An in vitro study of the effect of reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of mussel *Crenomytilus grayanus*

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Abstract

The study was performed to assess in vitro effects of reactive oxygen species (ROS, oxyradicals) on intracellular distribution of accumulated cadmium in digestive gland of the mussel *Crenomytilus grayanus*.

In vitro induction of ROS (by Fe/ascorbate reaction) in tissue homogenates of Cd-accumulated mussels led to a significant increase in lipid peroxidation (as conjugated dienes and malondialdehyde) and also to decrease in reduced glutathione and Cd-binding protein contents. Also fraction of MT-like proteins (20–22 kDa) has been shifted to a higher molecular weight area (40–45 kDa), which indicates dimerization of the protein. The level of intracellular vesicle-stored cadmium (within membrane compartments like lysosomes) was decreased significantly in oxyradicals-exposed tissue crude homogenate of mussels in comparison with controls. Additionally, Cd distribution among three weight classes of cytosol proteins has been significantly changed after ROS exposure.

Taken together the results, there is a clear indication that ROS induce an oxidative stress resulting in damaging of intracellular Cd-binding compartments that may trigger (or contribute) the toxicity of this metal. Thus, from our experimental results and reviewed information follows that under high "pressure" of heavy metals on marine environment the aquatic organisms can show higher sensitivity to normal variations of natural factors of the environment or even decrease the range of tolerance to their variations.
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Keywords: Cadmium; Accumulation of heavy metals; Reactive oxygen species; Oxidative stress; Marine invertebrates

1. Introduction

As it has been shown many marine bivalves, particularly *Mytilidae*, are able to accumulate relatively high concentrations of heavy metals including highly toxic

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cadmium and retain it for a quite long time (Simkiss et al., 1982). This process is a result of functioning of well coordinated complex biochemical system, which includes synthesis of specific proteins such as metallothioneins (MT) and metal-depositing membrane compartments such as lysosomes, spheroblasts granules, mineral concretions etc (George, 1982; Simkiss et al., 1982; Viarengo et al., 1987; Viarengo, 1989). This means that the process of bioaccumulation and detoxification of heavy metals has hierarchical structure and involve both the synthetic unit of cell biochemical machinery and mechanisms responsible for the biogenesis and recovery of biomembranes. Consequently, in course of a heavy metal accumulation–detoxification an enhanced energy expense occurs on top of the common metabolic costs. Potentially, this might lead to damage of the general cell structures. Moreover, complex structure of such hierarchical cause–effect links indicates its vulnerability to both exogenous and endogenous harmful factors. Reactive oxygen species (ROS or oxyradicals) are one of important among all such harmful factors. ROS are usual by-products of many oxidative reactions (Halliwell and Gutteridge, 1986; Sies, 1991) and its level in living cells depends on physiological and biochemical rearrangements during adaptation to fluctuating environment (Livingstone et al., 1990; Sheehan and Power, 1999; Filho et al., 2001; Livingstone, 2001). Taking in account the fact that different forms of deposited heavy metals (in MT which are stored in membrane compartments) may be a target for the ROS (Miura et al., 1997; Viarengo et al., 2000) we have carried out the experiment to elucidate the effect of ROS (induced by Fe^{2+}-ascorbat model) on subcellular distribution of deposited cadmium in digestive gland of mussels *Crenomytilus grayanus*.

2. Materials and methods

The flow chart of the experiment is presented at Fig. 1. Adult individuals ($n = 30$, 100 ± 10 mm) of mussels *C. grayanus* (Dunker, 1853) from Popov island (Peter The Great bay, Sea of Japan, Russia) were used for the experiments. Mussels were collected during August–September, 18–20 T °C, 35‰. Mussels were exposed 50 μg Cd^{2+} l^{-1} (in form of $CdCl_2$) during four weeks in 140 l aquariums with well aerated (>90%) fresh sea water (18–20 °C, 35‰). Aquarium water was

exchanged every second day to keep the cadmium concentration at constant level.

After exposure period the digestive gland has been extracted from mussels on ice and thoroughly cleaned from connective tissue. Then it was homogenized in ice-cold buffer 50 mM Tris–HCL pH 7.8, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2.0 mM dithiothreitol (DTT). Then the homogenate was centrifuged at $2000 \times g$ for 15 min to remove cell debris. Obtained homogenates were split onto two equals parts: control and experimental (Fig. 1).

The reactive oxygen species (ROS) were induced by adding Fe^{2+} (in form of $FeSO_4$) and ascorbic acid to the crude homogenate to the final concentration of 10 and 50 μM correspondingly (Kreps et al., 1987), for 90 min at room temperature (18 °C).

Then homogenates (control or experiment) were centrifuged at $15\,000 \times g$ for 40 min to separate cytosol and membrane fractions (Fig. 1). The aliquots of each fraction were taken for further biochemical and chromatographic analyses.

Protein separation was carried out by gel-filtration on chromatographic column (100 cm × 2.0 cm) with Sephadex G75 with mobile phase of 25 mM Tris–HCI, pH 7.8. The elution rate was 17 ml/h and 3.0 ml fraction volume. The optical density of the samples (control and experiment) at 254 nm has been equalized before separation. Protein detection was performed by flow-through UV detector (LKB, Sweden) at 254 nm. The column has been calibrated with protein molecular weigh standards (Pharmacia) ovalbumin (43 kDa), DNAase (31 kDa), tripsin (23.3 kDa) cytochrome *c* (11.7 kDa).

Cadmium content in homogenates and chromatographic fractions was determined by AAS after ashing with HNO_3 and $HClO_4$ (3:1, v/v) (Julshamn and Andersen, 1983). Additionally, after the protein separation the optical densities of all collected fractions (3 ml each) was measured again at 254 nm and Cd^{2+} content was expressed as μg Cd^{2+} OD_{254}^{-1} of a fraction. The protein content was quantified by Greenberg method (Greenberg and Gaddock, 1982) with bromephenol blue dye with human serum albumin as standard. Level of lipid peroxidation was evaluated by content of conjugated dienes (CoDi) and malondialdehyde (MDA) (Buege and Aust, 1978). Amounts of the reduced glutathione (GSH) and contents of reduced SH-groups in proteins were evaluated by quantitative colored reac-
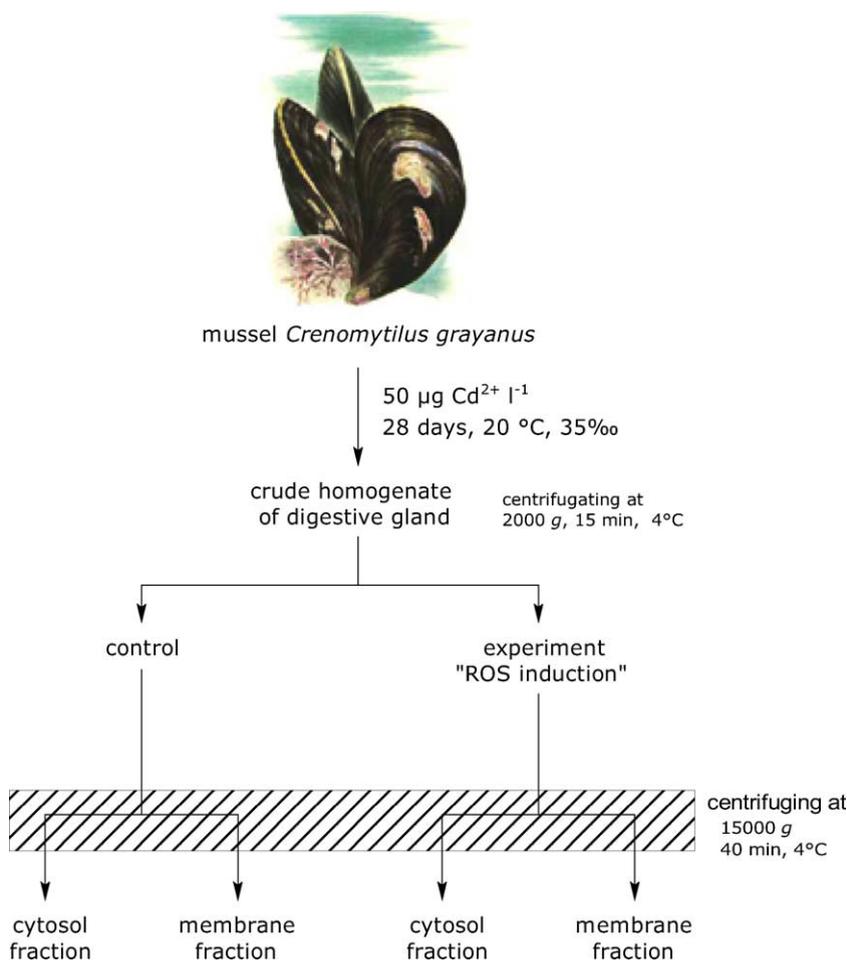
Fig. 1. Flow chart of the experiment. The membrane fraction means a fraction of intracellular membrane-formed compartments like mitochondria, lysosomes, different vesicles formed from debris of ER, Golgi and so on, with correspondent contents trapped inside.

tion with Ellman reagent (Moron et al., 1979). The metallothionein (MT) content has been quantified by mercury saturation method described by Lobel and Payne (1987).

Unpaired *t*-test with accepted significance level of $P < 0.05$ was used to compare the groups.

3. Results

The exposure of mussels *C. grayanus* to $50 \, \mu g \, l^{-1}$ during four weeks results in significant accumulation of cadmium in digestive gland ($117 \pm 22 \, \mu g \, g^{-1}$ dry weight). Accumulated Cd level is ten fold higher then

natural background. About 72% of accumulated cadmium is deposited in cytosol proteins and the rest 28% in the membrane fraction ("control" in Fig. 2).

Chromatographic profiles of cytoplasmic proteins (254 nm) and SH groups (412 nm) in digestive gland of Cd-exposed mussels (cytosol fraction from control group, Fig. 1) were aligned with cadmium profile and shown in Fig. 3. The cadmium that has been accumulated in cytoplasmic proteins was eluted from chromatographic column in three peaks. The peak I contents 5–7% of total accumulated cadmium and has been eluted from the column with dead volume, consequently this cadmium is associated with high molecular weight proteins (>60–75 kDa). The peak
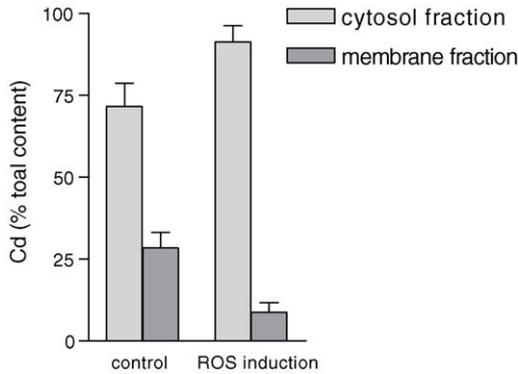
Fig. 2. In vitro effect of reactive oxygen species ("ROS induction") on cadmium distribution between subcellular fractions of mussel digestive gland. Conditions of the experiment are described in Fig. 1. Data are the average ± S.D. ($n = 8$).

II contents 60–65% of total accumulated cadmium and has been eluted from the column with 20–22 kDa proteins. The peak III contents about 30% of total accumulated cadmium and has been eluted from the column with the low molecular weight proteins (about 3 kDa). Interestingly, these peaks perfectly corresponded with the peaks of reduced SH-groups in proteins. Consequently, it is logic to conclude that the cadmium is concentrated in proteins that are enriched with sulfhydryl groups (Fig. 3). Addition of DTT to the separation buffer has not had any effect on separation profile of the cytosol proteins.

All three peaks (Fig. 3) have increased level of SH-groups, but only peak II shows SH-group to Cd stoichiometric ratio about 3 (SH/Cd ≈ 3), all the rest have <1. Moreover, peak II can be detected only at 254 nm in contrast to peaks I and III that also can be detected at 280 nm (results not shown). According to these characteristics the Cd-binding proteins from peak II can be classified as metallothionein-like proteins (Chelomin et al., 1998), which earlier were identified in *Mytilus edulis* (Julshamn and Andersen, 1983; Frazier et al., 1985).

Further, we have investigated effect of ROS induced by non-enzymatic Fe^{2+}-ascorbat system (Kreps et al., 1987) on crude digestive gland homogenates (cytosol and membrane fraction of experimental group, Fig. 1). Table 1 shows that in vitro generation of ROS stimulates of peroxidation processes in two major directions: (i) in lipid phase of biomembranes and (ii) in water soluble cytosol fraction. The first direction is indicated by significant increase in content of initial (conjugated dienes) and final (malondialdehyde) products of lipid peroxidation (Table 1). The second direction is indicated by significant decrease in content of reduced SH-groups of glutathione (about 4.6-folds) and metallothionein (>1.5-folds) (Table 1).

Moreover, ROS induction in crude homogenate of digestive gland has evoked cadmium redistribution among cytosolic and membrane fractions ("ROS induction" in Fig. 2). Cadmium content has been de-
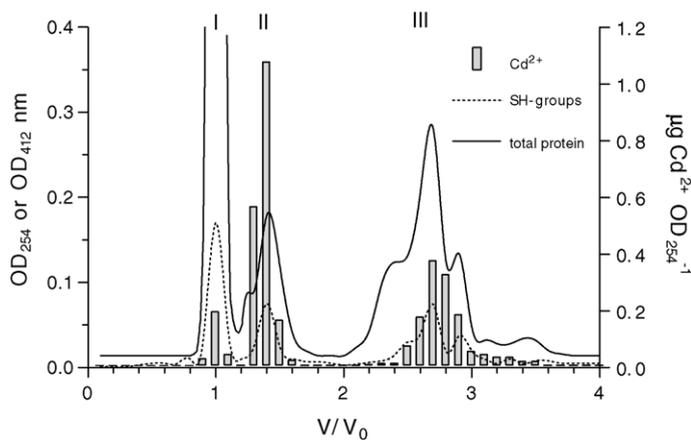

Fig. 3. Cadmium distribution among cytosolic total proteins in digestive gland of mussels *Crenomytilus grayanus* after four weeks exposure to 50 μg Cd^{2+} l^{-1}. Content of total proteins was monitored at 254 nm. Cadmium concentration (μg ml^{-1}) and concentration reduced SH-groups ($\lambda = 412$ nm) were measured in 3 ml elution chromatographic fractions. V/V_0 is relative retention volume, where V is elution volume and V_0 is dead volume of the column. I, II, III – peaks of cadmium allocation within protein elution profile.

Table 1
Change of biochemical parameters in cell homogenates of mussels *C. grayanus* digestive gland induced by reactive oxygen species (ROS)

Homogenate	MDA (nmol/mg total protein)	CoDi (nmol/mg total protein)	GSH (µg/mg total protein)	MT (µgHg/mg total protein)
Control	0.210 ± 0.018	0.54 ± 0.08	60.07 ± 0.4	18.20 ± 1.1
ROS induction	$0.437 \pm 0.032^*$	$2.47 \pm 0.11^*$	$13.73 \pm 0.35^*$	$11.70 \pm 0.9^*$

MDA: malondialdehyde; CoDi: conjugated dienes; GSH: reduced glutathione; MT: metallothionein. Values are mean $\pm$ S.D. (n = 8).
  * Significantly different (unpaired *t*-test, $P < 0.05$).

creased in membrane fraction in 3.2-folds (down to about 9%) and this cadmium has appeared in cytosolic fraction up to 91.3% (increase in about 1.3-folds). At the same time the ROS induces shift of elution profile of cytosolic proteins and cadmium distribution in that (Fig. 4A and B). The observed changes are: decrease in contents of high-molecular weight proteins by 30%; shift of peak II to a higher molecular weight (up to 40–45 kDa); shift of low-molecular proteins peak to a lower molecular weight (Fig. 4A). The same trend has been observed for the cadmium distribution (Fig. 4B).
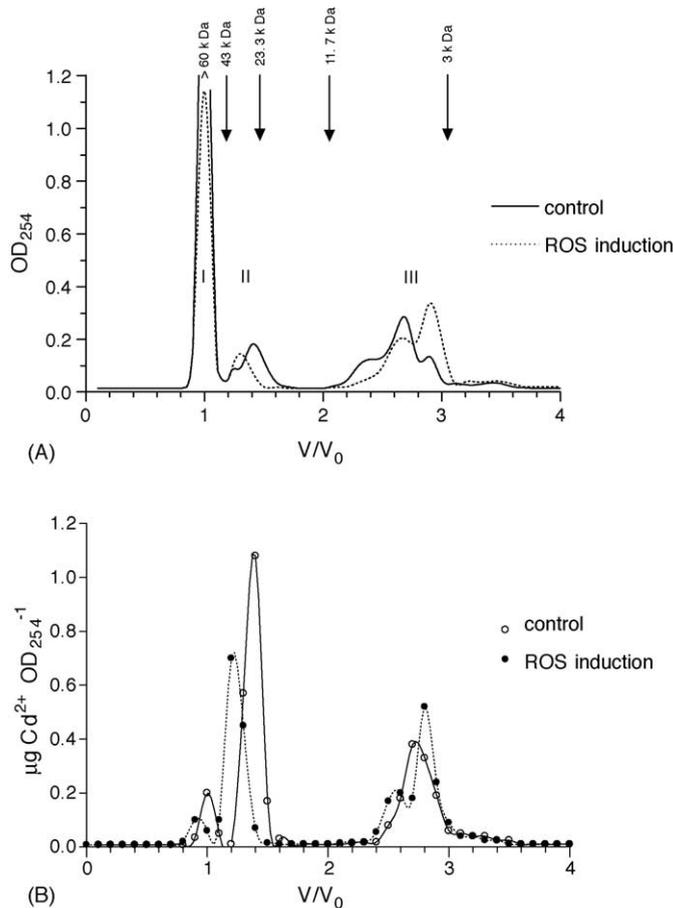

Fig. 4. Elution profile of cytosolic proteins (A) from digestive gland of mussel *Crenomytilus grayanus* and cadmium distribution (B) among them before (control) and after induction of reactive oxygen species (ROS induction) in crude cell homogenates. Symbols and axis units are the same as in Fig. 3.

4. Discussion

We have observed redistribution of the deposited cadmium among subcellular components: cytosol and membrane fractions in course of ROS induction (Fig. 2). It is quite likely that this pool of cadmium has come from membrane-depositing fraction, particularly from such as lysosomes. Lysosomes have crucial meaning for detoxification of heavy metals, especially in their biotrasformation (changing its bioavailability). Although an acidic media of the lysosomes is not perfect for depositing of the heavy metals the experimental data indicate that particularly this organelles of mussels' digestive gland "capture" and deposit on long-term scale most of the Cu^{2+}-, Zn^{2+}-, and Cd^{2+}-containing metallothioneins (Viarengo, 1985; Viarengo et al., 1985).

It is well known that allyl hydrogen atoms of polyunsaturated fatty acids from lipids of biomembrane are preferable target for the ROS (Storey, 1996). As a result of such attacks the process of lipid peroxidation in biomembrane is initiated, which result in structure destabilization of a biomembrane and release of the content of lysosomes into cytoplasm (Winston et al., 1991). Our assumption is supported by high content of products of lipid peroxidation (CoDi and MDA) after induction of the ROS (Table 1).

Moreover, we have observed significant change in protein elution profile after in vitro induction of ROS in the cytoplasmic fraction (Fig. 4A). The character of such shifts indicates significant stimulation of the oxidation processes, which on our opinion were initiated by lysosomes. Usually these organelle concentrates hydrolytic enzymes and consequently destabilization of its membranes with lipid peroxidation can result in "leakage" to the cytoplasm both hydrolytic enzymes and partially transformed molecules MT. Therefore, it is very likely that appearance of the MT with higher molecular weight and shifts of protein profile towards lower molecular weights is the result of lysosomes destruction. This hypothesis is supported by data of Winston et al. (1991), where they have linked change in protein elution profile with effect of hydrolytic enzymes from oxidatively damaged lysosomes.

Currently a lot of data are obtained about consequent effects of membrane lipid peroxidation and glutathione oxidation for a cell, for instance Christie and Costa (1984), Chatterjee et al. (1988). However, at the same time it has been shown that MT are "scavengers" for superoxide anion radical and hydroxyl radical (Thornalley and Vosak, 1985; Bremner and Beattie, 1990; Miura et al., 1997; Viarengo et al., 2000), but this aspect has not been taken adequately in account in terms of cell metabolism.

Our experiment has shown that direct induction of ROS in the crude cell homogenate results in decrease of MT levels in 1.5-folds (Table 1). There are two possible explanations of ROS effect on detectable MT concentration: (i) direct destructive effect and (ii) conformational change that results in decrease of MT availability for used detection method (mercury saturation method). This method uses an ability of mercury to replace any metal in native molecules of MT. Consequently, decrease of mercury level combined to the MT fraction might indicate change in accessibility of metal-coordinating clusters. Apparently, this is related to the oxidation by ROS of the SH-groups of cysteine residuals forming these clusters in MT molecules. Particularly these groups are very sensitive to the ROS (Thornalley and Vosak, 1985). Oxidation of SH-groups of cysteine residuals results in formation intra- or intermolecular disulfide bridges (Klein et al., 1994). In such a case, the affinity of MT to Cd significantly decreases sometimes down to complete loss of it (Klein et al., 1994). By the way, our gel-filtration chromatography (Fig. 4A) has shown that after induction of ROS the maximum of MT fraction has been shifted to a higher molecular weight area (40–45 kDa), which indicates forming of MT dimmers. Also it was found that the relative amount of cadmium decreases in this fraction. At the same time we would like to draw your attention to the fact that after the induction of ROS amount of reduced glutathione (GSH) in cell crude homogenate significantly reduces (Table 1). GSH as well as the MT participates in metal binding and it is important part of the cell detoxification system (Christie and Costa, 1984).

At the moment it is quite difficult to assess the complete consequences of formation of oxidized molecules of MT-like protein and decrease of glutathione level in cell cytoplasm on the cell metabolism as a whole. However, it is likely that this affects the biochemical system responsible for the depositing and removal of heavy metals from the cell.

Also ROS induction results is increase of cadmium content in area of low-molecular weight proteins (peak III, Fig. 4B). This is the elution area of free amino acids,

which normally presented in high concentration in bivalves, especially in mussels. Most free amino acids form relatively unstable complex with heavy metal ions. These complexes are characterized by relatively low stability constant, lower in a few magnitudes then those for GSH and MT (Christie and Costa, 1984; Roesijadi, 1992). Therefore, the pool of cadmium bound to free amino acids threatens the cell because it is quite labile and consequently has higher potential to be released and interact with intracellular structures.

4.1. Ecotoxicological consequences

Mussel is typical facultative aerobic species consequently most of redox reactions in their cells use molecular oxygen. A wide spectrum of enzymatic and nonenzymatic inductors of ROS (such as $O_2^\bullet$, (OH and H_2O_2) have been shown in mussel tissues (Livingstone et al., 1990; Winston et al., 1990; Livingstone and Pipe, 1992). Relatively high level of ROS is often causes the oxidative stress in cells due to their high reactivity, which usually results in oxidation of phospholipids, proteins and nucleic acids (Sies, 1991; Storey, 1996).

Aquatic invertebrates (especially intertidal) are regularly subjected to oxidative stress due to fluctuating environment they inhabit. Therefore, these organisms have exceptionally high antioxidant defence system that allows normal functioning of biochemical systems responsible for the season/age physiological adaptation and reproduction in conditions of fluctuating environment.

Fluctuating environmental parameters (such as temperature, salinity, oxygen availability, anthropogenic pollution, and etc) and seasonal metabolic adjustments that depend on nutrients availability and sexual maturity stage are natural sources of imbalance between pro- and antioxidant systems in most bivalves (Livingstone et al., 1990; Viarengo et al., 1991a, 1991b; Livingstone, 2001). As a result of such variations either the rise of ROS generation or decrease of antioxidant levels usually is observed. Also it is important to note that prooxidative processes normally are stimulated by oxygen availability in course of hyperoxia, hypoxia, fatigue musculature exercises, resuming of aerobic metabolic mode after anaerobic challenge (for instance low tide) (Livingstone et al., 1990, 1992; Livingstone and Pipe, 1992). Also it is known that organic xenobiotics (Livingstone et al., 1990; Ribera et al., 1991;

Livingstone and Pipe, 1992) as well as heavy metals (Chelomin and Belcheva, 1992) stimulate induction of the ROS in tissues of bivalve. Viarengo et al. (1991a) observed seasonal variation of antioxidant content in molluscs which is very likely linked to metabolic adjustments due to food availability and maturation stage. Gradual decrease of antioxidant levels can be the reason of higher predisposition of the molluscs to the oxidative stress with age (Viarengo et al., 1991b).

In course of heavy metal accumulation, especially cadmium, some marine bivalves indicate metabolic shifts, proved for instance by change in an enzyme concentrations (Zakhartsev et al., 2000). Also cadmium accumulation apparently evokes metabolic shifts in favour of prooxidative processes that will result in oxidative stress, which finally may lead to disturbance of existing balance among components of detoxification system (damage of metal depositing structures, transformation of metal-binding proteins, appearance of labile metal pool) and cases the toxic process.

Accumulation of toxic cadmium in MT-proteins and others intracellular compartments has potential threat that the metal can be released from that due to effect of high content of oxyradicals after challenging of the animal by varying environmental factors (variation of temperature, salinity, and oxygen availability) causing the "pollution" of the cell cytoplasm. Released heavy metals, especially such as copper, can exert secondary destabilizing effect on biomembrane stability (particularly on phospholipids asymmetric distribution) via oxidation of membrane protein sulfhydryl groups (Kurilenko et al., 2002).

Thus, from our experimental results and reviewed information follows that under high "pressure" of heavy metals on marine environment the aquatic organisms can show higher sensitivity to normal variations of natural factors of the environment or even decrease the range of tolerance to their variations.

References

Bremner, I., Beattie, J.H., 1990. Metallothionein and the trace minerals. Annu. Rev. Nutr. 10, 63–83.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Meth. Enzymol. 52, 302–310.

Chatterjee, S.N., Agarwal, S., Kumar, A., Bose, B., 1988. Membrane lipid peroxidation and its pathological consequences. Indian J. Biochem. Biophys. 25, 25–31.

Chelomin, V.P., Belcheva, N.N., 1992. The effect of heavy metals on processes of lipid peroxidation in microsomal membranes from the hepatopancreas of bivalve mollusks *Mizuhopecten yessoensis*. Comp. Biochem. Physiol. 103C, 419–422.

Chelomin, V.P., Belcheva, N.N., Zakhartsev, M.V., 1998. Biochemical mechanisms of adaptation to cadmium and copper ions in the mussel *Mytilus trossulus*. Russ. J. Mar. Biol., 330–336.

Christie, N.T., Costa, M., 1984. In vitro assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: interactions with membranes, glutathione, metallothionein and DNA. Biol. Trace Element Res. 6, 139–158.

Filho, D.W., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). Aquaculture 203, 149–158.

Frazier, M., George, S.S., Overnell, J., Coombs, T.L., Kagi, J., 1985. Characterization of two molecular weight classes of cadmium binding proteins from the mussel, *Mytilus edulis* (L.). Comp. Biochem. Physiol. 80, 257–262.

George, S., 1982. Subcellular accumulation and detoxification of metals in aquatic animals. In: Physiological Mechanisms of Marine Pollutant Toxicity. Academic Press, London, pp. 3–52.

Greenberg, C.S., Gaddock, P.R., 1982. Rapid single-step membrane protein assay. Anal. Chem. 28, 1725–1726.

Halliwell, B., Gutteridge, J.M.C., 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys. 246, 501–514.

Julshamn, K., Andersen, K.-J., 1983. Subcellular distribution of major and minor elements in unexposed mollusks in Western Norway. Comp. Biochem. Physiol. 75, 9–12.

Klein, D., Sato, S., Summer, K.H., 1994. Quantification of oxidized metallothionein in biological material by saturation method. Anal. Biochem. 221, 405–409.

Kreps, E.M., Tyurin, V.A., Chelomin, V.P., Gorbunov, N.V., et al., 1987. On mechanisms of initiation of lipid peroxidation in synaptosomes from the brain of marine teleosts. J. Evol. Biochem. Physiol. 23, 461–467.

Kurilenko, A.V., Zakhartsev, M.V., Chelomin, V.P., 2002. In vitro effect of copper ions on transbilayer distribution of aminophospholipids in synaptosomal membrane of walleye pollock (*Theragra chalcogramma*). Aquat. Toxicol. 58, 131–136.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Livingstone, D.R., Garcia, P.M., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other mollusks. Func. Ecol. 4, 415–424.

Livingstone, D.R., Lips, F., Martinez, P.G., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265–276.

Livingstone, D.R., Pipe, R.K., 1992. Mussels and environmental contaminants: molecular and cellular aspects. In: Gosling, E.M. (Ed.), The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Elsevier Science Publ., Amsterdam, pp. 425–464 (Developments in Aquaculture and Fisheries Science, no. 25).

Lobel, P.B., Payne, J.F., 1987. The mercury-203 method for evaluating metallothioneins: interference by copper, mercury, oxygen, silver and selenium. Comp. Biochem. Physiol. 86C, 37–39.

Miura, T., Muraoka, S., Ogiso, T., 1997. Antioxidant activity of metallothionein compared with reduced glutathione. Life Sci. 60, 301–309.

Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione reductase and glutathione *S*-transferase activities in rat lungs and liver. Biochim. Biophys. Acta 582, 67–78.

Ribera, D., Narbonne, J.F., Michel, X., Livingstone, D.R., O'Hara, S., 1991. Responses of antioxidants and lipid peroxidation in mussels to oxidative damage exposure. Comp. Biochem. Physiol. 100C, 177–181.

Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. 22, 81–114 (Review).

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comp. Biochem. Physiol. 123C, 193–199.

Sies, H., 1991. Oxidative Stress: Oxidants and Antioxidants. Academic Press, London, pp. 650.

Simkiss, K., Taylor, M., Mason, A.Z., 1982. Metal detoxification and bioaccumulation in molluscs. Mar. Biol. Lett. 3, 187–201.

Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715–1733.

Thornalley, P.I., Vosak, M., 1985. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta 827, 36–44.

Viarengo, A., 1985. Biochemical effects of trace metals. Mar. Pollut. Bull. 16, 153–158.

Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. CRC Crit. Rev. Aquat. Sci. 1, 295–317.

Viarengo, A., Burlando, B., Ceratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneins: a comparative overview. Cell. Mol. Biol. 46, 407–417.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991a. Seasonal variation in the antioxidant defense systems and lipid peroxidation of the digestive gland of mussels. Comp. Biochem. Physiol. 100C, 187–190.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., Orunesu, M., 1991b. Age-related lipid peroxidation in the digestive gland of mussels: the role of the antioxidants defense systems. Experientia 47, 454–457.

Viarengo, A., Moore, M.N., Mancinelli, G., Mazzucotelli, A., Pipe, R.K., Farrar, S.V., 1987. Metallothioneins and lysosomes in metal toxicity and accumulation in marine mussels: the effect of cadmium in the presence and absence of phenanthrene. Mar. Biol. 94, 251–257.

Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Zanocchi, G., Pipe, R.K., 1985. Detoxification of copper in the cells of the digestive gland of mussel: the role of lysosomes and thioneins. Sci. Total Environ. 44, 135–145.

Winston, G.W., Livingstone, D.R., Lips, F., 1990. Oxygen reduction metabolism by the digestive gland of the common marine mussel, *Mytilus edulis*. J. Exp. Zool. 255, 296–308.

Winston, G.W., Moore, M.N., Straatsburg, I., Kirchin, M.A., 1991. Decreased stability of digestive gland lysosomes from the common mussel *Mytilus edulis* L. by in vitro generation of oxygen-free radicals. Arch. Environ. Contam. Toxicol. 21, 401–408.

Zakhartsev, M.V., Chelomin, V.P., Belcheva, N.N., 2000. The adaptation of mussels *Crenomytilus grayanus* to cadmium accumulation result in alterations in organization of microsomal enzyme–membrane complex (non-specific phosphatase). Aquat. Toxicol. 50, 39–49.