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Cytogenetic damage in perch (*Perca fluviatilis* L.) and Duck mussel (*Anodonta anatina* L.) exposed to crude oil

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This study presents the data on the frequency of micronuclei, nuclear buds and fragmented cells in gill cells of freshwater bivalve molluscs (*Anodonta anatina* L.) and in blood erythrocytes of perch (*Perca fluviatilis* L.) exposed to crude oil. Bivalves and fish were exposed for ten days to 0.25, 0.5 and 1.0 ppm of crude oil from the Minija oil-well (Lithuania). In molluscs, a statistically significant increase of micronuclei was found after treatment with 0.25 (P = 0.0260) and 0.5 ppm of oil (P = 0.0022), in perch – after exposure to 0.5 ppm of oil (P = 0.0057). Exposure to 1 ppm of crude oil significantly elevated the frequency of bi-nucleated cells in mussels (P = 0.0411) and fragmented cells in perch (P = 0.0133).

Key words: micronuclei, nuclear abnormalities, genotoxicity, crude oil, perch, bivalve molluscs

INTRODUCTION

Thousands of chemical compounds have been released into aquatic ecosystems and can cause hazardous effects in marine and freshwater organisms. These substances (*e.g.*, heavy metals, oil products, chlorinated pesticides, halogenated aromatic hydrocarbons) have the ability to accumulate in water organisms (Woodhead et al., 1999). Oil products are among pollutants widely distributed in the hydrosphere, and hydrocarbons are a well known primary source of persistent toxicity in aquatic environment.

Hazardous effects of different PAHs arise mainly as a result of oxidative biotransformation producing highly DNA-reactive metabolites. These metabolites are recognized as carcinogenic and mutagenic compounds (Torres-Bugarin et al., 1998; Woodhead et al., 1999; Maria et al., 2002). The mechanisms of PAH metabolic transformation have been studied and the genotoxic potency of metabolites was confirmed in the European eel *Anquilla anquilla* (Pacheco, Santos, 1997, 2002; Maria et al., 2002a, 2002b; Teles et al., 2003). The genotoxicity of benzo[*a*]pyrene has been shown in some freshwater fish species in laboratory experiments (Al-Sabti, 1986; Metcalfe, 1988; Venier et al., 1997b). Some studies described an increase in environmental genotoxicity in oil spill zones (Parry et al., 1997; Harvey et al., 1999; Pietrapiana et al., 2002; Baršienė et al., 2004).

However, little attention has been paid to genotoxic effects in invertebrate aquatic organisms inhabiting contaminated environments. Invertebrates present over 90% of the species in aquatic communities and have a particularly important role in the ecosystem function (Dixon et al., 2002). Molluscs have been widely used as indicator organisms, because they are ubiquitous. Furthermore, they have highly conserved control and regulatory pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs. Comparatively high bioaccumulation factors for organic pollutants, relatively low metabolic detoxification rates and a sessile filter-feeding life style allow using the bivalves as sensitive organisms in biomonitoring studies (Depledge, 1994; Bolognesi, 2004).

The micronucleus (MN) test, based on the presence of micronuclei that occurs in actively dividing cell populations, has served as an index of cytogenetic damage for over 30 years (Ayllon, Garcia-Vazquez, 2000, 2001). It has been assessed in fish and molluscs as a biological indicator of pollution *in situ*, and also for genotoxicity evaluation of physical and chemical agents after direct or indirect exposure *in vivo* (Metcalfe, 1988; Bahari et al., 1994; Al-Sabti, Metcalfe, 1995; Ayllon et

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al., 2000; Ayllon, Garcia-Vazquez, 2001; Farah et al., 2003; Rodriguez-Cea et al., 2003). Micronuclei are produced in all cell types after irregular division process 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 main objective of this study was to evaluate the micronuclei formation in gill cells of freshwater bivalve molluscs and in blood erythrocytes of perch after treatment with different concentrations of crude oil. In order to assess the potential genotoxicity in the Minija oil drilling site, the native fish and mollusc species were used.

MATERIAL AND METHODS

An experimental group of 40 perch, three years old, 8–10 cm in length, reared under laboratory conditions was used. Forty specimens of the Duck mussel were sampled from the Verkiai water body, which according to previous studies (Baršienė, 1994) is characterized as comparatively unpolluted. Bivalves of similar sizes were subdivided into experimental groups (Table 1).

After a 3-day acclimation (molluscs) a 10-day experimental treatment was performed under laboratory conditions. The experimental groups of organisms were treated with 0.25, 0.5 or 1.0 ppm of crude oil. Oil from the Minija oil-well (western Lithuania) was used. Every day, water was changed and an adequate concentration of crude oil was added, the main hydrochemical parameters were measured. The content of dissolved oxygen, water pH and temperature were stable during the experimental time (Table 2).

At the end of exposure, a drop of blood from perch caudal vessels was directly smeared on slides. The slides were air-dried, fixed in methanol for 10 min and stained with 5% Giemsa solution in a phosphate buffer (pH = 6.8). The frequency of micronuclei, nuclear buds and fragmented cells was evaluated by scoring at a 1.250× magnification (Olympus BX51) of 5.000 mononucleated intact erythrocytes in each specimen.

In mussels, a gill cell suspension was prepared in a drop of 3:1 ethanol acetic acid and smeared on slides. The slides were air-dried, fixed in methanol for 10 min and stained with 4% of Giemsa (for 30–50 min) in phosphate buffer (pH 6.8), then rinsed with water and dried. The stained slides were analysed under a light microscope (Olympus BX51), and at a final magnification of 1.000 × 2.000 cells with intact cytoplasm were scored in each specimen of mussels.

Micronuclei were identified according to the following criteria: (1) spherical or ovoid-shaped extranuclear bodies in the cytoplasm, (2) a diameter of 1/3–1/20 (mussels) or 1/3–1/50 (fish) of the main nucleus, (3) non-refractory bodies, (4) colour, texture and optical features resembling those of the nucleus, and (5) the bodies are completely separated from the main nucleus (Fig. 1). Nuclear buds and fragmented cells were assessed following criteria presented in the Fenech et al.,

Table 1. **Morphological characteristics of Duck mussels**

Oil concentration/ Morphological characteristics	Length, cm	High, cm	Weight, g	Shell weight, g
Control	7.63 ± 0.34	4.37 ± 0.28	25.7 ± 4.12	4.2 ± 0.61
0.25 ppm	7.22 ± 0.58	4.2 ± 0.35	20.83 ± 4.48	3.46 ± 0.73
0.5 ppm	7.73 ± 0.48	4.44 ± 0.21	25.65 ± 5.67	4.28 ± 0.78
1 ppm	6.91 ± 1.02	4.16 ± 0.25	20.74 ± 3.9	3.51 ± 0.66

Table 2. **Hydrochemical parameters of the water in the experimental tanks**

Treatment / Date Parameters	22.07.2003			23.07.2003			24.07.2003			25.07.2003		
	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C
1.0 ppm	7.7	8.0	21.4	7.5	8.2	23.1	7.4	8.0	22.1	8.3	8.2	22,1
0.5 ppm	7.7	8.1	21.6	7.3	8.2	23.0	7.8	8.2	22.5	8.5	8.1	22.5
0.25 ppm	8.0	8.2	21.3	7.8	8.1	22.7	7.7	8.2	22.2	7.8	8.2	22.4
Control	8.2	8.1	21.1	7.9	8.1	22.6	7.7	8.1	22.0	7.8	8.1	22.0
Treatment / Date Parameters	28.07.2003			29.07.2003			30.07.2003			31.07.2003		
	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C	O_2, mg/l	pH	T, °C
1.0 ppm	7.6	8.2	22.3	7.6	8.2	23.5	7.4	8.1	23.8	7.4	8.1	23.8
0.5 ppm	7.0	8.1	22.5	7.7	8.2	23.4	7.6	8.1	23.4	7.6	8.1	23.4
0.25 ppm	7.5	8.1	22.8	7.7	8.2	23.1	7.7	8.2	23.0	7.7	8.2	23.0
Control	7.8	8.1	23.0	7.5	8.1	23.0	7.7	8.2	22.8	7.7	8.2	22.8

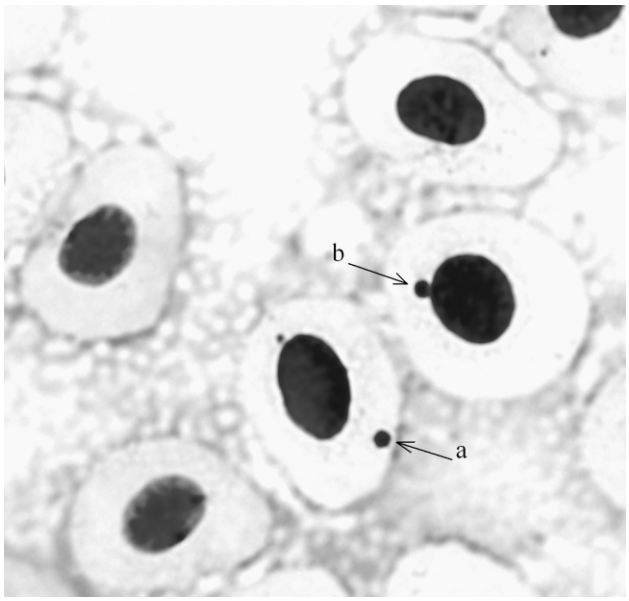

Fig. 1. Nuclear abnormalities in perch blood erythrocytes: micronucleus (*a*), nuclear bud (*b*)

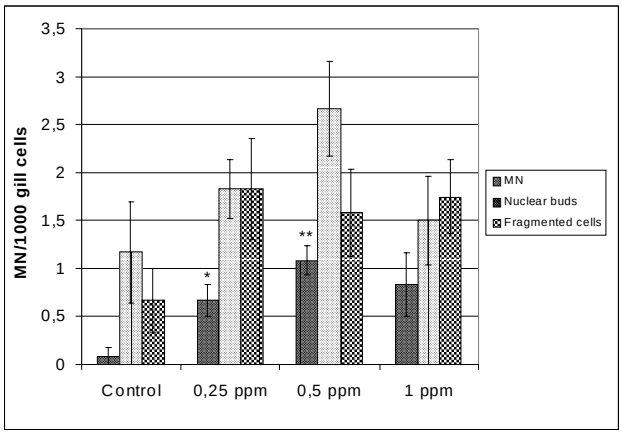

Fig. 2. Frequency of nuclear abnormalities in gill cells of Duck mussel. Asterisks show statistically significant differences compared to control group (one asterisk – P is at 0.01, two asterisks – P at 0.001 levels; Mann–Whitney U-test)

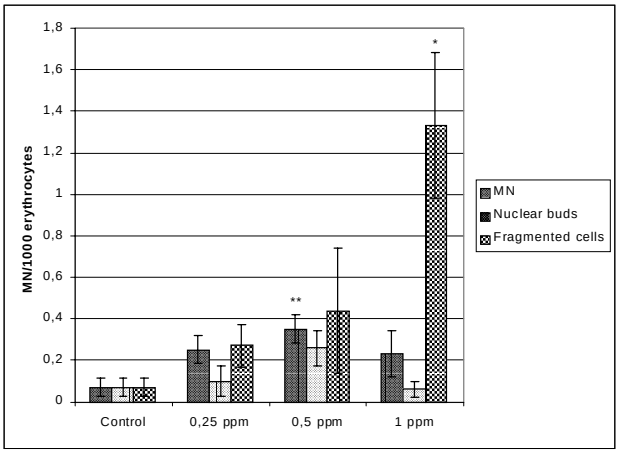

Fig. 3. The frequencies of nuclear abnormalities in perch blood erythrocytes. Asterisks show statistically significant differences compared to control group (one asterisk – P is at 0.01, two asterisks – P at 0.001 levels; Mann–Whitney U-test)

study (2003) and in our previous studies (Baršienė et al., 2005, 2006).

The frequencies of MN, nuclei buds, bi-nucleated and fragmented cells were expressed per 1.000 cells studied. The data were analyzed for statistical significance using the one-way analysis of variance (ANOVA). Differences between the control and experimental groups were evaluated by the non-parametric Mann–Whitney U-test. All the data were analyzed using the PRISM statistical software package. The level of significance was taken as $P < 0.05$.

RESULTS

Anodonta anatina L.

The frequency of micronuclei ranged from 0.08 MN/1000 gill cells (‰) in the control group to 1.08 MN/1000 gill cells of specimens treated with 0.5 ppm of crude oil. Lower response was observed in duck mussels exposed to 1 ppm (0.83 MN/1000 gill cells) or to 0.25 ppm of crude oil (0.67 MN/1000 gill cells) (Fig. 2). The incidence of bi-nucleated cells varied from 0.67‰ (control group) to 1.75‰ in mussels exposed to 1 ppm of oil. The highest induction of nuclear buds was observed in mussels treated with 0.5 ppm of oil. The level of fragmented gill cells in control mussels was equal to 0.67‰ and in treated molluscs 1.58–1.83‰ (Fig. 2).

The non-parametric Mann–Whitney U-test showed the largest differences in MN frequency between mussels from the control group and those exposed to 0.5 ppm of oil ($P = 0.0022$), or to 0.25 ppm of oil ($P = 0.0260$). Statistically significant differences were found in the induction of bi-nucleated cells in mussels from the control group compared to those treated with 1 ppm of oil ($P = 0.0411$). Experimental treatment did not show any significant induction of nuclear buds (P values varied in a range from 0.0931 to 0.4848). One-way analysis of variance (ANOVA) indicated significant differences in micronuclei induction ($P = 0.0176$; $F = 4.2620$; $R = 0.3900$).

Perca fluviatilis L.

The control group of perch showed the lowest value (0.07‰) of MN, nuclear bud and fragmented erythrocyte incidences. In treated fish groups, the frequency of micronuclei varied from 0.23 to 0.35 MN/1000 erythrocytes. Perch treated with 0.5 ppm of oil showed the highest induction of micronuclei and nuclear buds. The level of fragmented erythrocytes was highest after exposure to 1 ppm of oil (Fig. 3).

The observed levels of micronuclei in perch exposed to 0.5 ppm of oil were statistically significantly (Mann–Whitney U-test) different from the control group ($P = 0.0057$). Treatment with the highest (1.0 ppm) or with 0.25 ppm concentration of oil did not induced a significant elevation of micronuclei ($P = 0.4470$ and $P = 0.0653$). A significant increase of fragmented

erythrocytes was found after exposure to 1 ppm of oil (P = 0.0133). Nuclear buds were not significantly induced after the treatment with any of the used oil concentration (P values ranged from 0.1051 to 0.9705).

A comparison of induced genotoxicity levels in mussel and perch showed a higher elevation of cytogenetic damage in molluscs. After exposure to different concentrations of oil, the frequency of micronuclei in mussels was approximately 3-fold and the incidence of nuclear buds 10–15-fold higher than in fish (Figs. 2 and 3).

DISCUSSION

The results of this study revealed that after exposure to 0.25, 0.5 and 1.0 ppm of crude oil, the induction of nuclear abnormalities (MN and nuclear buds) was 3–15-fold higher in the filter-feeding Duck mussel (*Anodonta anatina* L.) than in the pelagic predator perch (*Perca fluviatilis* L.). Nuclear abnormalities (micronuclei, nuclear buds, fragmented and bi-nucleated cells) were analyzed in gill cells of molluscs, which were in direct contact with crude oil dispersed in water. Gills, as a respiratory and filtration organ, present the first barrier of contaminant intake and often have been used as a target tissue in biomonitoring studies (Baršienė et al., 1999; Baršienė, Bučinskienė, 2001; Bolognesi et al., 2004). In perch, the frequencies of nuclear abnormalities (micronuclei, nuclear buds and fragmented cells) were detected in blood erythrocytes. It is known that the frequency of micronuclei largely depends on the type of tissue analyzed (Hayashi et al., 1998). Since formation of micronuclei and other nuclear abnormalities take place in proliferating cells, this end-point in erythrocytes as non-proliferating cells is lower. Therefore, inter-tissue and interspecific differences in the induction of nuclear abnormalities by oil products were shown.

A higher level of micronuclei compared to fish was also observed in blue mussel from Lithuanian coastal areas of the Baltic Sea and from the North Sea (Baršienė et al., 2004) and exposed to crude oil from the North Sea (Baršienė et al., unpublished data). Petroleum hydrocarbons in the environment are known as DNA-damaging agents in filter-feeding bivalve populations (Hamoutene et al., 2002). A high level of bioaccumulation of water-soluble alkylated PAHs has been described in inter-tidal mussels inhabiting Halifax Harbors (Hellou et al., 2005), Venice Lagoon (Wetzel, Van Vleet, 2004). Significant elevation of micronuclei level in mussels after 30 days post-oil spill and persistence of the cytogenetic damage up to 100 days later have been described (Parry et al., 1997). Micronucleated cells were on the increase in the gills or hemolymph of marine molluscs treated with benzo[*a*]pyrene (Burgeot et al., 1995; Venier et al., 1997a; Siu et al., 2004), dimethylbenzol[*a*]anthracene (Bolognesi et al., 1996). The results of the Comet and MN assays clearly showed dose- and time-dependent responses to benzo[*a*]pyrene exposure in bivalve *Perna viridis* (Siu et al., 2004).

More frequent cytogenetic damage has been described in molluscs inhabiting the marine port and oil terminal areas in the Baltic Sea (Baršienė, Baršytė-Lovejoy, 2000; Baršienė, 2002), in French Mediterranean coast at Fos-sur Mer, affected by the oil-refinery industry (Burgeot et al., 1996). Elevated levels of the micronuclei were detected in the blue mussels eight months after oil spill from the Būtingė oil terminal in the Baltic Sea (Baršienė et al., 2004). A higher frequency of DNA adducts in marine organisms were observed 12–17 months after the Sea Empress oil spill (Harvey et al., 1999).

The presence of anaphase aberrations in fish embryos correlated with the concentrations of PAHs in the oil trajectory following the Exxon Valdez oil spill in Prince William Sound in March 1989 (Hose, Brown, 1998). More frequent chromosomal aberrations and malformations have been observed in cod (*Gadus morhua*) and pollock (*Pollachius virens*) embryos from the oil spill area (Longwell, 1977). Elevated levels of micronuclei were detected in flounder (*Platychthys flesus*) eight months after oil spill from the Būtingė oil terminal in the Baltic Sea (Baršienė et al., 2004). Higher frequency of DNA adducts has been described in fish 12–17 months after the Sea Empress oil spill (Harvey et al., 1999).

The results of this study revealed the highest induction of micronuclei and nuclear buds in mussels and perch after a 10-day treatment with 0.5 ppm of crude oil. The highest level of PAH concentrations in liver were observed in Atlantic cod 3 days after the start of exposure (Aas et al., 2000). It is known that oil contains potentially genotoxic components (Klekowski et al., 1994). Thus, the elevation of genotoxicity in mussels and perch after a 10-day exposure to crude oil is an obvious response to the genotoxic substances of crude oil. On the other hand, exposure to 1 ppm of crude oil showed a lower induction of genotoxicity (micronuclei and nuclear buds) in both organisms than the treatment with 0.5 ppm of oil. The analysis of chromosome aberrations and aneugenic effects in tissues of the same specimens showed a suppression of cell division in bivalves exposed to the highest concentration of crude oil (unpublished data); thus, the mitostatic effects caused by some of the components present in Lithuanian crude oil is evident. Crude oil is a complex mixture of various hydrocarbons, nitrogen-oxygen compounds and heavy metals. The content of components differs depending on the areas of oil drilling (Wake, 2005). The interaction of hazardous compounds in the mixtures of contaminants can change their genotoxic and toxic features (Ma et al., 1992). A detailed analytical study of Lithuanian crude oil content was not performed and it is unclear which compound contributes most to the mitostatic and genotoxic effect. Nevertheless, assessment of time-dose-dependent, tissue-species-specific peculiarities of oil genotoxicity in aquatic organisms should be further developed.

A dose-related increase in DNA damage has been studied in green-lipped mussels, *Perna viridis* exposed to 0.3, 3 and 30 μgl^{-1} benzo[*a*]pyrene (Ching et al., 2001). Treatment with 0.3 and 3 μgl^{-1} benzo[*a*]pyrene induced a significant increase in DNA strand breaks in mussel hepatopancreas after one day of exposure, followed by a gradual decrease in strand breaks after 3–6 days, and after 12 days the frequency of DNA strand breaks returned to the control level. Different kinds of genotoxic damage were observed after a 30 μgl^{-1} benzo[*a*]pyrene exposure. A significant increase in the damage was observed from day 12 to day 24 (Ching et al., 2001). Low concentrations of benzo[*a*]pyrene (0.5–100 µg/l) induce specific reproducible DNA adducts in the mussel *Mytilus galloprovincialis* gills (Venier, Canova, 1996). Consequently, the complex interactions between exposure doses and duration need to be verified.

Future research should focus on identifying both the short- and long-term consequences of genotoxicity in organisms exposed to crude oil compounds. An assessment of cytogenetic damage in early life stages and in mature organisms (including somatic and gonad cells) is desirable since the level of cytogenetic damage can vary depending on age, tissue, sex, season, temperature, oxygen factors (Brunetti et al., 1986).

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CITOGENETINIS ŽALIAVINĖS NAFTOS POVEIKIS EŠERIAMS (*PERCA FLUVIATILIS* L.) IR MOLIUSKAMS (*ANODONTA ANATINA* L.)

S a n t r a u k a

Darbe pateikiami duomenys apie žaliavinės naftos indukuotų mikrobranduolių (MB), branduolio pumpurų bei fragmentuojančių ląstelių dažnius gėlavandenių moliuskų (*Anodonta anatina* L.) žiaunų ląstelėse bei paprastojo ešerio (*Perca fluviatilis* L.) kraujo eritrocituose. Moliuskai ir žuvys 10 dienų buvo veikiami Minijos naftos gręžinio (Lietuva) 4, 8 ir 16 μg/l koncentracijos žaliavine nafta. Statistiškai patikimi mikrobranduolių dažnio pokyčiai nustatyti moliuskuose, paveiktuose 4 ir 8 μg/l koncentracijų (P = 0,0260 ir P = 0,0022); ešeriuose – paveiktuose 8 μg/l koncentracijos žaliavine nafta (P = 0,0057). 16 μg/l koncentracijos žaliavinė nafta indukavo dvibranduolių ląstelių susidarymą moliuskų žiaunų ląstelėse ir fragmentuojančių ląstelių susidarymą ešerio eritrocituose (skirtumai buvo statistiškai patikimi – P = 0,0411 bei P = 0,0133).

Raktažodžiai: mikrobranduoliai, genotoksiškumas, žaliavinė nafta, ešerys, dvigeldžiai moliuskai