Summary
Industrial wastes and effluents are undesirable by-products of economic development and technological advancement. Hazardous wastes include chemical compounds, effluents, discharges, sludges, and wastewaters from industrial sources, air borne pollutants released from industries or waste sites. When improperly handled and disposed, industrial wastes imperil both human health and environment.

Application of wastewater to agricultural lands for enhancing crop productivity is a common practice in India as well as in many other countries from several decades. However, long-term irrigation with industrial effluents mixed with municipal wastewater has resulted in the excessive accumulation of toxicants in agricultural soils (such as heavy metals and polychlorinated substances from domestic and industrial wastes that enters the sewer system) and has brought a potential risk to human health due to the accumulation of toxicants through the food chain.

Genetic recombination, and more specifically the horizontal transfer of genes within and between bacterial populations is commonly regarded as an important mechanism in the selective adaptation of bacteria to changes in the local environment. For the terrestrial environment (soil) the transfer of conjugative plasmids is probably the best studied and is argued to be the most important means of gene transfer because these genetic elements contain independent replicons, encode dedicated transfer mechanism, and can mobilize advantageous factors.

Conjugative gene transfer mediated by plasmids with broad-host-range is generally believed to be a common and widespread mechanism for the transfer of genes across a broad phylogenetic range of bacteria. Within the proteobacteria, broad-host-range (BHR) plasmids have been defined as those plasmids that can be introduced and stably maintained in bacterial species from at least two subgroups (e.g. between α and β-proteobacteria). The well known self transmissible BHR plasmids belong to the incompatibility groups IncP, IncN, IncW and Inc U. For instance, RP4 is an IncP plasmid, R388 is an IncW and the IncN plasmids are represented by pKM101, and RSF1010 is an IncQ plasmid. IncP, IncN, IncW and IncQ plasmids are commonly found in different soil samples and pig manure slurries.

Traditionally the impact of toxic pollutants discharged into water resources or soil have been evaluated by measurement of specific chemical pollutants or perhaps more commonly surrogate chemical parameters in the discharge. A major problem for this approach is the need for complex analytical technique in the identification and quantification of all the pollutants of interest. Furthermore, risk estimation derived
solely from the presence of specific pollutants requires detailed knowledge of the toxicological properties of these pollutants both singly or in combination. An alternative approach for testing contaminated soil or water involves the use of biological test system for determining the toxicological impact of industrial and municipal discharges.

Present study focused to assess the genotoxicity of wastewater and soil irrigated with wastewater of Chihnat industrial area, Lucknow (UP), India near pesticide industry to predict the putative hazards of wastewater. An additional aim of the study is to test the pesticide tolerance and multi-resistance in bacteria as well as the presence of conjugative plasmids in these bacterial isolates.

The findings of the study along with their explanations are summarized as under:

I. Characterization of conjugative plasmids in pesticide tolerant and multi-resistant bacteria

- Gas chromatography analysis of the soil samples revealed the presence of lindane at a concentration of 547 ng/g and α-endosulfan and β-endosulfan of 422 ng/g and 421 ng/g respectively.
- Atomic absorption spectrophotometry analysis of the soil sample was done and Cr, Zn, Fe, Ni, Cu and Cd were detected at the concentrations of 36.2, 42.5, 241, 43.2, 13.3 and 11.20 mg/kg respectively. Wastewater samples revealed the presence of heavy metals Cr, Zn, Fe, Ni, Cu and Cd at the concentrations of 21.3 μg/ml, 22.4 μg/ml, 221 μg/ml, 11.2 μg/ml, 8.3 μg/ml and 3.3 μg/ml of water respectively.
- More than 100 bacteria were isolated from agricultural soil irrigated with wastewater from Chinhait industrial area, Lucknow, India. A total of 35 bacterial isolates were finally selected on the basis of their MIC against pesticides and heavy metals for further study.
- Minimum inhibitory concentrations of all bacterial isolates were determined for pesticides and heavy metals. A maximum MIC of 3200 μg/ml was observed for the pesticides lindane, endosulfan, chlorpyriphos, monocrotophos and captan, whereas 1600 μg/ml was observed for folpet.
- All pesticide tolerant bacteria were also tested for their resistance to heavy metals (Cd, Zn, Pb, Ni, Cu and Cr). A maximum MIC of 100 μg/ml for Hg,
800 μg/ml for Cr, 400 μg/ml for Ni, Cu and Cd, and 3200 μg/ml for Pb was observed.

- Majority of the pesticide tolerant and metal resistant bacteria were also resistant to one or more antibiotics tested.
- All the multi-resistant/tolerant bacterial isolates were also tested for the presence of incompatibility (Inc) group IncP, IncN, IncW, IncQ plasmids and for rolling circle plasmids of the pMV158-type by PCR.
- PCR amplification of the bacterial isolates and soil DNA revealed the presence of IncP-specific sequences (trfA2 and oriT) which was confirmed by dot blot hybridization with RP4-derived DIG-labelled probes. Plasmids belonging to IncN, IncW and IncQ group were neither detected in the bacterial isolates nor in total soil DNA.
- Bacterial isolates were identified by 16S rDNA sequencing and isolates WR7, WR17, WR19, WR21, WR22, WR24, WR33 and WR35 were found to be most similar to *Pseudomonas aeruginosa* (accession no.GQ922652), *Pseudomonas aeruginosa* (accession no.GU384325), *Pseudomonas* sp.GS8 (accession no.GU384329), *Pseudomonas* sp.M9J918 (accession no.GU384328), *Citrobacter* sp.Azor-5 (accession no.GU384326), *Pseudomonas* sp.CL-3 (accession no.384327), *Acinetobacter Iwoffii* (accession no.GU384330) and *Enterobacter radicinitans* (accession no. GU384332) respectively.

These findings clearly indicated that the soil was highly contaminated with the pesticides lindane, α-endosulfan, and β-endosulfan as well as with several heavy metals. Maximum MICs of bacterial isolates for pesticides and heavy metals showed that bacteria surviving in these contaminated soil were highly tolerant or resistant to the pesticides/heavy metals and simultaneously resistant to antibiotics. IncP specific DNA sequences (trfA2 and oriT) were detected which revealed that IncP plasmids are prevalent in these bacteria isolated from soil irrigated with wastewater. The presence of IncP plasmids in these bacterial isolates suggest that pesticide contamination has applied selective pressure for proliferation of these plasmids, as IncP plasmids are involved in catabolic pathways of pollutants (pesticides/metals). Present findings suggest the role of conjugative plasmids as contributors to maintenance and spread of adaptive traits (pesticide tolerance/metal and antibiotic resistance) in microbial communities.
II. Exogenous isolation of conjugative plasmids from soil

- Exogenous plasmid isolation method was used to assess the conjugative plasmids from contaminated soil using bacteria detached from soil sample as donor and rifampicin resistant *E. coli* HMS as recipient strain on mineral salt agar medium supplemented with pesticides and antibiotics:

- Transconjugants were picked up from mineral salt agar (MSA) plates either with ampicillin (10 μg/ml) or tetracycline (20 μg/ml) supplemented with pesticide (γ-HCH 100 μg/ml) as a sole carbon source.

- The frequency of gene transfer of tetracycline resistant transconjugants was 9.2 x 10^-4 whereas, ampicillin was 7.2 x 10^-6.

- Each putative transconjugant was also tested on the basis of MIC against different pesticides and metals. A maximum MIC of 3200 μg/ml against lindane was observed, whereas MIC of endosulfan, chlorpyriphos, monocrotophos and captan was 1600 μg/ml. However, maximum MIC of 25 μg/ml for Hg, 400 μg/ml for Cr, Cu, Cd, Ni was observed in the selected transconjugants.

- PCR typing method was conducted to assess the presence of plasmids of the incompatibility groups IncP, IncN, IncW, IncQ and rolling circle pMV158 type in the DNA derived from transconjugants. IncP, IncW, and rolling circle plasmids of pMV158 type were detected in the transconjugants.

- Five of the transconjugants; 3 positive PCR for IncP_{orf12&ori7} specific sequence from tet and 2 positive PCR for IncW_{ori7} specific sequence from ampicillin plates were selected for plasmid isolation and it was found that transconjugants harbor one or more plasmids of different sizes when compared with standard DNA marker.

In this study, transconjugants isolated by exogenous plasmid isolation in biparental mating indicate that genes for the tolerance of pesticides and metals/antibiotic resistance can be distributed by conjugative plasmids in the natural environment. Consequently, the availability of different incompatibility group plasmids indicate that pesticide/metal contamination has applied selective pressure for proliferation of these different plasmids and diverse gene cassettes are assembled into catabolic pathways of pollutants having different origins. Our observations are also contributing to the understanding of gene exchange between bacteria in dynamically developing ecosystems and underline the importance of describing the succession of
bacterial populations indigenously present in such system due to contamination events.

III. Genotoxicity of wastewater

- Gas chromatography analysis of the wastewater revealed the presence of certain organochlorine and organophosphorus pesticides viz., lindane, α-endosulfan, β-endosulfan, chlorpyriphos and monocrotophos.
- The mutagenicity of the wastewater samples was evaluated with XAD concentrated and liquid-liquid extracted samples using Ames Salmonella/mammalian microsome test and it was found that the test samples revealed maximum response with TA98 strain in the presence and absence of S9 metabolic activation.
- In terms of mutagenic index, induction factor (Mi) and slope (m) of initial linear dose response curve, XAD concentrated sample was found to be more mutagenic than liquid-liquid extracted samples.
- A mixture of technical grade pesticides; lindane (57.55 ng/ml), α-endosulfan (80.90 ng/ml), β-endosulfan (220.9 ng/ml), chlorpyriphos (20.90 ng/ml) and monocrotophos (8.32 ng/ml) were prepared in the amounts and ratios comparable to their existence in the test sample to see their cumulative effect. Interestingly, we found a similar pattern of the responsiveness of various tester strains with the liquid-liquid extracted wastewater and pesticide mixture.
- On the basis of the toxicity; different test samples can be arranged in order of toxicity as follows: XAD-concentrated water sample > liquid-liquid extracted water sample > pesticides mixture.
- The recA, lexA, and polA mutants of E. coli K-12 were highly sensitive in presence of test samples. Damage occurred highest in the cell in presence of XAD concentrated sample (40 µl/ml) as compared to the liquid-liquid extracted wastewater and pesticide mixture.
- lexA and polA mutants were found to be most sensitive strains in the presence of test samples.
- Extracellular treatment of bacteriophage λ with test samples resulted significant loss of plaque forming units (PFUs) at a dose 40 µl/ml of culture (XAD concentrated) and 80 µl/ml of culture (liquid-liquid extract and pesticide mixture).
The different principles and biological end points of the *Salmonella* reversion test and of the *E. coli* DNA repair test, as well as the comparative data obtained in this study, clearly indicate that the two experimental models are not alternative but complementary, thus supporting the view that they should be conveniently combined in any battery of short-term tests predictive of carcinogenicity. Our results suggest that wastewater of industrial area contained certain genotoxic agents which are capable of inducing mutations. Moreover, the test samples also initiate the SOS response and thus bring about mutation in the bacterial DNA. Thus, the genotoxic effect of the test water samples would obviously pose a risk of neoplastic transformations in humans. Therefore, consistently high levels of mutagenicity of the wastewater thus call for serious attention and remedial measures.

IV. Genotoxicity of soil

- Soil samples were extracted with different solvents (hexane, acetonitrile, methanol, chloroform and acetone) and assayed for mutagenicity using Ames *Salmonella/mammalian microsome test.
- Agricultural soil irrigated with wastewater was found to be most responsive to the *Salmonella* tester strains as compared to that of ground water irrigated soil.
- All samples exhibited maximum mutagenicity with TA98 strain in the presence as well as in the absence of S9 metabolic activation. There was an increase in the reversion of tester strains with increasing doses up to 5 µl/plate and a decline at the dose of 10 µl/plate.
- Hexane extract of agricultural soil irrigated with wastewater was found to be more mutagenic than other extracts. Extraction of soil with acetonitrile, methanol, chloroform and acetone also displayed the maximum mutagenicity with TA98 strain both in the presence and absence of S9 metabolic activation.
- The order of mutagenicity of soil extracts (soil irrigated with wastewater) in terms of mutagenic potential of initial linear dose response for the most sensitive strain TA98 with and without S9 fraction is in the following order: hexane > acetonitrile > methanol > chloroform > acetone.
- Net revertants per gram of soil extracts (soil irrigated with wastewater) of hexane, acetonitrile, methanol, chloroform and acetone for the most responsive TA98 strain in the presence of S9 fraction was 46900, 45700, 42800, 26500, and 25200 revertants/gm respectively and in the absence of S9
fraction was 42000, 37300, 32500, 19400, and 17600 revertants/gm respectively.

- A significant decline in the survival of recA, lexA, and polA mutants of E. coli K-12 was observed as compared to their isogenic wild-type counterparts with different soil extracts.

- polA mutant was found to be most sensitive strains when tested with soil extracts but the decline was more pronounced when they were treated with wastewater irrigated soil extracts of soil than groundwater extracted soil.

- Survival was only 15.2% in polA mutant when treated with hexane extract of soil irrigated with wastewater after 6 h of treatment, while for acetonitrile, methanol, chloroform and acetone, it was 20.0%, 30.2%, 35.0% and 39.3% respectively.

- Survival was 65.4%, 66.0%, 70.3%, 72.4% and 75.7% in polA strain after 6 h of treatment when treated with ground water irrigated soil extracts of hexane, acetonitrile, methanol, chloroform and acetone respectively.

- Extracellular treatment of bacteriophage λ with soil extracts resulted significant loss of plaque forming units (PFUs) at a dose of 5 μl/ml of culture.

- The decline in plaque forming units (PFUs) was more pronounced in lexA strain as compared to their wild type counterparts in the presence of wastewater irrigated soil extracts.

Our study on mutagenicity assessment of contaminated soil in the vicinity of industrial area revealed that TA98 was the most responsive strain in terms of mutagenic index, mutagenic potential and induction factor. In Salmonella typhimurium strains, frame shift tester strain TA98 and base-pair substitution strain TA100 have strings of GC (guanine–cytosine) base pairs at the critical site for reversion (growth without histidine) in contrast to TA102 and TA104. These findings clearly indicated that the test samples preferentially act on G-C base pair mutants (frame shift) as compared to those having A-T base pairs (TA102 and TA104) at the site of mutation and also initiate the inducible error prone SOS response within the soil extracts treated E. coli mutants. In view of the common practice of application of untreated wastewater to agricultural land in the neighbouring area should be strictly prohibited as the pollutants might enter into the food chain and cause health hazards to humans.