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Evaluation of DNA Damage in the Marine Mussel *Crenomytilus grayanus* as a Genotoxic Biomarker of Pollution

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Abstract Marine pollution affects all life processes in aquatic organisms. The genotoxic effect of pollution on the mussel *Crenomytilus grayanus* was assessed. Bivalves were collected from the 'clean' (Vostochnaya Cove) and polluted (Nakhodka Bay) areas in the Peter the Great Bay. The degree of DNA damage in *C. grayanus* was determined by alkaline comet assay as mean percentage of DNA in tail, and the genetic damage index was calculated. Our results indicate that almost one-third of DNA in cells of gills and digestive gland of *C. grayanus* inhabiting the Nakhodka Bay had destructive changes compared to the individuals of this species from the Vostochnaya Cove. This study has shown that chronic pollution of the aquatic environment causes destructive changes to DNA in gill and digestive gland cells of *C. grayanus.*

Key words genotoxicity; DNA comet assay; *Crenomytilus grayanus*; pollution

1 Introduction

The sharp increase in the human population and industrial activity has caused chronic pollution of marine ecosystems worldwide, with the most pronounced impacts observed in coastal waters. Coastal ecosystems receive terrigenous runoff contaminated with various chemical compounds, including highly toxic petroleum hydrocarbons, pesticides, detergents, phenols, heavy metals, etc. Some of these pollutants are accumulated in bottom sediments and taken up by aquatic organisms. In addition, coastal waters are characterized by abrupt changes in hydrochemical parameters such as temperature, salinity, oxygen content, turbidity, etc., all of which have significant effects on life processes in marine species. In turn, the variations in physical and chemical factors may alter the bioavailability and, consequently, the toxicity of pollutants. Therefore, in coastal areas there is a variety of combinations of synergistic, antagonistic, and masking effects of anthropogenic factors that greatly complicate the use of traditional hydrobiological approaches to assess adverse changes in ecosystems. Moreover, the traditional hydrobiological approaches alone do not allow quick assessment of the ecotoxicological situation, and the data of ecological monitoring can often be analysed only when the effects have become apparent at the ecosystem level and irreversible (Sarkar *et al*., 2008; Belcheva, *et al*., 2013).

It is obviously necessary to introduce a new system of criteria, based on the key biochemical parameters (molecular markers), for ecotoxicological monitoring in coastal ecosystems with unstable environmental conditions and a wide range of potential chemical contaminants. This system would allow an integrated assessment of the physiological condition of living organisms under the effect of adverse environmental factors. The main advantages of using the non-specific molecular markers are the high sensitivity, accuracy, rapid detection, and, especially, the potential to identify causal relationships between the organisms and the environment. Non-specific markers make it possible to predict changes in populations and communities in the polluted areas. Currently, there is a popular idea among researchers, using molecular markers as early and sensitive indicators of the condition of organism, that assessing the risk of long-term effects of pollution can be more efficient if based on the results of genotoxicity analysis (Hamoutene *et al*., 2002; Slobodskova *et al*., 2015).

Structural damage to DNA molecules in genome is one of the most important biomarkers of genotoxic effects of environmental pollution due to the extremely important functional role of these molecules in biological systems. The comet assay can detect DNA damages, and, thus, it is

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widely used to assess genotoxicity of various contaminants: petroleum hydrocarbons (Hamoutene *et al*., 2002; Taban *et al*., 2004; Delunaro *et al*., 2013), polyaromatic hydrocarbons (Wessel *et al*., 2007), pesticides (Wessel *et al*., 2007; Akcha *et al*., 2012; Mai *et al*., 2012), and heavy metals (Hook and Lee, 2004; Slobodskova *et al.,* 2010a, 2010b; Mai *et al*., 2012; Goswami *et al.*, 2014). The practical application of the DNA comet assay in genotoxic monitoring of marine waters is still developing, as is evidenced by the relatively small number of recent works (Sarkar *et al.*, 2008; Siu *et al*., 2008; Raimundo *et al*., 2010; Goswami *et al*., 2014; Slobodskova *et al*., 2015). As this method is widely used in biomedical research and for evaluating the genome integrity of individual cells, more scientific publications on various aspects of the comet assay in marine research should be expected.

Bivalves are commonly used for genotoxic monitoring, with their analysis focused on the degree of DNA damage in somatic cells (of gills, digestive gland, and hemolymph) (Siu *et al*., 2008; Mai *et al*., 2012; Slobodskova *et al*., 2015). A destruction of the genome of somatic cells may gradually increase the dysfunction of individual cells, tissues, and organs (from reversible to irreversible), followed by the disruption of life processes and death of the organism.

Based on these considerations, we studied the level of the DNA damage to somatic cells (in gills and digestive gland) of marine mussels, *Crenomytilus grayanus*, exposed to polluted seawater. The studies were conducted in the Nakhodka Bay (Peter the Great Bay, Sea of Japan (East Sea)) with large sea ports and several industrial zones located on its shores and in the drainage basin. The discharges of untreated domestic and industrial wastewater, terrigenous runoff, dredging, and continuous leakage of petroleum products from port-based vessels exert various negative effects on the ecosystem in the bay (Naumov, 2006). The water was qualified as 'polluted' and, in some parts, as 'highly polluted'. The mussel *Crenomytilus grayanus* (Dunker, 1853) was used in the experiments as the most accessible and convenient bivalve species for biomarker research and ecological monitoring in coastal ecosystems of the Peter the Great Bay.

2 Materials and Methods

2.1 Sample Collection

Mussels and water samples were taken in August 2012 from three experimental sampling sites (waters off Cape Shvedova, Cape Krasny, and in Kozmina Cove) in the Nakhodka Bay (experimental area) (Fig.1). Besides, mussels were collected from their natural habitat off Rikorda Island (Vostochnaya Cove, Peter the Great Bay) characterized by a low anthropogenic impact (control site). From each sampling site 15 individuals of *C. grayanus* were collected by divers.

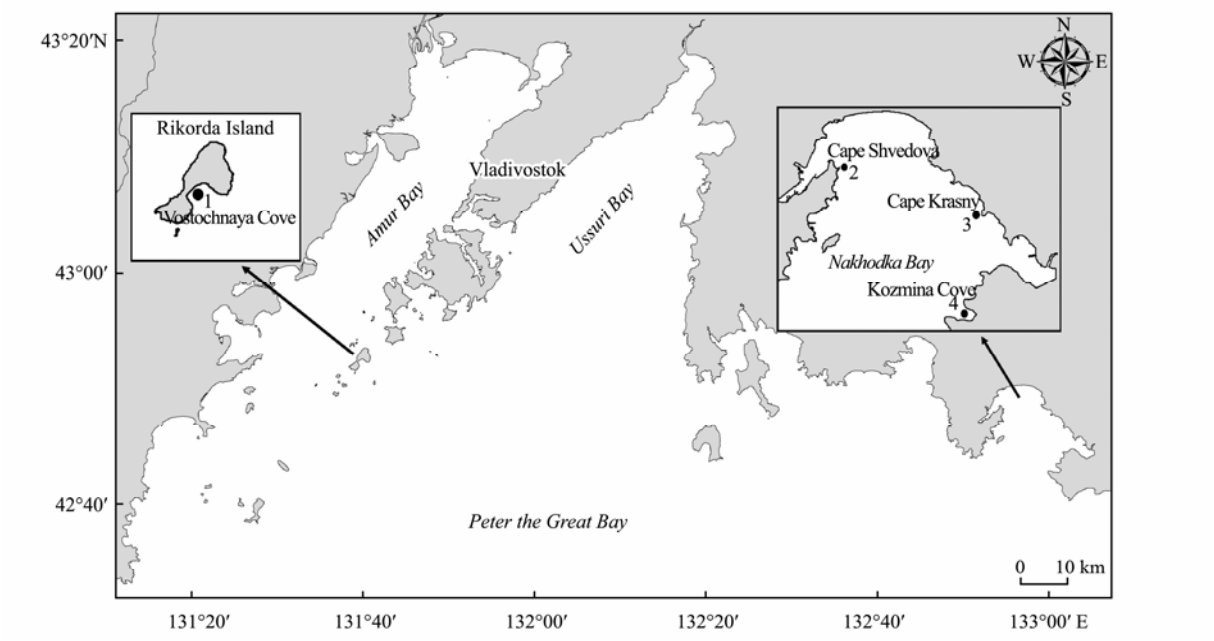

Fig.1 Study area and sites of *C. grayanus* sampling. 1, control site, Vostochnaya Cove; 2, Cape Shvedova; 3, Cape Krasny; 4, Kozmina Cove.

Hydrological (water temperature and salinity) and hydrochemical parameters (dissolved oxygen and biochemical oxygen demand) were measured using the standard techniques (Table 2). Seawater salinity was measured with an electromagnetic salinometer GM-65M (Russia). Dissolved oxygen (DO) was measured by the Winkler method. Samples for determination of biochemical oxygen demand (BOD_5) were incubated at 20℃ for 5 d. Chemical oxygen demand (COD) was measured by permanganate titration (Manual, 2003).

2.2 Alkaline Single-Cell Gel Electrophoresis Assay (Comet Assay)

The level of damage to the DNA molecules was assessed

using the comet assay method modified for marine organisms, which was described previously by the authors of the present study (Slobodskova *et al*., 2010a).

The DNA comets were visualized and registered using a scanning fluorescence microscope (Carl Zeiss, AxioImager A1) equipped with a digital camera AxioCam MRc. Digital images were processed using a CASP program (Conca *et al*., 2003), which allows calculations of various parameters of comets indicating the level of DNA damage. The percentage of DNA in the comet tail (% DNAt) was determined for each comet.

Moreover, cells were graded visually into five classes (C0, C1, C2, C3, C4) according to the amount of the DNA in the tail (Collins *et al.*, 1995), where C0 are undamaged cells and C4 are highly damaged cells. Based on the number of comets from each class (from C0 to C4) (Collins *et al*., 1995), the genetic damage index (GDI) was calculated for each of the experimental and control sampling sites:

$$(C1+2*C2+3*C3+4*C4)/(C0+C1+C2+C3+C4)$$

(Cavas and Konen, 2008).

For cells of the mussels from the experimental and control sampling sites we analyzed 15 slides (1 slide=1 mussel) each containing no less than 100 comets (n=100).

2.3 Data Analysis

All data were tested for normality. A one-way ANOVA was performed for data analysis. Dunett's test was applied to compare the results of the comet assay for the control and each of the experimental sites. Significance was set at $P<0.01$.

3 Results and Discussion

The genotoxicity analysis of mussels *C. grayanus* using the DNA comet assay revealed a significant damage to nuclear DNA in cells of gills and digestive gland of bivalves inhabiting the experimental sites. A visual analysis of comets microphotographs after electrophoresis clearly showed that the DNA molecules from the gill and digestive gland cells of the mussels from the control site (Vostochnaya Cove, Rikorda Island) did not have destructive changes and formed a symmetrical bright nucleus with a small halo. In contrast, DNA of the mussels from the experimental sampling sites in the Nakhodka Bay formed a distinct 'comet' after electrophoresis due to the degradation and migration of genomic DNA fragments.

If comets are classified based on the percentage of DNA that migrates from the nucleus to the comet 'tail' (% of DNA in the comet tail) (Collins *et al*., 1995), the comets of tissues of the mussels from the control site were represented by almost 90%–95% C0/C1 classes, characterizing the cells as intact and viable. Mussels from the experimental sites demonstrated an increase in the proportion of comets of C2 and C3 classes, indicating an increased level of DNA degradation. Fig.2 shows a quantitative parameter of the obtained comets: mean percentage of DNA in the comet tail. An estimation of this parameter showed that in cells of gills and digestive gland of the mussels from the Nakhodka Bay, the DNA damage was significantly higher than in the control mussels from unpolluted waters (Vostochnaya Cove).

The most evident genetic damage was revealed in tissues of the mussels collected off Cape Krasny and in Kozmina Cove. More than 30% of their DNA was subject to fragmentation, and GDI values were higher than 2 (Table 1).

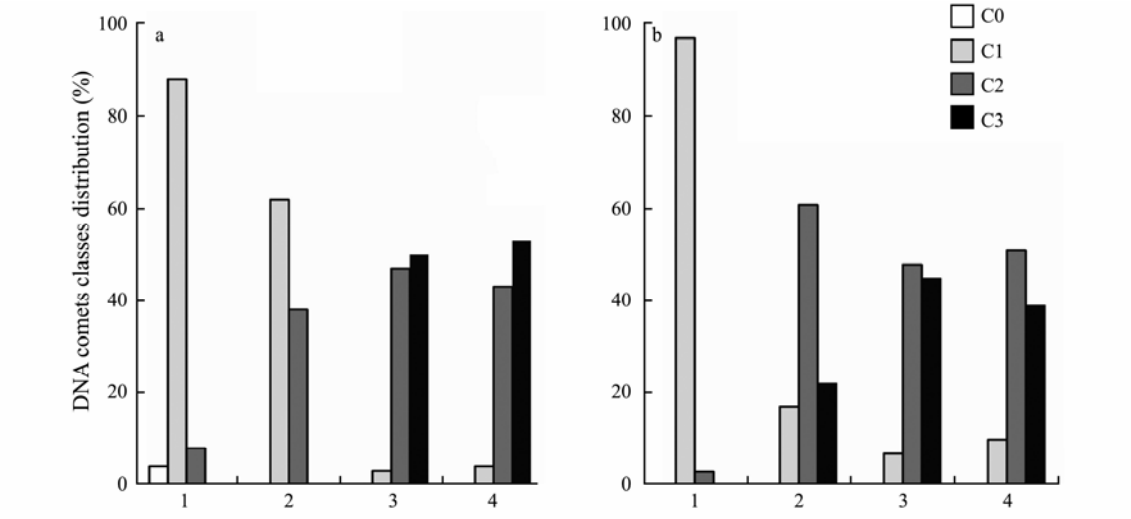

Fig.2 Distribution of the DNA comets classes (in %) for the gill (a) and digestive gland cells (b) of *C. grayanus*. Numerals 1–4 along the *X*-axis are the numbers of sampling sites (see explanation in Fig.1).

The hydrochemical regime of the Nakhodka Bay directly determines the condition of the biota, being influenced by a variety of meteorological factors, such as wind-driven waves, and, especially, by human impacts. The influence of human activity on the Nakhodka Bay ecosystem has been demonstrated by the results of comprehensive environmental studies (Ogorodnikova, 2001; Naumov, 2006). A wide range of hydrobiological works have been conducted in these waters, including tests of the reproductive function and descriptions of the structure

of benthic and planktonic communities, which have revealed a complex, 'mosaic' pattern of pollution in the Bay (Naumov, 2006). A comparison with the earlier embryotoxic experiments on the sea urchin *Strongylocentrotus intermedius* (Kashenko, 2000) and the sand dollar *Scaphechinus mirabilis* (Zhuravel *et al*., 2006) shows that the Nakhodka Bay is still exposed to significant negative impacts from human activities. Since the time of these earlier studies, the entire complex of anthropogenic impacts in the region has changed dramatically, which inevitably has an effect on the local geochemical and biological processes.

Table 1 The genetic damage index (GDI) for the gill and digestive gland cells of *C. grayanus* from different sampling sites (mean ± standard deviation, $n = 15$)

Tissue	Vostochnaya Cove	Nakhodka Bay		
		Cape Shvedova	Cape Krasny	Kozmina Cove
Gills	0.65 ± 0.07	1.34 ± 0.20*	2.45 ± 0.30*	2.49 ± 0.40*
Digestive gland	0.81 ± 0.15	2.35 ± 0.40*	2.05 ± 0.30*	2.34 ± 0.40*

Note: *Differences from the control are significant at $P \leq 0.01$.

In the process of improving the methodological basis for biological monitoring and searching for the highly sensitive and rapid responses of the biota, a direct correlation between embryotoxicity and genotoxicity was identified. In studies with the oyster *Crassostrea gigas*, after exposure to various chemical agents (including benzo (a) pyrene, a synthetic estrogen, pesticides, and herbicides) the impairment of fertilization and development was accompanied by the DNA damage (Wessel *et al*., 2007; Akcha *et al*., 2012).

The results of our study (Fig.3; Table 1) confirm the effect of water pollution on the DNA integrity. Large-scale destructive changes of DNA were previously recorded for mussels in Gornostay Cove, where an active landfill was operated on the shore (Belcheva *et al*., 2013). However, mussels living in the waters off Cape Krasny can manifest DNA degradation not only due to the presence of contaminants, but also due to the reduced oxygen availability, when the environmental oxygen concentration is close to the minimum permissible one (Table 2). It was previously shown for the molluscs *Mizuhopecten yessoensis* and *Corbicula japonica* that the destructive changes in the DNA molecule occur under oxygen-deficient conditions, which lead to genomic instability (Slobodskova *et al*., 2012, 2013).

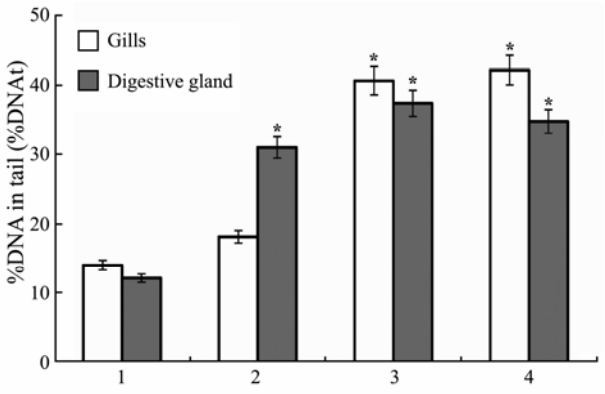

Fig.3 The level of the DNA damage in the gill and digestive gland cells of the mussel *C. grayanus* inhabiting different areas in the Peter the Great Bay (mean ± standard deviation, $n = 15$). Numerals 1–4 along the *X*-axis are sampling sites (see Fig.1). *Differences from the control are significant at $P \leq 0.01$.

Table 2 Hydrochemical parameters in the sites of *C. grayanus* sampling (mean ± standard deviation, $n = 3$)

Station	T (℃)	Salinity (‰)	O_2 (mg L^{-1})	O_2 (%)	BOD_5 (mg L^{-1})
Vostochnaya Cove	20.10	32.50	8.10±0.09	110	1.22±0.05
Cape Shvedova	20.20	32.50	8.14±0.12	110	1.23±0.17
Cape Krasny	20.30	32.58	6.13±0.10	83	1.12±0.02
Kozmina Cove	19.50	31.70	7.90±0.05	105	1.21±0.30
Maximum permissible concentrations (Water quality standards 2010)	-	-	6.00	-	3.00

The observed loss of the DNA integrity should be attributed to the most significant manifestations of the environmental toxicity. Therefore, when analyzing the experimental genotoxicity data, we should focus not on the fact that a genome damage has been detected, but rather on the fact that in the study area (Nakhodka Bay) there are some toxicants for which the DNA molecule is one of the main targets.

The Nakhodka Bay is one of the most industrially and economically developed regions in Primorsky Krai. Among the wide range of pollutants, petroleum hydrocarbons and heavy metals are the most dangerous and widely spread ones (Nigmatulina *et al.*, 2011). The levels of Fe, Zn, Cu, and Mn in soft tissues of the bivalves *Mytilus trossulus* and *C. grayanus* were 1.8–3.7 times as high as the background level determined for the North-East Pacific (Nigmatulina *et al.*, 2011). The concentrations of total petroleum hydrocarbons in the water and bottom sediments of different parts of the Nakhodka Bay in 2012 exceeded the permissible levels 3.4 and 6 times, respectively. The level of polyaromatic hydrocarbons (PAH) and polychlorinated biphenils (PSB) in bottom sediments was similar to that in low-polluted areas of the World Ocean (Zhuravel *et al.*, 2015), but the concentrations of hexachlorocyclohexanes and DDTs were higher than the maximum permissible level more than

4–8 times.

According to the previous experimental studies, dispersed crude oil, even at low concentrations, caused the DNA degradation in erythrocytes of the fish *Hippocampus reidi* (Delunardo *et al.*, 2013), in haemocytes of the mussel *Mytilus edulis*, the sea urchin *Strongylocentrotus droebachiensis* (Taban *et al*., 2004), and embryos of the sea urchin *Paracentrotus lividus* (Rial *et al.*, 2014). The water-soluble fraction of crude oil and diesel fuel also sharply increased the degree of DNA damage in haemocytes and digestive gland cells of two bivalve species, *M. edulis* and *Mya arenaria* (Hamoutene *et al*., 2002), erythrocytes of the amphipod *Quadrivisio aff. lutzi* (Weber *et al*., 2013) and the fish *Prochilodus lineatus* (Vanzella *et al*., 2007). It is noteworthy that the dispersing agents, widely used for oil spill cleanup, also have genotoxic properties (Rial *et al*., 2014). Crude oil and diesel fuel contain not only paraffins but also toxic PAH. Some PAH have a genotoxic effect and retain these properties up the food chain from mussels to laboratory rats (Lemiere *et al*., 2004). The genotoxic effect of PAH showed a significant correlation with the hydrochemical parameters such as dissolved oxygen, BOD_5 and nitrite concentration (Sarker *et al*., 2018).

It is well known that heavy metals have a high genotoxic potential (Slobodskova *et al*., 2010, 2012; Raimundo *et al*., 2010; Baker *et al.*, 2014). For example, cadmium, at concentrations occurring in coastal waters, induced disturbances in development and also apoptosis in sea urchin larvae by genotoxicity (Filosto *et al*., 2008). In similar experiments, this metal caused major disruptive DNA changes in early embryo development of the grass shrimp *Paleomonetes pugio* (Hook and Lee, 2004), in gill and digestive gland cells of the bivalves *M. yessoensis* (Slobodskova *et al*., 2010, 2012) and *C. japonica* (Slobodskova *et al*., 2010), as well as in various cells of benthic invertebrates including sea urchins, sponges, and corals (Schroder *et al*., 2005). It should be emphasized that in the above examples, the genome damage was recorded before the cytotoxic and morphological changes occurred.

The adverse consequences of pollution, identified based on the highly sensitive and relevant methods for determining genotoxicity (including those used in our work such as the DNA comet assay), were reported from the Hong Kong Bay (Siu *et al*., 2005), the Baltic Sea (Barsiene *et al.*, 2005), waters off the western coast of India (Sarkar *et al*., 2008, 2018), and waters adjacent to the cities of Tokyo, Osaka, and Kobe, Japan (Sasaki *et al*., 1997). Even a short list of examples, along with our experimental results, suggests the importance of determining the genotoxic properties in an ecotoxicological assessment of the environment.

In conclusion, it should be noted that our results have first shown that the pollution in the Nakhodka Bay affects aquatic organisms by causing hidden defects at the genome level (damage to the DNA structure) that may have uncontrolled and irreversible consequences. Previously, no similar studies were conducted here. It is obvious that a quantitative assessment of destructive genome changes in certain species of marine organisms, as indicated in this paper, can be considered as the initial stage of research. However, this study provides an opportunity to predict and assess the response of marine ecosystems to changes in environmental conditions and to pollution.

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