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The Application of the Umu Test System for Screening Mutagenicity of Surface Water

[Yüzey Sularının Mutajenite Taramasında Umu Test Sisteminin Uygulanması]

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ABSTRACT

The present study was conducted on the Meric delta which is highly polluted by the active industrial and agricultural wastes. The organic extracts in water were collected by the XAD-2 and XAD-4 resin column method at 3 locations (Meric, Ergene, Enez) throughout the year. Evaluation of the SOS inducing activity of the sample extracts was examined with the umu test system using *Salmonella typhimurium* TA1535/pSK1002 as a parental strain and *S. typhimurium* NM2009 (overexpressed O-acethyltransferase), *S. typhimurium* NM3009 (overexpressed nitroreductase and O-acethyltransferase) strains were used for the detection of the mutagenic nitroarenes.

The induction of the umuC gene expression we observed in the parental strain without S9 and in NM2009 by the extract of Ergene for the spring was high enough to define the sample as a weak inducer. The sample extract from Enez and Ergene stations caused an evident induction in all of the strains used for the summer samples but the high O-acetyltransferase activity in NM2009 and the high nitroreductase plus O-acethyltransferase activities in NM3009 did not significantly increase the genotoxic activation of the extract comparing to the parental strain, suggesting that the pollution in the Meric delta is not derived from nitroarenes but is due to the presence of other organic compounds. The extracts obtained from some of the stations for different seasons caused an evident decrease of the enzyme activity in all of the strains although there was a significant increase in the bacterial density. In view of these results we can say that the umu test can be a useful tool for genotoxicity assesment of surface water.

Key Words: mutagenicity, nitroarenes, surface water, umu test.

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ÖZET

Bu çalışma, endüstriyel ve tarımsal kaynaklı atıklar ile yoğun olarak kirletilen Meriç delta'sında yürütülmüştür. Yıl boyunca 3 yerden (Meriç, Ergene, Enez) alınan su örneklerinin organik ekstraktları XAD-2 ve XAD-4 reçinelerinin kullanıldığı kolon metoduyla toplanmıştır. Örnek ekstraktlarının SOS indükleme aktivitelerinin değerlendirildiği umu test sisteminde *Salmonella typhimurium* TA1535/pSK1002 atasal suş olarak, *S. typhimurium* NM2009 (O-asetiltransferaz enzimini normalin üzerinde ifade eden) ve *S. typhimurium* NM3009 (O-asetiltransferaz ve nitroredüktaz enzimlerini normalin üzerinde ifade eden) suşları mutajenik nitroarenlerin belirlenmesi amacıyla kullanılmıştır.

Baharda Ergene'den elde edilen ekstraktın NM2009 suşunda ve S9'suz atasal suşta neden olduğu umuC gen ifadesinin indüksiyonu, su örneğinin zayıf mutajen olarak tanımlanmasına yetecek kadar yüksekti. Yaz ayında Enez ve Ergene istasyonlarından alınan örnekler kullanılan tüm suşlarda belirgin bir indüksiyona neden olduğu halde NM2009 ve NM3009 suşlarındaki yüksek O-asetiltransferaz ve nitroredüktaz aktiviteleri atasal suşla kıyaslandığında ekstraktın genotoksik aktivitesini anlamlı bir şekilde yükseltmedi. Dolayısıyla Meriç deltasındaki kirliliğin nitroarenlerden değil diğer organik maddelerden olduğu ileri sürülebilir. Farklı mevsimlerde bazı istasyonlardan elde edilen ekstraktlar bakteri yoğunluğunu anlamlı biçimde arttırırken tüm suşların enzim aktivitesinde belirgin bir düşmeye neden oldu. Bu sonuçlar doğrultusunda, umu testinin yüzey sularının nitroaren kaynaklı genotoksisite değerlendirilmesinde uygun bir araç olabileceğini söyleyebiliriz.

Anahtar Kelimeler: mutajenite, nitroarenler, yüzey suları, umu test.

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INTRODUCTION

For humans, surface water is required as a source of drinking water, for agricultural activities, for industrial use and for sports and leisure as well. It is, there for, important to acquire knowledge on the quality of surface water. In urbanized areas the quality of surface water can be influenced by many toxic contaminants originating from industries, agriculture and private households. Toxic contaminants may disturb the biological condition of aquatic ecosystems and be harmful for humans. In the recent years, the several studies were published on the genotoxicity of the surface water in the various countries (1-3). The purpose of these investigations is to identify the pollution sources and to reduce the release of possibly hazardous compounds into the environment.

A number of short term bacterial test systems have been introduced for determining the genotoxic potential of aquatic ecosystems. Because of the complex chemical nature of aquatic ecosystems, standart chemical analyses are limited in their ability to characterize the chemical composition of the mixtures taken from the aquatic ecosystems and permit a subsequent chemical-specific genotoxicity assesment (4). The umu test that is specific for determining the mutagenic nitroarenes and is a potentially useful for screening the genotoxicity of waste waters is one of the bacterial test systems. Nitroarenes are widespread in the environment, because these chemicals are readily formed from products of incomplete combustion of polycyclic aromatic hydrocarbons and oxides of nitrogen (5,6). Some of them are reported to be potent mutagens and carcinogens (7,8). They are activated to mutagens by reduction to arylhydroxylamines by nitroreductase (NR) and then these arylhydroxylamines activated by O-acethyltransferase (O-AT) to form the ultimate reactive electrophiles, nitronium ions that bind to DNA and cause mutagenesis (9). Since nitroarenes are important risk factor for humans, the studies concerning their mutagenicity and genotoxicity are important. The umu test, developed by Oda et al (10) was based on the ability of DNA damaging agents to induce the expression of the umuC gene, which is also a SOS response gene. The tester strains used in the umu test carry an umuC'-'lac Z fusion gene on their chromosomes. In this strains, the structural gene for β-galactosidase activity, lacZ, is under the umuC control. The expression of the umuC gene, induced by DNA damage, is measured indirectly by the determination of β-galactosidase activity using a simple colorimetric assay. Umu test has been used extensively for monitoring the genotoxicity of the waste water in many countries (11-14).

This investigation is the first apply of the umu test to surface water in Turkey. In this study we aimed screening the genotoxic potential of the Meric delta which is the only water source in the region that used for irrigation. For that reason, it is thougt that determining the kind of pollution and the potential effects of pollutants and maintanence of water quality of the Meric delta will be important. The genotoxicity of the concentrated water samples were examined with the umu test system using S.typhimurium TA1535/pSK1002, NM2009 and NM3009 strains. The study was performed with and without S9 for the parental strain and without S9 for the other strains which do not require metabolic activation (15, 8).

MATERIALS AND METHODS

Chemicals, tester strains and rat liver S9 supernatant

The chemicals used were obtained from the following sources: Glucose-6-phosphate, NADP, o-nitrophenyl-β-D-galactopyranoside (ONPG), sodium dodecyl sulfate (SDS), 4-nitroquinoline-N-oxide (4-NQO) (CAS No:55-57-5; purity: >97%) from Sigma (USA); ampicillin and chloramphenicol from Fluka, dimethyl sulfoxide (DMSO) (CAS No: 67-71-0; purity: 99%), chloroform, β-mercaptoethanol, methanol (CAS No: 67-56-1; purity: ≥99,5%), acetone (CAS No: 67-64-1; purity: ≥99%) and dichloromethane (CAS No: 75-09-2; purity: ≥99,9%) from Merck; benzene (CAS No:71-43-2; purity: 99%), diethyl ether (CAS No: 60-29-7; purity: ≥95%) from BDH; Tris (hydroxymethyl) aminomethane and 3-methylcholanthrene (3-MCT) (CAS No: 56-49-5) from ICN (USA). Benzene was chosen as a solvent for 3-MCT. 4-NQO was used as a positive control for the parental strain without S9, NM2009 and NM3009 strains. 3-MCT (10) was used as a positive control for the parental strain with S9 because of restrictions on Aflatoxin B_1 import.

Salmonella typhimurium TA1535/pSK1002, NM2009 and NM3009 strains were kindly provided by Dr. Yoshimitsu Oda (Osaka Prefectural Institute of Public Health, Osaka, Japan). The tester strains used in the umu test are constructed by introducing a vector plasmid pACYC184 carrying only O-AT gene (NM2009) and both O-AT and NR genes (NM3009), into the parental strain *S. typhimurium* TA1535/pSK1002 harboring umuC'-'lacZ fusion gene (16-18). The genetic markers of these tester strains have been described elsewhere (19). Frozen permanent stocks of the tester strains were prepared from overnight cultures to which DMSO was added as a cryoprotective agent. The cultures were stored at –70 °C and the genetic markers of the strains were checked before mutagenicity performance tests according to Ames et al(20).

The S9 (microsomal) fraction used in the umu test was prepared from livers of male Sprague-Dawley rats pretreated with 3-methylcholanthrene and phenobarbital according to Ames et al. (20).

Sample collection and preparation

The river water was taken from the three sampling stations according to their pollution levels namely, Meric, Ergene and Enez of the Meric delta. The 5 L water sample taken from each station was filtered through Sartorious millipore filter. XAD-2 and XAD-4 resins were prepared according to Nakamuro and Junk (1, 21). Half of each sample taken from each station was run through packed XAD-2 column after adjusting the pH to 10 and the other half was run through packed XAD-4 column without pH adjustment at a flow rate of 10 mL/min. After removing residual water in the column by blowing nitrogen gas the organic pollutants in the column were consecutively eluted by using 100 mL each of dichloromethane, acetone, diethyl ether and methanol. Each fraction collected was evaporated to dryness in a water bath. Subsequently, all the residues were dissolved in 3 mL DMSO and the maintained concentration factor was observed to be 1600 which was kept constant throughout the study. 150 µL of this sample extract was used for each assay in the mutagenicity test.

Mutagenicity assay

The umu test was performed as recommended by Oda et al. (10). Overnight cultures of bacterial strains were prepared in Luria broth medium (1 % Bacto tryptone, 0,5 % Bacto yeast extract and 1 % NaCl) including ampicillin (25 µg/mL) and chloramphenicol (5 µg/mL). The overnight culture was diluted 50-fold with TGA medium (1% Bacto

Table 1 The induction of umuC gene in *S. typhimurium* TA1535/pSK1002, NM2009 and NM3009 strains by the spring and summer sample extracts[a].

Seasons	Chemicals	Concentration		TA1535/pSK1002		NM2009	NM3009
		Amount added/assay		S9(+) β-gal	S9(-) β-gal	β-gal	β-gal
		(μL)	(μmol/L)	(units)	(units)	(units)	(units)
Spring	Control (DMSO)	150		9 ± 1	14 ± 1	136 ± 7	115 ± 10
	Control (benzene)	10		4 ± 2	-	-	-
	4-NQO		26.2	-	414 ± 82	1707 ± 63	755 ± 65
			39.4	-	653 ± 224	1063 ± 32	899 ± 37
	3-MCT		5.6	6 ± 1	-	-	-
			11.2	11 ± 1	-	-	-
	Meric extract [b]	150		3 ± 1	5 ± 1	80 ± 15	36 ± 9
	Ergene extract [b]	150		11 ± 2	31 ± 2[c]	282 ± 22[c]	75 ± 2
	Enez extract [b]	150		9 ± 1	13 ± 1	109 ± 8	53 ± 4
Summer	Control (DMSO)	150		31 ± 2	39 ± 3	143 ± 4	117 ± 3
	Control (benzene)	10		18 ± 2	-	-	-
	4-NQO		26.2	-	468 ± 131	1628 ± 196	1202 ± 144
			39.4	-	652± 237	1098 ± 33	1086 ± 65
	3-MCT		5.6	21 ± 3	-	-	-
			11.2	41 ± 4	-	-	-
	Meric extract [b]	150		23 ± 1	33 ± 2	100 ± 15	111± 6
	Ergene extract [b]	150		45 ± 5	55 ± 3	216 ± 7	83 ± 16
	Enez extract [b]	150		47 ± 7	70 ± 5	226 ± 28	174 ± 7

[a] Each value is the mean ± S.D. of four replicates from 2 separate experiments. [b] Concentration factor is 1600. [c] Weak mutagen.

Table 2 The induction of umuC gene in *S. typhimurium* TA1535/pSK1002, NM2009 and NM3009 strains by the autumn and winter sample extracts[a].

Seasons	Chemicals	Concentration		TA1535/pSK1002		NM2009	NM3009
		Amount added/assay		S9(+) β-gal	S9(-) β-gal	β-gal	β-gal
		(μL)	(μmol/L)	(units)	(units)	(units)	(units)
Autumn	Control (DMSO)	150		33 ± 1	42 ± 1	209 ± 10	191 ± 7
	Control (benzene)	10		21 ± 1	-	-	-
	4-NQO		26.2	-	787 ± 21	1522 ± 233	2054 ± 191
			39.4	-	1043 ± 78	811 ± 91	1460 ± 40
	3-MCT		5.6	24 ± 1	-	-	-
			11.2	25 ± 2	-	-	-
	Meric extract [b]	150		26 ± 1	22 ± 1	109 ± 6	91 ± 12
	Ergene extract [b]	150		31 ± 1	26 ± 2	143 ± 6	122 ± 17
	Enez extract [b]	150		33 ± 2	42 ± 1	193 ± 4	163 ± 13
Winter	Control (DMSO)	150		63 ± 6	96 ± 13	183 ± 14	148 ± 19
	Control (benzene)	10		54 ± 4	-	-	-
	4-NQO		26.2	-	683 ± 94	3286 ± 228	1683 ± 127
			39.4	-	1012 ±116	2283 ± 93	1147 ± 52
	3-MCT		5.6	47 ± 4	-	-	-
			11.2	43 ± 6	-	-	-
	Meric extract [b]	150		54 ± 3	154 ± 36	208 ± 11	209 ± 43
	Ergene extract [b]	150		53 ± 6	119 ± 31	236 ± 12	205 ± 16
	Enez extract [b]	150		51 ± 7	117 ± 9	191 ± 12	191 ± 7

[a] Each value is the mean ± S.D. of four replicates from 2 separate experiments. [b] Concentration factor is 1600.

tryptone, 0.5 % NaCl, 0.2 % glucose and 20 µg/mL ampicillin) and was incubated at 37 °C until the absorbance at 600 nm of the bacterial culture reached 0.25-0.30. The number of viable bacteria was determined as described by Oda et al. (10) that a concentration of 5-6x10^{10} cells/L will correspond to 0.25-0.30 absorbance unit. The bacterial culture obtained, was divided into 2 mL fractions, nine in total. Over the prepared nine fractions, 150 µL of the sample concentrate prepared previously and other chemicals were added as shown in Table (1,2). After 5 hours of incubation at 37 °C with shaking, the bacterial density was determined by measuring A_{600}. The β-gal activity was measured by the method of Miller (22). The bacterial cultures (2 mL) were divided into 0.2 mL of fractions and were diluted by adding 1.8 mL of Z buffer (16.1g $Na_2HPO_4.7H_2O$, 5.5 g $NaH_2PO_4.H_2O$, 0.75 g KCl, 0.25 g $MgSO_4.7H_2O$ and 2.7 mL of β-mercaptoethanol/L of distilled water, pH 7). The bacterial cell permeability to the chromogenic substrate was attained by adding two drops of chloroform and 50 µL of 0.1 % SDS and mixing. The enzyme reaction was initiated by the addition of 0.2 mL of 4 mg/mL ONPG solution. The reaction was terminated when color development reached a spectroscopically observable level by the addition of 1 mL of 1 M Na_2CO_3 solution and the absorbances were measured at 420 and 550 nm against a blank which consisted of TGA medium of bacterial culture.

The level of enzyme activity in units was calculated according to the equation;

$$\beta\text{-gal activity (U)} = 1000\ (A_{420} - 1.75 \times A_{550}) / t.v.A_{600}$$

where t is the reaction time in minutes and v is the volume of culture used in the assay (mL).

At least a 2-fold or greater than 2-fold increase in β-gal activity above the control level was accepted as positive (10). Nakamura et al. (23) have classified the inducibility of chemicals, with increased umuC gene expression, as follows: The potent inducers, 6 fold; intermediate inducers, 3 fold; weak inducers, 2 fold.

RESULTS AND DISCUSSION

Table 1 and 2 show the effect of sample extracts of spring, summer, autumn and winter respectively, on the induction of the umuC gene in the SOS response in *S. typhimurium* TA1535/pSK1002 both in the presence and absence of the S9 fraction and in NM2009 and in NM3009 strains without S9.

The mutagenic and genotoxic effects of several nitroarenes arise following the reduction of nitro groups in bacterial or mammalian cell systems where they are tested (15, 24). It has been suggested that these two enzymes catalyze key steps in the mutagenic activation of nitroarenes and that strains which overexpress NR and/or O-AT might be particularly sensitive to the genotoxic activities of these chemicals (25, 26).

In the present study, the induction of the umuC gene expression we observed in the parental strain without S9 and in NM2009 by the extract of Ergene for the spring was high enough to define the sample as a weak inducer (Table1). The sample extract from Enez and Ergene stations caused an evident induction in all of the strains used for the summer sample. The induction of the umuC gene expression was found to be almost the same for both the parental and other strains (Table1). The high O-acetyltransferase and nitroreductase activity in the nitroarene sensitive strains did not significantly increase the genotoxic activation of the extracts comparing to the parental strain in both the spring and summer, suggesting that the pollution in the Meric delta is not derived from nitroarenes but from other organics. None of the extracts obtained from the sampling stations in the autumn and winter induced the umuC gene expression in any of the strains tested (Table 2).

While the organic concentrate obtained from Meric station in the spring, summer and autumn caused an evident decrease of the enzyme activity in all the tested strains (Table 1, 2), the sample from Enez station showed decrease during the spring (Table1), the sample from Ergene during the autumn (Table 2). It is interesting to note that although there was a significant increase in bacterial density, no induction of the umuC gene expression was observed in any of the strains. This finding suggests that, some kind of pollutants induce the bacterial growth while inhibiting the β-gal activity. The response in the umu test and the bacterial growth can be used to assess both genotoxic and toxic properties of compounds, enabling the detection of promutagenic and mutagenic compounds found at low concentration in water samples. 4NQO which is used as a positive control in the test system showed a dose-related induction in the parental strain without S9 but it did not show in the nitroarene sensitive strains (Table 1, 2). Because the induction levels in the nitroarene sensitive strains by the high concentration were less than low concentration in contrast to the results observed in the parental strain without S9, suggesting that the high O-AT activity in NM2009 and NR plus O-AT activities in NM3009 strains increase their sensitivity towards 4NQO application (27).

The results show that the Meric delta is highly polluted especially in the spring and summer. However, it is also confirmed that the pollutants are not derived from nitroarenes but from other organics. Furthermore, the increase of the pollution in the spring and summer at the Meric delta is caused by the active industrial and agricultural wastes from Turkey and Bulgaria which should be seriously considered.

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