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Subcellular Distribution of Heavy Metals in Organs of Bivalve *Modiolus Modiolus* Living Along a Metal Contamination Gradient

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Abstract – Concentration and distribution of Fe, Zn, Cu, Cd, Mn, Pb, Ni among subcellular fractions (cellular membrane structures and cytosol) and Zn, Cu, Cd among cytoplasmic proteins in the kidney and digestive gland of mussel *Modiolus modiolus* living along a polymetallic concentration gradient were studied. It was found in the kidney of *M. modiolus* from contaminated sites that the Fe percent increased in the "membrane" fraction, whereas Zn, Pb, Ni and Mn percent increased in the cytosol compared to the kidney of the control mussel. Note kidney cytosol of *M. modiolus* from clean and contaminated sites sequestered major parts of Cu and Cd. In the digestive gland of *M. modiolus* from contaminated sites Fe, Zn, Cd, Mn, Ni percent increased in the "membrane" fraction, whereas Cu, Pb percent increased in the cytosol compared to digestive gland of control mussel. Gel-filtration chromatography shows kidney of *M. modiolus* contains increased metallothionein-like protein levels irrespective of ambient dissolved metal concentrations. It was shown that the metal detoxification system in the kidney and digestive gland of *M. modiolus* was efficient under extremely high ambient metal levels. However, under complex environmental contamination in the kidney of *M. modiolus,* the metal detoxification capacity of metallothionein-like proteins was damaged.

Key words – heavy metals, subcellular distribution, metal-binding proteins, contamination

1. Introduction

Under ambient elevated level of heavy metals, bivalves are known to accumulate high metal concentrations without apparent damage to their metabolism. Metal tolerance of such organisms is result of metal sequestration system, involving cytoplasmic low molecular weight metal-binding proteins – metallothioneins MT (Isani *et al.* 2000), lysosomes, granules and mineral concretions (Vesk and Byrne 1999; Bonneris *et al.* 2005). Therefore alterations of subcellular distribution of metals reflect success in cellular detoxification and can provide valuable information whether or not accumulated metals induce toxic effects (Wallace *et al.* 2003).

Evaluation of success of heavy metal detoxification is often limited to study of metal distribution among subcellular fractions (Sallivan *et al.* 1988; Regoli and Orlando 1994) or cytoplasmic metal-binding proteins (Ponzano *et al.* 2001; Giguere *et al.* 2003; Riveros *et al.* 2003). However, bivalves have these processes interrelated, therefore metal binding by both subcellular fractions and cytoplasmic proteins should be studied simultaneously. But few works deal with metal subcellular distribution in detail on chronically exposed indigenous bivalves (Mouneyrac *et al.* 1999; Bebianno and Serafim 2003; Campbell *et al.* 2005).

Recently, we studied subcellular distribution of heavy metals in the organs of two mussels *Crenomylulis grayanus* and *Modiolus modiolus* at a site highly contaminated with heavy metals (Podgurskaya and Kavun 2005). *C. grayanus* prefers solid sediments, whereas *M. modiolus* prefers soft sediments (silty sand or silty sediment); however these bivalves can form combined settlement (Skarlato, 1981). Our work demonstrated that under high chronic contamination, *M. modiolus* has a metal detoxification system more successful than does *C. grayanus* likely due to induced tolerance to soft sediments enriched with heavy metals.

The aim of this study was to evaluate success of the

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metal sequestration system of the bivalve preferring soft sediment. Present work demonstrates concentration and distribution of Fe, Zn, Cu, Cd, Mn, Pb, Ni among subcellular fractions (cellular membrane structures and cytosol) and Zn, Cu, Cd among cytoplasmic proteins in the kidney and digestive gland of mussel *M. modiolus* living along a polymetallic contamination gradient.

2. Materials and Methods

Mussels (*M. modiolus*) were collected along a polymetallic contamination gradient (Fig. 1). Coastal waters of Reineke Is. (site 1) are considered as control area, as bivalves and sediments from this site contain minimal metal concentrations (Shulkin *et al.* 2003). Vostok Bay (site 2) is a slightly contaminated area (Shulkin 2004). There is domestic and industrial waste landfill in the Gornostay Bay (site 4). Due to liquid from the landfill, Gornostay Bay and the nearest Desuntnaya Bay (site 3) are highly contaminated areas, containing similar extremely high levels of Fe, Zn, Cd, and especially Cu, Pb in the coastal waters and sediments (Shulkin *et al.* 2003).

Mussels were collected in June 2002 (sites 2, 3, 4) and 2004 (site 1). At each sampling site, divers collected 15 adult mussels at a depth of 5 - 6 m. Size of collected samples was 10.4 ± 0.8 cm (site 1), 10.9 ± 1.4 cm (site 2), 10.5 ± 0.9 cm (site 3), 10.0 ± 1.0 cm (site 4). Mann-Whitney *U*-test showed no significant differences between age values of mussels. Before preparation mussels were kept in an aquarium containing natural seawater. The mussels were dissected to obtain digestive gland and kidney. Organs of five mussels per site were taken to determine Fe, Zn, Cu, Cd, Mn, Pb and Ni concentration. Digestive gland and kidney from ten mussels per site were divided into two pools (for separation of subcellular fractions and gel filtration chromatography). Separation of subcellular fractions and gel filtration chromatography were done on fresh material.

Separation of subcellular fractions

Tissues were homogenized at 0°C in 0.05M Tris-HCl buffer, pH 7.5, with 0.25M sucrose, 0.5M NaCl and 0.01 M $MgCl_2$ for membranes stabilization, the homogenate was divided into three sub-samples. Sub-samples were centrifuged at 20000 g (+4°C, 1.5 hour) to separate "membrane" fraction (nuclei, cellular debris, granular concretions, mitochondria, lysosomes, microsomes) and particle-free supernatant (cytosol).

Gel filtration chromatography

The metal distribution among the proteins with different molecular weight in the cytosol of the digestive gland and kidney was studied by gel filtration chromatography. Tissues were homogenized at 0°C in 0.05 M Tris-HCl buffer, pH 7.5, the homogenate was divided into three sub-samples. Sub-samples were centrifuged at 20000 g (+4°C, 1.5 h). The supernatant (< 1 ml) was applied to Sephadex-100 column (1.5 cm × 60 cm) equilibrated with the same buffer at a flow rate 15 ml h^{-1}. The void volume

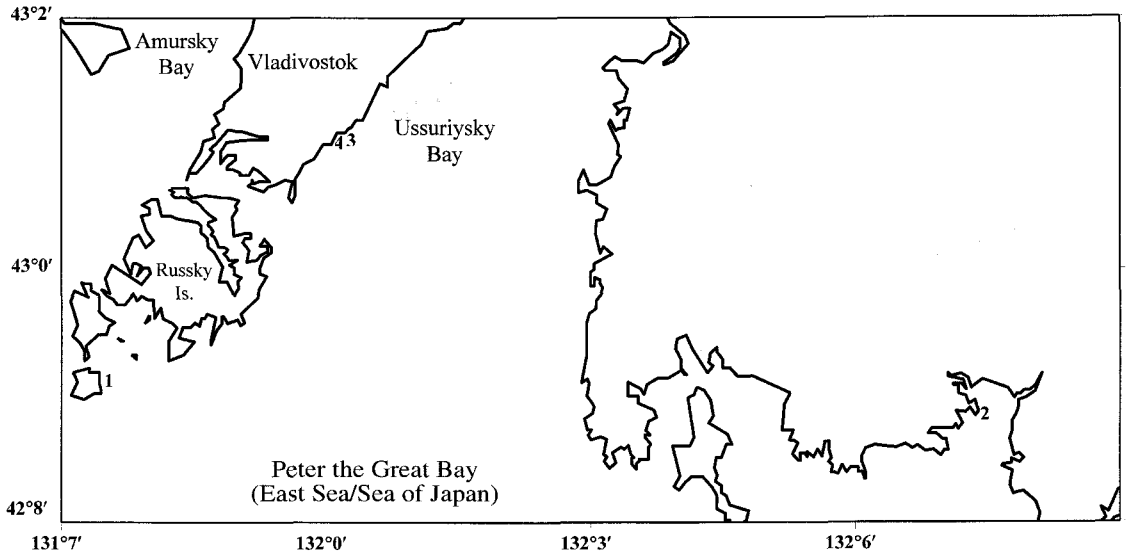

Fig. 1. Schematic map of the sampling sites in Peter the Great Bay (East Sea/Sea of Japan). 1, 2, 3, 4 are sites numbers.

of the column was determined with Dextran Blue and column was calibrated with standard molecular weight markers: bovine serum albumin (67 kDa), cytochrome C (12.5 kDa).

As MT have maximal absorption at wave-length (λ) = 254 nm due to high content of mercaptide bonds and minimal absorption at λ = 280 nm due to lack of aromatic aminoacids (Frazier *et al.* 1985), fractions (3 ml) from columns were monitored for absorbance at 254 nm and 280 nm using UV-260 spectrophotometer. Eluting fractions were combined into three pools (Fig. 2): a high molecular weight (HMW) pool (> 60 kDa, Ve/Vo = 1), eluted at the same retention time as bovine serum albumin; a MT-like proteins (MTLP) pool (≈ 12.5 kDa, Ve/Vo = 1.8), eluted at the same retention time as cytochrome C; a low molecular weight (LMW) pool (< 4 kDa, Ve/Vo = 2.8); Ve – effluent volume, Vo – void volume.

Heavy metal analysis

Tissues, subcellular fractions and combined fractions from Sephadex-100 column were dried at 85°C. The dried samples were wet acid mineralized (concentrated HNO_3) at 180°C, dry residues were dissolved in 0.1 N HNO_3. Metal concentrations were determined using Hitachi 180-70 flame atomic absorption spectrophotometer. The QA/QC procedures included measurement of duplicate samples, blanks and certified reference materials during each analytical set (Table 1). Statistical analysis (mean value, standard deviation, Mann-Whitney significance test) was performed using the Excel and Statistica programs.

3. Results

Metal concentrations

Metal concentrations (except Cu in the kidney and Mn in the digestive gland) in the organs of *M. modiolus* from Vostok Bay (site 1) were significantly higher compared to that in the mussel from coastal waters of Reineke Is. (control site 1) (Table 2). Concentrations of Zn, Cu, Cd, Mn, Pb in the kidney and Zn, Cu, Cd, Pb, Ni in the digestive gland of the mussel from Desuntnaya Bay (site 3) were significantly higher compared to that in the mussel from site 2; whereas Mn content in the kidney of the mussel from site 3 was significantly lower compared to that in the mussel from site 2. Heavy metal content in the kidney and digestive gland of the mussels from Desuntnaya Bay and Gornostay Bay (site 4) was similar. However, Cd concentration in the kidney and Cd, Ni concentrations in the digestive gland of the mussels from site 4 were significantly higher than that in the mussel from site 3 (Table 2).

Metal relative distribution among subcellular fraction

Our data show in the kidney of control *M. modiolus* Zn,

Table 1. Results of analysis of standard reference materials (μg g^{-1} dry weight) mean ± standart deviation

NBS 2976	Fe	Zn	Cu	Cd	Mn	Pb	Ni
certifed values	171 ± 4.9	137 ± 13	4.02 ± 0.33	0.82 ± 0.16	33 ± 2.0	1.19 ± 0.18	0.93 ± 0.12
values found	189 ± 15	139 ± 29	4.21 ± 0.04	0.88 ± 0.14	31 ± 0.49	0.99 ± 0.24	1.13 ± 0.06

Note: NBS 2976 – mussel tissue from NIST (National Institute of Standards and Technology, USA)

Table 2. Heavy metal concentrations (μg g^{-1} dry weight) in the organs of *Modiolus modiolus* (mean ± standard deviation) (N=5)

Site	Fe	Zn	Cu	Cd	Mn	Pb	Ni
				Kidney			
Site 1	216 ± 85	1340 ± 572	528 ± 112	44 ± 10.9	904 ± 240	46 ± 14.4	1.2 ± 0.9
Site 2	980 ± 422[a]	5021 ± 2129[a]	598 ± 92	92 ± 17.7[a]	2570 ± 653[a]	134 ± 45[a]	54 ± 22[a]
Site 3	525 ± 654	21238 ± 11196[a,b]	1783 ± 545[a,b]	203 ± 75[a,b]	1455 ± 1245	4513 ± 2443[a,b]	72 ± 36[a]
Site 4	1006 ± 208[a]	22875 ± 6594[a,b]	3687 ± 313[a,b,c]	254 ± 48[a,b]	2463 ± 439[a,b]	3826 ± 1328[a,b]	69 ± 15.1[a]
				Digestive gland			
Site 1	245 ± 93	44 ± 15.6	11.9 ± 6.2	1.0 ± 0.6	4.1 ± 1.2	1.8 ± 0.8	0.06 ± 0.03
Site 2	691 ± 416a	85 ± 22[a]	30 ± 7.89[a]	2.06 ± 0.72[a]	7.63 ± 3.34	4.59 ± 1.64[a]	1.70 ± 0.99[a]
Site 3	535 ± 131[a]	133 ± 18.8[a,b]	153 ± 53[a,b]	7.39 ± 1.0[a,b]	17.6 ± 2.6[a,b]	144 ± 22[a,b]	2.0 ± 0.3[a]
Site 4	701 ± 324[a]	172 ± 34[a,b]	221 ± 78[a,b]	9.42 ± 1.38[a,b,c]	18.1 ± 5.61[a,b]	148 ± 35[a,b]	7.28 ± 2.22[a,b,c]

Note: a - significant difference ($p \leq 0.05$) compared to the mussel from site 1; b - significant difference ($p \leq 0.05$) compared to the mussel from site 2; c - significant difference ($p \leq 0.05$) compared to the mussel from site 3. The significance between the values was tested by Mann-Whitney *U*-test

Table 3. Relative distribution of heavy metals (% of total metal content in a cell) among subcellular fractions of the kidney and digestive gland of mussel *Modiolus modiolus*.

Fraction	Kidney				Digestive gland			
	Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
					Fe			
"membrane"	44	54	65	57	58	79	74	74
cytosol	56	46	35	43	42	21	26	26
					Zn			
"membrane"	94	36	43	29	26	31	37	41
cytosol	6	64	57	71	74	69	63	59
					Cu			
"membrane"	11	21	22	10	49	53	37	34
cytosol	89	79	78	90	51	47	63	66
					Cd			
"membrane"	14	17	21	25	13	65	53	46
cytosol	87	83	79	75	87	35	47	54
					Mn			
"membrane"	71	48	28	20	19	48	51	55
cytosol	29	52	72	80	81	52	49	45
					Pb			
"membrane"	97	67	78	57	70	74	59	50
cytosol	3	33	22	43	30	26	41	50
					Ni			
"membrane"	65	56	44	58	41	76	77	62
cytosol	35	44	56	42	59	24	24	38

Note: Values are mean of three replicates. Error ± 3 %.

Mn, Pb, Ni were mainly sequestered by membrane fraction; Cu, Cd were mainly bound by cytosol; Fe was approximately evenly distributed among subcellular fractions. Whereas in the kidney of the mussels from contaminated sites 2, 3, 4, content of membrane-bound Zn, Mn, Pb, Ni decreased and content of membrane-bound Fe increased compared to control mussel (Table 3).

Digestive gland of the control mussel has Pb, Fe mainly sequestered by membrane structures and Zn, Cd, Mn, Ni mainly bound to cytosol. Whereas in the digestive gland of the *M. modiolus* from contaminated sites 2, 3, 4, percentage content of Fe, Zn, Cd, Mn, Ni increased in the membrane fraction, content of Cu, Pb increased in the cytosol compared to control mussel (Table 3).

Cd, Cu, Zn distribution among cytoplasmic protein pools

Our work considers Cd, Cu, Zn cytoplasmic distribution only. Gel filtration chromatography show three absorbance peaks in the cytosol of the *M. modiolus* kidney (as example shown in Fig. 2). MTLP peak was not isolated from cytosol of the digestive gland. But elution profiles of the digestive glands showed part of cytosolic Cu, Zn, Cd was eluted at a fraction corresponding in Ve/Vo index to MTLP (Fig. 2B). Clearly, MTLP content in the cytosol of *M. modiolus* digestive glands was below the detection limit of gel-chromatography.

Our data show MTLP bound major parts of Cu, Cd in the kidney cytosol of the mussels from sites 1, 2, 3. It should be noted, in the *M. modiolus* kidney from site 4, the percent of MT-bound Cd and Cu decreased (Table 4). Digestive gland cytosol of control mussel has about 90 % of Cu bound by HMW; in this organ of mussels from contaminated sites Cu percent increased in the MTLP pool. In digestive gland cytosol from all sites, Cd was mainly bound by HMW (Table 4).

4. Discussion

Elevated ambient heavy metal levels result in significant increase in the concentration of these toxicants in the digestive gland and especially the kidney of *M. modiolus* collected along environmental metals gradient (Table 2).

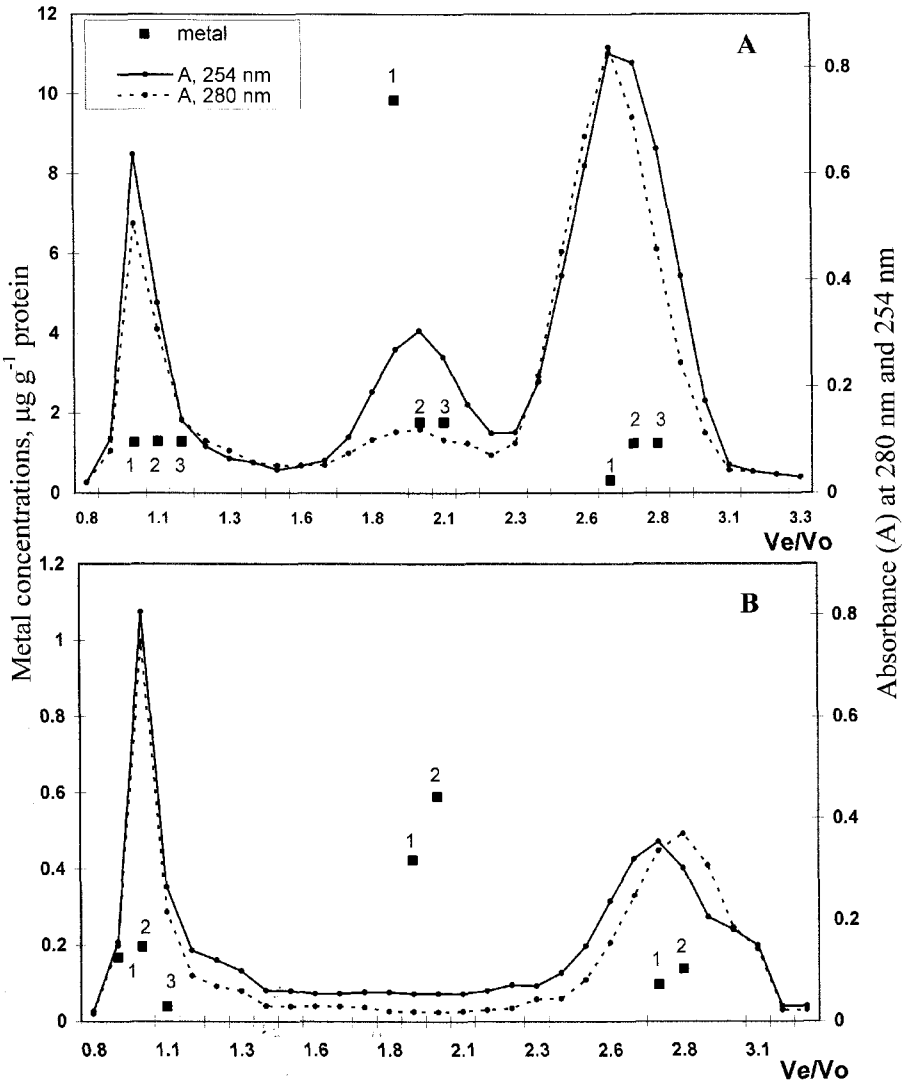

Fig. 2. Metal concentrations in cytoplasmic protein pools from the kidney (A) and the digestive gland (B) of the mussel *Modiolus modiolus* from Desuntnaya Bay (site 3). 1–copper, 2–zinc, 3–cadmium. Ve–effluent volume, Vo–void volume. Ve/Vo=1-HMW, Ve/Vo=1.9–MTLP, Ve/Vo =2.8–LMW.

The digestive gland of bivalves is the main centre for metabolic regulation, participating in the mechanisms of immune defense and homeostasis regulation of the internal medium (calcium, hemolimphatic pH, cell volume), as well as in the processes of detoxification and elimination of xenobiotics (Salvini-Plawen 1988). Therefore detoxification and elimination of heavy metal excesses from this organ are an important aim of defense system of living organisms. For bivalves, as a rule, this homeostasis is provided by metal redistribution into the kidney (Kavun and Shulkin 2005). However, total very high metal content in bivalves can result in kidney elimination capacity failure and metal accumulation in other organs. Thus, elevated Cu level was observed for the digestive gland of the *C. grayanus* transplanted from a clean area to contaminated Desuntnaya Bay (Kavun and Shulkin 2005). In contrast, *M. modiolus* from Desuntnaya Bay and Gornostay Bay has no similar damage of equilibrium of processes metal uptake and elimination (Table 2), confirming this mussel evolved tolerance to increased level of ambient dissolved metals.

All heavy metals have potential toxicity to living organisms due to their high affinity to macromolecules containing S and N (Nieboer and Richardson 1980), leading to damaging their function. Moreover metals can induce reactive oxygen species (oxyradicals) or overwhelm antioxidant enzymes

Table 4. Relative distribution of Zn, Cu, Cd (%) among cytoplasmic protein pools of the kidney and digestive gland of mussel *Modiolus modiolus*.

Fraction	Zn	Cu	Cd	Zn	Cu	Cd
	Kidney			Digestive gland		
			site 1			
HMW	33	3	16	9	89	94
MTLP	26	89	77	34	4	5
LMW	41	8	7	57	7	1
			site 2			
HMW	40	9	5	56	90	93
MTLP	39	85	90	23	6	7
LMW	21	6	5	21	4	0
			site 3			
HMW	26	13	11	42	47	100
MTLP	24	80	83	25	23	0
LMW	50	7	6	33	30	0
			site 4			
HMW	22	29	28	65	58	100
MTLP	8	57	49	18	30	0
LMW	70	14	23	17	12	0

Note: Values are mean of three replicates.

activities (Stohs and Bagchi 1995; Dovzhenko *et al.* 2005), leading to oxidative stress.

Essential Fe can generates oxyradicals; therefore defense cell systems tend toward elimination or immobilization of Fe excess. Increased Fe percentage in the membrane structures of the organs of *M. modiolus* from contaminated sites (Table 3) showed Fe immobilization into granules as ferric hydroxide (George *et al.* 1976) as well as in the kidney of another bivalve, *Mercenaria mercenaria*, preferring soft sediment (Sallivan *et al.* 1988). Such way of Fe sequestration likely results in not significant differences between Fe content in the digestive gland and kidney of *M. modiolus* (Table 2). In contrast, total Fe level increase in *C. grayanus* organs results in elevated Fe percent in the cytosol. In these cases this metal was mainly accumulated by mussel kidney (Podgurskaya *et al.* 2004; Podgurskaya and Kavun 2005).

Essential Zn mainly accumulated in cell membrane structures of control *M. modiolus* kidney; excess of this metal bound to cytosol (Table 3) as well as in other bivalves (Sallivan *et al.* 1988; Kaland *et al.* 1993; Podgurskaya *et al.* 2004). In contrast, the digestive glands of *M. modiolus* from contaminated sites has Zn percent increase in the "membrane" fraction (Table 3) probably due to metal excess sequestration as zinc pyrophosphate in granules (Pullen and Rainbow 1991).

Essential Cu was mainly found in the kidney cytosol of the mussels from all sites (Table 3). Similar Cu distribution was shown in the kidney of the *M. mercenaria* (Sallivan *et al.* 1988). Whereas in the kidney of the *Nassarius reticulatus* (Kaland *et al.* 1993) and *C. grayanus* (Podgurskaya *et al.* 2004) Cu mainly bound by "membrane" fraction under background conditions, and sequestration this metal by cytosol was observed under elevated total Cu level. Therefore, clearly, *M. modiolus* accumulates excess of this metal irrespective of ambient Cu level. Major Cu binding by MTLP in the kidney of mussels from all sites (Table 4) confirmed this hypothesis, as these proteins bind metal excess. It should be noted that main pollutant Cu clearly removed Mn from kidney of mussels from sites 3 and 4, as these metals compete for binding sites (Sunda and Huntsman 1983), and resulted in no significant differences between Mn concentrations in the kidney of *M. modiolus* from slightly and highly contaminated sites (Table 2). In the digestive gland (with low Cu level) of mussels from contaminated sites Mn content increase was observed, and this metal clearly bound to granules (Table 3) (Sallivan *et al.* 1988; Regoli and Orlando, 1994).

In bivalves, toxic Pb mainly accumulated by endocytosis in a colloidal or particulate form as sulfur or phosphate salts, bound by lysosomes (Regoli and Orlando 1994) and isolated with "membrane" fraction (Table 3). Pb in the cytosol of *M. modiolus* organs perhaps bound with specific

protein enriched with glutamine, asparagine, glicine, cysteine (Fowler 1998). Pb-bound proteins were found in mammals (Fowler and DuVal 1991) and fishes (Conner and Fowler 1994); probably bivalve have similar mechanisms for Pb detoxification. Table 3 showed Pb percent increase in the cytosol of *M. modiolus* kidney and digestive gland under elevated total Pb concentration. Clearly, Pb-bound proteins in the *M. modiolus* play a detoxification role under elevated level of ambient metals.

Experimental and field research showed toxic Cd mainly sequestered by cytosol (Sallivan *et al.* 1988; Kaland *et al.* 1993; Mouneyrac *et al.* 1999; Podgurskaya *et al.* 2004), as well as in the kidney of *M. modiolus* from all sites. In contrast, in the digestive gland of mussels from contaminated sites, Cd was likely sequestered by granules (Table 3). Major Cd accumulation by "membrane" fraction was shown, for example, in the marine bivalve *Laternula elliptica* (Choi *et al.* 2001, 2003) and in the digestive gland of *C. grayanus* (Podgurskaya *et al.* 2004) from regions with naturally elevated Cd. Under contamination more than 50 % of Cd was found in the calcium concretions of the gills of freshwater bivalve *Pyganodon drandis*, producing high level such granules (Bonneris *et al.* 2005).

Therefore, likely under contamination *M. modiolus* digestive gland has heavy metals bound by granules and lysosomes, as this detoxification mechanism clearly requires less energy than MTLP synthesis. This fact confirms that the defense route of *M. modiolus* tends to both prevent toxic effects of accumulated metals and reserve enough energy in these organs to provide normal metabolism. Whereas in the kidney, main organ for storage and elimination of toxicants, heavy metals are sequestered by cytosolic metal-binding proteins.

MT play central role in detoxification of Cd and storage of essential Cu, Zn (Roesijadi 1992; Isani *et al.* 2000). Cu and Zn are known to bind by cytoplasmic HMW and LMW, MT sequester excess of these metals. Whereas Cd is mainly bound by MT with HMW as initial ligands (Carpene and George 1981; Mouneyrac *et al.* 1999; Podgurskaya *et al.* 2004). It should be noted, MTLP were isolated from the kidney of all mussels (including from control areas), although such synthesis of these proteins are observed under very high level of ambient metals (Mouneyrac *et al.*, 1999; Podgurskaya *et al.* 2004). For example, MTLP were isolated from *C. grayanus* kidney from mighty upwelling region, whereas in kidney cytosol of the same bivalve from control site (Reineke Is.) and seasonal upwelling region MTLP peak was not observed (Podgurskaya *et al.* 2004). Increased synthesis MTLP in the *M. modiolus* kidney is likely due to induced tolerance arising from adaptation to soft sediments enriched with trace metals (Podgurskaya and Kavun 2005).

Table 4 showed kidney MTLP of *M. modiolus* from site 4 bound a lesser percentage of metals than in the mussels from other sites. Likely this fact is due to increased production of oxyradicals in bivalves. MT, being part of cell antiradicals system react with oxyradicals (Viarengo *et al.* 2000), resulting in SH-groups of MT being oxidized and their metal-binding capacity failing (Klein *et al.* 1994); moreover, bound metals remove from MT (Roesijadi *et al.* 1997). In the kidney of mussels from site 4, metals excess was found in the LMW pool, sequestered probably by glutathione (Chelomin *et al.* 1998), and in the HMW, showing in this case toxic stress. As earlier work has not showed significant differences between environmental metal concentrations in the sites 3 and 4 (Shulkin *et al.* 2003), probably increased production of oxyradicals in the bivalve from site 4 is due to other pollutants entering marine water with liquid from the landfill.

Thus, digestive gland of *M. modiolus* has an excess of metals bound with "membrane" fraction, whereas kidney bind with metal-binding proteins. Cell detoxification mechanisms of this bivalve are successful under high metal contamination; under high complex contamination, decreased metal-binding capacity of MTLP in the kidney was observed.

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