Environmental genotoxicity and cytotoxicity studies in mussels and fish inhabiting northern Atlantic zones impacted by aluminum industry

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Micronuclei (MN), nuclear buds (NB), fragmented-apoptotic (FA) and bi-nucleated (BN) cells were analyzed in *Mytilus edulis* gills and in kidney erythrocytes of Atlantic cod *Gadus morhua* collected from three boreal locations in the North Atlantic, representing the pollution gradient originating from aluminium smelter industry (Mosjøen, Norway). The highest MN, NB, FA and BN values were measured in mussels from the most polluted site, Halsøy, which is located close to the aluminium smelter works. In Halsøy, a 5.2-fold increase ($p < 0.0001$) in MN, a 3.4-fold increase in fragmented-apoptotic cells ($p = 0.0001$), a 1.8-fold increase in nuclear buds ($p = 0.0002$) and a 1.7-fold increase in bi-nucleated cells ($p = 0.0524$) was found as compared to the reference site. A difference in the incidence of MN ($p = 0.0052$) and FA ($p = 0.0003$) were found in mussels from the reference and an intermediate contaminated site (Hundålbukta). A significant difference in MN ($p = 0.0087$) and NB ($p = 0.0186$) frequencies were observed in fish kidney immature erythrocytes from Halsøy and Hundålbukta sites.

Key words: genotoxicity, cytotoxicity, micronuclei, mussels, cod, North Atlantic, aluminum industry

INTRODUCTION

Substances released from human activities are well known primary sources of persistent toxicity in aquatic environments. A large proportion of chemicals is recognized as potentially genotoxic and carcinogenic substances (Claxton et al., 1998). In recent years, there has been increased concern that certain environmental genotoxins show a very aggressive mode of action and are able to induce DNA damage in organisms at low levels of exposure (Regoli et al., 2004). Damaging the DNA of organisms, the genotoxins can initiate a cascade of impairments at the molecular, cellular, organ, whole organism, or population and community levels. DNA and cytogenetic alterations in aquatic organisms have been associated with an impaired enzyme function or general metabolism, cytotoxicity, immunotoxicity, abnormal development, reduced survival, growth, reproduction potency (Jha, 2004).

The feasibility of the current study appeared as there is a growing concern over the presence of genotoxic substances in the sub-arctic marine environment and a scarcity of data

on the genotoxicity of hazardous compounds in marine species inhabiting northern latitudes. At present, only limited information is available on environmental genotoxicity in marine organisms of sub-arctic waters under the influence of aluminum industry. The aluminum industry poses severe environmental hazards due to the large volumes of produced effluents and emission of hazardous substances originating from their technological process (Yumei et al., 1998). An exceptionally high level of micronuclei incidence has been found in mussels *Mytilus edulis* (up to 6.9‰) and wrasse *Symphodus melops* inhabiting the Høgevarde zone (North Sea, Karmsund fjord system) affected by the discharge from an aluminum smelter and from its considerable boat traffic (PAHs, TBT, and other organic pollutants), although in unpolluted Karmsund zones the reference levels of micronuclei in mussels and wrasse were 5–6 times lower than in those from the Høgevarde site (Baršienė et al., 2004). The Høgevarde study site is located close to the discharge from an old aluminum smelter producing about 200,000 tons of aluminium per year and discharging to the Karmsund strait about 450 kg of PAHs annually (Beyer et al., 1998). Significantly higher levels of both biliary PAH metabolites

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and hepatic DNA adducts in corkwing wrasse and Atlantic cod collected in the vicinity of the aluminium smelter in Karmsund have been reported by Aas with co-authors (2001). Furthermore, at the most contaminated site (Høgevarde), skin ulcers occur in about 70% of cod, and fin erosion was detected in 45% of the cod (Aas et al., 2001). Recently, an elevation of histopathological alterations has been described in mussels collected at Høgevarde. In the soft tissues of *Mytilus edulis* collected in this site, high concentrations of pyrene, chrysene, phenanthrene, benzo(*b,j*) fluoranthene, benzo(k)fluoranthene and dibenzo(*a*)pyrene were observed (Aarab et al., 2008).

The Mosjøen industrialized zone is located in the North Atlantic, close to the northern polar circle over the latitude of 65°. The aluminum smelter in this area was established in 1958 and produces 189,000 tons of aluminum per year in the form of rolling slabs, low iron foundry ingots, and it also produces its own pre-baked anodes. The Mosjøen aluminum smelter atmospheric emissions contain sulphur dioxide as well as a highly toxic fluoride compound containing dust that can dissolve in water and be carried into the aquatic environment (Vike, 1999). Moreover, in aluminum smelters, large amounts of PAHs are released through the loss of green coke during transportation to the smelters, airborne emissions, surface runoff and waterborne effluents (Sanderson et al., 2004; Frouin et al., 2007). Among individual compounds, in smelter discharge sediments, comparatively high concentrations of fluorene, phenanthrene, anthracene, benzo(*c*)phenanthrene, benzo(*a*)antracene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*) pyrene, dibenzo(*a,l*)pyrene, dibenzo(*a,h*)anthracene were detected (Frouin et al., 2007). Polyaromatic hydrocarbons in a marine environment can significantly impact DNA and induce mutagenic and cytotoxic effects in fish and mussels (Hamoutene et al., 2002; Gravato, Santos, 2003; Teles et al., 2003). A higher frequency of micronuclei has been detected in mussels from the zones of the Venice Lagoon polluted by aromatic hydrocarbons (Venier, Zampieron, 2005), in the Croatian coastal zone (Klobučar et al., 2008) or after dredging of sediments contaminated by PAHs and Pb (Bocchetti et al., 2008). A significant level increase of the micronuclei in mussels 30 days post-oil-spill and the persistence of the cytogenetic damage up to 100 days (Parry et al., 1997) or even 8 months (Baršienė et al., 2004, 2006a) have been described. A statistically significant increase of micronuclei levels has been observed in oysters and fish caged in the Haven oil spill zones 10 years after an oil spill (Bolognesi et al., 2006a).

In this paper, we report data on environmental genotoxicity and cytotoxicity in mussels and fish from the North Atlantic zones affected by discharges from the Mosjøen aluminum smelter in northern Norway. Induction of micronuclei and nuclear buds in gill cells of mussels and in kidney immature erythrocytes of cod was used as an environmental genotoxicity biomarker, and the incidence of fragmented-apoptotic

and bi-nucleated cells was employed as the endpoints of environmental cytotoxicity. The micronucleus (MN) test, one of the most popular and promising tests of environmental genotoxicity, has served as an index of cytogenetic damage for the last decades. Micronuclei formation correlates with pollution load, as it has been shown in a number of the latest studies in marine organisms (Bolognesi et al., 2004, 2006a, 2006b; Baršienė et al., 2004, 2006a, 2006b, 2006c, 2006d, 2006e, 2008; Çavaş, Ergene-Gözükara, 2005a, 2005b; Çavaş et al., 2005; Nigro et al., 2006; Baršienė, Andreikėnaitė, 2007; Ergene et al., 2007a). Micronuclei, as a sensitive endpoint, were used in developing the Bioeffect Assessment Index (BAI) and the Integrated Biomarker Index (IBR) to describe the toxin-induced stress levels in different sites of the Baltic Sea (Broeg, Lehtonen, 2006). The MN test was suggested in the toolbox of biomarkers for improving the risk assessments of chemicals considering the Water Framework Directive requirements (Hagger et al., 2008).

MATERIALS AND METHODS

Thirty specimens of mussels *Mytilus edulis* were sampled from three study locations: Krokvik, which was considered a reference site (66°03.671 N, 12°47.091 E), Halsøy (Elkem, 65°51.760 N, 13°10.990 E), which is situated in a zone heavily polluted by pyrogenic PAH (the most contaminated site close to the aluminium work) and Hundålbukta (65°55.500 N, 12°57.070 E), which is located in a pyrogenic PAH gradient (an intermediate contaminated site). The study locations can be described as fully marine; their water salinity ranges from 34 to 35 ppt and water temperatures from 4 to 6 °C. Eight specimens of cod (*Gadus morhua*) were collected in deep layers (up to 500 m) from Hundålbukta and six specimens from Halsøy (Elkem) sites. Only live specimens, all strictly of the same size, were processed for analysis.

Mussels were dissected, gills removed and two gill arches placed in a drop of 3 : 1 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. In fish, after the sacrifice, a small piece of cephalic kidney was dissected, softly dragged along the clean slide and allowed to dry for one or two hours. The dried smears were fixed in methanol for 10–15 min. Air-dried slides were stained with 5% Giemsa solution in phosphate buffer pH 6.8. The stained slides were analyzed under the Olympus BX51 light microscope at a final magnification of 1 000x. For each studied specimen of mussels, 2 000 gill cells with an intact cellular and nuclear membrane were analyzed (Baršienė et al., 2004). A total of 5 000 immature erythrocytes with intact cytoplasm were examined for each fish specimen.

A 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
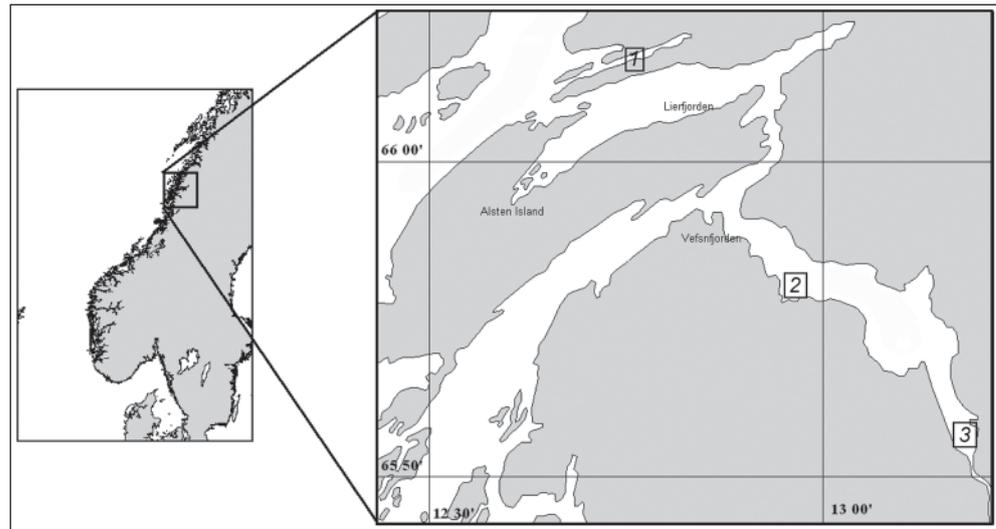
Fig. 1. Sampling locations in the Mosjøen zone of the North Atlantic: *1* – Krokvik, *2* – Hundålbukta, *3* – Halsøy (Elkem)

non-refractory particles in the cytoplasm, (2) colour and structure similar to those of chromatin, (3) a diameter of 1/3–1/20 of the main nucleus, (4) particles completely separated from the main nucleus (Fig. 2*a*) (Fenech et al., 2003). Nuclear buds, bi-nucleated and fragmented-apoptotic cells were identified using the criteria described by M. Fenech with co-authors (2003). The morphological features of the nuclear abnormalities studied are shown in Fig. 2.

The final results were expressed as a mean value (‰) of the sums for individual lesions scored in 1 000 cells per mussel or fish collected from each study location. The statistical analysis was carried out using PRISM statistical package. The mean and the standard error were calculated for each group of organisms. The non-parametric Mann–Whitney U-test was used to compare the frequencies of abnormalities in mussels from reference and contaminated sites.
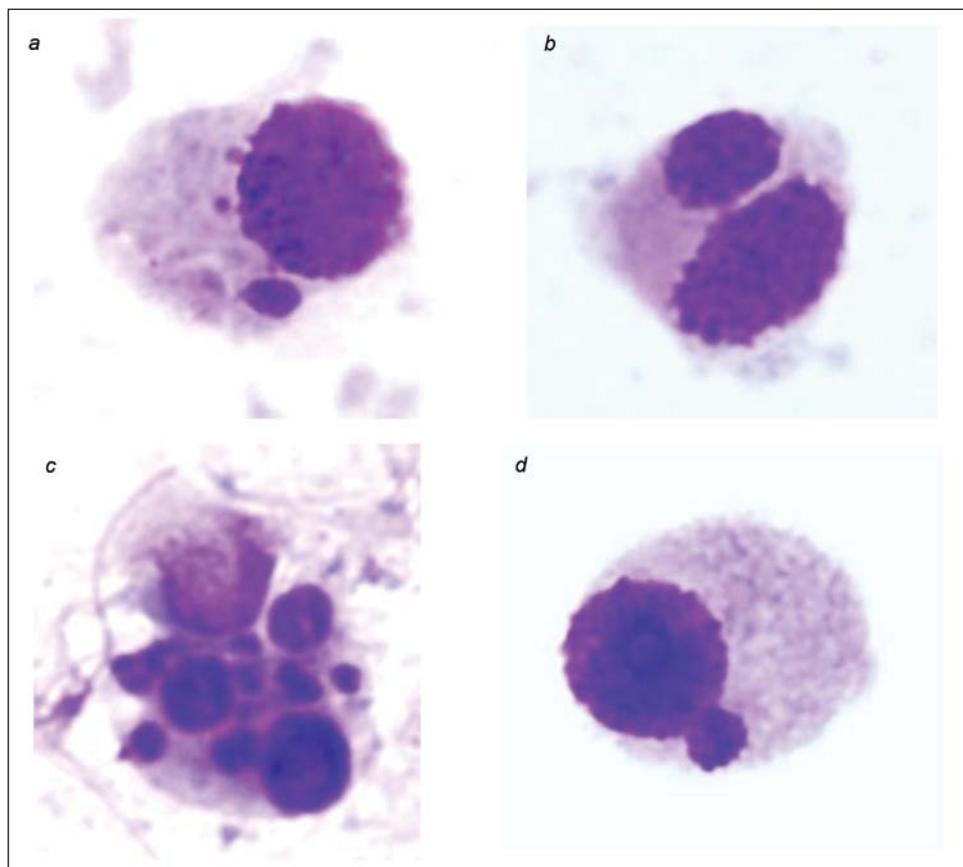

Fig. 2. Nuclear abnormalities in *Mytilus edulis* gills: (*a*) cell with micronuclei, (*b*) bi-nucleated cell, (*c*) fragmented-apoptotic cell, and (*d*) cell with nuclear bud

RESULTS

In mussels, the frequency of micronuclei (MN / 1 000 cells) varied from 0.65 to 3.35‰, of nuclear buds (NB / 1 000 cells) – from 1.85 to 3.40‰, of fragmented-apoptotic cells (FA / 1 000 cells) from 0.95 to 3.25‰, of bi-nucleated cells (BN / 1 000 cells) from 1.75 to 2.90‰. The lowest values of micronuclei, nuclear buds, fragmented-apoptotic and bi-nucleated cells were found in mussels from the reference Krokvik site. The highest induction of the cellular alterations studied was registered in mussels inhabiting the Halsøy station which is located close to the aluminum smelter (Fig. 3). In Halsøy, a 5.2-fold increase of MN, a 3.4-fold increase of fragmented-apoptotic cells, a 1.8-fold increase of nuclear buds and a 1.7-fold increase of bi-nucleated cells as compared to the reference Krokvik site were found.

Statistically significant differences were found between the incidence of micronuclei in mussels from the reference Krokvik and both contaminated sites (Hundålbukta, $p = 0.0052$ and Halsøy, $p < 0.0001$) as well as between mussels from Halsøy and Hundålbukta ($p < 0.0001$). A comparison of fragmented-apoptotic cell frequencies also showed a significant differentiation between mussels from the reference and from Hundålbukta ($p = 0.0003$) and Halsøy ($p < 0.0001$). The frequencies of nuclear buds in mussels from Krokvik and Halsøy were also statistically significantly different ($p = 0.0002$).
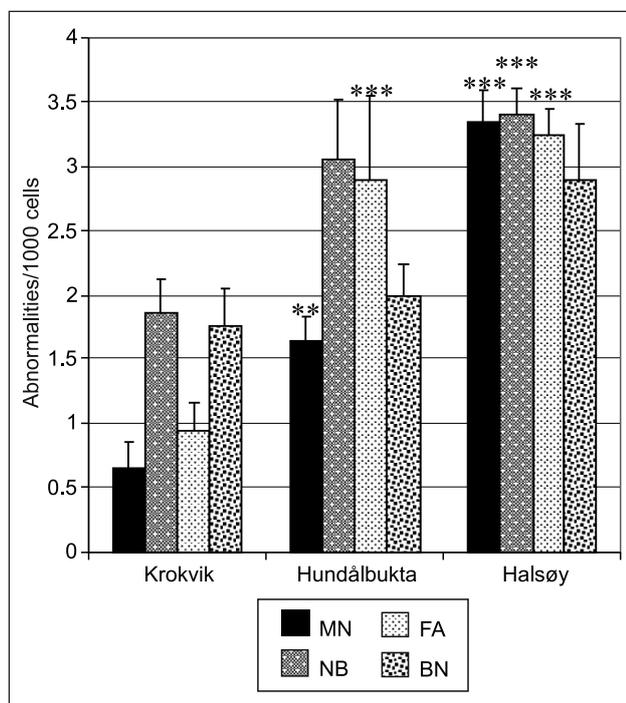

Fig. 4. Frequency of micronuclei (MN), nuclear buds (BD), fragmented-apoptotic (FA) and bi-nucleated (BN) erythrocytes in cod kidney immature erythrocytes. Asterisks show statistically significant inter-location differences compared to the reference Krokvik site: one asterisk at $p < 0.01$, two asterisks at $p < 0.001$

The difference between the incidence of bi-nucleated cells in mussels from the reference and Halsøy sites was not significant ($p = 0.0524$).

The frequency of fish kidney immature erythrocyte micronuclei in fish from Hundålbukta was 0.13‰, while in cod from Halsøy it was 0.60‰. The difference was statistically significant ($p = 0.0087$). The inter-site frequency of nuclear buds was also statistically significantly different. A comparison of cytotoxicity endpoints in cod didn't reveal any significant inter-site differences. The frequency of bi-nucleated erythrocytes was 0.5‰ in cod from Hundålbukta and 0.7‰ in cod from Halsøy. The induction levels of fragmented-apoptotic erythrocytes were the same in fish from both locations (Fig. 4).

DISCUSSION

There are a number of reports suggesting the use of the micronuclei test for assessing the genotoxicity of compounds released from industry into aquatic environments. The analysis of MN has been proven useful for assessing the genotoxicity of effluents from a pulp mill (Pacheco, Santos, 1999), petroleum refinery (Çavaş, Ergene-Gözükara, 2005a, 2005b; Da Silva Souza, Fontanetti, 2006), textile mill (Marlasca et al., 1998; Çavaş, Ergene-Gözükara, 2003), aluminium smelter industry (Baršienė et al., 2004). Besides MN, other nuclear abnormalities (nuclear buds, bi-nucleated, fragmented or 8-shaped cells) have also been used in environmental genotoxicity and cytotoxicity studies (Gravato, Santos, 2002; Pacheco et al., 2005).

In the present study, micronuclei and other nuclear abnormalities were analyzed in indigenous bivalve mollusk and

Fig. 3. Frequency of micronuclei (MN), nuclear buds (NB), fragmented-apoptotic cells (FA) and bi-nucleated cells (BN) in gills of mussels *M. edulis* from the reference area (Krokvik) and zones influenced by pollution from the Mosjøen aluminium smelter (Halsøy and Hundalbukta). Asterisks show statistically significant inter-location differences compared to the reference Krokvik site: two asterisks at $p < 0.001$, three asterisks at $p < 0.0001$
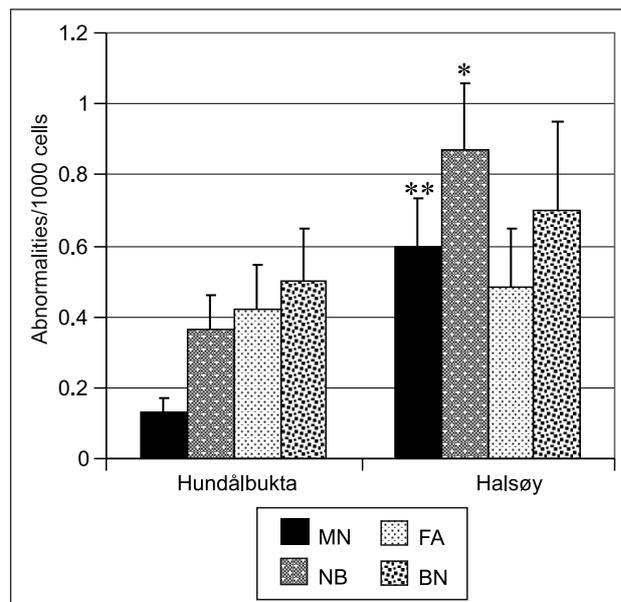
gadoid fish species inhabiting three sites in the North Atlantic, which differ in their contamination load originating from the Mosjøen aluminium smelter. The results revealed a significant increase of genotoxicity (5.2-fold increase of MN and 1.8-fold increase of nuclear buds) and cytotoxicity (3.4-fold increase of fragmented-apoptotic cells and 1.7-fold increase in bi-nucleated cells) in mussels collected from the Halsøy station located in the vicinity of the aluminium smelter. An increased level of genotoxicity and cytotoxicity was also detected in fish kidney immature erythrocytes. Thus, the enumeration of genetic and cytotoxic alterations was registered not only in bottom-dwelling, sedentary and filter-feeding mussels, but also in the Atlantic cod, a fish characterized by a high migration potential.

In soft tissues of mussels from the Halsoy location, the sum of 16 EPA PAHs reached 4682 μg/kg ww (161-fold increase), of CPAH 2739.9 μg/kg ww (326-fold increase), of B(*a*)P 335.8 μg/kg ww (672-fold increase); 2–3 ring PAHs comprised 232 μg/kg ww (116-fold increase) and 4–6 rings 5436 μg/kg ww (236-fold increase) versus the reference level in mussels from the Krokvik site. The concentrations of heavy metals were not increased. Catalase, TOSC protein expression and the micronuclei induction pattern in mussels from the Halsoy location, compared to those from the reference site, were statistically significantly (Bjørnstad et al., 2003).

Hazardous effects of PAHs arise as a result of oxidative biotransformation producing highly DNA-reactive metabolites recognized as carcinogenic, mutagenic and cytotoxic compounds (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). Naphtalene and benzo[*a*]pyrene (concentrations 0.1, 0.3, 0.9, or 2.7 μM for both compounds) were able to induce micronuclei in erythrocytes after a short-term exposure (2, 4, 6 and 8 h) in *Dicentrarchus labrax* juvenile fish (Gravato, Santos, 2002). In eel (*Anguilla anguilla*), treatment with 0.3, 0.9 and 2.7 μM of naphtalene induced formation of micronuclei and other nuclear abnormalities (Teles et al., 2003). An increased frequency of MN was observed in mussels after exposure to 100 ppb of dimethylbenz(*a*)antracene (Bolognesi et al., 1996), to 0.1 μg/L of phenanthrene (Koukouzika, Dmitriadis, 2008), and in zebra mussel *Dreissena polymorpha* after 2-, 3- and 4-day treatments with different concentrations (2 μg/L and 10 μg/L) of B[*a*]P (Binelli et al., 2009). Benzo[*a*]pyrene was classified as a progenotoxin which after metabolic activation becomes a very aggressive DNA-damaging agent (Johnson, 1992).

In our previous study, an exceptionally high level of environmental genotoxicity was detected in *M. edulis* mussels and *S. melops* wrasse from the Høgevarde site (the southwestern North Sea) impacted by effluents discharged from an aluminum smelter (Baršienė et al., 2004). The MN frequency

in blood erythrocytes of Atlantic cod *Gadus morhua* collected from this site was 0.36‰ (Baršienė et al., unpublished observations).

Induction of micronuclei in poikilotherm organisms is a temperature-dependent process, which could be due to seasonal changes in pollution load, differences in mitotic activity as well as to physiological changes related to metabolism, bioaccumulation of contaminants and reproduction cycle in bivalves (Bolognesi et al., 2004). Analysis of environmental genotoxicity and cytotoxicity in the Mosjøen area of the North Atlantic was performed late in autumn (at the end of October 2002) when water temperature at the bottom ranged from 4 to 6 °C. Despite the low environmental temperature, a significantly increased frequency of micronuclei was found in both studied species collected from the Halsøy site which is close to the contamination source. In addition, high frequencies of nuclear buds, fragmented-apoptotic and bi-nucleated cells were observed in mussels from the site.

A comparatively low MN level in mussels *M. galloprovincialis* haemocytes from six localities along the northern Mediterranean (Greece) coast – about 1‰ in June and about 2‰ of micronucleated haemocytes in October – was demonstrated in studies performed by Dailianis with colleagues (2003). MN frequencies in gill cells from unpolluted Mediterranean areas ranged from 1‰ to 3‰. When water temperature is above 20 °C, the MN baseline can reach 3‰, while at a temperature below 15 °C it equals to 1‰. The MN frequency of 2‰ was reported as a baseline level in mussels from an unpolluted zone of the Tyrrenian Sea (Brunetti et al., 1992). Besides, for the Mediterranean mussels, the reference values 1.42 and 0.73‰ haemocytes were presented by Dolcetti and Venier (2002). The lowest MN level in gill cells of *Mytilus edulis* from Norwegian fjords in the Karmsund area was equal to 1.1‰ (Baršienė et al., 2004).

According to our results, the appearance of other nuclear abnormalities (FA and BN cells) as the endpoints of cytotoxicity was quite frequent in mussels from the Halsøy site. In addition to the MN test, nuclear buds, bi-nucleated cells and other nuclear abnormalities were successfully used for assessing pollutant effects in marine fish and mollusks (Çavaş, Ergene-Gözükara, 2003, 2005a; Baršienė et al., 2006b, 2006c, 2006e, 2008; Baršienė, Rybakovas, 2006; Baršienė, Andreikėnaitė, 2007; Çavaş, Könen, 2007; Ergene et al., 2007a, 2007b). Significantly increased levels of micronuclei, nuclear buds and fragmented-apoptotic cells was found in bivalves inhabiting the Baltic Sea close to the Būtingė oil terminal and near the D-6 Russian oil platform (Baršienė et al., 2008) and in Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) after exposure to the North Sea crude oil (Baršienė et al., 2006b).

The formation of morphological nuclear abnormalities was described first in fish erythrocytes by Carrasco with co-authors (1990). Later, the induction of MN and other nuclear abnormalities in fish erythrocytes, as well as in gill and liver cells was suggested for the screening of genotoxic compounds

in aquatic ecosystems (Çavaş et al., 2005). The formation of bi-nucleated cells after exposure to high concentrations of contaminants could be suspected as a result of alterations in chromosome segregation and cytokinesis. Gene amplification via the breakage-fusion-bridge cycle can cause appearance of nuclear buds – the mechanism of amplified DNA elimination from the nucleus (Shimizu et al., 1998). Fragmented-apoptotic cells can be recognized as one of the main mechanisms in elimination of micronucleated damaged cells (Mičić et al., 2002). Since the assessment of cytotoxicity markers is quite helpful due to a relationship with the incidence of MN and nuclear buds, examination of the biomarkers should be included into the battery of the genotoxicity and cytotoxicity test with the aim to improve the estimation of sub-cellular damage caused by environmental pollution.

ACKNOWLEDGEMENTS

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APLINKOS GENOTOKSIŠKUMO IR CITOTOKSIŠKUMO TYRIMAI ŽUVYSE IR MOLIUSKUOSE IŠ ALIUMINIO PRAMONĖS ŠIAURĖS ATLANTE ZONŲ

S a n t r a u k a

Mikrobranduolių (MB), branduolio pumpurų (BP), fragmentuotų-apoptozinių (FA) ir dvibranduolių (DB) ląstelių dažniai įvertinti midijų *Mytilus edulis* žiaunų ląstelėse bei Atlanto menkių *Gadus morhua* inkstų eritrocituose. Tiriami organizmai surinkti trijose Šiaurės Atlanto vietose, aliuminio lydyklos (Mosjøen, Norvegija) taršos gradiente. Aukščiausi MB, BP, FA ir DB ląstelių dažniai nustatyti arčiausiai aliuminio lydyklos, labiausiai užterštos Halsøy tyrimų vietos moliuskuose. Palyginus su kontroline tyrimų vieta, Halsøy midijose nustatytas 5,2 karto (P < 0,0001) aukštesnis mikrobranduolių, 3,4 karto (P = 0,0001) didesnis fragmentuotų-apoptozinių ląstelių, 1,8 karto (P = 0,0002) didesnis branduolio pumpurų bei 1,7 karto (P = 0,0524) didesnis dvibranduolių ląstelių dažnis. Mažiau užterštos nei Halsøy, Hundålbukta tyrimų vietos midijų ląstelėse MB ir FA dažniai patikimai (atitinkamai P = 0,0052 ir P = 0,0003) skyrėsi nuo nustatytųjų kontrolinės vietos moliuskuose. Halsøy ir Hundålbukta menkių inkstų eritrocituose patikimai skyrėsi MB (P = 0,0087) ir BP (P = 0,0186) dažniai.

Raktažodžiai: genotoksiškumas, citotoksiškumas, mikrobranduoliai, moliuskai, menkės, Šiaurės Atlantas, aliuminio pramonė