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Spatial Distribution of DNA Integrity in Mussels, *Mytilus galloprovincialis*, from the Adriatic Sea, Croatia

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The marine environment is the major ultimate recipient of increasing range of anthropogenic contaminants, a large proportion of which are genotoxic. The first event in marine organisms following exposure to genotoxic contaminants is the impact on the integrity of cellular DNA. Therefore, DNA integrity in certain marine organism reflects the level of marine pollution by genotoxins and is widely accepted as a good biomarker (Shugart 2000). Genotoxicity biomarkers detected in mussel *Mytilus galloprovincialis* have been validated in the field and applied on a large scale in coastal monitoring studies (Steinert et al. 1998, Rank 1999, Izquierdo 2003). DNA single strand brakes, as the most used genotoxic biomarker, have been detected by methods such as the DNA unwinding test (Nacci et al. 1992), alkaline elution (Vukmirović et al. 1994), and the Comet assay (Lee and Steinert 2003), revealing difference in sensitivity and discriminatory power. However, these methods do not satisfy the need for a fast, routine and simple technique for large numbers and series of samples. The Fast Micromethod® (Batel et al. 1999) detects DNA damage (strand breaks, alkali-labile sites and incomplete excision repair) and determines DNA integrity in cell suspension or tissue homogenates in single microplates. The sensitivity and precision of the method is similar to the Comet assay (Bihari et al. 2002). It was successfully applied for DNA integrity determination in dab (Lacorn et al. 2001) and marine invertebrates (Jakšić and Batel 2003) as well as in a long-term field study (Jakšić et al. 2005). Here we presented the results of genotoxic biomonitoring by the Fast Micromethod® performed in June 2001 using gills as the target organ from a sentinel organism the mussel *Mytilus galloprovincialis*, collected along the Adriatic coast, Croatia.

MATERIALS AND METHODS

All the chemicals were molecular biology grade and purchased from Sigma (St. Louis, MO, USA). The fluorochrome dye PicoGreen®, P-7581, was from Molecular Probes Inc., Eugene, OR, USA. The monitoring network comprises 7 types of sampling sites including island pristine (19), island semiurban (16, 17, 18, 20, 21), mainland urban (1, 3, 7, 8, 9, 10, 11, 13, 14, 15, 22, 24, 25), mainland agriculture (23), industrial and urban (2, 6, 12), refinery and shipyard (5) and harbor (4) along the Adriatic coast, Croatia (Fig. 1.). Mussels *Mytilus galloprovincialis* (Mollusca: Bivalvia) were collected from each site in June 2001. Gills of mussels were removed from 10 g mussels at the place of collection, stored

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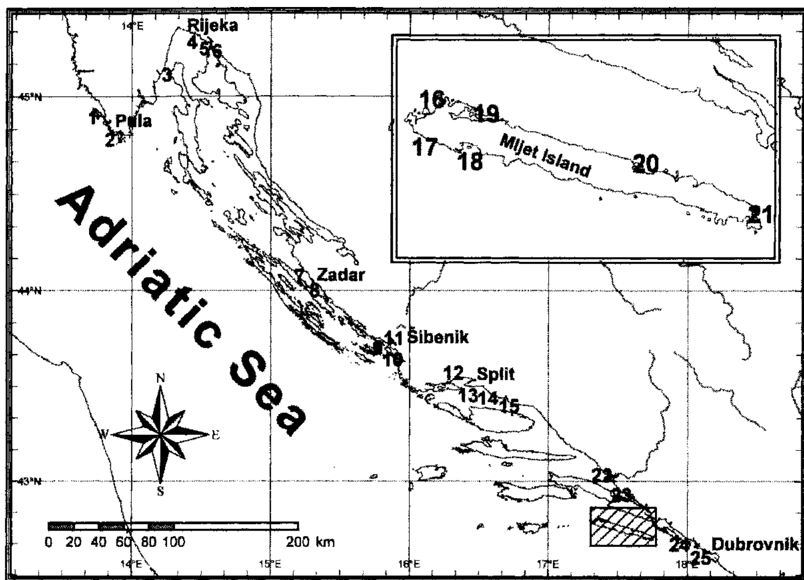


Figure 1. Study area.

in liquid nitrogen and transported to the laboratory. 100 mg of gill tissue were homogenized in 2 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and DMSO (9:1) in liquid nitrogen with a mortar and pestle pre-cooled in liquid nitrogen. DNA denaturation rate in mussel gill lysates at alkaline pH was measured by Fast Micromethod® (Batel et al. 1999, Bihari et al. 2002, Jakšić and Batel 2003) in a Fluoroscan Ascent microplate reader (Labsystems, Finland). Briefly, 25 µl of a lysing solution (4.5 M urea, 0.1% SDS, 0.2 M EDTA, pH 10) supplemented with PicoGreen (20 µl of the original stock dye/P-7581, Molecular Probes Inc., Eugene, Oregon / per ml of lysing solution) was added to 25 µl gill homogenate (100 ng DNA/ml). Analyses were performed in quadriplicates of minimum 5 mussels in each. The microplates were kept in darkness for 30 min. DNA denaturation conditions were achieved at pH 11.5 with 250 µl NaOH-EDTA solution. The extent of DNA denaturation was followed in the microplate by measuring the decline in fluorescence of the dsDNA-PicoGreen complex at room temperature. The number of strand breaks in DNA of mussel gills was expressed as the strand scission factor (SSF) calculated as: SSF = $\log_{10}$ (% $dsDNA_{site\ X\ sample}$ / % $dsDNA_{site\ 19\ sample}$) after 5 min denaturation. The SSF = - 0.5 and - 1.0 represents the 64.8 and 90.0 % lower DNA integrity than in mussels from referent site 19, respectively. Cluster analysis was used to group the sampling sites with similar DNA integrity distribution. Distance between objects was measured as Euclidian distances. Principal component analysis (PCA) was used to study the correlation

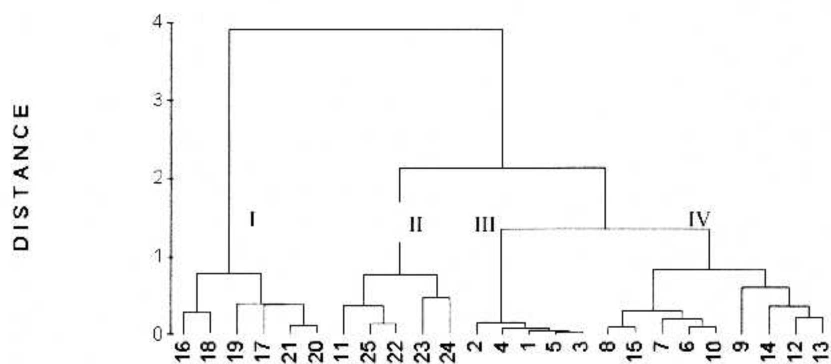
structure of sampling sites according to DNA integrity and type of DNA damage. All statistical procedures were performed using PRIMER 5 ver. 5.2.9, PRIMER–E Plymouth, UK

RESULTS AND DISCUSSION

A total of 125 mussels collected along the Adriatic coast in June 2001 were analysed for DNA integrity in gills. A hierarchical cluster analysis of the DNA integrity separated sampling sites into four groups (Figure 2a.). Group I comprised six sampling sites (16-21), group II had five sites (11, 22-25), group III five sites (1-5), and group IV nine sampling sites (6-10, 12-15). Statistical difference is significant between group I vs all others ($p = 0.005$), and between group II vs group III ($p = 0.005$). For further investigation of the correlation structure of sampling sites according to DNA integrity and type of damage, principle component analysis (PCA) was performed. PCA revealed that the first two principal components account for 99.4 % of the total variance (Figure 2b.). The first principal component (PC1) had significant negative loadings with DNA integrity while the second principal component (PC2) had significant negative loadings on single strand breaks (ssb). Sampling stations located in the upper left – hand part of the score plot are characterized with the highest DNA integrity and the lowest amount of single strand breaks reflecting the lowest level of genotoxins present in the mussel environment.

According to cluster and PCA results, DNA integrity in mussel gills was mainly correlated with geographical distribution of mussels and decreased in the order Island Mljet, Southern Adriatic together with station 11, Middle Adriatic together with station 6 and Northern Adriatic. In general, potential genotoxic load decreased from north to south and from mainland to island area. Such distribution could be explained by the semienclosed character of the Adriatic Sea with influence of big cities in the north and urban – industrial areas in the mainland zone. In general, circulation of the Adriatic sea is cyclonic with an inflow of "clean" Medierranean waters along the Croatian coast. During spring and summer closed circulation cells are formed in the northern part of the Adriatic (Krajcar 2003., Supić et al. 2003), retaining potential genotoxic contaminants in this region. The highest DNA integrity was detected in mussels collected at a pristine island area, sampling site 19. Therefore, sampling site 19, situated in a strongly protected area and characterised by no significant human activities and discharge water outflow, has been considered as undisturbed reference site for quantitative analysis of DNA integrity by calculation of strand scission factor (SSF). Higher absolute values of SSF represent lower DNA integrity (higher DNA damage) in mussel gills, thereby reflecting a higher influence of genotoxic compounds present in that environment. An increase in genotoxicity corresponded to the type of sampling sites as follows: island pristine < island semiurban < mainland urban < mainland agriculture < industrial and urban < refinery and shipyard < harbor (Fig. 3) reflecting the pollution status of the studied areas. Obtained results enabled definition of positive control sites with $SSF * (-1) > 0.50$ (industrial and urban, refinery and shipyard and harbor). It can be concluded that the response of

a



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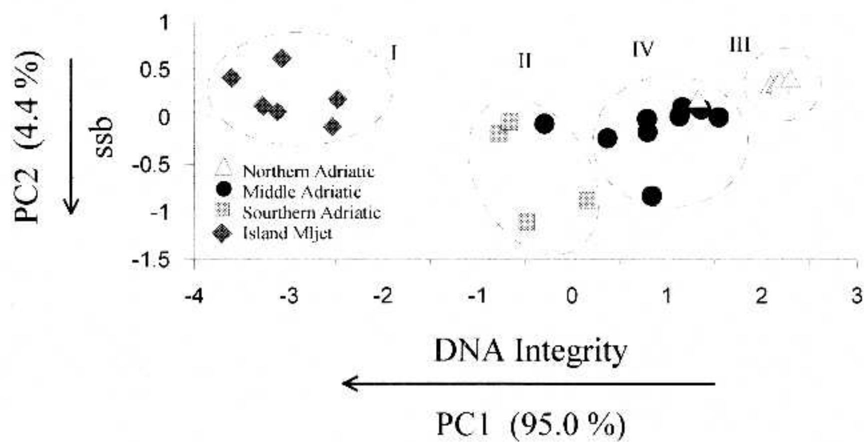

Figure 2. The results of cluster (a) and principal component (b) analysis of DNA integrity in mussel *Mytilus galloprovincialis* collected at different sampling sites along the Adriatic coast, Croatia.

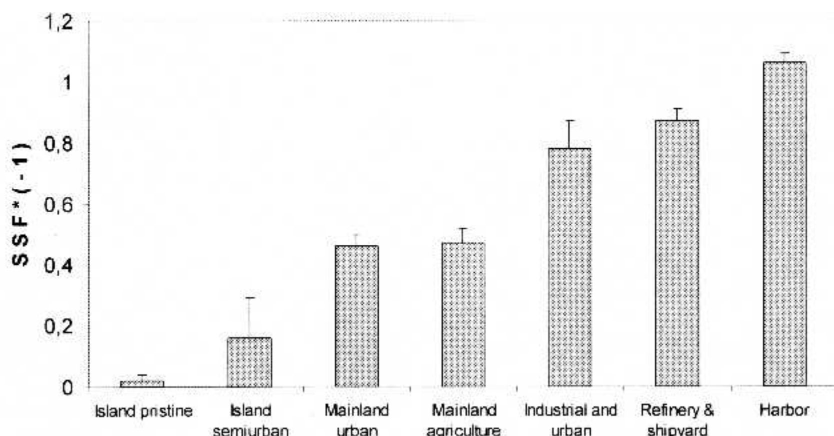

Figure 3. Comparison of DNA integrity expressed as strand scission factor (SSF) in gills of mussel *Mytilus galloprovincialis* collected at different types of sampling sites.

genotoxicity biomarker (DNA integrity) using mussels as a sensitive organism towards genotoxins showed a good discriminatory power allowing the classification of sampling sites along the Adriatic coast, Croatia and revealing the most threatened areas, so called "hot spots". However, the ecotoxicological significance of genotoxin - induced decreases in DNA integrity in gills of mussels collected at the "hot spots" remains an open question. Additional data of chemical analyses could allow better risk assessment and development of remediation strategies.

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