Polish J. of Environ. Stud. Vol. 17, No. 5 (2008), 765-771

Original Research

Seasonal Variability in Micronuclei Induction in Haemocytes of Mussels along the Eastern Adriatic Coast

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Received: 12 December, 2007
Accepted: 3 June, 2008

Abstract

The frequency of micronuclei (MN) was determined in haemocytes of native mussels, *Mytilus galloprovincialis*, collected at five sites along the Eastern Adriatic coast in Croatia, four times throughout the year to evaluate seasonal variability in connection with possible genotoxic pollution. Chosen sites were either near closed industrial facilities or in tourist resorts. Mussels from a mussel farm were used as control. Seasonal variations in MN frequency were not observed for the control mussels, but for other investigated sites clear seasonal variations were observed. The highest frequency of MN was observed in summer on all studied sites, elevated in comparison to the control, while the lowest frequency was detected in autumn except for one site. These results pointed out that seasonal changes are observed only at polluted sites, most probably caused by seasonality of pollution as well as by interaction between contaminants and higher metabolic and filtration rates in mussels, resulting in higher values of cytogenetic damage.

Keywords: micronucleus assay, haemocytes, mussels, genotoxicity, biomonitoring

Introduction

Pollution of the marine environment by industrial, agricultural and urban wastes is known to cause deleterious effects on organisms at different levels of their biological organization. Persistent pollution with genotoxic impact produces unpredictable long-term hazards and seriously damages the health of marine organisms affecting their physiology, reproductive status, population size and therefore, survival [1]. It is known that detection of hazardous chemicals in the environment by physico-chemical analyses does not provide enough information about their effects on living organisms and ecosystem. On the other hand, biomonitoring assays could effectively define health risk for the environment and man [2]. In the past few decades, different cellular and molecular biomarkers have been developed to detect an early sign of genotoxic effects in polluted environment [3-7]. The micronucleus assay is one of the biomarkers of effect used for identifying genetic changes in aquatic animals. Micronucleus (MN) is a small chromatin mass in the cytoplasm; it can be a consequence of clastogenic (chromosome break) or aneugenic (spindle failure) effect and it is visible after cell division therefore representing a delayed genotoxic effect. MN assay has been used successfully since the late 1980s for assessing genotoxic effect of pollutants in different aquatic organisms, marine [6-12] as well as freshwater [13-17]. In addition, it is used in environmental biomonitoring studies for

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detection of genotoxic pollution and its effect on DNA integrity in different aquatic organisms, including bivalves [1, 7, 8, 16, 18, 19].

Bivalves, particularly mussels of the genus *Mytilus*, are an ideal bioindicator species for biomonitoring studies because of their sessile and filter-feeding lifestyle, relatively low metabolic detoxification rates and ability to bioaccumulate many organic pollutants [20]. For MN assay, gill cells and haemocytes have been extensively used [5, 6, 8, 11]. Haemocytes have proven to be as sensitive, reliable and reproducible in MN assay as gill cells [15, 18, 21, 22]. Moreover, haemocytes can be collected more easily than gill cells, requiring no additional manipulation that can further damage DNA. In addition, the sampling does not require sacrificing the organisms, thus allowing repeatable tissue sampling of the same individuals.

Data on the relationships between seasonality, xenobiotic pollution and biomarkers of genotoxic effect in bivalves are of recent date [1, 6, 12, 17, 19, 23, 24]. Investigations of seasonal variability are of great importance in biomonitoring of genotoxic effects in order to avoid misinterpretation of obtained results.

In the present study we report on the results of MN assay on haemocytes of native mussels collected from different sites along the Eastern Adriatic coast in Croatia. Data on the quality of the marine environment and its impact on marine biota along the Adriatic coast in Croatia are scarce [25, 26]. Many coastal areas are under the influence of urban, industrial and harbour wastes. According to existing data on the coastal population size, seasonal influence of tourism and industrial activities several hot spots in the Adriatic Sea were identified [25, 26].

The MN frequency was analyzed in all four seasons of the year to evaluate seasonal variability of micronuclei induction in connection with possible genotoxic pollution of sea water. The aim of this research was to investigate whether seasonal changes can affect metabolic and filtration rates in mussels, and consequently their response to genotoxic pollution.

Material and Methods

Study Area

The Adriatic Sea is a temperate warm sea. Average surface monthly temperatures of the investigated coastal waters range from 11ºC (February) to 25ºC (August) with daily values in November and May of 17ºC and 17.5ºC, respectively (data from the State Meteorological and Hydrological Service; the city of Split).

Native Mediterranean mussels, *Mytilus galloprovincialis* Lamarck, were collected at five sites (Table 1, Fig. 1) along the coast in the Eastern Adriatic in Croatia, northwest and southeast from the city of Split. Split is the largest transportation, industrial and commercial centre of the Dalmatia region, a well-known tourist area.

The mussel farm Marina, situated on the western side of Trogir Bay, was used as the reference site (Fig. 1. site 5). Other monitoring sites were situated southeast from the city of Split, on the stretch Dugi Rat – Baška Voda (Fig. 1). For each site 10 individuals of the similar size (5.5-7.5 cm in length) were collected at 0.5 to 1 m depth.

The site Dugi Rat was situated near the ferroalloy factory that was shut down ten years ago (Fig. 1. site 1).

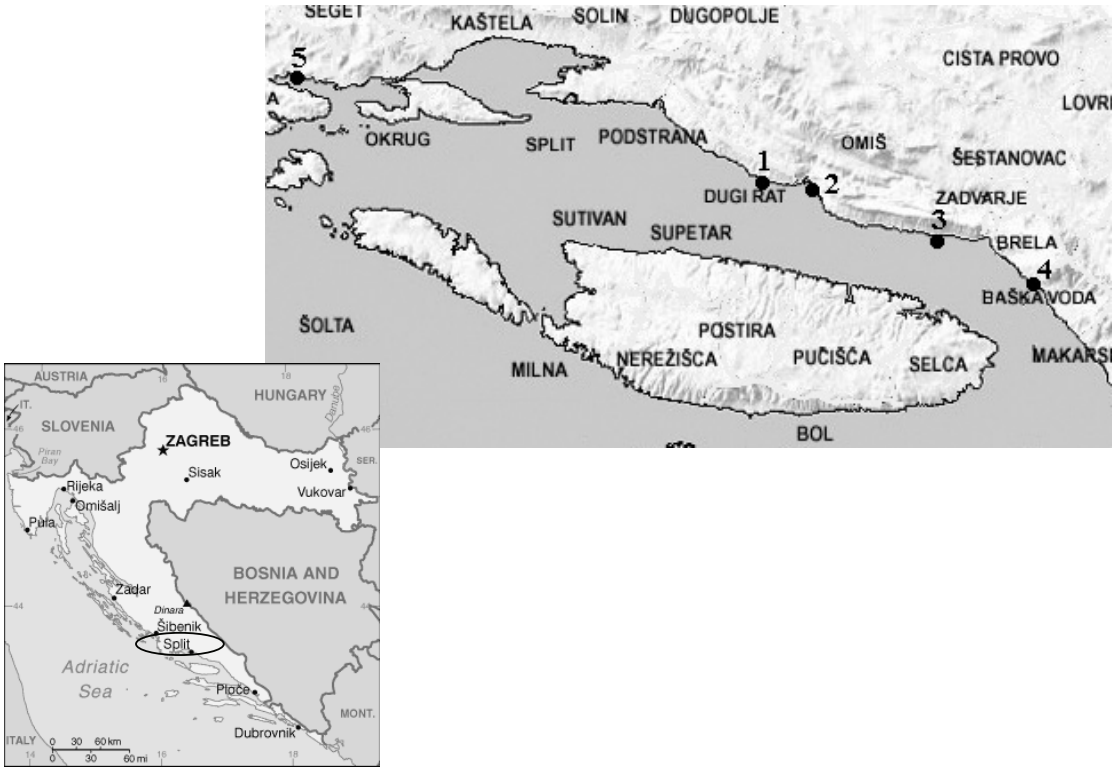

Fig. 1. Eastern Adriatic coast in Croatia with sampling sites: 1 Dugi Rat, 2 Ravnice, 3 Mimice, 4 Baška Voda, 5 Marina (control).

Table 1. Monitoring sites (numbers) and their description.

Monitoring site	Number	Site description
Dugi Rat	1	Ex-black metallurgic industry (ferroalloy factory); industrial and municipal wastes
Ravnice	2	Fishing settlement and port, in the vicinity of ex-cement plant, tourist centre; industrial, harbour and municipal wastes
Mimice	3	Tourist centre, fishing settlement and port; municipal and harbour wastewaters
Baška voda	4	Tourist centre; municipal wastewaters
Marina	5	Mussel farm - reference site

The black metallurgic industry was, in that area, active for almost 100 years, leaving strong pollution of organic and inorganic origins. Mussel specimens were collected from cement docks.

The second sampling site was the little port in the village Ravnice (Fig. 1. site 2). The sampling site was near the closed industrial facilities of a cement plant. The factory has been inactive for almost 10 years. Emissions from cement factories include cement dust from the grounding process, nitrogen oxides, sulphur dioxide, carbon dioxide, carbon monoxide, dioxins, furans, PCBs, PAHs and some trace elements like thallium, mercury and others [27]. Cement kiln dust is a by-product of cement manufacture and it can contaminate groundwater with toxic metals like lead, cadmium, mercury, chromium and thallium [28].

The third and fourth monitoring sites were the little ports in the popular tourist centres Mimice and Baška Voda (Fig. 1. sites 3 and 4). Baška voda is a well-known tourist centre so during the summer and early autumn the population rises two- or three-fold [29], causing elevated contamination of the sea due to all kinds of human activities (municipal waste, yachting, tourist boats, other recreational activities, etc.).

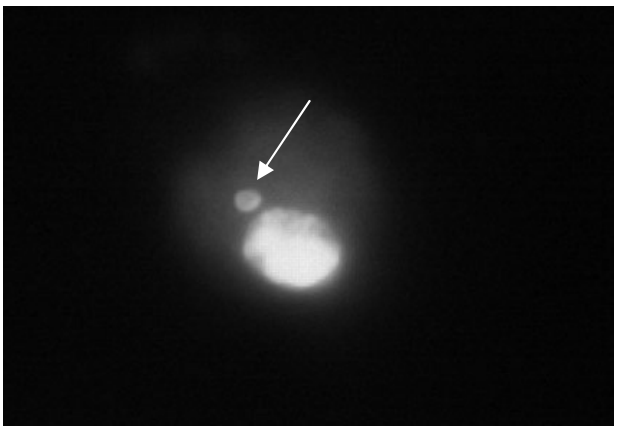

Fig. 2. Micronucleus (arrow) near the nucleus of mussel haemocyte (magnification 1000x).

Ravnice and Mimice are, besides being tourist resorts in summer, also fishing settlements with considerable amount of boating activities during the winter. This mainly includes coating the boats with antifouling paints and preparing them for the next fishing season.

In ports, mussels were collected from either buoys or boat ropes. Native mussels were collected at all sites four times throughout the year (summer and autumn in 2003, winter and spring in 2004) and MN assay was used to evaluate seasonal variability in MN frequency.

Micronucleus Assay

Haemolymph of mussels was collected from the posterior adductor muscle sinus with hypodermic syringe. Aliquots of 0.1 ml haemolymph and phosphate buffered saline (PBS) + 10 mM of ethylenediaminetetraacetic acid (EDTA) were placed on slides and left for 15 min in a humidified chamber for cells to settle down. The slides were then fixed with 1% glutaraldehyde in PBS for 5 min. After rinsing with PBS, the slides were stained with bisbenzimide 33258 (Hoechst) at final concentration 1 μg/ml for 5 min, washed and mounted in glycerol-McIlvaine buffer (1:1). The slides were stored in the dark at 4ºC prior to microscopic analyses. Analyses were done with an Olympus fluorescent microscope at 1,000x magnification. On each slide 400 cells with preserved cytoplasm and membranes were scored per mussel (4,000 cells per site) according to described criteria [30]. MN were defined as small round structures in the cytoplasm smaller than 1/3 of the nucleus diameter (Fig. 2). Also, MN has to be in the same optical plane as the main nucleus and its boundary should be distinguishable from that of main nucleus. According to Mersch and Beauvais [31], a minimum sample size of 500 haemocytes from each of 4 individuals/mussels giving a total of 2,000 cells, is sufficient for a qualitative discrimination between the spontaneous MN level and a toxicant-induced heightened MN frequency.

All results were expressed as means followed by corresponding standard errors. Data were analyzed by one-way analysis of variance (ANOVA). Duncan's test of multiple ranges was used to determine possible significant differences in MN frequency between monitoring sites and seasons as well (at $p<0.05$). All statistical analyses were done with Statistica 7.1. Software (StatSoft, Inc. USA, 2005).

Results

The frequency of MN at control site Marina ranged from 1.38‰ in autumn to 1.75‰ in spring (Figs. 3-6) with no statistical differences between the seasons. There was no seasonal effect on the background level of MN frequency in all seasons studied. The highest frequency of MN, significantly different from the control, was observed in summer on locations Ravnice and Baška Voda (5.75‰ and 6.50‰, respectively) (Fig. 3). At sites Mimice and Dugi Rat the MN incidence in summer was still elevated in comparison to the control, although lacking statistical significance.

In autumn the decrease in MN frequency was observed at all investigated sites except Baška Voda, and it was statistically significant (Fig. 4). In winter and spring no statistical difference in MN frequency was observed between the investigated sites, and higher MN frequencies than in autumn were observed for all sites except for the Baška Voda (Figs. 4-6). At this particular site the MN frequency decreased throughout the year, with the highest value observed in summer (Fig. 3) and the lowest in spring (Fig. 6), which was significantly different from summer and autumn values.

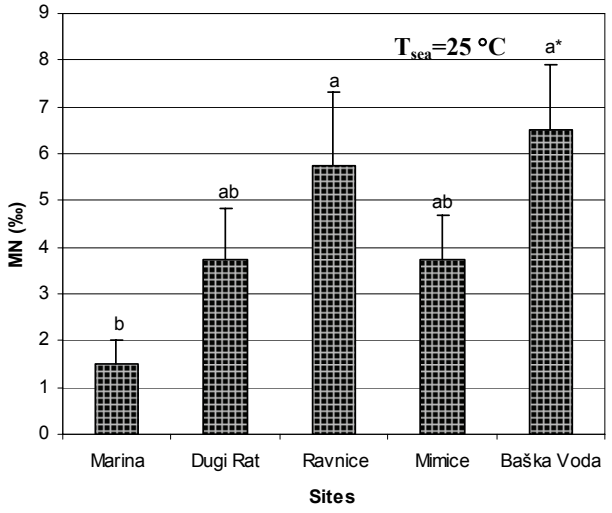

Fig. 3. MN frequency (mean + SE) in haemocytes of mussels at monitoring sites in summer 2003. Bars with different letters significantly differ (ANOVA: F = 3.0857, df = 4, p = 0.025; Duncan's post-hoc: p<0.05);
* indicates statistical difference from the spring mean (ANOVA: F = 2.9369, df = 3, p = 0.0462; Duncan's post-hoc: p<0.05).
T_{sea} represents the average surface monthly temperatures of the investigated coastal waters.

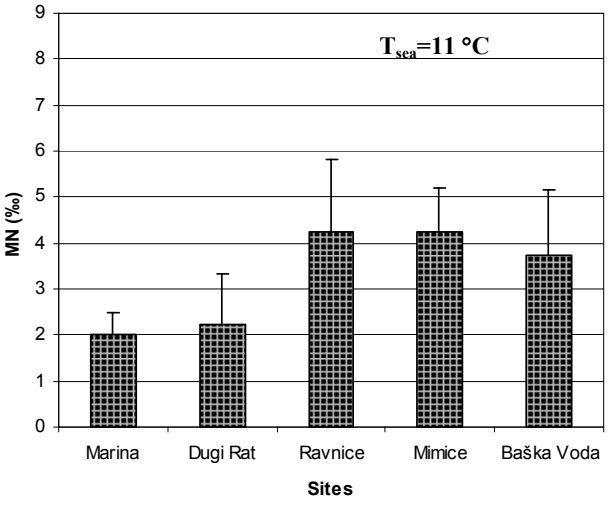

Fig. 5. MN frequency (mean + SE) in haemocytes of mussels at monitoring sites in winter 2004. (ANOVA: F = 0.8537, df = 4, p = 0.4989; Duncan's post-hoc p>0.05).
T_{sea} represents the average surface monthly temperatures of the investigated coastal waters.

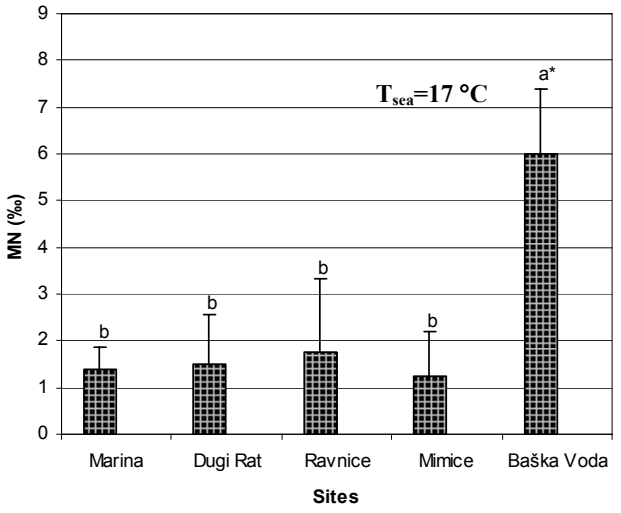

Fig. 4. MN frequency (mean + SE) in haemocytes of mussels at monitoring sites in autumn 2003. Bars with different letters significantly differ (ANOVA: F = 3.7903, df = 4, p = 0.0097; Duncan's post-hoc p<0.05);
* indicates statistical difference from the spring mean (ANOVA: F = 2.9369, df = 3, p = 0.0462; Duncan's post-hoc: p<0.05).
T_{sea} represents the average surface monthly temperatures of the investigated coastal waters.

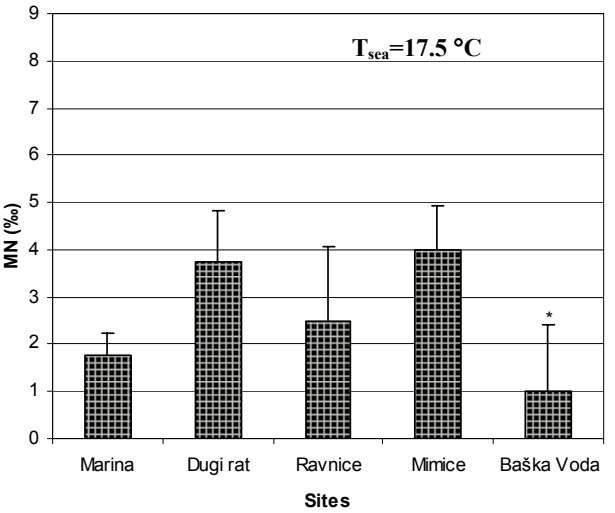

Fig. 6. MN frequency (mean + SE) in haemocytes of mussels at monitoring sites in spring 2004. (ANOVA: F = 1.2948, df = 4, p = 0.2863; Duncan's post-hoc p>0.05);
* indicates statistical difference from the summer and autumn means (ANOVA: F = 2.9369, df = 3, p = 0.0462; Duncan's post-hoc: p<0.05).
T_{sea} represents the average surface monthly temperatures of the investigated coastal waters.

Discussion

Seasonal variations in salinity and temperature and metabolic change in aquatic organisms can modulate the bio-availability of pollutants and the efficiency of the cellular defence mechanisms, thus stimulating or compensating for the pollution effect in aquatic environments [24]. This is probably the reason why some studies have detected different seasonal trends in micronucleus frequency [1, 6, 19, 24, 32].

The present study reports on the seasonal variability of micronuclei induction in haemocytes of native mussels collected from five sites with different pollution intensity.

Results showed elevated frequency of MN in summer on all investigated sites in comparison with the control site (Marina), although only values at sites Ravnice and Baška Voda reached statistical significance (Fig. 3). Two of selected sites were in the tourist resorts Mimice and Baška voda that are known to double the number of inhabitants during the summer, while the other two (Ravnice, Dugi Rat) are situated near closed industrial facilities (cement and ferroalloy factories) and could therefore have been polluted with genotoxic contaminants. Although both factories have been closed for more than 10 years, some genotoxic contaminants could still be present in the area, especially in the soil and most likely in the sea, because both factories are located on the shore. The coastal area Dugi Rat (black metallurgy) was, in the former Yugoslavia, known for the highest mortality rate of cancer and similar diseases in populations working in the factory or living nearby [33]. At Ravnice and Dugi Rat sites there is probably some genotoxic contamination left (heavy metals, PCBs, PAHs etc.) from cement and ferroalloy plant activities causing elevated MN frequencies. In addition, municipal waste waters which are released into the sea without any prior treatment most probably contribute to overall contamination. It is well known that due to high loading values of municipal wastewaters a strong relationship exists between surface water and its genotoxic impact on biota [34]. Along with genotoxic PAHs known to be present in many municipal wastewaters, human sanitary wastes contain other genotoxic substances such as N-nitroso compounds and aromatic amines (Hoffman et al. 1984, cited by [34]).

With higher temperatures of sea water there is a higher rate of metabolism in mussels so the induction of DNA damage by genotoxic pollutants is more obvious than in other seasonal periods. Since it is known that mussel tolerance to pollution is regulated by seasonal changes, especially temperature [13], temperature-dependent metabolic and filtration rates can modulate exposure to toxicants. Higher temperatures together with lower oxygen concentrations may decrease tolerance for toxicants due to limitations in energy supply for detoxification, elimination of the toxicant and/or repair of the toxicant-induced cellular damage [35]. In addition, an increase in MN frequency observed in summer could be related to a higher mitotic activity of haemocytes at higher temperatures [6, 32].

In autumn, the frequency of MN at all sites, except Baška Voda, decreased to control values (Fig. 4). This is in agreement with other studies that observed higher MN number in summer than in autumn [1, 5, 19]. Bolognesi et al. [1] observed higher values of MN frequency in mussel gill cells in May than in September, which correlated to higher levels of heavy metals Hg and Cd in May samplings. This could also be explained on the basis of changes in sea temperature. It is known that mussels, as poikilothermic organisms, at temperature within the optimal temperature range show a higher resistance to xenobiotic treatment at low doses, probably due to more efficient detoxifying and repair mechanisms [13]. Interestingly, on the site Baška Voda MN incidence decreased in winter and further in spring. This may be linked to reduced genotoxic burden in the environment out of the tourist season, as municipal wastewaters are the main source of pollution at this site.

During the winter observed MN frequency was also higher than in autumn and somewhat in the spring – especially for the sites Ravnice and Mimice (Figs. 5 and 6). This may be linked to the changes in pollution input level due to winter boating activities, while it is well known that many applied antifouling paints and oils may have genotoxic properties [36, 37].

It is evident from our results that biomarker response (in this case MN frequency) is related to seasonal effects, mainly temperature changes, contaminant exposure or most probably their interaction.

Conclusions

In the present study MN assay confirmed its usefulness as a biomarker for monitoring genotoxic pollution in coastal waters of the Adriatic Sea. Monitoring was done throughout one year to evaluate if seasonal changes (sea temperature) modulate response to pollution in mussels. Seasonal variations in MN frequency were not observed for the control mussels, but for other investigated sites clear seasonal variations were observed. The highest MN frequency was observed in summer, while the lowest was observed in autumn. These results point out that seasonal changes observed only at polluted sites were caused by seasonality of pollution, as well as by interaction between contaminants and higher metabolic and filtration rates in mussels, resulting in higher values of cytogenetic damage.

Acknowledgements

The authors wish to thank the Scientific Research Council of the Ministry of Science, Education and Sports, Republic of Croatia, for funding this investigation within the framework of projects No. 0119-112 and 0119-128.

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