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THE PRESENCE OF GENOTOXIC SUBSTANCES IN THE RIVER DANUBE FIVE MONTHS AFTER BOMBARDMENT OF THE INDUSTRIAL ZONE OF PANČEVO AS REVEALED BY THE *ALLIUM* ANAPHASE-TELOPHASE GENOTOXICITY ASSAY

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The consequence of aerial bombardment of the petrochemical complex, oil refinery and chemical industry including the HIP "Azotara" fertilizer factory in the vicinity of Pančevo in April 1999 was that a large amount of dangerous chemicals leaked into the canal collecting industrial wastewater and flowed pouring out into the Danube. As the level of water in the Danube was high at that time, leaking of chemicals from the canal into the river lasted for a long time. Therefore the aim of this work was to check if toxic and genotoxic effects were present 5 months after the bombardment. Three samples of Danube water (upstream from the canal, just after the opening of the canal and downstream from the canal) were used for this purpose. All three samples plus a sample from the canal were also chemically analyzed. The Allium anaphase-telophase test was used to examine the toxicity and genotoxicity of water samples because it can be used without any concentration or purification of samples before testing. Clear inhibition of growth, compared to the control sample, was produced by the second sample. This sample, taken just after entry of the canal was the only one which produced a statistically significant increase in the number of chromosome aberrations in comparison with the control sample ($X^2_{(1)}=10.7, p<0.001$). The results of our test and the chemical analyses of the water samples, show that both toxic and genotoxic effects of the Danube water may occur due to water coming in from the canal even 5 months after the chemicals had leaked into the canal.

Key words: genotoxicity, toxicity, Allium anaphase-telophase test, water samples, chemical analyses, Danube

INTRODUCTION

Pančevo is an industrial town located 15 kilometers from the capital city Belgrade. The consequence of the bombing of the petrochemical complex, oil refinery and chemical industry near Pančevo including the - HIP "Azotara" fertilizer factory in April 1999 was that a large amount of dangerous chemicals entered into the canal collecting industrial wastewater. This canal flows pouring out into the Danube and there is no weir at its end.

From the southern industrial zone of Pančevo, through the wastewater canal the Danube was loaded with large amounts of toxic materials: 200 t of ammonia, 1,000 t of 1,2-dichloroethane (EDC), 70 t of 33% hydrochloric acid, 0.2 t of mercury, 40 t of sodium hypochlorite and 85 t of polyvinyl chloride (PVC). Around 62 t of crude oil and other derivatives burned for the most part or leaked into the canal (37,700 t of crude oil, 6,200 t of different types of petrol, 350 t of diesel fuel, 7,480 t of heating oil, 6,600 t of heavy vacuum oil, about 230 t of liquid petroleum gas, 80 t of benzene, 1,200 t of kerosene and 1,900 t of other derivatives and unspecified products mixed with hundreds of kg of substances for fire extinction). After the bombing, undetermined amounts of ammonium nitrate and mono ammonium phosphate as well as other raw materials from the fertilizer factory (NPK plant) were flushed into the wastewater canal due to the heavy rain.

Owing to the bombing, the wastewater treatment plant in Pančevo's southern industrial zone stopped working. It is located in the petrochemical complex and wasdesigned to treat wastewater mechanically, chemically and biologically before its release into the canal. This plant released 98,229 m^3 of wastewater from the petrochemical complex and 69,761 m^3 of wastewater from the oil refinery into the canal. This water contained hazardous and harmful substances from these factories (Tanasković et al., 1999; Tošović, ed., 2000).

As the level of water in the Danube was high at that time the leaking of chemicals from the canal into the river lasted for a long time, so we decided to check if the toxic and genotoxic effects were still present 5 months after the bombardment.

In recent years increasing concern about the introduction of mutagenic substances into water has resulted in the development of tests to assess the potential effects of these aquatic pollutants on public health. A number of studies have shown the presence of genotoxic compounds in waste water (Rannug et al., 1981; Al-Sabti and Kurelec, x1985; McGeorge et al., 1985; Metcalfe et al., 1985; Somashekar, 1987; van der Gaag et al., 1990; Doeger et al., 1992; Houk, 1992; Rank and Nielsen, 1993; Nielsen and Rank, 1994; Odiegah et al., 1997). Since it was introduced by Levan (1938) the *Allium* test has been used many times, not only for studying the effects of different chemicals but also for investigating the effects of complex chemical mixtures and samples of river and lake water. The common onion, *Allium cepa*, has proved to be an excellent material for short-term studies of genotoxic effects of environmental mutagens. In addition the *Allium* test combines two targets: general toxicity and genotoxicity. This test is especially suitable for testing water samples as it can be used without any concentration or purification of the samples before testing. Furthermore, the *Allium* test shows good correlation with mammalian test systems (Fiskesj, 1985). Therefore this test was recommended by the Royal Swedish Academy of Science (1973) and the GENE-TOX Program (Grant, 1982).

MATERIAL AND METHODS

Chemical analyses of water and sediments

Water and sediment samples for chemical analysis were collected on 15.10.1999 at four localities in the Danube (3 samples) and in the wastewater canal (1 sample):

I - Danube - 50 m upstream of the main (navigational) canal of the fertilizer factory -("HIP-Azotara") and petrochemical complex

II - Danube - 100 m downstream of the wastewater canal (small canal parallel to the main canal)

III - Danube - 1 km downstream of the wastewater canal of the "HIP-Azotara" fertilizer factory and petrochemical complex

IV - The wastewater canal of the southern industrial zone of Pančevo.

Water samples were taken 0.5 m under the surface and 0.5 m above the bottom, except in the wastewater canal where the sample was taken 0.5 m under the surface only. Sediment samples were dried and all results are expressed in mg or g per kg of dry matter.

Samples taken for physico-chemical examinations were analyzed using standard procedures approved by APHA (1980). All chemical analyses were performed in the laboratories of the Public Health Institute, Belgrade.

For testing for toxicity and genotoxicity three undiluted samples from the Danube (I, II and III) were used. The modified procedure of Fiskesj (1985, 1993) and Rank and Nielsen (1993) known as the *Allium* anaphase-telophase genotoxicity assay was employed. The test organism was the common onion *Allium cepa*. Onions weighing between 2-4 grams were selected for study.

Test procedure for general toxicity

Twelve onions were used for testing each sample and the control. The onions were grown at room temperature for five days. Each day fresh test solution was added. After five days the length of 10 root bundles in each test-group was measured (the two onions with poorest growth were eliminated). Exceptionally short or long roots were not measured. The mean root length for each sample was calculated.

Test procedure for genotoxicity

Six onions were exposed to each testi sample and the control. For the first 24 hours the onions were grown in fresh synthetic water and afterwards exposed for 48 hours to the samples of river water. Fresh samples were added each 24 hours. Synthetic water used as the control instead of tap water in which onion growth is normal. For chromosome preparations 5 onions per group were used (the one with the poorest growth was eliminated).

Root tips (10 mm in length) were fixed in acetic acid/HCl solution, heated and macerated. Five root tips (1-2 mm in length) from one onion were placed on each slide, stained with 2% orcein and squashed. Slides were coded and examined blind.

Chromosome aberrations were scored on slides after calculating the mitotic index. If the mitotic index was above 1% the slide was used for scoring aberrations. Aberrations taken into consideration were: bridges and fragments in anaphase and telophase but vagrant chromosomes, multipolarity and c-mitoses were scored also.

RESULTS

The results of the chemical analysis of water and sediment samples from the Danube and the wastewater canal are given in Tables 1 and 2.

Laboratory testing indicated that the concentrations of mineral oil, polycyclic aromatic hydrocarbons - PAHs, polychlorinated biphenyls - PCBs, 1,2-dichloroethane - EDC and mercury in the Danube water, as well as other parameters, did not exceed MPCs for class II river water. This does not hold for the values of physico-chemical parameters of the water in the canal (Table 1).

The consequences of pollution by mercury, oil and derivatives are evident in the sediment of the Danube and the canal (Table 2). Concerning interactions between the canal and the Danube, the canal is acting as point source of pollution of the Danube.

The degree of toxicity of water samples was assessed by means of root length values. Samples one and three did not show toxic effects. Clear inhibition of growth, compared to the control sample, was produced by sample 2 (Figure 1).

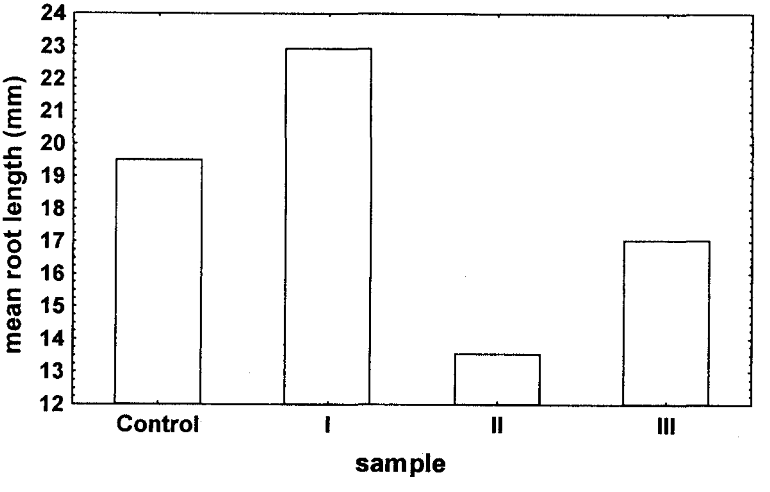

Figure 1. Mean root length in three water samples from the Danube (I, II, III) and control

Genotoxic effects are shown in Table 3. The percent of chromosome aberrations (Fig. 2) was the smallest in the upstream sample, even smaller than in the control. The second sample was the only one which produced statistically significant increase in the number of chromosome aberrations in comparison with control sample ($X^2_{(1)}=10.7$, $p<0.001$) as revealed using two-by-two frequency tables. However the upstream sample was significantly different from both the second sample ($X^2_{(1)}=11.19$, $p<0.001$) and the third sample ($X^2_{(1)}=4.60$, $p<0.05$). The second and the third samples did not differ significantly ($X^2_{(1)}=3.00$, n.s.).

Table 1. Results of analysis of physico-chemical parameters of water samples from the Danube (I, II, III) and wastewater canal (IV)

	I		II		III		IV
	1	2	1	2	1	2	1
Water temperature, °C	13.2	13.2	13.2	13.2	13.2	13.2	13.2
pH	7.7	7.7	7.9	7.9	7.8	7.8	8.6
Consumption of $KMnO_4$,mg/l	13.5	14.5	13.2	13.9	11.6	14.2	472.5
Elec. conductivity, μS/cm	461	433	428	421	417	419	718
Ammonium, mgN/l	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	13.97
Nitrite, mgN/l	0.030	0.031	0.046	0.037	0.034	0.037	0.912
Nitrate, mgN/l	1.58	1.36	1.36	1.36	1.36	1.13	1.36
Chloride, mgCl/l	26.8	27.5	26.8	28.2	25.4	26.1	3.5
Sulphate, $mgSO_4$/l	40.8	47.6	44.0	40.8	47.6	48.0	119.3
Phosphate, mgP/l	0.16	0.15	0.11	0.16	0.55	0.12	0.36
Cyanide, mgCN/l	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Mineral oil, mg/l	0.045	<0.005	<0.005	<0.005	<0.005	0.045	8.50

Table 1. (Continued) Results of analysis of physico-chemical parameters of water samples from the Danube (I, II, III) and wastewater canal (IV)

	I		II		III		IV
	1	2	1	2	1	2	1
Iron, mgFe/l	0.30	0.45	0.40	0.40	0.30	0.40	3.80
Manganese, mgMn/l	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.17
Lead, mgPb/l	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.03
Cadmium, mgCd/l	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Zinc, mgZn/l	<0.010	<0.010	<0.010	<0.010	<0.010	0.035	0.280
Nickel, mgNi/l	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.085
Chromium, mgCr/l	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	0.023
Mercury, mgHg/l	<0.005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	<0.0015
Arsenic, mgAs/l	0.002	0.002	0.002	0.002	0.002	0.002	0.004
Copper, mgCu/l	<0.005	0.008	<0.005	0.010	<0.005	0.024	0.074
1,2-dichloroethane, μg/l	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	97.5
Toluene, μg/l	2.8	<0.1	4.2	<0.1	9.7	4.5	12.9
Xylene, μg/l	2.8	1.2	5.9	<0.1	3.1	<0.1	42.8
PCB, μg/l	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.092
PAH, μg/l	0.02	0.01	0.04	0.02	0.01	0.02	133.84

1- samples taken 0.5 m under the surface
2- samples taken 0.5 m above the bottom

Table 2. Results of analysis of physico-chemical parameters of sediment samples from the Danube and wastewater canal

	I	II	III	IV
pH	7.2	7.3	7.2	8.0
Phosphorus,%	0.07	0.07	0.08	0.10
Nitrogen, %	0.14	0.10	0.17	0.13
Fluoride, mg/kg	188.9	194.53	219.57	248.85
Sulphate, mg/kg	<5	<5	<5	19.67
1,2-dichloroethane, mg/kg	<1.0	10.2	1.8	39.5
Toluene, μg/kg	1.8	5.6	4.2	14.7
Xylene, μg/kg	2.1	9.8	7.6	22.5
PCB, μg/kg	4.48	83.8	<1.0	344.4
PAH, μg/kg	155.8	1079.1	765.4	3094.2
Total hydrocarbons, μg/kg	229.8	1125.9	694.8	10251.2
Lead, mgPb/kg	25.4	26.10	54.6	27.62
Cadmium, mgCd/kg	<0.85	<0.87	<0.0	1.10
Zinc, mgZn/kg	139.4	147.90	185.25	149.17
Nickel, mgNi/kg	20.28	21.75	27.3	25.97
Chromium, mgCr/kg	21.12	19.57	31.17	15.47
Mercury, mgHg/kg	0.17	0.17	<0.19	0.66
Arsenic, mgAs/kg	5.07	6.09	7.80	3.31

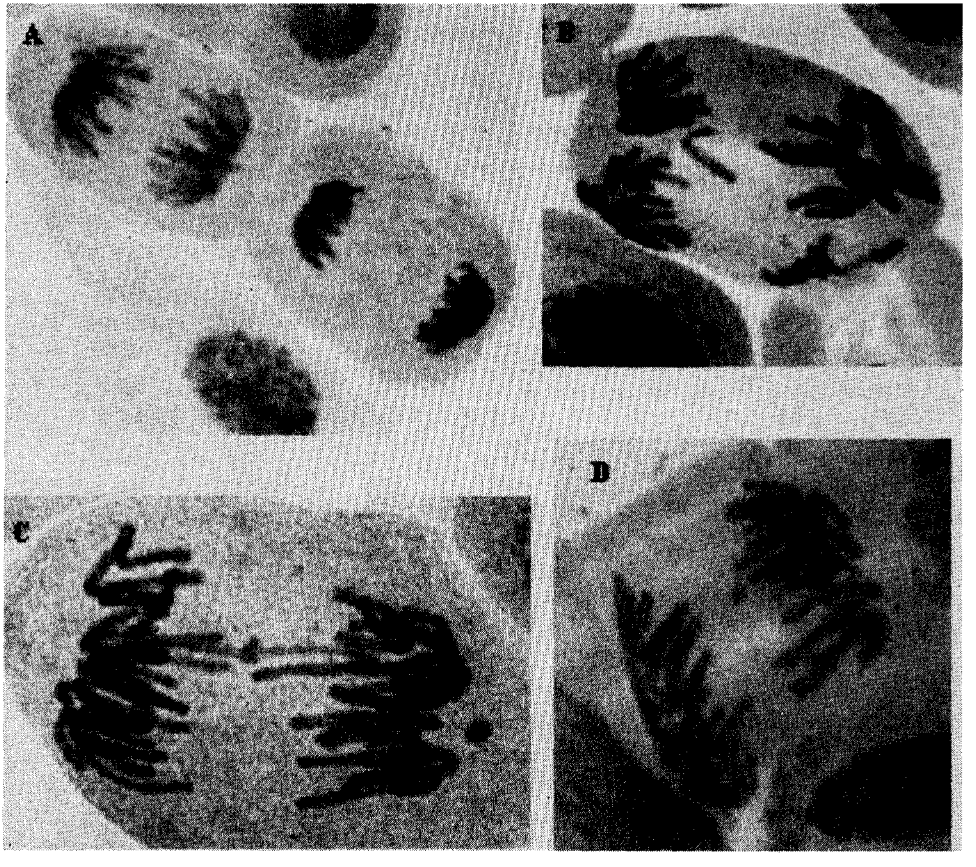

Figure 2. A - normal anaphase and early telophase; B - Multipolar anaphase; C - Anaphase bridge; D - anaphase fragment

Table 3. Results of cytological analysis. N – number of anaphases and telophases analyzed; * Other aberrations include: multipolar anaphases, vagrant chromosomes and polyploid cells

Sample	N	Bridges	Fragments	Other aberrations*	% of aberrations
I	687	1	-	-	0.15
II	1153	8	2	13	1.99
III	884	5	1	3	1.02
Control	888	2	-	1	0.34

DISCUSSION

The presence of chemicals with toxic and genotoxic effects in wastewater has been scored repeatedly (Houk, 1992). Rivers and lakes are commonly used as recipients of these waters so, depending on the amount of wastewater the same effects could be expected in lakes and rivers.

Tracing the source of contamination can be done by the *Allium* test on samples of river water. The shortest roots will occur in the samples located nearest to the damaging outlet (Fiskesj, 1985). The fate of toxicants in water cannot be predicted from knowledge of their single known effects because in the aquatic environment they make complex mixtures. Therefore, only testing samples of the polluted water could reveal the actual effects.

From the results of our tests it is clear that both toxic and genotoxic effects of the Danube water are caused by water coming from the canal even 5 months after the chemicals had leaked into the canal. The absence of these effects in upstream sample confirms this. Positive results obtained in the test should be considered as a warning of the risk present both for human health and for the water environment. With about 50,000 m^3 of contaminated sediment, the wastewater canal of the southern industrial zone of Pančevo, is a latent danger for aquatic ecosystems of the Danube and its flooded regions.

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ANALIZA PRISUSTVA TOKSIČNIH I GENOTOKSIČNIH SUPSTANCI U DUNAVU PET MESECI POSLE BOMBARDOVANJA INDUSTRIJSKE ZONE PANČEVA ALLIUM ANAFAZNO -TELOFAZNIM TESTOM

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SADRŽAJ

Kao posledica bombardovanja Petrohemijskog kompleksa, Rafinerije nafte i hemijske industrije Pančevo - HIP Azotara u blizini Pančeva u aprilu 1999. godine izlile su se velike količine opasnih hemikalija u kanal za industrijsku otpadnu vodu, a njime u Dunav. Pošto je u to vreme nivo vode Dunava bio visok izlivanje hemikalija u reku trajalo je dugo vremena, tako da je cilj našeg rada bio da se proveri da li su toksični i genotoksični efekti prisutni 5 meseci nakon bombardovanja. Za tu svrhu analizirana su tri uzorka Dunavske vode (uzvodno od kanala, na ušću kanala i nizvodno od kanala). Sva tri uzorka kao i uzorak vode iz kanala hemijski su analizirani. Za ispitivanje toksičnosti i genotoksičnosti uzoraka vode korišćen je *Allium* anaphase-telophase test zbog činjenice da se uzorci mogu ispitivati bez koncentrisanja ili prečišćavanja. Drugi uzorak pokazao je jasnu inhibiciju rasta korenčića luka u odnosu na kontrolni uzorak (toksični efekat). Isti uzorak je bio jedini koji je produkovao statistički značajan porast broja hromozomskih aberacija u poređenju sa kontrolom ($X^2{}_{(1)}=10.7$, $p<0.001$). Rezultati testiranja kao i rezultati hemijske analize pokazuju da su tokskični i genotoksični efekati u vodi Dunava poreklom iz vode koja dolazi iz kanala.