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Toxic and Genotoxic Activity of Water Samples from the River Ljubljanica

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Water pollution by organic micropollutants is one of the most critical problems facing resources of drinking water, and in the realm of environmental protection of water as a whole. It is extremely difficult to quantify the risks associated with xenobiotics in general, because they usually occur in concentrations too low to allow analytical determination, and often the single or combined biological effects for most of the micropollutants are unknown. The best way of approaching the problem of risk assessment is to use biological tests that give a global response to the pool of micropollutants present. Testing the toxicity to aquatic organisms and genotoxicity to bacteria can be recommended for simple and effective initial screening. If the biological tests can be accomplished in conjunction with the chemical characterisation of toxic pollutants, the effectiveness of measures for pollution reduction will be improved.

Many studies have reported on the mutagenicity of river water (Sayato et al., 1987, 1990, Pelon et al., 1977, Kool et al., 1981), lake water (Hartlein et al., 1981) and groundwater (Tye, 1986). Usually the mutagenic effect of the water was only revealed after concentration of the sample. Genotoxic substances are concentrated using XAD-2 column extraction, solvent extraction or lyophilisation (Sayato et al., 1987, Kool et al., 1981).

In the present study XAD-2 column extraction was used to isolate a fraction of the organic micropollutants in samples from the Ljubljanica river. Extracts were tested for toxicity with the Microtox assay, and for genotoxicity with the modified Ames test including a pre-incubation step (Maron and Ames, 1983). Two strains of Salmonella typhimurium were used in the mutagenicity tests. Strain TA98 detects mutagens inducing frameshift mutations and strain TA100 detects mutagens inducing base pair substitution mutations. The responses of the two strains to treatment with the samples in the absence or presence of exogenous metabolic activation were the basis for determining the toxic profile of each sample, yielding rough information on the kind of mutagens present in the sample.

MATERIALS AND METHODS

Samples were taken from the River Ljubljanica at four sites in spring 1992 and autumn 1992. Sampling site 1 was at the spring where the Ljubljanica rises. Sampling site 2 was

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at the confluence of the Ljubljanica and the River Borovniščica, which contains contaminants from the leather and chemical industries. Sampling site 3 was at theapproach to the city of Ljubljana, and sampling site 4 was just downstream of the city where contamination from different industries (chemical, food and feed industry, etc.) and domestic discharges can be expected. The samples taken on each occasion for toxicity and mutagenicity testing were 10 litres in volume. Each sample was transported to the laboratory within two hours, where it was chilled to 4°C, filtered through a glass filter, and stored at -18øC until extraction. Simultaneously, appropriate samples were taken for chemical analysis.

The chemical parameters to be examined in addition to the results of biological tests were chosen according to Slovenian regulatory requirements regarding the control of surface and wastewaters (Official Gazette of the SFRY, 1978). Most were determined according to Standard Methods for the Examination of Water and Wastewater (Standard Methods, 1992). The fixed solids ignited at 500øC (2540 E) and the total suspended solids dried at 103-105øC (2540 D) (Standard Methods, 1992) were measured. Chemical oxygen demand (COD) was determined titrimetrically (5220 B) (Standard Methods, 1992). Biochemical oxygen demand (BOD) was determined with a 5-day BOD test (5210 B) (Standard Methods, 1992). Concentrations of ammonia (4500-NH3 C), nitrites (4500-NO2 B) and nitrates (4500-NO3 B) were determined spectrophotometrically (Standard Methods, 1992) on an LKB-Ultrospec II spectrophotometer. Sulphate concentration was determined titrimetrically using barium perchlorate and thorin indicator (Merck, 1974). Cyanide concentration was determined spectrophotometrically with a standard solution of pyridine barbituric acid and an LKB-Ultrospec II spectrophotometer. The anionic surfactants were determined as MBAS (5540 C) (Standard Methods, 1992), and cationic surfactants were determined according to the method described by Merck which is suitable for determination of cationic surfactants in concentrations from 0.025 to 0.20 mg/l, using cetylmethylammonium bromide (Merck, 1974). Phenol levels were determined by the liquid-liquid extraction gas chromatographic method (6420 B) (Standard Methods, 1992). Mineral oils were analysed by gas chromatography using a Hewlett Packard HP 5890 Series II on an HP-1 capillary column with an FID detector. To measure metal levels, water samples were concentrated by evaporation and then subjected to flame atomic absorption spectrometry on a Varian AA575 spectrophotometer (3111 B) (Standard Methods, 1992). Trihalomethane levels were determined directly by heating the water samples in sealed bottles in a Head Space Sampler HP 19395 with direct introduction of volatile compounds into a HP 5890 gas chromatograph with capillary column and ECD detector. Polyaromatic hydrocarbons and triazines were extracted from the water samples with dichloromethane, cleaned, and separated with an HPLC with UV detector. Comparative analysis was performed by mass spectroscopy on an HP 5889 Series II gas chromatograph with an MSD detector. Hexane was used to extract pesticides from water prior to their analysis on a Varian 3700 gas chromatograph equipped with an ECD, Ni63 detector. The analysis was carried out on fused silica capillary columns (i.d. 250 nm), with an SE-S4 stationary phase and using nitrogen as the carrier gas (Pesticide Analytical Manual, 1982).

Surface waters are classified into two groups and four quality classes by the Official Gazette of the SFRY (1978) on the basis of maximum permissible levels (MPL) of pollutants allowed in water as given in Table 1. The first group containing class I and class II surface waters, comprises waters that may be directly or after conditioning used for drinking or in food processing. The second group (class III and class IV surface

waters) comprises water for other purposes after appropriate conditioning. According to this regulation, on the basis of chemical analysis, summation of toxicity was calculated as:

$$TOX = Ca/La + Cb/Lb + ... + Cn/Ln < 1$$

where Ca, Cb,...Cn are the concentrations of the different toxic chemicals in the surface water and La, Lb,...Ln are the respective MPL concentrations (Official Gazette of the SFRY, 1978).

Dissolved organics were removed from the water by adsorption on XAD-2 resin (Sigma) and elution using acetone. This was carried out by first passing 10 L of pH 7 filtered raw water through 20 cm^3 of XAD-2 resin (the diameter of the column was 1.4 cm, the height of the column was 13 cm), and then passing it through a second XAD-2 column at pH 2. Adsorbed organics were removed from each resin column using 200 mL of acetone, which was evaporated to a small volume at 40°C under reduced pressure, and finally to dryness under a nitrogen stream. The dry residue was dissolved in 1 mL of dimethylsulfoxide (DMSO) and then diluted with distilled water to a volume of 10 mL (1000-fold concentration). The same procedure of extraction was performed with distilled water (as a control) to assess if the method of extraction per se produced toxic or genotoxic substances.

The toxicity of the water extracts was determined by Microtox assay. Microtox assay uses freeze-dried cultures of the bioluminescent bacterium Phosphobacterium phosphoreum (Bulich, 1986). The assay is based on the measurement of the inhibition of natural light emission from luminescent bacterium when challenged by a toxic compound. The analysis was carried out with a Microtox Model 2055 Toxicity Analyser System (Beckman Inc., Carlsbad, CA). The assay procedure was as described by Bulich et al. (1981). Sample extracts were primary diluted five-fold with Microtox diluent. Further dilution was made so that 12, 25, 50 and 100 times concentrates of raw samples were tested. The toxicity endpoint was measured as the effective concentration (EC) producing toxic effect. EC values were computed by an equation provided by the manufacturer after calculating Γ (a ratio of light loss to the light remaining). EC50 was determined from the concentration factor corresponding to a 50% normalised light loss. Phenol was used as a positive control of the performance of the Microtox assay.

Mutagenicity was measured with a modified Ames test using bacterial strains of Salmonella typhimurium TA98 and TA100 with and without exogenous metabolic activation (S9 mix). The plate incorporation procedure was performed using a pre-incubation step. The bacterial suspension was prepared by overnight growth in nutrient broth Oxoid No2. To 100 µL of bacterial suspension, 200 µL of appropriately diluted sample extract was added. In the case of metabolic activation, 500 µL of S9 mix was also added. The mixture was incubated on a rotary shaker for 90 min at 37°C. At the end of the incubation period, 2 mL of 0.6% top agar containing a limited quantity of histidine was added, and poured on minimal agar plates. Three concentration points per sample were tested ranging from 50 to 200 µL/plate. The positive control for testing without activation was N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma), and for testing with activation benz(a)pyren (Sigma). Two plates per concentration point were used, and at least two independent tests were performed on each sample. Mutagenicity is expressed as the mutation factor (Q) which is the ratio between the number of revertants in the presence of the sample and the number of spontaneous revertants. For clear positive response two criteria were taken into account: dose response and a more than two-fold increase of the

number of revertants in the presence of the sample over the number of spontaneous revertants (Q > 2) in at least one concentration.

RESULTS AND DISCUSSION

None of the chemical parameters measured exceeded the maximum permissible level (MPL) for a contaminant. The results are shown in Table 1.

Table 1. Results of chemical analyses of River Ljubljanica water samples taken at defined sampling sites in spring 1992 and autumn 1992

Parameter	Spring samples				Autumn samples				MPL for water classes *	
	1	2	3	4	1	2	3	4	I and II	III. and IV
pH	7.9	8.0	8.0	8.0	7.9	7.8	8.0	7.7	6.8-8.5	6.0-9.0
fixed sol. mg/L	98.0	85.0	114.5	134.0	94.6	108.2	127.9	114.7	n.r.	n.r.
tot.susp.s.(mg/L)	<0.1	0.1	0.2	0.5	2.2	6.4	9.2	5.2	8.50	9.00
COD (mg O2/L)	8.7	17.6	21.9	17.5	7.9	6.5	5.8	9.5	10.00	20.00
BOD (mgO2/L)	4.0	4.0	6.0	12.0	1.2	2.6	1.6	9.0	2.00	7.00
NH_3 (mg N/L)	0.02	0.02	0.02	0.02	0.80	0.41	0.16	1.17	0.01	0.10
NO_2 (mg N/L)	0.001	0.026	0.028	0.120	0.001	0.007	0.066	0.026	0.05	0.50
NO_3 (mg N/L)	1.6	1.6	2.0	2.0	1.7	1.7	1.8	1.9	10.00	15.00
SO_4 (mg/L)	5.0	10.0	15.0	10.0	8.4	11.0	14.0	10.1	n.r.	n.r.
surf.cat.. (mg/L)	0.10	0.10	0.40	0.40	0.10	0.10	0.10	0.10	n.r.	n.r.
surf.an. (mg/L)	0.05	0.18	0.05	0.10	0.21	<0.05	<0.05	0.25	n.r.	n.r.
min. oils (mg/L)	1.00	5.00	5.00	8.00	5.00	6.00	15.00	16.00	n.r.	n.r.
atrazine (µg/L)	0.010	0.010	0.022	0.052	0.010	0.018	0.050	0.019	n.r.	n.r.
PAH (µg/L):	0.500	0.450	0.778	0.858	0.045	0.059	0.597	0.111	0.20	n.r.
fluorene (µg/L)	<0.005	<0.005	0.055	0.077	n.d.	0.012	0.180	0.052	n.r.	n.r.
phenanthrene(µg/L)	0.250	0.290	0.554	0.525	0.010	0.009	0.150	0.042	n.r.	n.r.
anthracene(µg/L)	0.080	0.012	0.075	0.062	0.010	<0.005	<0.005	<0.005	n.r.	n.r.
benz(a)pyrene(µg/L)	0.125	0.149	0.259	0.288	0.016	n.d.	0.006	0.007	n.r.	n.r.
benz(a)anthra(µg/L)	0.022	0.025	0.028	0.029	n.d.	<0.005	<0.005	<0.005	n.r.	n.r.
chrysene(µg/L)	0.010	0.018	0.011	0.010	0.010	0.018	0.011	0.010	n.r.	n.r.
metals(mg/L)Al	0.100	0.050	0.500	58.000	0.528	0.284	1.455	0.054	n.r.	n.r.
Ba	0.040	0.040	0.050	0.040	0.500	0.440	0.480	0.920	1.00	4.00
Pb	0.012	n.d.	0.021	0.071	0.059	0.060	0.079	0.040	0.05	0.10
Ni	0.003	<0.001	0.008	0.021	0.001	<0.001	<0.001	0.004	0.05	0.10
Fe	0.088	0.005	0.466	0.525	0.050	0.085	<0.005	0.068	0.30	1.00
Zn	0.081	0.005	0.023	0.030	0.010	0.021	<0.005	0.050	0.20	1.00
Sn	0.020	0.080	0.070	0.070	0.050	0.005	0.040	0.020	n.r.	n.r.
Calculated TOX:										
PAH's	2.47	2.53	3.88	4.32	0.22	0.20	1.98	0.55		
metals w.cl. I, II	1.09	0.13	0.95	0.75	0.32	0.59	0.87	0.36		
metals w.cl.III,IV	0.55	0.05	0.26	0.56	0.15	0.16	0.17	0.14		

Trihalomethanes, chlorinated pesticides, phenols, cyanides and of metals beryllium, cadmium, chromium, mercury and silver were below detectable limits.

* - MPLs as regulated in Slovenia (Official Gazette of SFRY, 1978)

n.r.- MPL not regulated,

n.d.- not defined

The levels of BOD, nitrites and mineral oils increased from sample 1 to sample 4 with the exception of the BOD of sample 3 taken in autumn. The BOD/COD relation indicates that the Ljubljanica gradually receives more and more wastewater from domestic discharges and, in the last sample, from the food processing industry. The concentration of PAHs (with the exception of chrysene) was significantly higher during the spring due to atmospheric fall out during the winter heating months. In all spring samples the total content of PAHs exceeded the regulatory value.

Unconcentrated water samples were not toxic in a Daphnia magna test, and in the Microtox assay none was mutagenic in S. typhimurium TA98 or TA100 (data not shown).

The toxicities of sample extracts are shown in Table 2. Comparing spring and autumn samples, the neutral fractions of spring samples are less toxic (except sample 2) than autumn samples. In the acid fraction the differences are less marked, but the autumn samples are less toxic (except sample 1). In most toxicity studies involving water pollutants, the neutral fractions are usually the only fractions tested (Galassi et al., 1992). The present study suggests that both neutral and acid fractions should be tested to cover a broader range of toxicants present in water. The Microtox assay can only give information about the presence of toxic substances. In the case of toxic response, further chemical analysis should be performed to determine the exact toxic components.

Table 2. Toxicity of extracts of water samples from River Ljubljanica river determined by Microtox assay.

	Spring samples				Autumn samples			
Fraction:	1	2	3	4	1	2	3	4
Neutral	>100*	29.15	95.75	86.47	44.49	25.70	>100*	24.97
Acid	>100*	29.46	27.42	26.26	56.29	47.74	55.29	67.91

EC50 is expressed for magnitude of concentration of the original sample
* no toxic effect was observed at maximum concentration (X 100)

Mutagenicity results are shown in Figure 1. Six of the eight water samples contained mutagenic chemicals in detectable concentrations. The difference in the mutagenic responses of the two strains to extract fractions of the samples and the difference in responses depending on the presence or absence of metabolic activation were the basis for defining the mutagenic profiles for each water sample. These profiles can be the basis for further chemical identification of micropollutants.

The mutagenic profiles of samples 1 and 3 taken in spring are similar. These two samples were not mutagenic. In sample 2 (Panel 2 in Figure 1), the acid fraction was highly mutagenic to TA100 with metabolic activation (Q=6.6), suggesting the presence of promutagens inducing base pair substitution. Further chemical analyses of this fraction are necessary to identify the chemicals responsible for the mutagenic response.

The main characteristic of sample 4 (Panel 4 in Figure 1) is the positive response of the neutral (Q=4.9) and acid (Q=3.2) fractions in strain TA100 without metabolic activation. In the spring sample, only the neutral fraction was positive (Q=2.3) according to the

criteria by which samples were judged. The acid fraction also gave dose response (data not shown) but the mutation factor did not exceed 2, indicating that this sample contained direct acting mutagenic chemical(s) causing base pair substitution mutations detected by strain TA100. This type of mutagenic response was not observed in other samples. Further chemical analysis of this sample may identify the mutagens and help trace their source.

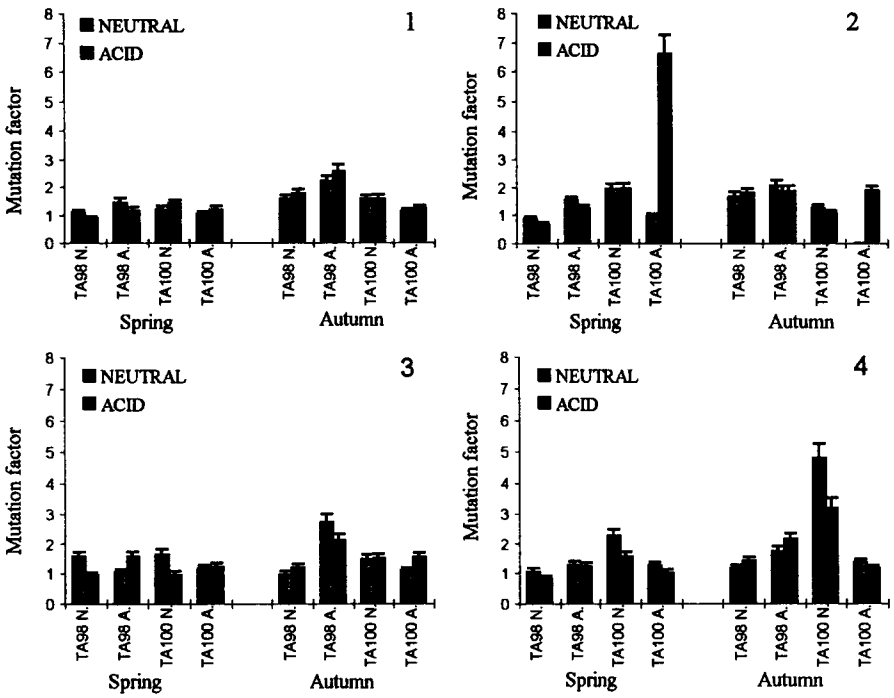

Figure 1. Mutagenic profiles of River Ljubljanica water samples. Panels 1 to 4 correspond to sampling sites 1 to 4. On each panel spring and autumn samples are shown. TA98 N and TA100 N indicate testing without metabolic activation, TA98 A and TA100 A testing with metabolic activation.

All four samples taken in autumn gave a weak positive response in strain TA98 with metabolic activation. In samples 1 and 3 both the neutral and acid fractions were mutagenic, in sample 2 only the neutral fraction, and in sample 4 only the acid fraction was mutagenic. In autumn, contaminants indirectly inducing frameshift mutation were present along practically the entire river. From the literature data, it appears surface waters and groundwaters often contain indirect mutagens detectable with S. typhimurium TA98 (Kool et al., 1989, Galassi et al., 1992, Doereger et al., 1992). Mutagenic activity of this type can be partly attributed to mutagens of natural origin, as it is also be observable in ground waters (Kool and van Kreyl, 1988).

None of the measured chemical parameters correlated with the results of the mutagenicity testing, and therefore no conclusion can be made as to which components specific mutagenicity could be attributed. The extraction with XAD-2 resin isolated only a fraction of the organic compounds present in the samples. In six of the eight samples, mutagenic compounds were isolated, but to identify these mutagenic compounds clearly, further chemical and biological investigations will be necessary. The biological tests indicate roughly where to search for specific pollutants that were responsible for the mutagenic effects.

The results show that the Ljubljanica is not polluted by high concentrations of single identified and regulated pollutants. None of the compounds gave a result exceeding MPL. Nevertheless, two samples showed specific unexplainable mutagenic activity and six of the eight samples contained mutagenic chemicals. This suggests that neither chemical analysis alone nor biological assays alone are able to give enough information for risk assessment of water pollution, but in conjunction it may be possible to determine the mutagenic and toxic components, track them and stop pollution at the source.

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