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Effect of Marine Environment Remediation on Oxidative Stress Indicators and Metals in the Digestive Gland of the Mussel *Crenomytilus grayanus*

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Abstract

The marine environment quality in Gornostai Bay of the Peter the Great Bay, East Sea/Sea of Japan (Russia's Primorski Krai) was assessed based on oxidative stress biomarkers and the content of heavy metals in the digestive gland of the mussel *Crenomytilus grayanus*. Mussels were collected in Gornostai Bay in 2013, three years after the remediation of a domestic solid waste dump site located on the shore of the bay and in a relatively clean area at Reineke Island (Peter the Great Bay). The results were compared with the data from the studies performed on *C. grayanus* from the two locations in 1999 and 2011. It is concluded that the marine environment quality in Gornostai Bay is progressively (gradually) improving three years after the remediation of the domestic solid waste dump site compared to 1999 and 2011.

Introduction

Aerobic species have evolved the capacity to use oxygen for the efficient release of energy. However, *in vitro* studies suggest that 1-3% of the oxygen molecules used are converted to reactive oxygen species (ROS), including both radical and non-radical species. Therefore, the ROS are continually produced as toxic bi-products of normal metabolism from various endogenous processes [1]. Key biological molecules, notably DNA, proteins, lipids, can all be adversely affected by ROS [2,3]. Furthermore, the reaction of ROS with these macromolecules generates additional ROS, setting in train a cascade of damage if left unchecked [4]. An antioxidant system (represented by low molecular weight free radical scavengers and specific antioxidant enzymes) is present in the body of all aerobes in order to retard the ROS generation processes and to protect the cellular components from oxidation and to repair the oxidized biomolecules [5].

The accumulation of heavy metals, petroleum hydrocarbons and polychlorinated phenols in the tissues promotes the formation of ROS [1,6,7]. Molecular biomarkers of oxidative stress (oxidative degradation products of biological molecules and the levels of antioxidants) have been widely used in recent decades to identify the toxic effect of pollutants [8,9].

The aim of this study was to use biochemical indicators to assess changes in the marine environment quality after remediation of the anthropogenically polluted coastal zone. For this purpose, the content of heavy metals (Fe, Zn, Cu, Cd and Pb) and indicators of oxidative stress (activity of antioxidant enzymes and levels of reduced glutathione and lipid peroxidation products - diene conjugates, TBA-reactive products) as well as total lipid content were determined in the digestive gland of *Crenomytilus grayanus* (Dunker 1853). Furthermore, studies focused both on the dynamics of release of heavy metals from digestive gland tissue of mussel and the dynamics of changes in biochemical indicators after remediation of the anthropogenically polluted coastal zone are of significant interest.

Therefore, a change in the marine environment quality in the Gornostai Bay in 2013 was evaluated based on the changes in contents of metals and biochemical indicators in the digestive gland of mussel by comparison of the obtained results with the data of studies conducted on *C. grayanus* from the same regions in 1999 [10,11] and 2011 [12].

Materials and Methods

Studied areas

Aquatic areas of the Gornostai Bay (Site 1) and Reineke Island

Figure 1: Location of the sampling sites in Peter the Great Bay of the East Sea/Sea of Japan (Russia): Site 1 - Gornostai Bay; Site 2 - Reineke Island.

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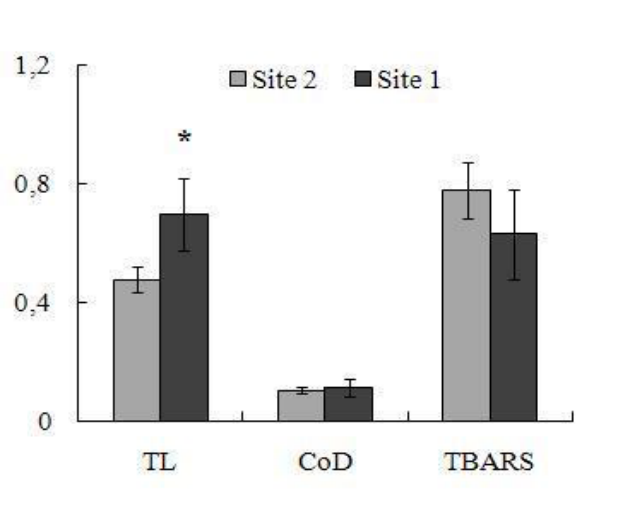

Figure 2: Total lipids (TL, lipids/g tissue/100), conjugated dienes (CoD, μmol/mg lipids) and TBA-reactive products (TBARS, nmol/mg protein) levels in digestive gland of the mussel C. grayanus from the reference (Site 2) and polluted (Site 1) sites.
* Indicates statistical differences (n = 4, p < 0.05).

(Site 2) (Figure 1), selected as the mussel sampling sites, are located at a distance of about 44 km from each other and virtually do not differ in their main hydrochemical (salinity, oxygen concentration) and hydrological (temperature, light intensity) parameters [13]. Coastal waters of Reineke Island were used as the reference site in the study of water pollution in the Peter the Great Gulf. It should be mentioned that there are no factories polluting the environment on the Shore of Reineke Island. Bottom sediments and the water column of this region are characterized by low content of heavy metals and chlorinated hydrocarbons (pesticides) [14-16].

The domestic solid waste landfill of Vladivostok was located on the shore of the Gornostai Bay in 1967-2010, which led to the pollution of contiguous waters. For example, according to a study by Shulkin [16], the mean content of heavy metals in sediment samples from Site 1 and contiguous waters collected at a distance of 50-100 m from the shore was 3739 μg/g dry wt for Zn, 2659 μg/g dry wt for Cu, and 2344 μg/g dry wt for Pb in 1996-1999. At the same time, the content of these metals in sediments of waters contiguous to Site 2 were 21 μg/g dry wt for Zn, 3 μg/g dry wt for Cu, and 10 μg/g dry wt for Pb [14]. The total concentration of organochlorine pesticides (such as HCH_s, DDT, and their metabolites) in bottom sediments of Site 1 was 5 times higher compared to that in Site 2 and amounted to 1.67 ng g^{-1} dry weight [15]. In 2008 and 2009 the concentration of oil products and phenols on the surface and in near-bottom layers of Site 1 exceeded the maximum permissible concentrations by 2- and 5-fold, respectively [17].

Remediation of the landfill was completed by the end of 2010. Annual monitoring conducted by the Far Eastern Regional Hydrometeorological Research Institute (FERHRI) in the water area of the Peter the Great Gulf, 500-800 m off the shore, revealed a steady downward tendency in the content of metals and petroleum products in sediments of the Site 1 region by 2012 [18].

Sampling

Mussels were manually collected at 3-4 m depth at a distance of 100 m from both sites in July 2013. In each region 18 individuals were selected. Their age was determined using the prominent growth retardation rings [19]. The shell length of mussels from Site 1 was 10.36 ± 0.83 cm, their age amounted to 8-16 years; mussels from Site 2 were characterized by the shell length of 11.63 ± 0.75 cm and the age of 10-15 years. We suppose that the individuals from the two water areas were in the similar physiological state since *C. grayanus* is a long-living mollusk [20].

In order to determine the biochemical parameters, the digestive gland tissues from three individuals were combined into a single sample (total of 4 samples for each population), which was immediately frozen in liquid nitrogen and stored at -80 °C. To determine the metal content, the tissues from six individuals were divided into three replicates (two specimens per replicate) and dried until a constant weight was achieved at +75 °C.

Sample preparation

Digestive gland of mussels was homogenized (1:10, w/v) at 0 °C in 50 mM Tris-HCl buffer (pH 8.0), containing 0.1 mM of phenyl methyl sulphonyl fluoride (PMSF). A portion of the homogenate obtained was used to detect TBA-reactive products. The remainder of the homogenate was centrifuged for 20 min at 5,000 g and then for 40 min at 10,800 at 4 °C. The supernatant was used to determine the activity of antioxidant enzymes.

Biochemical analyses

Antioxidant enzymes were assayed at standard assay temperatures of 20 °C. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured according to Paoletti et al. [21]. Catalase (CAT) (EC 1.11.1.6) activity was determined by the hydrogen peroxide decomposition rate. Glutathione reductase (GR) (EC 1.6.4.2) activity was measured by following the decrease in absorbance at 340 nm due to the oxidation of NADPH [6]. Glutathione peroxidase (GP) activities were measured in a coupled enzyme system where the

Table 1: Mean concentrations of metals ± standard deviation (μg/g dry wt, n=3) in the digestive gland of *C. grayanus* from the polluted (Site 1) and reference (Site 2) sites.

	Year	Fe	Zn	Cu	Cd	Pb
Site 1	1999[a]	620 ± 211	229 ± 74	61 ± 5.8	9.73 ± 2.47	169 ± 50
	2011[b]	153.1 ± 17.6	90.4 ± 15.1	89.3 ± 10.9	2.7 ± 0.8	28.9 ± 12.9
	2013	144.5±19.4	100.8 ± 12.8*	24.14 ± 2.96	4.19 ± 2.28	26.31 ± 4.71*
Site 2	1999[a]	123 ± 27	108 ± 50	12 ± 1.1	3.76 ± 2.28	n.d.
	2011[b]	119.7 ± 14.7	97.1 ± 13.6	24.9 ± 0.4	4.6 ± 0.9	n.d.
	2013	194.8 ± 27.8	137.3 ± 13.5	19.36 ± 0.69	11.02 ± 6.22	1.70 ± 0.61

[a]Kavun, Shulkin, 2005
[b]Belcheva et al. 2013
Note: n.d. - not determined
*Indicates significance levels of differences between tissue of reference mussels and tissue of polluted mussels in 2013 (n = 3, $p < 0.05$).

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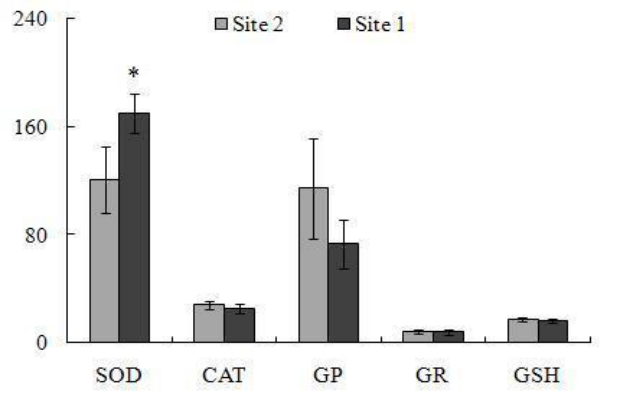

Figure 3: Antioxidant levels in the digestive gland of the mussel C. grayanus from the reference (Site 2) and polluted (Site 1) sites: SOD: Superoxide Dismutase, U/mg; CAT: Catalase, μmol/min/mg; GP: Glutathione Peroxidase, nmol/min/mg*10; GR: Glutathione Reductase, nmol/min/mg; GSH: Glutathione, nmol/g wet weight/100.
* Indicates statistical differences (n = 4, p < 0.05).

formed oxidized glutathione is converted to its reduced form by GR [22]; cumenehydroperoxide was used as substrate (respectively for the sum of Se-dependent, EC 1.11.1.9, and Se-independent, EC 2.5.1.18, activities). The reduced glutathione (GSH) concentration was determined according to Moron et al. [23]. The TBA-reactive products (TBARS) concentration was determined via color reaction with TBA (2-thiobarbituric acid) [24]. Conjugated dienes (CoD) was determined spectrophotometrically [25]. The total lipid (TL) content was determined using the method of Amenta after extraction of lipids with a chloroform-methanol mixture (2:1, v/v) [26]. Protein concentration was determined using the modified Lowry method [27]. All measurements were performed using spectrophotometer Shimadzu UV-2550.

Metal analyses

Dried samples of the digestive gland were weighed and digested in Teflon bottles with nitric acid (16 M HNO_3). Contents of metals Fe, Zn, Cu, Cd and Pb were analyzed using an atomic absorption spectrophotometer with flame atomization and deuterium background correction (Shimadzu AA-6800). Control of the analysis quality included measurement of the content of metals in the certified standard sample (ERM-CEZ78 mussel tissue). The standard sample analysis precision was above 90%.

Statistical analyses

Data were statistically compared using an unpaired *t*-test at the $p < 0.05$ significance level (STATISTICA; StatSoft, USA).

Results

The contents of metals in the digestive gland of the mussels from the two water areas collected in 2013 (Table 1) showed that Fe, Cu and toxic Cd between the two populations of mussels. At the same time, the content of Pb in the digestive gland of mussels from Site 1 was 15 times higher.

As seen from the results, the content of lipid peroxidation products CoD and TBARS in the tissues of the mussels from both biotopes did not differ significantly (Figure 2). However increase (by 45%) in TL content was observed in the mussels from Site 1.

Among the studied antioxidants, only the SOD activity in the mussels from Site 1 was nearly 1.5 times higher than in the mussels from the clean area (Figure 3). The activity levels of the remaining antioxidant enzymes (CAT, GP and GR) and the levels of the low molecular weight antioxidant GSH did not differ significantly between the mussels from these regions (Figure 3).

Discussion

Overall, the comparison of data on metals in the digestive gland of mussels collected in different years in Site 1 showed that the contents of all metals decreased by 2013 in comparison with 1999, e.g. the content of Fe reduced 4 times, Zn and Cd 2 times, Cu 2.5 times, and Pb 6.5 times (see Table 1). It should be noted that the contents of Fe, Zn, Cd and Pb decreased as soon as a half year after the remediation [12] in comparison with 1999; in this case, the content of Fe, Zn, Cd reached levels comparable to the levels of these metals in the digestive gland of mussels from Site 2. By 2013, the Cu content also reached the level typical of mussels from Site 2 (see Table 1). However, the Pb content in 2013 was not changed compared to that in 2011. Since the content of metals in the tissues of mussels is a sensitive indicator of the content of bioavailable forms of metals in the environment, the decrease in the content of metals in the digestive gland of mussels from Site 1 in 2011 and 2013 reflects a decrease in metal content in the marine environment. We suppose that the reduction in the content of metals in the digestive gland of mussels collected in Site 1 in 2011 and 2013 compared to that in 1999 is the result of remediation of the domestic solid waste dump site located on the Bay Shore. Nevertheless, the content of highly toxic metal - Pb - remains at a higher level in the mussels from Site 1 as compared to mussels from Site 2, probably due to the migration of previously accumulated toxins from the bottom sediments into the water [28] and reabsorption from the mussel valves. Certain metals, such as lead, can accumulate to a considerable extent in the shells of mollusks [29]. It has been shown that mussel *Mytilus edulis* can eliminate Pb from the tissues, but at very low rates [30,31]. Riget et al. investigated the loss of lead in whole soft tissues of mussel *Mytilus edulis* [32]. Thus Mussels transplanted from a polluted site to a clean one revealed that Pb depuration is not complete, and about a 50% of the Pb originally present in the mussel cannot be excreted even after 5 years of depuration.

Lipid peroxidation products, CoD and TBARS, are widely used as markers of oxidative stress in aquatic organisms caused by pollution [9,33]. In 1999, the content of these products in mussels from Site 1 was 2 times higher compared to that in mussels from Site 2 [10]; while in 2011, only the TBARS content was 85% higher [12]. It should be noted that, in 2013, the CoD and TBARS content of the digestive gland of mussels did not differ significantly between specimens from the two water areas. At the same time, higher levels of TL in the digestive gland of mussels from Site 1 may indicate a continuing negative impact of the waste landfill. Accumulation of neutral lipids in the mollusk digestive gland and fish liver was demonstrated to be induced by the influence of toxic substances [34].

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The antioxidant defense system maintains the organism integrity and chemical balance of its interior. Induction of antioxidant enzymes is known to be an important compensatory response to stress caused by environmental pollution [8,9]. Therefore it can be assumed that the increased SOD activity prevents lipid peroxidation in mussels from Site 1 and is a consequence of the mussels' adaptation to chronic pollution. We had obtained similar results in 2011 [12]. Thus, the digestive glands of the mussels also collected from Site 1 in July, showed an increase in the activity of only SOD; the other antioxidants (CAT, GP, GR and GSH) did not show significant differences when compared with the mussels from Site 2 [12].

In general, the comparative analysis of the data obtained in 1999 [10,11], 2011 [12], and 2013 (data of the present study) demonstrated that a reduction in the level of human-induced load on Site 1 due to remediation of the waste landfill is accompanied by a tendency towards decreasing the content of metals in mussel tissues and recovering a number of oxidative stress parameters to the background level. However, according to our data, elevated levels of SOD and TL in the digestive gland of the mussel from Site 1 indicate the continuing negative impact of pollutants, probably due to mobilization and migration of previously accumulated toxicants from sediments into water.

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