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Genotoxic and cytotoxic effects in the bivalve mollusks *Macoma balthica* and *Mytilus edulis* from the Baltic Sea

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Micronuclei (MN), nuclear buds (NB), fragmented-apoptotic (FA) and bi-nucleated (BN) cells were analysed in gills of bivalve mollusks *Macoma balthica* and *Mytilus edulis* collected from five study locations in Lithuanian territorial waters of the Baltic Sea. The frequency of micronuclei varied from 1.28 to 3.63‰ (MN/1000 cells), of nuclear buds – from 0.50 to 1.49‰, fragmented-apoptotic cells from 0.53 to 1.72‰ and of bi-nucleated cells from 1.51 to 2.23‰. The highest level of MN and bi-nucleated cells was determined in mollusks from the offshore Station 65, nuclear buds and fragmented-apoptotic cells from Station 1B located close to the Būtingė oil terminal. A comparatively high frequency of MN was observed in bivalves from the Būtingė oil terminal area – 3.38‰ in Station 1B, 2.85‰ in Station B06 in June and in mollusks from Station N-4 (2.66‰) located nearby the oil platform D-6.

Key words: micronuclei, mollusks, *Macoma balthica, Mytilus edulis*, genotoxicity, the Baltic Sea

INTRODUCTION

A large number of chemical compounds are known to cause hazardous effects in aquatic organisms. Among those, heavy metals, oil products, chlorinated pesticides, halogenated aromatic hydrocarbons and other substances found in aquatic ecosystems have the ability to accumulate in organisms. Many contaminants exert their effects via genotoxic and metabolically toxic mechanisms, simultaneously causing carcinogenesis, embryotoxicity and inflict a long-term damage to organisms (Jha et al., 2000).

The Lithuanian coastal zone in the Baltic Sea is impacted by pollution from industrial, agricultural and municipal activities. Oil products represent one of the most widespread and rather harmful pollutants practically in the whole area. Oil compounds could be released due to spills caused by the Būtingė oil terminal which is situated in the northern part, from the Klaipėda port and Klaipėda oil terminal in the central part and from the Russian oil platform D-6 in the southern region of the Lithuanian economic zone. In addition, oil compounds reach the marine environment via rivers and rainwater, marine traffic, and transportation of oil products. More recently ecological risk of spills have become relevant in the area due to increased oil export and import through the Klaipėda and Būtingė oil terminals (Pupienis et al., 2007).

Environmental monitoring based exclusively on chemical analysis of water and sediment is not suitable for the assessment of biological injuries and prediction of ecological effects of contaminants. Biological effects caused by oil pollution can be displayed at different levels, but first of all they can be observed at the molecular–biochemical level, and later at higher biological levels including reproductive dysfunctions (Depledge, 1994). Biomarker approaches *in situ* provide data on biological effects in target organisms and can be successfully used to predict the ecological impacts of accidental oil spills and release of hazardous contaminants.

Previous studies on biomarker responses in fish and mussels from the Lithuanian coastal area have shown a significant increase of genotoxicity, cytotoxicity, immunotoxicity, PAH metabolites in fish bile and neoplastic lesions after an accidental oil spill in the Būtingė oil terminal in November 2001 (Baršienė et al., 2004, 2005, 2006a, 2006b). Increased genotoxicity and cytotoxicity levels have been found at sites in the Lithuanain economic zone located close to the oil platform D-6 (our unpublished data).

Petroleum hydrocarbons occurring in the marine environment can significantly impact the DNA of filter-feeding bivalve populations (Hamoutene et al., 2002). A higher frequency of micronuclei has been detected in mussels from the Būtingė oil terminal and Klaipėda marine port zones in the Baltic Sea (Baršienė, Baršytė Lovejoy, 2000; Baršienė, 2002), in Mediterranean commercial port zone (Magni et al., 2006), in the Venice Lagoon polluted by aromatic hydrocarbons (Venier, Zampieron, 2005). In different studies, a significant elevation of micronuclei levels has been described for mussels 30 days after an oil spill, and the cy-

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togenetic damage persisted up to 100 days (Parry et al., 1997) or even 8 months (Baršienė et al., 2004, 2006a). A statistically significant increase of the micronuclei level has also been observed in oysters and fish caged in Haven oil spill zones 10 years after an oil spill (Bolognesi et al., 2006).

The main objective of the present study was to evaluate the level of environmental genotoxicity and cytotoxicity at different sites within the Lithuanian economic zone of the Baltic Sea. Micronuclei and nuclear buds formation in gill cells of the bivalve mollusks *Macoma balthica* and *Mytilus edulis* was measured as endpoint of environmental genotoxicity. The presence of fragmented-apoptotic and bi-nucleated gill cells was used as a marker of environmental cytotoxicity.

MATERIALS AND METHODS

In May 2006, clams (*Macoma balthica*) and blue mussels (*Mytilus edulis*) were sampled from six locations. Bivalves *M. balthica* were collected at Station N-8 which was considered as a reference site, and at Station N-4 which is situated comparatively close to the Russian oil platform D-6. In addition, clams were collected at Station 20M (dumping zone) and at the offshore Station 65. Since *M. balthica* did not inhabit the shallow coastal area near the Būtingė oil terminal, blue mussels were sampled from the area in May and August 2006 at Station 1B and in June 2006 at Station B06 (Fig. 1). At each study location, 8–11 clam or mussel specimens were collected to study micronuclei and other nuclear abnormalities.

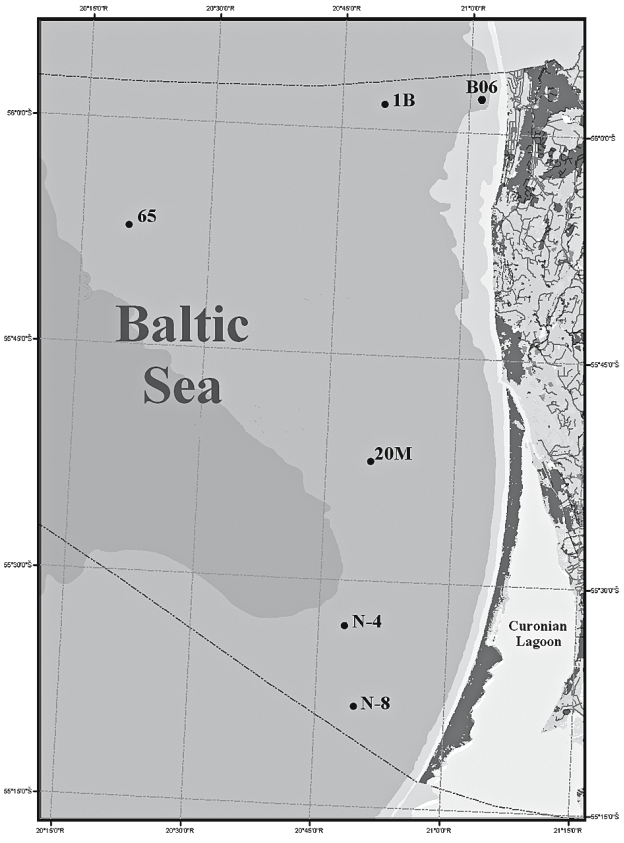

Fig. 1. Sampling stations in the Lithuanian economic zone of the Baltic Sea

Clams and mussels were dissected, gills removed and two gill arches placed in a drop of 3 : 1 ethanol acetic acid (or methanol acetic acid) solution separately on two clean microscope slides and gently nipped with tweezers for 2–3 min. The produced cell suspension was softly smeared on both slides and air-dried. Dried smears were subsequently fixed in methanol for 10 min. Air-dried slides were stained with 4% Giemsa solution in phosphate buffer pH 6.8. The stained slides were analysed under the Olympus BX51 light microscope at a final magnification of 1000x. For each studied specimen of clams and mussels, 2000 gill cells with the intact cytoplasm were scored (Baršienė et al., 2004).

The blind scoring of micronuclei and other nuclear abnormalities was performed on coded slides without knowing the origin of samples. Micronuclei (MN) were identified according to the following criteria: (1) round and ovoid-shaped non-refractory particles in the cytoplasm, (2) colour and structure similar to chromatin, (3) a diameter of 1/3–1/20 of the main nucleus, (4) particles completely separated from the main nucleus (Fig. 2). Nuclear buds, bi-nucleated and fragmented-apoptotic cells were identified following criteria described by Fenech et al. (2003).

The final results were expressed as the mean value (‰) of the sums for the individual lesions scored in 1000 cells per mussel collected from every study location. The statistical analysis was carried out using PRISM statistical package. The mean and the standard error were calculated for each group of bivalves. The non-parametric Mann–Whitney U-test was used to compare MN frequencies in mollusks from the reference and contaminated sites.

RESULTS

The frequency of micronuclei (MN/1000 cells) varied in clams *M. balthica* from 1.28 to 3.63‰ and in mussels from 1.74 to 3.38‰ (Fig. 3). A low level of micronuclei was observed in clams inhabiting Station N-8 (reference site). A significantly increased MN incidence was found in clams from location N-4 ($P = 0.0155$) and in clams from location 65 ($P = 0.0003$) (Fig. 3).

Similar levels of nuclear abnormalities were found in mussels collected in the Būtingė oil terminal area. MN frequency in mussels from Station 1B was analysed in May and August 2006. In August, the mean of MN (3.38‰) was two-fold higher than in May (1.74‰). The value of this parameter in mussels from B06 in June was equal to 2.86‰ (Fig. 3).

The occurrence of other nuclear abnormalities was also analysed in clams and mussels. In *M. balthica* the frequency of nuclear buds (BD/1000 cells) varied from 0.50 to 1.49‰, fragmented-apoptotic cells (FA/1000 cells) from 0.53 to 1.72‰, bi-nucleated cells (BN/1000 cells) from 1.51 to 2.23‰. The highest levels of nuclear buds were observed in mussels collected in May at Station 1B, of fragmented-apoptotic cells in mussels collected at the same station in August, and of bi-nucleated cells in those mussels inhabiting Station 65 (Fig. 4).

Analysis of heavy metals in the tissues showed increased levels of Zn, Pb and Cu in bivalves collected at Station 20M, of Cd in specimens from Station 1B, and Cr in those from Station 65 (Table).

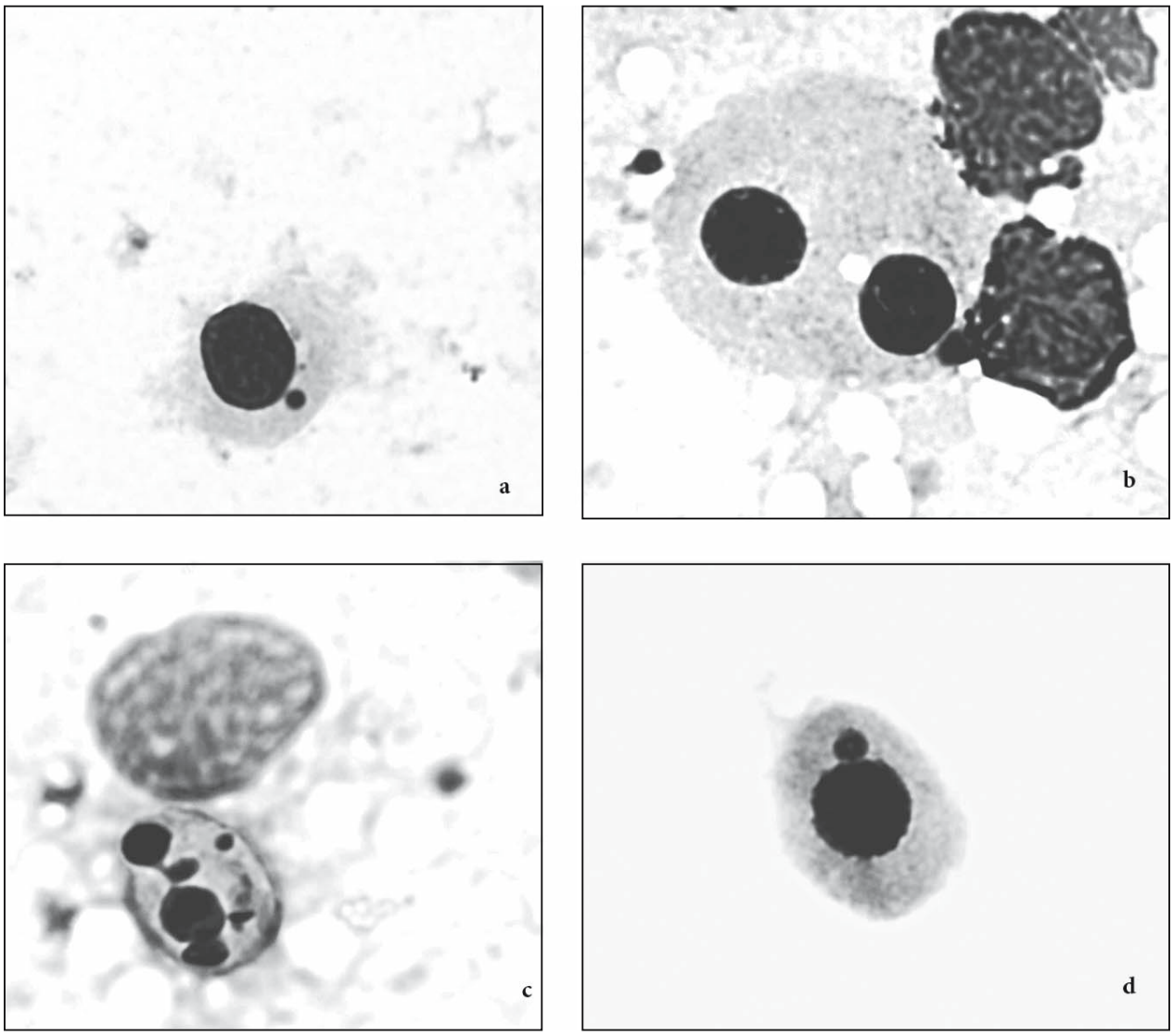

Fig. 2. Nuclear abnormalities in *Macoma balthica* gills: (a) cell with micronuclei, (b) bi-nucleated cell, (c) fragmented-apoptotic cell and (d) nuclear bud in *Mytilus edulis* gill cell

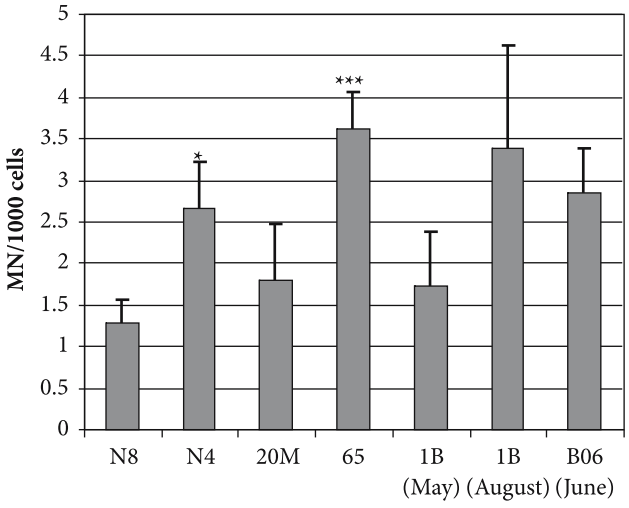

Fig. 3. Frequency of micronuclei in gill cells of clams *M. balthica* collected at Stations N8, N4, 20M and 65 and blue mussels *M. edulis* from Stations 1B and B06 in the Lithuanian economic zone of the Baltic Sea. Significant differences as compared with *M. balthica* clams from the reference station (N8) and the other locations are indicated with one asterisk ($P < 0.05$) and three asterisks ($P < 0.0001$)

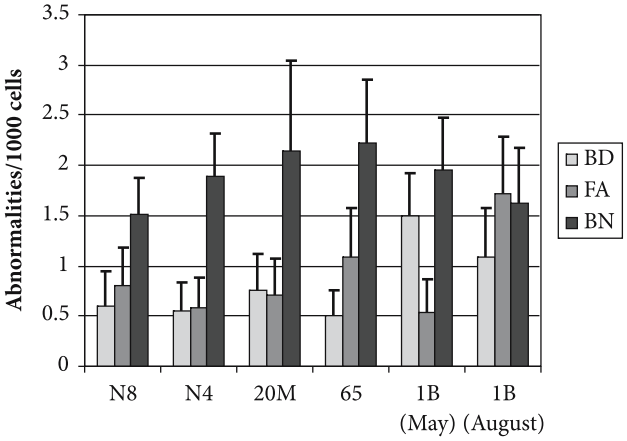

Fig. 4. Frequency of nuclear buds (BD), fragmented-apoptotic (FA) and bi-nucleated (BN) cells of clams collected at Stations N8, N4, 20M and 65 and blue mussels from Stations 1B and B06 in the Lithuanian economic zone of the Baltic Sea

Table. **Concentrations of heavy metals in blue mussel *(Mytilus edulis) and clam (Macoma balthica)*. The tissues number of samples is shown in brackets**

Year	Zn	Pb	Cd	Cu	Cr
	mg/kg wet weight				
Mytilus edulis, Station 1B					
2006 (6)***	<5.15–42.8	<0.13–0.21	0.29–1.03	0.77–1.10	<0.27–0.40
Macoma balthica, Station 65					
2005*–2006 (3)	76.5–154	0.21–0.50	0.10–0.18	25,7–35,3	0.28–0.54
Macoma balthica, Station 20M					
2006 (2)**	164–250	0.38–0.41	0.13–0.20	34.9–53.8	–
Macoma balthica, Station N4					
2006 (2)**	124–135	<0.13	0.07–0.10	2.81–3.57	–
Macoma balthica, Station N8					
2006 (2)**	127–181	0.22–0.30	0.11–0.18	8.33–11.9	–

* FIMR data; ** LIFE project data; *** HELCOM monitoring and LIFE project data.

DISCUSSION

The micronucleus test (MN) is one of the most popular and promising approaches in environmental genotoxicity studies; it has served as an index of cytogenetic damage for over 30 years. The application of the micronucleus test is based on the presence of micronuclei that occur in actively dividing cell populations (Fenech et al., 2003). Micronuclei, small fragments of chromatin, are separated from the main cell nucleus and are an evidence of chromosome breaking or mitotic spindle dysfunction. They are frequently caused by clastogenic and aneugenic agents. Micronuclei are produced in all cell types after irregular division processes when a chromosome fragment or a whole chromosome is not lost during the anaphase, but is delayed with respect to the rest of chromosomes, constituting a small secondary nucleus in an interphase (Heddle et al., 1991). The MN test has been used successfully in marine mollusks as a biological indicator of pollution *in situ* (Brunetti et al., 1992; Burgeot et al., 1995; Bolognesi et al., 1996, 2004, 2006; Izquierdo et al., 2003; Baršienė et al., 2004, 2005, 2006a, 2006b; Magni et al., 2006; Schiedek et al., 2006).

In the present study, micronuclei and other nuclear abnormalities were analysed in two indigenous bivalve mollusk species inhabiting five different sites in the Lithuanian economic zone of the Baltic Sea. The study revealed the highest level of micronuclei in clams inhabiting the offshore Station 65. A comparatively high frequency of micronuclei was found in blue mussels from station 1B in August 2006 and in clams from Station N-4. The reference level of micronuclei incidences was observed in clams inhabiting the comparatively uncontaminated coastal Station N-8. Analysis of other nuclear abnormalities revealed no significant differences among the bivalves from the study sites.

Marine traffic is the main source of pollution at the offshore Station 65. Long-term data on total oil hydrocarbons and heavy metal contamination disclosed elevated oil hydrocarbon concentrations in water in 2003. Especially high total oil hydrocarbon concentrations were observed in autumn 2005. In sediments from Station 65, we have detected elevated concentrations of Cu, Pb and Zn in 2006. The concentration of TBT reached 6.0 µg/kg dry weight, the concentration of total PAHs being 120 µg/kg dry weight (FIMR report, 2007). In 2006, DDT concentrations in clam tissues were lower than the average level (Fig. 5).

The study station 1B is situated near the Būtingė oil terminal area, where increased levels of total oil hydrocarbons have been observed episodically. Elevated concentrations of Cd were detected in mussels in the present environmental genotoxicity study, but DDT concentrations were lower than the average level (Fig. 5). The station N-4 is located close to the Russian D-6 oil platform.

A higher frequency of micronuclei has been detected earlier in mussels taken close from the oil terminal and marine port zones in the Baltic Sea (Baršienė, Baršytė Lovejoy, 2000; Baršienė, 2002). A number of studies have reported an increase of environmental genotoxicity in zones affected by accidental oil spills (Parry et al., 1997; Harvey et al., 1999; Pietrapiana et al., 2002; Perez-Cadahia et al., 2004; Baršienė et al., 2004; 2006a, 2006b; Frenzilli et al., 2004; Laffon et al., 2006; Bolognesi et al., 2006; Martinez-Gomez et al., 2006). Higher incidences of micronu-

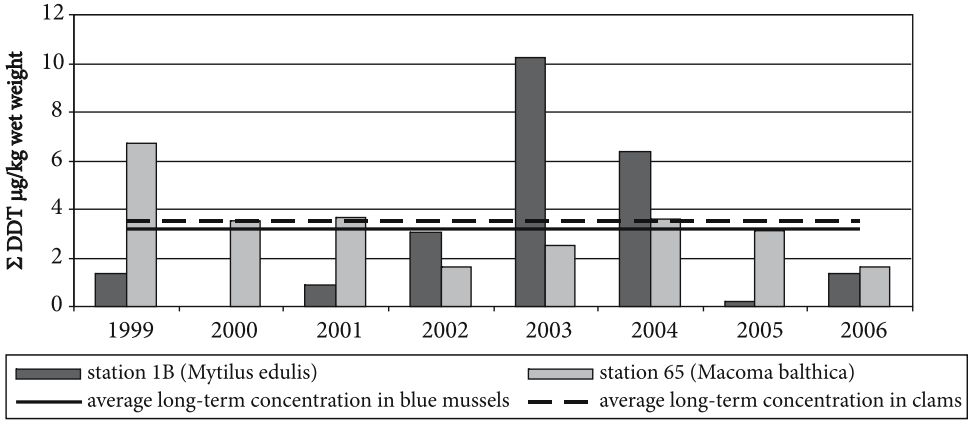

Fig. 5. Σ DDT concentration in *M. balthica* tissues (Station 65) and *M. edulis* (1B) collected in 1999–2006

clei and nuclear abnormalities were found in fish from areas affected by effluents from a shale processing plant (da Silva Souza, Fontanetti, 2006). Significantly increased levels of micronuclei and other nuclear abnormalities were observed in mussel gills after exposure to 0.5 ppm of Statfjord B (the North Sea) crude oil and to 0.5 ppm of spiked oil (Baršienė, Andreikėnaitė, 2007).

Hazardous effects of PAHs arise as a result of oxidative biotransformation producing highly DNA-reactive metabolites which have been recognized as carcinogenic, mutagenic and cytotoxic compounds (Torres-Bugarin et al., 1998; Woodhead et al., 1999). The genotoxic potency of ten polycyclic aromatic hydrocarbons (anthracene, 7,12-dimethylbenz[*a*]anthracene, benz[*a*]anthracene, dibenz[*a,h*]anthracene, dibenz[*a,c*]anthracene, 3-methylcholanthrene, benzo[*a*]pyrene, benzo[*e*]pyrene, chrysene and pyrene) has been demonstrated (Nishikawa et al., 2005). Cells with micronuclei were found to increase in the gills or hemolymph of marine mollusks treated with benzo(a)pyrene (Burgeot et al., 1995; Venier et al., 1997; Siu et al., 2004), and with dimethylbenz(a)antracene (Bolognesi et al., 1996).

Analysis of heavy metals in bivalve tissues showed increased levels of Cd, Cr, Zn, Pb and Cu in bivalves collected from the study sites of the Lithuanian economy zone of the Baltic Sea. The genotoxic properties of heavy metals are related to accumulation of DNA-damaging free radicals, clastogenic process or simultaneously to the clastogenic and aneugenic action in aquatic organisms (Nepomuceno et al. 1997). MN elevation has been described in eel (*Anguilla anguilla*) after treatment with cadmium and mercury (Sanchez-Galan et al., 2001; Teles et al., 2005), in *Carassius auratus gibelio* treated with chromium (Al-Sabti, Härdig, 1990). A statistically significant increase in micronuclei was observed in rainbow trout *Oncorhynchus mykiss* after exposure to a model mixture composed of Cu, Zn, Pb, Ni, Cr and Mn (Andreikėnaitė et al., 2007). The genotoxicity of chromium, cadmium and copper has been detected in different tissues of common carp (*Cyprinus carpio*), Prussian carp (*Carassius gibelio*) and Peppered corydoras (*Corydoras paleatus*), and the MN test was proposed as a suitable approach for the screening of genotoxic compounds in aquatic ecosystems *in situ* (Cavas et al., 2005).

There is a growing concern over the presence of genotoxic compounds in the marine environment and a rising need to elaborate appropriate methods for the assessment of genotoxicity in indigenous marine organisms. Nevertheless, there is a shortage in our knowledge on short- and long-term implications of mutagenic complex mixtures in wild marine populations (Moore et al., 2004). The application of cytogenetic assays using ecologically relevant species offers a chance to perform early tests on ecosystem health in relation to exposure to contaminants (Jha, 2004). Chemicals with a genotoxic potential for the aquatic environment are of serious concern since they can bind to DNA molecules and provoke a damaging chain of biological changes such as impaired enzyme function or general metabolism, cytotoxicity, immunotoxicity, disturbances in reproduction, inhibition of growth, or carcinogenesis (Ohe et al., 2004).

Nevertheless, a comparatively low response in indigenous organisms could arise as a result of adaptation to chronically contaminated habitats such as dumping sites (Station 20M) in Lithuanian economic zone of the Baltic Sea. Therefore, adaptation to chronically contaminated habitats should be one of the priorities for the future studies of environmental genotoxicity in dumping areas, in marine port zones, in the oil refinery or drilling sites, dredging and other areas with a long history of anthropogenic influence. Deployment of organisms from uncontaminated sites and caging in these ecologically stressful areas, as well as in zones of accidental spills of contaminants should be applied for assessing changes in genotoxicity of environments.

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GENOTOKSINIO IR CITOTOKSINIO POVEIKIO ĮVERTINIMAS BALTIJOS JŪROS DVIGELDŽIŲ MOLIUSKŲ *MACOMA BALTHICA* IR *MYTILUS EDULIS* LĄSTELĖSE

S a n t r a u k a

Mikrobranduolių (MN), branduolio pumpurų (NB), fragmentuotų-apoptozinių (FA) ir dvibranduolių (BN) ląstelių dažniai buvo tirti dvigeldžių moliuskų *Macoma balthica* ir *Mytilus edulis* žiaunose. Moliuskai surinkti iš penkių Baltijos jūros Lietuvos teritorinių vandenų vietų. Tyrimų vietos pasirinktos atsižvelgiant į taršos šaltinį. Stotis N-4 yra netoliese D-6 naftos platformos, 20M – Klaipėdos uosto grunto sąvartos zonoje, 1B ir B06 – Būtingės terminalo poveikio vietoje, 65 – intensyvios laivybos zonoje, N-8 – kontrolinė vieta.

Mikrobranduolių dažnis kito nuo 1,28 iki 3,63‰ (MN/1000 ląstelių), branduolio pumpurų – nuo 0,50 iki 1,49‰, fragmentuotų-apoptozinių ląstelių – nuo 0,53 iki 1,72‰, dvibranduolių ląstelių – nuo 1,51 iki 2,23‰. Didžiausi MN ir BN dažniai rasti moliuskuose, kurie gyveno atviroje jūroje, 65 stotyje, o NB ir FA – moliuskuose iš 1B stoties, kuri yra netoli Būtingės naftos terminalo. Santykinai didelis MN dažnis aptiktas moliuskuose iš Būtingės naftos terminalo zonos ir iš stoties N-4, kuri yra netoli D-6 naftos platformos.

Raktažodžiai: moliuskas, *Macoma balthica*, *Mytilus edulis*, genotoksinis, Baltijos jūra