Cadmium accumulation and Cd-binding proteins in marine invertebrates – a radiotracer study

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Abstract

Tissue and subcellular accumulation of cadmium were studied in different tissues of three marine invertebrates (blue mussel *Mytilus edulis*, the tunicate *Ciona intestinalis* and the sea star *Asterias rubens*) using radioactive ^{109}Cd as a tracer. The organisms were exposed to 0.05, 2 and 50 µg Cd L^{-1} for 21 days. Quantitative data were obtained by dissecting, weighing and subsequently measuring radioactivity in organs and tissues. Differences between each exposure and each tissue with regard to the amount of radioactivity and metallothionein (MT) content were evaluated. Obvious interspecies differences in Cd accumulation were observed, as well as differences between tissues of the three species. The highest concentrations of Cd in all exposure treatments were found in the hepatopancreas of *M. edulis* and body wall of *A. rubens*. Taking all treatments into account, Cd accumulation in the tunic of *C. intestinalis* was high compared to other tissues from this species. Over 60% of Cd was present in the S50 fraction in all treatments in all three species. Metallothionein levels were increased at the highest Cd-exposure in all species and tissues, except in branchial pharynx of *C. intestinalis* where the highest MT level was reached following exposure to 2 µg Cd L^{-1}. The most surprising finding was that even the lowest Cd exposure concentration (0.05 µg Cd L^{-1}) caused MT induction in pyloric caeca of *A. rubens*, but there was no dose-dependent increase in MT at higher exposure levels.

Key words: blue mussel *Mytilus edulis*, sea star *Asterias rubens*, sea squirt *Ciona intestinalis*, cadmium, metallothioneins

Introduction

Cadmium is a heavy metal of high environmental concern due to its high toxicity, general usage pattern, industrial production and emissions from fossil fuel combustion. Although it is present in the seawater at a trace levels it is readily accumulated by marine invertebrates. Severe cellular damage may result from uptake of the Cd^{2+} ion in the tissues of marine organisms. One of the most important mechanisms of effect is through substitution of essential cations (Zn^{2+} and Cu^{2+}), which serve as co-factors in a number of enzymes. Other intracellular ligands acting as Cd-binding sites are low molecular mass proteins such as metallothioneins (MTs). Metallothioneins are present in a range of aquatic organisms and are important in the response of an organism to Cd exposure (Roesijadi, 1994). They may be involved in the detoxification of Cd ions entering an organism, but most importantly regulate intracellular availability of essential metals such as Zn and Cu (Viarengo and Nott, 1993). Molluscs, crustaceans and other marine invertebrates are known to accumulate high levels of heavy metals in their tissues and yet survive in polluted environments (Rainbow, 1997). Their tolerance of high tissue levels of the metal at least partly depends on the ability of these organisms to regulate the heavy metal cation concentration inside the cell and to accumulate excess metal in non-toxic forms (Rainbow, 2002).

To assess the toxicity of a metal it is necessary to determine both the transfer of a metal from the medium to the organism and the effects. Blue mussels (*Mytilus spp.*) have been used extensively as bioindicators in biomonitoring studies. Mussels generally accumulate and tolerate high levels of heavy metals, including cadmium, and consequently they have often been chosen as indicator organisms for pollutants (Goldberg et al., 1978). In order to have a more comprehensive picture of the impact of contaminants, organisms at other trophic levels, or belonging to other taxa with different physiology, need to be studied (e.g. Coteur et al., 2003; den Besten et al., 2001).

The sea star *Asterias rubens* (Echinodermata, Asteroidea) is an interesting test organism because it is a key predator in coastal marine food chains and it appears to be a valuable bioindicator of spatial and temporal trends of Pb and Cd contamination in the field. Also, in this species it appears possible to differentiate a long-term bioindicator (skeleton) from a short-term bioindicator (pyloric caeca) of Cd exposure (Temara et al., 1998).

The sea squirt *Ciona intestinalis* (Chordata, Urochordata, Ascidiacea) is widely distributed, shallow water, solitary, sessile filter feeder. Its body is coated by a cellular exoskeleton (tunic) that is soft and gelatinous and made of a fibrous network containing mucopolysaccharides with blood vessels passing through the posterior pedicel. The branchial pharynx is a sac-like structure that occupies most of the interior part of the body. It is considered as serving the dual roles of a food collecting apparatus and a site of gaseous exchange with the water. The pharynx walls are thick and perforated by abundant tiny oval stigmata. The simple heart, the gonads (in adult specimens), the short oesophagus, the stomach and the tubular intestine are placed in the abdominal part of the organism. The stomach walls are covered with orange glandular tissue which forms a simple liver or hepatic organ (Goodbody, 1974).

Research on tunicates has shown that these organisms selectively accumulate certain trace elements from the marine environment. The high concentration factors found for some elements (iron, cobalt, zinc, selenium, vanadium) support the use of tunicates as models in environmental studies of trace metals (Papadopoulou and Kanias, 1977).

There is limited data on cadmium accumulation in tunicates in general and *C. intestinalis* in particular. Some data does however exist on the toxicity of Hg, Cu, Cd and Cr to early developmental stages of *C. intestinalis* (Bellas et al., 2001).

The present study gave an opportunity to compare accumulation of cadmium in different tissues and organs of marine organisms at different trophic levels, as well as compartmentalisation of this toxic metal in different subcellular fractions in those species.

The objectives of the study were:

- to clarify tissue-, species- and concentration-dependent accumulation of cadmium
- to quantify the response to elevated Cd concentrations using metallothioneins

Three concentrations of Cd in seawater were used. The lowest exposure concentration of dissolved Cd (0.05 μg Cd L^{-1}) corresponds to background level in seawater, the intermediate exposure concentration (2 μg Cd L^{-1}) corresponds to highly polluted seawater, while the highest exposure concentration (50 μg Cd L^{-1}) is encountered only very rarely in nature. In addition, a control group was exposed to filtered seawater, not spiked with Cd (see below).

Materials and Methods

Organisms and exposure experiments

Blue mussels (*Mytilus edulis*, shell length 48-71 mm), sea stars (*Asterias rubens*, arm length 35-50 mm), and sea squirts (*Ciona intestinalis*, length 38-95 mm) were collected in the outer Oslofjord near NIVA's marine research station Solbergstrand. The organisms were held in seawater of salinity 35 from 40 m depth in the outer Oslofjord. Temperature for maintenance and exposures was 10°C. The organisms were acclimated to the holding conditions for 1 week before the start of the experiments.

Groups of 3 organisms were separately exposed to various concentrations of waterborne cadmium: 0.05 μg Cd L^{-1}, 2 μg Cd L^{-1} and 50 μg Cd L^{-1} containing ^{109}Cd (0.12 MBq L^{-1}, 0.0045 μg ^{109}Cd L^{-1}; added as a tracer) for 21 days. A fourth group was kept in clean seawater (control, *i.e.* without the addition of ^{109}Cd). Prey species (*M. edulis, C. intestinalis*) and predator species (*A. rubens*) were hold in the separate aquaria, designated Aquaria I and Aquaria II, respectively (Table 1). The organisms were not fed during the exposure period. Radioactive cadmium in $^{109}CdCl_2$ form (1.25 μgCd mL^{-1}, 32.56 MBq mL^{-1}) was purchased from Amersham, UK. Three replicate aquaria were used for control and each treatment. Exposure solutions were changed once (after 9 or 11 days of exposure). Stock Cd-solutions were prepared using $Cd(NO_3)_2 \times 4H_2O$ (p.a., Merck, Germany), which was dissolved in filtered seawater (salinity 35) collected from 40 m depth in the outer Oslofjord, and solution of radioactive ^{109}Cd was added to make the final activity of 0.12 MBq L^{-1} in the aquaria. The concentrations of Cd in aquaria during the exposure period are provided in Table 1. It is assumed that Cd concentrations in water are equated with nominal concentrations, *i.e.* that the isotope behaves as stable Cd. Cadmium concentrations in water were calculated using the relationship between known Cd concentration in Cd-stock solutions and respective γ-activity of water sample.

Determination of ^{109}Cd tissue distribution

Quantitative data were obtained by dissecting and weighing organs and tissues from three organisms per aquarium and by measuring radioactivity in organs and tissues. At the end of the exposure period, the organisms were rinsed in clean water and dissected. Tissues were snap frozen in liquid nitrogen, transported to laboratory and stored at -80°C. The analyses were completed within 4 months. Each organism from each exposure group was analysed individually. Gills, digestive gland (hepatopancreas), mantle, muscle and foot were dissected from blue mussels. Three body compartments were dissected from sea stars: pyloric caeca,

body wall, and the remaining tissues (mainly skeleton). Branchial pharynx, intestinal part (containing stomach, digestive gland and intestine) and tunic were dissected from sea squirts. Each tissue from each organism was weighed. The γ-emmision of ^{109}Cd (22 keV and 88 keV via ^{109m}Ag) in each tissue from each organism was determined using an automatic NaI(Tl) Packard detector equipped with an automatic sample changer (Packard Cobra II Auto-gamma Counting Systems). Energy windows were set taking into account both energy maxima. Counting time was adjusted to 1 min, giving counting efficiency 17%. Optimal geometry was ensured by setting an elevator position to 3, which is recommended for use with sample volumes between 0.5 and 1.5 ml. The activity of all samples was corrected for background. The amount of Cd associated with each tissue was calculated using the relationship between known Cd concentration in Cd-stock solutions and respective γ-activity.

(insert Table 1 about here)

Determination of ^{109}Cd subcellular distribution

Nine blue mussels (3 per aquarium) were dissected to quantify intracellular ^{109}Cd distribution in the digestive gland and gills. For the same purpose a part of the branchial pharynx and the intestine of nine sea squirts were separated. A part of the hepatopancreas of nine sea stars was also excised. These tissues were homogenised in three volumes of ice-cold 100mM Tris buffer, pH 8.0 (at 4ºC) with 1mM dithiotreitol and 1 Complete® protease inhibitor tablet per 200 ml of buffer. Since the same tissue sample was processed for impulse counting and MT determination it was necessary to prevent oxidation in the sample and inhibit the activity of proteases. During the isolation process oxidation leads to polymerization of MT molecules jeopardizing the integrity of MT molecule and its metal content (Cosson, 2000). The samples were homogenised in a Potter-Elvehjem type homogeniser. The homogenates were centrifuged at 10 000×g for 30 minutes (at 4ºC) to obtain particulate fraction P1, which contains membranes, nuclei, mitochondria, cells and cell debris. Resulting supernatants (S9 fraction) were subsequently centrifuged at 50000×g for 120 minutes (at 4ºC) to obtain microsomal (P2) and cytosolic fractions (S50).

The particulate fraction, the microsomal fraction and an aliquot of the cytosolic fraction were placed in γ-vials and counted immediately for γ-emission. The reminder of the cytosol was divided into aliquots and frozen at -80ºC. Care was taken to avoid thawing-refreezing cycles and cytosols used for further processing and analyses were only frozen once.

An aliquot of S50 was used for chromatography (HPLC) on a Superdex 75 column (Amersham Biosciences); dimensions: diameter 10 mm, height 30 mm; total bed volume 24 ml; flow rate 0.2 mL/min; elution buffer 100 mM Tris, pH 8.0 at 4ºC. Column calibration was performed using low molecular weight gel filtration calibration kit comprising Ribonuclease A, Chymotrypsinogen A, Ovalbumin and Albumin (Amersham Biosciences). HPLC was performed using HPLC Pump Model 590 with auto-sampler Model 717 (Waters^{TM}). Elution profiles were obtained by continuous absorbance measurements at 280 and 254 nm using programmable multiwavelength detector (Waters^{TM}, type 490). Each chromatographic fraction (1 ml) was analysed for ^{109}Cd in order to determine the distribution of radioactivity in different molecular weight fractions of the solutes present in cytosol.

Protein measurement

The protein contents in the cytosols were determined by the method described by Lowry et al. (1951) using a kit from Bio-Rad (on microplate reader THERMOmax, Molecular Devices).

Isolation and measurement of metallothioneins

The S50 fraction from each tissue and exposure group was diluted (10 times for blue mussels and sea stars, and 5 times for sea squirts) in 0.9% NaCl. This solution was heated at 85ºC for 10 minutes, immediately cooled in ice and centrifuged at 10000×g for 15 minutes in order to remove high molecular weight (HMW) components. Dilution of S50 prior to the heat treatment was found to be beneficial, because the co-precipitation of MTs with HMW proteins was reduced (Cosson, 2000), and it was not necessary to dilute the sample prior to electrochemical measurement (Erk et al., 2002). The resulting supernatant contained heat stable metallothionein like proteins that were determined by the method described by Brdička (1933). Compared to untreated S50 fraction, it has been shown that heat treatment effectively removed HMW proteins from S50 supernatant, which would otherwise interfere with the electrochemical measurement, while MT10 isoform remained unchanged, and MT20 isoform was significantly reduced (Erk et al., 2002). Furthermore, using gel filtration chromatography it has been shown that in the heat stable fraction, the only cytosolic -SH rich compounds had an apparent low molecular mass, which could correspond to metallothioneins (Hamza-Chaffai et al., 2000).

Voltammetric measurements were performed using μAutolab Type II with IME663 (EcoChemie, the Netherlands) and automatic mercury electrode 663VA Stand (Metrohm, Switzerland), that was run by PC software package 757VA Computrace Ver. 1.0 (Metrohm, Switzerland). In the absence of commercially available standards of bivalve MTs and other

invertebrate MTs, commercially available rabbit liver MT I+II (Sigma M7641, Lot 20K7000) was used as the calibrant. Some problems have been reported regarding the use of commercial MT standards, the composition of which is far from being constant in terms of purity and metal content (Cosson, 2000), but this is of little importance if control and exposed organisms are analyzed using the same working solution of the MT standard as it was done in our study. Reliable data on MT content rely on the consistent and reproducible isolation, purification and quantification procedures. From a quantification point of view, it is necessary to strictly follow the experimental conditions which influence the catalytic signal height like buffer, depolarizer concentration, pH value, temperature, type of calibrant, and linear calibration range (Dabrio et al., 2002).

Statistical analyses

Differences between groups were tested using ANOVA. Prior to tests, homogeneity of variances was tested and variables log-transformed if required. Whenever it was not possible to achieve homogenous variances, non-parametric Kruskal-Wallis multiple comparison tests were used. Post-hoc multiple comparisons between exposure groups were performed with the use of Bonferroni's test subsequent to ANOVA, and Dunn's test subsequent to Kruskal-Wallis test (Zar, 1999). A significance level of α=0.05 was chosen for the rejection of H_0: no difference between groups. All statistical analyses were performed using SigmaStat for Windows, Version 1.0.

Results and Discussion

Tissue distribution of ^{109}Cd

In blue mussels, hepatopancreas contained the highest concentrations of Cd following all exposure treatments. Cadmium accumulated significantly following all exposures in all organs (compared to control). The relative increase in Cd concentration was somewhat less in mantle, muscle and foot than in hepatopancreas and gills (Figure 1A). Log/log transformed data gave a linear relationship between Cd concentrations in tissues and in seawater for all studied mussel tissues. Slope coefficients were close to 1 for all studied tissues, with the highest value for the digestive gland (1.094), indicating similar accumulation rates in all organs.

When the Cd content of each organ was taken into account and the percent tissue burden of Cd was calculated, the importance of the mantle became evident, since it contained a larger part of the body burden than the gills (Fig. 1B). Furthermore, there were no statistically significant differences between treatments in gills, muscle and foot, but the digestive gland burden was higher at the highest exposure, while the mantle burden was lower at the highest exposure.

(insert Figure 1 about here)

Tissues are quite specific in their ability to accumulate metals and radionuclides, and many tissues or organs also incorporate their element burdens through direct absorption from water or translocation from other tissues (Fisher, 2002). For suspension feeders such as mussels, uptake from the dissolved phase and food ingestion can be equally important to metal accumulation (Wang and Fisher, 1999). In aquatic molluscs, gills constitute a key interface for dissolved metal uptake, where metals are bound to MT, incorporated into lysosomes, and released basally towards the blood plasma and circulating hemocytes (Marigomez et al., 2002). It has earlier been found that Cd accumulation in *Mytilus galloprovincialis* determined after 1 week of exposure to 500 μg Cd L^{-1} was tissue dependent. In that study Cd concentration was highest in the gills and decreased in the order: gills > viscera > mantle > adductor muscle (Serra et al., 1999). In the present study hepatopancreas (viscera) was found to accumulate higher levels than the gills at all exposure levels, presumably due to a longer exposure period. Correspondingly to the present study, it has been shown earlier that cadmium concentrations in gills were higher than in foot after 7 days of exposure to 0.6, 1.0

and 1,6 μg Cd L^{-1}, *i.e.* gills respond more readily than foot to the elevated concentrations in the surrounding marine environment (Odžak et al., 1994).

In the field study of metal accumulation in the Lagoon of Venice, in *M. galloprovincialis* the examined heavy metals were generally more concentrated in the digestive gland than in the gills. In particular, the concentrations of Mn, Fe, and Cd were always significantly higher in the digestive gland (Irato et al., 2003). Similarly, it has been observed that in the mussels from the Mar Grande of Taranto the hepatopancreas was the preferential organ for accumulation of metals, while gills and mantle contained lower concentrations which were comparable (Cardellicchio et al., 1988). The comparison of the accumulation of metals in the mussels caged in the field and maintained in the laboratory was possible, since it has been shown that they generally display a similar metal absorption efficiencies (Fisher et al., 1996).

In sea stars, the body wall contained the highest concentrations of Cd at all exposure treatments, although the relative increase in its concentrations was less than in pyloric ceaca (hepatopancreas). Furthermore, it appeared that the relative Cd accumulation in the body wall decreased at the highest exposure level. The skeleton showed similar Cd accumulation pattern as the body wall (Figure 2A). Log/log transformation gave a linear relationship between Cd concentrations in tissue and seawater for pyloric caeca only, (slope coefficient close to 1), indicating different accumulation capacity between the studied tissues, with the highest accumulation capacity in pyloric caeca.

(insert Figure 2 about here)

The lowest Cd body burdens were found in pyloric caeca in all exposure treatments, with a significant increase up to 21.5% (median value) at the highest exposure level. The highest Cd body burden was found in external tissues (body wall), although a marked decrease (down to 52%, median value) was observed at the highest exposure level (Figure 2B). There were no statistically significant differences between treatments in Cd accumulation in the skeleton. The results are in agreement with Temara et al. (1998), in which uptake of Pb and Cd in the body compartments of *A. rubens* was found to be directly related to the concentration of the same metals in seawater. It has earlier been found that waterborne Cd significantly accumulated in the body wall but not in the pyloric caeca or the skeleton, while dietary Cd accumulation occurred in all body compartments (Temara et al., 1996). Den Besten et al. (1990) studied Cd accumulation in sea stars during 4 months of exposure (50 μg Cd L^{-1}) and found the highest accumulation rates for body wall and pyloric caeca. The body wall also

contained the majority of the accumulated cadmium, which can presumably be attributed to the high calcium concentration in this tissue. The pyloric caeca have much lower calcium content. Cadmium accumulation in the caeca may involve interactions or exchanges with both calcium and zinc, but the metal will be expected to bind to sulphydryl groups on proteins in general (including metallothioneins). Pyloric ceaca will be the tissues with the highest metabolic activity and thus the organ most likely to accumulate non-essential metals. However, it has also been found that the body wall is a metabolically active organ, and its energetic requirement is a significant component of the energetic requirements of the organism (Saito and Watts, 1989).

The tunic of sea squirts, which represents a cellular exoskeleton, contained the highest Cd concentration in the lowest exposure treatment, but in the intermediate and the highest exposure treatment it contained lower Cd concentrations than the other tissues (intestine and branchial pharynx). Taking into account all treatments, Cd accumulation in the tunic was comparatively high. In the intermediate exposure treatment Cd concentration was significantly lower ($P<0.05$) in tunic than in other analysed tissues. The intestine had the highest relative Cd accumulation, although in the intermediate exposure treatment Cd concentrations were similar in the intestine and the branchial pharynx. In the highest exposure group, Cd concentration was significantly higher ($P<0.05$) in intestine compared to other analyzed tissues. Log/log transformed data gave a linear relationship between Cd concentrations in tissues and in seawater with a slope of 0.862 and 1.012 in tunic and intestinal organs, respectively. It indicated higher accumulation rate in intestine than in tunic. In the lowest exposure treatment the branchial pharynx contained the lowest Cd concentration, and the relative accumulation of Cd decreased in the highest exposure treatment (Figure 3A). The observed pattern could indicate lower Cd accumulation capacity in the branchial pharynx compared to the intestine and the tunic.

(insert Figure 3 about here)

The highest Cd tissue burden was found in tunic in all exposure treatments, but there were no statistically significant differences between the treatments. Cadmium burdens in the intestine and the branchial pharynx were similar with no significant differences between the three treatments (Figure 3B).

Similarly, distribution of Cd levels between different tissues of Cd exposed *Pyura stolonifera* showed the highest Cd concentration in the liver, the lowest in the tunic and intermediate Cd concentration in the branchial tissue. Furthermore, Cd levels in the hepatic organ of *P. stolonifera* were found to be low compared to other marine invertebrates (Liebrich et al., 1995).

Comparing the three species, the highest accumulation of ^{109}Cd was found in blue mussels, followed by sea stars and sea squirts (Figs. 1A, 2A and 3A). Although sea squirts are filter feeders as are blue mussels, ^{109}Cd accumulation in their tissues was significantly lower than in the tissues of blue mussels. This result could be due to slower filtration rates, a less efficient uptake of cadmium over the gills, a regulation mechanism for cellular cadmium accumulation or through more effective excretion mechanisms in the sea squirts. In another ascidian species, it has been suggested that it responds to the adverse conditions by closing the siphonal apertures, which would decrease uptake (Agell et al., 2004). Differences in tissue levels of Cd on a wet weight basis could also be due to a higher water content in one of the tissues. Reported water content of liver tissue in *P. stolonifera* was 85.7% (Liebrich et al., 1995). Macroscopical examination of sea squirt tissues did suggest a less compact tissue than mussel gills. Therefore, in future studies it would be preferable to obtain this type of results on a dry weight basis.

During the exposure experiment a significant decrease of radioactivity in aquaria with blue mussels was observed (even down to 30% of initial radioactivity measured at the zero-day of exposure), which was caused by the large filtration capacity of the mussels (Table 1). Since the sea squirts were present in these aquaria, they could also be responsible for removal of ^{109}Cd, but not as much as the mussels. Reported filtration rates of *C. intestinalis* range from about 2 to 5.5 L g^{-1} d^{-1} (Goodbody, 1974), which is a factor 26 to 36 lower than reported for *M. edulis* (52 to 196 L g^{-1} d^{-1}) (Widdows et al., 1995). Given their high filtration rates, mussels will process large volumes of water, and so uptake from the dissolved phase may contribute significantly to metal accumulation in mussels. In aquaria with sea stars ^{109}Cd radioactivity measured on the 9th day of exposure was in the range from 61% to 75% of initial radioactivity (Table 1).

In organisms exposed to waterborne cadmium several pathways of uptake could be involved: across the entire body surface of the organism, across specialised respiratory structures, or across the digestive epithelium. Although it cannot be based on the current evidence, most

probably the predominant pathway of uptake would involve the structures which would be in direct contact with seawater, like gills in *M. edulis*, or branchial pharynx in *C. intestinalis*. The route of metal uptake will influence both distribution of metals in tissues of an organism and toxicity of the metal (Brown and Depledge, 1998; Wang and Fisher, 1999). The uptake of heavy metals from solution is generally thought to be a passive process not requiring energy (Phillips and Rainbow, 1993). In addition to ion channels, some non-essential metals such as Cd may also be taken up through active transport pumps for essential metals. This will occur for heavy metals with similar free ion radius as a major metal. Calcium pumps may, therefore, be a significant uptake route for cadmium (Simkiss, 1998).

Cadmium is a non-essential metal and is, therefore, accumulated from the surrounding water by all aquatic organisms that depend on water for the exchange of ions and gases (Rainbow, 1985). Even under similar ambient conditions, variations in surface area available for absorption, permeability of cells/tissues, number and nature of binding sites (intra- and extracellular) and metabolic rate can result in differences in metal uptake between species and even between individuals of the same species (Brown and Depledge, 1998).

In some cases, rather than being excreted to the surrounding environment, metal complexes are secreted into mineralised and organic extracellular stuctures such as shells, exocuticle or byssal threads (Brown and Depledge, 1998), as was seen for the skeleton of *A. rubens* in the present study. A similar process could be the explanation for the high accumulation of Cd in the tunic of *C. intestinalis*, but it could also be the consequence of direct incorporation from the water.

Subcellular compartmentalisation of ^{109}Cd

Cadmium partitioning was determined for three compartments: the P1 fraction (contains membranes, nuclei, mitochondria, cells and cell debris), P2 fraction (microsomal fraction) and S50 fraction (cytosol). In blue mussels, sea stars and sea squirts exposed to waterborne Cd, the metal was primarily found in the soluble fraction (Table 2). Cadmium increased in the P1 fraction of the digestive gland of blue mussels at the highest exposure concentration, and decreased in ^{109}Cd in the S50 fraction relative to the lowest exposure. A similar pattern of Cd partitioning was found in the branchial pharynx of sea squirts with a statistically significant increase of Cd in the P1 fraction, and a decrease of ^{109}Cd in the S50 fraction at the highest exposure level relative to the lowest exposure (Table 2). No statistically significant

differences between treatments in subcellular distribution of Cd were found in gills of blue mussels, pyloric caeca of sea stars or intestinal organs of sea squirts.

(insert Table 2 about here)

Contrary to the present findings, Cd was found to be distributed equally between soluble and insoluble fractions in the soft tissues of oysters (*Crassostera gigas*) experimentally exposed to 20 μg Cd L^{-1} of waterborne cadmium for 21 days (Ettajani et al., 2001). In another study, Cd was primarily found in the insoluble fraction of oysters (*Crassostrea gigas*) exposed chronically *in situ* (Mouneyrac et al., 1999), where the percentage of Cd bound to the insoluble fraction was higher in the transplanted oysters than in the native oysters. In many invertebrates detoxification processes also involve an insolubilisation of the metal *via* the formation of (or incorporation into) the mineral concretions.

In accordance with the results found here for Cd partitioning in intestinal organs of *C. intestinalis*, heat-treated *P. stolonifera* liver supernatant contained about half of the total amount of cadmium found in the crude homogenate (Liebrich et al., 1995). Loss of cadmium from the soluble cell fraction could most easily be explained by insufficient homogenisation and hence co-sedimentation of soluble material within the particulate fraction. However, it is also possible that cadmium is associated with membrane fractions or with insoluble granules (Viarengo and Nott, 1993).

In order to detect distribution of Cd in different molecular mass fractions of cytosol from Cd-exposed organisms, gel filtration was performed. In Figure 4A-D the typical elution profiles after gel filtration (on a Superdex 75) of the cytosols from the Cd exposed organisms are shown. In the elution profile of the digestive gland of blue mussels exposed to 0.05 μg Cd L^{-1}, a single peak of proteins with molecular weight (MW) ~6 kDa contained 65% of Cd (Figure 4A).

(insert Figure 4 about here)

Cytosolic distribution of Cd between proteins of different MW was affected during exposure to higher Cd concentrations. A more complex elution profile was observed for the digestive gland of mussels exposed to 2 μg Cd L^{-1}, with three peaks of protein with MW~6 kDa, ~18 kDa and ~54 kDa (HMW proteins), containing 45%, 8% and 4% of Cd, respectively. After

the highest Cd exposure, the amount of Cd associated with proteins of apparent size 18 kDa increased to up to 20%, while the HMW proteins bound 5% of Cd. A decrease in the Cd amount in the peak of 6 kDa was the most striking feature of the elution profiles. The decrease was from 65% and down to 24%.

Contrary to the results for digestive gland, three peaks of proteins were present in the elution profiles of gills of blue mussel exposed to 0.05 μg Cd L^{-1} (Figure 4B). This showed that even in the low Cd exposure considerable amount of Cd (about 25%) was associated with both proteins of apparent size14 kDa, and HMW proteins (Figure 4A). At the highest Cd exposure only 8% of the Cd was associated with 6 kDa proteins, while the major part of the Cd was associated with the broad peak of proteins with MW between 10 and 25 kDa.

According to the elution profile of commercially available rabbit liver metallothionein (Sigma, M7641), which has a 254 nm absorbance peak at approximately 11 kDa, peaks of proteins with 6 kDa and 18 kDa could represent monomer and dimer forms of metallothioneins in the digestive gland of mussels. Redistribution of the amount of radioactivity between these two fractions in the intermediate and the highest exposure treatment (data not shown), corroborates this statement. These results for MT components isolated by liquid chromatography are in correspondence with data published by George et al. (1979) and Mackay et al. (1993). Those authors reported the existence of two molecular-mass classes, which were separable by the conventional gel-filtration (Sephadex G-75) chromatography. They were designated as MT-10 (monomer) and MT-20 (dimer), following the terminology proposed by Mackay et al. (1993). Using cloning and characterisation of metallothionein cDNA in *M. edulis*, Baršyte et al. (1999) found that the MT-20 isoform represents a Cd-inducible form of MT, whereas MT-10 is a basally expressed form. Applying the same techniques, Lemoine et al. (2000) concluded that MT-20 was induced by Cd exposure, while MT-10 was induced by Zn exposure.

Studies on polymorphism of metallothioneins in the digestive gland of indigenous mussels and experimentally Cd-exposed mussels *M. galloprovincialis* (200 μg Cd L^{-1}, 14 days) have shown that in Cd-exposed mussels the larger proportion of Cd was bound to the MT-20 than to the MT-10 component, suggesting that the dimeric component may be considered as a primarily inducible metallothionein (Ivanković et al., 2002). The occurrence of a specific cadmium-binding isoform in the gills of cadmium-exposed mussels (200 μg Cd L^{-1}, 21 days) has been reported, and according to the detection by DEAE-HPLC it represents the MT-II

pool, *i.e.* MT dimer (Geret and Cosson, 2002). Similar results were found for other bivalve molluscs, *e.g.* oysters. In the soft tissues of the oyster *Crassostera gigas* exposed to waterborne cadmium, cytosolic Cd was present predominantly in the heat-stable fraction and mainly bound to compounds of molecular weight equal to 13.5 kDa. Furthermore, MT levels were positively correlated with total Cd (Ettajani et al., 2001). Increased ability to bind additional Cd atoms has also been found in rabbit liver and horse kidney MTs as a result of oligomerization or development of aggregates in metal binding situations at physiological pH values in both species (Wilhelmsen et al., 2002).

In elution profiles of the cytosol of pyloric caeca from sea stars exposed to 0.05 μg Cd L^{-1}, the peak of proteins with MW~6 kDa contained the largest part of the accumulated cadmium (Figure 4C), while in the highest exposure the major part of Cd was associated with the proteins of approximately 18 kDa (data not shown).

The amount of Cd in these peaks was about the same in the lowest and the highest exposure treatments. At both exposures a minor part of Cd was associated with the HMW pool of MW~54 kDa.

According to results based on cytosolic distribution of Cd, heat-treatment and thiol content, MT in the pyloric caeca of *A. rubens* has apparent molecular weights of approximately 13 and 22 kDa (Temara et al., 1997). Temara and co-workers (1997) found an increase in Cd cytosolic concentrations in pyloric caeca of sea stars exposed to 20 or 200 μg Cd L^{-1} for 21 days, but this increase was not dose-dependent. Cadmium was mainly incorporated into the low molecular weight pool of the heat-treated cytosol and Cd cytosolic concentrations in sea stars exposed to 20 μg Cd L^{-1} were not different from those in sea stars exposed to 200 μg Cd L^{-1}, indicating a possible saturation of the system.

The striking feature of the elution profiles of the cytosol of intestinal organs from sea squirts exposed to low and high cadmium concentration is the redistribution of Cd between two protein pools.

At low exposure (0.05 μg Cd L^{-1}) the major part of Cd was associated with the protein with approximate MW of 5 kDa (Figure 4D). Cytosolic distribution of Cd was affected during exposure to higher Cd concentration with the majority of Cd being associated with ~11 kDa proteins.

Studies of sea squirts in relation to accumulation of metals from the environment have mainly concerned themselves with vanadium (Michibata and Kanamori, 1998) and the literature on *e.g.* cadmium accumulation and Cd-binding proteins is scarce (Liebrich et al., 1995). In a study performed on a large solitary ascidian *Pyura stolonifera*, the authors found the highest concentrations and the highest accumulation of Cd in hepatic organ. They used this tissue to isolate and characterise Cd-binding proteins, which were heat-stable, cystein-rich and resembled fish metallothionein. Separation of heat-treated *P. stolonifera* supernatant on a Sephadex G-75 column produced only one cadmium-containing peak with a molecular weight of about 6 kDa. In untreated controls the cadmium peak was much smaller, whereas the remaining elution pattern was comparable (Liebrich et al., 1995).

Cadmium-binding proteins in heat-stable fraction

Metallothionein (MT) content in selected tissues of blue mussels, sea stars and sea squirts are shown in Figure 5.

(insert Figure 5 about here)

In both tissues from blue mussel there was a statistically significant difference between the control group and the group exposed to 50 μg Cd L^{-1}. The levels of MT were higher in digestive gland than in gills (Figure 5A and 5B).

The measurement of metallothionein induction in mussels has been shown to be a viable method for determining biological response to metal contamination (Bebianno and Langston, 1991). A number of laboratory studies have been performed on MT induction using cadmium in *Mytilus* species, but most commonly very high Cd concentrations have been used (often higher than 100 μg L^{-1}). Such concentrations are unrealistic compared to levels seen in the environment and physiological responses difficult to interpret as a range of cellular mechanisms will be triggered. The present study has confirmed that even low Cd exposure concentrations, which are closer to true environmental levels, are capable of inducing an increase in MT concentrations. These results could be used to explain the natural situation only to some extent, since the real environmental situation is complicated by the presence of a mixture of contaminants and blue mussels will be affected by other factors, e.g. food availability, temperature and salinity.

In the pyloric caeca of the sea star a comparison of all groups *vs.* control group showed statistically significant differences between all of them (Figure 5E). This was the most striking result, where exposure of only 0.05 μg Cd L^{-1} during 21 days resulted in a relative increase of MT of 1.9 times, and furthermore 1000 times higher exposure concentration caused the same increase in MT level (Figure 5E).

Den Besten et al. (1990) performed partial characterisation of Cd-binding proteins in *A. rubens* and found that Cd-binding proteins in pyloric caeca have metallothionein-like characteristics. Their molecular weight was of about 10.8 kDa, which is within the range of molecular weights reported for metallothioneins in other marine invertebrates; they are heat-stable, have high Cd content, and high content of thiols. They also reported that after 16 weeks of exposure, the amount of MT-like proteins hardly increased, while the Cd-binding capacity of the MT-like protein fraction was only slightly increased.

Basal levels of metallotioneins have been reported for asteroids collected from unpolluted sites (SW England, SE Netherlands and SW Norway) of 2.5-4.5 mg MT g^{-1} dw, as well as values from the heavy metal polluted site (Sørfjord, SW Norway) of 5-5.6 mg MT g^{-1} dw (Temara et al., 1997). It is clear that this subject needs to be further investigated, in which the characterisation of the MT in sea star should be prioritised.

In the intestinal organs of *C. intestinalis* there was a statistically significant difference between control group and group exposed to 50 μg Cd L^{-1} (Figure 5C), while in the branchial pharynx the highest exposure concentration did not induce the highest MT level (Figure 5D). On the contrary, in the branchial pharynx of *C. intestinalis* there was a statistically significant difference between control group and group exposed to 2 μg Cd L^{-1} (Figure 5D).

The highest MT level between all of the control groups was found in the digestive gland of the blue mussel, but the relative increase in MT level in the highest Cd exposure was the lowest in this tissue (1.5 times higher). This is in agreement with the literature data, where high levels of MT were reported for the digestive gland of transplanted mussels (Raspor et al., 2004). The relative increase in MT level was the highest in the gills of blue mussel and in the intestinal organs of sea squirt (2 times), while in the pyloric caeca of sea stars, the increase was between 1.7 and 1.9 times (Figure 5A-E). It is interesting to notice that the values of the MT content expressed to cytosolic protein for all studied species are distributed in the range from 1.12 up to 37.8 mg(MT)/$g_{(protein)}$, *i.e.* they do not show high variability between species.

Conclusions

An expected bioaccumulation of cadmium was observed, although with obvious interspecies, as well as inter-tissue, differences in accumulation kinetics. The highest Cd accumulation was found in *Mytilus edulis*, which filters large volumes of seawater for the purpose of respiration, ion exchange and feeding. No concentration-dependent differences in Cd body burden were found for the studied species and tissues.

The highest concentration of MT of any organism or tissue was found in the digestive gland of *M. edulis*. Intestinal MT in sea squirt, *C. intestinalis*, increased in a dose-dependent fashion with increasing Cd exposure, although with a drop at the highest concentration. The most surprising finding was that even the lowest Cd exposure concentration (0.05 μg Cd L^{-1}) caused MT induction in pyloric caeca of *A. rubens*, although with no increase at higher exposure levels.

Acknowledgement

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Table 1. Concentrations of Cd (μg L^{-1}) in aquaria during 21 days of the exposure period (median values for three replicate aquaria). Grey highlight indicates a day when the exposure solution was changed.

	0 day	1st day	2nd day	3rd day	4th day	7th day	8th day	9th day	10th day	11th day	14th day	15th day	16th day	17th day	18th day	21st day
Aquaria I: *M.edulis C.intestinalis*	0.05	0.05	0.04	0.04	0.03	0.02	0.02	0.02	0.01	0.05	0.02	0.02	0.02	0.01	0.01	0.01
Aquaria II: *A. rubens*	0.05	0.05	0.05	0.04	0.04	0.03	0.03	0.05	0.04	0.04	0.03	0.03	0.03	0.03	0.05	0.04
Aquaria I: *M.edulis C.intestinalis*	2.0	1.9	1.7	1.5	1.2	0.9	0.9	0.7	0.6	2.0	1.4	1.2	1.0	1.0	0.9	0.7
Aquaria II: *A. rubens*	2.0	2.0	2.0	1.8	1.6	1.3	1.2	2.0	1.8	1.6	1.3	1.2	1.1	1.0	1.6	1.3
Aquaria I: *M.edulis C.intestinalis*	50.0	50.0	42.2	37.2	32.6	25.5	25.4	17.9	19.9	50.0	34.7	31.2	28.5	26.5	24.7	19.1
Aquaria II: *A. rubens*	50.0	48.5	47.4	44.1	43.8	38.6	39.1	50.0	49.3	47.2	43.6	41.6	41.6	40.7	38.1	37.3

Table 2. Percent ^{109}Cd (%) in different subcellular fractions obtained by differential centrifugation. Underlined are the statistically significant increased or decreased values in the P1 fraction or S50 fraction relative to 0.05 µg Cd L^{-1} exposure, respectively.

			0.05 µg Cd L^{-1}		2 µg Cd L^{-1}		50 µg Cd L^{-1}	
			mean	(SD)	mean	(SD)	mean	(SD)
Mytilus edulis	digestive gland	P1	17.7	(2.2)	16.4	(2.5)	<u>25.5</u>	(8.7)
		P2	6.8	(2.7)	7.4	(3.0)	6.3	(2.5)
		S50	75.7	(3.0)	76.2	(4.1)	<u>68.2</u>	(9.8)
	gills	P1	20.2	(3.2)	22.0	(4.1)	20.5	(3.2)
		P2	5.0	(1.8)	5.0	(1.7)	4.2	(0.6)
		S50	74.8	(4.3)	73.0	(5.1)	75.3	(3.5)
Ciona intestinlis	intestinal organs	P1	29.1	(14.0)	33.5	(6.5)	32.6	(11.6)
		P2	4.1	(1.9)	8.4	(3.7)	5.3	(1.9)
		S50	66.9	(13.9)	58.1	(4.6)	62.1	(10.3)
	branchial pharynx	P1	18.5	(4.2)	25.8	(6.5)	<u>33.9</u>	(10.9)
		P2	2.1	(1.1)	1.7	(1.0)	1.9	(0.6)
		S50	79.3	(4.1)	72.4	(7.0)	<u>64.2</u>	(10.9)
A.rubens	pyloric cacca	P1	30.0	(6.8)	27.7	(4.7)	30.2	(8.7)
		P2	10.2	(2.6)	11.9	(3.4)	9.4	(3.5)
		S50	59.7	(7.0)	60.4	(3.8)	60.4	(9.4)

Figure legends

Figure 1. (**A**) Accumulation of cadmium into the organs of *Mytilus edulis* exposed to Cd through water for 21 days (mean±SD, n=9 in each treatment); (**B**) Percent Cd body burden in the organs of *Mytilus edulis* exposed to $^{109}CdCl_2$ through water for 21 days (boundaries of the box indicate the 25^{th} and 75^{th} percentiles, a line within the box marks the median, the whiskers indicate 90^{th} and 10^{th} percentiles, and the symbol "+" indicates 5^{th} and 95^{th} percentiles, n=9 in each treatment; letter "a" indicates statistically significant difference between treatments, $P<0.05$)

Figure 2. (**A**) Accumulation of cadmium into the organs/tissues of *Asterias rubens* exposed to Cd through water for 21 days (mean±SD, n=9 in each treatment); (**B**) Percent Cd body burden in organs and tissues of *Asterias rubens* exposed to $^{109}CdCl_2$ through water for 21 days, n=9 in each treatment (box description as in Fig. 1B, letters "a", "b", "c" and "d" indicate statistically significant differences between treatments, $P<0.05$).

Figure 3. (**A**) Accumulation of cadmium into the tissues of *Ciona intestinalis* exposed to Cd through water for 21 days (mean±SD, $n_{0.05}=9$; $n_2=7$; $n_{50}=8$); (**B**) Percent Cd body burden in the tissues of *Ciona intestinalis* exposed to $^{109}CdCl_2$ through water for 21 days, $n_{0.05}=9$; $n_2=7$; $n_{50}=8$ (box description as in Fig. 1B, asterisk indicates statistically significant difference between treatments, $P<0.05$).

Figure 4. Superdex 75 chomatogram of a cytosol of: **(A)** digestive gland of *M. edulis*, **(B)** gills of *M. edulis*, **(C)** pyloric caeca of *A. rubens*, **(D)** of intestinal organs of *C. intestinalis*, all exposed to 0.05 μg Cd L^{-1} with ^{109}Cd added as a tracer (gray line: absorbance at 280 nm, black line: absorbance at 254 nm, black circles and line: cpm ^{109}Cd).

Figure 5. Metallothioneins expressed per gram of the total proteins in heat-treated cytosolic fraction: (**A**) *M. edulis* - digestive gland, (**B**) *M. edulis* – gills, (**C**) *C. intestinalis* – intestinal organs, (**D**) *C. intestinalis* – branchial pharynx, (**E**) *A. rubens* – pyloric caeca (box description as in Fig. 1B, letter "a" indicates treatments significantly different from controls, $P<0.05$).

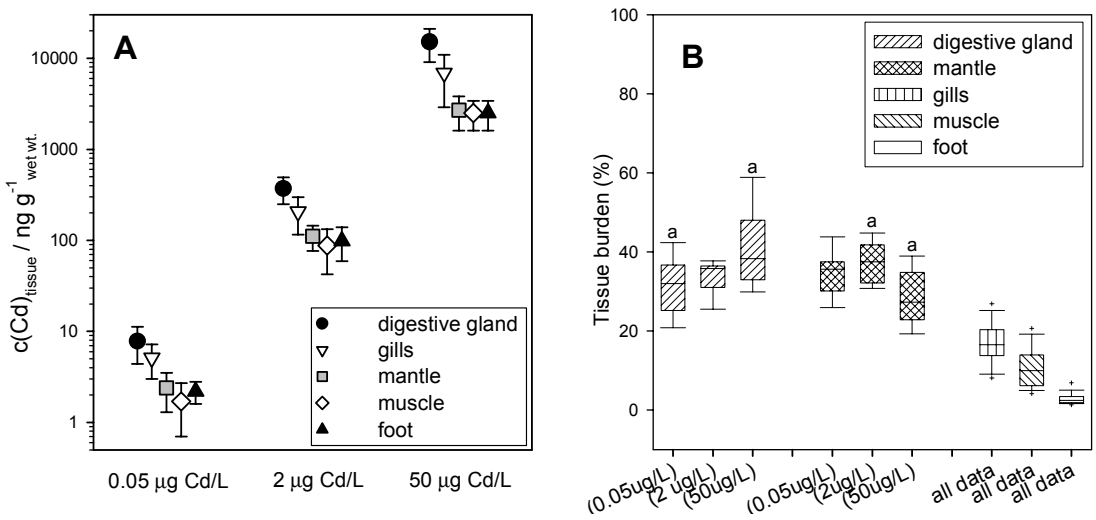

Figure 1.

M. Erk, A. Ruus, K. Ingebrigtsen, K. Hylland

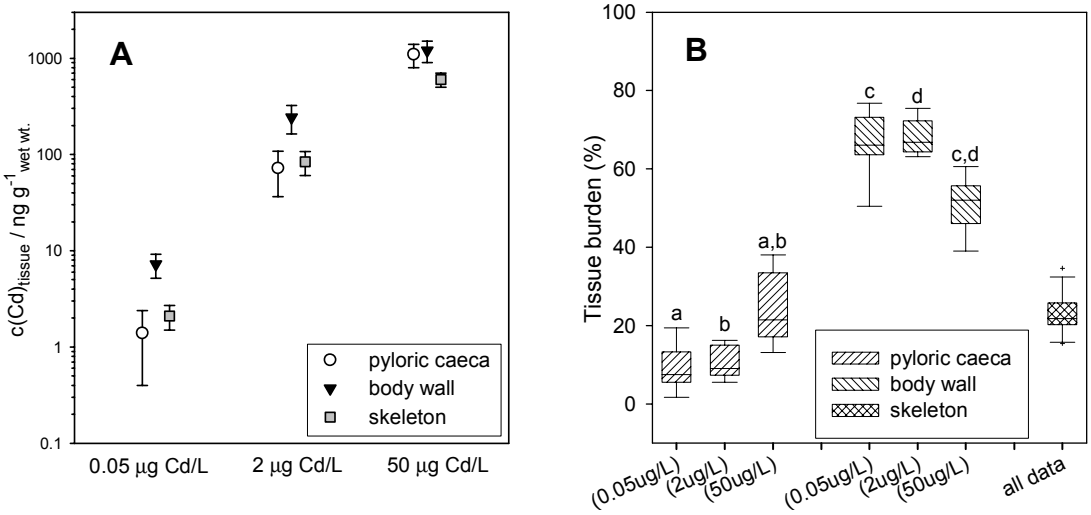

Figure 2.

M. Erk, A. Ruus, K. Ingebrigtsen, K. Hylland

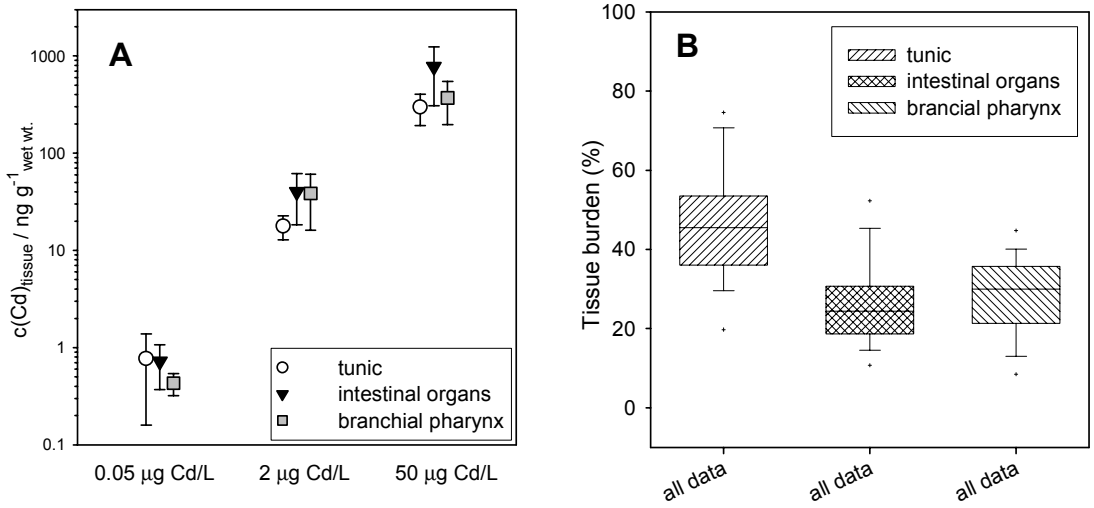

Figure 3.

M. Erk, A. Ruus, K. Ingebrigtsen, K. Hylland

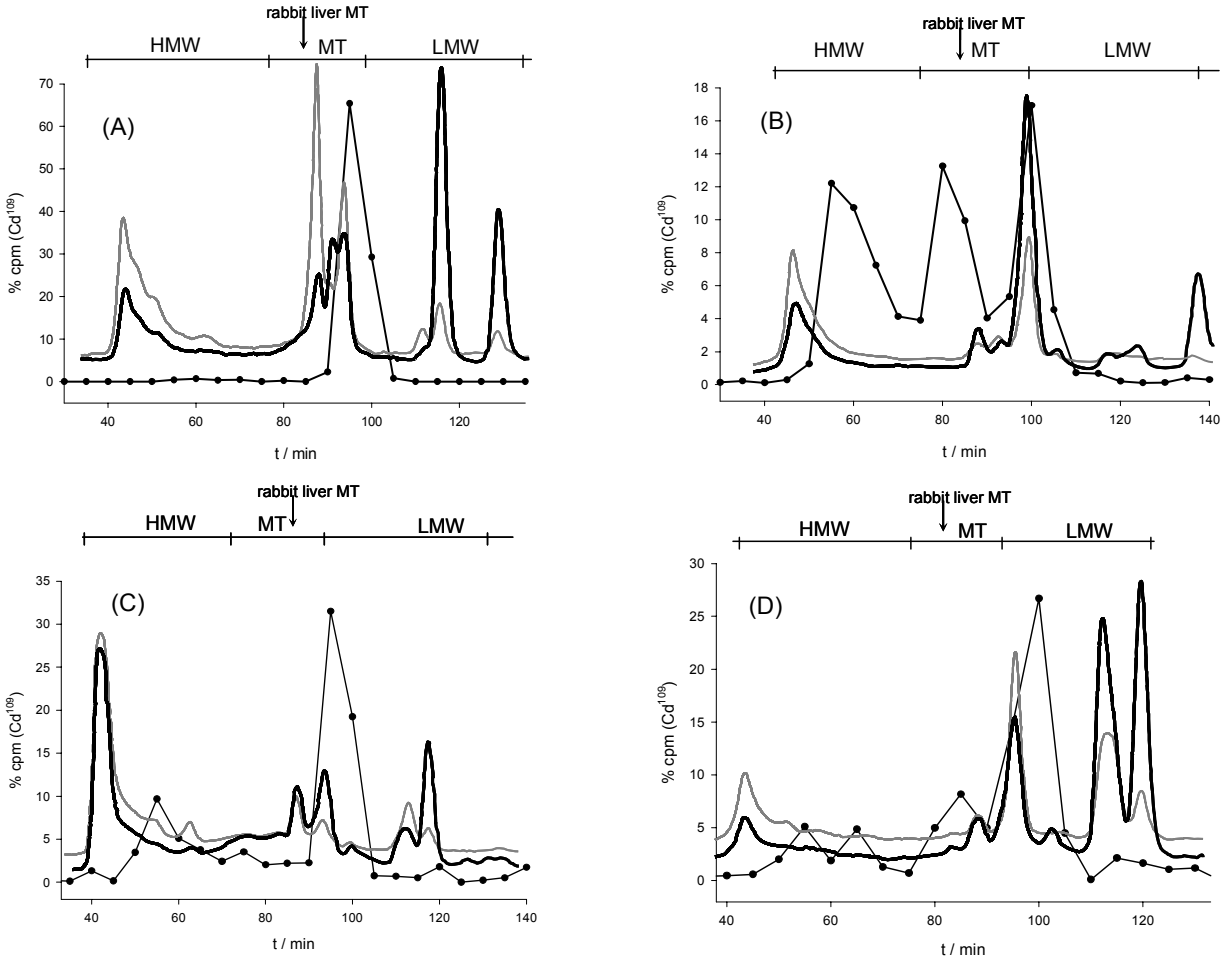

Figure 4.

M. Erk, A. Ruus, K. Ingebrigtsen, K. Hylland

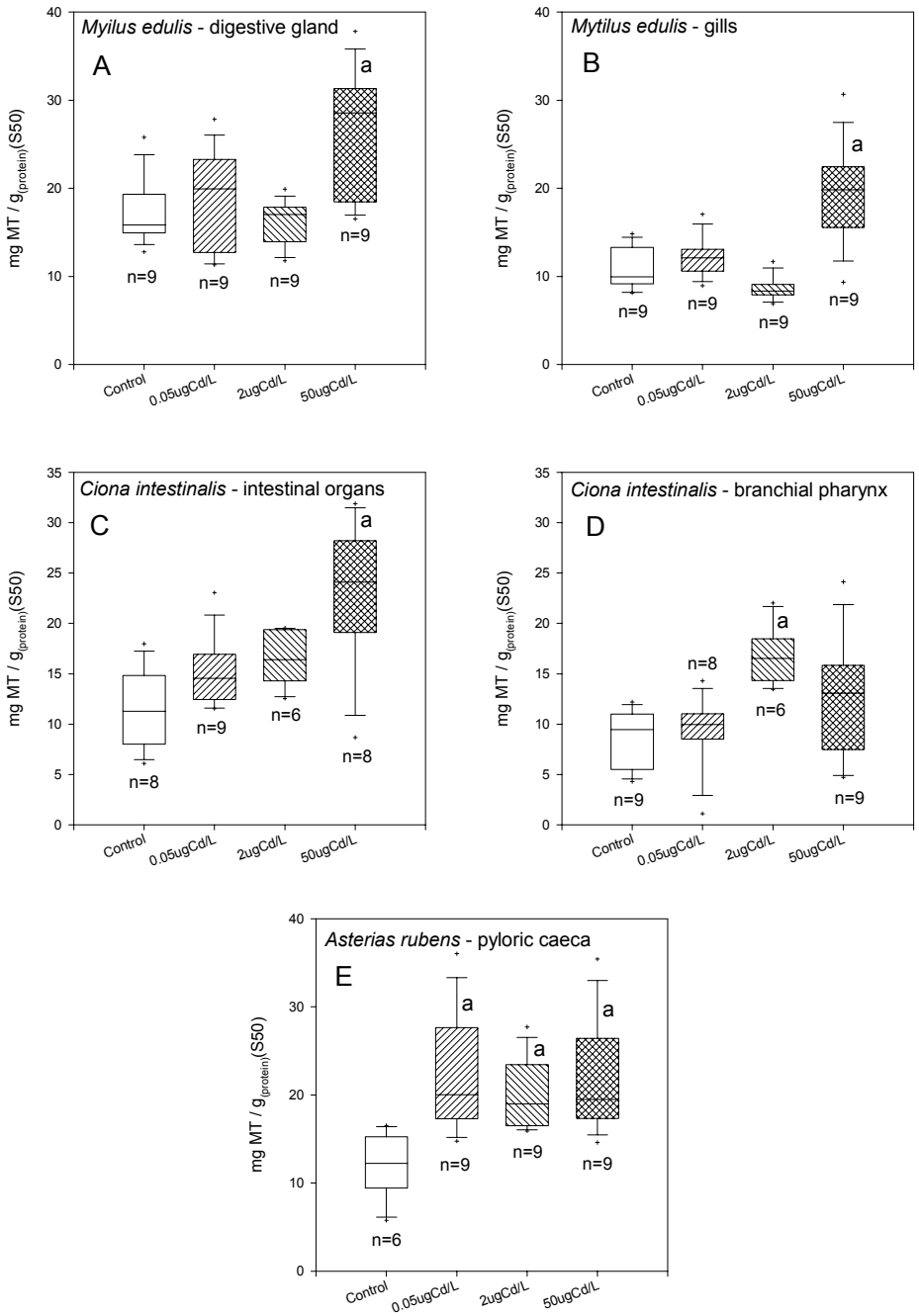

Figure 5.

M. Erk, A. Ruus, K. Ingebrigtsen, K. Hylland