Chapter VI: Genotoxicity of soil
Introduction

Soil contaminants are widespread in industrialized countries, causing direct pollution of the soil and indirect pollution of the ground water and food. Among the toxic compounds, particular attention should be paid to soil mutagens and carcinogens due to their potentially detrimental effects on animal populations and human health (Monarca et al., 2002).

In most towns of India, the raw sewage is used either directly to irrigate the agricultural land as a supplement of essential plant nutrients or disposed into fresh water streams which again can be used for agricultural purposes. This treatment increases crop production and results in the accumulation of toxic substances in soil and in the crop as sewage is usually contaminated with pollutants such as heavy metals and polychlorinated substances from domestic and industrial wastes that enters into the sewer system (Kansal, 1994, Singh et al., 2004).

It is expected that the results of genotoxicity assays of complex mixtures like soil are strongly influenced by the method of sample preparations and extractions, because chemical and physical properties of constituents, including major genotoxic compounds, in the mixtures differ greatly. In most of the studies assessing the genotoxicity, leachates of soil samples were prepared prior to the assay. The leachates from soil samples were generally made by shaking soil samples with aqueous (Smith, 1982; Ehrlichmann et al., 2000; Cabrera and Rodriguez, 1999) and/or organic solvents (Knize et al., 1987; Goto et al., 2000; Courty et al., 2004).

Several workers have studied the genotoxicity of soil irrigated with wastewater using plant bioassays. They demonstrated that the extracts of the soil showed differential sensitivity in these bioassays (Ma, 1995; Chroust et al., 1997; Kong and Ma, 1999; Grover and Kaur, 1999; Cotelle et al., 1999 Monarca et al., 2002).

Present study focuses on the genotoxicity of agricultural soil irrigated with wastewater from factories and domestic sewage about a decade and ground water irrigated soil; using three different bioassays namely Ames Salmonella/mammalian microsome test, survival of SOS defective E. coli K-12 mutants and bacteriophage lambda systems. Few studies have been concerned in testing potentially contaminated soils of India by these methods (Malik and Ahmad, 1995).
Materials and methods

Sample collection

Soil samples were collected as described earlier (Chapter II: General Materials and methods).

Extraction of soil samples with different solvents

Extraction of soil with different solvents (methanol, acetonitrile and acetone) was done according to the method of Knize et al. (1987). 10 g of soil was extracted with 10 ml of the extraction solvent. The extracts were centrifuged at 7000 rpm for 10 min. The extracts were evaporated to dryness and then re-dissolved in 1 ml of DMSO. These extracts were filtered sterilized through 0.45 µm filters and stored at -20°C until testing was completed.

Salmonella mutagenicity testing

The preincubation test was performed as described by Maron and Ames (1983) with some modifications (Pagano and Zeiger, 1992). Five increasing doses (20, 40, 60, 80 and 100 µl) of each soil extract were plated in duplicate with 0.1 ml bacterial culture. After incubating the test sample and bacterial culture for 30 min at 37°C, 2.0 ml top agar containing traces of histidine and biotin were added, and contents were poured on minimal glucose agar plates. Plates were incubated at 37°C for 48-72 h. Negative and positive controls were included in each assay. The negative control plates had bacteria and solvent (DMSO) but no test sample. Methyl methane sulphonate and sodium azide were used as positive controls. All the soil extracts were also tested in the presence of (+S9) microsomal fraction, to which 20 µl of S9 liver homogenate mix per plate was added. The criterion used to classify the results as positive was similar to those of Vargas et al. (1993; 1995b) i.e. number of revertants double the spontaneous yields accompanied by a reproducible dose-response curve.

Treatment of E. coli K-12 strains with soil extracts

The SOS defective recA, lexA and polA mutants of E. coli K-12 as well as the isogenic wild type strains were harvested by centrifugation from an exponentially growing culture (1-3x10⁸ viable counts/ml). The pellets so obtained were suspended in 0.01M MgSO₄ solution and treated with 80 µl of each soil extract. Samples were
withdrawn at regular intervals, suitably diluted and plated to assay the colony forming ability. Plates were incubated overnight at 37°C. Solvent control was also run simultaneously.

**Extracellular treatment of Bacteriophage λ with the test samples**

Purified bacteriophage λ (10^{10} PFU/ml) was incubated at 37°C with 80 μl/ml of test samples. Aliquots of 0.1ml were withdrawn at regular intervals, suitably diluted in 0.01M MgSO₄ solution of pH 8.0 and allowed to adsorb on DNA repair defective and wild type hosts of *E. coli* K-12 strains at 37°C. The infective centers were plated on nutrient agar by double layer method. Plaques were counted after overnight incubation at 37°C.

**Results**

The reversion of Ames tester strains in the presence of soil extracts are summarized in Tables 1-3. Extracts of soil with methanol were found to be more mutagenic in agricultural soil irrigated with wastewater than acetonitrile and acetone extracts. However, agricultural soil irrigated with wastewater was found to be most responsive to the tester strains compared to that of ground water irrigated soil. Extracted soil samples (methanolic) exhibited a significant degree of mutagenicity with TA98 strains (218 net revertants) at the dose level of 80 μl/plate. The reversion property which displayed an increasing trend up to 80 μl/plate declined sharply at 100 μl/plate. Significantly enough the presence of S9 liver microsomal fraction exhibited an inhibitory effect on appearance of his* revertants. The strains could be listed in order of their responsiveness with methanolic extract of soil in the absence of S9 fraction as follows:

TA98> TA100> TA97a> TA102, TA104.

Extraction of soil with acetonitrile and acetone also displayed the maximum mutagenicity with TA98 strain both in the presence and absence of S9 fractions. In the absence of S9 fraction, the mutagenic indexes of TA97a strain towards the acetonitrile extracts of the soil were different to those of acetone extracts. In the presence of S9 fraction the mutagenic indexes of TA97a towards the acetone extracts from soil were higher than TA100. Moreover, significant difference was not found among the mutagenic indexes of TA102 and TA104 strains towards the acetonitrile and acetone extracts. Here
again maximum mutagenicity was found in agricultural soil irrigated with wastewater compared to that of ground water irrigated soil (Tables. 2-3).

Survival pattern of recA, lexA and polA mutants of E. coli K-12 and their isogenic wild-type counterparts in the presence of soil extracts is shown in Fig. 1. It was observed that the polA mutant was the most sensitive strain when tested with soil extracts but the decline was more pronounced when they were treated with extracts of soil irrigated with wastewater than ground water extracted soil. The survival was 16.5%, 21.0% and 33.7% in polA strain after 6 hours of treatment when tested with wastewater irrigated soil extracts of methanol, acetonitrile and acetone respectively. However, the survival was 45.2%, 49.8% and 55.9% in polA strain after 6 hours of treatment when tested with ground water irrigated soil extracts of methanol, acetonitrile and acetone respectively.

Fig. 2 shows the survival of bacteriophage λ with the solvent extracts of soil. The decline in plaque forming units was more pronounced in lexA mutants as compared to their wild type counterparts. The survival was 17.7%, 24.5% and 31.1% in lexA strain after 6 hours of treatment when tested with wastewater irrigated soil extracts of methanol, acetonitrile and acetone respectively. However, survival was 47.7%, 53.1% and 66.4% in lexA strain under the same experimental condition when tested with ground water irrigated soil extracts of methanol, acetonitrile and acetone respectively.

Discussion

There are a large number of short-term bioassays for detecting genetic toxicity. These assays utilize a wide range of organisms and cell types and measure a variety of different genetic changes. The genetic damage detected represents DNA damage from point mutations to chromosomal mutations. However, there is no single test that adequately detects the types of genetic damage that may be induced by all chemical classes of genotoxic compounds and/or complex chemical mixtures. Furthermore, only a limited number tests can be utilized for detecting genetic damage from hazardous industrial waste sites under field conditions (Cabrera and Rodriguez, 1999).

The assay developed by Ames (Ames, 1971) is the most commonly used mutagenicity test. The Salmonella assay has been widely used throughout the world to detect the mutagenic activity of complex environmental mixtures (Umbuzeiro et al., 2001). It was modified and improved several times (Maron and Ames. 1983; Ames et al.,
1973; Ames et al., 1975; Mortelmans and Zeiger, 2000). The Ames test is very useful for detecting mutagens under laboratory conditions. It was specifically designed to detect chemically induced mutagenesis. In fact, it is commonly used in the initial screening to determine the mutagenic potential of new chemicals and drugs (Mortelmans and Zeiger, 2000).

Soil irrigated with wastewater contains certain agents capable of inducing mutations. We have found differential pattern of his\(^{+}\) reversion of Ames tester strains with different solvents used for the extraction of mutagenic substances from soil irrigated with wastewater. Methanol extract was being the most mutagenic and producing 218 net revertants at the dose of 80 \(\mu l/plate\) with TA98 strains while the net number of his\(^{-}\) revertants in the presence of acetonitrile and acetone extracts of the same soil with TA98 strain were 145 and 62 respectively. Cabrera and Rodriguez (1999) tested the genotoxicity of soil from farmland irrigated with wastewater using three plant bioassays and they also reported that contaminated soils contained certain agents which are capable of inducing mutations.

Present study indicated that the samples collected from ground water irrigated soil was also mutagenic to the TA98 strain (Mutagenic index = 2.1) when extracted with methanol at the dose level of 80 \(\mu l/plate\). Several workers have also reported that agricultural soil was found to be mutagenic in *Salmonella* mutagenicity assay both in the presence and absence of S9 mix (Smith, 1982; Goggleman and Spitzauer, 1982; Brown et al., 1985; Edenharder et al., 2000). Goggleman and Spitzauer (1982) examined \(n\)-hexane/acetone extracts of soil from several agricultural fields on which crops such as hops, asparagus, rye, oat pasture and meadow grew and they showed that all soil samples were mutagenic to *Salmonella typhimurium* TA98 and TA100 strains with some difference in potency. Mutagenicity of soil is related not only to farming but also to industrial and vehicular emissions and fires (Knize et al., 1987). Microorganisms in soil could have a worldwide distribution and may account for the mutagen-depth profile by living and producing mutagenic products at an optimum depth of 4-10 cm. The mutagenicity per gram of soil is variable in different locations and with the type of plant grown (Goggleman and Spitzauer, 1982). Products of microorganisms such as aflatoxins are known to be potent mutagens. This type of mutagen, like the major response in soil extracts, requires activation and responds better to strain TA98. Soil near some cities in
Asia (Tokyo, Bangkok, Chaing Mai and Manila) has been found to have mutagenic effect (Matsushita et al., 1983).

We have extracted 10 g of soil sample with 10 ml of extraction solvents and evaporated it to dryness under reduced pressure and finally made up the volume to 1 ml in DMSO so that 1 g of the soil is equivalent to 100 μl of the extract. The data presented in the Tables 1-3 for the Ames tester strains at the doses of 20, 40, 60, 80 and 100 μl/plate (extract) is equivalent to 0.2, 0.4, 0.6, 0.8 and 1.0 g of soil respectively. The number of net histidine revertants per kg of soil for the TA98 strain with methanolic extract at linear doses were 286000 in the absence of S9 mix and therefore demonstrated significant mutagenicity in the soil samples. Knize et al. (1987) tested the genotoxicity of soil using different solvents employing Ames test and found that the strain TA98 was most sensitive to acetonitrile extracts producing 298000 his\textsuperscript{+} revertants per kilogram of soil. While the strain TA100 was less sensitive producing 73000 his\textsuperscript{+} revertants per kilogram of soil.

Our data is indicative of the presence of methanol soluble substances in the soil to be more mutagenic than those of acetonitrile and acetone. It is obvious that under the experimental conditions only organic substances would exclusively be extracted. For the past several decades organochlorine pesticides have been widely used for both agricultural and public health purposes and there is always a tendency to use them in excess. For examples, the use of DDT has now been restricted in many countries including India, people are still using it; probably because it is economical. Even though there is restriction on DDT and HCH, they have already become universal pollutants and are reported from virtually every component of the environment (Lal et al., 1989). The field survey conducted by the Aligarh Muslim University, Aligarh showed that most of the organochlorine pesticides are predominantly used by farmers in the vicinity of Aligarh region and these pesticides account for over 97% of the total insecticides and pesticides used and thus agricultural soil may become contaminated with these pesticides. A number of organic and inorganic pollutants from the wastewater might enter into the soil as a result of irrigation. This treatment increases crop production and results in the accumulation of toxic substances in soil and in the crop as sewage is usually contaminated with pollutants such as heavy metals and polychlorinated substances from domestic and industrial wastes that enters into the sewer system. However, it is difficult to isolate and identify all the pollutants because it needs the complex analytical
techniques and therefore we have performed genotoxicity of the soil extract (complex mixtures) using biological test system for determining the toxicological impact of the industrial and municipal discharges. Mutagenic response of the organic extracts of the soil would not necessarily reflect the mutagenicity of the pesticides because some of the other organic pollutants might also come along with the pesticides in the extracts.

The necessity of using different solvents to isolate mutagenic constituents from complex environmental mixtures was demonstrated by Barbee et al. (1996). This may be particularly important when microbial transformation impact the soil detoxification process since bio-transformation and/or biodegradation frequently produce intermediate breakdown products which are more polar, mutagenic, and mobile than the parent compound (Abbott and Sims, 1989).

Despite confusion about the properties of genotoxins in surface water and municipal wastewater extracts, many studies discuss the genotoxicity of substances known to be present in mixed municipal wastewater. These includes sanitary wastes, pesticides for lawn and garden care, combustion by-products that enter the municipal system via surface run off from roadways, commercial and industrial areas, as well as discharges from hospitals and research institutions that use antineoplastic drugs and experimental genotoxins (White and Rasmussen, 1998).

The survival pattern of lexA, recA and polA mutants of E. coli as well as their isogenic wild type counter parts in presence of soil extracts is shown in Fig 1. It is postulated that the inducible error prone repair pathway presumably involving the recA and lexA genes which could potentially operate on several types of lesions in DNA, whether produced by radiation or environmental chemicals or by other agents (Strauss, 1989; Malik and Ahmad, 1995; Rehana et al., 1996; White and Rasmussen, 1998)

Extracellular treatment of bacteriophage λ with the soil extracts is shown in Fig. 2. Soil extract gave significant loss of plaque forming units (PFUs) at the dose of 80 μl/ml of phage. The decline was more pronounced in the lexA mutants in the presence of methanolic extract than acetonitrile and acetone extracts of the same soil. The survival was 47.7% in lexA strain when the phage was treated with ground water irrigated methanolic soil extracts after 6 h of treatment and whereas, it was only 17.7% when tested with the extract of soil irrigated with wastewater.

Our results suggest that the agricultural soil irrigated with wastewater contained certain genotoxic agents which are capable of inducing mutations. In view of the
common practice of application of untreated wastewater to agricultural land in the neighboring area should be strictly prohibited as the pollutants might enter into the food chain and causing health hazards to humans.
Table 1. Reversion of *Salmonella* tester strains in the presence of methanol extracts of soil

<table>
<thead>
<tr>
<th>Soils</th>
<th>Strain designation</th>
<th>S9 Spontaneous reversion</th>
<th>Doses (μl/plate)</th>
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<tr>
<td>Agricultural soil irrigated with ground water</td>
<td>TA97a - 116±9.0</td>
<td>122±10.2(1.1)</td>
<td>126±9.4(1.1)</td>
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<td>+ 112±8.6</td>
<td>117±6.3(1.0)</td>
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<td>46±7.0</td>
<td>59±7.2(1.3)</td>
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<td>+ 45±5.6</td>
<td>56±5.2(1.2)</td>
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<td>TA98 - 168±10.2</td>
<td>177±5.2(1.1)</td>
<td>185±7.6(1.1)</td>
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<td>+ 178±8.6</td>
<td>186±6.4(1.0)</td>
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<td>TA100 - 256±17.2</td>
<td>262±7.6(1.0)</td>
<td>272±9.4(1.1)</td>
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<td>+ 282±12.5</td>
<td>272±8.2(1.0)</td>
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<td>TA102 - 326±13.4</td>
<td>334±11.6(1.0)</td>
<td>346±12.2(1.1)</td>
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<td>+ 336±11.5</td>
<td>338±5.2(1.0)</td>
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<td>TA104 - 95±6.5</td>
<td>129±16.8(1.4)</td>
<td>152±9.0(1.6)</td>
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<td>+ 99±7.1</td>
<td>148±4.2(1.5)</td>
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<td>Agricultural soil irrigated with wastewater</td>
<td>TA97a - 38±6.5</td>
<td>98±6.2(2.6)</td>
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<td>+ 44±4.5</td>
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<td>TA98 - 168±11.4</td>
<td>295±9.7(1.8)</td>
<td>334±9.2(2.0)</td>
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<td>+ 189±10.5</td>
<td>298±11.0(1.6)</td>
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<td></td>
<td>TA100 - 272±15.5</td>
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<td>308±10.2(1.1)</td>
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<td>+ 290±9.5</td>
<td>312±14.0(1.1)</td>
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<td></td>
<td>TA102 - 316±14.2</td>
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<td></td>
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<td>+ 339±21.0</td>
<td>356±7.2(1.1)</td>
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The numbers represent histidine revertants in terms of mean ± standard deviation

Abbreviations: S9, liver microsomal fraction; - , in the absence of S9; + , in the presence of S9

Values in parentheses indicate the mutagenic index

Mutagenic index = No. of his' revertants induced in the sample/No. of spontaneous his' revertants in the negative control
Table 2. Reversion of Salmonella tester strains in the presence of acetonitrile extracts of soil

<table>
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<tr>
<th>Soils</th>
<th>Strain designation</th>
<th>S9 Spontaneous reversion</th>
<th>Doses (μl/plate)</th>
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<td>125±14.2 (1.4)</td>
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<td>149±9.0 (1.6)</td>
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<td>78±6.6 (1.6)</td>
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<td>+</td>
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<td>241±12.0 (1.2)</td>
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<td>278±28.2 (1.4)</td>
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<td>374±7.0 (1.2)</td>
<td>370±5.0 (1.2)</td>
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</table>

The numbers represent histidine revertants in terms of mean ± standard deviation
Abbreviations: S9, liver microsomal fraction; - , in the absence of S9; + , in the presence of S9
Values in parentheses indicate the mutagenic index
Mutagenic index = No. of his' revertants induced in the sample/No. of spontaneous his' revertants in the negative control
Table 3. Reversion of *Salmonella* tester strains in the presence of acetone extracts of soil

<table>
<thead>
<tr>
<th>Soils</th>
<th>Strain designation</th>
<th>S9</th>
<th>Spontaneous reversion</th>
<th>Doses (µl/plate)</th>
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<td>20</td>
<td>40</td>
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<tr>
<td>Agricultural soil irrigated with ground water</td>
<td>TA97a</td>
<td>-</td>
<td>92±9.1</td>
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The numbers represent histidine revertants in terms of mean ± standard deviation
Abbreviations: S9, liver microsomal fraction; -, in the absence of S9; +, in the presence of S9
Values in parentheses indicate the mutagenic index
Mutagenic index = No. of his revertants induced in the sample/No. of spontaneous his revertants in the negative control
Figure 1. Survival of E. coli K-12 strains exposed to soil extracts (a) methanol extract of soil integrated with ground water (b) acelone extract of soil integrated with wastewater (c) acelone extract of soil integrated with ground water (d) acelone extract of soil integrated with wastewater (e) wild-type (f) acelone extract of soil integrated with wastewater (g) acelone extract of soil integrated with ground water (h) acelone extract of soil integrated with wastewater.
Fig. 2. Survival of Bacteriophage λ exposed to soil extracts (a) methanol extract of soil irrigated with ground water (a') methanol extract of soil irrigated with wastewater (b) acetonitrile extract of soil irrigated with ground water (b') acetonitrile extract of soil irrigated with wastewater (c) acetone extract of soil irrigated with ground water (c') acetone extract of soil irrigated with wastewater. Wild-type (recA+, lexA+) (○-○); recA (⊗-⊗); lexA (●-●).