DNA damage in marine mussel *Mytilus galloprovincialis* as a biomarker of environmental contamination

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ABSTRACT: Primary DNA damage (single-strand breaks and crosslinks) was measured in haemolymph of mussel *Mytilus galloprovincialis* using alkaline filter elution, and turned out to be a useful biomarker for genotoxic environmental contamination. Increased frequencies of single-strand breaks and DNA crosslinks in natural mussel populations under the influence of urban and industrial wastes enable the classification of sampling sites in the Rovinj area (Istrian coast, Northern Adriatic) according to the genotoxic impact on haemolymph DNA.

KEY WORDS: DNA damage · Pollution · Mussel

INTRODUCTION

The use of naturally occurring mussels is a very direct approach to *in situ* biomonitoring investigations and is favorable in several aspects (Viarengo & Canesi 1991). Mussels are good indicator organisms for identification of environmental changes whose cause may be unknown, or which may be the result of a varying mixture of contaminants, e.g. industrial effluents. Exposure of mussels to environmental contamination gives rise to DNA damage comprising chromosomal aberrations (Al-Sabti & Kurelec 1985), sister-chromatid exchange (Dixon et al. 1985), micronuclei formation (Brunetti et al. 1988, Wrisberg et al. 1992) and strand breaks (Nacci et al. 1992). All these lesions may contribute to cell death, mutagenesis and/or carcinogenesis, but their relative contributions are likely to be different. It is important, therefore, to study various damages with respect to their abundance and persistence.

Continuous pollution impacts of genotoxins at discriminated sites in the investigated Rovinj area (Istrian coast, Northern Adriatic) were determined earlier by several experimental approaches. Increase in benzo(a)pyrene monooxygenase activity in fish liver at these contaminated sites was established successfully (Kurelec et al. 1977, 1979), as well as significant increase in numbers of salmonella revertants in the Ames test caused by water extracts (Zahn et al. 1982). Activation of premutagens in the SOS-*umu* test by liver S9 fractions from fish collected at different sampling sites is also correlated with environmental contamination (Bihari et al. 1990). Frequencies of secondary DNA lesions, chromosomal aberrations, were increased in gills of mussel populations living in polluted Rovinj areas (Al-Sabti & Kurelec 1985), and primary DNA lesions measured as strand breaks in DNA of gills could be correlated to pollution effects in mussels (Herbert & Zahn 1990).

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In order to detect the environmentally induced DNA damage in mussel haemolymph, biomonitoring of the Rovinj area using mussels was conducted from February 1991 until February 1992. The alkaline elution technique, as a promising genotoxicity test for purposes of biomonitoring in the marine environment, was applied. It was important to find out whether the method is suitable for detecting DNA alterations resulting from unknown mixed environmental pollution among natural mussel populations, and can thus be used for routine monitoring studies. For this purpose DNA single-strand breaks and crosslinks in mussel haemolymph were measured. Haemolymph was considered for routine evaluation in this study because of its potential exposure to environmental genotoxins through the gills and open vascular system. It is drained without disturbance of the whole animal and easily applied to the filter, avoiding additional strand breaks during the rapid handling procedure (Bihari et al. 1991).

The results of this investigation could help in ranking of sampling sites according to predominance of certain types of primary DNA lesions, giving insights into the genotoxic risk for mussels exposed to mixed environmental pollution.

MATERIALS AND METHODS

Chemicals. Tetraethyl ammonium hydroxide was obtained from Sigma (St. Louis, MO, USA), N-lauryl-sarkosine 35 % Na salt from Serva (Heidelberg, Germany), Hoechst 33258 from Riedel de Haen AG Seelze (Hannover, Germany), and proteinase K from Merck (Darmstadt, Germany). Bleomycinum, a clinical preparation of bleomycins containing 1400 to 2100 µg mg^{-1} bleomycin sulphate was a gift from Mack, Jllertissen, Germany.

Animals and treatment. Mussels *Mytilus galloprovincialis* L. (Mollusca: Bivalvia), average weight 4.0 g, were collected at 5 different locations (Fig. 1). Stns S-1, S-2, and S-3 are under the influence of industrial and/or urban runoff from a fish cannery, tobacco factory and urban waste, respectively. Stns S-4 and S-5 (maricultural area) are uncontaminated sampling sites (Fig. 1). Mussels were transported in seawater tanks to the laboratory and the haemolymph was taken from the adductor muscle within 1 h of collection. Composite samples of haemolymph from 5 mussels were applied on the filters.

Alkaline elution. Alkaline elution was performed according to Kohn et al. (1981) with some modifications (Bihari et al. 1991, 1992). Filter holders with membrane filters (PVF, 25 mm diameter, 0.22 µm pore size, GVWP 02500, Millipore Corp., Bedford, MA, USA) were used. Composite samples of 1.5 ml mussel haemolymph, containing 2×10^6 cells, were deposited on filters. This was then lysed with 5 ml of 0.2 % sodium lauryl-sarkosine, 2 M NaCl and 0.02 M EDTA, pH 10. In the case of DNA-protein crosslinks determination, the lysing solution contained 0.5 mg ml^{-1} proteinase K (Kohn et al. 1981). Filters were then washed with 10 ml of 0.02 M EDTA, pH 10. To detect total DNA crosslinks, cell-free DNA was washed with 3 ml of washing solution, followed by 3 ml iron-bleomycin solution and by 4 ml washing solution again as described earlier (Batel et al. 1993). The iron-bleomycin solution was used instead of X-irradiation to introduce reproducible amounts of DNA strand breaks. After washing, 18 ml of alkaline solution (0.02 M EDTA buffered to pH 12.3 with tetraethyl ammonium hydroxide) was added. The single-strand DNA was eluted at a flow
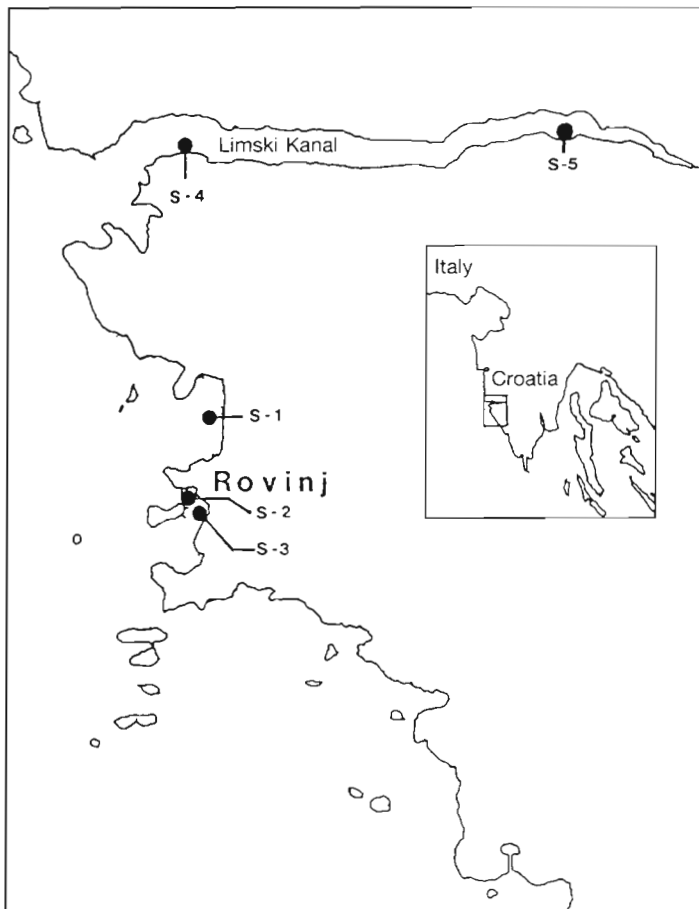

Fig. 1. Sampling sites in the Rovinj area, Istrian coast, Northern Adriatic

rate of 0.05 ml min^{-1}. The DNA contents of 2 ml aliquots from 6 fractions (3 ml h^{-1}), filters and filter assembly washes were determined according to Stout & Becker (1982), using bisbenzimide (Hoechst 33258) as fluorescent stain.

Expression of the data. The results were plotted as the logarithm of the fraction of DNA retained on the filter versus the elution volume. A value characterizing a relative number of DNA strand breaks, referred to as a 'strand scission factor' (SSF; Meyn & Jenkins 1983), was calculated by:

$$SSF = \log_{10}\left(\frac{\% \ DNA_{sample}}{\% \ DNA_{control}}\right)$$

where % DNA$_{sample}$ = % DNA retained in the treated sample after 9 ml eluted, and % DNA$_{control}$ = % DNA retained in the control after 9 ml eluted. Therefore a SSF = 0 assumes absence of DNA strand breaks, while SSF < 0 indicates a relative value for DNA breaks in the treated mussels. Results are expressed as absolute values of SSF together with standard deviations or percent DNA retained after 9 ml calculated from the linear part of the elution curve.

DNA damage expressed as crosslink frequencies or single-strand break frequencies was calculated according to Kohn et al. (1981) with minor modifications. In order to avoid any possible influence of internal standards on independent DNA strand elution, replicate assays were performed. To correct the time scale, DNA from mussels collected at reference Stn S-5 and treated with 100 nM iron-bleomycin complex on the filter was used. Results are expressed in frequency equivalents of iron-bleomycin complex concentrations.

Statistical analysis. Two-way analysis of variance (ANOVA) was performed according to Dowdy & Weardon (1983). Statistical significance of the means was also evaluated using the Welsch test (Welsch 1977).

RESULTS

To detect the influence of seasonal changes on haemolymph DNA, mussels were collected from February 1991 to February 1992 at 4 different sampling sites. Single-strand breaks were measured in composite samples of haemolymph from at least 5 mussels per filter immediately after collection. Results are represented in Fig. 2. At Stn S-4 (Limski Kanal, uncontaminated area), the variation of the percentage of DNA retained on the filter through the season was negligible, suggesting the presence of constant amounts of DNA damage in mussel haemolymph. Variations in amounts of single-strand breaks could be explained as
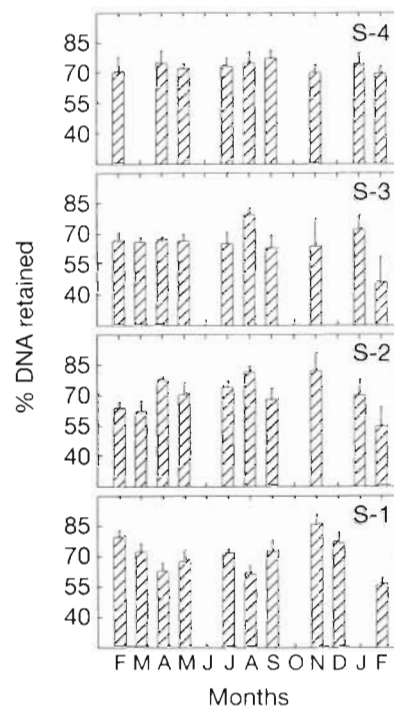

Fig. 2. *Mytilus galloprovincialis*. Seasonal variations in the percentage of mussel haemolymph DNA retained on the filter. Mussels were collected at 4 stations: S-1, fish cannery waste; S-2, harbor and urban waste; S-3, tobacco factory and graphic industry waste; S-4, uncontaminated area. Results represent means ± SD of at least 3 measurements. Statistical significance by 2-way analysis of variance was p < 0.001, p = 0.026 and p < 0.001 for month, site and month × site respectively

a result of normal cellular events present in mussel haemolymph during the whole year. However, at Stns S-1, S-2 and S-3, under the influence of industrial and urban wastes, the percentage of DNA retained on the filter depended on the time and site of collection. Variations in the percentage of DNA retained indicate the presence of different amounts and/or types of damage in mussel DNA during the year. Lower amounts of DNA retained indicate the presence of higher amounts of single-strand breaks, while higher amounts of DNA retained on the filter indicate lower amounts of single-strand breaks in DNA or their compensation with crosslinks present in the DNA.

The shapes of DNA elution curves from mussels collected at different sampling sites were compared in Fig. 3. DNA elution rates from mussels collected at the site under the influence of fish cannery wastes (Stn S-1) in August was higher than in February, indicating the presence of more single-strand breaks in haemolymph DNA. However, DNA elution rate for mussels collected in the harbor (Stn S-2) in August was lower than in February. The decrease in DNA elution rate could have been the result of reduced amounts of
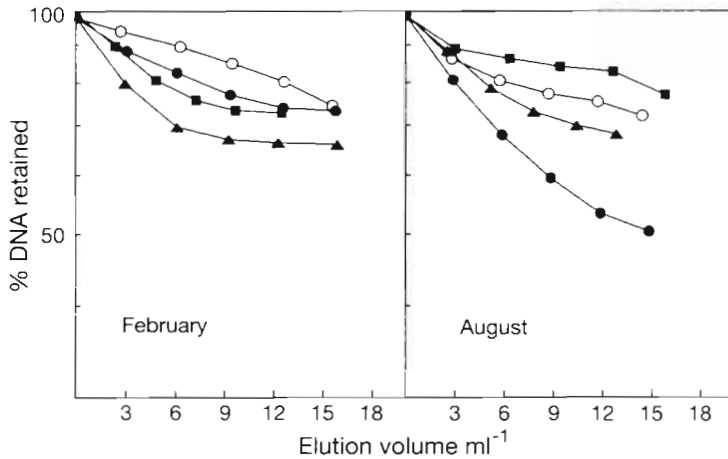
Fig. 3. *Mytilus galloprovincialis.* Comparison of DNA elution profiles from mussels collected at different sampling sites in February and August 1991. ●: S-1; ■: S-2; ▲: S-3; O: S-4

single-strand breaks in DNA or the result of the presence of crosslinks which retain more DNA on the filter. Concomitantly, higher DNA elution rate of mussels from the harbor (Stn S-2) compared to mussels from the uncontaminated Stn S-4 favors the second explanation. Satisfactory explanation of the reported results is difficult without detailed investigations into the type and amount of DNA damage. However, the amount and type of DNA damage in mussel haemolymph depended on the collection site, since mussels used in experiments had the same biometric characteristics and were in the same developmental stage. We can conclude that other factors besides normal cellular activity influenced mussel haemolymph DNA at these sampling sites, throughout the year. For further confirmation of this statement, different types of DNA damage in mussels from different sampling sites were analyzed.

To establish the presence of DNA crosslinks in mussel haemolymph, a reproducible amount of single-strand breaks in cell-free DNA was introduced by iron-bleomycin complex in the washing step of the alkaline elution procedure, as we described previously (Batel et al. 1993). If DNA crosslinks are present in mussel haemolymph, increase of DNA elution rate caused by increased concentrations of iron-bleomycin complex will not occur. Fig. 4 shows the influence of increasing concentrations of iron-bleomycin on DNA from haemolymph of mussels from different sampling sites. In haemolymph DNA from mussels collected at Stn S-4, an increase in iron-bleomycin concentration caused a proportional increase of additional single-strand breaks, expressed as strand scission factor (SSF). In contrast, in mussels collected at all other sampling sites (Stns S-1, S-2 and S-3), the proportional increase in

amount of single-strand breaks introduced by increasing concentrations of iron-bleomycin complex could not be detected. This could be explained by the presence of DNA crosslinks, which retain more DNA on the filter, in mussels from sampling sites under the influence of industrial and urban wastes. The highest amount of total DNA crosslinks could be attributed to mussels from Stn S-3 due to the lowest effect of 800 nM of iron-bleomycin complex to elution of cell-free DNA.

When the presence of DNA crosslinks was confirmed, DNA-protein crosslinks were distinguished by introducing proteinase K in the lysing step of the alkaline elution procedure (Kohn et al. 1981). If DNA-protein crosslinks are present in mussel haemolymph, proteinase K would remove proteins from DNA and an increase in the DNA elution rate would result in a lower percentage of DNA retained on the filter. If DNA-DNA crosslinks are present in mussel haemolymph, proteinase K would have no effect on DNA elution rates, and a higher percentage of DNA retained would appear. Since in natural mussel populations all 3 types of damage occur simultaneously, estimation of one will have an influence on the estimation of others. Although proteinase K did not induce any measurable change in the DNA elution rate in mussels from all collection sites, its effect became
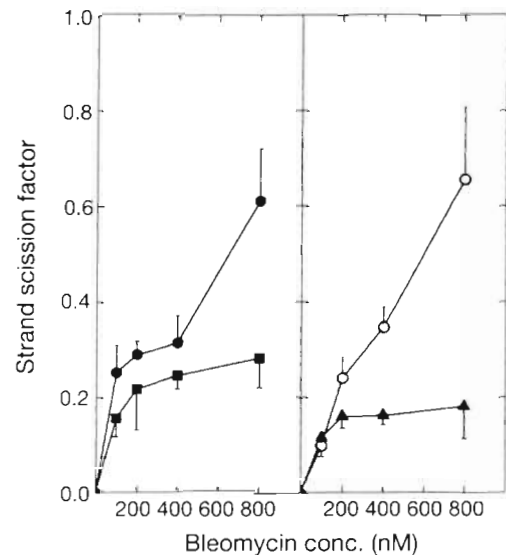

Fig. 4. *Mytilus galloprovincialis.* Effect of iron-bleomycin complex on DNA from mussels collected at sites with different pollutional loads in May 1991. ●: S-1; ■: S-2; ▲: S-3; O: S-4. Strand scission factor was calculated as described in 'Materials and methods' Results represent means ± SD of 3 measurements
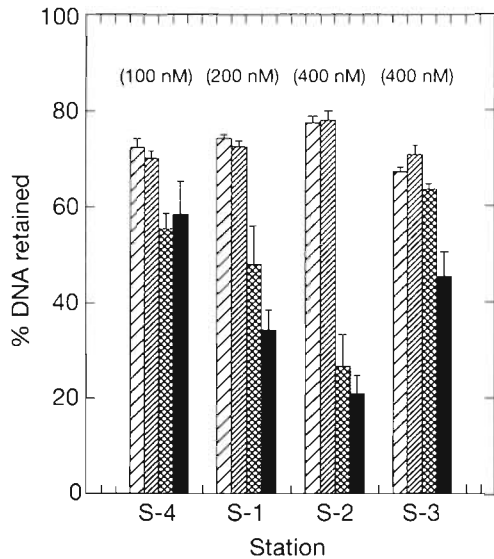
Fig. 5. *Mytilus galloprovincialis*. Effect of iron-bleomycin complex and proteinase K on the percentage of DNA retained in mussels collected at different sampling sites in April 1991. ▨: untreated DNA; ▨: DNA lysed with proteinase K; ▨: DNA treated with iron-bleomycin complex; ■: DNA lysed with proteinase K and treated with iron-bleomycin complex. Numbers in parentheses indicate the different concentrations of iron-bleomycin complex used for each sampling site (100, 200, 400 and 400 nM respectively). Results represent means ± SD of 3 measurements

DNA-crosslinks, mainly DNA-protein crosslinks.

– Stn S-3: 400 nM iron-bleomycin complex had practically no effect on the percentage of DNA retained. Proteinase K in combination with iron-bleomycin complex decreased the percentage of DNA retained. This indicates the presence of single-strand breaks and DNA-protein crosslinks together with DNA-DNA crosslinks.

DNA damage expressed as crosslink frequency or single-strand break frequency was calculated according to Kohn et al. (1981). Total DNA damage was calculated as a sum of both crosslink and single-strand break frequency. Frequency equivalents were expressed relative to DNA damage in mussels from the reference, uncontaminated Stn S-5, assuming its zero value (Fig. 6). Mussels from Stn S-4, with the lowest amount of single-strand breaks in haemolymph DNA during the whole year, also showed the lowest amounts of crosslinks. Additional DNA damage in mussels from Stns S-1, S-2 and S-3 could be attributed to human-related contamination. Consequently, sampling sites can be ranked according to induced single-strand break frequencies in the order S-2, S-3, S-4 < S-1, according to crosslink frequencies in order S-4 << S-1 < S-2 < S-3, and according to total DNA damage S-4 << S-2 < S-1, S-3.

obvious after treatment of cell-free DNA with iron-bleomycin complex (Fig. 5). Taking together the decrease in the percentage of DNA retained by increased concentrations of iron-bleomycin complex and by proteinase K, detection of DNA-protein crosslinks became possible. The higher the concentration of iron-bleomycin complex needed to decrease the percentage of DNA retained, the higher the number of total DNA-crosslinks present in mussel haemolymph DNA.

Consequently, the predominance of certain types of DNA damage in mussels from different sampling sites could be described in the following way:

– Stn S-4: the lowest concentration of iron-bleomycin complex (100 nM) caused a significant decrease in the percentage of DNA retained. Proteinase K in combination with iron-bleomycin complex had no influence on the percentage of DNA retained. This indicates the presence of low amounts of single-strand breaks and DNA crosslinks.

– Stns S-1 and S-2: higher concentrations of iron-bleomycin complex (200 nM and 400 nM respectively) were needed for a significant decrease in the percentage of DNA retained (100 nM had no effect). Proteinase K in combination with iron-bleomycin complex further decreased the percentage of DNA retained. This indicates the presence of single-strand breaks and
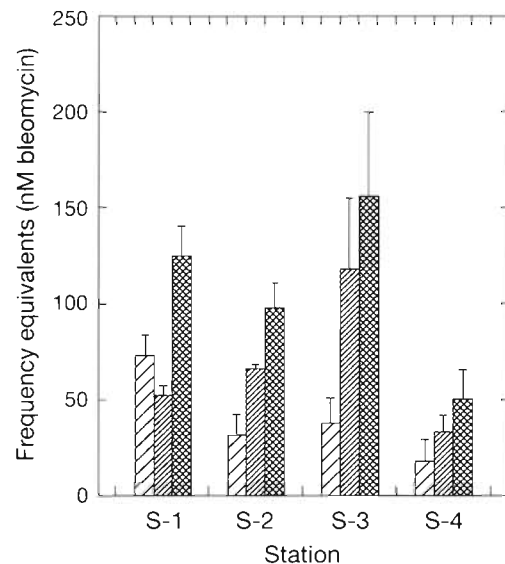

Fig. 6. *Mytilus galloprovincialis*. Increase in frequency of DNA lesions in mussels collected in May 1991, relative to DNA lesions in mussels from reference, uncontaminated Stn S-5, expressed in bleomycin equivalents. ▨: single-strand breaks; ▨: DNA crosslinks; ▨: total DNA damage. Results represent means ± SD of 3 measurements. Differences of the mean values were statistically significant (Welsch test: $p < 0.05$) for single-strand break frequencies Stn S-1 vs Stn S-4, for DNA crosslinks Stns S-1, S-2, S-3 vs Stn S-4 and for total DNA damage Stns S-1, S-2, S-3 vs Stn S-4

DISCUSSION

The experimental data reported here indicate that the alkaline filter elution technique is suitable for determination of DNA damage in mussels with the aim of monitoring for possible exposures to environmental genotoxins. The technique has the advantage of detecting different types and amounts of DNA damage, giving the possibility for ranking of sampling sites according to genotoxic effects on mussel DNA. It enabled us for the first time to classify sampling sites according to the genotoxic effect of bulk pollutants to mussels.

At the uncontaminated site (Limski Kanal, Stn S-4) the effects on mussel DNA can be related to normal cellular activities. Mixed pollution at sites under influence of urban and industrial wastes additionally induce different types and amounts of DNA damage, such as DNA-protein crosslinks in addition to single-strand breaks at Stns S-1 (fish-cannery waste) and S-2 (urban wastes), together with DNA-DNA crosslinks at Stn S-3 (tobacco factory and graphic industry wastes). However, those types and amounts of DNA damage in mussel haemolymph are not persistent throughout the year. This could be explained by seasonal fluctuations in pollution caused by industrial and urban wastes, and by modulation of DNA repair induced by pollutants, as we reported for mussels treated in laboratory conditions with known carcinogens (Bihari et al. 1991).

We proved also that simple measurements of strand breaks are not always sufficient to detect genotoxic effects in the marine environment without additional informations about total crosslinks in the DNA. Simple measurements of only single-strand breaks are not sufficient to distinguish Stns S-2 and S-3 from the uncontaminated Stn S-4, while measurements of total DNA damage gave rise to a clear difference. According to measurements of only single-strand breaks in mussels by alkaline unwinding, as reported by Herbert & Zahn (1990), pollution works in 2 ways: it can reduce the number of DNA strand nicks due to changes in transcriptional events caused by pollutants, and it can increase the number of DNA lesions. By the alkaline elution method we showed the presence of DNA-crosslinks in mussels in their natural habitats caused by pollution, rather than a reduction in the number of DNA strand nicks.

Measurement of other types of DNA damage in mussels, such as DNA-adducts, cannot reveal by itself enough information about genotoxic pollution at our sampling sites. Kurelec et al. (1990) suggested that the lack of pollution-related DNA adduct should depend upon the type of pollutants at particular sampling sites. However mixed pollution operates at different levels of cellular and genomic organization thus inducing direct and indirect damages to the DNA (Eastman & Barry 1992). Besides binding of pollutants to DNA bases, they can inhibit DNA repair or induce strand breaks and crosslinks in the DNA strands. In previous investigations Al-Sabti & Kurelec (1985) demonstrated that morphological changes occurred in chromosomes from mussels at these polluted sites. Our results are consistent with their findings since chromosomal aberrations are consequences of primary DNA damage such as single-strand breaks and DNA crosslinks (Olivieri & Micheli 1983, Natarajan et al. 1986, Šlotova et al. 1986).

Field and laboratory data on genotoxic effects of pollutants accentuate the complexity of biological impact of mixed pollution in the marine environment. Because of actions of different pollutants, the biological responses may be very complex. Genotoxic effects of pollutants are further complicated by the presence of groups of pollutants that may interact with DNA or can modify DNA synthesis and repair. Direct chemical analysis of pollutants and their speciation are time consuming and very complex. Their single effects on mussels are certainly modulated in mixed pollution, and could hardly give satisfactory insights about their genotoxic properties. The use of DNA damage (single-strand breaks and DNA crosslinks) as a final biomarker of environmental pollution enabled us to classify polluted locations according to their genotoxic impact on mussel DNA. At the same time it gave additional information about the genotoxic risk for mussels exposed to mixed environmental pollution.

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