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Testing quality of sea water from the Adriatic coast of Croatia with toxicity, genotoxicity and DNA integrity tests

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The quality of sea water along the Adriatic coast of Croatia was evaluated three times during 1.5 years at 16 sites located in protected, urban, industrial, harbor or brackish areas. Samples of sea water (30 l) were tested by the Microtox test for toxicity and the umu-test for genotoxicity. Samples of mussel gills (5 individuals, Mytilus galloprovincialis*) were collected from each sampling site and the Fast Micromethod® was used to test DNA integrity, which may have been altered by water contaminants. According to the Microtox text, 75% of the water samples were potentially toxic. According to the* umu*-test, 40% were potentially genotoxic. Alteration of DNA integrity was detected in 40% of the mussel gill samples.*

Key words: Toxicity, genotoxicity, DNA damage, Microtox, SOS/umu-test, Fast Micromethod®

INTRODUCTION

The need to detect and assess the impact of pollutants, particularly low concentrations of complex mixtures, on environmental quality has led to the development of several assay methods. Monitoring of water by chemical analysis requires a precise knowledge of the contaminants being looked for, is limited by sensitivity of the detection method, measures only some xenobiotics, and gives no information about biological effects. In contrast, biomonitoring takes advantage of the fact that organisms combat pollution by accumulating xenobiotics under less toxic forms or in spatial compartments and/or by inducing biotransformation mechanisms that can be assayed to show the presence and effects of known and unknown contaminants.

In this study, the Microtox test for toxicity measurements (BULICH & ISENBERG, 1981; BIHARI *et al.,* 1989), the *umu*-test for genotoxicity measurements (ODA *et al*., 1985; HAMER *et al*., 2000) and the Fast Micromethod® for DNA integrity measurements (BATEL *et al*., 1999) were performed. They appeared to be simple, fast and inexpensive methods for *in vitro* and *in vivo* evaluation of pollution in the marine environment. The Microtox test is based on the fact that luminescence of the marine bacterium *Vibrio fischeri* is inhibited when the organism is exposed to toxic substances. The *umu*-test is based on the ability of genotoxins to induce expression of the *umu*C gene, measured as the increase of ß-galactosidase activity in *Salmonella typhimurium* (TA1535/pSK1002 tester strain). The data obtained by these tests indicate water quality at the time the water samples were collected. Reflection of long-term genotoxicity

in the marine environment can be detected by *in vivo* tests such as the Fast Micromethod®, which estimates potential genotoxicity by measuring genetic endpoints (such as strand breaks) that show primary DNA damage. This is a quick and convenient procedure for determining DNA damage in cell suspensions and solid tissues on single microplates. However, because the Fast Micromethod® measures only single strand breaks and alkali-labile sites, it can miss DNA alterations involving processes such as cross-linkage induction, nucleotide substitution and adduct formation. Mussels (*Mytilus galloprovincialis*) are good indicator organisms for identifying environmental changes of unknown origin resulting from mixtures of contaminants, e.g., industrial effluents (VUKMIROVIĆ *et al*., 1994). Earlier studies confirm that *Mytilus* spp. is a useful organism for studying pollution (BAYNE, 1976; GOSLING, 1992). In the present study, DNA strand breaks were measured in mussel gills by the Fast Micromethod®.

There is scarcity of data regarding the quality of the marine environment along the Croatian Adriatic coast. Some areas are influenced by urban, industrial or harbor wastes, therefore toxic and genotoxic effects on the marine environment can be expected. Such so-called "hot spots", as well as presumably pristine waters in protected areas, were monitored from August 1998 to October 1999 under a national monitoring program. Preliminary results are presented in this paper. Analyses for contaminants were not performed since, due to complex biological responses to mixed pollution, they would reveal nothing about potential adverse effects of the water quality. Our approach in this study was to collect information on water quality in "hot spots" along the Adriatic coast of Croatia that would allow us to focus on chemical analysis in the future.

MATERIALS AND METHODS

Sampling sites

Seawater quality along the Adriatic coast of Croatia was evaluated at 16 sites in protected (sites 1,2), harbor (sites 3,4,5,6,16), urban (sites 7,8,9,10,11,13), industrial (site 12) or brackish (site no 14,15) areas (Fig. 1). Seawater samples (30 l) and mussels (5 individuals) were collected from each site in August 1998, August 1999 and October 1999.

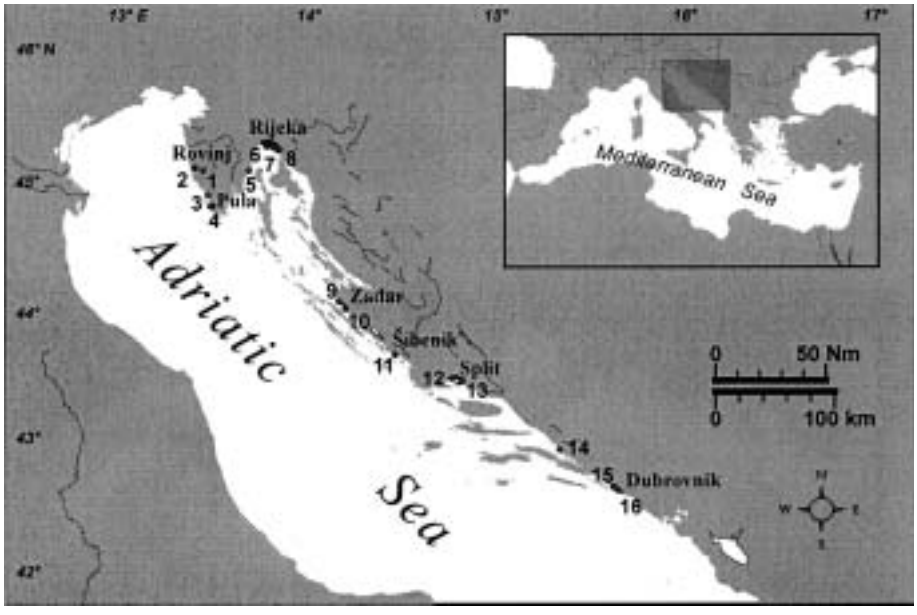

Fig. 1. Sampling sites along the Adriatic coast, Croatia

Seawater extraction

Non-polar compounds from seawater samples (30 l) were concentrated at the time of collection on amberlite XAD-7 resin columns (20 ml) by gravity at a flow rate of 200 ml min^{-1} and transported to the laboratories of the Center for Marine Research in Rovinj. Adsorbed compounds were eluted with 150 ml acetone, evaporated until nearly dry and extracted with 70 ml dichloromethane. Dichloromethane extracts were evaporated to dryness, dissolved in 300 μl dimethylsulfoxide (DMSO) and stored at -20°C prior to toxicity (Microtox test) and genotoxicity (*umu*-test) assays. 12.5 μl of total extracts (300 μl), diluted in 0.5 ml 2% saline, were used as starting sample concentrations for the toxicity assay while 20 μl aliquots were used in the genotoxicity assay. Higher amounts were not tested due to possibly false positive results induced by the solvent (DMSO).

Tissue preparation

Gills of mussels *Mytilus galloprovincialis* Lmk. (Mollusca: Bivalvia) were removed from 10-g mussels at the place of collection, stored 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. The pellets were collected in test tubes and stored at -80°C.

Microtox test

The luminescence of marine bacterium *Vibrio fischeri* NRRL B-11177 (DIN EN ISO 11348-3, BioFix® Lumi, MACHERY-NAGEL, Düren, Germany), exposed to a series of dilutions with water extracts, was measured in a PACKARD Tri-Carb 2500 TR liquid scintillation analyzer for toxicity assay (Microtox test). Briefly, 0.5 ml of a 2% saline suspension containing 10^6 exponentially growing luminescent bacteria was diluted (1:2) with 0.5 ml of extracted sea water, starting with 12.5 μl extract in 0.5 ml 2% saline. Eight dilutions corresponding to 1250, 625, 312, 156, 78, 39, 20 and 10 ml of sea water and a saline blank were tested. Equivalent volumes (ml) of sea water for each dilution were obtained from the equation: Equivalent volume of sea water (in ml) = original volume of water collected (30 l) x volume of extract (in μl) total extract volume (300 μl), where volume of extract = volume of extract in starting dilution (12.5 μl)/dilution factor. After 15 min incubation in the extract, the bioluminescence of the bacteria was measured, compared to the blank samples and corrected for spontaneous decline in photoactivity. Results are expressed as ml of sea water that caused a 50% reduction of bioluminescence in 15 min (EC_{50}). Estimates of EC_{50} were obtained using linear regression analysis (SCHIEWE *et al*., 1985; BIHARI *et al*., 1989). 2.5 $\mu g ml^{-1}$ 4-nitroquinoline-N-oxide reduced bacterial bioluminescence by 50% and was, therefore, used as a positive control. The toxic potential of seawater samples was reported in cases where less then 1250 ml of seawater caused the same effect.

SOS/*umu*-test

The SOS/*umu* test was performed according to ODA *et al*. (1985) and HAMER *et al*. (2000) with a slight modification. Briefly, an overnight culture was diluted 50-fold with TGA medium and incubated at 37°C until the bacteria reached the exponential growth stage. The incubation mixture consisted of 800 μl bacterial culture, 180 μl 0.1 M phosphate buffer pH 7.4 and 20 μl of the seawater sample. After 2 h incubation at 37°C, the bacterial suspension was diluted 5-fold with warm TGA medium and incubated at 37°C for an additional two hours. Bacterial growth was measured as turbidity at 600 nm and as the level of ß-galactosidase activity at 420 nm, using *o*-nitrophenyl-ß-D-galactopyranoside as the substrate (MILLER, 1972). Genotoxic activity was expressed as the ratio of induction of enzyme units in the tested sample to induction of enzyme units in the control, which contained only DMSO. Induction ratios above 1.5 are considered sufficiently positive by DIN

and ISO standards. Genotoxicity results are expressed as ml of sea water that resulted in an induction ratio of at least 1.5. Six serial dilutions (1:2) with seawater extracts corresponding to 2000, 1000, 500, 250, 125, and 63 ml of sea water and the blank (DMSO) were tested. As a positive control, 30 $ngml^{-1}$ 4-nitroquinoline-N-oxide was used.

Fast Micromethod®

The DNA integrity assay (Fast Micromethod®) detected changes in the pattern of DNA denaturation. Directly altering DNA integrity in mussel gills lysates at alkaline pH and the time dependence were measured 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). 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 every 30 sec for at least 20 min. The number of strand breaks in DNA of mussel gills was expressed as strand scission factors (SSF) and calculated as: $SSF = \log_{10}(\%\ dsDNA_{site\ x\ sample}/\%\ dsDNA_{site\ 1\ sample})$ after 5 min denaturation. DNA integrity was expressed as the increase in number of DNA strand breaks, relative to the effect of 1 μg 4-nitroquinoline-N-oxide/g mussel, considered 1.0 (SSF = 0.17).

RESULTS

Forty-eight seawater samples taken along the Adriatic coast of Croatia were analyzed for toxic and genotoxic potential. Two hundred and forty mussels were analyzed for DNA integrity (5 mussels per sampling per site). The results for all samples are shown in Table 1. Sampling sites were grouped according to characteristic contamination into five groups: protected, harbor, urban, industrial and brackish areas. The toxicity, genotoxicity and DNA integrity results did not follow the grouping according to type of contamination.

DISCUSSION

Toxicity, genotoxicity and DNA integrity differed with time of collection and site. Since significant recovery of pollutants can be achieved by concentrating seawater on XAD columns (PAOLA *et al.*, 1993), we used this procedure to analyze toxicity in up to 1250 ml and genotoxicity in up to 2000 ml of sea water. According to the Microtox assay, 75% of the analyzed samples were potentially toxic. According to the SOS/*umu*-test, 40% of the samples were potentially genotoxic. Alteration of DNA integrity in mussel gills was detected in 40% of the samples with the Fast Micromethod®. Half of the affected mussel samples were collected in October 1999.

Correlation analysis between toxicity, genotoxicity and changes in DNA integrity was performed. Values that were not toxic at the highest concentration tested were excluded from the calculation and the correlation assessment was limited to samples with quantifiably definable endpoints. No correlation (toxicity vs genotoxicity $r^2 = 0.029$, n = 11; toxicity vs DNA integrity $r^2 = 0.003$, n = 31; genotoxicity vs DNA integrity $r^2 = 0.11$, n = 18) was observed, which can be explained by the collective effect of the pollutants that differed from site to site (BEG *et al.*, 2001). Further, water genotoxicity data depend on sampling time and site which are influenced by currents, winds, tides, frequency of wastewater inputs, etc., while DNA integrity in mussel gills reflects the acute and chronic effects of exposure. This may be the reason that there was no correlation between genotoxicity and DNA integrity.

According to the Microtox test, extractable organic compounds of 12 seawater samples (from sites 1,2,6,7,9,10 in August 1998, 5, 7, 8, 15 in August 1999 and 2,12 in October 1999)

Table 1. Toxicity, genotoxicity and DNA integrity data for sampling sites along the Adriatic coast of Croatia

Site type	Site No	Toxicity[1]			Genotoxicity[2]			DNA integrity[3]		
		Aug 98	Aug 99	Oct 99	Aug 98	Aug 99	Oct 99	Aug 98	Aug 99	Oct 99
Protected	1	>1250	208	547	>2000	890	896	0.0	0.0	0.1
	2	>1250	60	>1250	387	968	953	1.0	0.7	2.6
Harbor	3	184	154	56	>2000	>2000	>2000	1.3	0.3	0.3
	4	625	76	763	>2000	>2000	416	1.1	0.6	2.5
	5	1000	>1250	++	>2000	>2000	209	0.6	0.3	2.1
	6	>1250	52	187	>2000	>2000	1466	2.0	1.3	2.4
	16	123	103	226	89	>2000	>2000	0.7	0.8	2.5
Urban	7	>1250	>1250	++	1461	>2000	>2000	1.8	0.1	2.4
	8	++	>1250	++	105	>2000	>2000	0.6	1.0	0.7
	9	>1250	572	1243	>2000	>2000	444	0.4	1.5	2.1
	10	>1250	817	73	96	>2000	>2000	0.0	0.7	3.4
	11	718	1200	1080	>2000	>2000	762	2.3	1.4	1.8
	13	131	617	112	>2000	>2000	976	0.6	1.5	2.6
Industrial	12	297	749	>1250	107	>2000	>2000	0.8	0.1	3.0
Brackish	14	325	1100	863	87	>2000	>2000	0.7	0.6	2.2
	15	869	>1250	++	>2000	>2000	888	0.2	1.2	0.5

[1] Microtox test: ml sea water that caused a 50% reduction of bacterial bioluminescence in 15 min (EC_{50}). ++ = increase in bacterial luminescence

[2] *umu*-test: ml sea water that caused a 1.5-fold increase in bacterial ß-galactosidase activity compared to control samples

[3] Fast Micromethod®: increase in number of DNA strand breaks in mussel gills, relative to the effect of 1 μg/g NQO, considered 1.0

were not potentially toxic (EC_{50} >1250 ml). The highest toxicity (EC_{50} <100 ml) was detected at sites 2, 4, 6 in August 1999 and 3, 10 in October 1999 while bacterial luminescence increased in five samples (site 8 in August 1998 and 5,7,8,15 in October 1999). Unexpectedly, the extracts from protected areas were highly toxic, raising questions regarding the pristine status of protected waters that could serve as reference or control sites. Toxicity testing could be improved by determining the total organic material content commonly present at each sampling site.

According to the SOS/*umu*-test, extractable organic compounds from less than 100 ml of sea water that induced a 1.5-fold increase in ß-galactosidase activity were detected in three samples (sites 10,14, 16 in August 1998). For comparison, 160 ml of waste-water taken near a spill from a tobacco factory, and viewed as industrial pollution, caused more than a 1.5-fold increase in ß-galactosidase activity (HAMER *et al*., 2000). In August 1999,

only two samples (sites 1,2) were genotoxic but two months later, in October 1999, eight samples (sites 1,2,4,5,9,11,13,15) contained sufficient genotoxins in less than 1000 ml of sea water to be genotoxic. Such irregular and weak mutagenic effects of seawater samples on *Salmonella typhimurium* have already been reported (PAOLA *et al.*, 1993).

The gills of 240 mussels were examined for DNA integrity. DNA was altered in mussel gills collected at five sites (3,4,6,7,11) in August 1998 and five sites (6,9,11,13,15) in August 1999. An increase in the level of DNA damage two months later (October 1999) at 12 of the 16 sites indicates seasonal influences on DNA integrity in mussel gills. DNA damage in samples from sites 6 and 11 was detected at all three sampling times. It appears that the input of contaminants at those sites was stable throughout the study period. The cause(s) of the variability in DNA damage related to a specific site or time of mussel collection could not be determined by this study.

Our findings, although preliminary, show the potential usefulness of toxicity, genotoxicity and DNA integrity testing for seawater quality assessment. They enhance the necessity for further and more frequent monitoring that is currently being undertaken in our laboratory. More frequent monitoring will allow better statistical analysis and we expect that variations in toxicity, genotoxicity and DNA integrity data among sampling areas will differ significantly from the variations at the same sampling sites.

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Kvaliteta morske vode uzduž jadranske obale bazirane na osnovi rezultata toksičnosti, genotoksičnosti i cjelovitosti DNA

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SAŽETAK

Uzorci morske vode i škrge školjkaša uzimani su sa 16 lokacija zaštićenog i bočatog područja, kao i s područja s urbanim, industrijskim i lučkim utjecajem uzduž jadranske obale. Za određivanje toksičnosti koristio se Microtox test, za određivanje genotoksičnosti SOS/*umu* - test, te za određivanje cjelovitosti DNA molekule tzv. Fast Micromethod® test. 75% analiziranih uzoraka vode pokazalo je toksičnost, 40% uzoraka vode pokazalo je genotoksičnost, dok je 40 % uzoraka škrga dagnji pokazalo oštećenje DNA.

Ključne riječi: Toksičnost, genotoksičnost, oštećenje DNA, Microtox, SOS/umu-test, Fast Micromethod®