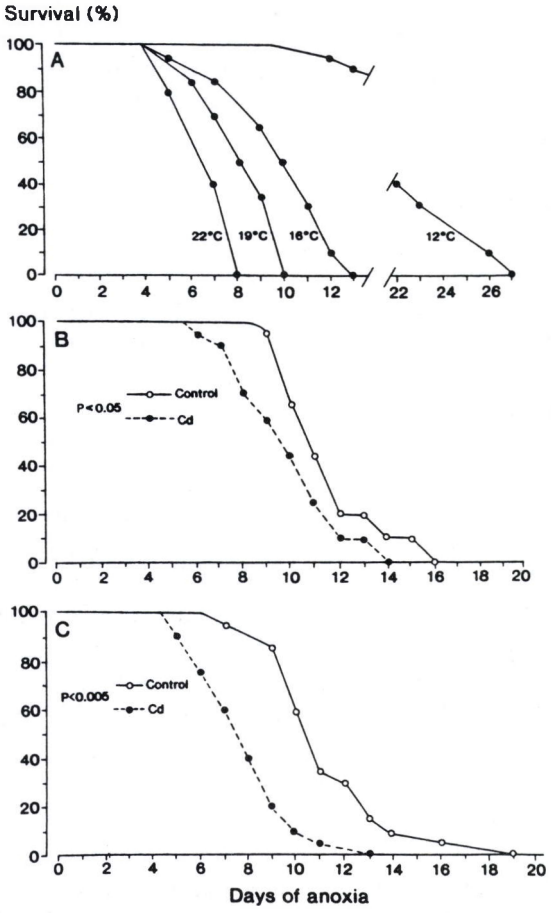


Fig. 1. Anoxic survival time of mussels in laboratory experiments (January-February '87). A. Effect of temperature; B. Effect of Cd exposure for 2 weeks; C. Effect of Cd exposure for 4 weeks. Groups initially consisted of 20 animals.

The 'anoxic survival' test was applied to mussels exposed to cadmium or polychlorinated biphenyls under semi-field conditions. Three months of cadmium exposure resulted in a significant reduction of anoxia tolerance (LT_{50} = 9.3 days) (Figure 2A). Neither survival curve nor LT_{50} of the PCB-exposed group differed significantly from those of the control group, although the initial death rate was higher in the PCB-exposed group (Figure 2A). When the exposure time was extended to 6 months, the shift of curves towards diminished anoxic survival was significant both in the Cd- and in the PCB-exposed group (Figure 2B). LT_{50} of control animals was 9.7 days while those of the Cd- and PCB-exposed groups were 8.6 days. After 10 months of exposure, the anoxia tolerance of mussels was significantly decreased by cadmium (Figure 2C). The anoxia tolerance of non-exposed animals fluctuated through the year (Figure 2D). The control LT_{50} had the highest value in the fall, declined during the winter and reached the lowest value in the late spring. The observed difference between LT_{50}'s of control and Cd-exposed groups was most conspicuous when the control LT_{50} value reached the maximum (Figures 2, A-D).

Accumulation of both pollutants proceeded almost linearly, increasing from 0.45 to 18.7 ± 6.1 (mean of 20 mussels, ± SD)µg Cd/g dry wt and from 4.2 to 62.9 ± 4.2 (mean of 2 samples composed of 3 mussels, ± SD) µg PCBs/g lipid after 3 months of exposure, and 40.3 ± 11.4 (ibid.) µg Cd/g dry wt and 150.5 ± 53.7 (ibid.) µg PCBs/g lipid after 6 months of exposure.

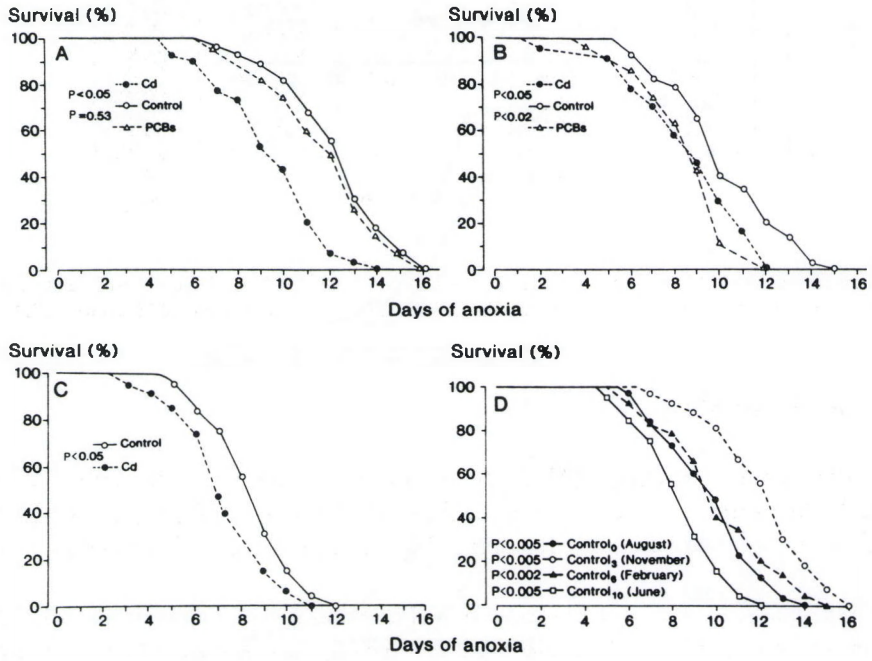


Fig. 2. Anoxic survival time of mussels in semi-field experiments (August '88-June '89). A. Exposure to Cd or PCBs for 3 months; B. Exposure to Cd or PCBs for 6 months; C. Exposure to Cd for 10 months; D. Anoxic survival time of control animals during 10 months of experiments. Groups initially consisted of 30 animals.

Condition indices

Two condition indices (CI_1 and CI_2) were determined to estimate changes in soft tissue and shell weights, and in shell length in response to 3 and 6 months of exposure to cadmium or PCBs under semi-field conditions. In agreement with the finding of Zandee *et al.* (1980), both indices (in all three groups) exhibited a seasonal dependence, having higher values in the fall than in the winter. However, neither cadmium nor PCBs altered CI_1 and CI_2 (Figure 3).

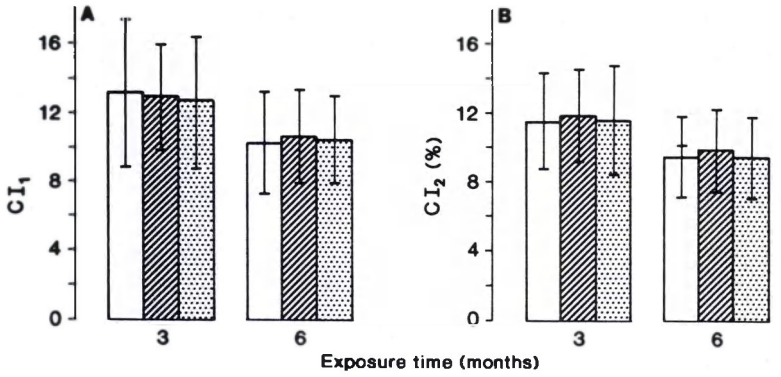


Fig. 3. Condition indices 1 (A) and 2 (B) of mussels exposed to cadmium or PCBs for 3 and 6 months under semi-field conditions. □ - control group; ▨ - Cd-exposed group; ▩ - PCB-exposed group. Mean of 30 animals ± SD.

Metabolic parameters

The adenylate energy charge (AEC) derives from the cellular concentrations of ATP, ADP and AMP and is given by the relationship: ([ATP] + ½ [ADP]) : ([ATP] + [ADP] + [AMP]). AEC was proposed by Atkinson (1968) as an indicator of the cellular "energy status".

The AEC values of the control group and after 6 months of exposure to cadmium or PCBs are summarized in Table 1. No significant changes were observed between exposed and unexposed animals. Six hours of anoxia resulted in a decrease of AEC that was most conspicuous in the PCB-exposed group. The AEC value of anoxic PCB-exposed animals was significantly lower than that of anoxic control mussels, while the AEC value of anoxic Cd-exposed animals was at the anoxic control level.

Exposure to cadmium did not affect the glycogen content (Table 1). Glycogen decreased in all three groups during aerial exposure for 6 h. A tendency towards lowered glycogen level was observed in either normoxic or anoxic PCB-exposed mussels, but this observation was not statistically significant.

Succinate levels were significantly higher in exposed mussels compared to the controls (Table 1). Anoxia for 6 h caused an increase in succinate level in the control and exposed animals. The increase was stronger in the Cd- and PCB-exposed groups, but the difference was not significant due to substantial variability of succinate values in the anoxic control animals.

Table 1 Metabolic parameters in normoxic and anoxic mussels exposed to Cd or PCBs for 6 months under semi-field conditions: adenylate energy charge (AEC), glycogen (μmol/g dry wt) and succinate (μmol/g dry wt). Mean of n mussels ± SEM.

	Normoxic groups		
	Control	Cd-group	PCB-group
AEC	0.807 ± 0.007	0.803 ± 0.006	0.794 ± 0.013
n	7	6	7
Glycogen	825.5 ± 83.9	866.8 ± 54.3	675.0 ± 69.4
n	7	6	7
Succinate	0.013 ± 0.003	0.147* ± 0.052	0.130* ± 0.017
n	7	6	7

* $P < 0.02$ with respect to the normoxic control

	After 6 hr of anoxia		
	Control	Cd-group	PCB-group
AEC	0.750 ± 0.008	0.743 ± 0.014	0.703** ± 0.009
n	7	6	6
Glycogen	756.8 ± 95.2	766.3 ± 93.1	605.7 ± 72.6
n	7	6	7
Succinate	2.215 ± 0.649	2.853 ± 0.147	3.402 ± 0.262
n	7	6	7

** $P < 0.05$ with respect to the anoxic control

Cadmium-binding proteins

The cadmium profile in gel permeation chromatography of cytosol obtained from mussels exposed to cadmium for 3 months is shown in Figure 4. The major part of cyto-

solic cadmium (88%) was bound to the low molecular weight (LMW) fraction, and a smaller part (8.7%) was associated with the high molecular weight (HMW) fraction (Figure 5). The LMW protein fraction was partly resolved into two peaks with apparent molecular weights of approximately 19 and 13.5 kDa (Figure 4). The percentage of cytosolic cadmium associated with this protein fraction appeared to plateau with extended time of exposure (10 months), while cadmium bound to HMW proteins increased to 13.4% (Figure 5). Gel permeation chromatography of control cytosols did not show any detectable cadmium profile (not shown). LMW protein fractions of control and Cd-exposed mussels were analyzed by SDS-PAGE (Figure 6). The protein band patterns were identical in both groups, but the intensity of Coomassie Blue staining was higher in the control fractions. SDS-PAGE analysis of column fractions, derived from mussels exposed to cadmium for 10 months and control animals, gave similar results (not shown).

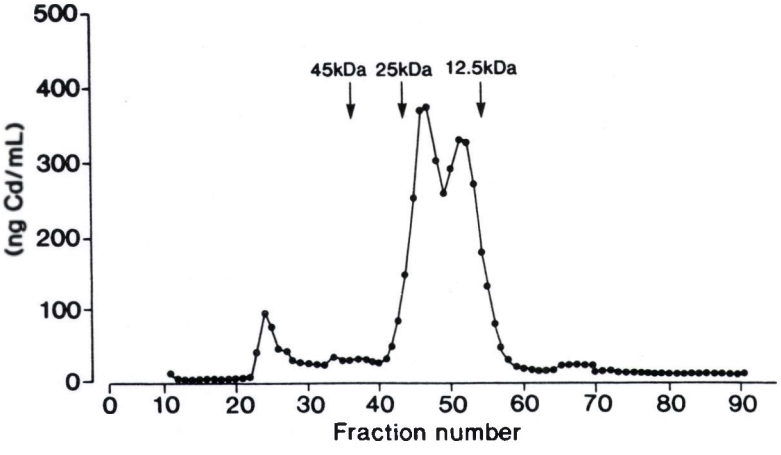


Fig. 4. Sephadex G-75 elution profile of cytosolic cadmium from mussels exposed to cadmium for 3 months under semi-field conditions. The arrows indicate the position of the peaks of molecular weight markers: egg albumin (45 kDa), chymotrypsin (25 kDa), and cytochrome c (12.5 kDa).

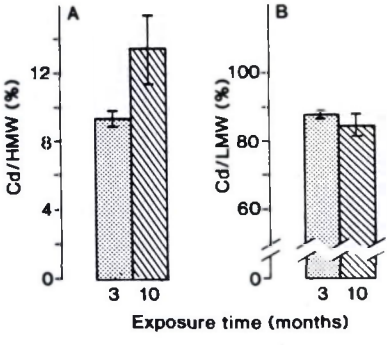


Fig. 5. Distribution of cytosolic cadmium between HMW (A) and LMW (B) protein fractions, resolved by Sephadex G-75, in mussels exposed to cadmium for 3 and 10 months under semi-field conditions. Mean of two runs ± SD.

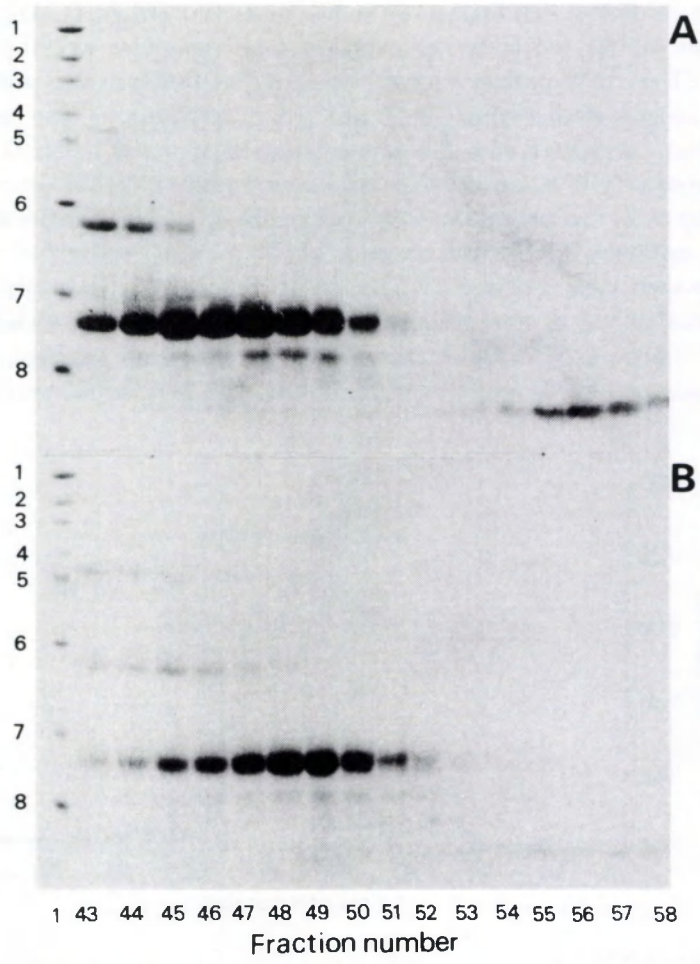


Fig. 6. SDS-PAGE analysis of LMW protein fractions from control mussels (A) and mussels exposed to cadmium for 3 months (B). Lane numbers correspond to column fraction numbers in Figure 4. Lane 1 - molecular weight protein markers: 1 - phosphorylase a (94 kDa); 2 - bovine serum albumin (68 kDa); 3 - catalase (60 kDa); 4 - 3'-phosphoglycerate kinase (47 kDa); 5 - aldolase (40 kDa); 6 - carbonic anhydrase (29 kDa); 7 - trypsin inhibitor (21.5 kDa); 8 - cytochrome c (12.5 kDa).

Discussion

Our study provides evidence that cadmium and polychlorinated biphenyls are toxic to *M. edulis*, as both pollutants significantly diminished the capacity of mussels to survive environmental anoxia. This effect was established at relatively low concentrations of cadmium and PCBs in the soft tissue of *M. edulis*. These concentrations were not far above contaminant levels in *M. edulis* in polluted marine environments (Martin *et al.* 1984; De Kock 1986).

Upon short-term laboratory exposure, cadmium reduced the anoxia tolerance in a time-dependent manner. Upon semi-field exposure to a lower cadmium concentration, the reduction of the anoxic survival time was most pronounced after 3 months, when the control LT_{50} of anoxic survival was at its maximum. With prolonged time of exposure, the effect of cadmium remained significant in spite of a reduction of the anoxic survival time of the control mussels. Under semi-field conditions the anoxia tolerance underwent seasonal variations reaching a maximum in the fall and being drastically reduced after spawning in the late spring. In *M. edulis*, seasonal variations in biochemical composition linked to the reproductive cycle have been shown to strongly influence the anaerobic energy metabolism (Zandee *et al.* 1980, 1986). The latter is the main biochemical strategy which underlies the anoxia tolerance of mussels.

The effect of PCBs on the anoxic survival time was discernible after 3 months of semi-field exposure. The PCB effect became significant and did not differ from that of cadmium with prolonged time of exposure. As the tissue concentration of both pollutants was of comparable order (taking into account a ratio dry wt/wet wt of 1:5, and total lipid content in tissues of 1.5%), the delay of the PCB effect can be ascribed to different mechanisms of toxic action of cadmium and PCBs. A recent study (Sunila 1988) on acute histological responses in the gill of *M. edulis* indicated that cadmium has a higher order of toxicity than PCBs after short-term (24 h) exposure to either pollutant at 5 mg/L.

In our study condition indices were not altered by cadmium or PCB exposure. Upon laboratory exposure to cadmium (33 days), the condition index (dry wt/shell wt) of juvenile *M. edulis* was influenced by food availability but not by cadmium (Borchardt 1983). In a field study, the same condition index was significantly negatively correlated with heavy metal concentrations (Cd, Zn, Hg) in *M. edulis* (Borchardt *et al.* 1988). Martin *et al.* (1984) reported a declined condition index (CI_2) in *M. edulis* transplanted along an increasing pollution gradient in San Francisco Bay. As condition indices did not change under the constant conditions of laboratory or semi-field exposure, the field data imply that condition indices are modified by variable environmental factors, *e.g.*, food availability, and/or by the synergistic toxic action of pollutants.

Several metabolic parameters were assessed in normoxic and anoxic mussels after 6 months of exposure when the reduction in anoxic survival time was significant for both exposed groups. The parameter of adenylate energy charge (AEC) has been proposed as a possible biochemical index of sublethal stress (Ivanovici 1980). Glycogen and succinate were used as indicators of the anaerobic energy metabolism. Under normoxic conditions AEC values were not affected by cadmium, but were slightly decreased by PCBs. The tendency to glycogen decrease in the PCB-exposed group, that was observed both in the normoxic and in the anoxic group, indicates an increased energy demand during exposure. Exposure to cadmium had no effect on the glycogen content. The elevated succinate level in both Cd- and PCB-exposed groups might reflect that exposure to these pollutants induces a partial switch to anaerobic metabolism, as succinate is one of the anaerobic metabolic end products (De Zwaan 1977). This observation supports the idea of Zandee *et al.* (1986) that pollutant stress promotes shell closure in *M. edulis*, thus causing a (partial) switch to anaerobic metabolism. In a recent study with the freshwater

clam *Anodonta cygnea*, Hemelraad *et al.* (1990) observed that Cd exposure resulted in depletion of glycogen, decline of AEC and accumulation of succinate. The authors have hypothesized that the observed phenomena indicate a partial switch to anaerobic metabolism, induced by closure of the valves in the toxic environment. Alternatively, cadmium could inhibit succinate dehydrogenase, thus causing impairment of oxidative metabolism and accumulation of succinate. As PCBs are unlikely to exercise their toxic action via inhibition of dehydrogenase-like enzymes, the present data corroborate the former possibility.

The 6-hours anoxic stress resulted in decreased values of AEC and glycogen in the control and pollutant-exposed groups, and increased levels of succinate. The metabolic parameters of PCB-exposed mussels exhibited the clearest changes under anoxic stress. The AEC value was significantly lower than in the anoxic control group, the glycogen level was the lowest, and the succinate level was the highest. No obvious alterations were observed in the Cd-exposed group, except a slightly higher succinate level than in the anoxic controls.

It has been suggested (De Zwaan and De Kock 1988) that general stress and pollution cause an elevated metabolic rate of anaerobic energy metabolism. During environmental anoxia mussels can reduce their metabolic rate about 75 times (De Zwaan and Putzer 1985) thus decreasing the energy expenditure. Additional stress may require extra energy, *e.g.*, for the induction and/or maintenance of detoxification systems (metallothioneins, mixed-function oxygenase system), therefore, creating an energy shortage. In the present study the metabolic parameters of the anoxic PCB-exposed group indeed signified an enhanced rate of anaerobic metabolism and increased energy expenditure. Neither of these parameters was apparently changed in the anoxic Cd-exposed group. This is surprising as the effect of cadmium on the anoxic survival time was significant and of comparable magnitude with that of PCBs. Further investigation is required to elucidate the biochemical mechanism of pollution action that underlies the reduction of the anoxic survival time of mussels.

Cadmium-binding, metallothionein-like proteins have been previously demonstrated in *M. edulis* (Scholz 1980; Nolan and Duke 1983; Frazier 1986; Harrison *et al.* 1988). Metallothioneins (MTs) are cysteine-rich proteins with low molecular weight of approximately 10 and 20 kDa. They play a major role in the detoxification of heavy metals. MTs have been proposed as a powerful, specific indicator of heavy metal pollution (Hennig, 1986).

Upon semi-field exposure for 3 months, cytosolic cadmium was divided among two protein fractions. The LMW protein fraction contained about 80% of the cadmium and consisted of two peaks with an apparent molecular weight of 19 and 13.5 kDa. With prolonged exposure time, the percentage of cytosolic cadmium bound to the LMW protein fraction remained unchanged, while cadmium bound to the HMW protein fraction increased. It appears that the detoxifying capacity in *M. edulis* becomes exceeded during long-term exposure to low cadmium concentration. This observation is consistent with results of Harrison *et al.* (1988). They have found that under long-term laboratory or environmental exposure to heavy metals the capacity of *M. edulis* to produce metallothioneins is limited.

SDS-PAGE analysis of the LMW protein fraction did not reveal induction of cadmi-

um-binding proteins. However, during the same semi-field exposure experiments, induction of metallothionein-like proteins in the gills of *M. edulis* was demonstrated by means of ^{35}S-cysteine labelling, followed by gel permeation chromatography and SDS-PAGE (Chapter 7).

The observed decrease in the intensity of Coomassie Blue staining of the gel might indicate that protein levels declined in the cytosols of Cd-exposed mussels. In SDS-PAGE resolved cytosols of Cd-treated fish, the same phenomenon was observed (Baksi *et al.* 1988). The obvious discrepancy between the induction of Cd-binding proteins and a decreased staining of the LMW band on gel was clarified by two-dimensional gel analysis of the fish cytosols. It was shown that staining of several LMW proteins was decreased, while there was an increase in two proteins that might correspond to the major Cd-binding proteins (Baksi *et al.* 1988).

In conclusion, the "stress approach" can be a profitable method for detecting noxious effects of environmental pollutants. The present data have shown that the anoxic survival time of mussels was significantly reduced by cadmium or PCB exposure, whereas other proposed stress parameters, *e.g.*, AEC, condition indices, remained still unchanged. Tedengren and Kautsky (1987) have also reported that diesel oil stress in *M. edulis*, measured as a decreased O/N ratio, was aggravated by the additional stress of a lowered salinity. The reduction of the anoxic survival time in *M. edulis* is a general stress index, as it is affected by several environmental factors, such as temperature and seasonal variations. However, under the applied constant (temperature) condition, this index is useful to uncover the effect of cadmium or PCBs. The ease of measurement and the practicability of this method should be taken into consideration. Recently, a field study was initiated in which the new stress index will be applied to mussels transplanted along pollution sites in the Western Scheldt.

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Effect of cadmium on protein synthesis in gill tissue of the sea mussel *Mytilus edulis*

M.B. Veldhuizen-Tsoerkan[*], *D.A. Holwerda*[*], *C.A. van der Mast*[**] *and D.I. Zandee*[*]

Departments of [*]*Experimental Zoology and of* [**]*Molecular Cell Biology, University of Utrecht, 8 Padualaan, NL-3584 CH Utrecht, The Netherlands*

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Effect of cadmium on protein synthesis in gill tissue of the sea mussel *Mytilus edulis*

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Abstract

Cellular toxicity of cadmium was studied in gill tissue of the sea mussel, *Mytilus edulis*. Mussels were exposed to cadmium chloride at 50 or 250 μg Cd/L for short periods. Then the gills were excised and incubated with ^{35}S-methionine or cysteine for 4 hr. Uptake of radiolabelled amino acids by isolated gills was not affected by cadmium, whereas the incorporation of label was significantly decreased after Cd exposure. Two dimensional gel electrophoresis was used to analyze the *de novo* synthesized gill proteins. It revealed that the expression of particular proteins was differentially altered by cadmium. One dimensional gel analysis by ^{35}S-cysteine labelled gill proteins demonstrated that cadmium induced, in a concentration-dependent manner, a cysteine-rich protein with molecular weight of approximately 13 kDa, consisting of two isomers with low isoelectric points.

Introduction

Sea mussels, *Mytilus edulis*, have a high capacity to accumulate cadmium and other heavy metals without notable toxic effects (1). Induction of metal-binding, metallothionein-like proteins by cadmium exposure has been demonstrated in *M. edulis* (2-5). Metallothioneins are specific metal-binding proteins of low molecular weight, having a high cysteine content. They play a crucial role in the cellular pathways and detoxification of heavy metals. Recent data indicate that the capacity of *M. edulis* to produce metal-binding proteins is limited, thereby restricting the organism's ability to tolerate a further increase in metal concentration (6). We have recently found that cadmium is toxic to *M. edulis* at a relatively low concentration; the survival time during environmental anoxia was markedly diminished after only 2 weeks of exposure to 50 μg Cd/L (Chapter 2). Based on this finding, a study was started on the toxic effects of cadmium at the macromolecular level.

Cadmium is a ubiquitous environmental pollutant and may be expected to exert its toxic effects either by complexing directly with cellular macromolecules or indirectly by interfering with the metabolism of essential metals. In a variety of organisms and cells in culture, cadmium was found to interfere with DNA, RNA, and protein metabolism. Cadmium induced DNA-single-strand breaks (7,8) and inhibited the processes of DNA repair and replication (8-11). At higher concentrations, cadmium inhibited the incorporation of radioactive precursors into RNA and proteins (9,10,12-15).

With marine invertebrates, the effects of cadmium at the molecular level are poorly known. Recently, Sanders (16) found that both heat shock and cadmium can induce heat shock proteins in *M. edulis*. At the same time, the expression of "normal" cellular proteins was decreased. However, according to Steinert and Pickwell (17) cadmium appeared to be a poor inducer of heat shock proteins in *M. edulis* and did not inhibit the incorporation of amino acids into cellular proteins. In fish hepatocytes, cadmium was found to affect the pattern of protein synthesis (18,19).

The present study was designed to assess possible effects of cadmium on protein synthesis in isolated gill tissue of *M. edulis*. The gills were chosen for these experiments, since they are ideally constructed for an absorptive role, in having a high surface area, a cell monolayer and a rich vascularization, and because they are known as cadmium-accumulating tissue (20,21). Moreover, morphological cadmium-induced changes have been found in molluscan gill tissue (22-24) that are apparently linked to physiological and biochemical alterations.

The experiments were carried out with gills isolated from mussels that had been exposed to cadmium chloride for short periods of time. The excised gills were incubated with ^{35}S-methionine or -cysteine in order to estimate the rate of amino acid incorporation into cellular proteins. The *de novo* synthesized gill proteins were analyzed by means of one and two dimensional gel electrophoresis.

Materials and methods

Animals

Mussels, *Mytilus edulis*, were collected in the Eastern Scheldt in October 1987 and February 1988. Mean shell length was 6.0 ± 0.5 cm. After transportation to Utrecht, the animals were kept in aquaria with recirculating sea water at 12^{0}C and were not fed.

Exposure system

Mussels were exposed to approximately 50 or 250 µg Cd/L in glass aquaria of 80 L to which sea water and metal solution were supplied with pumps at rates of 1 L/hr and 10 mL/hr, respectively. The animals were kept under natural light regime. The sea water temperature was 12^{0}C. Cadmium was added as $CdCl_2$ (Merck No. 2011). Prior to the further experimental procedure, the animals were kept overnight in unspiked sea water to eliminate adherent cadmium.

Metal analysis

Cadmium in gill tissue was assayed by decomposing lyophilized tissue in 65% (w/v) nitric acid (Merck No. 456) at 80^{0}C for 1.5 hr, using teflon (PTFE) bombs placed in a sandbath (25). Cd concentration was measured by atomic absorption spectrophotometry, using a Varian SpectrAA-10, equipped with a deuterium lamp for background correction.

Incubation of isolated gills with radiolabelled amino acid

Gills were isolated from mussels exposed to cadmium for 4, 7, or 15 days. The middle parts of the outer gill lamella were incubated individually in 0.5 mL of standard medium (0.45 µm filtered sea water, 32 mM imidazole, pH 7.6, 25µg/mL chloramphenicol, and 1 µM each of 19 unlabelled amino acids) with 12.5 or 25 µCi/mL ^{35}S-methionine or ^{35}S-cysteine (Amersham). Incubations were carried out in the wells of multidishes (24 wells, Nunclon) for 4 hr at room temperature, using a rotation shaker plate (Brouwer Scientific). Incubations were stopped by placing on ice for 5 min. Gill tissue was removed from the incubation medium, rinsed, and washed twice with standard medium. After centrifugation for 2 min at 16,000 g at room temperature the supernatants were discarded. Pellets were weighed and resuspended 1:5 (w/v) in buffer (5 mM Tris·HCl, pH 7.1, 0.1 mM PMSF and 1 mM DTT). Pellets were disrupted by sonification for 2 x 30 sec. The homogenate was centrifuged at 16,000 g for 60 min at 4^{0}C. A 10 µL aliquot of supernatant (SN) from each sample was used for protein determination according to Bradford (26). SN aliquots of 5 L were transferred to Whatman 3 MM filters to measure incorporation of the label by hot 10% trichloroacetic acid precipitation. Label uptake was determined as total radioactivity present in the SN aliquots of 5 µL. Radioactivity was counted in a Beckman LS 75 liquid scintillation counter.

Label incorporation is expressed as counts per minute (cpm) per microgram cellular protein or as percentage of total radioactivity. Label uptake is expressed as cpm per microliter of supernatant. Statistical analysis of the data was performed by Student's t-test.

Two dimensional gel electrophoresis

Two dimensional isoelectric focusing in polyacrylamide gels (IEF/SDS-PAGE) was employed to resolve the ^{35}S-methionine or -cysteine labelled proteins, as described by O'Farrell *et al.* (27) and Garrels (28). SN-samples, containing 75 µg of protein, were mixed with an equal volume of lysis buffer: 9.95 M urea 4%, Nonidet P-40, 2% Bio-Rad ampholytes (1.3% pH range 5-7 and 0.7% pH range 3-10), 0.1 M DTT and 0.3% SDS and were brought into the cathodic end of the gel for isoelectric focusing. The first dimension gels were cast from the mixture: 8.5 M urea, 2% Nonidet P-40, 3.5% acrylamide/bisacrylamide (ratio 30:1.5%) and 2% ampholytes (1.3 pH range pH range 5-7

and 0.3% pH range 3-10). The gels were polymerized with 2 µL 10% ammonium persulfate and 1.5 µL TEMED per milliliter of gel mixture. The gels were loaded in glass tubes (12 mm x 2.5 mm inside diameter) and allowed the polymerize for 2 hr. They were prerun for ½ hr at 200 V, ½ hr at 400 V in a Bio-Rad model-175 tube cell. The lower reservoir was filled with 25 mM H_3PO_4 (anode electrode solution) and the upper reservoir with 50 mM NaOH (cathode electrode solution). After the samples were loaded and the reservoirs refilled, the gels were run for 20 hr at 800 V. The gels were then equilibrated for 2 x 15 min with gentle shaking in 125 mM Tris· HCl, pH 6.8, 3% SDS, 50 mM DTT and 0.0001% bromophenol blue and loaded on the second dimension. Analysis in the second dimension was performed by SDS-PAGE on 10-20% polyacrylamide gradient gels (see below).

Following electrophoresis, the gels were stained in 40% methanol and 10% acetic acid with 0.25% Coomassie Brilliant blue R and treated with Enhance (New England Nuclear), according to the instructions of the manufacturer. The gels were dried and fluorographed with Hyper-film-MP (Amersham) at -70^{0}C.

Computer analysis

The fluorographed protein spots were analyzed with the IBAS image analysis system (Zeiss/Kontron, Eching, FRG). This system is organized around a fast pipeline-structural array processor (cycle time 100 ns). Images were digitized 10 times and averaged to improved signal to noise ratio (frame size 512 x 512 pixels, 8 bits = 256 grey levels). With the digital filter operation TRACKG (filter size 30 x 30 pix) the grey values of the spots are replaced by the locally most likely background values. The resulting image served as shading correction image for the original. Measurements were carried out on the corrected image. To delimit the individual spots, the procedure DISDYN was applied. This dynamic discrimination method operates with a local threshold which is dependent on the local neighborhood region. The local thresholds are derived from the local background grey level. Before determination of the integrated optical density as a measure for the amount of protein in the spots of interest, the input grey range is calibrated according to the corresponding optical density values.

One dimensional gel electrophoresis

^{35}S-cysteine labelled gill proteins were carboxymethylated by incubation in 0.2 M iodoacetate for 1 hr at 37^{0}C in the dark, and resolved either by SDS-PAGE or by one dimensional gel isoelectric focusing.

SDS-PAGE was performed by the method of Laemmli (29). Samples containing 70 µg of protein were mixed 2:1 with loading buffer (0.125 M Tris·HCL, pH 6.8, 5% SDS, 25% glycerol, 25% β-mercaptoethanol and 20 µM bromophenol blue), incubated at 95^{0}C for 10 min and applied to 10-20% gradient gel. Gels were run at 80 V for 16 hr in a Bio-Rad Proteon II slab cell. Phosphorylase B (92 kDa), serum albumin (67 kDa),

ovalbumin (45 kDa), catalase (4 subunits, 40 kDa) and cytochrome c (12.5 kDa) were used as standard markers.

One dimensional denaturating isoelectric focusing in a vertical mini gel system (Bio-Rad mini Proteon II slab cell) was performed essentially after Robertson *et al.* (30). Samples of 20 µg protein were mixed with an equal volume of lysis buffer. Lysis buffer and gel mixture were composed as described for IEF/SDS-PAGE, except that ampholytes were used of pH range 3-10. Samples were applied to the cathodic part of the gel. Electrophoresis was performed at room temperature for 3 hr at 200 V. After electrophoresis was completed, gels were fixed with 10% trichloroacetic acid for 10 min and then transferred to 1% trichloroacetic acid for 16 hr to remove ampholines.

Staining and fluorography of SDS-PAGE and IEF-gels were carried out as described above.

Results

Cadmium accumulation

The *in vivo* exposure resulted in accumulation of cadmium in the gills of *M. edulis*. Cadmium concentration both increased with dosage (Fig. 1A) and time (Fig. 1B).

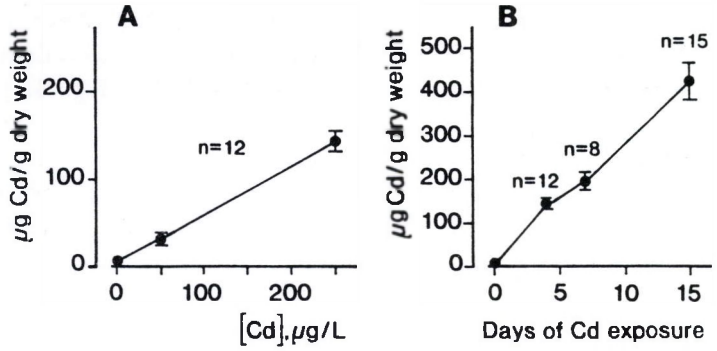


Fig. 1. Cadmium accumulation in gills of mussels exposed (A) to 50 and 250 µg Cd/L for 4 days, (B) to 250 µg Cd/L. Mean ± SEM.

^{35}S-methionine incorporation

In preliminary experiments the optimal conditions for incorporation of label by isolated gills were assessed. Addition of chloramphenicol, an inhibitor of bacterial protein synthesis, and of 19 unlabelled amino acids to the incubation medium had a positive

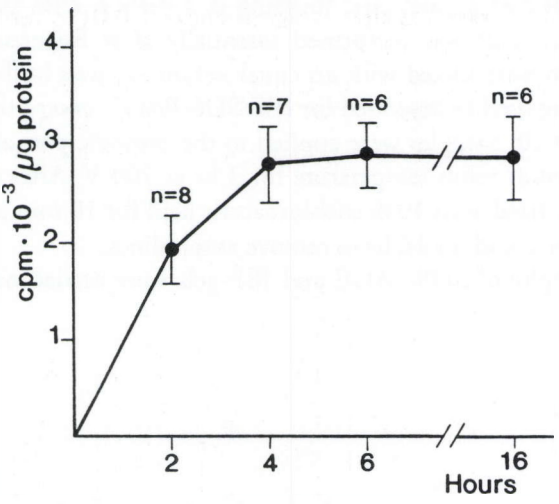


Fig. 2. Time course of ^{35}S-methionine incorporation. Isolated gills were incubated with 12.5 µCi ^{35}S-methionine at 22.5°C. Mean ± SEM.

effect on the incorporation of label. Maximal incorporation in the gills was reached after 4 hr of incubation in standard medium at room temperature (Fig. 2).

Exposure to 250 µg Cd/L for 7 days caused a significant ($p < 0.02$) decrease of 30% in the rate of methionine incorporation (mussels from October 1987). When the exposure time was extended to 15 days, the incorporation rate in gills of the exposed groups continued to decline, attaining a significant ($p < 0.001$) decrease of 40% (Fig. 3).

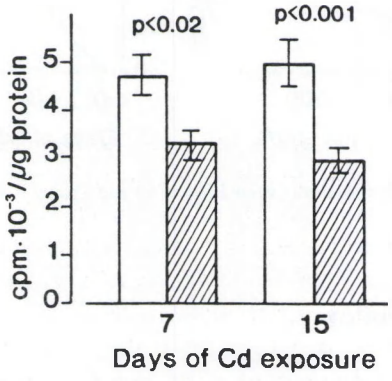


Fig. 3. Effect of cadmium on the ^{35}S-methionine incorporation. Isolated gills were incubated with 25 µCi ^{35}S-methionine for 4 hr at 20°C. □, gills from control mussels; ▨, gills from mussels exposed to 250 µg Cd/L. Mean of 10 animals ± SEM.

The experiment was duplicated on mussels collected in February 1988. Uptake of ^{35}S-methionine by the isolated gills was not affected by cadmium (Fig. 4A). Inhibition (34%) of the protein synthesis rate was already significant ($p < 0.01$) after 4 days of exposure to 250 µg Cd/L. The previously observed significant decrease in methionine incorporation rate following 7 and 15 days of exposure to 250 µg Cd/L was confirmed but, in this experiment, extension of the exposure time to 7 and 15 days did not result in an additional inhibition of the incorporation rate that remained at a level of approximately 76% of the control (Fig. 4B,C).

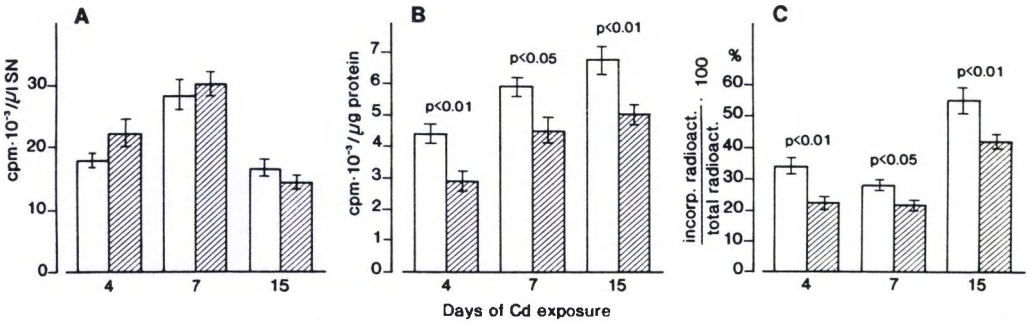


Fig. 4. Effect of cadmium on uptake (A) and incorporation rate (B,C) of ^{35}S-methionine in gill tissue. Isolated gills were incubated with 25 µCi ^{35}S-methionine for 4 hr at 20°C. □, gills from control mussels; ▨, gills from mussels exposed to 250 µg Cd/L. Mean of 10 mussels ± SEM.

Two dimensional gel electrophoresis of *de novo* synthesized gill proteins, followed by image analysis, revealed that protein synthesis was differentially affected by 7 days of exposure to 250 µg Cd/L (Fig. 5, Table 1). The majority of protein spots showed a decreased integrated optical density (Table 1). Synthesis of several proteins was almost completely inhibited (number 7-9, 30-32, 40-42; Table 1, Fig. 5), whereas synthesis of others was increased (number 3, 5, 6, 11, 22 and 38; Table 1, Fig. 5).

35S-cysteine incorporation

Experiments with ^{35}S-cysteine were carried out on mussels from February 1988. Uptake of ^{35}S-cysteine by the isolated gills appeared to be stimulated by cadmium (Fig. 6A). A significant decrease of label incorporation rate of 34% was found after 4 days of exposure to 50 µg Cd/L. The inhibition of protein synthesis rate increased to 45% when the cadmium concentration was raised to 250 µg Cd/L. The effect of cadmium became more conspicuous when the incorporated radioactivity was expressed as percentage of the total radioactivity was expressed as percentage of the total radioactivity (Fig. 6C). In a duplicate experiment, no significant effect of cadmium on label uptake was found (Fig. 7A). Exposure for 4 days to 50 and 250 µg Cd/L resulted in a significant inhibition of 32% and 40%, respectively, of the protein synthesis rate (Fig. 7B). In the next experiment, the exposure to 250 µg Cd/L was extended to 15 days. Uptake of cysteine was not affected by cadmium (Fig. 8A), whereas the rate of label incorporation was significantly

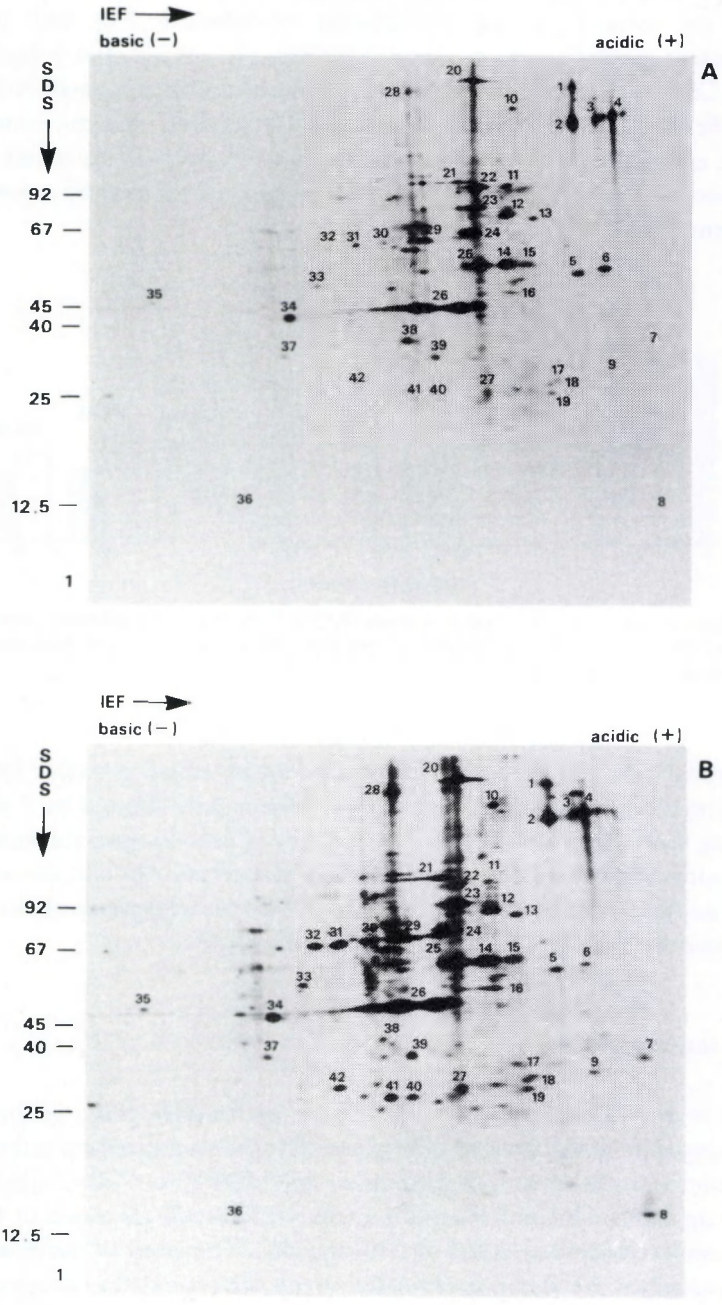


Fig. 5. Two dimensional IEF/PAGE patterns of ^{35}S-methionine labelled gill proteins from mussels exposed to 250 µg Cd/L for 7 days. A-exposed group; B-control group. Fluorography was for 30 days. Lane 1: molecular weight markers. Numbers indicate the most conspicuous spots. Integrated optical density of these spots is given in Table 1.

Table 1. Differential effect of Cd exposure on the synthesis of gill proteins.

Protein spot No.	Integrated Optical Density[a] Cd-exposed group	Control group	Ratio[b]	Protein spot No.	Integrated Optical Density[a] Cd-exposed group	Control group	Ratio[b]
1	1.01	1.19	85	22	2.36	1.87	126
2	3.64	3.70	98	23	1.65	4.23	39
3	1.06	0.88	121	24	3.58	5.33	67
4	1.43	1.68	85	25	2.87	6.13	47
5	0.78	0.69	113	26	17.3	29.5	59
6	1.09	0.24	454	27	0.41	1.07	38
7	0.00	0.40	0	28	0.56	1.65	34
8	0.00	0.45	0	29	1.57	3.93	40
9	0.02	0.23	9	30	0.13	1.26	10
10	0.36	0.98	40	31	0.33	3.37	10
11	0.66	0.34	194	32	0.05	2.01	3
12	2.07	1.99	104	33	0.16	0.60	27
13	0.37	0.83	45	34	1.20	2.40	50
14	2.79	5.07	55	35	0.08	0.26	31
15	0.59	1.34	44	36	0.10	0.41	24
16	0.15	0.77	20	37	0.13	0.40	33
17	0.04	0.11	36	38	0.71	0.28	254
18	0.07	0.25	28	39	0.37	1.19	31
19	0.11	0.32	34	40	0.05	0.71	7
20	1.44	2.08	69	41	0.07	1.39	5
21	0.86	2.23	39	42	0.07	1.05	7

[a]Integrated optical density of IEF/SDS-PAGE patterns (Fig. 5) of ^{35}S-methionine labelled gill proteins.
[b]Integrated optical density of Cd exposed group as a percentage of integrated optical density of control group.

decreased (Fig. 8B). The observed decrease of 33% became less conspicuous than that after 4 days of exposure to 250 µg Cd/L.

Two dimensional gel electrophoresis of ^{35}S-cysteine labelled gill proteins, followed by image analysis, indicated that 4 days of exposure to 50 µgCd/L differentially changed the protein synthesis pattern (Fig. 9, Table 2). The majority of protein spots had a decreased integrated optical density, while the synthesis of several proteins was almost completely inhibited (numbers 3-5, 13, 20-21, 24, 27, and 29; Fig. 9, Table 2).

One dimensional SDS-PAGE of the carboxymethylated, ^{35}S-cysteine labelled gill proteins revealed that cadmium induced the synthesis of a cysteine-rich protein with molecular weight of approximately 13-14 kDa (Fig. 10). The induction of this protein increased with dosage (Fig. 10, lanes 3 and 4) and time (Fig. 10, lanes 4 and 5) of Cd exposure.

One dimensional isoelectric focusing of the same ^{35}S-cysteine labelled samples showed that this newly induced, cysteine-rich protein consists of two isomers with isoelectric points between pH 3 and 5 (Fig. 11). Induction of these proteins was strongly dependent on the Cd concentration: the higher the Cd concentration, the more pronounced the expression of these proteins (Fig. 11, lanes 3, 4, and 5).

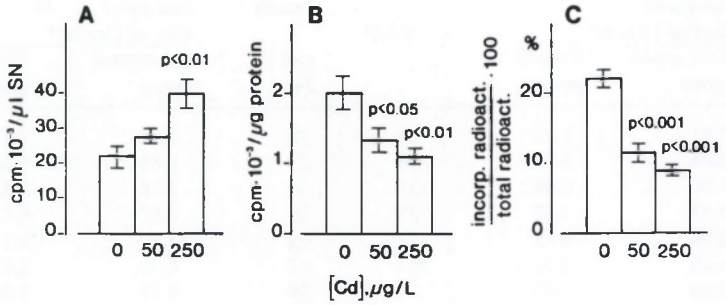


Fig. 6. Effect of cadmium on uptake (A) and incorporation rate (B,C) of ^{35}S-cysteine in gill tissue from mussels exposed to 0, 50, and 250 µg Cd/L for 4 days. Isolated gills were incubated with 25 µCi ^{35}S-cysteine for 4 hr at 20°C. Mean of 5 animals ± SEM.

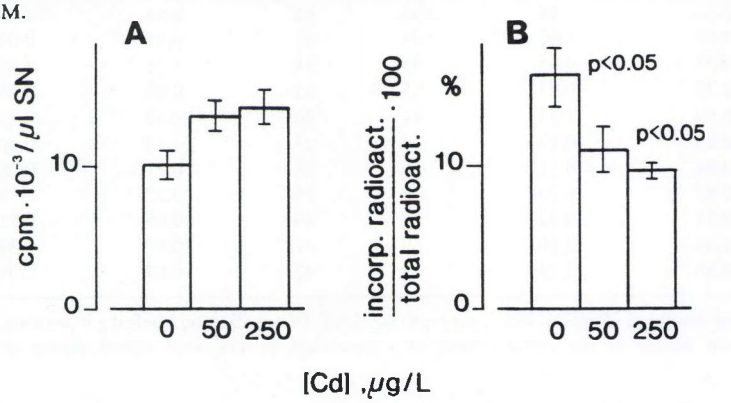


Fig. 7. Effect of cadmium on uptake (A) and incorporation rate (B) of ^{35}S-cysteine in gill tissue from mussels exposed to 0, 50, and 250 µg Cd/L for 4 days. Gills were incubated with 12.5 µCi ^{35}S-cysteine for 4 hr at 20°C. Mean of 5 animals ± SEM.

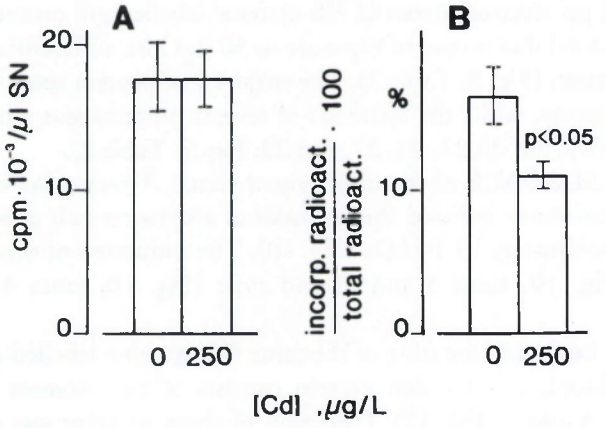


Fig. 8. Effect of cadmium on uptake (A) and incorporation rate (B) of ^{35}S-cysteine in gill tissue from mussels exposed to 0 and 250 µg Cd/L for 15 days. Gills were incubated with 25 µCi ^{35}S-cysteine for 4 hr at 22°C. Mean of 5 animals ± SEM.

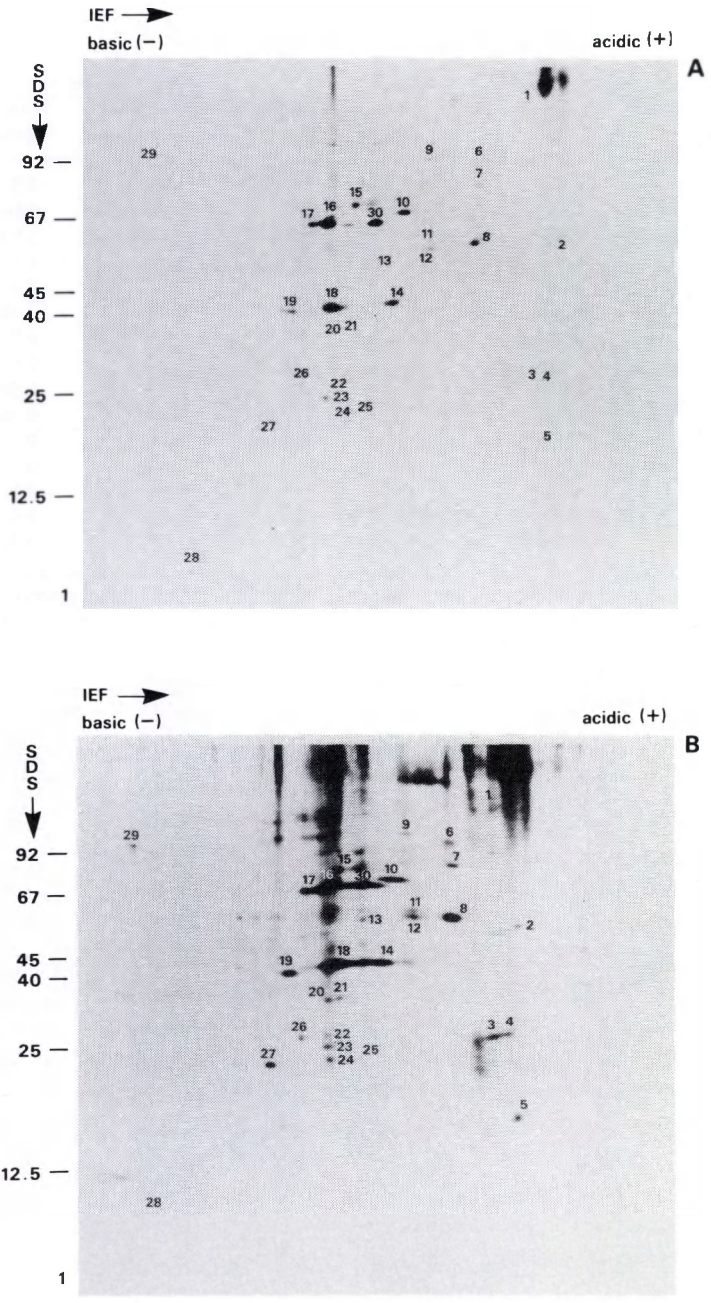


Fig. 9. Two dimensional IEF/PAGE patterns of ^{35}S-cysteine labelled gill proteins from mussels exposed to 50 µg Cd/L for 4 days. A-exposed group; B-control group. Fluorography was for 45 days. Lane 1: molecular weight markers. Numbers indicate the most conspicuous spots. Integrated optical density of these spots is given in Table 2.

Table 2. Differential effect of Cd exposure on the synthesis of gill proteins.

Protein spot No.	Integrated Optical Density[a] Cd-exposed group	Control group	Ratio[b]	Protein spot No.	Integrated Optical Density[a] Cd-exposed group	Control group	Ratio[b]
1	2.81	3.84	73	16	2.78	3.35	83
2	0.05	0.27	19	17	0.54	0.86	63
3	0.00	0.56	0	18	2.23	5.53	40
4	0.01	0.42	2	19	0.50	1.17	43
5	0.03	0.76	4	20	0.01	0.31	3
6	0.07	0.46	15	21	0.01	0.19	5
7	0.14	0.51	27	22	0.00	0.29	0
8	0.55	2.23	25	23	0.24	0.37	65
9	0.09	0.16	56	24	0.00	0.38	0
10	0.87	1.65	53	25	0.10	0.18	56
11	0.07	0.28	25	26	0.09	0.30	30
12	0.19	0.66	29	27	0.00	0.79	0
13	0.01	0.22	5	28	0.25	0.17	147
14	0.88	3.36	26	29	0.01	0.42	2
15	0.38	0.54	70	30	1.79	3.31	54

[a]Integrated optical density of IEF/SDS-PAGE patterns (Fig. 5) of ^{35}S-methionine labelled gill proteins.
[b]Integrated optical density of Cd exposed group as a percentage of integrated optical density of control group.

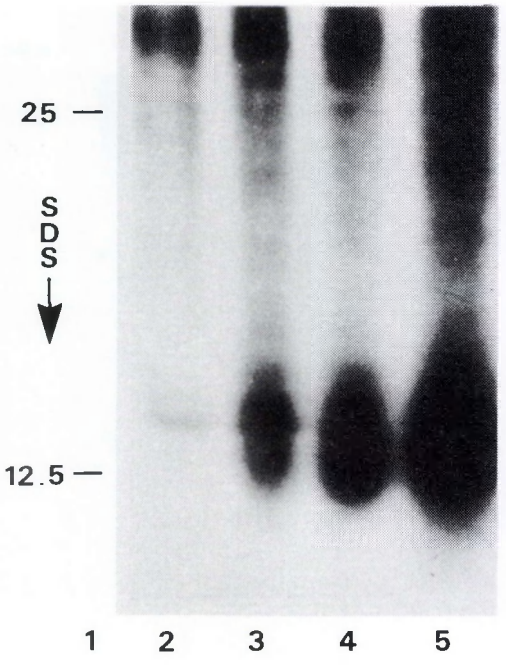


Fig. 10. Induction of low molecular weight, cysteine-rich protein in the gills of Cd-exposed mussels. SDS-PAGE of ^{35}S-cysteine labelled gill proteins. Fluorography was for 14 days. Only the low molecular weight part of the gel fluorograph is shown. Lanes: 1-molecular protein markers; 2-control group; 3-group exposed to 50 µg Cd/L for 4 days; 4-group exposed to 250 µg Cd/L for 4 days; 5-group exposed to 250 µg Cd/L for 15 days.

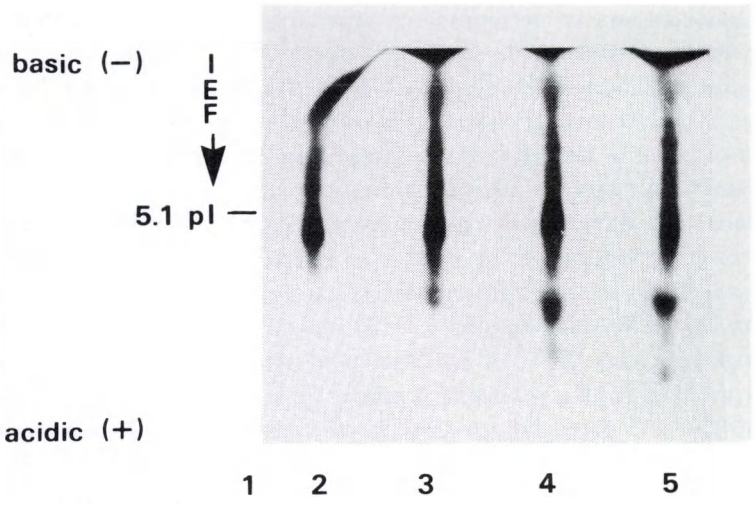


Fig. 11. Induction of two cysteine-rich proteins with low isoelectric point in the gills of Cd exposed mussels. One dimensional IEF of ^{35}S-cysteine labelled gill proteins. Fluorography was for 7 days. Only the acidic part of the gel fluorograph is shown. Lanes: 1-IEF standard, β-lactoglobulin B, pI 5.1; 2-control group; 3-group exposed to 50 µg Cd/L for 4 days; 4-group exposed to 250 µg Cd/L for 4 days; 5-group exposed to 250 µg Cd/L for 15 days.

Discussion

Cadmium-binding proteins in the sea mussel, *Mytilus edulis*, have been reported to contain a small amount of methionine residues and a high cysteine content, up to about 25 residue-% (5). Therefore, ^{35}S-cysteine labelling of gill proteins was applied to specifically trace the induction of thioneins by Cd exposure, whereas ^{35}S-methionine labelling was used to study the general effect of Cd on total protein synthesis. Amino acid incorporation is commonly used to indicate cytotoxicity (31) and it reflects both the cellular uptake of amino acid and the protein synthesis activity.

The present data show that the rate of protein synthesis in gills is significantly decreased by short-term *in vivo* Cd exposure. The effect of cadmium was most pronounced after 4 days and remained significant after 15 days of exposure. Inhibition of cysteine incorporation (32-34%) was observed at a Cd concentration of 50 µg/L (Fig. 6B, C-7B), while an equal decrease (34%) of methionine incorporation was found at a Cd concentration 5 times higher after 4 days of exposure (Fig. 4B, C). Prolonged exposure to cadmium (250 µg/L, 15 days) did not result in a further decrease of incorporation rate, that amounted to 33% for ^{35}S-cysteine and 24% for ^{35}S-methionine (Figs. 4B, C and 8B). Stabilization of the cadmium effect at a certain level may indicate that the inhibition of label incorporation is an acute response to Cd exposure which is counteracted by, *e.g.*, synthesis of metal-binding proteins after longer exposure times.

Two dimensional gel analysis revealed that expression of gill proteins is differentially affected by cadmium. Synthesis of methionine-labelled proteins was generally decreased, however, to a different extent and the expression of specific proteins was even enhanced (Fig. 5, Table 1). The majority of cysteine-labelled proteins had a decreased integrated optical density and the expression of several proteins was beyond the detection level (Fig. 9, Table 2). Induction of thioneins, as cysteine-rich proteins, by cadmium was not apparent on a two dimensional gel, as the protein samples were not subjected to prior carboxymethylation. The latter resulted in poor two dimensional resolution (results not shown). Carboxymethylation is usually applied to block SH-residues of thioneins, that can be smeared all over the gel, presumably through formation of S-S-linkages with other proteins during electrophoresis (32). Carboxymethylation followed by one dimensional gel analysis of ^{35}S-cysteine labelled gill proteins showed that cadmium induced, in a concentration-dependent manner, cysteine-rich proteins with low molecular weight (13-14 kDa), consisting of two isomers with low isoelectric points (Fig. 10 and 11). These proteins are presumably metallothioneins, but proof of their cadmium-binding capacity is lacking in this study.

The observed differences between cysteine and methionine incorporation into the gill proteins may reflect different cellular mechanisms of Cd toxicity. The decrease of cysteine incorporation is probably related to the cellular metabolism of cysteine. The synthesis of metallothioneins by metal stress may cause a decrease in the intracellular level of cysteine available for the synthesis of other molecules, *e.g.*, high molecular weight proteins and glutathione. In mammalian cells, the glutathione level was shown to be markedly decreased by Cd treatment (11). In fishes, cadmium depressed the ^{35}S-cysteine incorpora-

tion into high molecular weight proteins, whereas synthesis of cysteine-rich, low molecular weight, metal-binding proteins was changed (19).

Depression of the ^{35}S-methionine incorporation rate may represent a general cellular stress response. A recent study with *M. edulis* showed that both heat-shock stress and cadmium caused a repression of "normal" cellular proteins, although 72 hr of exposure to cadmium at 0.1 - 10 µg/L inhibited the incorporation of ^{35}S-methionine into gill proteins to a lesser degree than did heat shock (16). On the other hand, in the experiments of Steinert and Pickwell (17) mussels were subjected to Cd exposure at 200 and 600 µg/L for 4 or 28 hr, but no inhibitory effect on methionine incorporation into gill proteins was observed (17). In both studies, however, label incorporation was measured by densitometry of gel fluorographs, while in our experiments the incorporation rate was assessed in individual gill pieces by hot trichloroacetic acid precipitation.

Cadmium was also shown to inhibit protein synthesis, as measured by a decreased incorporation of labelled amino acids other than cysteine, in mammalian cells (9,10,13-15). In these experiments, an effect of cadmium on protein synthesis was observed at Cd concentration of 200-220 µg/L after several hours of exposure.

To make sure that inhibition of amino acid incorporation was not due to a decreased membrane permeability, uptake of amino acid by the isolated gills was also measured. The uptake of ^{35}S-methionine appeared not to be affected by *in vivo* exposure (Fig. 4A), while a tendency to an enhanced uptake of ^{35}S-cysteine was observed in some experiments (Figs. 6A and 7A).

Possible mechanisms for a decrease of protein synthesis may include disfunction at the level of replication, transcription, or translation. In mammalian cells, cadmium is known to cause DNA damage (7,8), to inhibit the processed of DNA replication and repair (8-11), and to depress RNA synthesis (9,10). It has also been reported that cadmium inhibits protein synthesis *in vitro* by phosphorylation of the α-subunit of the eucaryotic initiation factor 2, the loss of reversing factor activity, and the disaggregation of polyribosomes (33). It is worthwhile to note that the initial phase of the stress response is characterized by a shutdown of normal protein synthesis due to inhibition of initiation (34,35).

In order to get more insight into the level and the mechanism of the inhibitory action of cadmium on protein synthesis in the sea mussel, *M. edulis*, additional research is required.

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Effects of cadmium exposure and heat shock on protein synthesis in gill tissue of the sea mussels *Mytilus edulis* L.

M.B. Veldhuizen-Tsoerkan[*], *D.A. Holwerda*[*], *C.A. van der Mast*[**] *and D.I. Zandee*[*]

Departments of [*] *Experimental Zoology and of* [**] *Molecular Cell Biology, University of Utrecht, 8 Padualaan, NL-3584 CH Utrecht, The Netherlands*

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Abstract

Sea mussels were exposed to different concentrations of Cd^{2+} for short periods of time. The gills were then isolated and incubated with radiolabelled methionine or cysteine. The exposure to cadmium resulted in a dose-dependent inhibition of amino acid incorporation into gill soluble proteins. The inhibition was independent of the exposure time. Cd^{2+} induced the expression of two types of specific proteins: metallothionein-like proteins and heat shock proteins of the high molecular weight. *In vivo* heat shock caused a slight decrease of methionine incorporation by the gills but induced a set of heat shock proteins with molecular weight of 30, 32, 68, 70, 80, 82, 90 and 110 kDa. Exposure of the mussels to cadmium followed by heat shock resulted in a strong inhibition of the incorporation of methionine. The pre-exposure to cadmium differentially enhanced the expression of the heat shock proteins.

Introduction

In a variety of organisms and cultured cells cadmium ions inhibit the overall protein synthesis, while the synthesis of specific proteins can be enhanced (Hidalgo *et al.*, 1976; Din and Frazier, 1985; Beattie *et al.*, 1987). The cytotoxic influence of cadmium also includes a change in DNA and RNA synthesis (Ochi *et al.*, 1984; Burkart and Ogorek, 1986; Nocentini, 1987).

The first group of metal stress-induced specific proteins are the metallothioneins (MTs), cysteine-rich proteins of rather low molecular weight involved in the binding of metal ions (Hamer, 1986; Kägi and Schäffer, 1988). The MTs are not only involved in the detoxification of heavy metal ions but also in the metabolism of essential trace metals such as zinc and copper. It is of interest that MTs are also induced by many forms of chemical and physical stress (Oh *et al.*, 1978; Kägi and Schäffer, 1988).

The second group of induced polypeptides are the heat shock proteins (hsps) or stress proteins. A rise in temperature results in a shut-off of the synthesis of the normal cellular proteins and a concomitant rapid induction of a small set of proteins with molecular weights ranging from 22 to 100 kDa. A variety of other stimuli, *e.g.*, heavy metal ions, ethanol, anoxia and viral infection, elicit a similar set of proteins (Craig, 1985; Lindquist, 1986).

MTs and stress proteins are highly conserved polypeptides which occur in all eukaryotic and some prokaryotic cells. Their regulation is primarily effected at the transcriptional

level (Lindquist, 1986; Hamer, 1986). Both groups are believed to afford protection against the adverse effects of stress. In contrast to the metallothioneins the precise physiological mechanism by which the heat shock proteins enhance the tolerance to stress is not yet known.

Sea mussels, *Mytilus edulis*, are capable of accumulating cadmium- and other heavy metal ions without noticeable toxic effects due to a high capacity to synthesize metallothionein-like proteins (Poulsen *et al.*, 1982; Nolan and Duke, 1983; Frazier, 1986). Nevertheless, cadmium ions induce morphological changes in the gill tissue of *M. edulis* (Sunila, 1981; Sunila and Lindström, 1985). The deleterious effects here could be exerted at the transcriptional level as up to 30% of the accumulated cadmium in the gills was found in the nuclear fraction (Nolan and Duke, 1983). Our previous study (Veldhuizen-Tsoerkan *et al.*, 1990) has shown that cadmium ions repress the incorporation of amino acids into cellular protein, while the synthesis of specific proteins is enhanced. Sanders (1988) and Steinert and Pickwell (1988) observed the induction of stress proteins in mollusc tissues in response to cadmium ions or heat exposure. In the tidal zone sessile organisms are often confronted with multiple stresses such as elevated temperatures and heavy metal pollution. However, the stress response of these organisms at the molecular level has hardly been studied. That kind of studies could not only reveal the mechanism of the stress response but could also lead to manageable parameters for measuring the polluted state of the marine abiota and biota. The present work further explores the dose- and time-dependent effects of cadmium on the overall and specific protein synthesis in the gills of *M. edulis*. In addition, the effect of a consecutive exposure to metal and elevated temperature was examined.

Materials and methods

Animals

Sea mussels, *Mytilus edulis*, were collected from the Eastern Scheldt in different seasons during 1988. Mean shell length was 5.0 ± 0.5 cm. The animals were kept in aquaria with recirculating sea water at 12°C without feeding.

Exposure to cadmium and elevated temperature

Mussels were exposed to cadmium in a semistatic system. Groups of 8 animals were placed in plastic buckets with 5 L of aerated sea water. Cadmium was added as $CdCl_2$ (Merck No. 2011) to a final concentration ranging from 25 to 500 µg Cd/L. Sea water and metal solution were changed every second day. Water temperature was 12°C. The animals were kept under a natural light regime. Prior to the further experimental proce-

dure, mussels were kept 2 hr in unspiked sea water to eliminate adherent cadmium. For heat shock experiments, the mussels were kept in unspiked sea water at 29.5-30°C for 4 or 16 hr.

Metal analysis

For Cd analysis tissue samples were composed from the gills of 6 mussels. Cadmium was assayed by decomposing lyophilized tissue in 65% (w/v) nitric acid (Merck No. 456) at 80°C for 1.5 hr, using teflon (PTFE) bombs placed in a sandbath (Hemelraad *et al.*, 1986). Cd concentration was measured by atomic absorption spectrophotometry, using a Varian AA-10, equipped with a deuterium lamp for background correction.

Amino acid incorporation

Gills were isolated from exposed and control animals. The middle parts of the outer gill lamellae were individually incubated in 0.5 mL standard medium which consisted of millipore (0.22 µm) filtered sea water, 32 mM imidazole buffer, pH 7.6, 25 µg/mL chloramphenicol, 1 µM each of 19 unlabelled amino acids and 20 or 40 µCi/mL ^{35}S-methionine or ^{35}S-cysteine (specific activity > 1000 Ci/mmol Amersham). The incubations were carried out in the wells of multidishes (24 wells, Nunclon) at 12°C for 22 hr using a rotation shaker plate. The parts were blotted dry and weighed. A buffer consisting of 5 mM Tris·HCl, pH 7.1, 0.1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma) and 1 mM dithiothreitol (DTT, Boehringer) was added in an amount of 5 times the weight of the gill parts. The tissue was disrupted by sonification for 2x30 sec under cooling. The homogenate was centrifuged at 16000 g for 1 hr at 4°C. The total amount of radioactive amino acid present in the supernatant was determined by taking an aliquot of 5µL which was mixed with 2 mL of Aqualuma (Lumac) and counted in a Beckman LS 75 counter. Incorporation of labelled amino acids into protein was determined in another 5-µL aliquot which was transferred to a Whatman 3 MM filter and precipitated with hot trichloroacetic acid (10%) as described by Van der Mast *et al.* (1977).

The incorporation of radioactive amino acids in the gill parts is given in the figures as relative incorporation, *i.e.*, acid precipitable radioactivity (= incorporation into protein) divided by the total uptake of radioactive amino acids. Statistical analysis of the data was performed with Student's t-test.

Gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The protein concentration of the samples was determined by the method of Bradford (1976).

Prior to SDS-PAGE ^{35}S-cysteine labelled proteins were carboxymethylated with 0.2 M iodoacetate (pH 8.0 adjusted with Tris base) for 1 hr at 37°C in the dark. Samples containing 70 µg of protein were mixed 2:1 with loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 12.5% 2-mercaptoethanol, 25% glycerol and 0.05% bromophenol blue), incubated at 95°C for 10 min and applied to 10-20% gradient gels (^{35}S-cysteine labelled proteins) or to 12.5% gels (^{35}S-methionine labelled proteins). As molecular weight markers were used: phosphorylase a (94 kDa), bovine albumin (68 kDa), catalase (60 kDa), 3'-phosphoglycerate kinase (47 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21.5 kDa) and cytochrome c (12.5 kDa).

Two-dimensional electrophoresis (IEF/SDS-PAGE) was performed according to O'Farrell *et al.* (1977) and Garrels (1979). Supernatant samples containing 80 µg of protein were mixed with an equal volume of lysis buffer (9.95 M urea, 4% Nonidet P-40, 1.3% Bio-Rad ampholytes pH range 5-7 and 0.7% pH range 3-10, 0.1 M DTT and 0.3% SDS) and loaded at the cathodic end of the isoelectric focusing gel. Further details of this method were described earlier (Veldhuizen-Tsoerkan *et al.*, 1990). The second dimension was performed with polyacrylamide gel of 12.5%. Following electrophoresis the gels were fixed in 40% methanol-10% acetic acid mixture and stained with 0.25% Coomassie Brilliant Blue R in the same mixture. Prior to fluorography the gels were treated with Enhance (New England Nuclear) according to the instructions of the manufacturer. Hyperfilm-MP (Amersham) was exposed at -70°C without the use of intensifying screens. The incorporation of radioactive amino acids into protein was quantified from the fluorographs by image analysis.

Computer analysis

The integrated optical density (IOD) of the fluorographed proteins was determined with the IBAS image system (Zeiss/Kontron, Eching, FRG). Images were digitized ten times and averaged to improve the signal to noise ratio (frame size 512 x 512 pixels, 8 bits = 256 grey levels). In the case of one-dimensional gel fluorographs noise was reduced by application of a 3 x 3 median filter. Following shading correction the EMPHASIZE-filter was used to enhance the different bands in the gel. To enable discrimination of bands of interest on the basis of grey level the enhanced image was modified by its low-pass version. The size of the lowpass filter was 3 x 10 pixels, the long axis being the length direction of the lanes. The actual measurements were carried out on the original shading-corrected image.

For two-dimensional fluorographs the grey-values of the spots are replaced by the locally most likely background values with the help of the digital filter operation TRACKG (filter size 30 x 30 pixels). The resulting image served as a shading correction image for the original. Measurements were carried out on the corrected image. To delimit the individual spots the DISDYN procedure was applied. This dynamic discrimination method operates with a local threshold that is dependent on the local neighbourhood region. The local thresholds are derived from the local background grey-level.

Prior to the determinations of IOD as a measure for the amounts of protein in the

spots of interest the input grey range was calibrated according to the corresponding optical densities.

IODs were determined from several fluorographs of one gel and only the exposures within the linear range of the film were used. The metallothionein synthesis was estimated from the fluorographs of ^{35}S-cysteine labelled proteins and expressed, according to Sone *et al.* (1987), as 100 x IOD of metallothioneins divided by the IOD of the total peptides minus the IOD of the thioneins.

Results

Cadmium accumulation

As can be seen in Fig. 1A, exposure of mussels to various concentrations of Cd for 4 days resulted in a linear accumulation of cadmium in the gills of the animals. In Fig. 1B the results are given for mussels exposed to 50 µg Cd/L. Cd uptake progressed linearly with the exposure time.

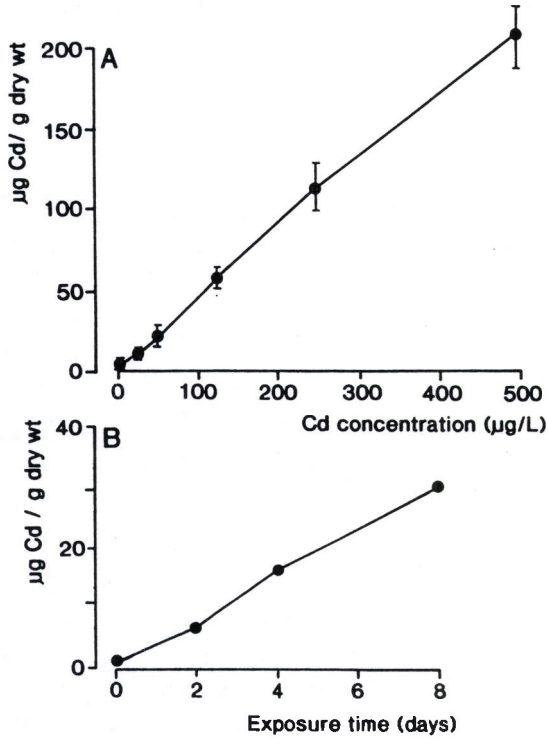


Fig. 1. Cadmium accumulation in the gills of *M. edulis.* A - Dose-dependency in mussels exposed for 4 days; mean of 3 samples of 6 gills ± SEM. B - Time-dependency in mussels exposed to 50 µg Cd/L; 6 gills per sample.

^{35}S-Methionine incorporation

To examine the effect of cadmium on the protein synthesis in gill tissue, mussels were exposed to various Cd concentrations for 4 days. The results which are given in Fig. 2 show a significant dose-dependent inhibition of the incorporation of methionine into gill proteins. The experiment was repeated with mussels collected at a later date. A similar decrease of methionine incorporation was noted (results not shown). Measurements of the integrated optical density (IOD) of the radiolabelled actin band showed that synthesis of this protein declined in parallel with overall protein synthesis (Fig. 2).

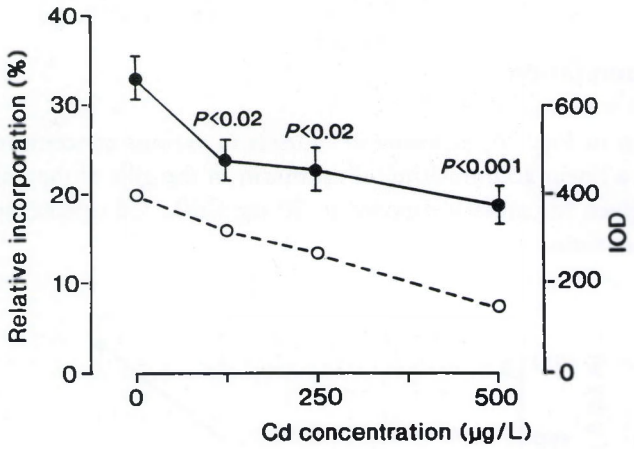


Fig. 2. Dose effect of cadmium on the ^{35}S-methionine incorporation into gill proteins. -●-, relative incorporation measured as acid precipitable radioactivity divided by the total amount of radioactive amino acid present in the supernatant; mean of 6 mussels ± SEM; P-values are given with respect to the control; animals collected in March '89. -o-, IOD of the actin band (molecular weight ≈ 45 kDa), estimated from the gel fluorograph shown in Fig. 3, lanes 2-4; mussels collected in August '89.

Electrophoresis further revealed the induction of several proteins with approximate molecular weight of 68, 70, 82 and 90 kDa (Fig. 3, lanes 2-5). This induction is seen most clearly at the highest Cd concentration used (lane 5) and is even evident when the exposure time was halved to 48 hr (lane 6). The enhancing effect of cadmium is also discernible in the 70 kDa protein and in the low molecular weight (20-25 kDa) region.

In the heat shock experiments, exposure of mussels to a higher temperature resulted in a decrease of the total incorporation of methionine into gill proteins (Fig. 4). Even after 16 hr of heat exposure the decrease was not yet significant, but already after 4 hr SDS-PAGE showed the presence of prominent bands of heat shock proteins with molecular weight of 30, 32, 68 and 70 kDa (Fig. 3, lanes 2, 7 and 8). The expression of heat shock proteins in the region 72-110 kDa is especially pronounced when exposure time to the higher temperature was increased from 4 to 16 hr (lane 7). The LMW region (20-25 kDa) did not show increased density in response to heat shock (lanes 2, 7 and 8). Two-

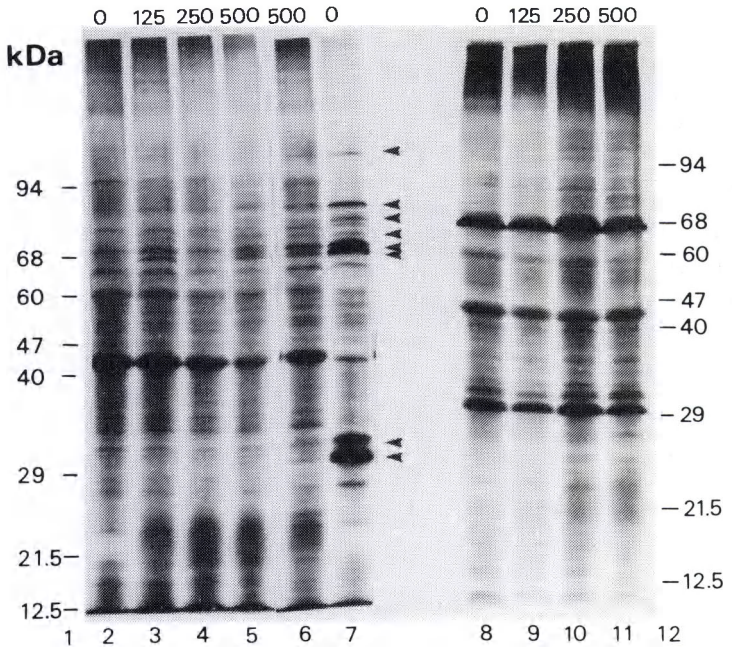


Fig. 3. SDS-PAGE patterns of ^{35}S-methionine labelled gill proteins from mussels exposed to cadmium and/or heat shock. Cd concentrations (µg Cd/L) are indicated at the top of each lane. Animal treatments were as follows: lane 2, control; lanes 3-5, Cd exposure for 96 hr; lane 6, Cd exposure for 48 hr; lanes 7 and 8, heat shock for 16 and 4 hr, respectively; lanes 9-11, Cd exposure for 96 hr, followed by 4 hr of heat shock. Lanes 1 and 12, molecular weight markers. Arrows indicate heat shock induced proteins. Fluorography was for 72 hr.

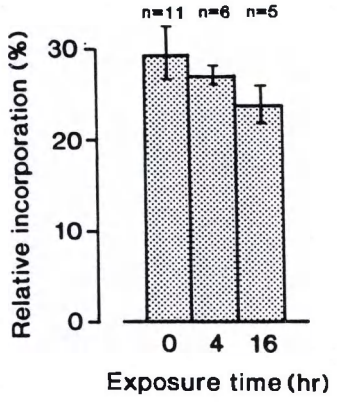


Fig. 4. Effect of heat shock on the ^{35}S-methionine incorporation into gill proteins. Mussels (collected in August '89) were exposed to elevated temperature (29.5^{0}C) for 4 and 16 hr. Mean of n mussels ± SEM.

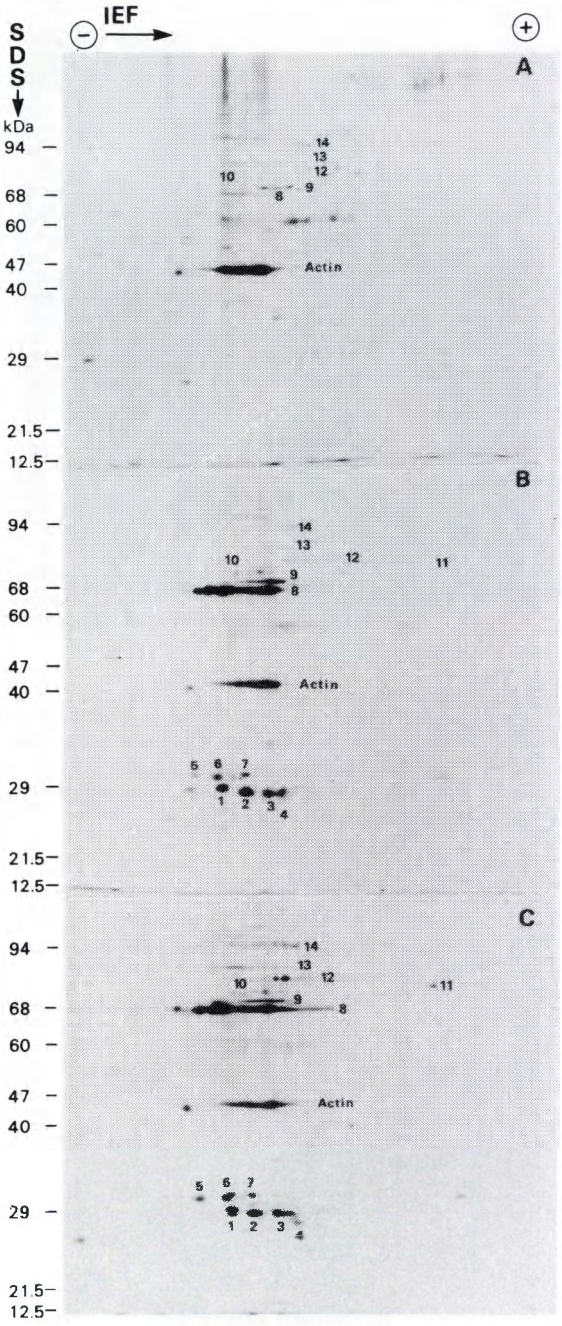


Fig. 5. Two-dimensional IEF/SDS-PAGE patterns of ^{35}S-methionine labelled gill proteins. A - control group; B - group exposed to elevated temperature for 4 hr; C - group exposed to 500 µg Cd/L for 96 hr, followed by 4 hr of heat shock. Fluorography was for 11 days. Lane 1: molecular weight markers. Numbers indicate the proteins induced by heat shock and cadmium treatment. IOD of these spots (B and C) is given in Table 1.

dimensional gel analysis of the proteins from animals heat shocked for 4 hr confirmed the induction of hsps of 30 and 32 kDa (Fig. 5A-B). These hsps seem to be produced in an array of charge isomers (Fig. 5B, spots 1-7). The proteins of 68 kDa and higher are constitutively synthesized at a low level (Fig. 5A, spots 8-14) but heat shock greatly increased the synthesis of all charge isomers of hsps with molecular weights of 68 and 70 kDa (Fig. 5B, spots 8 and 9).

Exposure of mussels to cadmium followed by a heat shock of 4 hr resulted in a significant inhibition of the incorporation of methionine into gill proteins (Fig. 6). This regime especially enhanced the synthesis of heat shock proteins of the high molecular weight class (Fig. 3, lanes 8 to 11). In order to correct the enhanced synthesis of hsps in the gills of Cd-exposed animals for the inhibition of total protein synthesis, IODs were normalized to equal amounts of acid precipitable radioactivity. As shown in Fig. 7, this normalization leads to equal amounts of actin at each Cd concentration tested. The IOD of hsps 30 and 32 increases to a maximum followed by a decrease which, however, stays above the level of the control. The expression of hsps 68 and 70 reaches a maximum and then declines almost to the level of this control. Only the hsps in the 72 to 110 kDa range show a continuous increase in a dose-dependent manner. These alterations in the expression of the high molecular weight hsps when mussels were exposed to cadmium followed by heat shock were confirmed by two-dimensional gel electrophoresis, as shown in Fig. 5B-C, spots 11 - 14. Image analysis of the fluorographs of these gels was performed to quantitate the increased synthesis of the various proteins. The results are given in Table 1.

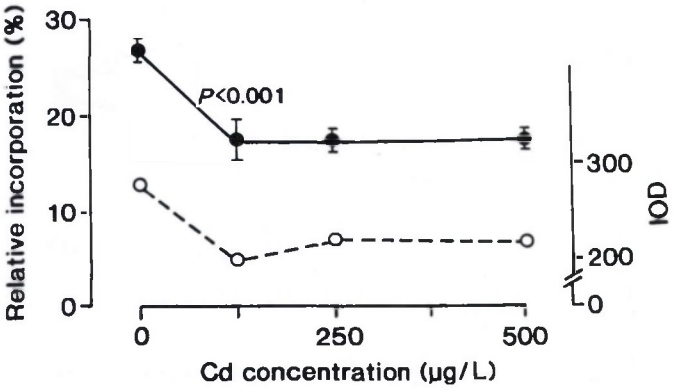


Fig. 6. Effect of consecutive exposure to cadmium and elevated temperature on the ^{35}S-methionine incorporation into gill proteins. Prior to heat shock at 29.5°C for 4 hr, mussels (August '89) had been exposed to cadmium for 96 hr. -●-, relative incorporation; mean of 6 mussels ± SEM; P-value is given with respect to the control. -o-, IOD of the actin band, estimated from the gel fluorograph shown in Fig. 3, lanes 8-11.

Table 1. Effect of Cd pre-exposure on the synthesis of heat shock proteins in gill tissue of mussels exposed to 29.5°C for 4 hr.

Protein spot No.	Molecular weight (kDa)	IOD[a]		Ratio[b]
		Control	Cd-exposed	
1	30	84.7	109.3	1.3
2	30	145.2	137.2	0.95
3	30	131.0	118.5	0.91
4	28	2.0	28.5	14.3
5	32	8.5	31.7	3.7
6	32	37.7	82.7	2.2
7	32	24.5	33.1	1.4
8	68	1068.0	1231.0	1.2
9	70	130.0	139.7	1.1
10	72-74	30.0	44.0	1.5
11	76	2.8	14.2	5.1
12	80	15.3	98.9	6.5
13	84	8.1	42.5	5.3
14	95	6.4	72.3	11.3

[a] IOD of the protein spots depicted in Fig. 5B and C. Values were normalized to an equal IOD of the actin spot.
[b] IOD (Cd-exposed group): IOD (control group).

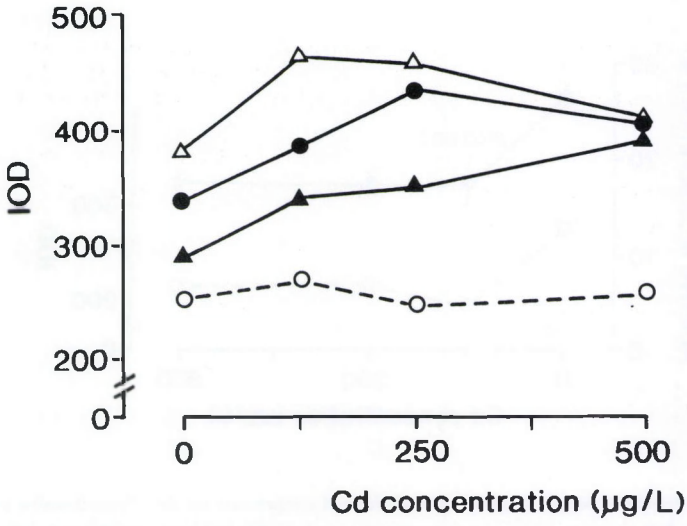


Fig. 7. Effect of consecutive exposure to cadmium and elevated temperature on the synthesis of heat shock proteins (hsps). Mussels were treated as described in Fig. 6. IOD of hsps and actin was estimated from the gel fluorograph (Fig. 3, lanes 8-11) and normalized to equal amounts of acid precipitable radioactivity. -o-, actin; -▲-, hsps 72-110; -●-, hsps 30 and 32; -Δ-, hsps 68 and 70.

^{35}S-Cysteine incorporation

Figs 8 and 9 show the effect of 96 hr of Cd exposure on the incorporation of cysteine into gill proteins and the induction of specific cysteine-rich proteins (thioneins). Protein synthesis was inhibited by cadmium in a dose-dependent manner, as measured via both total cysteine incorporation and IOD of total polypeptide bands (Fig. 8). Decrease of label incorporation was evident at 25 µg Cd/L, became significant at 50 µg Cd/L and continued to decline at higher Cd concentrations.

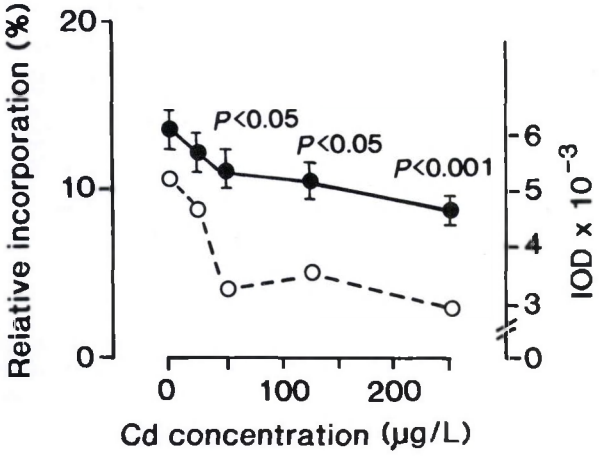


Fig. 8. Dose effect of cadmium on the ^{35}S-cysteine incorporation into gill proteins. -●-, relative incorporation; mean of 6 mussels ± SEM; P-values are given with respect to the control. -o-, IOD of total polypeptide bands (without the induced thioneins), estimated from the gel fluorograph (not shown). Mussels collected in November '88.

Relative thionein synthesis was estimated from the gel fluorograph (not shown) of the gill proteins. It increased strongly at the lower Cd concentrations, up to 125 µg Cd/L, and levelled off thereafter (Fig. 9).

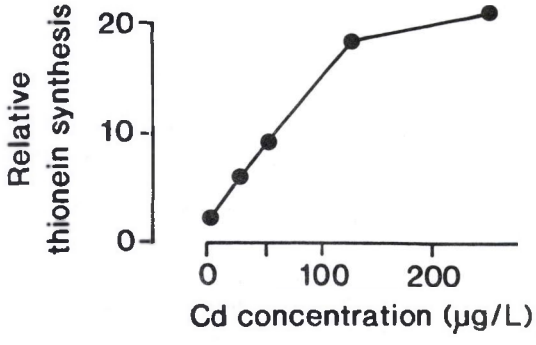


Fig. 9. Dose effect of cadmium on the relative of thionein synthesis. Mussels (November '88) were exposed to a range of Cd concentrations for 96 hr. Thionein synthesis was expressed as 100 x IOD of thioneins divided by the IOD of total polypeptides minus the IOD of thioneins. IODs were estimated from the gel fluorograph of ^{35}S-cysteine labelled gill proteins.

The time-dependence of the effect of cadmium on the total protein synthesis in gill is shown in Fig. 10. Incorporation of cysteine was significantly inhibited after 4 days of exposure at 50 µg Cd/L, but did not decrease further after longer exposure time. SDS-PAGE of the carboxymethylated, ^{35}S-cysteine labelled gill proteins revealed a time-dependent induction of a LMW (13-14 kDa), cysteine-rich protein (Fig. 11). The relative thionein synthesis, estimated from the gel fluorograph, increased with increasing duration of Cd exposure (Fig. 12).

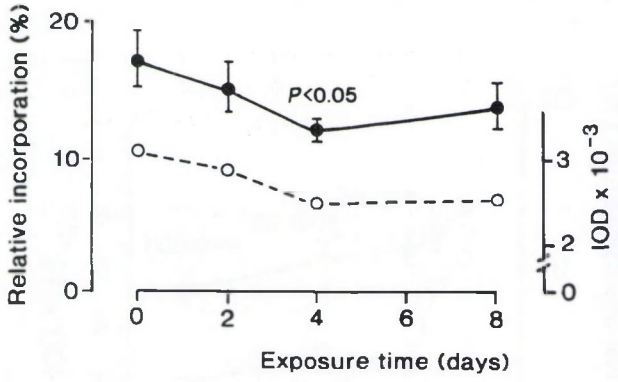


Fig. 10. Effect of cadmium on the ^{35}S-cysteine incorporation into gill proteins from mussels exposed to 50 µg Cd/L for different periods of time. -●-, relative incorporation; mean of 6 mussels ± SEM; P-value is given with respect to the control. -o-, IOD of total polypeptide bands (without the induced thionein) estimated from the gel fluorograph shown in Fig. 11. Animals were collected in August '89.

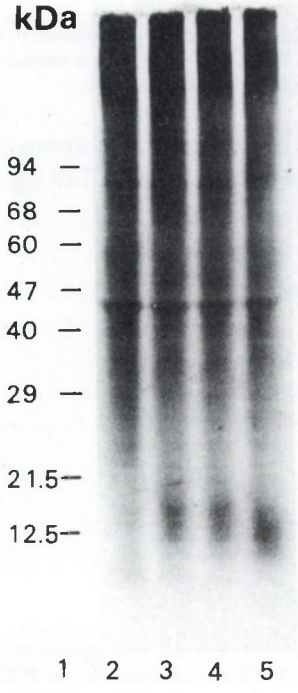


Fig. 11. SDS-PAGE patterns of ^{35}S-cysteine labelled gill proteins from mussels exposed to 50 µg Cd/L. Lane 1, molecular weight markers; lane 2, control mussels; lanes 3-5, mussels exposed to cadmium for 2, 4, and 8 days, respectively. Fluorography was for 10 days.

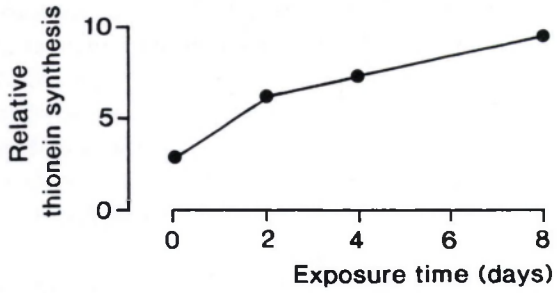


Fig. 12. Time course of relative thionein synthesis in gill tissue of mussels exposed to 50 µg Cd/L. Relative thionein synthesis was estimated from the gel fluorograph shown in Fig. 11.

Discussion

In this study a toxic effect of cadmium in gill tissue of *M. edulis* has been established. After 96 hr of in vivo exposure to cadmium the synthesis of gill proteins, measured by methionine and cysteine incorporation, was inhibited in a dose-dependent manner. A dose-dependent inhibition of protein synthesis was also found in mammalian cells after several hours of Cd exposure (Din and Frazier, 1985; Beattie *et al.*, 1987). In fish, injection of cadmium decreased the ^{35}S-cysteine incorporation into HMW proteins (Baksi *et al.*, 1988). In mussels and limpets, exposure to cadmium for 72 hr caused a repression of protein synthesis (Sanders, 1988).

Labelling of the proteins with methionine or cysteine appears to be a sensitive method to reveal the effect of cadmium on protein synthesis. The two amino acids did not show equal sensitivity with regard to the threshold concentration of cadmium. Inhibition of cysteine incorporation was observed at Cd concentrations (25-50 µg/L) not giving an effect on the methionine incorporation (results not shown). This difference in sensitivity might be related to the different metabolism of these amino acids. Induction of MTs and decreased synthesis of glutathione (Ochi *et al.*, 1987) indicate that cadmium affects the metabolic pathway(s) of cysteine. In the foregoing study (Veldhuizen-Tsoerkan *et al.*, 1990), an equal decrease of label incorporation was found at a 5 times higher Cd concentration for methionine than for cysteine. The same study indicated that prolonging the exposure to 15 days did not further decrease the incorporation of amino acids into gill proteins. In the present experiments, the incorporation of cysteine declined with time of exposure up to 4 days, but no longer thereafter. This suggests that inhibition of amino acid incorporation into gill proteins is an acute, dose-dependent response to cadmium insult.

Repression of protein synthesis is considered to be a general cellular response to stress. In many cell types, elevation of temperature elicits a decrease in the overall protein syn-

thesis and induction of specific stress proteins (Craig, 1985; Lindquist, 1986). In our experiments, a 4-hours heat shock caused only a slight, insignificant decrease of methionine incorporation. This could be due to a partial recovery of the stressed cells during the rather long (22 hr) procedure of labelling. SDS-PAGE analysis indicated that some protein bands were more strongly labelled in the control sample, whereas the synthesis of several other proteins (with molecular weight of 30, 32, 68, 70, 80, 82, 90 and 110 kDa) was enhanced by heat exposure. In *M. edulis*, heat shock has already been reported to decrease the overall protein synthesis and to induce the synthesis of hsps with molecular weight of 28, 50 and 70 kDa (Steinert and Pickwell, 1988) or molecular weight of 29, 32, 42, 43, 47, 61, 68, 70 and 90 kDa (Sanders, 1988). Small discrepancies in molecular weights can be attributed to differences in the employed gel systems, while the differences in the group with molecular weight around 50 kDa could be due to genetic or seasonal variations.

Exposure to cadmium induced the expression of several HMW proteins that comigrated with hsps synthesized at the elevated temperature. The increased synthesis of these proteins was most evident at the highest Cd concentration (500 µg/L) after 48 or 96 hr of exposure. Short-term exposure to cadmium has also been reported to induce hsp synthesis in avian and mammalian cells (Levinson *et al.*, 1980; Li *et al.*, 1982; Caltabiano *et al.*, 1986). Treatment of fish cells with 550 µg Cd/L caused an increased expression of hsps (Heikkila *et al.*, 1982). Sanders (1988) observed an hsp response in mussels upon 72 hr of exposure to 0.1-10 µg Cd/L, while Steinert and Pickwell (1988) could detect hsp induction only at higher Cd concentration (600 µg/L) after 48 hr of exposure.

Pre-exposure to cadmium, followed by heat shock augmented the inhibition of methionine incorporation. This synergistic effect was also evident from the gel analysis. The hsp synthesis was differentially increased in Cd-pretreated animals. The effect of the consecutive exposure to metal and heat was most evident for the hsps of high molecular weight.

The same regime with mammalian cells also resulted in an enhanced synthesis of hsps with molecular weight of 70, 87 and 97 kDa and in increased thermotolerance (Li *et al.*, 1982). Li and Laszlo (1985) pointed out that cadmium could induce thermotolerance, but heat did not induce protection against cadmium. Therefore, the enhanced expression of hsps, especially with higher molecular weight, indicates the severity of cadmium stress.

Application of hsp analysis in environmental pollution research has already been considered (Sanders, 1988), since the current parameters, such as scope for growth, metal accumulation, metabolite contents, condition indices, are not always successful to pinpoint the deleterious effects of heavy metals in such low-metabolic animals like mussels. However, the cadmium hsp response is believed to be a transient phenomenon. For example, Heikkila *et al.* (1982) observed that the hsp level in fish declined towards the control value after prolonged exposure to cadmium ions. Considering this fact, a cadmium hsp response may not be easy to detect (without immunochemical assay) in chronically exposed animals. Then, the consecutive short-term exposure to elevated temperature can be a useful tool - by disclosing the enlarged hsp expression - in monitoring stress during environmental exposure to metal and perhaps to other pollutants.

Short-term exposure to cadmium also induced the synthesis of a cysteine-rich protein of low molecular weight that was already detectable after 2 days of exposure at 50 µg Cd/L. It was previously shown to consist of two charge isomers of low isoelectric points (VeldhuizenTsoerkan *et al.*, 1990). Its cadmium-binding capability was assessed by gel chromatography (results to be published). These observations are in accordance with those of Nolan and Duke (1983) who showed induction of metallothionein-like proteins in the gills of *M. edulis* within 48 hr of exposure at 100 µg Cd/L, and with those of Roesijadi and Klerks (1989) who observed 'MT' induction in the oyster gill between 1 and 4 days of exposure at 50 µg Cd/L.

Induction of the MT-like protein could also be detected by methionine labelling (Fig. 3, lanes 3-6). The bands around molecular weight of 20 to 25 kDa were not present in the control or heat shocked groups (lanes 2 and 7). Methionine has been shown to serve as an SH-source for MT synthesis in rat hepatocytes (Stein *et al.*, 1987). Heat shock didn't induce the MT synthesis in fish and mussels (Heikkila *et al.*, 1982; Steinert and Pickwell, 1988; Misra *et al.*, 1989). The relatively high apparent molecular weight (compare Figs 3 and 11) is due to the fact that the methionine labelled proteins were not carboxymethylated prior to SDS-PAGE.

The relative rate of synthesis of the cysteine-rich protein increased with concentration and time of Cd exposure. There was, however, no linear relation between the rate of the thionein synthesis and the Cd content in the tissue. This would mean that the rate of thionein synthesis is regulated by the strength of the metal insult rather than by the total concentration of cadmium in the tissue.

The presented data show that the effects of cadmium ions form a complex phenomenon which includes modulation of gene expression of various proteins. The similarity in some aspects between the cadmium and the heat shock response and their synergistic action could indicate that both inducers act via a mechanism of general stress response.

Acknowledgements

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Cadmium-induced changes in macromolecular synthesis at transcriptional and translational level in gill tissue of sea mussels, *Mytilus Edulis* L.

M.B. Veldhuizen-Tsoerkan[*], *C.A. van der Mast*[**] *and D.A. Holwerda*[*]

[*]*Department of Experimental Zoology and* [**]*Department of Molecular Cell Biology, University of Utrecht, 8 Padualaan, NL-3584 CH Utrecht, The Netherlands.*

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Cadmium-induced changes in macromolecular synthesis at transcriptional and translational level in gill tissue of sea mussels, *Mytilus Edulis*l L.

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Abstract

Sea mussels, *Mytilus edulis*, were exposed to cadmium chloride at 0 - 500 µg Cd/l for 48 hours. The gills were excised and incubated with protein- and RNA precursors. The exposure resulted in a concentration-dependent inhibition of the synthesis of proteins and of RNA. The inhibitory effect was most pronounced in RNA synthesis. RNA was extracted from the gills as total RNA or as polyadenylated RNA. The translational activity of RNAs and the induction of mRNA for metallothionein-like proteins were studied by translation in a cell-free system. Exposure of the animals to cadmium at 500 µg/l caused a five-fold increase in proto-oncogene c-*fos* mRNA.

Introduction

Cadmium is a widespread pollutant with a broad range of toxic actions which include mutagenic (Ochi and Ohsawa, 1983), teratogenic (Swapan *et al.*, 1990) and carcinogenic (Terracio and Nachtigal, 1988) effects. At the molecular level cadmium elicits inhibition of DNA-, RNA- and protein synthesis (Ochi *et al.*, 1984; Nocentini, 1987; Kishimoto *et al.*, 1990). Metal ions can bind directly to macromolecules (Waalkes and Poirier, 1984) thus inducing conformational changes (Koizumi and Waalkes, 1990) and structural damage (Ochi and Ohsawa, 1983).

Interaction of cadmium with the metabolism of essential divalent cations such as zinc and calcium, and their binding proteins, is considered to be one of the major mechanisms of cadmium toxicity. Both zinc and calcium are essential elements at the level of the synthesis of various macromolecules. Zinc plays a role in the functioning of several enzymatical metalloproteins, which include RNA- and DNA polymerases (Vallee and Falchuk, 1981) and in transcriptional regulators, DNA-binding Zn-finger containing proteins (Klug and Rhodes, 1988; Evans and Hollenberg, 1988). Calcium is required for the regulation of protein synthesis (Brostrom and Brostrom, 1990). Complexed with calmodulin, its binding protein, calcium governs a large number of cellular functions (Cheung, 1980).

Cadmium activates transcription of the genes coding for heat shock proteins (Lindquist, 1986) and metallothioneins (MTs) (Hamer, 1986). Both protein groups are related to the cellular defence mechanism under stress conditions. In a recent study, Jin and Ringertz (1990) have shown that cadmium also activates transcription of several

proto-oncogenes whose products, DNA-binding proteins, are involved in transduction of mitogenic signals elicited by growth factors to the nucleus (Rozengurt, 1986).

The present work was designed to correlate the effects of cadmium on both the synthesis of proteins and of RNA after a short-term exposure of the sea mussels to the metal. After extraction of RNA, the translational activity of cellular mRNAs and the induction of mRNA for MT-like proteins were studied by translation in a cell-free system. The activation of the proto-oncogene c-*fos* expression was also examined in *M. edulis*.

Materials and methods

Animals and animal treatments

Sea mussels, *Mytilus edulis*, were collected from the Eastern Scheldt in October 1989 and March 1990. Mean shell length was 5.5 ± 0.4 cm. The animals were kept in aquaria with recirculating sea water at 12^0C without feeding under a natural light regime.

Mussels were exposed to cadmium chloride (Merck) in a semistatic system as described in Veldhuizen-Tsoerkan *et al.* (1990b). The concentrations of cadmium mentioned refer to metal part of the compound. Groups of 10 mussels (collection 1989) and 6 mussels (collection 1990) were exposed to 500 µg Cd/l and to a range of cadmium concentrations (50 - 500 µg/l), respectively, for 48 hr. Prior to experimental procedures, mussels were kept in unspiked sea water to eliminate adherent cadmium.

Incorporation of ribonucleosides and amino acids

Gills were excised from Cd-exposed and control animals. The middle parts of the outer gill lamellae were individually incubated with 50 µCi/ml ^{35}S-cysteine or 60 µCi/ml ^{3}H-uridine (specific activity 1000 Ci/mmol and 39 Ci/mmol, respectively, Amersham, Bristol, U.K.) for 24 hr at 11.5^0C. The incubation medium and processing of ^{35}S-cysteine labelled gill tissue as well as the determination of incorporated label have been described earlier (Veldhuizen-Tsoerkan *et al.*, 1990a,b).

After washing and dry-blotting, the ^{3}H-uridine labelled gills were lysed in a solution containing 5 M guanidinium isothiocyanate, 5 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol and 0.5% sodium dodecyl sulphate (SDS). Per 1 g wet weight 10 ml of this solution was used. Gill lysates were extracted twice with equal volumes of phenol/chloroform/isoamylalcohol (24:24:1) at 65^0C, followed by two extractions with chloroform/isoamylalcohol (24:1). To the water phase sodium acetate (pH 5.2) was added to a concentration of 0.1 M and the nucleic acids were then precipitated twice with 2.5 volumes of 96% ethanol at -20^0C for 24 hr. The pellets were dried and dissolved in sterile bidistilled water. The incorporation of uridine into nucleic acids was determined in 10-

µl aliquotes in a Beckman LS 6000 liquid scintillation counter and expressed as counts per minute (cpm)/µg nucleic acids. The nucleic acid concentration was assessed as 25 A_{260} absorbance units being 1 mg/ml.

Statistical analysis of the data was performed with Student's t-test.

Extraction of total and polyadenylated RNA

Total RNA was extracted by the guanidinium/cesium chloride method (Maniatis *et al.*, 1982). Gills of 6 control or Cd-exposed mussels were pooled and, after washing, homogenized in 7 volumes (w/v) of the guanidinium isothiocyanate mixture mentioned earlier. The homogenates were layered onto 15 ml cushions of 5.7 M CsCl-0.1 M EDTA (pH 7.0) and centrifuged at 140,000 x g in a Beckman Ti 60 rotor at 20^0C for 25 hr. The RNA pellets were extracted with phenol/chloroform/isoamylalcohol and precipitated as described above. The resulting RNA pellets were taken up in sterile water, to which the RNase inhibitor methylmercuric hydroxide (Ventron) was added to a final concentration of 1 mM.

To select polyadenylated RNA, total RNA from 10 gills of Cd-exposed or control mussels was subjected to affinity chromatography on oligo-(dT)-cellulose (Sigma, St.Louis MO, U.S.A.) (Maniatis *et al.*, 1982). Unbound poly A^- RNA was removed with 20 mM TRIS-HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA and 0.1% SDS at 37^0C. Poly A^+ RNA was then eluted with sterile bidistilled water at 37^0C. Both RNA fractions were precipitated as described above and stored in 1 mM methylmercuric hydroxide at -80^0C.

RNA translation *in vitro*

RNA translational activity was assayed in a mRNA-dependent cell-free system (Thomas *et al.*, 1979). Incubation mixtures of 25µl contained 20 mM Hepes/KOH, pH 7.6, 1 mM ATP-$TRIS_3$, 0.4 mM GTP-$TRIS_3$, 5 mM creatine phosphate-$TRIS_3$, 0.6 unit of creatine kinase (EC 2.7.3.2), 1 mM dithiothreitol, 2 mM magnesium acetate, 120 mM potassium acetate, 100 µM spermine-HCl_4, 50 µM each of 19 unlabelled amino acids, 5 µCi ^{35}S-cysteine, 1.5 µg tRNA (rat liver), 5 pmol each of 40S and 60S subunits (rat liver), 6 µg "pH 5" enzymes, 6 µg of crude initiation factor fraction A (0 to 40% ammonium sulphate fraction of a ribosomal wash of rabbit reticulocytes) and 24 µg of fraction BC (40 to 70% ammonium sulphate fraction, *ibid.*). Optimal amounts, determined by RNA titration, were 5 µg of total RNA, 5 µg of poly A^- RNA and 0.25 µg of poly A^+ RNA for translation in the above-mentioned systems. The mixtures were incubated for 90 min at 30^0C. Incorporation of ^{35}S-cysteine was measured as hot trichloroacetic acid (TCA) insoluble radioactivity as described by Van der Mast *et al.* (1977). The translational products were analyzed by SDS-PAGE.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Cytosolic ^{35}S-cysteine labelled proteins (30 µg) and translational products (20 µl of the incubation mixtures) were carboxymethylated as described previously (Veldhuizen-Tsoerkan *et al.*, 1990a,b) and applied to 12.5% and 15% polyacrylamide gels (ratio of acrylamide to bisacrylamide = 30:0.8). Molecular weight markers used were: phosphorylase a (92 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), 3'-phosphoglycerate kinase (47 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21.5 kDa) and cytochrome c (12.5 kDa). Following Coomassie Brilliant Blue R staining and destaining, the gels were dried and fluorographed (Veldhuizen-Tsoerkan *et al.*, 1990b). The incorporation of ^{35}S-cysteine into gill proteins was quantified from the gel fluorographs by determining the integrated optical density (IOD) of polypeptide bands with the IBAS image analysis system (Zeiss/Kontron, Eching, F.R.G.) as described earlier (Veldhuizen-Tsoerkan *et al.*, 1990a,b). The relative amount of MT-like proteins (relative thionein synthesis) was estimated as (IOD of metallothioneins/ IOD of total polypeptides) x 100%.

The integrity of the purified RNA was checked by agarose gel electrophoresis in the presence of methylmercuric hydroxide (Maniatis *et al.*, 1982). RNA samples (15 µg) were applied to a 1.5% agarose gel and run at 4.5 V/cm for 1.5 hr. Markers used were: rat liver tRNA (4S), rabbit reticulocyte globin mRNA (9S) and rat rRNA (18S and 28S).

Northern blot analysis

Northern blot analysis was performed as described by Tuijl *et al.* (1991). RNA samples (15 µg) were denatured by glyoxal and run in 1.1% agarose gels (4.5 V/cm). The resolved RNA was blotted to nylon (Hybond N, Amersham) in 10 x SSPE (1.8 M NaCl, 0.1 M sodium phosphate, 10 mM EDTA, pH 7.0). Filters were hybridized overnight at 50^{0}C in 50% formamide, 5 x SSPE, 5 x Denhardt's solution (50 x Denhardt's solution contains 1% BSA, 1% Ficoll, 1% polyvinylpyrrolidone with molecular weight of 25 - 30 kDa), 0.2 mg of sonicated herring sperm DNA per ml and 0.5% SDS, (pH 7.0). The DNA probe coding for full-length c-*fos* mRNA (human cDNA, 1.6 kb EcoRI fragment) was randomly primed with [α-^{32}P]dCTP (Amersham), yielding a specific activity of 5 x 10^8 - 2 x 10^9 cpm/µg DNA. Subsequently, the blot was washed in 0.2 x SSPE-0.1% SDS at 50^{0}C and exposed to X-ray film (Hyperfilm MP, Amersham) between two intensifying screens (Kodak X-Omatic, Kodak) for 3 days. IOD's of the hybridized probes were determined from the gel autoradiogaphs by IBAS image analysis.

Results and discussion

RNA- and protein synthesis

The effect of cadmium on RNA- and protein synthesis in gill tissue was investigated after *in vivo* exposure for 48 hr. Fig. 1 shows that cadmium inhibited the incorporation of ^{3}H-uridine into gill ribonucleic acids. RNA synthesis decreased by 33% at the lowest Cd concentration (50 µg/l) and almost by 50% at 500 µg Cd/l compared to the control values.

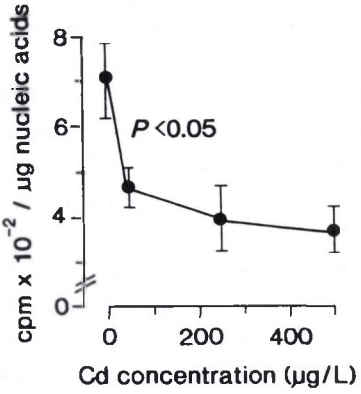


Fig. 1. Dose effect of cadmium on the incorporation of ^{3}H-uridine into the nucleic acids of excised gills. Mean of 5 mussels ± SEM; P-value is given with respect to the control.

The inhibition of the total incorporation of ^{35}S-cysteine into gill proteins was less pronounced at the lowest Cd concentration but became significant at the higher concentrations (Fig. 2). However, the inhibitory effect of cadmium on protein synthesis was already substantial at the lowest Cd concentration when only the integrated optical density (IOD) of polypeptides other than the Cd-induced, MT-like proteins was taken into account (Fig. 2).

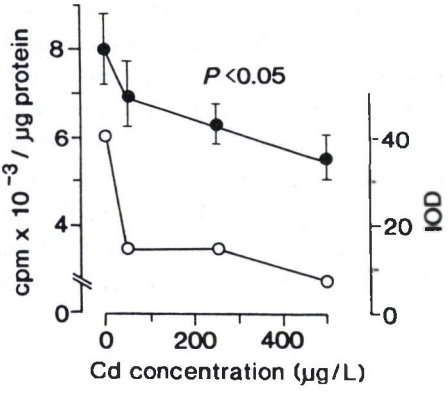


Fig. 2. Dose effect of cadmium on the incorporation of ^{35}S- cysteine into gill proteins. -●-, Incorporated cysteine measured as trichloroacetic acid insoluble radioactivity (counts per minute/µg protein); Mean of 6 mussels ± SEM; P-value is given with respect to the control. -o-, Integrated optical density (IOD) of total polypeptides minus IOD of the Cd-induced LMW thioneins, estimated from the fluorograph shown in Fig. 5.

These data are in agreement with our previous observations of a significant inhibition of protein synthesis in gill tissue of *M. edulis* after exposure to 50 µg Cd/l for 96 hr but not for 48 hr (Veldhuizen-Tsoerkan *et al.*, 1990a,b). Evidence of an inhibitory effect of cadmium on the synthesis of various types of macromolecules has also been obtained in experiments with cultured cells. In Chinese hamster cells (Ochi *et al.*, 1984), simian cells (Nocentini, 1987) and HeLa S_3 cells (Kishimoto *et al.*, 1990) Cd concentrations of 10^{-2} - 10^{-6} M repressed the incorporation of DNA-, RNA- and protein precursors after treatments for 2 - 3 hr. DNA synthesis was already influenced at the lowest Cd concentrations while the formation of RNA and proteins was less sensitive (Ochi *et al.*, 1984; Nocentini, 1987).

The cytotoxicity of cadmium may result from interaction with a variety of cellular pathways where essential cations as zinc and calcium play a role in sustaining normal cellular functions. Cadmium is capable to replace and/or compete with these essential ions for binding sites, which could lead to structural and, hence, functional changes of macromolecules. Cadmium was shown to bind *in vitro* to bases of DNA, causing significant conformational changes (Waalkes and Poirier, 1984; Koizumi and Waalkes, 1990) and to induce single strand breaks (Ochi and Ohsawa, 1983). The protective effect of zinc on the stability of DNA structure (Waalkes and Poirier, 1984; Koizumi and Waalkes, 1990) and on DNA synthesis (Nocentini, 1987) suggests that the primary targets for cadmium at the level of nucleic acids are molecules that require zinc for their function. Zinc is an important component of DNA and RNA polymerases (Vallee and Falchuk, 1981). Addition of cadmium to *in vitro* DNA synthesizing systems caused infidelity of this synthesis (Sirover and Loeb, 1976). RNA polymerase was inhibited by cadmium *in vivo* in rat liver (Hidalgo *et al.*, 1976). Also heavy metals decreased the activities of RNA- and DNA polymerases in the digestive gland of *Mytilus galloprovincialis* (Viarengo *et al.*, 1982; Accomando *et al.*, 1990).

Zinc also serves as a structural component of the Zn-finger domains in DNA-binding proteins which act as transcriptional regulators with a wide variety of functions (Klug and Rhodes, 1988; Evans and Hollenberg, 1988). Malfunction of these proteins might occur when cadmium is bound instead of zinc (Sunderman and Barber, 1988). In experiments with 7S particles of *Xenopus laevis* oocytes, cadmium induced the dissociation of this complex into a 5S RNA and a Zn-finger containing 40 kDa protein (transcription factor IIIA), apparently by displacing zinc (Miller *et al.*, 1985).

It is conceivable that inhibition of protein synthesis by cadmium is, at least partly, related to a repressed synthesis of the nucleic acids. Protein synthesis is a complex process that involves a major expenditure of cell energy, affects all the cellular activities and requires calcium for its regulation (Brostrom and Brostrom, 1990). Interference of cadmium with calcium and Ca-binding proteins, particularly calmodulin, is considered to be one of the major mechanisms of cadmium toxicity (Verbost *et al.*, 1987; Sutoo *et al.*, 1990).

RNA isolation and translation

Total cellular RNA was isolated from gills of control and Cd-exposed mussels. Fig. 3 shows the preparations of RNA after analysis by agarose gel electrophoresis. An influence of cadmium on the separated RNA species could not be expected due to the low resolution of agarose gel electrophoresis, but as can be seen in Fig. 3 (lanes 4 and 5) one RNA band around the 28S value was present at higher Cd concentrations. It is noteworthy that these results and the data from other RNA isolations (not shown) indicate that rRNA of the large ribosomal subunit of *M. edulis* appears to have a lower molecular weight than that of mammalian (rat) 28S rRNA.

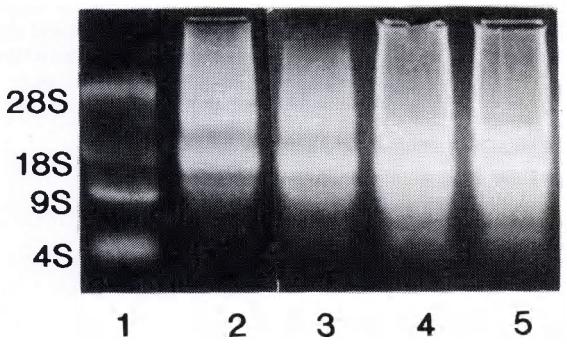


Fig. 3. Analysis of total RNA by agarose gel electrophoresis. Lane 1: markers; lane 2: control group; lanes 3-5: groups exposed to 50, 250 and 500 µg Cd/l, respectively.

In order to investigate the induction of MT-like proteins, total cellular RNA was translated in a cell-free system and the translational products were analyzed by SDS-PAGE. The induction of MT mRNAs as visualized by their proteineous products became apparent only at the highest Cd concentration (Fig. 4, lane 5) due to the obscuring presence of cysteine-containing, LMW proteins which were also translated from RNA isolated from the control animals (lane 2). By contrast, the induction of MT-like proteins in ^{35}S-cysteine labelled gills displayed a relatively stronger dependence on Cd concentration (Fig. 5). It should be kept in mind that in the translation experiments the total cellular complement of proteins is produced, while in the experiments of gill labelling the selection procedure of cytosolic proteins favoured the MT-like proteins relative to the others (Figs 4 *vs.* 5). Moreover, the inhibition of total protein synthesis (Fig. 2) enhanced this effect (Fig. 5), while the translation of RNA *in vitro* was not decreased as a consequence of cadmium exposure (Fig. 4). This could explain the discrepancy observed between the *in vitro* (Fig.4) and "*in vivo*" (Fig. 5) relative synthesis of MT-like proteins.

In the next experiment, total RNA was resolved into poly A$^+$ and poly A$^-$ RNA fractions by affinity chromatography. The sub-fractions were used for *in vitro* translations. Fig. 6 shows that the poly A$^+$ RNA fraction contained the major translational activity for MT-like proteins.

Although the *in vitro* synthesis of MT-like proteins was enhanced by cadmium (Fig. 6,

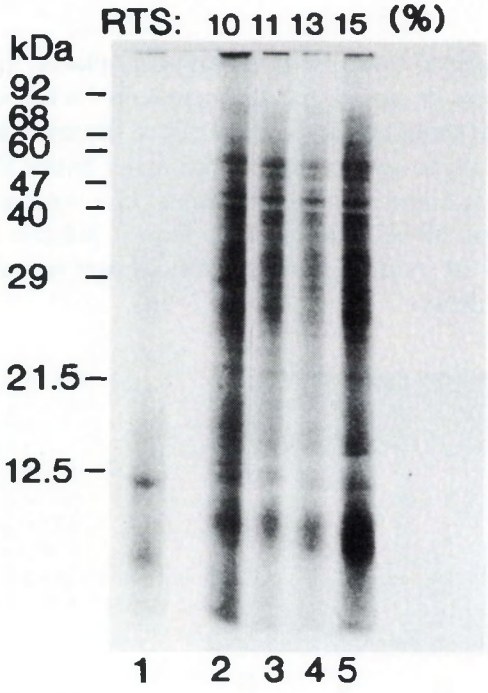


Fig. 4. SDS-PAGE patterns of ^{35}S-cysteine labelled gill proteins synthesized *in vitro*. Total cellular RNA was translated in cell-free systems. Aliquots of these cell-free systems (20 µl) containing 4.4 x 10^5, 1.5 x 10^6, 0.9 x 10^6, 0.8 x 10^6 and 1.5 x 10^6 cpm were applied to lanes 1-5, respectively. Lane 1: control cell-free system without added RNA; lanes 2-5: translational products of RNA from groups exposed to 0, 50, 250 and 500 µg Cd/l, respectively. The position of the molecular weight markers is indicated on the left side. Relative thionein synthesis (RTS) is shown at the top of the lanes 2-5, and is expressed as 100% x (IOD of LMW thioneins/IOD of total polypeptides). Fluorography time was 24 hr.

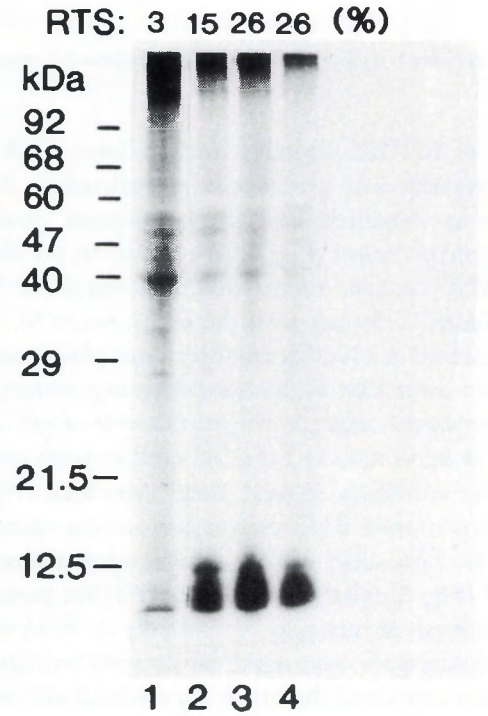


Fig. 5. SDS-PAGE patterns of ^{35}S-cysteine labelled gill proteins synthesized *"in vivo"*. Lane 1: control group; lanes 2-4: groups exposed to 50, 250 and 500 µg Cd/l, respectively. Relative thionein synthesis (RTS) is shown at the top of the lanes, and is expressed as 100% x (IOD of LMW thioneins/IOD of total polypeptides). Fluorography time was 48 hr.

lane 5) LMW cysteine-containing proteins were also observed in the control (lane 4).

The present data do not permit to conclude whether these polypeptides belong to the MT-like protein family. It is possible that control animals contain MT mRNA in an inactive state as an untranslatable mRNP which becomes active after removal of the adherent proteins by phenol extraction. Although unwarranted, it is tempting to speculate that the presence of inactive MT mRNPs in control mussels might be a mechanism for quick-response in physiological adaptation to varying environmental conditions.

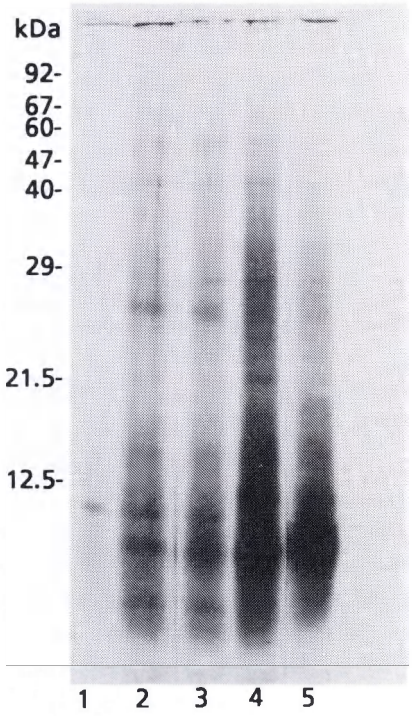


Fig. 6. SDS-PAGE analysis of translational products. Total cellular RNA was extracted from gills of control mussels (lanes 2 and 4) and from mussels exposed to 500 µg Cd/l (lanes 3 and 5). The RNA was resolved into poly A^- (lanes 2 and 3) and poly A^+(lanes 4 and 5) RNA fractions which were translated in cell-free systems with ^{35}S-cysteine. Aliquots of cell-free systems (20 µl) containing 3.1 x 10^5, 1.8 x 10^6, 1.8 x 10^6, 2.3 x 10^6 and 1.6 x 10^6 cpm were applied to lanes 1-5, respectively. Lane 1: control cell-free system without added RNA. Fluorography time was 24 hr.

Induction of MT-like proteins by cadmium in *M. edulis* has been demonstrated by George *et al.* (1979) and Frazier (1986) and in our previous investigations (Veldhuizen-Tsoerkan *et al.*, 1990a,b, 1991a). The metallothioneins are considered to constitute the major cellular defence system against heavy metals. However, the inertness of the Cd-MT complex has recently been doubted by Waalkes and Goering (1990). Müller *et al.* (1991) have indeed demonstrated that Cd/Zn-MT can induce DNA breaks *in vitro*. Kägi and Schäffer (1988) emphasized that the similarities in metal-binding sequence motifs between regulatory Zn-finger containing proteins and metallothioneins could imply an interaction of the latter with nucleic acids. In this context, an adverse effect of the Cd-MT complex on macromolecular synthesis cannot be excluded.

Expression of c-fos

In order to investigate the expression of proto-oncogene c-*fos* in gill tissue after 48 hr of *in vivo* exposure to 500 g Cd/l, the accumulation of c-*fos* mRNA was examined by Northern blot analysis. Human c-*fos* cDNA was used as a hybridization probe. A basal c-*fos* mRNA synthesis was detected in the gills of control animals (Fig. 7). Exposure to cadmium caused a 5-fold increase of c-*fos* mRNA.

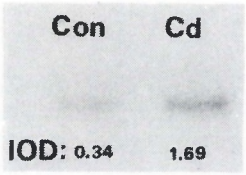


Fig. 7. Effect of cadmium on the induction of c-*fos* mRNA. Total cellular RNA was extracted from gills of control animals (lane Con) and of mussels exposed to 500 µg Cd/l (lane Cd). RNA was subjected to Northern blot analysis. Integrated optical density (IOD) of the autoradiograph of ^{32}P-labelled RNA-cDNA hybrids is given at the bottom.

In rat L6 myoblasts, cadmium has already been shown to induce proto-oncogene (c-*jun* and c-*myc*) mRNAs with maximum expression after 2-4 hr of exposure to 5 M $CdCl_2$ (Jin and Ringertz, 1990). However, in this case, the Cd-induced accumulation of c-*fos* mRNA was only observed in the presence of cycloheximide.

Altogether, the above-mentioned findings indicate that cadmium has the ability to alter the expression of proto-oncogenes. The aberrant expression of a variety of cellular proto-oncogenes can lead to a malignant transformation of the cell, as the products of these genes (c-*fos*, c-*myc* and c-*jun*) are thought to play an important role in cell growth and differentiation (Sambucetti and Curran, 1986). The c-*fos* genes code for nuclear phosphoproteins that participate in nucleoprotein complexes that regulate the expression of other genes (Distel *et al.*, 1987; Curran and Franza, 1988). The rapid and transient induction of c-*fos* and c-*myc* by growth factors suggests that they are involved in signal transmission of external stimuli to the transcriptional machinery of the nucleus (Rozengurt, 1986; Chiu *et al.*, 1988). Binding of the growth factors to their receptors rapidly induces a number of processes in the cell, *e.g.*, mobilization of Ca^{2+} from intracellular stores and an increased phosphorylation of several proteins (Rozengurt, 1986). These cellular processes can also be triggered by cadmium (Verbost *et al.*, 1987; Dwyer *et al.*, 1990; Behra and Gall, 1991; Veldhuizen-Tsoerkan *et al.*, 1991b), which may indicate a conceivable mechanism of proto-oncogene induction by cadmium. Another possibility of the modification of proto-oncogene expression may be the interference of cadmium with Zn-finger containing proteins (as mentioned above) that are thought to be involved in regulation of the proto-oncogene expression (Sukhatme *et al.*, 1988).

In addition, a direct relation has been found between the induction of c-*fos* mRNA and the inhibition of protein synthesis by heat shock (Tuijl *et al.*, 1991). Therefore, the enhanced expression of c-*fos* at a cadmium concentration inhibiting protein synthesis

supports our previous suggestion (Veldhuizen-Tsoerkan *et al.*, 1990b) that cadmium may act via a mechanism of general stress response.

Acknowledgements

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C H A P T E R 6

Short-term exposure to cadmium modifies phosphorylation of gill proteins in the sea mussel

M.B. Veldhuizen-Tsoerkan[*], *C.A. van der Mast*[**] *and D.A. Holwerda*[*]

[*]*Department of Experimental Zoology and* [**]*Department of Molecular Cell Biology, University of Utrecht, 8 Padualaan, NL-3584 CH Utrecht, The Netherlands*

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Short-term exposure to cadmium modifies phosphorylation of gill proteins in the sea mussel

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Abstract

Sea mussels were exposed to cadmium for short periods of time. The excised gills were incubated with radioactive orthophosphate. The gill proteins were separated by one- and two-dimensional gel electrophoresis and the phosphorylation state of the proteins was determined by image analysis of autoradiographs.

One-dimensional gel electrophoresis revealed that exposure of the animals to cadmium stimulated phosphorylation of the gill proteins in a cadmium concentration-dependent manner.

Two-dimensional gel electrophoresis showed that cadmium differentially affected the phosphorylation of various proteins. Major alterations were observed in the basic, high molecular weight proteins and in the acidic, low molecular weight polypeptides.

Introduction

Protein phosphorylation/dephosphorylation is involved in the regulation of a large number of cellular processes. This reversible covalent modification of proteins is catalyzed by diverse protein kinases and phosphoprotein phosphatases, the activities of which are controlled by second messenger systems such as cAMP and Ca^{2+}/calmodulin (see for reviews Rasmussen and Goodman, 1977; Krebs and Beavo, 1979; Cheung 1980; Klee and Vanaman 1982).

Alterations in protein phosphorylation have been observed in response to heat stress in eukaryotic and prokaryotic cells (Landry *et al.*, 1988; Balodimos *et al.*, 1990). The heat shock response is characterized by repression of the synthesis of various proteins and an induction of heat shock proteins (Lindquist, 1986), and is correlated with changes in the phosphorylation state of several structural and regulatory proteins (Glover, 1982; Kennedy *et al.*, 1984; Duncan and Hershey, 1984; Duncan *et al.*, 1987; Sorger and Pelham, 1988; Larson *et al.*, 1988).

Exposure to heavy metals can elicit a similar stress response in different types of cells (Levinson *et al.*, 1980; Heikkila *et al.*, 1982; Caltabiano *et al.*, 1986; Lindquist, 1986). Furthermore, heavy metal ions (Cd^{2+}, Hg^{2+}, Pb^{2+}) inhibit protein synthesis *in vitro* by enhancing phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (Hurst *et al.*, 1987). Cadmium has been shown to alter phosphorylation of proteins in several in vitro systems, *e.g.*, in homogenates of the rat caudal artery (Mazzei *et al.*, 1984) and in

the cytosol of cultured fish cells (Behra and Gall, 1991). Also phosphorylation of human erythrocyte membrane proteins was stimulated by Cd^{2+} (Suzuki *et al.*, 1985a).

Our previous studies (Veldhuizen-Tsoerkan *et al.*, 1990a,b) have established that short-term, *in vivo* exposure to cadmium represses the overall protein synthesis and evokes the expression of stress proteins in gills of the sea mussel, *Mytilus edulis*. The present study was initiated to investigate whether protein phosphorylation is involved in the response of *M. edulis* to metal stress.

Materials and methods

Animals and animal treatment

Sea mussels, *Mytilus edulis*, were collected from the Eastern Scheldt in October 1989 and March 1990. Mean shell length was 5.5 ± 0.4 cm. The animals were kept in aquaria with recirculating sea water at 12°C under a natural light regime, without feeding. Mussels were exposed to cadmium in a semistatic system for 48 or 72 hr. Groups of 6 animals were placed in plastic buckets with 5 l of aerated sea water. Cadmium was added as $CdCl_2$ (Merck) to a final concentration ranging from 50 to 900 µg Cd^{2+}/l (0.5 - 8 µM). Prior to dissection, the animals were kept for 2 hr in unspiked sea water to remove adherent cadmium.

Phosphate incorporation

Gills were excised from 6 control and 6 Cd-exposed mussels. The middle parts of the outer gill lamellae were incubated with 100 µCi/ml ^{32}P-orthophosphate (carrier-free, specific activity 4000 Ci/mmol, Amersham, Bristol, U.K.) at 11.5°C for 2 hr (collection 1989) or 24 hr (collection 1990). The incubation medium and processing of the labelled gill tissues have been described earlier (Veldhuizen-Tsoerkan *et al.*, 1990b). To remove unincorporated label, the gill supernatant (100 µl) was chromatographed on Sephadex G-25 Fine (Pharmacia), column dimensions: 10 x 0.4 cm. Fractions of 100 µl were collected. The protein content of the fractions was determined in a 10-µl aliquot according to Bradford (1976). Incorporated phosphate was measured as trichloroacetic acid (TCA)-insoluble radioactivity in 5 µl-aliquots as described in Veldhuizen-Tsoerkan *et al.* (1990b). Column fractions with the highest incorporation levels were immediately mixed with loading or lysis buffer (see for details Veldhuizen-Tsoerkan *et al.*, 1990a,b) and stored overnight at -80°C.

Gel electrophoresis and computer analysis

One-dimensional electrophoresis (SDS-PAGE) according to Laemmli (1970) was performed on 12.5% and 15% polyacrylamide slab gels (ratio of acrylamide to bisacrylamide = 30:0.18 and 30:0.8, respectively). All samples contained 30 µg of protein. Molecular weight markers used were: phosphorylase a (92 kDa), bovine albumin (68 kDa), catalase (60 kDa), 3'-phosphoglycerate kinase (47 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21.5 kDa) and cytochrome c (12.5 kDa).

Samples containing 40 µg of protein were subjected to two-dimensional electrophoresis (IEF/SDS-PAGE) according to methods of O'Farrell *et al.* (1977) and Garrels (1979). The details of this method are given in our preceding studies (Veldhuizen-Tsoerkan *et al.*, 1990a,b). The second dimension was performed on 12.5% slab gels. The gels were stained with Coomassie Brilliant Blue R, dried and autoradiographed with X-ray film (Hyperfilm-MP, Amersham) at -70^{0}C with the use of intensifying screens (Kodak X-Omatic, Kodak, Rochester NY, USA). The exposure times of two-dimensional gels given in the legends are referring to day 0, at which time the specific activity of the labelled compound was rated. The pH gradient was determined from 1-cm sections of tube gels, which were incubated in 0.5 ml of 5 mM KCl for 2 hr.

The incorporation of ^{32}P into gill proteins was quantified from gel autoradiographs by determining the integrated optical density (IOD) of protein bands and spots with the IBAS image analysis system (Zeiss/Kontron, Eching, F.R.G.). The IODs were determined from several autoradiographs of one gel and the exposures within the linear range of the film were used. The autoradiographs were scanned with a Panasonic CCD camera (WW-CD50) and digitized 10 times and averaged to improve the signal to noise ratio (frame size 512 x 512 pixels, 8 bits = 256 grey levels).

The two-dimensional autoradiographs were subjected to the digital filter operation TRACKG. This filter replaces the grey values of the spots by the locally most likely background value. The square filter has a size larger than the largest spots in the image. The resulting image is substracted from the original images, in this way correcting for shading and inequalities in the background of the autoradiographs. Measurements were carried out on the corrected image. To delimit the individual spots the DISDYN procedure was applied. This dynamic discrimination method operates with a local threshold that is dependent on the local neighbourhood region. The local thresholds are derived from the local background grey level. Interactively, the delineated spots of interest were indicated and in overlay projected over the corrected image in order to verify visually that the spot detection is correct. The gel images were photographed with a Polaroid Freeze-Frame Video Recorder. Further details of the method have been given in Veldhuizen-Tsoerkan *et al.* (1990b).

Results and discussion

In vivo exposure of mussels to cadmium for 48 hr, followed by ^{32}P-labelling of the isolated gills for 2 hr, resulted in a cadmium concentration-dependent increase of ^{32}P-incorporation into gill proteins. This enhancing effect of cadmium was most evident for the low molecular weight (LMW) proteins (Fig. 1).

In the next experiment, mussels were exposed to cadmium for 72 hr. Subsequently the gills were incubated with ^{32}P for 24 hr. The stimulatory effect of cadmium on the phosphorylation of proteins with molecular weight of 12.5 - 110 kDa (Fig. 2) was virtually identical to that found in the first experiment. The ^{32}P-incorporation into these proteins increased almost linearly up to 300 µg Cd/l and remained above the control level at the higher Cd-concentration.

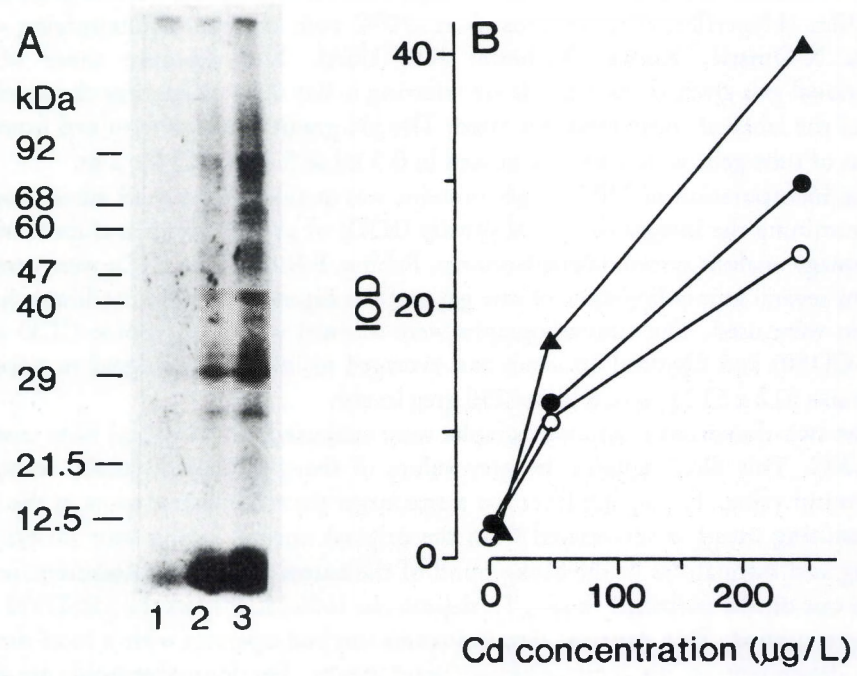


Fig. 1. Effect of *in vivo* exposure to cadmium for 48 hr on the phosphorylation state of gill proteins. Excised gills were incubated with ^{32}P for 2 hr. A. SDS-PAGE patterns of labelled gill proteins. Lane 1, control group; lanes 2 and 3, mussels exposed to 50 and 250 µg Cd/l, respectively. Proteins were resolved on a 12.5% gel. The position of molecular weight markers is indicated. Autoradiography time was 22 days. B. Integrated optical density (IOD), estimated from the gel autoradiograph shown in A. Proteins with molecular weight: -▲-, < 12.5 kDa; -o-, 12.5 - 40 kDa; -●-, 40 - 110 kDa.

In this experiment, the extended time of the labelling procedure resulted in higher phosphorylation levels of the LMW proteins, which hampered the detection of a cadmium effect by one-dimensional resolution. Only a slight increase in phosphorylation of the LMW proteins could be detected at the lowest Cd-concentration (Fig. 2), but the increase can be clearly observed in Fig. 3.

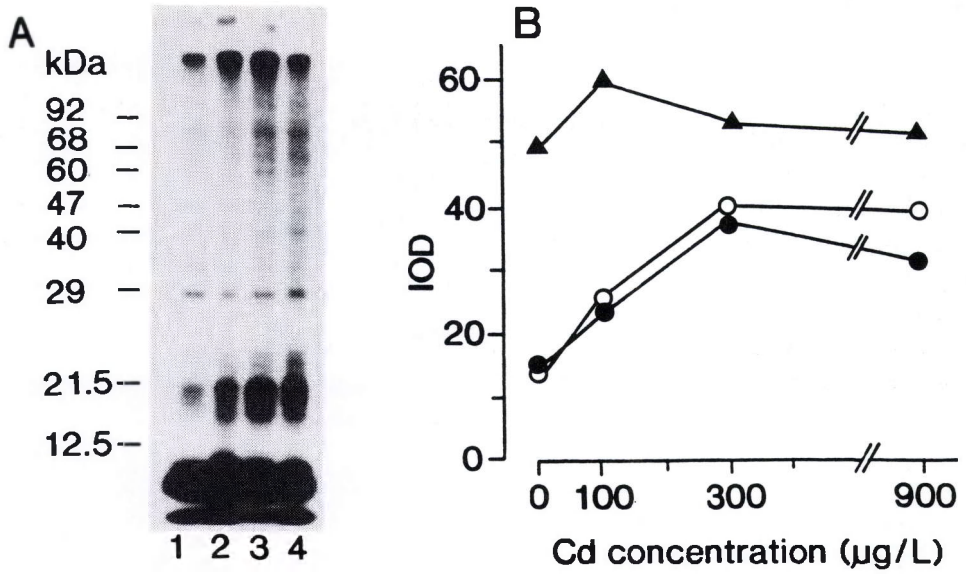


Fig. 2. Effect of *in vivo* exposure to cadmium for 72 hr on the phosphorylation state of gill proteins. Excised gills were incubated with ^{32}P for 24 hr. A. SDS-PAGE patterns of labelled gill proteins. Lane 1, control group; lanes 2, 3 and 4, mussels exposed to 100, 300 and 900 µg Cd/l, respectively. Proteins were resolved on a 15% gel. Autoradiography time was 20 hr. B. Integrated optical density (IOD), estimated from the gel autoradiograph shown in A. Proteins with molecular weight: -▲-, < 12.5 kDa; -o-, 12.5 - 40 kDa; -●-, 40 - 110 kDa.

The present data are in agreement with results of *in vitro* studies in which cadmium has been shown to stimulate the phosphorylation of rat vascular smooth muscle proteins (Mazzei *et al.*, 1984), of human erythrocyte membrane proteins (Suzuki *et al.*, 1985a), of smooth muscle myosin (Kostrzewska and Sobieszek, 1990), and of cytosolic proteins of fish gonadal cells (Behra and Gall, 1991). The enhancing effect of cadmium has been observed at Cd concentrations (0.5 - 10 µM) comparable to those used in our work, while at Cd concentrations of 50 - 200 µM the protein phosphorylation was inhibited (Mazzei *et al.*, 1984; Behra and Gall, 1991).

To characterize the effect of cadmium on phosphorylation of individual gill proteins, two-dimensional gel electrophoresis was applied. Fig. 3A shows the pattern of ^{32}P-incorporation into gill proteins of the control animals. Fig. 3B and C show the changes in the phosphorylation patterns induced by cadmium. A quantitative analysis of the most conspicuous changes, measured in the linear range of the exposed films, revealed that several proteins were more heavily labelled with ^{32}P in Cd-exposed animals than in the controls (spots 1, 3 and 10, Fig. 3, Table 1). The phosphorylation of other proteins increased up to the highest Cd-concentration (spots 4, 6 and 7). Several proteins, however, appeared to be less phosphorylated (spots 2, 5, 9 and 11) or remained unchanged (spot 8). It is of interest to note that spots 4 and 5 are probably charge isomers whose phosphorylation was conversely affected by cadmium.

Fig. 4 represents the computer images of the two-dimensional gels shown in Fig. 3 that were made from overexposed autoradiographs after background substraction. This

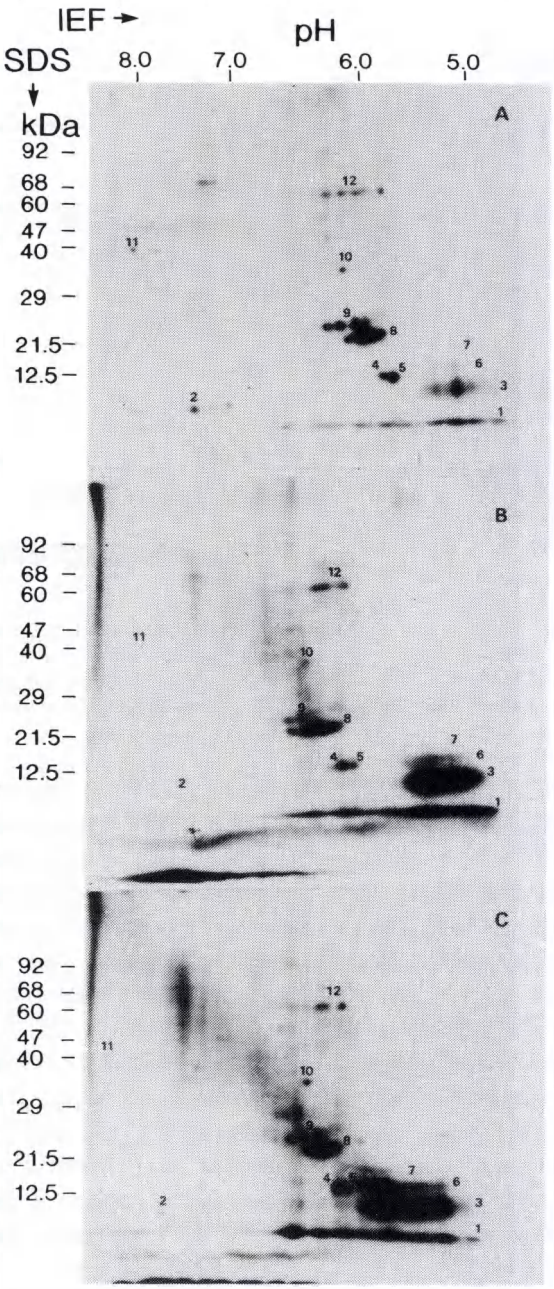


Fig. 3. Two-dimensional IEF/SDS-PAGE patterns of ^{32}P-labelled gill proteins. Animal treatment and labelling procedure were as described in the legend to Fig.2. A. Control group. B. and C. Groups exposed to 100 and 900 µg Cd/l, respectively. Forty micrograms of protein containing 7.3 x 10^3 (A), 10.5 x 10^3 (B) and 11 x 10^3 (C) cpm were applied to each gel. The pH gradient is given. Integrated optical density (IOD) of the numbered phosphoproteins is given in Table 1. Film exposure time was from day 16 to day 32.

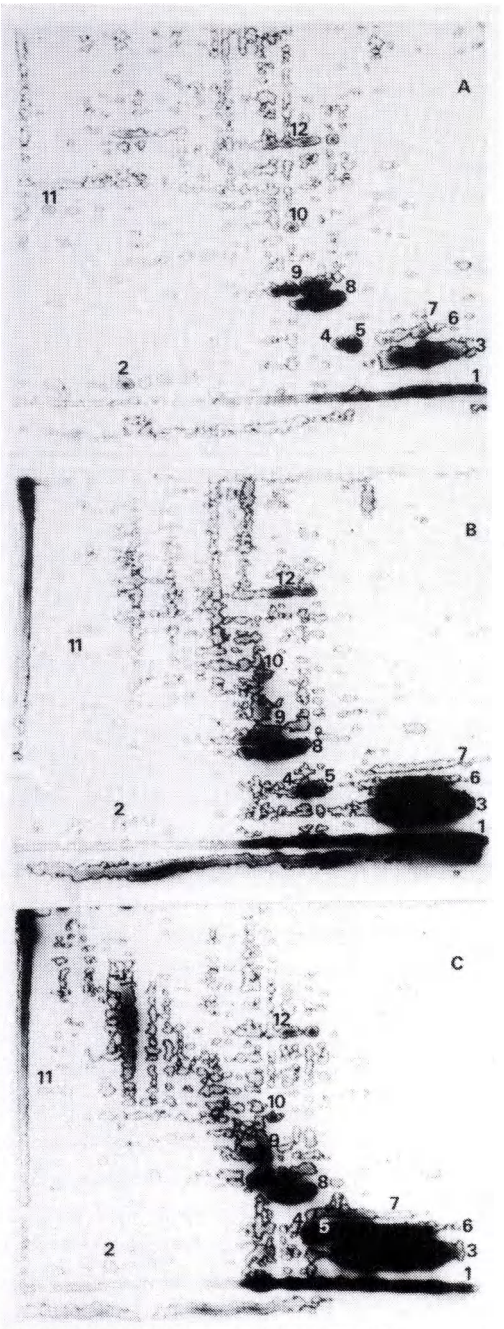


Fig. 4. Gel images of gill phosphoproteins. Images were made from overexposed autoradiographs of the gels shown in Fig. 3. Film exposure time was from day 8 to day 16. Images were taken from the computer screen after background substraction as described in Materials and methods. Letters (A, B, and C) and numbers correspond to those shown in Fig. 3.

Table 1: Effect of cadmium on the phosphorylation of gill cytosolic proteins. Integrated optical density (IOD) of ^{32}P-labelled proteins shown in Fig. 3. Values in parentheses are percentages of the control

Protein	IOD		
Spot No.	Control group	100 µg Cd/l group	900 µg Cd/l group
1	8.5 (100)	34 (400)	34 (400)
2	0.64 (100)	n.d.	0.12 (19)
3	6.4	45 (703)	45 (703)
4	0.79 (100)	1.62 (205)	2.84 (359)
5	2.57 (100)	0.55 (21)	1.05 (41)
6	0.38 (100)	5.3 (1394)	13.1 (3447)
7	n.d.	0.49	1.7
8	14.3 (100)	13.2 (92)	13.6 (95)
9	6.4 (100)	2.5 (39)	4.9 (77)
10	0.35 (100)	0.45 (129)	0.47 (134)
11	0.18 (100)	0.06 (33)	0.07 (39)
12	1.8 (100)	2.4 (133)	1.9 (105)

n.d., not detected.

procedure allows the detection of a larger number of distinct phosphoproteins (compare Figs 3 and 4), and thus accentuates the stimulatory effect of cadmium. The most prominent and consistent changes caused by cadmium are the enhanced ^{32}P-labelling of proteins in three distinct regions of the two-dimensional gel: high molecular weight (HMW) proteins with high pI's (pH range 6.8 - 8.2), proteins with lower molecular weight in the pH range of 5.8 - 6.8, and LMW proteins with low pI's (pH 4.5 - 6.5).

The observed increase of protein phosphorylation is not due to an enhanced synthesis of phosphoproteins, as short-term exposure to cadmium has previously been shown to inhibit the synthesis of gill proteins under similar *in vivo* exposure conditions (Veldhuizen-Tsoerkan *et al.*, 1990a,b). The repression of protein synthesis in gill tissue can at least partly account for the observed decrease in phosphorylation of several proteins.

Short-term exposure to cadmium induces and/or enhances the synthesis of stress proteins such as metallothionein(MT)-like proteins and heat shock proteins (hsps) in gill tissue of *M. edulis* (Sanders, 1988; Veldhuizen-Tsoerkan *et al.*, 1990b). Therefore, the high degree of phosphate incorporation into such proteins could be ascribed to their increased synthesis. The phosphorylation of several hsps has been documented. For example, constitutive hsp 90 is highly phosphorylated at serine and threonine residues in the unstressed cell (Schlesinger, 1990), and hsp 26-28 is phosphorylated at serines upon heat shock (Landry *et al.*, 1988; Tomasovic, 1989). Whereas indirect evidence exists that the phosphorylation process is involved in the regulation of MT expression (Imbra and Karin, 1987), there are no reports on this covalent modification of MTs. The phosphorylation of the latter cannot be completely ruled out since MT-like proteins, *e.g.*, of *M. edulis* contain serine and threonine in mol% of 7.9 and 5.9, respectively (Frazier, 1986). The strongly phosphorylated LMW proteins with low pI's occupy a similar position as the gill MT-like on the two-dimensional gel (Veldhuizen-Tsoerkan *et al.*, 1991). Moreo-

ver, in the study of Endresen *et al.* (1984) two-dimensional resolution of non-carboxymethylated MTs resulted in a wide horizontal band which could correspond to spot 1 (Fig. 3). Whether phosphorylation of MT occurs and that this could play a role in MT functioning remains obscure.

Protein phosphorylation is a process generally mediated by calcium. Cadmium can substitute for calcium on calmodulin, a ubiquitous Ca^{2+}-binding protein that promotes many effects of calcium (Chao *et al.*, 1984; Suzuki *et al.*, 1985b; Mills and Johnson, 1985). Cadmium is also known to increase the cytosolic free Ca^{2+} level (Verbost *et al.*, 1987; Dwyer *et al.*, 1991), and this increase could activate Ca^{2+}/calmodulin-dependent processes. Stimulation of the Ca^{2+}/calmodulin-dependent protein phosphorylation by cadmium has been reported in several *in vitro* systems (Mazzei *et al.*, 1984; Kostrzewska and Sobieszek, 1990; Behra and Gall, 1991).

The observation of changes in protein phosphorylation induced *in vivo* (and *in vitro*) by cadmium may provide a clue to the question of how toxic metals function in the general stress response. Heat shock appears to affect the same cellular processes as those influenced by cadmium. For example, heat shock has also been reported to elevate the level of cytosolic free Ca^{2+}, to induce Ca^{2+}/calmodulin-dependent processes and to change the level of ^{32}P-incorporation into a number of proteins (Wiegant *et al.*, 1985; Stevenson *et al.*, 1986; Landry *et al.*, 1988; Balodimos *et al.*, 1990). Furthermore, the inhibition of protein synthesis under heat stress conditions is correlated with changes in the phosphorylation state of ribosomal proteins and eukaryotic initiation factors (Glover, 1982; Kennedy *et al.*, 1984; Duncan and Hershey, 1984; Duncan *et al.*, 1987). Hurst *et al.* (1987) recently confirmed that cadmium inhibits protein synthesis *in vitro* by stimulating phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF-2). In addition, the induction of hsp expression under stress conditions involves phosphorylation of a heat shock gene transcription factor (Sorger and Pelham, 1988; Larson *et al.*, 1988).

In conclusion, the observed cadmium-dependent changes in ^{32}P-incorporation into gill proteins may represent the harmful cellular perturbations underlying the toxic mechanism of heavy metal stress in *M. edulis*.

Acknowledgements

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C H A P T E R 7

Synthesis of stress proteins under normal and heat shock conditions in gill tissue of sea mussels after chronic exposure to cadmium

M.B. Veldhuizen-Tsoerkan[*], *D.A. Holwerda*[*], *C.A. van der Mast*[**] *and D.I. Zandee*[*]

[*]*Department of Experimental Zoology and* [**]*Department of Molecular Cell Biology, University of Utrecht, 8 Padualaan, NL-3584 CH Utrecht, The Netherlands*

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Synthesis of stress proteins under normal and heat shock conditions in gill tissue of sea mussels after chronic exposure to cadmium

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Abstract

Sea mussels were exposed to 16.5 µg Cd/l under semi-field conditions for almost one year. The isolated gills were incubated with ^{35}S-methionine or -cysteine. Chronic exposure to cadmium neither altered the rate of amino acid incorporation nor induced expression of heat shock proteins in the gills. Heat shock imposed after chronic exposure to cadmium resulted in an increased synthesis of heat shock proteins, especially those of high molecular weight. Synthesis of cadmium-binding, low molecular weight proteins was observed at any point of the exposure time. Their cadmium-binding capacity and rate of synthesis, after the initial increase, remained unchanged throughout the exposure.

Introduction

In the gills of sea mussels acute heavy metal exposure modulates the gene expression of various proteins. The phenomenon includes repression of the overall protein synthesis, and induction of stress proteins, *viz.* specific metal-binding proteins and heat shock proteins (Viarengo *et al.*, 1980; Sanders, 1988; Steinert and Pickwell, 1988; Veldhuizen-Tsoerkan *et al.*, 1990a,b). Both groups of stress proteins provide tolerance to a number of different stresses at the molecular level. Metallothionein (MT)-like proteins prevent cellular damage by sequestering the toxic metal ions (Kägi and Schäffer, 1988; Waalkes and Goering, 1990), while heat shock proteins (hsps) form complexes with various proteins in order to protect them under stress conditions. Some of the hsps are thought to act as chaperonines to induce a correct folding of heat-denatured proteins (Tomasovic, 1989; Schlesinger, 1990).

In recent reviews the utility of measurement of MT-like proteins and hsps as stress indicators in biomonitoring of marine environmental contamination has been discussed (Viarengo, 1989; Benson *et al.*, 1990; Garvey, 1990; Sanders, 1990). Our preceding studies (Veldhuizen-Tsoerkan *et al.*, 1990b, 1991) have shown that the "stress approach" is a useful method in disclosing acute effects of cadmium. Both the anoxic and heat shock response in the sea mussel were altered by short-term exposure to cadmium. Upon long-term, semi-field exposure of sea mussels to cadmium, commonly used stress parameters such as condition indices, adenylate energy charge and metabolite contents appeared to remain unchanged. However, the anoxic tolerance was clearly diminished after chronic

exposure to cadmium (Veldhuizen-Tsoerkan *et al.*, 1991).

In the light of these findings, the present study was undertaken to assess the effects of long-term, semi-field exposure to a low Cd concentration on the total and specific protein synthesis in the gills of *Mytilus edulis* and to examine the response to heat shock. Induction of hsps and MT-like proteins was measured via the incorporation of radiolabelled amino acids, followed by electrophoretic and chromatographic analysis.

Materials and methods

Animals and animal treatments

Sea mussels, *Mytilus edulis*, were collected from the Eastern Scheldt (a relatively unpolluted area) in August 1988 and October 1989. Mean shell length was 5.5 ± 0.4 cm. The animals were exposed to cadmium from August 1988 to July 1989 and from October 1989 to July 1990, respectively, at the field laboratory of Tidal Waters Division (Ministry of Transport and Public Works). Mussels were kept in 1000-l tanks to which sea water from the Eastern Scheldt (salinity 28$^{o}/_{oo}$, ambient temperature) was pumped at a flow rate of 41 l/hr. Cadmium was added as $CdCl_2$ (BHD Chemicals Ltd) to give an actual concentration of 16.5 µg/l (0.15 µM). The mussels were fed *Phaeodactylum tricornutum* at a concentration of $50x10^6$ cells/l.

Prior to further experiments, the mussels were kept in unspiked sea water for 24 hr to eliminate adherent cadmium.

In some experiments control and Cd-exposed mussels were subjected to a heat shock of 29.5°C for 4 hr.

Metal analysis

For Cd analysis tissue samples were composed from the gills of 6 mussels. Tissue decomposition and determination of Cd tissue concentration were performed as described previously (Veldhuizen-Tsoerkan *et al.*, 1990b).

Amino acid incorporation

Gills were isolated from 6 Cd-exposed or control animals. The middle parts of the outer gill lamellae were incubated with 25 or 40 µCi/ml ^{35}S-methionine or ^{35}S-cysteine (specific activity > 1000 Ci/mmol, Amersham) at 11.5°C for 20 hr (see for details Veldhuizen-Tsoerkan *et al.*, 1990b). Incorporation of radiolabelled amino acids into the gill proteins was determined in a 5-µl sample of gill supernatant which was transferred to a

Whatman 3MM filter and precipitated with trichloroacetic acid (10%), as described by Van der Mast *et al.* (1977). A 10-µl aliquot of the supernatant was used for a protein determination according to Bradford (1976). Amino acid incorporation into the gill proteins of exposed and control animals was estimated as cpm (counts per minute)/µg protein.

Prior to further analysis, ^{35}S-cysteine labelled proteins were carboxymethylated with 0.2 M iodoacetate (adjusted to pH 8.0 with Tris base) for 1 hr at 37°C in the dark.

Labelled gill supernatants were immediately mixed 2:1 with loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 2.5% 2-mercaptoethanol, 25% glycerol and 0.05% bromophenol blue) and 1:1 with lysis buffer (9.95 M urea, 4% Nonidet P-40, 1.3% Bio-Rad ampholytes pH range 5-7 and 0.7% ampholytes pH range 1-10, 0.1 M DTT and 0.3% SDS) for one- and two-dimensional electrophoretic resolution, respectively. Samples were stored at - 80°C.

Gel filtration chromatography

Analysis of Cd-binding proteins was performed on a Sephadex G-75 Superfine column (Pharmacia, 1.4 x 54 cm). A sample of 120 mg dry wt, composed of 6 gills, was processed and chromatographed as described previously (Veldhuizen-Tsoerkan *et al.*, 1991). Column fractions of 1 ml were assessed for Cd content with atomic absorption spectrometry.

^{35}S-cysteine labelled supernatants, containing equal amounts of radioactivity (cpm/µg protein), were recentrifuged for 1 hr at 100,000 g and chromatographed on a Sephadex G-75 Superfine column (0.9 x 50 cm). An aliquot (100 µl) of each column fraction was mixed with 2 ml of Aqualuma (Lumac) and counted in a Beckman LS 6000SE counter. Fractions (1 ml) of the LMW column range were pooled, desalted on a Sephadex G-25 Medium column (PD-10, Pharmacia) and concentrated by lyophilization. Samples were dissolved in 5 mM Tris-HCl buffer (pH 7.0), mixed with loading or lysis buffer and stored at - 80°C.

Gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970). SDS-PAGE of ^{35}S-methionine labelled proteins was carried out as described earlier (Veldhuizen-Tsoerkan *et al.*, 1990b). ^{35}S-cysteine labelled proteins were run on gels with varying concentrations of acrylamide and bisacrylamide (see Results). As molecular weight markers were used: phosphorylase a (92 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), 3'-phosphoglycerate kinase (47 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21.5 kDa) and cytochrome c (12.5 kDa).

Two-dimensional electrophoresis (IEF/SDS-PAGE) was conducted according to O'Farrell *et al.* (1977) and Garrels (1979). The details of IEF/SDS-PAGE of ^{35}S-

methionine labelled proteins can be found in our preceding studies (Veldhuizen-Tsoerkan *et al.*, 1990a,b).

The LMW, ^{35}S-cysteine labelled proteins, resolved by Sephadex G-75, were applied to the cathodic part of the IEF gel. Only the acidic part of the IEF gel (2/3 of gel tube with pH range 3-6) was used. The second dimension was performed on 15% polyacrylamide:0.4% bisacrylamide gel for 3 hr at 150 V in a BioRad mini Proteon II slab cell.

Following electrophoresis, gels were stained, dried and fluorographed as described in Veldhuizen-Tsoerkan *et al.* (1990b). Incorporation of amino acids into specific proteins was quantified from the gel fluorographs. The integrated optical density (IOD) of protein spots or bands was determined with the IBAS image system (Zeiss/Kontron, Eching, F.R.G.). A detailed description of the image analysis is given in Veldhuizen-Tsoerkan *et al.* (1990a,b). IODs were determined from several fluorographs of one gel and the exposures within the linear range of the film were used. Thionein synthesis was estimated from the fluorographs of ^{35}S-cysteine labelled proteins according to Sone *et al.* (1987).

Results

Synthesis of heat shock proteins

Chronic, semi-field exposure to 16.5 µg Cd/l neither induced expression of heat shock proteins (hsps) (Fig. 1, lanes 1 and 2), nor changed the rate of incorporation of methionine into the gill proteins (data not shown). By contrast, after a heat shock treatment for 4 hr, the Cd-exposed mussels showed an enhanced synthesis of hsps compared to heat-shocked, unexposed animals. This stimulation was most apparent in the expression of hsps with a molecular weight exceeding 70 kDa (Fig.1, lanes 3 *vs.* 4, and 5 *vs.* 6).

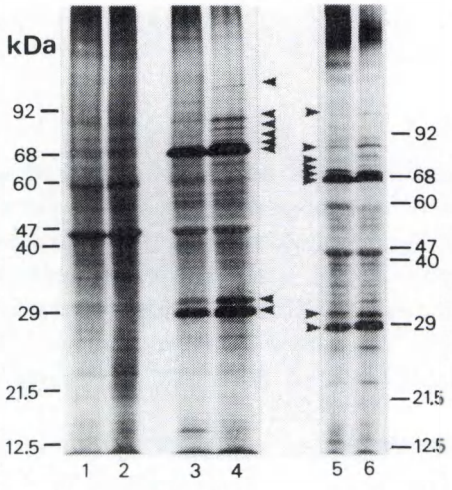


Fig. 1. SDS-PAGE patterns of ^{35}S-methionine labelled gill proteins from mussels exposed to cadmium (16.5 µg/l) under semi-field conditions (August'88 - July'89) with or without an ensuing heat shock. Animal treatment: lane 1, control; lane 2, Cd exposure for 9 months; lanes 3 and 5, heat shock for 4 hr; lanes 4 and 6, Cd exposure for 9 and 11 months, respectively, followed by 4 hr of heat shock. The position of molecular weight markers is separately indicated for lanes 1-4 and lanes 5-6. The arrows show the position of heat shock proteins. Fluorography was for 96 hr.

Two-dimensional gel electrophoresis was applied to analyze the synthesis of individual hsps. As can be seen in Figs 2 and 3, alterations in hsp synthesis occurred throughout the whole range of molecular weights. After 9 months of Cd exposure, the synthesis of most low molecular weight (LMW) hsps increased by a factor of 1.2 to 3.9 (Fig. 2, spots 1-3, 6, 7 and 16; Table 1) and of 27 (spot 4), whereas the expression of two proteins was inhibited (spots 5 and 8). In the high molecular weight (HMW) range, the stimulation of hsp synthesis by heat shock was more conspicuous after chronic exposure to cadmium. The expression of these hsps increased by a factor of 4.3 to 10.5 (Fig. 2, spots 10-15; Table 1). Imposing of the additional heat stress after a more prolonged exposure time (11 months) evoked similar alterations in the hsp expression (Fig. 3). The synthesis of LMW hsps was enhanced 1.5 to 3.5 times (spots 1, 2, 5-7; Table 2), while the expression of HMW hsps increased 3.4 to 17.1 times (spots 11-14; Table 2). The inhibition of one LMW hsp (spot 8) was confirmed.

It appears that some hsps (Fig. 2, spots 16-18) are not synthesized later in the season (July) as they are lacking in the experiment depicted in Fig. 3. It is also of interest to note that spots 17 and 18 were observed only in sexually maturing animals and especially before spawning (May, this experiment). This phenomenon was proved for several mussel populations examined. For example, these proteins were not found in animals collected in August-November (Veldhuizen-Tsoerkan *et al.*, 1990b), but they were present in mussels collected in February-April (Chapter 8).

Synthesis of cadmium-binding, cysteine-rich proteins

Chronic exposure to 16.5 µg Cd/l did not affect the rate of incorporation of cysteine into the gill proteins (data not shown). SDS-PAGE analysis of ^{35}S-labelled gill proteins revealed the induction of an LMW, cysteine-rich protein (Fig. 4), which migrated as a broad diffuse band. The electrophoretic mobility of this protein seemed to be dependent on the amount of cross-linker used. On gels with a low percentage of bisacrylamide, the protein showed an apparent molecular weight between 12.5 and 21.5 kDa (Fig. 4, lane 2), while in a high cross-linked gel this was somewhat lower than 12.5 kDa (Fig. 4, lanes 4 and 5). An anomalous electrophoretic behaviour has also been reported for vertebrate MTs (Koizumi *et al.*, 1982; Sone *et al.*, 1987; Hidalgo *et al.*, 1988). Fig. 5 shows the Sephadex G-75 cadmium profile of the gill cytosol after 11 months of exposure. The greater part of cytosolic cadmium was bound to proteins with an apparent molecular weight of 25 to 12.5 kDa. When, in the same experiment, ^{35}S-cysteine labelled cytosolic gill proteins were resolved by gel permeation chromatography (Fig. 6), the Cd-induced cysteine-rich peak was found in the same molecular weight range as the major Cd-binding protein (Fig. 5). This LMW, ^{35}S-labelled peak was further analyzed by electrophoresis on a high cross-linked gel and was found to contain a cysteine-rich protein with molecular weight < 12.5 kDa (Fig. 7). Two-dimensional electrophoretic resolution of this protein resulted in three "smeared" spots with low isoelectric points (pI's of 3-4, Fig. 8). Nordberg *et al.* (1972) reported pI's of 4-5 for mammalian MTs. However, the poor two-dimensional resolution of MTs, as observed by Endresen *et al.* (1984), necessitates carboxymethylation of the protein samples. In our experiments this was per formed with

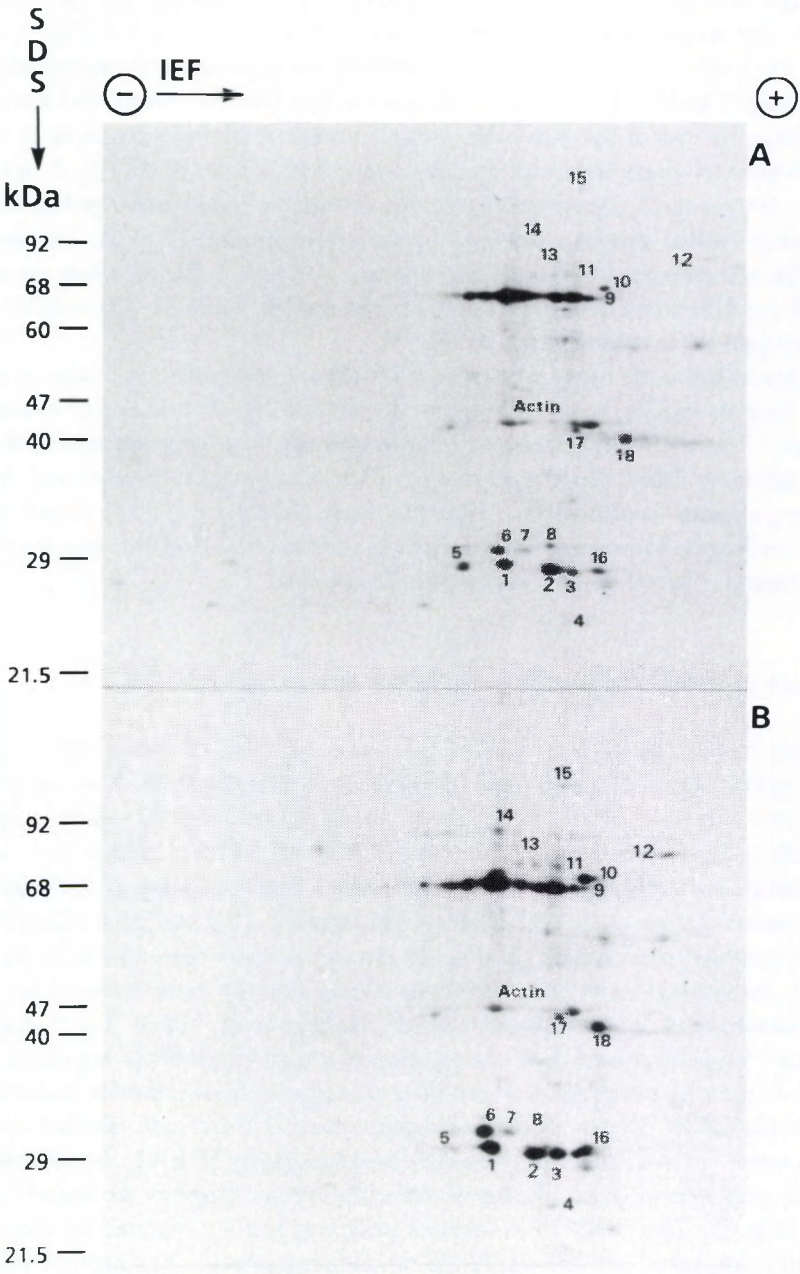


Fig. 2. Two-dimensional electrophoretic patterns of ^{35}S-methionine labelled gill proteins from heat-shocked control animals and from heat-shocked Cd-exposed mussels. (A) control group, 4 hr of heat shock; (B) heat shock of the same duration after 9 months of Cd exposure. The numbers indicate heat shock proteins. The integrated optical densities of these proteins are given in Table 1. Fluorography was for 11 days.

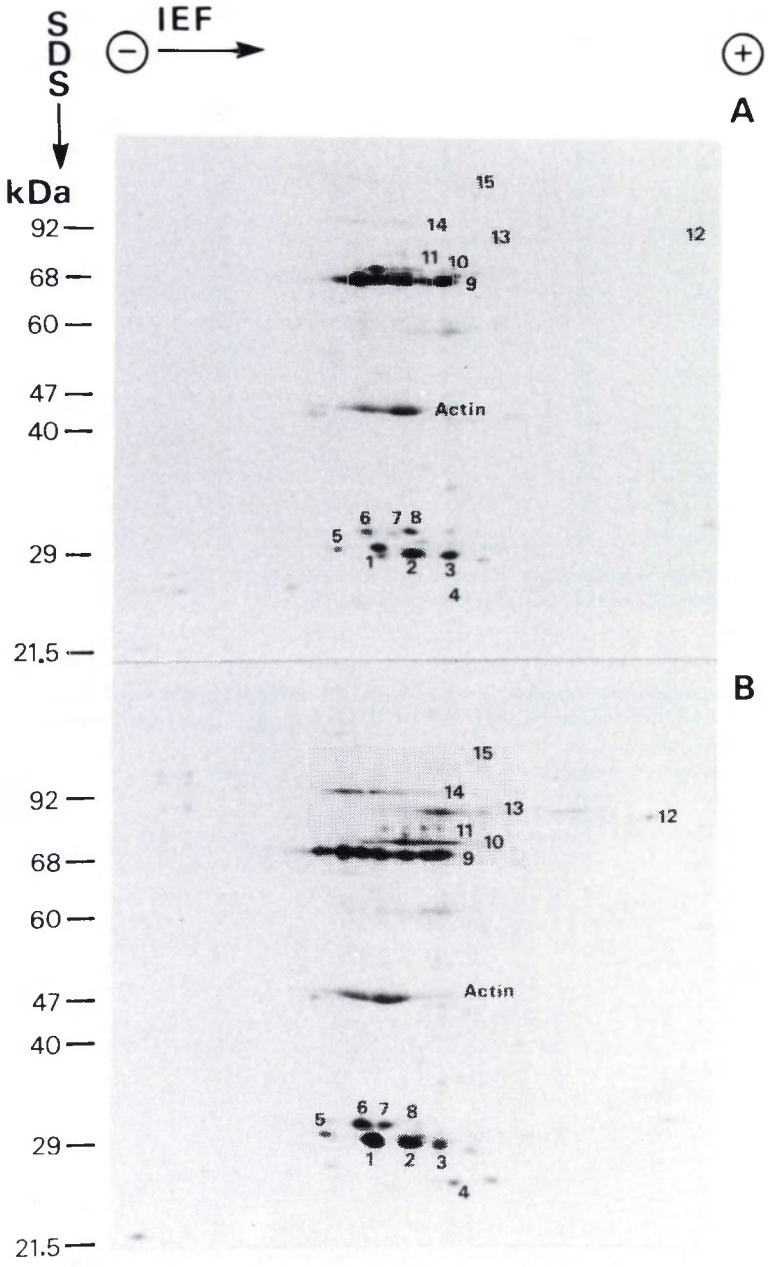


Fig. 3. Two-dimensional electrophoretic pattern as mentioned in the legend of Fig. 2, but with the (B) group exposed to cadmium for 11 months. The integrated optical densities of the heat shock proteins are given in Table 2. Fluorography was for 11 days.

Table 1. Effect of 9 months of Cd exposure on the synthesis of heat shock proteins in gill tissue of mussels heat-shocked at 29.5°C for 4 hr. The integrated optical density (IOD) was determined from protein spots shown in Fig. 2.

Protein spot No.	Molecular weight (kDa)	IOD[a]		Ratio[b]
		Control	Cd-exposed	
1	30	135	241	1.8
2	30	204	236	1.2
3	30	61	170	2.8
4	25	0.2	5.3	27
5	30	69	26	0.4
6	32	78	184	2.4
7	32	11	39	3.6
8	32	23	2.1	0.1
9	68	1281	1433	1.1
10	70	38	185	4.9
11	72-74	15	68	4.5
12	76	8.5	59	6.9
13	84	5.1	22	4.3
14	94	9.8	103	10.5
15	105	3.5	14	4.0
16	30	53	208	3.9
17	43	149	211	1.4
18	40	21	39	1.9

a) Values were normalized to equal IOD's of the actin spots.
b) IOD (Cd-exposed group): IOD (control group).

Table 2. Effect of 11 months of Cd exposure on the synthesis of heat shock proteins in gill tissue of mussels heat-shocked at 29.5°C for 4 hr. The integrated optical density (IOD) was determined from protein spots shown in Fig. 3.

Protein spot No.	Molecular weight (kDa)	IOD[a]		Ratio[b]
		Control	Cd-exposed	
1	30	141	299	2.1
2	30	186	287	1.5
3	30	118	103	0.9
4	25	0	37	
5	30	38	51	1.3
6	32	59	161	2.7
7	32	24	85	3.5
8	32	65	7.5	0.1
9	68	1117	1157	1.0
10	70	148	255	1.7
11	72-74	16	61	3.8
12	76	5.6	19	3.4
13	84	12	206	17.2
14	94	18	139	7.7
15	105	3.4	5.4	1.6

a) Values were normalized to equal IOD's of the actin spots.
b) IOD (Cd-exposed group): IOD (control group).

iodoacetic acid which introduces additional negative charges. The partial smearing effect found in the second dimension can still be attributed to aggregation of MTs as described by the authors mentioned.

The relative synthesis of the Cd-induced proteins was estimated from gel fluorographs as depicted in Fig. 9. It can be inferred that the relative synthesis of these MT-like proteins reached a steady state level after exposure to cadmium for 3 months.

As shown in Fig. 10, the relative amounts of cytosolic cadmium bound to the HMW and LMW peaks did not change essentially in dependence of the exposure time, although the absolute amount of cytosolic, protein-bound cadmium increased proportionally to the metal concentration in whole gill tissue. The latter concentration increased linearly with time, reaching values of 44.8 ± 15.6, 133 ± 30 and 182 ± 36 µg Cd/g dry wt (mean of six animals ± SD) after 3, 8 and 11 months, respectively.

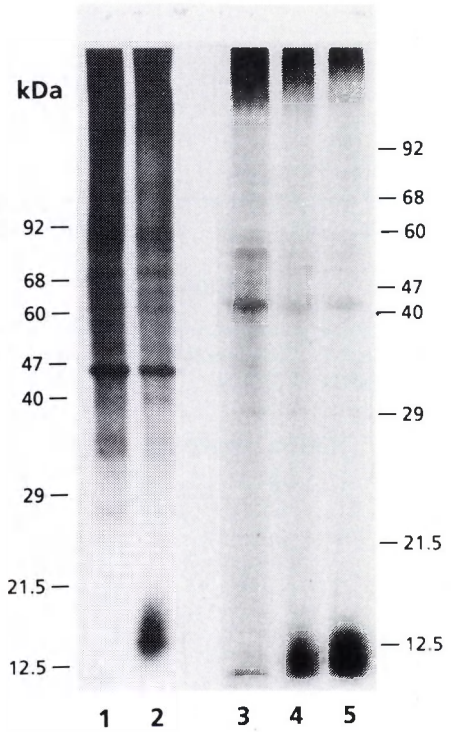


Fig. 4. Induction of a cysteine-rich protein in gills of mussels exposed to cadmium (16.5 µg/l). Lanes 1 and 2: 10-20% polyacrylamide gel (0.06-0.12% bisacrylamide); and lanes 3-5: 12.5% polyacrylamide (0.33% bisacrylamide). Lanes 1 and 3, unexposed animals; lanes 2, 4 and 5, mussels exposed to cadmium for 3 (August - November '88), 4 (March - July '90) and 9 (October '89 - July' 90) months, respectively. Fluorography was for 6 and 3 days, respectively.

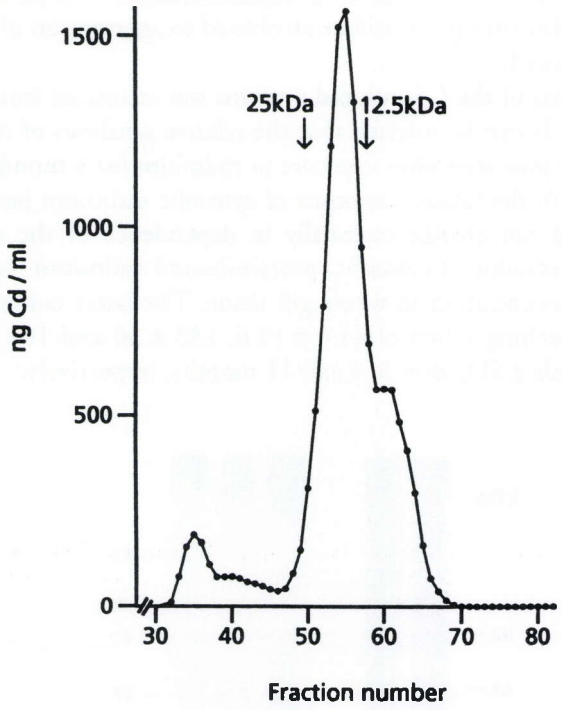


Fig. 5. Sephadex G-75 elution profile of cytosolic protein-bound cadmium from gills of mussels exposed to cadmium (16.5 µg/l) for 11 months (August '88 - July '89). The arrows indicate the position of separately run molecular weight markers: chymotrypsin (25 kDa) and cytochrome c (12.5 kDa).

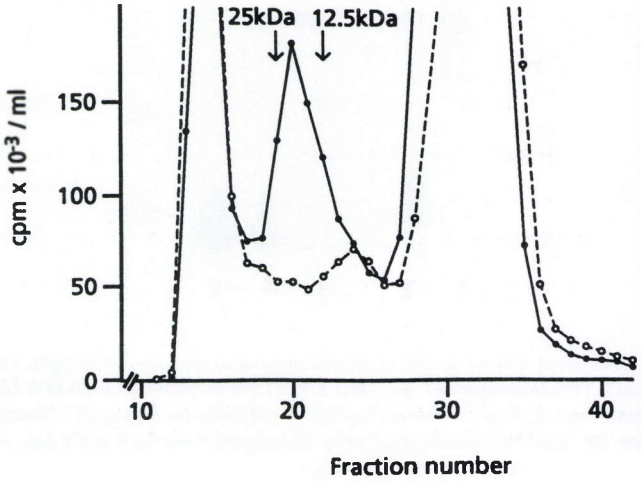


Fig. 6. Sephadex G-75 elution profile of ^{35}S-cysteine labelled cytosolic gill proteins of mussels exposed to cadmium (16.5 µg/l) for 11 months (August '88 - July '89). Control group, -o-o-; Cd-exposed group, -●-●-. The arrows indicate the position of separately run molecular weight markers: chymotrypsin (25 kDa) and cytochrome c (12.5 kDa).

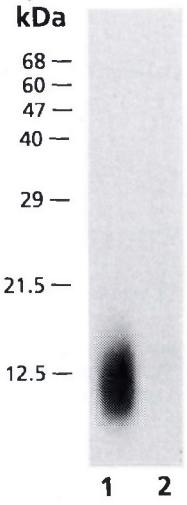


Fig. 7. SDS-PAGE analysis of column fractions 19 - 23 (see Fig. 6) of control and Cd-exposed groups of mussels. The fractions of each group were pooled, concentrated and electrophoresed on a 15% polyacrylamide (0.4% bisacrylamide) gel. Lane 1, Cd-exposed group; lane 2, control group.

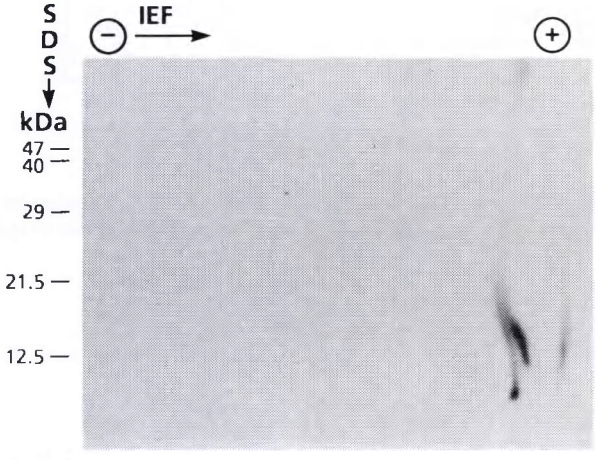


Fig. 8. Two-dimensional electrophoretic analysis of the Cd-induced, ^{35}S-cysteine labelled column peak, resolved by Sephadex G-75 (Fig. 6). One-dimensional resolution of these pooled column fractions is shown in Fig. 7 (lane 1). The slab gel of the second dimension contained 15% acrylamide with 0.4% bisacrylamide. Fluorography was for 14 days.

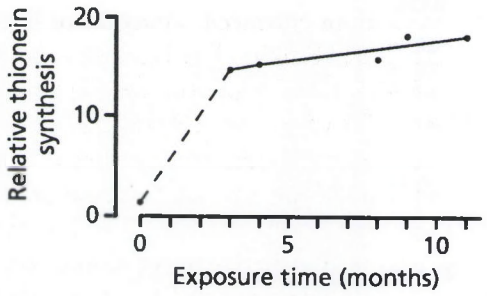


Fig. 9. Relative synthesis of thioneins in gills of mussels exposed to 16.5 µg Cd/l for 11 months. The relative synthesis was estimated from fluorographs of 15% polyacrylamide (0.4% bisacrylamide) gels, and is expressed as 100 x IOD of thioneins divided by the sum of IOD of total polypeptides minus IOD of thioneins. Data derived from two semi-field experiments (August '88 - July '89 and October '89 - July '90).

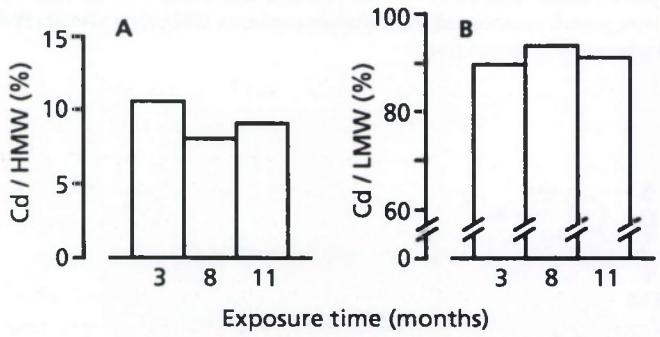


Fig. 10. Distribution of cytosolic cadmium between HMW (A) and LMW (B) proteins, resolved by Sephadex G-75, in the gills of mussels exposed to cadmium (16.5 µg/l) for 11 months under semi-field conditions (August '88 - July '89).

Discussion

In contrast to the short-term exposure of mussels to high Cd concentrations, the long-term, semi-field exposure to a low concentration of cadmium did not affect the overall protein synthesis in gill tissue. Neither was an induction of hsps apparent. Sanders (1990) also emphasized the transient nature of a detectable hsp expression under conditions of continuous moderate stress.

Due to their sessile mode of life in the tidal zone, mussels have to deal with multiple environmental stresses. It appears a promising approach to investigate the effects of pollutants under the condition of a natural stress, such as anoxia or elevated temperature. In

the same semi-field experiment (Veldhuizen-Tsoerkan *et al.*, 1991), adverse effects of cadmium were most clearly manifested under anoxic conditions. Under laboratory conditions, Cd-exposed animals showed an enhanced induction of hsps after a heat shock treatment (Veldhuizen-Tsoerkan *et al.*, 1990b). The increase amounted to a factor of 5 to 11 for several HMW hsps (76-95 kDa). A similar effect has now been found in the gills of mussels exposed to 16.5 µg Cd/l for 9 or 11 months (Figs 2 and 3; Tables 1 and 2). Li *et al.* (1982) have also reported an enhanced synthesis of hsps with molecular weight of 70, 87 and 97 kDa in mammalian cells after a consecutive exposure to cadmium and heat shock. These data could imply that cadmium interferes with the cellular mechanism of the hsp response. A conceivable site of metal interference is formed by the calmodulin system. Sutoo *et al.*(1990) have suggested that the inability of calmodulin to distinguish between Ca^{2+} and Cd^{2+} ions underlies the toxicity of cadmium. Heat shock, on the other hand, induces some Ca^{2+}-dependent processes (Landry *et al.*, 1988). Evans and Tomasovic (1989,1990) suggested that perturbation of calmodulin-regulated processes by hyperthermia may contribute to the cell death at increased temperature. The same authors also showed that hsps 90, 70 and 26 have the capacity to bind calmodulin. The influence of cadmium might, therefore, be exerted at this level. Further research at the molecular level is required to disclose the cellular mechanism of cadmium toxicity in *M. edulis*.

Induction of MT-like proteins in the gills of *M. edulis* was observed at any point of the chronic exposure to cadmium. After an initial increase, the rate of their synthesis was maintained at a steady state level from 3 to 11 months of exposure. This corroborates our previous conclusion (Veldhuizen-Tsoerkan *et al.*, 1990b) that the rate of relative thionein synthesis depends on the strength of the metal insult rather than on the actual metal concentration in the tissue.

The cadmium binding capacity of MT-like proteins was not exceeded in the gills up to 11 months of exposure. By contrast, in the same semi-field experiment a spillover of cadmium to the HMW protein fraction was observed for total tissue of mussels after 10 months of exposure (Veldhuizen-Tsoerkan *et al.*, 1991). Interorgan transport of cadmium out of the gills to internal tissues (Roesijadi and Klerks, 1989) will contribute to the fact that this spillover occurs in the other organs. For example, a limited capacity of MT production was found in the digestive gland of *M. edulis* upon 21 weeks of exposure to copper at 25 µg/l (Harrison *et al.*, 1988).

Upon chronic exposure, cadmium continued to be stored in the gills of *M. edulis* mainly in a non-toxic form. This could account for the high adaptive capability of sea mussels to metal stress, as neither the established stress parameters (Veldhuizen-Tsoerkan *et al.*, 1991) nor the protein synthesis in gill tissue (this study) were altered in the semi-field experiment. However, an additional stress, such as anoxia (Veldhuizen-Tsoerkan *et al.*, 1991) or elevated temperature (this study), reveals the animals' vulnerability caused by the chronic exposure to cadmium.

Acknowledgements

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A field study on stress indices in the sea mussel, *Mytilus edulis*: application of the "stress approach" in biomonitoring

M.B. Veldhuizen-Tsoerkan[*], *D.A. Holwerda*[*], *A.M.T. de Bont*[*], *A.C. Smaal*[**] *and D.I. Zandee*[*]

[*]*Department of Experimental Zoology, University of Utrecht, 8 Padualaan, 3584 CH Utrecht, The Netherlands*
and [**]*Tidal Waters Division, Ministry of Transport and Public Works, P.O. Box 8039, 4330 EA Middelburg, The Netherlands*

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Abstract

Sea mussels, *Mytilus edulis*, collected from a relatively unpolluted area of the Eastern Scheldt, were transplanted along contaminated sites of the Western Scheldt for 2½ and 5 months. Several established stress indices were determined such as accumulation of pollutants, adenylate energy charge (AEC) and condition index. Following field exposure, mussels were also subjected to an additional lethal or sublethal stress. The data show that environmental exposure alters the mussel's response to stress, *viz.* aerial exposure and increased temperature, at the organismal (anoxic survival time), biochemical (AEC) and molecular (heat shock protein synthesis) level. The "stress approach" to assessment of environmental contamination appears to be a promising method to disclose early changes in the organism at a stage when conventional parameters (condition index, AEC) remain still unchanged.

Introduction

In the last decades, there is a great concern about the environmental impact of the vast amounts of industrial pollutants discharged into the coastal waters. In the estuary of the Western Scheldt, which is loaded with polluted water of the Scheldt river from Belgium and receives discharges from several Dutch industries, elevated levels of cadmium, copper, zinc, nickel, chromium, lead, PCBs and pesticides like dieldrin and DTTs have been detected (Akkerman *et al.* 1989).

Wide distribution, abundance, sedentary behavior, ease of collection and high ability to accumulate heavy metals and organic xenobiotics have made sea mussels, *Mytilus edulis*, to a sentinel species in the biomonitoring of marine environmental contamination (Stephenson *et al.* 1980, 1982; De Kock 1986; Luten *et al.* 1986; Akkerman *et al.* 1989). However, the consequences of environmental contamination are not yet clear for mussel populations. Evident toxic effects in *M. edulis* have been found at high concentration of pollutants (Poulsen *et al.* 1982; Sunila 1988) but are often difficult to translate to the field situation. Effects of pollutants are usually displayed first at the molecular/biochemical level where the functioning of important macromolecules and biochemical pathways can be affected. At a later stage, the deleterious changes can be manifested at higher biological levels, affecting the ability of organisms to grow, to reproduce or to survive (McCarthy and Shugart 1990). Thus, a set of parameters, ranging from the molecular to the organismal and community level, has to be used to adequately assess the biolo-

gical impact of environmental contamination.

In estuarine areas, the survival of a mussel population largely depends on its ability to tolerate the strong fluctuations of natural environmental factors. This ability can be affected by additional pollutant stress. Our foregoing studies (Veldhuizen-Tsoerkan *et al.* 1990a, 1991a,b) have shown that exposure to cadmium or PCBs altered the mussel's ability to adjust to natural stress(es). Both the anoxic and heat shock response in mussels were affected by short- and long-term exposure to cadmium. The anoxia tolerance was also diminished by chronic exposure to PCBs.

In the present study, sea mussels collected from a relatively unpolluted area were transplanted along contaminated sites in the Western Scheldt. The transplantation method allows to accurately define the exposure time and to eliminate possible differences that could arise from a varying origin of the mussels. Measured parameters ranged from the molecular to the organismal level. Several established stress indices such as accumulation of pollutants, adenylate energy charge and condition index were examined. Following field exposure, mussels were also subjected to natural, lethal or sublethal stress. It appears that field exposure at the contaminated sites alters the response of mussels to natural stress at the molecular, biochemical, and organismal level.

Materials and methods

Animals and animal treatments

Sea mussels, *M. edulis*, were collected in a relatively unpolluted area of the Eastern Scheldt in October 1989. Animals with shell length of 5.1 ± 0.1 cm were selected. The mussels were placed in cages (50 animals per cage) that were attached to buoy systems at four locations in the Eastern and Western Scheldt as shown in Figure 1. Jacoba harbor (Eastern Scheldt) was used as a reference point. The degree of pollution was roughly Walsoorden > Terneuzen > Honte > Jacoba harbor, while the salinity gradient was converse for the first three locations, with Jacoba harbor having about the same high salinity as Honte (Akkerman *et al.* 1989). Field exposure was initiated in November 1989 and terminated in April 1990. In the laboratory, the animals were acclimated for 8 days in aquaria with recirculating sea water at 9°C under a natural light regime. Sea water salinity was 2.8%. Mussels were not fed.

Metal and PCB analysis

Mussels were examined individually for the metal content. Whole tissue was decomposed in 65% (w/v) nitric acid as described previously (Veldhuizen-Tsoerkan *et al.* 1991a). Concentrations of cadmium and other metals were determined by inductively coupled

plasma atomic emission spectrophotometry (ICP-AES). The apparatus (Applied Research Laboratories ARL, type 34000) measures thirty channels simultaneously. An argon plasma (1200 W) was used with a crystal-controlled frequency of 27.12 mHz. The spectrophotometer has a light path of 1 m and the spectrum is scattered by a Rowland grating (1080 lines/mm). The measurement was corrected for background and inter-element effects.

Polychlorinated biphenyls in whole soft tissue were analyzed by the Netherlands Organization for Applied Scientific Research (TNO) as described in Den Besten *et al.* (1990). Eight PCB congeners (CB-52, 87, 101, 105 118, 138, 153 and 180) were measured which represent about 30% of the technical PCB mixture by weight (Den Besten *et al.* 1990). PCB concentration in the tissue is expressed as μg Σ_8PCB/gram total extractable lipid.

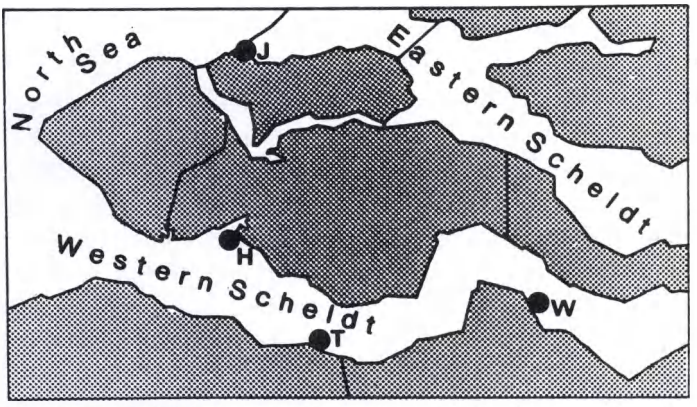


Fig. 1. Map of the Eastern and Western Scheldt. Letters indicate the sites at which mussels were transplanted: Jacoba harbor (J), Honte (H), Terneuzen (T), Walsoorden (W).

Condition index

The index of body condition was determined as formulated by Fischer (1988): CI = 100% x (soft tissue dry weight)/(soft tissue dry weight + shell weight).

Adenylate energy charge

Groups of 7 mussels were subjected to sublethal anoxia by keeping them just above the water surface for 6 h at 12°C. The AEC values were determined in anoxic and 'normoxic' controls (animals taken directly from the aquarium). Processing of soft tissues and determination of the cellular concentrations of ATP, ADP and AMP were carried out as described earlier (Veldhuizen-Tsoerkan *et al.* 1991a). The AEC value is given by the relationship: ([ATP] + ½[ADP]) : ([ATP] + [ADP] + [AMP]).

Anoxic survival test

Groups of 30 mussels were subjected to anoxia. Mussels were exposed to air at 16°C in closed humid boxes. Survival was assessed daily. Death symptoms were considered to be a specific smell, absence of any muscular activity and open valves.

Statistical analysis

The Kaplan-Meier curve estimate was applied in the statistical treatment of the anoxic survival time. Data from other experiments were analyzed for significance of difference with Student's t-test, taking a probability limit $P < 0.05$ as significant.

Synthesis of heat shock proteins

Groups of 6 mussels were subjected to elevated temperature (heat shock) by keeping them in sea water at 29°C for 4 h. Gills were excised from heat-shocked and control mussels (animals kept at 9°C). The middle parts of the outer gill lamellae were incubated with 40 µCi/ml ^{35}S-methionine (specific activity > 1300 Ci/mmol, Amersham) at 11°C for 20 h. The incubation medium, processing of ^{35}S-methionine labelled gill tissue and determination of methionine incorporation into the gill proteins were described previously (Veldhuizen-Tsoerkan *et al.* 1990a,b). Cytosolic ^{35}S-methionine labelled gill proteins were immediately mixed with loading or lysis buffer for electrophoretic analysis (see for details Veldhuizen-Tsoerkan *et al.* 1990a).

One-dimensional gel electrophoresis (SDS-PAGE) of samples containing 30 µg protein was performed on 12.5% polyacrylamide slab gels (ratio of acrylamide to bisacrylamide = 30:0.18) according to Laemmli (1970). Molecular weight markers used were: phosphorylase a (92 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), 3'-phosphoglycerate kinase (47 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21.5 kDa) and cytochrome c (12.5 kDa).

Samples containing 40 µg protein were subjected to two-dimensional gel electrophoresis (IEF/SDS-PAGE) by the methods of O'Farrell *et al.* (1977) and Garrels (1979). The details of IEF/SDS-PAGE of ^{35}S-methionine labelled proteins can be found in preceding studies (Veldhuizen-Tsoerkan *et al.* 1990a,b). The second dimension was performed on 12.5% slab gels. The pH gradient was determined from 1-cm sections of the tube gels, which were incubated in 0.5 ml of 5 mM KCl for 2 h.

Following electrophoresis, the gels were stained, dried and fluorographed as described in Veldhuizen-Tsoerkan *et al.* (1990a). Incorporation of methionine into specific proteins was quantified from gel fluorographs. The integrated optical density (IOD) of protein spots was determined with the IBAS image analysis system (Zeiss/Kontron, Eching, F.R.G.). A detailed description of the image analysis is given in Veldhuizen-Tsoerkan *et al.* (1990a,b). IODs were determined from several gel fluorographs of one gel and exposures within the linear range of the film were used.

Results

Accumulation of pollutants

Of several metals determined only cadmium had accumulated significantly after an exposure period of 2½ months (Figure 2). Cadmium tissue concentration indicated an increasing gradient of metal contamination of inward sites. After 5 months of field exposure the accumulation of cadmium remained essentially unchanged (not shown). Accumulation of polychlorinated biphenyls was measured after 5 months of exposure. Mean tissue concentrations of Σ_8PCB at locations Jacoba harbor, Honte, Terneuzen and Walsoorden were 4.07 ± 1.41, 3.99 ± 1.94, 8.85 ± 2.61 and 9.92 ± 0.40 (mean of 2 samples composed of 3 mussels ± SD) µg PCBs/g lipid, respectively.

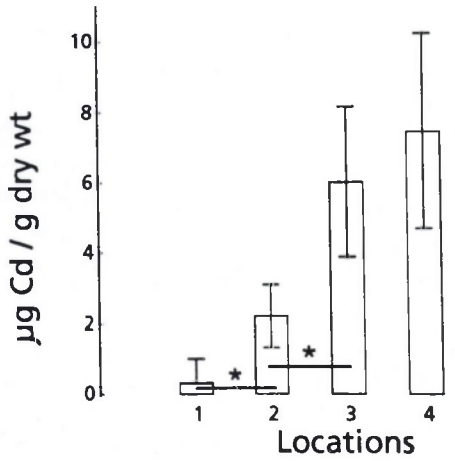


Fig. 2. Cd concentration in whole animal tissues after 2½ months of field exposure at: Jacoba harbor (1), Honte (2), Terneuzen (3), Walsoorden (4). Mean of 5 mussels ± SD; *: $P < 0.001$.

Condition index

Field exposure for 2½ months did not alter the condition index (Figure 3A). After 5 months, a significant decrease of this index was found (Figure 3B). Mussels from the Eastern Scheldt had the highest values of condition index that gradually decreased in mussels transplanted in the Western Scheldt. It is noteworthy that a diminished dry weight of the soft tissues had greatly contributed to the alterations of condition index.

Adenylate energy charge

The adenylate energy charge (AEC) indicates the cellular "energy status" and is often used as an index of sublethal stress.

AEC values were measured in normoxic mussels and mussels subjected to 6 h of

anoxia (Figure 4). Neither 2½ nor 5 months of field exposure altered the AEC values in normoxic animals (Figure 4, A and C). Anoxic animals from one location in the Western Scheldt (Terneuzen) displayed decreased AEC values after 2½ months of exposure (Figure 4B). Five months of exposure resulted in a significant decrease of AEC in anoxic mussels derived from the Western Scheldt locations (Figure 4D). The lowest AEC value was observed in animals that had been transplanted in Terneuzen.

Anoxic survival time

After 2½ months of field exposure the anoxic survival time (LT_{50}'s = 5.7 and 5.1 days) of mussels from the polluted areas (Terneuzen and Walsoorden) was significantly lower than that (LT_{50}'s = 7.6 and 6.6 days) of animals from the reference site in the Eastern Scheldt and the outwards location (Honte) in the Western Scheldt (Figure 5A). Anoxic tolerance of the control animals (Jacoba harbor) was reduced later in the season as LT_{50} became 5.3 days (Figure 5B). This is in good agreement with our earlier observation that the anoxic tolerance of mussels shows seasonal dependence, having the lowest values in the late spring (Veldhuizen-Tsoerkan *et al.* 1991a). Field exposure for 5 months at the Western Scheldt locations caused a dramatic shift of curves towards diminished anoxic survival (Figure 5B). LT_{50} of animals from Honte was 3.1 day, whereas LT_{50}'s of mussels from Terneuzen and Walsoorden were reduced to 2.2 and 1.9 days. Over 10% of animals from the latter groups were hardly able to survive anoxia even for one day.

Synthesis of heat shock proteins

The incorporation of ^{35}S-methionine into gill proteins was studied in mussels under normal and heat shock conditions after field exposure. Field exposure alone neither altered incorporation of methionine nor induced the expression of specific proteins such as heat shock proteins (hsps) (Figure 6A). Heat shock treatment for 4 h induced synthesis of hsps in gills of mussels derived from the relatively unpolluted site (Jacoba harbor) (Figures 6B and 7A). These proteins seem to be produced in an array of charge isomers (Figure 7A-D, protein spots 1, 2 and 4-10). Heat shock imposed *after* field exposure of mussels derived from the contaminated locations in the Western Scheldt revealed alterations in hsp synthesis in gills (Figures 7 and 8, Tables 1 and 2). After 2½ months of exposure, an enhanced expression of several hsps was observed (Figure 7, proteins 7, 9 and 10). The synthesis of other hsps decreased (proteins 1, 4-6 and 8) or showed irregular changes (protein 2). It appears that exposure at the contaminated locations increased the number of charge isomers of low molecular weight (LMW) hsps (Fig. 7, protein 2). Interestingly, protein 3 was synthesized under normal conditions and its expression was repressed by heat shock in control animals (Figures 6B and 7A), but heat-shocked animals from the contaminated locations seemed to increasingly synthesize this protein (Figure 7, C and D). Five months of field exposure enhanced the expression of several LMW hsps (Figure 8, protein spots 1 and 2) and high molecular weight (HMW) hsps (proteins 5, 7-

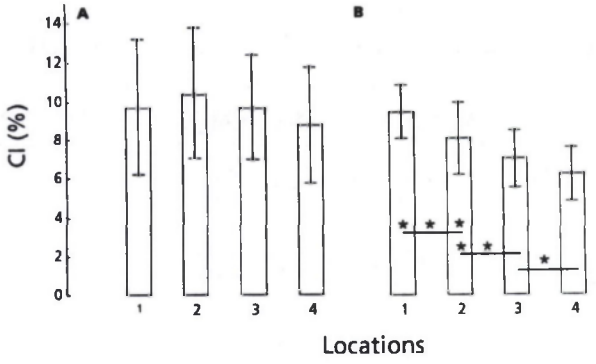


Fig. 3. Condition index of mussels after 2½ months (A) and 5 months (B) of field exposure at: Jacoba harbor (1), Honte (2), Terneuzen (3), Walsoorden (4). Mean of 30 mussels ± SD; *, ** and ***: P < 0.05, 0.01, and 0.001, respectively.

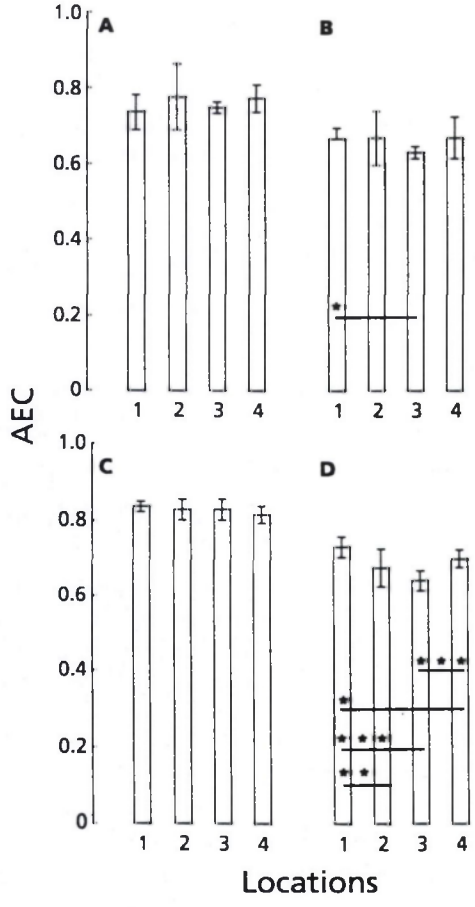


Fig. 4. Adenylate energy charge of mussels after 2½ months (A,B) and 5 months (C,D) of field exposure at: Jacoba harbor (1), Honte (2), Terneuzen (3), Walsoorden (4). A and C: normoxic group; B and D: mussels subjected to 6 h of anoxia. Mean of 7 mussels ± SD; *, ** and ***: P < 0.05, 0.01, and 0.001, respectively.

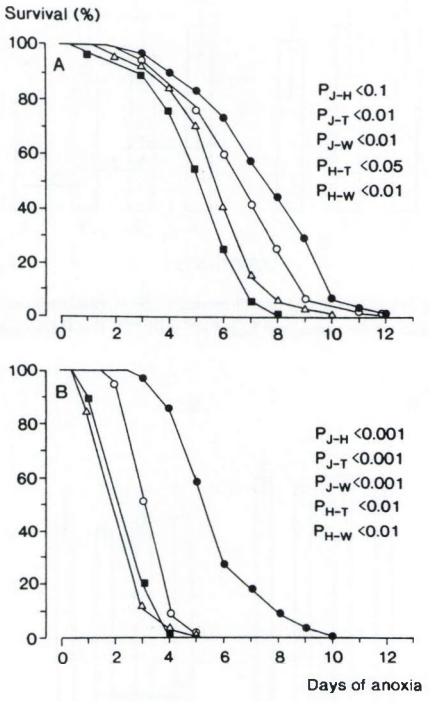


Fig. 5. Anoxic survival time of mussels after 22 months (A) and 5 months (B) of field exposure at: Jacoba harbor (-●-), Honte (-o-), Terneuzen (-Δ-), Walsoorden (-■-). Groups initially consisted of 30 animals.

10). LMW hsps appeared again to consist of a higher number of charge isomers after exposure in the polluted areas (Figure 8, D and C, proteins 1-2). Expression of HMW hsps increased even to a higher degree (proteins 9 and 10). Synthesis of protein 3 displayed changes similar to those observed after 2½ months of exposure, although its expression was stimulated to an even higher extent in animals from the contaminated locations. As noted after 22 months of exposure time, an inhibition of synthesis of hsps of 43 kDa and of 70 kDa was observed after 5 months of exposure (proteins 4 and 6).

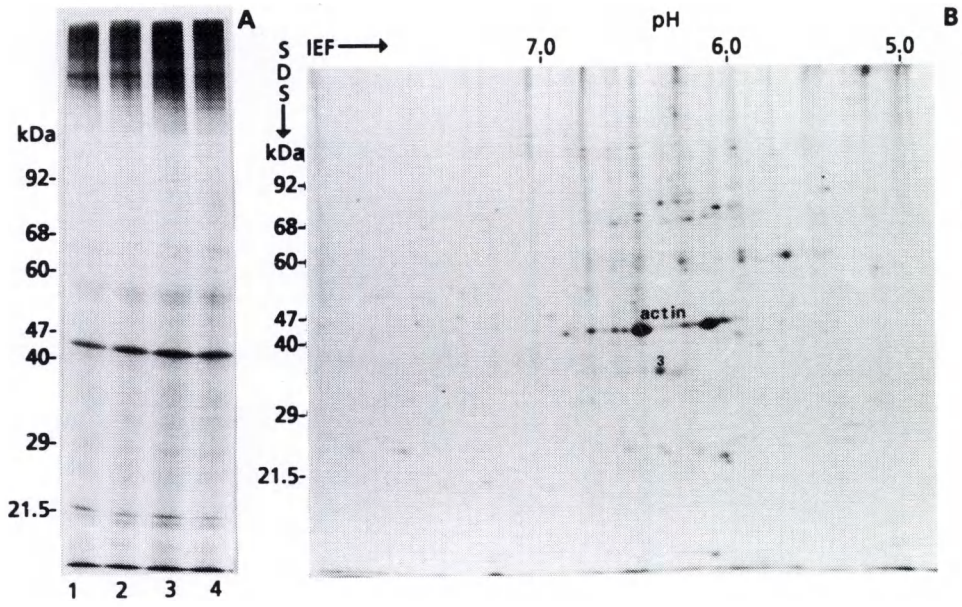


Fig. 6. One- and two-dimensional gel electrophoretic patterns of ^{35}S-methionine labelled gill proteins from mussels after 2½ months of field exposure. A. SDS-PAGE; lanes 1-4: locations Jacoba harbor (1), Honte (2), Terneuzen (3), Walsoorden (4). The position of molecular weight markers is indicated on the left side. B. IEF/SDS-PAGE of gill proteins from mussels transplanted at Jacoba harbor. The pH gradient is shown at the top.

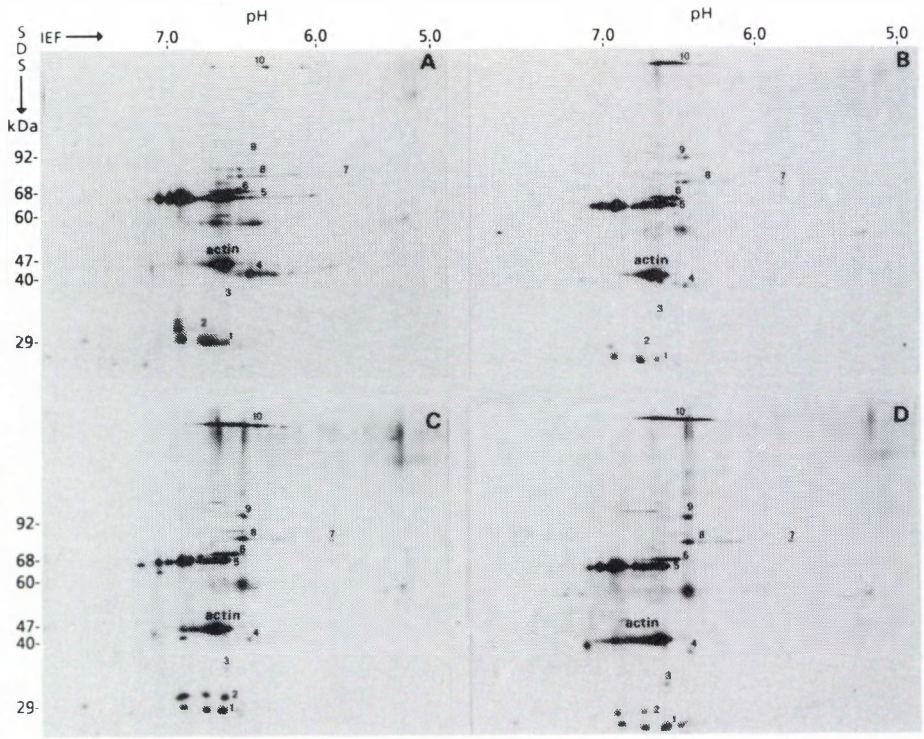


Fig. 7. Two-dimensional gel electrophoretic patterns of ^{35}S-methionine labelled gill proteins from mussels after 2½ months of field exposure, followed by 4 h of heat shock at 29°C. Locations: Jacoba harbor (A), Honte (B), Terneuzen (C), and Walsoorden (D). The position of molecular weight markers is indicated on the left side. The pH gradient is shown at the top. Integrated optical density (IOD) of numbered proteins (arrays of charge isomers) is given in Table 1.

Table 1: Effect of 2½ months of field exposure on the synthesis of heat shock proteins in gill tissue of mussels, heat-shocked at 29°C for 4 h. The integrated optical density (IOD) was determined for protein spots shown in Fig. 7

Protein spot no.	Molecular weight (kDa)	IOD[a]			
		J[b]	H[b]	T[b]	W[b]
1	28	100	29	29	23
2	30	100	23	135	33
3	36	100	248	225	331
4	43	100	16	8	4
5	68	100	55	40	41
6	70	100	46	45	36
7	74	100	161	168	188
8	76	100	60	85	78
9	94	100	44	473	487
10	>100	100	161	278	223

a) IOD values were normalized to an equal IOD of the actin spot, and expressed as percentages of the control.
b) Letters indicate the locations: Jacoba harbor, Honte, Terneuzen and Walsoorden.

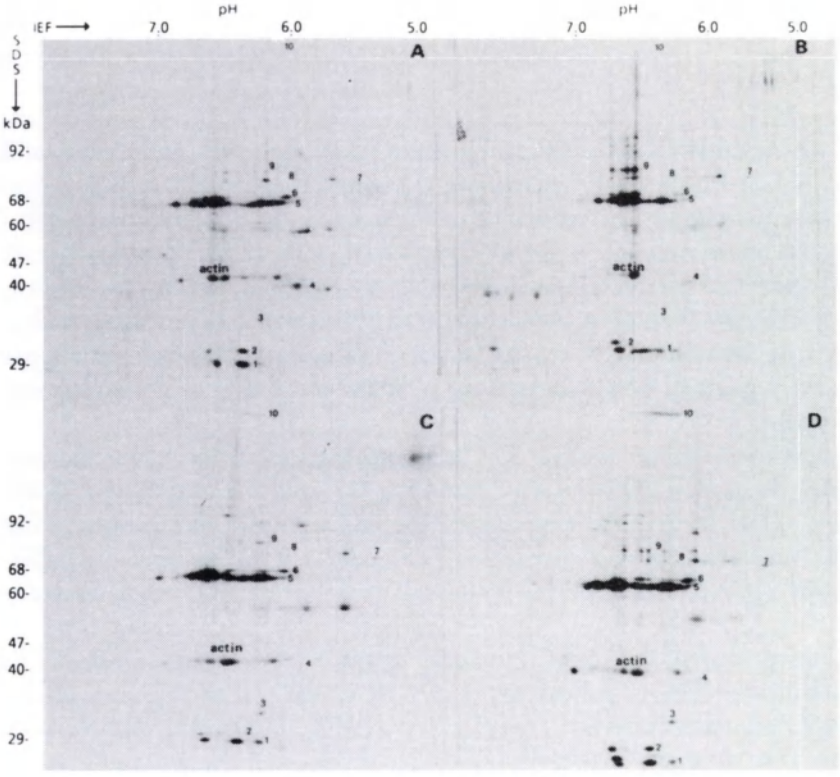


Fig. 8. Two-dimensional gel electrophoretic patterns of ^{35}S-methionine labelled gill proteins from mussels after 5 months of field exposure, followed by 4 h of heat shock at 29°C. Locations: Jacoba harbor (A), Honte (B), Terneuzen (C), and Walsoorden (D). The position of molecular weight markers is indicated on the left side. The pH gradient is shown at the top. Integrated optical density (IOD) of numbered proteins (arrays of charge isomers) is given in Table 2.

Table 2: Effect of 5 months of field exposure on the synthesis of heat shock proteins in gill tissue of mussels, heat-shocked at 29°C for 4 h. The integrated optical density (IOD) was determined for protein spots shown in Fig. 8

Protein spot no.	Molecular weight (kDa)	IOD[a]			
		J[b]	H[b]	T[b]	W[b]
1	28	100	167	72	146
2	30	100	110	39	178
3	36	100	81	412	641
4	43	100	32	4	6
5	68	100	142	107	164
6	70	100	88	35	64
7	74	100	163	86	211
8	76	100	228	67	127
9	84	100	302	164	207
10	>100	100	70	222	1148

a) IOD-values were normalized to an equal IOD of the actin spot, and expressed as percentages of the control.
b) Letters indicate the locations: Jacoba harbor, Honte, Terneuzen and Walsoorden.

Discussion

Field exposure resulted in increased concentrations of pollutants such as cadmium and polychlorinated biphenyls in mussels transplanted at contaminated locations in the Western Scheldt. These findings are in accordance with results of earlier biomonitoring studies of the Dutch coastal waters (De Kock 1986; Luten *et al.* 1986; Akkerman *et al.* 1989). In the Western Scheldt the concentration of heavy metals in the sea water decreased in the direction of the North Sea (Akkerman *et al.* 1989) which is adequately reflected by the cadmium concentration of mussel tissues (Figure 2). By contrast, tissue levels of polychlorinated biphenyls were increased by a factor of 2 only in Terneuzen and Walsoorden locations.

Upon field exposure, changes measured in the physiologic condition of an organism will integrate the effects of all stressors (*e.g.*, pollutants and variable environmental factors) acting on it. Thus, in order to discriminate the pollutant effects, a parameter has first to be elaborated under laboratory conditions. Secondly, a set of parameters at different levels must be applied to measure the biological impact of environmental contamination.

In our preceding studies, the "stress approach" appeared to be a useful method to pinpoint the deleterious effects of pollutants (Cd, PCBs) in short- and long-term laboratory exposure experiments (Veldhuizen-Tsoerkan *et al.* 1990a, 1991a,b). This method is based on the idea that pollutants may affect the special mechanism that mussels have acquired to survive environmental fluctuations in the tidal zone of coastal and estuarine waters. Moreover, this approach fits one of the important criteria of stress index which has to display a detrimental effect on the organism's capacity to resist environmental changes (Bayne 1980).

Anoxia tolerance (or ability to survive exposure to air) and the underlying biochemical mechanism are well-studied in *M. edulis* (Zandee *et al.* 1986). Field exposure at the contaminated sites significantly reduced the anoxia tolerance of mussels (Figure 5) although the levels of pollutants (Cd, PCBs) were much lower than those of Cd or PCBs that had been effective under laboratory conditions (Veldhuizen-Tsoerkan *et al.* 1991a). After prolonged exposure, a significant change in the anoxic survival time was observed even between locations (Jacoba harbor and Honte, Figure 5B) where the difference in tissue pollutant content and in environmental conditions, such as food availability and salinity, was minimal (Akkerman *et al.* 1989). The drastic reduction of anoxic survival time (Figure 5B) indicates a diminished ability of the mussel populations in Terneuzen and Walsoorden to cope with natural stress.

Sublethal anoxic stress (6 h) caused an evident decrease in the adenylate energy charge (AEC) values of the animals exposed in the Western Scheldt for 5 months (Figure 4D). AEC has been considered to be an important parameter in the regulation of biochemical pathways related to the energy metabolism (Ivanovici 1980). The declined AEC values confirm the idea (De Zwaan and De Kock 1988) that natural stress and pollution cause an increased energy expenditure. Under laboratory conditions, sublethal anoxic stress resulted in a decrease of AEC in mussels after long-term exposure to PCBs, but not

to cadmium (Veldhuizen-Tsoerkan *et al.* 1991a). As tissue levels of cadmium did not significantly differ and accumulation of PCBs was of similar order in the animals from Terneuzen and Walsoorden, the lowered AEC values (Figure 4, B and D) may imply the presence of other, unidentified pollutants in the Terneuzen area. However, the higher concentration of nutrients in Walsoorden (Akkerman *et al.* 1989) could have counteracted a decrease of AEC value.

When exposed to elevated temperatures, a wide range of cells and organisms respond by synthesizing a limited set of proteins that are believed to provide a tolerance to stress at the molecular level and that, therefore, are called stress or heat shock proteins (hsps) (Schlesinger 1990). As the specific hsp response can be elicited by a number of stresses (Lindquist 1986), its utility as a stress index in the biomonitoring of environmental contamination has already been discussed (Sanders 1990). In *M. edulis*, hsps are synthesized in response to heat or cadmium exposure (Sanders 1988; Veldhuizen-Tsoerkan *et al.* 1990a). Furthermore, short- and long-term pre-exposure to cadmium enhanced the hsp expression during heat shock (Veldhuizen-Tsoerkan *et al.* 1990a, 1991b). Imposing a heat shock following field exposure caused differential alterations of the hsp response in mussels from contaminated locations (Figures 7 and 8), thus emphasizing the complexity of the field impact. The increased synthesis of hsps, especially of those with high molecular weight (proteins 1, 2, 7-10), could be ascribed to the accumulation of cadmium and, perhaps, of other heavy metals. The increased number of charge isomers of low molecular weight hsps (proteins 1 and 2) and the enhanced synthesis of protein 3 are intriguing phenomena, but their interpretation requires further research. Hsp of 70 kDa appears to be synthesized as a general response to stress (Misra *et al.* 1989). Thus, the inhibition of its synthesis (protein 6) upon heat shock could indicate a declined ability of the animals to resist stress after the exposure at contaminated sites. The consistent and striking inhibition of the synthesis of hsp of 43 kDa (protein 4) in the course of field exposure makes it a good candidate for a stress index. Moreover, hsp 43 has previously been found in heat-shocked animals only during the period in which the gonads are developed and especially before spawning (Veldhuizen-Tsoerkan *et al.* 1991b). Therefore, it is tempting to speculate that perturbation of its synthesis upon stress conditions (heat shock) could be related to disturbances in gonad development and/or sexual maturation. However, histological analysis of mussel mantles revealed pathological follicles only in male animals from the Walsoorden location after 5 months of exposure (not shown).

AEC and body condition index are used as parameters to assess the physiological condition of mussels. In the present study neither parameter was altered in normoxic animals by 2½ months of field exposure (Figures 3A and 4A). After 5 months of exposure, AEC values were slightly higher, but did not differ within locations (Figure 4C). By contrast, the condition index indicated a significant, gradient-like decrease in the physiological condition of mussels from the Western Scheldt (Figure 3B). It is noteworthy that neither AEC nor condition index was affected by long-term exposure to cadmium or PCBs (Veldhuizen-Tsoerkan *et al.* 1991a). A similar insensibility of the condition index to cadmium exposure of mussels under laboratory conditions has been reported by Borchardt (1983). However, field exposure at polluted areas significantly decreased condition indi-

ces of mussels (Martin *et al.* 1984; Borchardt *et al.* 1988).

A comparison of field and laboratory data accentuates the complexity of the biological impact of marine environmental contamination. Because of a simultaneous accumulation of different pollutants, the biological responses may be expected to be very complex. Biological effects of pollutants are further complicated by the presence of variable environmental factors that may interact with pollutants, thus modifying their effect. The pollutant stress superimposed on the already existing natural stresses may prove to be more harmful. Recent laboratory studies have indicated that in marine invertebrates a combination of pollutant stress (cadmium or diesel oil) with natural stresses (hypoxia, decreased salinity, elevated temperature) results in severe adverse effects that are induced by neither stressor alone (Tedengren and Kautsky 1987; Johnson 1988; Howard and Hacker 1990).

The present data demonstrate that the "stress approach" to assessment of environmental contamination has both a conceptual and a practical appeal. Field exposure at contaminated sites altered the response of mussels to stress, such as anoxia and elevated temperature, at organismal (anoxic survival time), biochemical (anoxic AEC values) and molecular (hsp synthesis) level. These alterations were detected at an exposure time, when conventional parameters (condition index, normoxic AEC values) still remained unchanged. At the late stage of field exposure, these changes upon stress conditions appeared to be related to a decreased fitness of the mussels at the contaminated sites. Therefore, the "stress approach" may constitute an early warning system and offer some potential for linking the decreased tolerance with aspects of population survival.

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C H A P T E R 9

Final discussion

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The rapid technological progress in our century has on the shadow side resulted in an increasing environmental contamination of global size. The first ecological studies on this subject were soon followed by toxicological approaches as it became apparent that organisms were affected by a range of hazardous waste chemicals. The research on the subject has now moved to the physiological and molecular level in the investigation of the mechanisms through which the toxicants exert their action. The work presented here is an extension of this approach, aimed at the development of sensitive indices to estimate the damage caused to organisms and populations by the contamination of the marine environment. The parameters concerned were devised under laboratory conditions, verified in long-term semi-field experiments and applied in a field study.

It has been emphasized by Bayne (1988) that there are no simple means by which the biological impact of pollution in the marine environment can be measured. In the present study, a set of newly developed and of already established stress indices was employed ranging from the molecular to the organismal level. From this, extrapolation to the population and even ecosystem level could be pursued.

The concept on which our work is based has been the "stress approach". In this method alterations in the response of the sea mussel, *Mytilus edulis*, to two forms of natural stress were utilized as indices of pollution.

Response of the Sea Mussel to Forms of Natural Stress

Mussels in the intertidal zone are liable to variation of environmental conditions such as fluctuation in salinity, food and oxygen availability, and in temperature. Aerial exposure, at low tide, ensues a sustained closure of the valves which is accompanied by oxygen depletion. *M. edulis* can withstand anoxic conditions for days or even weeks. The biochemical mechanism underlying this anoxic tolerance is a strong reduction of the energy demand and an activation of highly efficient pathways of anaerobic energy metabolism (Zandee *et al.*, 1986). In contrast to anoxic vertebrate tissues, the degradation of glycogen as the substrate of anaerobic metabolism leads to the formation of multiple end products, *viz.*, alanine, succinate, propionate and acetate (De Zwaan, 1977). Aerial exposure

for a period of six hours indeed diminished the glycogen content and decreased the adenylate energy charge (AEC) while the level of succinate increased (Chapter 2). The anoxic survival time of mussels was found to be strongly dependent on the season, reaching a maximum value in the fall and being drastically reduced after spawning in late spring (Chapter 2). This is in accordance with the results of Zandee *et al.* (1980, 1986) that seasonal variations in the biochemical composition, linked to the reproductive cycle, modify the anaerobic energy metabolism in *M. edulis*. Furthermore, interaction of different forms of natural stress was observed when elevated temperature reduced the anoxic tolerance of mussels (Chapter 2).

The response of mussels to elevated temperature was examined at the molecular level. *In vivo* heat shock at 30^{0}C for four hours inhibited the overall protein synthesis in the excised gills and induced the expression of heat shock proteins (hsps) with molecular weights of 30, 32, 68, 70, 80, 82, 90 and 110 kDa (Chapter 4). The hsps were produced in an array of charge isomers. Several high-molecular-weight (HMW) hsps were found to be constitutively synthesized in minor amounts in the unstressed cells but their expression was greatly enhanced, especially after a prolonged heat shock. Synthesis of hsps was also observed after a mild *in vitro* heat shock by incubating the excised gills at 20^{0}C for four hours (Chapter 3) although the two-dimensional hsp patterns differed from those induced by the *in vivo* heat shock at 30^{0}C (Chapters 4, 7-8). In *M. edulis*, Steinert and Pickwell (1988) identified three gill hsps (70, 50 and 28 kDa) after 24 hours of *in vivo* heat shock at 25^{0}C or 30^{0}C. Sanders (1988) reported the induction of at least nine hsps in mussel gills already after two hours at 31^{0}C. In Chapter 4, the absence of hsps with molecular weights around 50 kDa is ascribed to possible genetic or seasonal variations. Our subsequent investigations confirmed the latter assumption as synthesis of hsps 40-43 was observed upon heat shock in mussels during sexual maturation and shortly before spawning (Chapters 7-8). These findings led to the speculation that expression of these hsps might be associated with the reproductive cycle of animals as their metabolism also undergoes seasonal changes linked to gametogenesis (Zandee *et al.*, 1980, 1986).

It is worthy to mention that anoxic stress for six hours induced the expression of several HMW hsps (Veldhuizen-Tsoerkan, unpublished data). Spector *et al.* (1986) also observed that anoxia triggered hsp synthesis in *Salmonella typhimurium*. Therefore, the responses to both natural stressors, anoxia and elevated temperature, are evidently interconnected at the molecular level.

Impact of pollutants

Bioaccumulation

Due to their high capacity to accumulate heavy metals and organic micropollutants, mussels are widely used as a sentinel organism in biomonitoring of marine environmen-

tal contamination (De Kock, 1986; Akkerman *et al.*, 1989; Zandee and Herwig, 1990). Numerous data on the accumulation of cadmium and other heavy metals in *M. edulis* under different conditions have been published (Elliott *et al.*, 1986; Luten *et al.*, 1986; Borchardt *et al.*, 1988; Fischer, 1988; Everaarts, 1990). The present studies show that in short-term, laboratory experiments or long-term, semi-field exposure the accumulation of cadmium proceeded in a linear mode in either whole animals or gill tissue (Chapters 2-4 and 7). This process was also found to be dependent on the metal concentration to which the mussels were exposed (Chapters 3-4). Upon field exposure, the cadmium content in the mussel tissues reflected the extent of metal contamination at the exposure sites (Chapter 8).

Accumulation of PCBs in sea mussels under laboratory conditions has been reported by Suteau *et al.* (1987) who suggested that the rapid absorption of organochlorines is mainly conducted through digestive pathways rather than by gill filtration. In the present semi-field experiments mussels were exposed to PCBs by feeding them with PCB-loaded algae. This resulted in a linear accumulation of PCBs in whole soft tissue during 6 months of exposure (Chapter 2). Field exposure caused a significant increase of PCB levels in whole soft tissue of the mussels from the polluted areas (Chapter 8). This is in good agreement with the results of other biomonitoring studies (De Kock, 1986; Martin *et al.*, 1984; Akkerman *et al.*, 1989).

Effects at the organismal level

Cadmium and PCBs are known to be highly toxic pollutants, but their adverse effects in sea mussels are observed at considerably high concentrations of pollutants. For example, body burdens of cadmium up to 150 ppm did not affect the clearance rate, ingestion, assimilation or growth of *M. edulis* (Poulsen *et al.*, 1982).

In our studies, condition indices were determined as a measure of the animals' fitness after semi-field and field exposure. These indices were not altered by long-term, semi-field exposure to cadmium or PCBs (Chapter 2), thus corroborating the idea that mussels are rather insensitive to pollutant insult. By contrast, the simultaneous accumulation of cadmium, PCBs and, most probably, of other pollutants significantly reduced the condition index of mussels after a 5-months exposure at the contaminated sites (Chapter 8). Studies by others have shown that laboratory exposure did not influence the condition index of juvenile *M. edulis* (Borchardt, 1983), whereas field exposure at polluted areas significantly decreased these indices for mussels (Martin *et al.*, 1984; Borchardt *et al.*, 1988). Decrease of condition index upon field exposure may reflect a simultaneous action of several pollutants and/or environmental factors.

Effects at the cellular level

The concept of adenylate energy charge (AEC) indicates the cellular "energy status," and has been proposed as a biochemical index of sublethal stress (Ivanovici, 1980). Decrease of the AEC value has been reported for mussels exposed to high concentrations of cadmium (1-2 mg/l), whereas exposure for four days to a low cadmium concentration (40 µg/l) caused a significant increase of AEC (Viarengo, 1989). The presented data here reveal that the AEC was neither altered by chronic semi-field exposure to cadmium and PCBs (Chapter 2) nor by field exposure at the contaminated sites (Chapter 8).

Glycogen and succinate contents were measured as possible biochemical indicators of pollutant effects on the energy metabolism in *M. edulis*. In the semi-field experiments, the glycogen content was slightly decreased upon exposure to PCBs (Chapter 2), but field exposure only caused irregular changes in the glycogen levels (unpublished results).

Chronic semi-field exposure did elevate the succinate levels in both Cd- and PCB-exposed animals (Chapter 2). This observation supports the idea of Zandee *et al.* (1986) that pollutant stress promotes shell closure in *M. edulis*, thus causing a partial switch to anaerobic metabolism. However, the field results (unpublished) did not indicate significant changes in succinate levels of mussels exposed at the contaminated sites.

It has to be concluded that neither of the above-mentioned biochemical parameters is directly suitable for monitoring the biological impact of environmental contamination.

Molecular effects of cadmium

The effects of cadmium at the molecular level are well established in a variety of organisms and in cultured cells. Cadmium is known to inhibit the synthesis of various macromolecules such as proteins, RNA and DNA (Hidalgo *et al.*, 1976; Norton and Kench, 1976; Ochi *et al.*, 1984; Beattie *et al.*, 1987; Nocentini, 1987; Kishimoto *et al.*, 1990). Cadmium can complex with macromolecules, and thus induces structural changes (Ochi *et al.*, 1983; Koizumi and Waalkes, 1990). The metal can also exert a toxic action by interference with the metabolism of essential divalent cations, especially Ca^{2+} and Zn^{2+}, which play an important regulatory role in the processes of macromolecular synthesis (Vallee and Falchuk, 1981; Sunderman and Barber, 1988; Brostrom and Brostrom, 1990). Alterations induced at this level are believed to underlie the mutagenic (Ochi and Ohsawa, 1983), teratogenic (Swapan *et al.*, 1990) and carcinogenic (Waalkes *et al.*, 1989) effects of cadmium.

In *M. edulis*, short-term exposure to cadmium significantly inhibited the synthesis of proteins in gill tissue (Chapters 3-5). This effect was dependent on the cadmium concentration and could be detected during 14 days of exposure only at a high metal concentration (250 µg/l). An earlier study of Viarengo *et al.* (1980) has shown that short-term exposure to copper at 80 µg/l resulted in a 50-70% decrease of protein synthesis in gills and other organs of *M. galloprovincialis*. The same authors suggested that this decrease in protein synthesis in Cu-exposed mussels can be used as a biochemical index of stress, exerting a detrimental effect on growth and survival. However, our data indicate that nei-

ther long-term, semi-field exposure to a relatively low concentration of cadmium nor field exposure at the contaminated sites could induce an inhibition of protein synthesis (Chapters 7-8).

In the short-term experiments the decreased protein synthesis was associated with an inhibition of RNA synthesis. The latter appeared to be more sensitive to cadmium than the synthesis of proteins (Chapter 5). In the long-term experiments some evidence was obtained that chronic exposure to cadmium had an inhibitory effect on RNA synthesis in gill tissue (unpublished data), but additional examination is required to state this finding.

Cadmium is further known to induce and/or enhance the expression of several types of specific proteins, *viz.*, metallothioneins (Hamer, 1986), heat shock proteins (Lindquist, 1986) and proto-oncogene products (Jin and Ringertz, 1990).

Metallothioneins (MTs) prevent cellular damage by sequestering heavy metal ions (Waalkes and Goering, 1990). Induction of MT-like proteins in *M. edulis* upon exposure to cadmium was demonstrated by George *et al.* (1979) and Frazier (1986). Harrison *et al.* (1988) have indicated that the capacity of *M. edulis* to produce these proteins is limited, thus restricting the organism's ability to tolerate any further increase in metal concentration. Our results confirmed the induction of Cd-binding proteins in whole animal tissue (Chapter 2) and in gills (Chapter 7). In the gills the Cd-binding capacity of MT-like proteins was not exceeded up to 11 months of semi-field exposure, while in the same experiment for whole tissue a spillover of cadmium to the HMW protein fraction was found after 10 months of exposure. This discrepancy could be explained by transport of cadmium from the gills to the internal organs. The Cd-induced synthesis of MT-like proteins was readily detected by ^{35}S-cysteine labelling of the excised gills (Chapters 3-5 and 7). These cysteine-rich, low-molecular-weight (LMW) proteins consist of two (Chapter 3) or three (Chapter 7) charge isomers with low isoelectric points. At the initial stage of exposure to cadmium, the rate of MT synthesis increased linearly with exposure time and metal concentration (Chapter 4), but remained unchanged during prolonged time of chronic exposure (Chapter 7). Induction of MT-like proteins depended on the synthesis of polyadenylated RNA, although the putative presence of inactive MT mRNPs was considered (Chapter 5).

Short-term exposure to cadmium induced the expression of several HMW heat shock proteins (hsps) (Chapter 4). HMW hsp families (hsp 60, hsp 70 and hsp 90) provide tolerance to a stress by preserving and recovering the functions of various protein complexes (Tomasovic, 1989; Schlesinger, 1990). Induction of hsps has been suggested to be a measure of chemical cytotoxicity (Edwards *et al.*, 1990), although the hsp response to stress is thought to be of a transient nature (Heikkila *et al.*, 1982). In fact, long-term exposure of mussels to cadmium did not induce hsp expression (Chapter 7).

An enhanced expression of the proto-oncogene c-*fos* was observed in response to acute exposure to cadmium (Chapter 5). Cadmium has been shown to activate the transcription of other proto-oncogenes, c-*myc* and c-*jun*, in rat myoblasts (Jin and Ringertz, 1990). The conceivable mechanism of activation of these genes by cadmium and the role of proto-oncogene products are discussed in Chapter 5. Interestingly, Sunderman and Barber (1988) have identified Zn^{2+} finger-loop domains in several transforming oncproteins (*e.g.*, *myc*, *src*, *fms*). These authors suggested that Zn^{2+}-binding sites in the finger

loops are potential targets of metal toxicity, and that replacement of Zn^{2+} by Cd^{2+}, Ni^{2+} or Co^{2+} in these loops of onc-proteins may form the molecular mechanism for a metal-induced carcinogenesis. Activation of c-K-*ras* oncogene has been observed in livers of the winter flounder, *Pseudopleuronectes americanus*, from polluted areas of Boston harbor (McMahon *et al.*, 1990). Results from a single experiment indicated that chronic exposure to a low concentration of cadmium for three months caused a 1.5-fold increase of c-*fos* mRNA (Veldhuizen-Tsoerkan, unpublished data). Thus, activation of proto-oncogenes could be used for assessment of detrimental repercussions of field exposure in which animals are subjected to complex mixtures of toxic chemicals.

The cadmium-induced alteration in the phosphorylation state of gill proteins is perhaps one of the most intriguing results of our investigations (Chapter 6). Protein phosphorylation is involved in the regulation of a large number of important cellular processes. As protein phosphorylation is often mediated by Ca^{2+}, the observed changes may signify an interference of Cd^{2+} with the metabolism of calcium. Cadmium has been shown to substitute for Ca^{2+} on calmodulin, a ubiquitous Ca^{2+}-binding protein, that promotes many Ca^{2+}-dependent effects (Chao *et al.*, 1984; Suzuki *et al.*, 1985). Sutoo *et al.* (1990) postulated that the inability of calmodulin to distinguish between Ca^{2+} and Cd^{2+} underlies the toxicity of cadmium. Stimulation of the Ca^{2+}/calmodulin-dependent protein phosphorylation by cadmium has indeed been reported in several *in vitro* systems (Mazzei *et al.*, 1984; Behra and Gall, 1991).

Changes in the degree of protein phosphorylation could provide a plausible explanation of how cadmium triggers a specific protein response. For example, the induction of hsp expression under stress conditions involves phosphorylation of a heat shock gene transcription factor (Sorger and Pelham, 1988; Larson *et al.*, 1988), while an increased phosphorylation of several proteins is associated with the expression of proto-oncogenes (Rozengurt, 1986).

When considered together, the effects of cadmium at the molecular level imply that the metal, at least at an initial stage of exposure, may act via the pathways of a general stress response. A number of similar changes occur in the heat-stressed cell. Heat shock typically triggers an inhibition of total protein synthesis, expression of hsps, and alterations in the phosphorylation state of proteins (Schlesinger, 1990). Moreover, a direct relation has been found between induction of c-*fos* mRNA and inhibition of protein synthesis by heat shock (Tuijl *et al.*, 1991). Heat shock also induced a twofold increase in the rate of synthesis of c-*myc* protein (Lüscher and Eisenman, 1988).

Effects of Pollutants under Stress Conditions

The tolerance of *M. edulis* to forms of natural stress may be challenged by pollution. The toxic effect of contaminants could cause a significant deterioration of the ecological fitness of populations in terms of surviving natural stress, and could, therefore, upset the natural ecological balance.

Laboratory studies have established that the combination of pollutant-induced and natural stress exerts deleterious effects in marine invertebrates (Tedengren and Kautsky,

1987; Johnson, 1988; Howard and Hacker, 1990). The present studies examined the alterations in the response of *M. edulis* to natural stress, induced by preceding exposures to pollutants.

Anoxia

In these experiments, the life span of mussels was limited to the survival time at aerial exposure. Short-term pre-exposure to cadmium reduced the anoxic survival time in a time-dependent manner (Chapter 2). Long-term exposure to cadmium or PCBs also diminished the ability of mussels to survive aerial exposure, although the PCB effect was delayed (Chapter 2). The "stress approach" by anoxia was successfully applied in the field study (Chapter 8). A decreased anoxic tolerance of mussels was already manifested after 2½months of exposure at the contaminated sites in the Western Scheldt. The ease of measurement and the practicability of this method must be emphasized.

Sublethal anoxic stress of six hours of aerial exposure was applied to look for disturbances in energy metabolism possibly underlying the reduction of anoxic survival time by pollutants. In the semi-field experiments the observed changes in the anoxic values of glycogen and succinate caused by exposure to cadmium or PCBs (Chapter 2) were not significant. So, as yet, available data do not permit to assign the diminished anoxic tolerance to disturbed (anaerobic) pathways. The AEC value of 6-hours anoxic animals did not respond to chronic cadmium exposure. Thus, at least in the first hours of anoxia, these animals were able to maintain the normal cellular energy status despite of possible changes in the anaerobic metabolism. At the same time, PCB-exposure (for 6 months) clearly affected the control of energy status during anoxia (Chapter 2). This fact and the delay of the PCB effect on the anoxic survival time (as compared to the cadmium effect) reflect the different mechanisms of action of the two toxicants.

The AEC of anoxic animals did respond to field exposure (Chapter 8). Whereas normoxic AEC values were independent of the site of exposure, the anoxic ones indicate a decrease of animal fitness along the west-east gradient in the Western Scheldt.

Heat shock

Changes in the response of *M. edulis* to elevated temperature were investigated at the molecular level. Pre-exposure to cadmium followed by mild *in vitro* heat shock enhanced the expression of several HMW proteins (Chapter 3) which were identified as hsps (Chapter 4). *In vivo* heat shock, imposed after short-term or chronic exposure to cadmium, resulted in an increased synthesis of hsps (Chapters 4 and 7). An increase of 4 to 17 times was established for the individual HMW hsps.

Heat shock is known to elevate the level of cytosolic free Ca^{2+} and to induce Ca^{2+}/calmodulin-dependent processes (Wiegant *et al.*, 1985; Stevenson *et al.*, 1986; Landry *et al.*, 1988). Evans and Tomasovic (1989, 1990) have shown that some hsps are calmodulin-binding proteins, and suggested that a perturbation of the calmodulin interaction with its binding proteins may contribute to cell death at increased temperature. In sea

mussels, exposure to elevated temperature increased the total Ca^{2+} (Viarengo *et al.*, 1988) and Cu^{2+} (Simkiss and Watkins, 1988) contents, while oscillating temperatures caused a change in the subcellular distribution of Zn^{2+} (Simkiss and Watkins, 1988). Although experiments were not conducted on the subject, it is tempting to speculate that upon heat shock alterations in the state of non-toxic, protein-bound cadmium could modify the hsp response. In this respect, a study of the subcellular distribution of cadmium during heat shock is of great interest, especially in animals after chronic exposure to the metal.

Imposing of *in vivo* heat shock following field exposure induced differential changes in the hsp response in mussels from contaminated sites (Chapter 8). An enhanced expression of several hsps occurred that was ascribed to accumulation of cadmium in the animals, although a decisive influence of other pollutants and/or natural factors can not be excluded. The synthesis of two hsps (hsp 43 and hsp 70) displayed changes similar to those of the condition index and anoxic AEC values that were observed at the later stage of field exposure. It is suggested that the inhibition of expression of hsp 70 can reflect a declined resistance to stress in mussels from the contaminated sites, whereas the decreased synthesis of hsp 43 may be related to perturbations of gonad development caused by field exposure (Chapter 8).

In conclusion, the described "stress approach" is a sensitive method to reveal the impact of pollutants in *M. edulis*. This approach pinpoints changes in the animal, at different levels of organization, at stages of exposure when more conventional parameters still remain unchanged and may, therefore, constitute an early warning system. The method can be recommended for mussel biomonitoring of the contamination in the marine environment.

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Samenvatting

Samenvatting

De zeemossel, *Mytilus edulis*, heeft een groot vermogen om zware metalen en organische verbindingen als PCB's, PAK's e.d. op te hopen. In de regel leidt dit tot veel hogere concentraties van genoemde stoffen in het dier dan in het omringende water. Het maakt deze sessiele dieren uitermate geschikt om de concentratie en bio-beschikbaarheid van chemische verontreinigingen over langere tijd te vervolgen. In internationale "Musselwatch" programma's worden mosselen gebruikt als indicator organisme om de mate van vervuiling vast te stellen. Ook in de Nederlandse situatie wordt de zeemossel door beheerders van estuaria en kustwateren veelvuldig ingezet voor de bewaking van de waterkwaliteit.

Minder duidelijk is of de zeemossel ook geschikt is om de *gevolgen* van verontreinigingen te registreren. Zijn er indicatoren die, in een vroegtijdig stadium, aangeven dat het fysiologisch functioneren van de dieren nadelig beïnvloed wordt als gevolg van verontreiniging?

De zeemossel komt onder andere voor in de getijdenzone. In deze habitat zijn de dieren blootgesteld aan fluctuaties in abiotische factoren zoals het droogvallen bij eb en schommelingen in temperatuur en saliniteit. Mosselen zijn aangepast aan deze situatie, en vertonen een grote mate van tolerantie voor deze natuurlijke vormen van stress.

In het hier beschreven onderzoek is door middel van laboratoriumproeven, semiveld- en veldexperimenten getracht om inzicht te verkrijgen in de effecten van cadmium en, zij het in mindere mate, van PCB's op het fysiologisch functioneren van de mossel. Hierbij stond ons vooral het vinden van gemakkelijk te hanteren stressparameters voor ogen, om daarmee schade aan de dieren door cadmium- en PCB-verontreiniging in een vroeg stadium te kunnen onderkennen. Het onderzoek vond zowel plaats op het niveau van het hele organisme als op cellulair en moleculair niveau.

Uit de te bespreken resultaten zal blijken dat het hanteren van één, enkelvoudige parameter niet voldoende is. Wordt daarentegen een combinatie van parameters toegepast, in onze studie de stress-benadering genoemd, dan blijken de effecten van verontreiniging op de fysiologische toestand van de mossel goed te volgen te zijn. Een duidlijke combinatie bestaat uit het onderwerpen van de met toxicanten belaste dieren aan een vorm van natuurlijke stress.

Het onderzoek omvatte zowel acute belasting van de mossel met cadmium als ook

chronische belasting met cadmium of PCB's. In het eerste geval werd bij een belastingsduur tot 4 dagen een statisch systeem gebruikt met relatief hoge cadmiumconcentraties (tot 500 µg/l). In het tweede geval vond de belasting (tot 11 maanden) plaats in een doorstroomsysteem, de zogenoemde semi-veld situatie, met een relatief lage concentratie cadmium dan wel PCB's. De resultaten werden gevalideerd in een veldsituatie. Hierbij werden mosselen die waren opgevist uit de betrekkelijk schone Oosterschelde enkele maanden lang uitgezet in de Westerschelde.

Van cadmium is bekend dat het aspecifieke interacties met macromoleculen aangaat waardoor deze hun functie kunnen verliezen. Daarnaast berust de toxiciteit van cadmium, als Cd^{2+}, in belangrijke mate op z'n gelijkenis met essentiële kationen, in het bijzonder die van calcium en zink, welke beide een regulerende rol in de cel vervullen. De genoemde verschijnselen leiden tot verstoring van de normale cellulaire functies, wat kan resulteren in diverse ziektebeelden zoals de beruchte Itai-itai ziekte, nierbeschadiging en kanker bij de mens en andere hogere zoogdieren.

Ons onderzoek heeft aangetoond dat bij kortdurende belasting van mosselen met cadmium de synthese van belangrijke macromoleculen, te weten eiwitten en RNA, wordt geremd. Tegelijk neemt toe de synthese van twee specifieke eiwitgroepen die beide de cel beschermen tegen de schadelijke gevolgen van stress, en die daarom stressproteïnen genoemd worden (Hoofdstukken 3 - 5).

De eerste groep van stressproteïnen wordt gevormd door de metallothioneinen (MT's). Dit zijn laagmoleculaire eiwitten met een hoog gehalte aan cysteine, waardoor ze zwaar-metaalionen kunnen binden en aldus voor de cel onschadelijk maken. De inductie van MT's en hun synthese-snelheid zijn afhankelijk van de cadmiumconcentratie in het medium (Hoofdstuk 3 en 4). Na langdurige belasting (10 maanden) van dieren onder semi-veld condities blijkt echter het cadmiumgehalte in de hoogmoleculaire eiwitten, het potentieel toxische cadmium dus, verhoogd te zijn (Hoofdstuk 2).

Een tweede groep door cadmium geïnduceerde eiwitten zijn de "heat shock" proteïnen (HSP's). HSP's worden door alle organismen aangemaakt als gevolg van een "heat shock". Ze vormen een groep eiwitten die sterk in grootte verschillen (ca. 20 - 100 kDa). De belangrijkste functie van deze HSP's is waarschijnlijk bescherming van andere eiwitmoleculen en herstel daarvan wanneer deze tengevolge van hitte schok beschadigd zijn. In de zeemossel worden HSP's zowel bij temperatuurverhoging gesynthetiseerd als bij acute blootstelling aan cadmium.

Kortdurende blootstelling aan cadmium leidt voorts tot verhoogde expressie van het proto-oncogen c-*fos* (Hoofdstuk 5). Dit proto-oncogen behoort tot een familie van genen waarvan de produkten het aan- en uitzetten van andere genen, betrokken bij de celdeling, beïnvloeden. Verhoogde expressie van deze genen kan leiden tot celtransformaties.

Een andere waarneming betreft de door cadmium geïnduceerde verandering in het fosforylerings-patroon en de fosforylerings-graad van eiwitten in de kieuw (Hoofdstuk 6). Waar fosforylering meestal tot stand komt via Ca^{2+}-ionen is dit waarschijnlijk een gevolg van het onvermogen van het calmoduline-systeem om tussen Ca^{2+} en Cd^{2+} te discrimineren.

Evaluatie van de genoemde fenomenen doet vermoeden dat cadmium in de eerste fase van belasting een reactie oproept die sterk op de "heat shock" respons lijkt.

Zeemosselen zijn aangepast om te kunnen reageren op de snelle veranderingen in de getijdenzone zoals fluctuaties in temperatuur, in zuurstof- en zoutgehalte. De overleving van mosselpopulaties is dus afhankelijk van het goed functioneren van de aanpassingsmechanismen. Er wordt in de literatuur verondersteld dat de door toxicanten veroorzaakte stress dieren kwetsbaar zou maken voor het ondergaan van natuurlijke stress. Inderdaad, worden beide stressors gecombineerd, bijvoorbeeld kortdurende blootstelling aan cadmium gevolgd door een temperatuurverhoging, dan blijkt de synthese van HSP's afhankelijk van de cadmiumconcentratie te stijgen (Hoofdstuk 4). Na chronische belasting met een lagere cadmiumconcentratie wordt deze HSP-vorming niet waargenomen (Hoofdstuk 7). Toch heeft wel degelijk een verandering op moleculair niveau plaatsgevonden, want wanneer aldus belaste dieren blootgesteld worden aan verhoogde temperatuur treedt een duidelijk hogere synthese van HSP's op dan in onbelaste dieren (Hoofdstuk 7). In de veldstudie bleek dat de dieren die uitgezet waren op verontreinigde lokaties in de Westerschelde een veranderde respons op temperatuursverhoging te zien gaven (Hoofdstuk 8).

Mosselen kunnen blootstelling aan de lucht dagenlang, tot soms weken overleven. Ons onderzoek heeft aangetoond dat kortdurende belasting met cadmium een significante verkorting van deze anoxische overlevingstijd veroorzaakt (Hoofdstuk 2). In semi-veld experimenten was de anoxische overlevingstijd eveneens verlaagd na chronische belasting, zowel met cadmium als met PCB's (Hoofdstuk 2). De toepassing van deze benadering in het veld bleek een gunstige methode te zijn om vroegtijdig de verslechterde conditie van mosselen aan te tonen, terwijl een andere, meer klassieke stressparameter als de conditie-index in die periode nog niet veranderd was (Hoofdstuk 8).

Het onderzoek heeft duidelijk gemaakt dat mosselen een belasting met cadmium tot op zekere hoogte zonder aanwijsbare schade kunnen doorstaan, tenzij de dieren tevens worden blootgesteld aan, overigens natuurlijke, stress-condities als verhoogde temperatuur en droogliggen. Een belangrijke fysiologische parameter als de "adenylate energy charge" (AEC) bijvoorbeeld bleef zowel in de semi-veld experimenten als in de veldstudie zeer constant (Hoofdstuk 2 en 8). Tegelijk blijkt uit onze studie dat het de tolerantie is die tengevolge van pollutie-stress vermindert. De weerstand van het dier tegen een natuurlijke stress wordt aangetast door belasting met toxicanten: de respons op temperatuurverhoging is veranderd; de daling van de AEC-waarde bij anoxie is groter en daarmee ongunstiger; de overlevingstijd onder anoxie is bekort bij de met toxicanten belaste dieren.

Samenvattend kunnen we stellen dat chemische verontreiniging het tolerantiegebied versmalt en daarmee de overlevingscapaciteit van de dieren vermindert. De "stressbenadering" toegepast op de mossel verschaft een goede en algemene methode om de gevolgen van verontreiniging vast te stellen. Deze methode zou wellicht ook benut kunnen worden voor andere diersoorten, in hetzelfde of in andere biotopen, die men zou willen gebruiken om de invloed van vervuiling na te gaan.

Nawoord

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Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 16 februari 1954 te Kiev (USSR). In 1971 behaalde zij het VWO diploma (cum laude) aan de Engelse School No.49 te Kiev. In 1978 studeerde zij af (cum laude) aan de Staatsuniversiteit te Kiev, faculteit Biologie, afdeling Biochemie. Van 1978 tot 1980 was zij werkzaam als ingenieur-biochemicus bij de afdeling "Biochemie van lagere planten", Academisch Instituut der Plantkunde te Kiev.

In 1981 heeft zij het verzoek ingediend om toegelaten te worden tot de doctoraalfase van de studierichting Biologie aan de Rijksuniversiteit te Utrecht. Na het afleggen van negen toelatingsexamens in 1982, kon zij beginnen met de doctoraalstudie Biologie. Het doctoraalexamen werd afgelegd op 25 augustus 1986 (cum laude) met als hoofdvakken Scheikundige Dierfysiologie en Moleculaire Celbiologie, en als bijvak Celbiologie (richting electronenmicroscopie).

Van oktober 1986 tot oktober 1991 was zij als toegevoegd onderzoeker werkzaam bij de vakgroep Experimentele Dierkunde (projectgroep Aquatische Toxicologie) van de Rijksuniversiteit te Utrecht, waar het in dit proefschrift beschreven onderzoek werd uitgevoerd.