

Tissue-specific accumulation of cadmium in subcellular compartments of eastern oysters *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae)

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Abstract

Cadmium distribution was studied in different subcellular fractions of gill and hepatopancreas tissues of eastern oysters *Crassostrea virginica*. Oysters were exposed for up to 21 days to low sublethal Cd concentrations ($25 \mu\text{g L}^{-1}$). Gill and hepatopancreas tissues were sampled and divided into organelle fractions and cytosol by differential centrifugation. Organelle content of different fractions was verified by activities of marker enzymes, citrate synthase and acid phosphatase for mitochondria and lysosomes, respectively. In both tissue types, there was a significant accumulation of cadmium in cytosol reaching $230\text{--}350 \text{ ng mg}^{-1}$ protein. Among organelles, mitochondria were the main target for Cd bioaccumulation in gills ($250\text{--}300 \text{ ng mg}^{-1}$ protein), whereas in hepatopancreas tissues, the highest cadmium accumulation occurred in lysosomes ($90\text{--}94 \text{ ng mg}^{-1}$ protein). Although 75–83% of total cadmium burden was associated with the cytosol reflecting high volume fraction of this compartment, Cd concentrations in organelle fractions reached levels that could cause dysfunction of mitochondria and lysosomes. Organ- and organelle-specific patterns of cadmium bioaccumulation support our previous *in vivo* studies, which showed adverse effects of cadmium exposures on mitochondrial oxidation in gills and on the lysosomal system of hepatopancreas. This may have important implications for the development of biomarkers of effect for heavy metals and for understanding the mechanisms of toxic effects of metals.

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Cadmium is a trace metal with no known biological function in animals, which is widespread in estuarine and coastal environments. At high concentrations, cadmium is extremely toxic to aquatic organisms, but

even low levels may adversely affect their physiology (Byczkowski and Sorenson, 1984; Viarengo, 1994; Stohs and Bagchi, 1995; Sokolova, 2004; Sokolova et al., 2004). Cadmium availability may greatly vary in different habitats due to natural and anthropogenic sources (GESAMP, 1987; Nriagu and Sprague, 1987). Anthropogenic sources of cadmium in estuarine and coastal habitats due to pollution of the estuaries have

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received the most attention (Roesijadi, 1996); however, natural ones are also very important and may be due to the river run-off from cadmium-rich soils, leaching of the rocks or diatom deposition in marine sediment (GESAMP, 1987; Frew et al., 1997).

Marine bivalves including oysters are exposed to varying levels of cadmium in their natural habitats in estuaries and coastal areas. They have the ability to concentrate cadmium in soft tissues from water and sediments several orders of magnitude over exposure levels (Roesijadi, 1996a; Frew et al., 1997; Engel, 1999). Body levels of cadmium in natural oyster populations range from 0.4 to 40 $\mu\text{g g}^{-1}$ dry weight (Roesijadi, 1996a; Frew et al., 1997; Lauenstein et al., 2002). During acute exposures to elevated cadmium concentrations in water or sediments, oysters can accumulate even higher loads of this metal up to 300–400 $\mu\text{g g}^{-1}$ dry weight (Roesijadi, 1996a). Although high Cd concentrations may cause acute toxicity, adverse effects on physiological and cellular processes are observed at low, environmentally realistic concentrations (Anderson et al., 1992; Roesijadi et al., 1997; Auffret et al., 2002; Sokolova, 2004; Sokolova et al., 2004 and references therein).

Physiological effects and toxicity of trace metals strongly depend on their intracellular localization and binding to organelles and ligands. Mitochondria are key intracellular targets for metal toxicity, which are very sensitive to metal exposures. Cadmium affects mitochondrial bioenergetics of oysters *in vitro* and *in vivo* at concentrations as low as 10^{-7} – 10^{-6} M leading to reduced coupling and impaired ability to produce ATP (Skulsky et al., 1988; Kessler and Brand, 1994, 1995; Ye et al., 2001; Sokolova, 2004). Consequently, cadmium accumulation in mitochondria may result in serious disturbances of tissue energy balance and eventually cell death (Sokolova, 2004; Sokolova et al., 2004; Sokolova et al., 2005).

Other key intracellular compartments involved in Cd accumulation are lysosomes and the cytoplasm. Lysosomes are an important organelle in which metals are sequestered in mollusks, especially in lysosomal-rich hepatopancreas tissues (Marigomez et al., 2002). Accumulated metals can be incorporated into lysosome-derived insoluble granules and either stored intracellularly or excreted from the cell and thus detoxified (Marigomez et al., 2002). Therefore, localization of the accumulated cadmium into lysoso-

mal fractions can function as a detoxification mechanism alleviating potential toxic effects on oyster physiology. Increases in lysosomal numbers in response to metal exposures have been reported in mollusks (Giambérini and Cajaraville, *in press*), which reflect compensatory changes to increased cellular metal concentrations. In the cytoplasm, metallothioneins (MT), which are low molecular weight, cysteine-rich metal-binding proteins, constitute another important detoxification mechanism that can serve to minimize the availability of metal ions to cytosolic components (Roesijadi, 1996b; Jenny et al., 2004). Recent studies have suggested that MTs may also be involved in transfer of metals to lysosomes thus serving as a link between the cytosolic and lysosomal systems of metal sequestration (Kurasaki et al., 1998; Okabe et al., 1999; Hahn et al., 2001). However, toxicity is observed when lysosomal or MT detoxification systems are overwhelmed, which causes destabilization of lysosomal membranes, reduced ATPase function, and interactions of free metal ions with essential enzyme systems (Moore, 1985; Regoli, 1992; Roesijadi, 1996b; Klaassen et al., 1999; Ringwood et al., 2004).

Most studies on subcellular distribution of trace metals in oysters and other mollusks have focused primarily on the distribution between cytosolic components (e.g. between MTs and high molecular weight or other low molecular weight proteins) and non-cytosolic components such as granular deposits (Julshamn and Andersen, 1983; Bebianno et al., 1993; Blackmore and Wang, 2002; Marigomez et al., 2002; Ng and Wang, 2004). Few studies have addressed cadmium accumulation in different organelles, in particular in mitochondria, which are the primary site of ATP production and a key target for cadmium toxicity (Evtushenko et al., 1986; Sokolova, 2004), and in lysosomes, another important target organelle for metal toxicity (Ringwood et al., 1998a,b). Clearly, more studies are required to investigate the dynamics of cadmium accumulation in different organelles, which is a key in understanding of the mechanisms of toxicity of this metal. The aim of this study was to characterize time-dependent accumulation of cadmium in different intracellular compartments in gill and hepatopancreas tissues of the eastern oyster, *Crassostrea virginica*. An important approach in this work is the use of marker enzymes to determine organelle content of various sub-

cellular fractions obtained by quantitative differential centrifugation.

1. Materials and methods

1.1. Animal collection and maintenance

Wild-cultured adult oysters (e.g. hatchery-reared oyster spat which were planted and cultivated in natural estuarine systems by J & B Aquafood Inc.) were collected in Stump Sound, NC in April 2004. The unpolluted area has very low background concentrations of heavy metals and organic pollutants (Mallin et al., 1999; Swartzenberg, personal communication). Water temperature at the time of collection was 18–20 °C and salinity varied between 22 and 30‰. Oysters (18 months old, 95–120 mm mean shell length) were transported to the University of North Carolina at Charlotte within 8 h after collection and placed in recirculating aquaria with artificial sea water (ASW) (Instant Ocean[®], Kent Marine, Acworth, GA) at 20 ± 1 °C and 650 ± 20 mOsm. Animals were acclimated in the laboratory for 1 week prior to experimentation. During the acclimation period, oysters were fed on alternate days with a commercial algal blend (0.5 mL L⁻¹) containing *Nannochloropsis*, *Tetraselmis* and *Isochrysis* spp. ranging in size from 2 to 15 µm (PhytoPlex[®], Kent Marine, Acworth, GA). No mortalities occurred during the preliminary acclimation period. After the preliminary acclimation, oysters were randomly assigned to polypropylene trays (six oysters per tray), and incubated in 5 L of ASW (control) or ASW with 25 µg L⁻¹ cadmium (Cd-exposed oysters). Oysters were fed with PhytoPlex[®] blend on alternate days, and water was changed 18 h after feeding. Control and Cd-exposed oysters were sampled after 2 days, 1 week and 3 weeks of incubation. Less than 2% mortality occurred during the experimental incubations, and there were no significant differences in mortality between control and Cd-exposed oysters.

1.2. Subcellular fractionation

After experimental incubations, oysters were dissected, and gill and hepatopancreas tissues were homogenized in 5 × (v/w) of homogenization medium (HM, containing 62.5 mmol L⁻¹ KCl, 62.5 mmol L⁻¹

NaCl, 250 mmol L⁻¹ sucrose, 30 mmol L⁻¹ HEPES, 5 mmol L⁻¹ MgCl₂, 0.1 mmol L⁻¹ of protease inhibitor phenylmethylsulfonyl fluoride, PMSF, and 1 mmol L⁻¹ β-mercaptoethanol; pH 7.5). Previous studies have shown that use of high ionic strength, slightly hypoosmotic media minimizes organelle damage during homogenization of oyster tissues (Sokolova, 2004). Tissues were homogenized on ice using a Potter-Elvehjem homogenizer equipped with a teflon pestle at 200 rpm, and the homogenate was filtered through a double layer of cheesecloth. Subcellular fractions were obtained by differential centrifugation and two-step gradient centrifugation of the homogenate using standard methods modified from Fleischer and Kervina (1974) and Hinton and Mullock (1997). This method yields less pure organelle fractions than centrifugation on density gradients, but it results in the minimal loss of material and allows quantitative recovery of subcellular fractions, which was critical for our study (Hinton and Mullock, 1997).

A schematic representation of the cellular fractionation procedure is given on Fig. 1. Briefly, tissue homogenates were centrifuged for 10 min at 1000 × g to remove unbroken tissue fragments, cell debris and nuclei. The pellet was washed in 5 ml of HM, re-centrifuged for 10 min at 1000 × g, and the supernatants were pooled (S1). Quantitative recovery of nuclei was not attempted in this study because it is very time- and effort-consuming, and earlier studies on cadmium accumulation in nuclei have indicated that these organelles account for only ca. 1% of the total intracellular cadmium pool (Evtushenko et al., 1986; Bracken et al., 1984).

A 0.5 mL subsample of the crude supernatant (e.g. S1) was collected to determine enzyme activities and cadmium concentration in the total tissue homogenates (e.g. total fraction). The rest of the supernatant was centrifuged for 4 min at 6700 × g, the pellet washed in 5 mL of HM and re-suspended in 1.5 mL of HM to give the heavy organelle fraction (H fraction). Supernatants were pooled (S2), and centrifuged for 10 min at 22,000 × g. The obtained pellet was washed in 5 mL of HM, and re-suspended in 1.5 mL of HM to yield the light organelle fraction (L fraction). The supernatant (S3) was pooled and centrifuged for 70 min at 70,900 × g to divide it into the particulate fraction (P fraction, pellet) and soluble, or cytosolic fraction (S

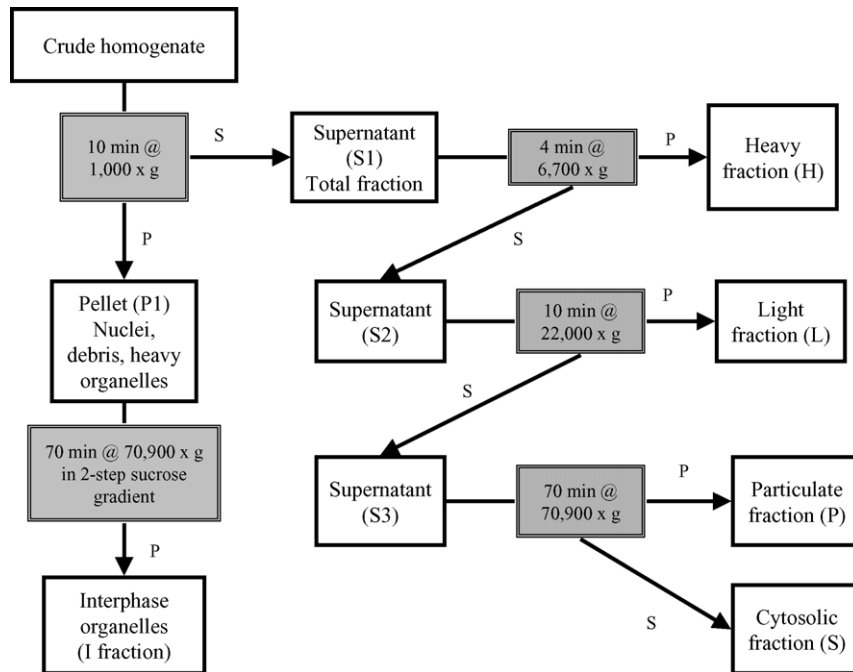


Fig. 1. Schematic diagram of subcellular fractionation of oyster tissues by differential centrifugation. Shaded boxes show details of centrifugation used to obtain the particular fraction. P, pellet and S, supernatant. Other details are in Section 2.

fraction, supernatant). The pellet was re-suspended in 1.5 mL of HM (P fraction).

Pilot studies indicated that some of the heavy organelles may co-precipitate with the nuclear and debris fraction during the first centrifugation. To recover these organelles, we re-suspended the pellet obtained after the second centrifugation at $1000 \times g$ in HM and adjusted sucrose concentration to 1.6 mol L^{-1} with the high density sucrose medium containing 2.4 mol L^{-1} sucrose, 10 mmol L^{-1} HEPES and 5 mmol L^{-1} MgCl_2 . The two-step gradient was set up by overlaying the pellet re-suspended in the high density medium with the lighter HM. The two-step gradient was centrifuged for 70 min at $70,900 \times g$, and the heavy organelle fraction (interphase, or I fraction) was collected from the interphase between the two media (Fleischer and Kervina, 1974).

1.3. Activity of marker enzymes

In order to analyze mitochondrial and lysosomal content of different organelle fractions, activities of the marker enzymes for mitochondria and lysosomes, cit-

rate synthase (CS) and acid phosphatase (AP), respectively, were determined in the total homogenates and all subcellular fractions at 25°C . Citrate synthase and acid phosphatase activity were measured spectrophotometrically as described previously (Sokolova and Pörtner, 2001; Bergmeyer, 1985). Samples were diluted to keep sucrose concentration in the assay below 0.01 mol L^{-1} in order to prevent inhibition of enzyme activities.

1.4. Cadmium determination

All fractions were acidified with 70% nitric acid (trace metal grade, Fisher Scientific, Suwanee GA, USA). An equal volume of nitric acid was added to re-suspended organelle or cytoplasmic fractions and incubated for 4–6 h at 65°C in a water bath until the tissues were digested. Cadmium concentrations were determined in tissue digests with an atomic absorption spectrometer (Perkin-Elmer AAnalyst 800), equipped with a graphite furnace and Zeeman background correction. NIST oyster tissue (1566b) was analyzed with the samples to verify the metal analyses; the percent recoveries over all batches were $94.6 \pm 6.6\%$ (mean \pm S.D.).

Protein concentrations in subcellular fractions were measured using a modified Biuret method with 1% Triton-X added to solubilize the organelles (Bergmeyer, 1985). BSA was used as the standard. Enzyme activities were expressed as U g^{-1} dry weight or U g^{-1} protein. Cadmium concentrations were expressed in ng Cd mg^{-1} protein. Tissue concentrations were also determined in different organelle fractions and used to convert cadmium loads (ng Cd mg^{-1} protein) into μM of cadmium assuming tissue water content of 80%, which was determined for *C. virginica* in our pilot studies.

1.5. Statistics

The effects of the factors “cadmium exposure”, “tissue” and “fraction” and their interactions were analyzed using split-plot generalized linear model ANOVAs after testing the assumptions of normality of data distribution and homogeneity of variances. Dunnett’s tests were used for post-hoc pairwise comparisons, and Fisher’s least square difference test (LSD)—for planned comparisons. Statistical analyses were performed using SAS 8.2 software (SAS Institute, Cary NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05.

2. Results

2.1. Characterization of subcellular fractions by activities of marker enzymes

Specific activities of citrate synthase (CS) per g tissue weight were similar in gill and hepatopancreas tissues, while specific activity of acid phosphatase (AP) was considerably higher in hepatopancreas, reflecting enrichment of this tissue with lysosomes (Fig. 2). Enzyme activities in subcellular fractions indicated that H and I fractions of the gill tissue were predominantly enriched in mitochondria while L fraction was enriched in lysosomes (Fig. 3A). Particulate fraction or microsomal fraction (P) in the gill also contained some lysosomes as indicated by intermediate acid phosphatase activity, but few mitochondria as indicated by low levels of CS activity (Fig. 3A). In hepatopancreas, H fraction was mixed and contained mitochondria and

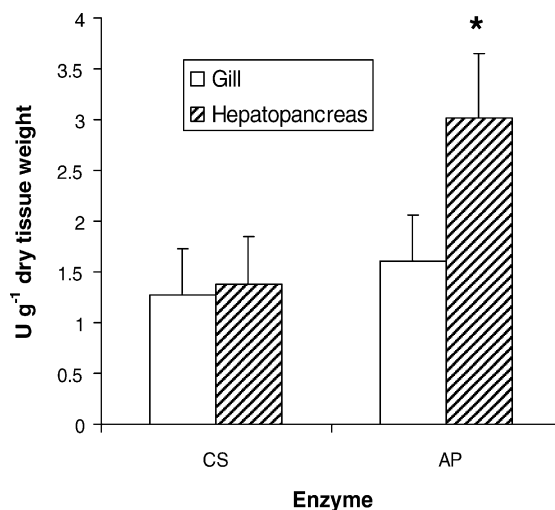


Fig. 2. Specific activities of mitochondrial and lysosomal marker enzymes in oyster tissues. CS, citrate synthase and AP, acid phosphatase. Asterisk denotes a statistically significant difference between the tissues ($P < 0.05$).

lysosomes as indicated by similar specific activities of the respective marker enzymes, while fractions I, L, and P were enriched in lysosomes (Fig. 3B). Levels of CS and AP activities in soluble fraction (S) were very low, close to the detection limit of the respective methods indicating minimal disruption of organelle integrity during homogenization. Lactate dehydrogenase activity (LDH), which is a cytosolic marker enzyme, was not detected in any of the organelle fractions indicating minimal contamination with cytoplasm.

2.2. Cadmium distribution in subcellular fractions

Average protein content in oyster hepatopancreas was higher than in the gills (12.5 mg g^{-1} versus 7.9 mg g^{-1} tissue weight, respectively), and there was no change in protein content in hepatopancreas of control or Cd-exposed oysters during 21 days of experimental exposure ($P > 0.08$). Similarly, no significant change in tissue protein content with exposure time was detected in the gills of control oysters ($P > 0.36$). In contrast, in Cd-exposed oysters there was a significant decrease in tissue protein content of gills after 21 days of exposure to cadmium compared to 2–7 days of exposure (3.5 mg g^{-1} versus $9.4\text{--}11.7 \text{ mg g}^{-1}$ tissue weight, respectively) ($P = 0.006$).

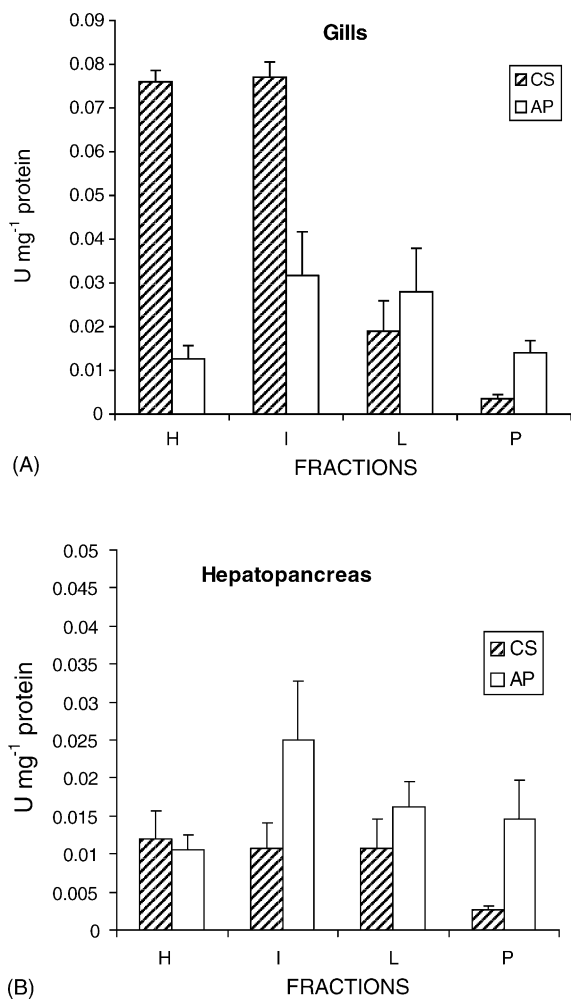


Fig. 3. Specific activities of mitochondrial and lysosomal markers enzymes in subcellular fractions of gills and hepatopancreas of *C. virginica*. CS, citrate synthase, and AP, acid phosphatase. Fractions: H, heavy; I, interphase; L, light; and P, particulate.

Exposure to cadmium resulted in a significant time-dependent accumulation in gills and hepatopancreas of *C. virginica*, which significantly differed between different subcellular fractions (Table 1, Fig. 4). In all fractions, cadmium levels were significantly higher in cadmium-exposed oysters as compared to their control counterparts (Fig. 5). In gills, cadmium accumulation was particularly strong in mitochondrial fractions (H and I) and cytoplasmic fraction (S) (Fig. 5). On average, up to 250–300 ng Cd mg⁻¹ protein was bound to

Table 1

Repeated measures ANOVA: effects of cadmium concentration in the water (exposure: 0 or 25 $\mu\text{g L}^{-1}$ cadmium), exposure time (time: 2, 7 or 21 days) and tissue (gill or hepatopancreas) on cadmium concentration in different subcellular fractions of *C. virginica*

Factor	D.F.	<i>F</i> -value	<i>P</i> -value
Exposure	1	80.35	<0.001
Time	2	28.14	<0.001
Tissue	1	4.94	0.026
Fraction	5	6.99	<0.001

DF, degrees of freedom of respective factors. Degrees of freedom for error were 369. Significant effects are highlighted in bold.

mitochondrial fractions in oyster gills, and ca. 350 ng Cd mg⁻¹ protein was found in the cytoplasm after 21 days of exposure. Cadmium levels in lysosomal (L) and lysosomal/microsomal fractions (P) of gill tissues were lower and corresponded to ca. 86–90 ng Cd mg⁻¹ protein. In hepatopancreas tissues after 7–21 days of exposure, the highest levels of cadmium were found in the cytoplasm (230–250 ng mg⁻¹ protein), while cadmium concentrations in all organelle fractions were significantly lower (55–90 ng Cd mg⁻¹ protein) (Fig. 5). Analysis of the proportional distribution of the total cadmium levels between different fractions indicates that 75–83% of total cadmium is found in cytoplasmic fraction of gills and hepatopancreas, which reflects the highest volume fraction of cytoplasm compared to other subcellular compartments (Fig. 6).

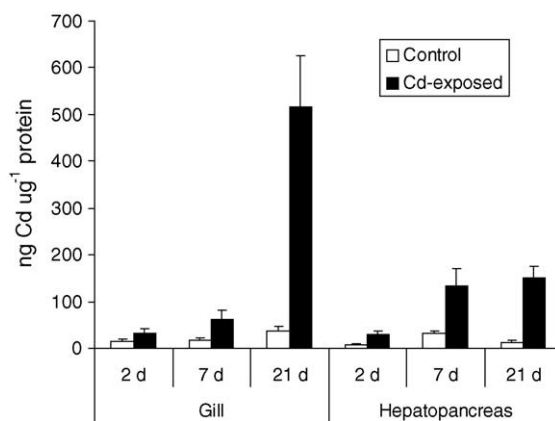


Fig. 4. Accumulation of cadmium in gills and hepatopancreas of *C. virginica* exposed to 25 $\mu\text{g L}^{-1}$ cadmium over time. Cd levels per mg protein of the total tissue homogenate is given. Exposure time: 2, 7 and 21 day.

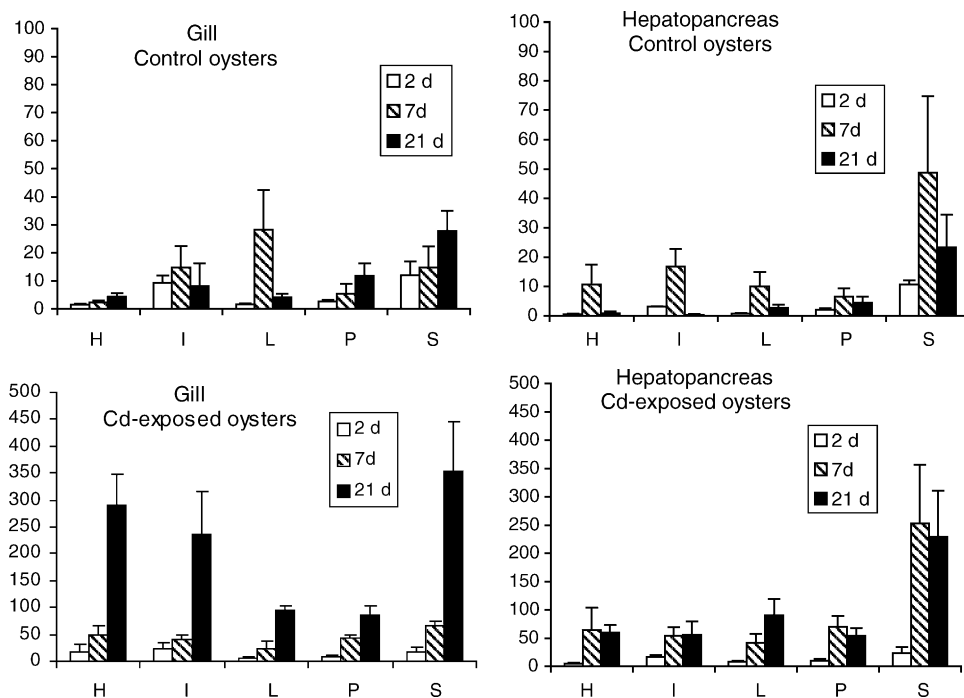


Fig. 5. Accumulation of cadmium in subcellular fractions of gills and hepatopancreas of *C. virginica* exposed to $25 \mu\text{g L}^{-1}$ cadmium over time. X-axis, fractions and Y-axis, cadmium concentrations (ng Cd mg^{-1} protein). Note differences in the scales of Y-axis for control and Cd-exposed animals. Fractions: H, heavy; I, interphase; L, light; P, particulate; and S, cytosol. Exposure time: 2, 7 and 21 day.

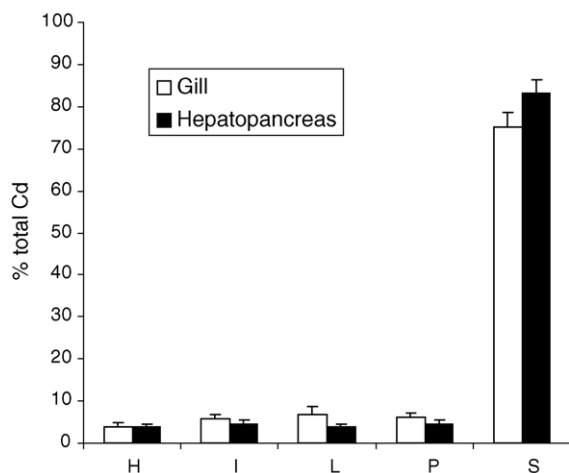


Fig. 6. Distribution (%) of cadmium burden in different subcellular fractions of gills and hepatopancreas of *C. virginica* exposed for 21 day to $25 \mu\text{g L}^{-1}$ cadmium. Fractions: H, heavy; I, interphase; L, light; P, particulate; and S, cytosol.

3. Discussion

This study demonstrates that oyster mitochondria can accumulate high levels of cadmium (up to $250\text{--}300 \text{ ng Cd mg}^{-1}$ protein in the gills) during 3 weeks of exposure to low sublethal concentration of cadmium ($25 \mu\text{g L}^{-1}$ or $0.22 \mu\text{M}$). These concentrations correspond to $25\text{--}30 \mu\text{M}$ of cadmium in mitochondrial fractions. It is worth noting that tissue burdens of cadmium in hepatopancreas and gills (31.9 ± 4.12 and $33.7 \pm 4.87 \mu\text{g g}^{-1}$ dry weight, respectively) were close to the levels found in oysters from polluted sites in the nature ($20\text{--}40 \mu\text{g g}^{-1}$ dry weight) (Roesijadi, 1996; Frew et al., 1997) suggesting that our exposure regime resulted in environmentally relevant tissue cadmium burdens. Strong cadmium accumulation in mitochondria of Cd-exposed oysters is consistent with the previously published findings of extensive Cd^{2+} uptake mediated by Ca^{2+} voltage-gated channels in isolated mitochondria (Li et al., 2000, 2003). Particularly high levels of cadmium

accumulated by gill mitochondria may be due to the fact that gills are the primary site of dissolved ion uptake in oysters (Kennedy et al., 1996), and thus gill mitochondria may be among the first organelles encountering elevated intracellular Cd^{2+} levels. In contrast, cadmium levels in the mitochondria-enriched H fraction of hepatopancreas were significantly lower than in gills (60 ng mg^{-1} protein). This may reflect a lower exposure of hepatopancreas mitochondria to cadmium and/or the mixed nature of this fraction, which contained a significant portion of lysosomes in addition to mitochondria.

Mitochondrial cadmium levels such as found in the present study are sufficiently high to deleteriously affect mitochondrial metabolism in oysters. Our earlier study has shown that cadmium concentrations as low as $5 \mu\text{M}$ can result in a significant decrease of oxidation capacity and decreased coupling in oyster mitochondria (Sokolova, 2004). It was also found that the negative effects on mitochondrial oxidation increase in a dose-dependent manner with increasing Cd^{2+} concentrations (Brierley, 1977; Skulsky et al., 1988; Kessler and Brand, 1994, 1995; Korotkov et al., 1999; Sokolova, 2004; Wang et al., 2004). Indeed, gill mitochondria of oysters exposed for 3 weeks to $25 \mu\text{g L}^{-1}$ of cadmium showed a significant decrease in ADP-stimulated (state 3) respiration rate by 35–40% (Sokolova et al., 2005). This level of inhibition of ADP-stimulated respiration closely corresponds to that expected from accumulation of 25–30 μM of cadmium, suggesting that this inhibition may be due to the direct effects of cadmium on mitochondria (Sokolova, 2004; Sokolova et al., 2005). Inhibition of state 3 respiration indicates a negative impact of cadmium on mitochondrial capacity for ATP production, which may lead to ATP deficiency under conditions of high energy demand such as spawning, temperature stress, etc. Proton leak was also inhibited in gill mitochondria from Cd-exposed oysters by 30–40% indicating a general decrease in mitochondrial oxidation rates due to cadmium (Sokolova et al., 2005). Cadmium-induced impairment of gill mitochondria could have serious consequences for the whole-organism metabolism and survival of oysters because gills are the major site for oxygen uptake and for various energy-requiring processes such as ion exchange (review in Kennedy et al., 1996). It is not known whether lower cadmium levels accumulated in mitochondria of hepatopancreas are

sufficient to affect their function, and further research is needed to determine sensitivity of these organelles to cadmium *in vivo* and *in vitro*.

Lysosomal fractions in gills and hepatopancreas of oysters also accumulated significant levels of cadmium ($90\text{--}94 \text{ ng mg}^{-1}$ protein). Lysosomal cadmium uptake may reflect sequestration and detoxification of this metal, but can also eventually result in adverse effects on lysosomal functions when detoxification capacity of the lysosomal system is overwhelmed. It has been previously shown that exposure to cadmium results in destabilization and damage of the lysosomal membranes in oysters and other marine bivalves (Sarasquete et al., 1992; Bolognesi et al., 1999; Ringwood et al., 1999a,b). Destabilization of lysosomal membranes and the subsequent release of proteolytic enzymes into the cytoplasm may contribute to cadmium cytotoxicity (Moore, 1985; Ringwood et al., 1999a,b). Recent studies have shown that lysosomes from hepatopancreas are particularly sensitive to the Cd-induced damage, and that lysosomal destabilization in hepatopancreas may occur at low sublethal cadmium concentrations, often prior to the onset of other symptoms of cadmium cytotoxicity (Sarasquete et al., 1992; Bolognesi et al., 1999; Ringwood et al., 1999a,b). This suggests that the target organelle for cadmium toxicity may vary depending on the organ, and that in oyster gills, the primary target may be mitochondria, whereas in hepatopancreas the main intracellular target for cadmium are lysosomes.

The cytoplasm is another major site of cadmium accumulation in oysters, both in gills and hepatopancreas. Cadmium concentrations in this compartment reached $230\text{--}350 \text{ ng mg}^{-1}$ protein after 21 days of exposure to $25 \mu\text{g L}^{-1}$ of cadmium. Most of the cytosolic cadmium in bivalves is typically bound to metallothioneins, which remove free metal ions and thus reduce their toxicity (Bracken et al., 1984; Roesijadi, 1996a; Bolognesi et al., 1999; Engel, 1999; Giguere et al., 2003). In this study, we did not analyze the proportion of metallothionein-bound cadmium in cytoplasm relative to the total cadmium load. However, the fact that metal-sensitive organelles such as mitochondria and lysosomes accumulated high levels of cadmium, which could significantly compromise their function suggests that the cytosolic detoxification system could not efficiently remove all the metal. Interestingly, current research also indicates that metallothionein-bound metals such as cadmium and zinc may be more phys-

iologically active than previously believed; in particular, they can transport metals into mitochondria and thus exert strong effects on mitochondrial function (Simpkins et al., 1994, 1998). This emphasizes the necessity of further investigations on physiological roles of metals bound to different intracellular ligands and compartments.

In general, our results suggest that the actual concentrations of cadmium associated with key intracellular organelles could be more important than % of the total load accumulated in each fraction. The percent of cadmium load associated with different intracellular compartments may reflect the relative volume of these compartments rather than physiologically relevant concentrations of cadmium. Indeed, in our study cytosolic cadmium accounted for 75–83% of the total tissue cadmium load, and less than 10% of total cadmium was associated with each of the organelle fractions. This distribution agrees with the previously reported findings on other bivalves, where the highest percentage of total cadmium (70–98%) was found in cytosol (Julshamn and Andersen, 1983; Evtushenko et al., 1986; Bebianno et al., 1993). Predominant accumulation of cadmium in cytoplasm and relatively low proportion in organelles is often interpreted as an indication of efficient detoxification of cadmium in aquatic organisms (Julshamn and Andersen, 1983; Evtushenko et al., 1986; Block et al., 1991; Bebianno et al., 1993; Blackmore and Wang, 2002). However, our study clearly showed that even the relatively low percentage of cadmium associated with organelles (less than 10%) can still be accompanied by high organelle-specific cadmium concentrations that are sufficient to cause dysfunction of such metal-sensitive organelles as mitochondria and lysosomes.

In summary, our study showed that exposure of oysters to low sublethal amounts of cadmium resulted in accumulation of high levels of this metal in organelle fractions as well as in cytoplasm. In gill tissues, mitochondria were the primary target organelle for bioaccumulation, which agrees with the results of our previous studies showing that mitochondrial dysfunction is an early toxic event in gills of Cd-exposed animals (Sokolova, 2004; Sokolova et al., 2005). In hepatopancreas, lysosomes were the primary target for bioaccumulation, supporting results of our previous work showing high sensitivity of hepatopancreas lysosomes to metals (Ringwood et al., 2002, 2004). It is

likely that this pattern of predominantly mitochondrial effects in gills and lysosomal effects in hepatopancreas tissues will be observed with other metals or pollutants in other species. This work has important implications for the development of biomarkers of exposure and effect for heavy metals and for understanding the mechanisms of toxic effects of metals. Organ- and organelle-specific differences may be related to the route and duration of exposure. The incorporation of these kinds of biomarker measures into environmental assessments and bioavailability models (e.g. biotic ligand model) should serve to improve the diagnostic capability for identifying toxicity.

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