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Induction of micronuclei and other nuclear abnormalities in blue mussels exposed to crude oil from the North Sea

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Micronuclei (MN), nuclear buds (NB), bi-nucleated (BN) and fragmented-apoptotic (FA) cells were investigated in blue mussels exposed for 3 weeks to crude oil processed in the Statfjord B oil platform (the North Sea, Norway). In one flow-through system, mussels from a shellfish farm were exposed to 0.5 ppm of crude oil, to 0.5 ppm oil spiked with a mixture of alkylphenols (Σ = 0.1 ppm) and to 30 ppb of nonylphenol. In the second experiment, wild mussels from the reference Forlandsfjorden site (Norway) were exposed to 0.5 ppm of crude oil and to 0.5 ppm crude oil spiked with a mixture of alkylphenols (Σ = 0.1 ppm) and 0.1 ppm of PAHs (the spike mixture was simulating the content of compounds found in water produced during oil drilling). Control groups of mussels were maintained in filtered seawater. In the first experiment (September–October 2002), a comparatively high level of genotoxicity and cytotoxicity was observed in the control group of mussels. Their gills were infected by microorganisms, which evidently increased approximately 3-fold the frequency of MN, FA and BN cells. Nonylphenol and 0.5 ppm of crude oil induced a similar level of nuclear alterations. In the second experiment (November–December 2002), treatment with 0.5 ppm of oil increased significantly the incidences of MN (P = 0.0288) and FA cells (P = 0.0115). The co-exposure to 0.5 ppm of oil spiked with PAHs and alkylphenols induced the highest level of MN (up to 3.58 MN/1000 gill cells; 2.8-fold increase versus the control level), NB (up to 1.50 MN/1000 gill cells; 4-fold increase versus the control level) and FA cells (up to 2.39 MN/1000 gill cells; 4-fold increase versus the control level) incidences.

Key words: micronuclei; nuclear abnormalities; crude oil; blue mussel

INTRODUCTION

Different types of bioassays have been widely utilized for assessing the genotoxic, mutagenic and cancerogenic potencies of a range of substances. Markers of genotoxic effects are at high priority due to reflection of damage to genetic material of organisms (Moore et al., 2004). Different methods have been developed for detection of both double- and single-strand breaks of DNA, DNA-adducts, micronuclei formation, and chromosome aberrations. The assessment of cytogenetic damage has been presented as a very important assay in identification of pollution effects in marine environments (Dixon et al., 2002).

Marine organisms can be exposed to mutagenic compounds via several routes. From the environmental perspective, oil pollution is a matter of great ecological concern. Hazardous effects of different polyaromatic hydrocarbons (PAHs) arise typically as a result of oxidative biotransformation producing highly DNA-reactive metabolites. These metabolites are recognized as carcinogenic and mutagenic compounds (Johnson, 1992; Torres-Bugarin et al., 1998; Woodhead et al., 1999).

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Oil spills can result in a wide distribution of petroleum hydrocarbons in marine environments, seriously impacting the DNA of filter-feeding bivalve populations (Hamoutene et al., 2002). The increase of genotoxicity in mussels from oil spill areas primarily depends on the action of water soluble components (Carls et al., 2001). High levels of bioaccumulation of water-soluble alkylated PAHs hava been described in inter-tidal mussels inhabiting Halifax Harbor (Hellou et al., 2005), Venice Lagoon (Wetzel, Van Vleet, 2004). There are studies describing the increase of environmental genotoxicity in zones affected by oil spill (Parry et al., 1997; Harvey et al., 1999; Baršienė et al., 2004, 2006a, 2006b). Significantly elevated levels of micronuclei in mussels 30 days post-oil spill and persistence of the cytogenetic damage up to 100 days (Parry et al., 1997) or even 7 months later (Baršienė et al., 2006a) have been described.

The main objective of the present study was to evaluate induction of micronuclei and other nuclear abnormalities (nuclear buds, bi-nucleated and fragmented-apoptotic cells) in gills of blue mussels treated with crude oil and a mixture of alkylphenols and PAHs processed from the Statfjord B oil platform (the North Sea, Norway). Model mixture of alkylphenols and different PAHs was used as contaminants usually appearing in

water produced as a by-product from offshore oil drilling platforms. About 400 million tons of produced water effluents, including approximately 8,000 tons of dispersed oil, are annually released from the Norwegian and UK oil platforms in the North Sea (Utvik, 1999). Therefore, laboratory-controlled assessment of genotoxic and cytotoxic effects caused by a complex mixture of crude oil and produced water effluents should provide new information on the validation of environmental risk estimates of discharges from offshore oil industry.

METHODS

Blue mussels

Blue mussels (*Mytilus edulis*) were collected from a shellfish farm and transported to the RF Akvamiljo experimental laboratory (Stavanger, Norway). Mussels for the second experimental exposure were collected from the uncontaminated habitat Forlandsfjorden, which had been used as a reference site in our earlier field investigations (Baršienė et al., 2004). After an intensive microorganism infection had been identified in gill cell smears prepared from control mussels (reared in farm), wild mussels were explored in the second exposure.

Experimental treatment with crude oil

In the first experiment (September–October 2002), 41 specimens of blue mussels were exposed for 3 weeks to: (a) 0.5 ppm of Statfjord B crude oil, (b) 0.5 ppm of the crude oil spiked with a mixture of alkylphenols (Σ = 0.1 ppm) and to 30 ppb of nonylphenol. Eleven specimens were used from spiked oil group and ten specimens from each of other groups.

In the second experiment (November–December 2002), wild mussels from the reference Forlandsfjorden site (Southern Norway) were exposed for 3 weeks to: (a) 0.5 ppm of the North Sea crude oil, (b) 0.5 ppm of the crude oil spiked with a mixture of alkylphenols (Σ = 0.1 ppm) and 0.1 ppm of PAHs mixture. The spike mixture was simulating the content and concentrations of compounds found in water produced during Statfjord B oil drilling. Nominal concentrations of spikes were 0.0915 μg/L for total PAH mixture and 0.1 μg/L for total alkylphenol mixture (Sundt et al., 2006). Ten specimens were used for the analysis from each experimental group. Control groups were maintained in the filtered seawater.

Preparation of slides

Two branches of mussel gills were placed in a big drop of 3:1 ethanol acetic acid (or methanol acetic acid) solution separately on two clean microscopic slides and gently nipped with tweezers for 2–3 minutes (until cells spread within a drop). Then the cell suspension was softly smeared on the whole surface (except label place) of both slides. Dried slides were fixed in methanol for 10 min. and stained with 5% Giemsa solution in phosphate buffer pH = 6.8. The stained slides were analyzed under the light microscope Olympus BX51 at a final magnification of 1000×.

Analysis of micronuclei and other nuclear abnormalities

To minimize technical variation, blind scoring of micronuclei and other nuclear abnormalities was performed on coded slides without knowing the origin of samples. Only the cells with intact cellular and nuclear membrane were scored. Round or ovoid-shaped non-refractory particles with colour and structure similar to chromatin, with a diameter 1/3–1/20 of the main nucleus and clearly detached from it were interpreted as micronuclei (Fig. 1). In general, colour intensity of MN should be the same or less than that of the main nuclei. Particles with colour intensity higher in comparison with the main nuclei were not counted as MN. Nuclear buds, bi-nucleated and fragmented-apoptotic cells were identified following the criteria described by M. Fenech with co-authors (2003). For each studied specimen of mussels, 2000 cells with intact cytoplasm were scored (Baršienė et al., 2004). The frequency of micronuclei and other nuclear abnormalities was evaluated as the number of injuries per 1000 cells scored.

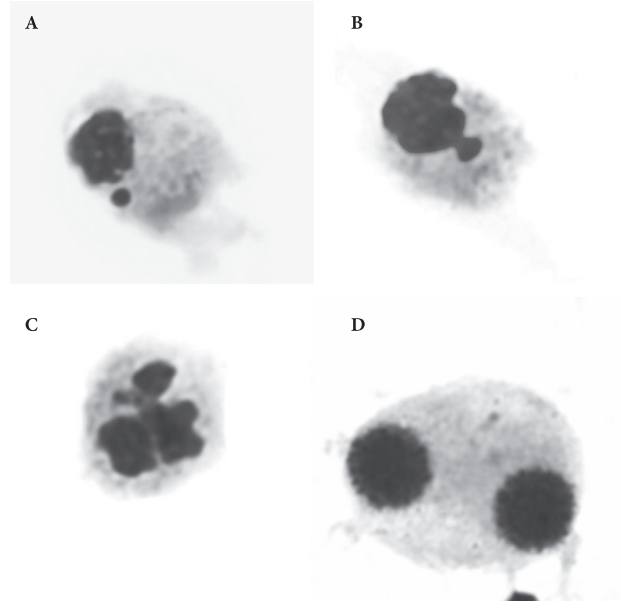

Fig. 1. Mussel gill cells with micronuclei (*a*), nuclear bud (*b*), fragmented-apoptotic cell (*c*) and bi-nucleated cell (*d*)

Statistical analysis was carried out using PRISM statistical package. Mean and standard error was calculated for each experimental group. Non-parametric Mann-Whitney U-test was used to compare alteration frequencies between the control and treatment groups.

RESULTS

Induction of MN

The MN frequencies of the control and treated mussel groups from the first experiment remained comparatively high (ranging from 2.40 to 3.58 MN/1000 cells) during the entire 3 weeks of exposure, and were statistically similar ($P > 0.05$). The highest frequency of MN (3.58 MN/1000 cells) was registered in molluscs from the spiked oil treatment. Nevertheless, as seen in the study results, there were significant spike-dependent differences (P = 0.0346) between 0.5 ppm crude oil and 0.5 ppm spiked oil treatment groups. Very similar values of MN were detected in mussels treated with 30 ppb of nonylphenol and 0.5 ppm of crude oil (Fig. 2). The control group in the first experiment showed 3.7-fold higher response (3.15 MN/1000 cells)

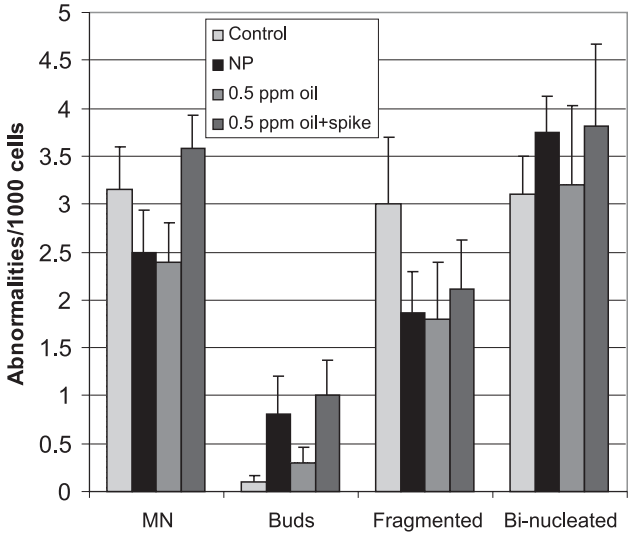

Fig. 2. *The first exposure experiment.* Frequency of micronuclei (MN), nuclear buds (Buds), fragmented-apoptotic (Fragmented) and bi-nucleated cells in mussel gills exposed to 0.5 ppm of crude oil, to 0.5 ppm of crude oil spiked with mixture of alkylphenols and treated with 30 ppb of nonylphenol

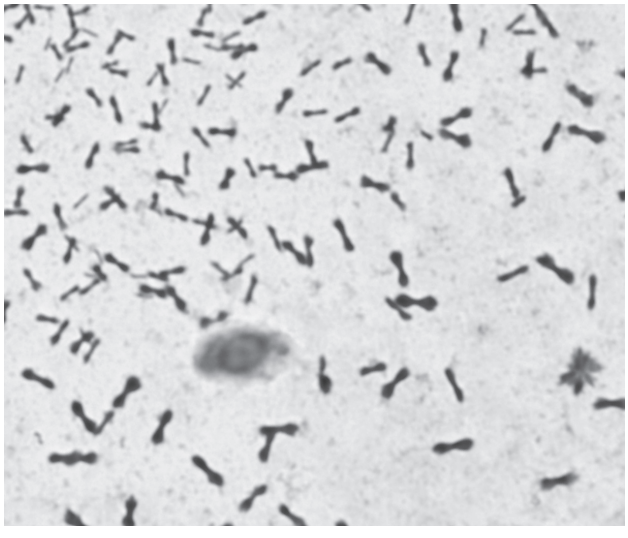

Fig. 3. *The first exposure experiment.* Microorganisms on the smears of mussel gill cells

compared to mussels from the control group of the second experiment. Their gill cell smears showed an extensive bacterial infection (Fig. 3).

In mussels from the second exposure experiment, the lowest mean of 0.85 MN/1000 cells was detected in the control group, and the highest MN frequency (2.39 MN/1000 cells) was recorded in mussels exposed to spiked oil. After the treatment with 0.5 ppm of crude oil, the level of micronuclei was elevated up to 1.91 MN/1000 cells (Fig. 4). The non-parametric Mann-Whitney U-test showed significant differences between the control and exposed groups of mussels (Table).

Induction of other nuclear abnormalities

Analysis of samples collected during the first experiment showed comparatively high levels of fragmented-apoptotic and bi-nucleated cells and low frequency of nuclear buds in mussels from the control group. Spiked oil induced greater incidences of these cells than exposure to 0.5 ppm of crude oil (Fig. 2). However, elevation after the treatment was not at a statistically significant level compared to control.

In the second experiment, control level of nuclear buds was 0.37 buds/1000 cells. After exposure to 0.5 ppm of oil, the parameter was 3-fold and after exposure to spiked oil 4-fold greater than in mussels from the control group (Fig. 4). A statistically significant increase of nuclear buds was found after the treatment with spiked oil. A significant elevation of fragmented-apoptotic cells was observed in both crude oil treatments (Table).

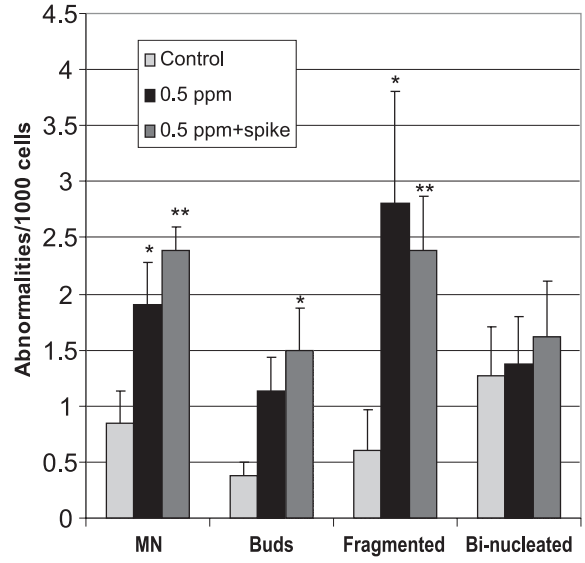

Fig. 4. *The second exposure experiment.* Frequency of micronuclei (MN), nuclear buds (Buds), fragmented-apoptotic (Fragmented) and bi-nucleated cells in mussel gills exposed to 0.5 ppm of crude oil and to 0.5 ppm of crude oil spiked with mixture of alkylphenols and PAHs. Differences between the control and exposed groups shown: one asterisk at level $P < 0.05$, two asterisks – $P < 0.001$

Table. **The levels of statistical significance (P values; Mann-Whitney U-test) between frequencies of MN and other nuclear abnormalities in gill cells of the control mussels and after exposure to dispersed crude oil (the second experiment)**

Nuclear abnormalities / exposure	0.5 ppm crude oil	0.5 ppm crude oil + spike
Micronuclei	0.0288	0.0015
Nuclear buds	0.0630	0.0232
Fragmented-apoptotic cells	0.0115	0.0029
Bi-nucleated cells	0.6305	0.6381

DISCUSSION

The blue mussel, cosmopolitan bivalve, was used as a target species to perform genotoxicity and cytotoxicity tests for crude oil from the North Sea. The results of the study showed a causative role for 0.5 ppm of crude oil from the Statfjord B platform to increase alterations following a 3-week exposure. Several methods were developed and used for the detection of the DNA damage in the mussel and reviewed as reliable tools in identification of a potential genetic hazard of xenobiotics (Dixon et al., 2002). Induction of nuclear buds, bi-nucleated and apoptotic cells has also been considered as a reliable approach in assessing genotoxic effects of certain compounds in

molluscs (Venier et al., 1997; Dailianis et al., 2003; Baršienė et al., 2006a, 2006c, 2006g).

Comparison of responses between mussels reared in a shellfish farm (the first exposure experiment) and wild ones (second exposure experiment) to 0.5 ppm and 0.5 ppm of spiked crude oil treatment did not reveal any significant differences. However, three times higher frequencies of MN, bi-nucleated and fragmented-apoptotic cells were recorded in the control group of the first exposure than in the control group of the second one. Since extensive microorganism (possibly bacterial / fungal) infection appeared in the reared mussel gills, increased levels of MN, bi-nucleated and fragmented-apoptotic cells were evidently provoked by the infective agents. Similar pattern was observed in the infected mussel specimens from comparatively uncontaminated locations of the North Sea (our unpublished data). Consequently, the finding suggests that infective agents could influence the level of analyzed alterations in marine organisms, even in those inhabiting uncontaminated sites. On the other hand, since the infected specimens appeared mainly in the control group, there is a presumption about the capability of crude oil to eliminate bacterial infection from the mussel gills.

In the second experimental exposure, statistically significant induction of MN and fragmented-apoptotic cells were registered in mussels treated with 0.5 ppm of crude oil and to spiked 0.5 ppm of the oil. A significant increase of nuclear buds was observed after exposure to spiked 0.5 ppm of crude oil. Therefore, the present study revealed existence of genotoxic and cytotoxic compounds in the crude oil processed at the Statfjord B platform in the North Sea. Moreover, induction of micronuclei significantly differed between the groups treated with 0.5 ppm of oil and with 0.5 ppm of spiked oil. Consequently, it shows a spike-dependent increase of genotoxicity in mussel gills related to mixtures of alkylphenols and PAHs usually appearing in the produced waters, a by-product from offshore oil platforms.

Phenolic molecules are widely distributed in the environment and can show genotoxic influence, however, the mechanism of their genotoxicity is not completely understood. Their potency in disruption of the cytoplasmic microtubules complex, mitotic spindle and induction of micronuclei has been demonstrated in Chinese hamster cells *in vitro*. Interestingly, all the induced MN possessed centromeric regions and were derived after aneuploidogenic metaphase arrest (Pfeiffer et al., 1997). A dose-response relationship between micronuclei frequency and concentration of pentachlorophenol has been detected in fish *Channa punctatus* after exposure of 72 and 96 hours (Farah et al., 2003). The highest incidence of micronuclei induced by pentachlorophenol (0.1, 0.2, 0.3 and 0.4 ppm) was recorded in fish *Heteropneustes fossilis* after 4-day exposure (Ahmad et al., 2002). High induction of micronuclei and other nuclear abnormalities were shown in mature erythrocytes of peripheral blood and immature erythrocytes of cephalic kidney in turbot (*Scophthalmus maximus*) and in the Atlantic cod exposed to 0.5 ppm of spiked crude oil from the Statfjord B platform (Baršienė et al., 2006d).

Casini et al. (2006) have developed the multi-biomarker approach to assess the toxicity of produced water (PW) from the Mediterranean "Aquila" oil field in the off-shore zone of the South Adriatic Sea. Elevated micronuclei induction was shown in peripheral blood of mosquitofish females exposed to PW before conventional treatment. Increased accumulation of carcinogenic PAHs (benzo(*a*)pyrene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, chryzene, benzo(*a*)anthracene, dibenzo-(*ah*)anthracene, indeno(1,2,3*cd*)pyrene) and naphthalene-type PAH bile metabolites was detected (Casini et al., 2006).

A time-related potency of naphtalenes to induce micronuclei in liver has been observed in juvenile *Dicentrarchus labrax* (Gravato, Santos, 2002). In eel (*Anguilla anguilla)*, treatment with 0.3, 0.9 and 2.7 μM of naphtalene resulted in the induction of micronuclei and other nuclear abnormalities (Teles et al., 2003).

Genotoxic effects of benzo(a)pyrene and dimethylbenz(a)antracene were earlier shown in the gills and hemolymph of marine molluscs (Burgeot et al., 1995; Bolognesi et al., 1996; Venier et al., 1997; Siu et al., 2004). Comet and MN assays presented clear dose- and time-dependent responses to benzo(a)pyrene exposure in *Mytilidae* bivalve *Perna viridis* (Siu et al., 2004). Cytogenetic damage was more frequently described in molluscs inhabiting marine port and oil terminal areas in the Baltic Sea (Baršienė, Baršytė-Lovejoy, 2000; Baršienė, 2002). Elevated levels of micronuclei incidences were detected in blue mussels in 8 months after oil spill from the Būtingė oil terminal in the Baltic Sea (Baršienė et al., 2004, 2006a, 2006b).

On the other hand, useful information could also be gained from the application of MN assay together with detection of other nuclear abnormalities. Genotoxic effects of environmental contaminants in organisms could be removed through the DNA repair, or other mechanisms. Elimination of cytogenetic damage through the apoptosis and necrosis is a key process, which occurs at different rates in various organisms (Mičic et al., 2002).

This study demonstrated that after exposure of mussels to crude oil the induction of fragmented-apoptotic cells and nuclear buds was also observed in mussel gills. Compared to other organisms, there were limited investigations of nuclear morphological abnormalities, like nuclear buds or bi-nucleated cells in mussels (Venier et al., 1997; Dolcetti, Venier, 2002; Dailianis et al., 2003; Izquierdo et al., 2003). Apoptotic cells in mussels *Mytilus galloprovincialis* have been investigated after treatment with TBT and other marine contaminants (Batel et al., 1993; Mičic et al., 2001, 2002), with cadmium (Pruski, Dixon, 2002). After TBT injection, apoptotic cells were identified in gills of *M. galloprovincialis* using TUNEL and agarose gel techniques (Mičic et al., 2002). Occurrence of apoptotic cells was shown in hemolymph of blue mussels after deployment to contaminated sites (Steinert et al., 1998), or inhabiting Adriatic coastal areas (Bihari et al., 2003). Rapid appearance of apoptotic cells in mussels was attributed to environmental exposure (Steinert, 1996).

The extensive development of oil industry in marine environment requires development of environmentally acceptable endpoints for an integrated approach to estimate the ecological risk of oil contamination in marine ecosystems. However, there is lack of validated tests, simple, cost-effective screening methods which can be applied effectively, taking into consideration environmentally realistic routes of contamination and presence of genotoxins in oil platform areas. In the present study, genotoxic and cytotoxic activity of crude oil and mixture of crude oil with produced water

components in mussels, cosmopolitan and ecologically relevant organisms, was demonstrated. Very similar responses were found out in parallel exposure of the Atlantic cod and turbot, which also are target species, considering oil and produced water spills in the oil platform areas (Baršienė et al., 2006d). An increase of genotoxicity and cytotoxicity was observed after caging of mussels and the Atlantic cod in surroundings of oil platforms in the North Sea (our unpublished data). Therefore, the approach developed and information generated should also help to fill in our knowledge gaps in understanding the ecological significance of crude oil pollution in marine ecosystems. A substantial progress in the area should be achieved using integrated approach for the assessment of genetic damage induction-repair system. A comprehensive assessment of chromosomal aberrations, induction of micronuclei, apoptosis, DNA repair in target species should be developed in future (Jha et al., 2004). Multi-species laboratory-controlled experiments, assessment of damage in various tissues (including gonads) of caged and indigenous species will uncover the pattern of early responses as well as short- or long-term adaptations to chronic pollution from petroleum industry.

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ŠIAURĖS JŪROS NAFTOS ĮTAKA MIKROBRANDUOLIŲ BEI KITŲ BRANDUOLIO PAŽAIDŲ SUSIFORMAVIMUI MIDIJOSE

S a n t r a u k a

Midijos (*Mytilus edulis*) tris savaites buvo veikiamos žaliavine Statfjordo B platformos nafta (Šiaurės jūra, Norvegija). Akvakultūroje užauginti moliuskai buvo veikiami 0,5 ppm žaliavine nafta, 0,5 ppm žaliavinės naftos ir alkylfenolių mišiniu (Σ = 0,1 ppm) bei 30 ppb nonylfenoliu. Antro eksperimento metu moliuskai iš sąlyginai švarios vietos (Forlansfjorden, Norvegija) buvo veikiami 0,5 ppm žaliavinės naftos; 0,5 ppm žaliavinės naftos ir alkylfenolių (Σ = 0,1 ppm) bei 0,1 ppm poliaromatinių angliavandenilių (PAA) mišiniu. Mišinio sudėtis pasirinkta atsižvelgiant į alkylfenolių ir PAA koncentracijas šio naftos grę-

žinio produkuojamuose vandenyse. Pirmo eksperimento kontrolinėje grupėje buvo dažni genotoksiniai ir citotoksiniai efektai, kurie, matyt, atsirado dėl moliuskų žiaunose gyvenusių mikroorganizmų poveikio. Panašūs genotoksinių ir citotoksinių efektų dažniai buvo aptikti midijas paveikus nonylfenoliu bei 0,5 ppm žaliavine nafta.

Antro eksperimento metu (2002 m. lapkritį–gruodį) moliuskuose po poveikio 0,5 ppm žaliavine nafta padidėjo mikrobranduolių ir fragmentuotų-apototinių ląstelių dažniai ($P = 0,0288$ ir $P = 0,0115$). Paveikus moliuskus 0,5 ppm žaliavinės naftos bei PAA ir alkylfenolių mišiniu buvo registruotas didžiausias mikrobranduolių (MB) dažnis (3,58 MB/1000 ląstelių), t. y. 2,8 karto didesnis negu kontrolinėje moliuskų grupėje. Taip pat šioje eksperimentinėje grupėje aptikti dideli branduolio pumpurų (1,5 MB/1000 ląstelių) bei fragmentuotų-apoptotinių (2,39 MB/1000) ląstelių dažniai.