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Genotoxicity of vegetables irrigated by industrial wastewater

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Abstract: Wastewater effluents from textile dyeing and printing industries of Sanganer are discharged directly, without any treatment, into Amani Shah Nallah drainage. The drainage water takes the dissolved toxicants to flora and fauna, including crops and seasonal vegetables, being grown in the land adjoining the Nallah drainage. Thus mutagenic potential of vegetables irrigated by the water of Amani Shah Nallah drainage was investigated in the present study. The vegetables irrigated by ground water from Sanganer have also been analyzed to determine possible adverse effects of these wastewater effluents on aqua duct.
Keywords: sanganer; textile wastewaters; mutagenicity; vegetables; *Salmonella typhimurium*; microsome bioassay

Introduction

Pollution of natural waters with waste effluents arising from various industries has become a serious problem in India. In Rajasthan particularly, textile mills represent an important economic sector. Effluents from these textile and other dye-related industries have considerable impact on water quality, primarily because of the large volume of wastewater they generate and the physical and chemical properties of their effluents. These effluents, if directly discharged into nearby water bodies, can cause health hazards and disturb the ecological balance. The wastes released from such industries cause soil, surface and ground water pollution, besides causing a number of adverse effects on agricultural products, animals and health of people living in that area.

Sanganer is known for its old form of block printing, a heritage craft. There are estimated to be around 500 block and screen-printing units in Sanganer. About 205 small-scale, 125 medium-scale and 15 large-scale textile industries are located in the vicinity of Amani Shah Ka Nallah drainage, which flows through Sanganer. Besides, few blue pottery and handmade paper mills are also located in this area.

These industries use a variety of synthetic chemicals and dyes during processing and finishing of raw materials. A large variety of chemicals are used by textile industries, however, toxicity of only a few has been identified. The effluent water released during industrial processes is untreated and discharged directly, into Amani Shah Nallah drainage. Further, the effluents sediment in the drainage bed can percolate down and contaminate underground water.

The situation further aggravates as seasonal vegetables are grown in the surrounding area using this drainage water. Khan *et al.* (1995) reported the effect of these textile industry effluents on physico-chemical characteristics of the water from Amani Shah Nallah drainage. Analyses of Sanganer tie and dye industry effluents also revealed heavy metal contamination (Ni, Zn, Pb, Cd, Fe), quite above permissible limits.

The picture becomes more alarming as these waters are directly being used for agriculture, in the land adjoining the drainage. In fact, during summer months, when very little amount of water is present in Amani Shah Ka Nallah drainage, the entire drainage area is converted into agricultural field.

Vegetables are a very important part of our daily diet. These can accumulate genotoxic compounds present in soil. This study was thus planned to test vegetables that have been grown at Sanganer Nallah for their genotoxicity, using Ames bioassay (The *Salmonella*/Microsome assay).

The short-term mutagenic bacterial bioassays are used as screens for detecting potential carcinogens. Whole animal tests for carcinogenicity are expensive and lengthy experiments lasting the lifetime of the animal, usually two to three years for rodent species. Whereas, the short-term mutagenicity assays, such as the *Salmonella typhimurium* reversion assay, are less expensive, with results in 1—2 weeks. Further, animal studies, due to cost and facility requirement involve a rather limited exposure population. In contrast, millions of bacteria are exposed to a chemical in the *Salmonella* assay. This large population exposure increases the overall likelihood of detecting weak genotoxicants.

The present study is thus aimed at studying the mutagenicty of vegetables irrigated by the water of Sanganer drainage. The vegetables irrigated by ground water from Sanganer have also been analyzed to determine possible adverse effects of these wastewater effluents on aqua duct.

1 Materials and methods

1.1 Study area: Sanganer

Sanganer is situated 26° 49'—26° 59'N and 75° 46'—75°50'E. The total area of Sanganer is 635.5 km^2.

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Out of which 12.9 km^2 comprise urban area and the rest 622.6 km^2 is rural area. Most of the industries of Sanganer are situated in the urban area (Fig.1).

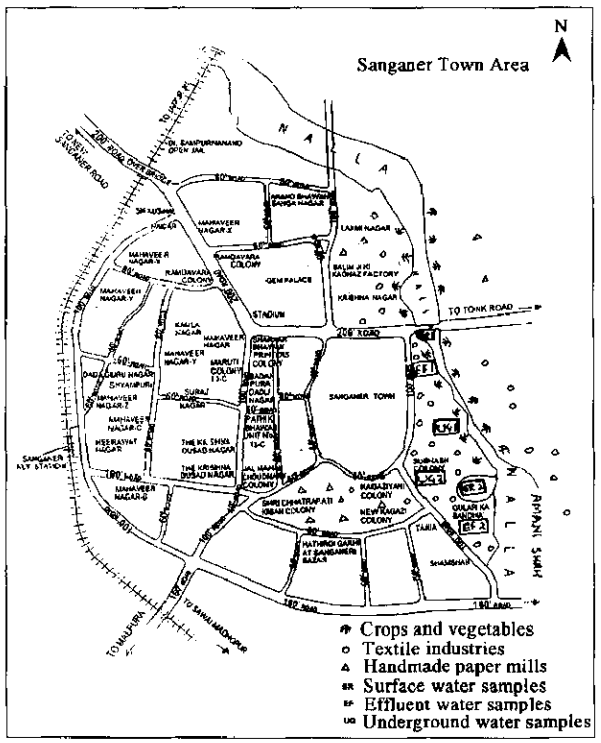

Fig.1 Site map of Sanganer (Map provided by Jaipur Development Authority, Rajasthan, India).

1.2 Sampling

All the samples were collected in June 2005. Vegetables were collected from Sanganer Amani Shah nallah and from Lal Kothi where the vegetables are supplied from Sanganer. Two vegetables were analyzed for mutagenicity: *Lagenarian siceraria* (Lauki) and *Abelmoschus esculentus* (Lady Finger). Vegetable samples were collected in clean, sterile bags and refrigerated at 4℃.

1.3 Preparation of sample extract

Vegetables were cut in cubes and grinded in a Blender. Shearing in a Blender made paste of vegetables that was diluted by adding double distilled autoclaved water. These sample solutions were labeled and kept in a refrigerator for further use.

1.4 Spontaneous revertants

Spontaneous reversion of the tester strains, to histidine independence was routinely measured in all mutagenicity experiments. The revertant colonies were clearly visible in a uniform background lawn of auxotrophic bacteria. Spontaneous reversions were expressed as the number of spontaneous revertants per plate. The following values were obtained for the two strains: Revertant/plate: without metabolic activation TA98 (42), TA100 (165); with metabolic activation, slightly higher values were obtained: TA98 (44), TA100 (168).

1.5 Exogenous metabolic activation

A deficiency of Prokaryotic assays is that bacteria lack many of the metabolic enzymes present in the mammals. Some of these enzymes activate chemicals into electrophilic compounds that bind covalently to DNA. Mammalian metabolism can be imitated to an extent, *in vitro*, by adding rodent liver homogenate, called S9 fraction, to the assay. The enzymes in this homogenate, along with cofactors, can activate many compounds that would otherwise be non-mutagenic in, *in vitro* bioassays.

The tissue homogenate used was 9000 ×g supernatant (S9) of Swiss albino mice. The homogenate and cofactor mixture were prepared in accordance with published methods (Maron and Ames, 1983). The protocol used, differs from the standard Ames Salmonella plate incorporation assay in a way that uninduced rat liver S9 has been used. This modification is based on the protocol given by Prival and Mitchell (1982) for testing of azo dyes. Concentration of S9 used was 10%.

1.6 Control

Autoclaved distilled water was used as a negative control. In each experiment positive mutagenesis controls were also routinely included. These were diagnostic mutagens used to confirm the reversion properties and specificity of each strain. The positive controls used in the assay were sodium azide, 2-nitrofluorene and 2-anthramine. Fresh solutions of the reference mutagens were prepared immediately before the beginning of each experiment.

1.7 Plates incorporation test

The assay method employed in this study was the plate incorporation test procedure. The protocol followed was the one described by Maron and Ames (1983). *Salmonella typhimurium* strains TA98 and TA100 were used in the present study. The strains were obtained from Microbial Type Culture Collection and Gene Bank (MMTC), Institute of Microbial Technology (IMTech), Chandigarh (India). They were stored as glycerol stocks at -20℃. All tester strains were maintained and stored according to the standard methods (Ames *et al.*, 1975; Maron and Ames, 1983). The tester strain genotypes (Histidine requirement, *rfa* mutation, *uvr* B and R-factor) were confirmed immediately after receiving the cultures and every time a new set of frozen permanents were prepared or used.

Tester strain cultures were grown in Oxoid nutrient broth No. 2 for 10 h. In this study, cultures were inoculated in a 10-ml flask, containing 5 ml of nutrient broth. To ensure adequate aeration, cultures (*S. typhimurium* strains TA98 and TA100) were shaken at 120 r/min. The cultures were removed after 10 h of incubation as soon as they had reached the specified density, determined by the turbidity

measurements at 650 nm.

0.1 ml of this fresh culture was mixed with 0.2 ml of his/bio solution, 0.1 ml or less of the test chemical, 0.5 ml of buffer or 0.5 ml of S9 mix and the total volume were made up to 1.0 ml by autoclaved distilled water. This mixture was gently shaken and poured on plates containing about 25 ml of minimal glucose agar medium. The test concentrations were selected from a set of standard test doses for liquids i. e. 2, 5, 10, 50 and 100 μl (Hayes, 1982).

The plates were immediately covered with paper to protect photosensitive chemicals present in the test compounds. Plates were inverted and placed in a dark incubator for 48 h at 37℃. After 48 h the revertant colonies on the test and control plates were counted manually and presence of the background lawn on all the plates was confirmed. All assays were performed in duplicate and each sample was tested on at least two occasions. All glassware, reagents, media and Petri plates used were sterile. All chemicals used were of analytical grade

1.8 Data analysis

Several recommendations for data presentation and evaluation have been reported (Krewski and Franklin, 1991). However, the most common method of evaluation of data from the *Salmonella* assay is the arbitrary rule that a doubling of spontaneous reversion rate at one or two test chemical concentrations constitutes a positive response. This non-statistical procedure used to evaluate the results of the *Salmonella* experiments (Cariello and Piegorsch, 1996; Mortelmans and Zeiger, 2000), also popularly known as "two fold rule", has been used in the present study. This rule specifies that if a test compounds doubles or more than doubles, the mean spontaneous mutation frequency obtained on the day of testing, and then the compound is considered significantly mutagenic. Using this procedure the following criteria are used to interpret results:

(1) Positive: A compound is considered a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains of *Salmonella typhimurium*. A compound is considered a weak mutagen if it produces a reproducible dose-related increase in the number of revertant colonies in one or more strains but the number of revertants is not double the background number of colonies.

(2) Negative: A compound is considered a non-mutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments.

(3) Inconclusive: If a compound cannot be identified clearly as a mutagen or a non-mutagen, the results are classified as inconclusive (e.g. if there is one elevated count).

For this analysis the dose related increases in the number of revertant colonies are observed for the test compounds and Mutagenicity ratios are calculated. Mutagenicity ratio is the ratio of average induced revertants on test plates (spontaneous revertants plus induced revertants) to average spontaneous revertants on negative control plates (spontaneous revertants). Mutagenicity ratio of 2.0 or more is regarded as a significant indication of mutagenicity.

2 Results and discussion

Dye related industries, in particular, appear to produce consistently genotoxic effluents that have been shown by many scientists, to be potent, relative to other industrial discharges. Studies of plants and animals indigenous to environments laden with such hazardous wastes or industrial effluents provide evidence of genotoxic effects. Dye industry waste-water discharged into a sewage canal in India has been shown to induce chromosomal abnormalities in two plant systems (Somashekar *et al.*, 1985; Somashekar, 1987). Further, Ravindran and Ravindran (1978) in a similar analysis of textile factory effluents found increased frequency of chromosomal abnormalities in plants treated with undiluted, polluted river water.

Toxic effluents from the Sanganer textile dyeing and printing industries being discharged directly, without any treatment, into Amani Shah Nallah drainage have been reported to contain highly mutagenic compounds. These compounds have also contaminated the surface and even underground water, thereby, making it unfit for irrigation and drinking (Mathur *et al.*, 2005a). Further case studies done by Mathur *et al.* (2005b, c), using an *in-vitro* Ames assay showed that some of the dyes (Black RL and Green 6B dyes) being used in textile industries at Sanganer are mutagenic.

The drainage water can take these dissolved toxicants to flora and fauna, including crops and seasonal vegetables, being grown in the land adjoining the Nallah drainage. Toxicants, in the form of chemicals and dyes used in Sanganer textile industries can thus enter the food chain. Therefore these seasonal vegetables were analyzed using Ames bioassay.

The response of the Ames tester strains are expressed as the Mutagenicity ratio, which is the ratio of average, induced revertants on test plates (Spontaneous revertants + induced revertants) to average spontaneous revertants on negative control plates (Spontaneous revertants). Mutagenicity ratio of 2.0 or more is regarded as a significant indication of mutagenicity provided all controls confirm to specifications (Mortelman and Zeiger, 2000).

Both the vegetables irrigated by surface waters of Amani Shah Ka Nallah drainage showed Mutagenicity ratios of more than 2.0 indicating positive

mutagenicity with both strain TA98 and strain TA100 (Table 1).

Table 1 Mutagenicity ratio of *Lageneria siceraria* and *Abelmoschus esculentus* from Sanganer with *Salmonella typhimurium* strain TA98 and TA100

Vegetable	Sample dose, μl	Mutagenicity ratio			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Lageneria siceraria	2	+	+	+	+
	5	+	+	+	+
	10	+	+	+	+
	50	+	+	+	+
	100	+	+	+	+
Abelmoschus esculentus	2	+	+	+	-
	5	+	+	+	+
	10	+	+	+	+
	50	+	+	+	+
	100	+	+	+	+

In the case of vegetables irrigated with ground water from Sanganer (Lal Kothi) mutagenicity ratio of less than 2.0 were obtained, in case of without S9 mix, indicating absence of mutagenicity. However on addition of hepatic fraction S9 positive mutagenicity was obtained (Table 2).

Table 2 Mutagenicity ratio of *Lageneria siceraria* and *Abelmoschus esculentus* from Lal Kothi with *Salmonella typhimurium* strain TA98 and TA100

Vegetable	Sample dose, μl	Mutagenicity ratio			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Lageneria siceraria	2	-	+	-	-
	5	+	+	-	-
	10	+	+	+	+
	50	+	+	+	+
	100	+	+	+	+
Abelmoschus esculentus	2	-	+	-	+
	5	-	+	-	+
	10	-	+	-	+
	50	-	+	-	+
	100	-	+	-	+

The results were more emphatically seen in the dose-response curves (Fig.2). The numbers of induced revertants, obtained for *Lageneria siceraria* with strain TA98 were 3882-8636 induced revertants and with strain TA100, 2153-5349 induced revertants, in absence of S9 hepatic fraction (Fig.2a).

The number of induced revertants, obtained for

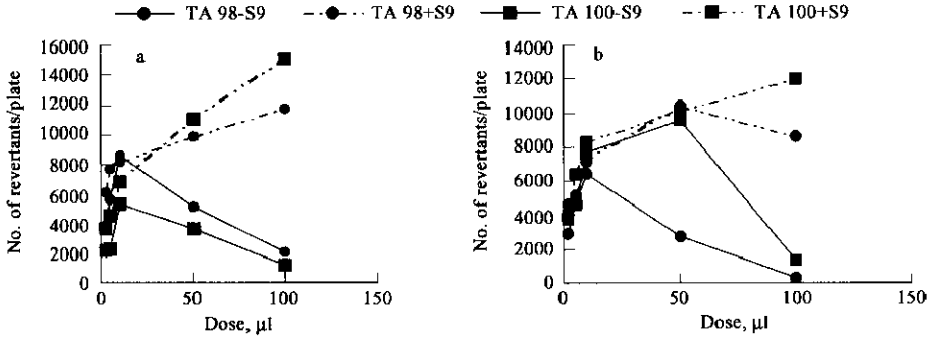

Fig.2 Dose response curve with *Salmonella typhimurium* tester strain TA98 and TA100 from Sanganer industrial area with and without S9 mix.
a. *Lageneria siceraria*; b. *Abelmoschus esculentus*

Abelmoschus esculentus with strain TA98 were 2924—6437 induced revertants, in absence of S9 hepatic fraction while with strain TA100, 3762—9633 induced revertants, in the absence of S9 hepatic fraction were obtained (Fig.2b).

In the case of both vegetables from Sanganer industrial area a clear dose related increase in the number of induced revertants was obtained indicating positive mutagenicity. Besides the increase in number of revertant colonies is double the value of spontaneous revertants, indicating positive mutagenicity.

Further addition of hepatic fraction, S9 mix, observed plates again showed increase in the number of revertants (Figs.2a and 2b). At higher dose levels, however, a sharp decrease in number of revertants was observed as seen from Fig.2a. Thus at higher concentrations the samples are showing toxicity. Similar results were observed with strain TA100 also (Fig.2a).

In the case of both vegetables from Lal Kothi a dose dependent increase in the number of revertants with strain TA98 was observed indicating presence of frame shift mutagens. Most of the dyes used in textile industry, are known to cause frame shift mutations. Therefore it can be correlated that the textile wastewaters, predominantly containing dyes (frame shift mutagens) are possibly percolating down into the aqua duct and subsequently entering the food chain.

Absence of mutagenic response with strain TA100 is, however, indicating absence of base-pair substitution mutagens in these waters (Fig.3).

Both vegetables *Lageneria siceraria* and

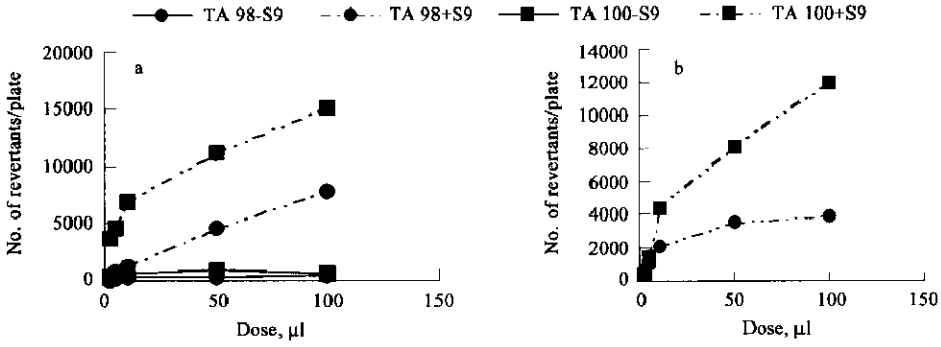

Fig.3 Dose response curve with *Salmonella typhimurium* tester strain TA 98 and TA100 from Lal Kothi area with and without S9 mix
a. *Lageneria siceraria*; b. *Abelmoschus esculentus*

Abelmoschus esculentus were also tested for their mutagenicity using S9 hepatic fraction. Interestingly, the addition of the hepatic fraction has increased the number of revertants, both in the case of strain TA98 and TA100 indicating that, mammalian enzymes are possibly converting some of the promutagenic compounds into active mutagenic metabolites.

The textile industry effluents reaching the agricultural fields take their own toll. Kidney beans and ladyfinger seeds were affected adversely when 75% and 100% concentrations of effluents were used (Mohammed and Ullah, 1985). Germination of *Bengal gram* and *Cicer arietinum* seeds, were adversely affected even when as low as 5% textile effluent was applied (Dayama, 1987). Khan and Jain (1995) have reported the adverse effects of Amani Shah drainage waters on the growth of *Triticum aestivum.*

From the above discussion it is clear that both types of point mutations i.e. Frame shift mutations (detected by TA98 strain) and base pair substitution (detected by TA100 strain) were detected in both vegetable sample analyzed with and without the hepatic S9 fraction. Yet frame shift mutation is more dominant. Results of Ames test in the study thus showed that both *Lageneria siceraria* and *Abelmoscus esculentus* have components that are mutagenic. Thus it can be concluded from this study that the vegetable being grown in Sanganer industrial area can induce genotoxic responses. A large fraction of Lal Kothi Vegetable markets vegetables are being supplied from agricultural fields of Sanganer Industrial Area.

Results of this study are clearly supporting the concern expressed by residents of Sanganer and reported by several local newspapers, about the critical pollution situation in Sanganer. The use of waters of Sanganer Nallah for irrigation might have deleterious effects on human health due to consumption of vegetables containing mutagenic compounds. Thus establishment of a treatment plant for the effluent water before discharging into the nallah is needed.

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