TOXICITY OF TRICHLOROETHYLENE (TCE) UNDER THE NATURAL MILIEU WITH SPECIAL REFERENCE TO THE BIOMARKERS AND GENOTOXICITY STUDIES

SUMMARY OF THE THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF Doctor of Philosophy IN BIOCHEMISTRY

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DEPARTMENT OF BIOCHEMISTRY FACULTY OF LIFE SCIENCES ALIGARH MUSLIM UNIVERSITY ALIGARH (INDIA) 2010
Summary
SUMMARY

Due to the ever increasing pollution of water bodies by TCE and other xenobiotics, there is a growing demand for robust and cost effective monitoring strategies. In addition to the routine analytical techniques, biomarkers are being increasingly used for the detection of aquatic pollution (Galloway et al. 2002; Ferrat et al. 2003; Nazar et al. 2008). Biomarkers have the advantage that they can predict a change much before it occurs at higher levels of biological organization (Jimenez and Stegeman, 1990; Pretti and Cognetti-Varriale, 2001).

The work presented in this thesis was carried out to study the utility of the different enzymes of rat tissues and Allium cepa as biomarkers of TCE pollution and different toxicants. Most of the toxicity testing systems rely on small mammals such as rats or mice, and hence are time consuming, expensive and attract considerable ethical criticism (Tsuda et al. 2001; Fatima and Ahmad, 2006). For these reasons, plant based toxicity biomarker studies was also under taken to compare the efficacy of both the system. Allium cepa system was selected since the Allium cepa test is a cost effective toxicity bioassay routinely used in water monitoring studies (Fiskesjo, 1985; Rank and Nielsen, 1993). It was thought that the efficacy of this test would increase greatly if enzymatic studies are carried out in the same onion bulbs used in the toxicity bioassay (Fatima and Ahmad, 2005). Moreover, the Allium cepa test can also be used to study the genotoxicity of TCE containing test samples.

Trichloroethylene, heavy metals and phenolics are considered to be the major water pollutants present in northern India especially of the region under study (Malik and Ahmad, 1995; Datta, 1999; Dua, 2003; Fatima and Ahmad, 2005; Chiya Nivaran Samithi, 2009). Test water samples were collected from the industrial areas of Aligarh and Saharanpur cities of northern India. These samples were especially found to contain TCE, heavy metals and phenolics. Keeping in mind the contradictory reports on the genotoxicity of TCE, It was our contention to clear this issue once and for all whether TCE is genotoxic or promotes genotoxicity under certain environmental conditions. For this purpose we have supplemented different concentration of TCE into the test water sample and performed genotoxicity assays employing Ames fluctuation test and Allium cepa genotoxicity test.
The genotoxicity/mutagenicity of the test water samples was estimated using Ames plate incorporation test, Ames fluctuation test, Allium cepa test and the plasmid nicking assay. Moreover, the quantitation of various ROS in the test waste waters was also carried out.

The significant findings along with their possible explanations are summarized as under:

(I) **Enzymatic and non-enzymatic antioxidants as biomarker of TCE, ethyl alcohol and heavy metals toxicity in rat tissues**

1. MDA levels seemed to be the best biomarker for the test toxicants in both renal and hepatic tissues.
2. SOD can be considered as a prominent biomarker of heavy metals induced hepatotoxicity.
3. GST and GR can serve as potential biomarkers of TCE and heavy metals induced renal toxicity.

(II) **Antioxidants and the tissue marker enzymes as the biomarkers of TCE contaminated wastewaters from Aligarh and Saharanpur in rat tissues**

It is to emphasize here that the water samples were not supplemented with any additional amount of TCE rather it was present as an essential contaminant of the wastewaters.

1. The most striking effect of the test wastewaters intake in rats was also found to be on MDA level.
2. A remarkable rise to the extent of 5 and 2.5 fold in MDA level was recorded in the liver and kidney respectively as a result of oral administration of the test wastewaters to the rats.
3. SOD and GR can act as a potential biomarker of toxicity for the test water samples in the rat liver.
4. Relatively higher levels of ALT in hepatic and AST in renal tissues of treated rats also suggests that intake of the test water samples might have caused injury to liver and kidney tissues.
(III) Antioxidant enzymes of *Allium cepa* as biomarkers for the detection of TCE pollution in water samples

1. GST and GR in *Allium cepa* system can serve as the appropriate biomarkers of TCE contamination in water. Other enzymes like APx and CAT could also serve as sensitive biomarkers of TCE pollution.

2. The increase in the activities of all the test antioxidant enzymes was at the level of synthesis.

3. All the test enzymes displaying variation in the activities consequent upon exposure to TCE are synthesized in the cytosolic compartment of the cell.

These findings suggest that the *Allium cepa* system can be used for monitoring the changes in antioxidant enzymes in case of suspected TCE pollution in water bodies.

(IV) Some CYP450 isozymes of plant and animal origin as the potential biomarker of TCE in natural milieu

1. PROD and NDMA-d can act as potent biomarkers in *A. cepa* system for assessing the TCE pollution and its hazard.

2. NDMA-d can serve as a potential biomarker of TCE and/or alcohol intake in rats both in the liver and kidney tissues.

(V) Genotoxicity testing by Ames plate incorporation test, Ames fluctuation test and *Allium cepa* test as well as plasmid nicking assay for *in vitro* DNA damage conducted on the wastewater sample

1. The test water samples were found to be highly mutagenic by both Salmonella mutagenicity assay namely Ames plate test as well as Ames fluctuation test.

2. Both the Ames tests were able to differentiate the two test samples qualitatively and quantitatively.

3. TA100 was found to be the most sensitive strain for SWW sample by both *Ames plate incorporation* and *Ames fluctuation tests in the presence* or absence of S9. However, TA98 strain was found to be the most
responsive strain in the absence of metabolic activation towards AWW while TA102 was recorded to be the most sensitive strain in the presence of S9 by Ames plate test.

4. The test water samples collected from Aligarh exhibited an increase in mutagenicity upon metabolic activation evaluated by both tests. However, Saharanpur waste water invariably displayed a decrease in mutagenicity upon metabolic activation.

5. A slight increase in mutagenicity of the test water samples supplemented with 100 ppm of TCE was observed in TA100 strain by Ames fluctuation test.

6. Both the test samples induced a mitodepressive effect in the root cells of *Allium cepa*, though the intensity of depression was quite different.

7. Fragmentation of chromosomes was the predominant effect of AWW while SWW led to the bridge formation to a large extent.

8. The plasmid nicking assay was also quite effective in detecting the genotoxicity of the test samples.

9. Addition of 100 ppm of TCE in these test water samples resulted in significant increase in total aberration frequency of chromosomes in *A. cepa* system. However, supplementation of 50 ppm of TCE in the water samples did not exhibit any significant change in the total chromosomal aberration.

10. As far as the reactive species in the test water samples are concerned, hydroxyl radicals were the prominent species in Aligarh sample and superoxide radicals were the major species in paper mill effluents.

The test water samples were highly genotoxic and could induce mutations in the exposed organisms. The antioxidant/ detoxification enzymes of *Allium cepa* showed significant induction upon exposure to TCE. Therefore, these enzymes would act as suitable biomarkers of TCE pollution. Based on the various findings obtained in this study, it is quite evident that biomarker studies in *Allium cepa* are very useful in assessing TCE pollution. They can complement well established analytical techniques like GC, HPLC and at the same time provide valuable information about the effects of toxicants on the living organisms.
The various biomarkers that could be utilized for the effective monitoring of TCE and heavy metal pollution in water samples are:

1. *Allium cepa* GST and GR can be utilized as the biomarkers of TCE pollution.
2. SOD can also be used as a biomarker of heavy metal stress in rats with special reference to liver.
3. PROD and NDMA-d activity analysis may be successfully used for the detection of the TCE pollution in *Allium cepa* system.
4. The generation of different ROS may also be utilized as an indicator of a particular contaminant. As far as the typical Indian surface waters are concerned, hydroxyl radical generation is an indicator of heavy metal pollution and superoxide radical can be a marker of paper mill contaminants.

Investigation of enzymatic biomarkers of genotoxicity was not included in our plan of work because the genotoxicity in quantitative and qualitative terms as examined by the *Allium cepa* test as well as by other genotoxicity tests, was quite efficiently determined and differentiated for the test water samples.

The results from our studies on toxicity and genotoxicity of TCE especially in the natural milieu as well as test wastewater samples demand that necessary action should be taken without delay to prevent unregulated discharge of TCE and other organic and inorganic xenobiotics into water bodies from the industries flourishing in these areas.
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ALIGARH (INDIA)
2010
Dedicated
To
My parents and
My Wife
I certify that the work presented in this thesis entitled “Toxicity of trichloroethylene (TCE) under the natural milieu with special reference to the biomarkers and genotoxicity studies” has been carried out by Mr. Shams Tabrez under my supervision. It is original in nature and has not been submitted for any other degree.

(Masood Ahmad)
Professor
Acknowledgements

All the admirations are for Almighty Allah, who helped me in difficulties and gave me enough strength and ability to accomplish this research work. All devotions, salat and salam are for Allah’s Prophet, Hazrat Mohammad (PBUH) who enabled us to recognize our Creator.

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I also had the luck of being in the cohort of some magnificent human beings, my friends. Words fall short to describe Shahnawaz’s contribution to this thesis. He has worn my coat to de load me from worries, stepped into my shoes to feel the pinches and drank in the same cup to bear the bitterness. He has been through all this. Shahnawaz you are unmatched and indispensable. Wasim’s presence has been rejuvenating though short timed.

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Last but not the least, my wife Nazia, for her moral support, sacrifice and help enabling me to fulfill my parents and my dream.

I want to end with these lines:

"Two roads diverged in yellow wood and I-
I took the one less travelled by
and that has made all the difference"

Indeed, the "start" culminating to writing acknowledgement begins with a long road seeming all uphill taken on by a lone traveller but when it "ends" its like all is well.

(Shams Tabrez)
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<td>ALP</td>
<td>Alkaline phosphatise</td>
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<td>Dissolved Oxygen</td>
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<td>DHAR</td>
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<td>EDTA</td>
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<td>Saharanpur wastewater</td>
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<td>TCE</td>
<td>Trichloroethylene</td>
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<td>USEPA</td>
<td>United states environment protection agency</td>
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<tr>
<th>Symbol</th>
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<td>µg</td>
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<td>Vogel-Bonner salts</td>
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**VI**

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<th>Activity profile of EROD following oral administration of the test toxicants to rats in the liver (panel a) and kidney tissues (panel b)</th>
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TCE pollution has been one of the key environmental problems of Aligarh (a city of India) for the last one decade. Although, sensitive monitoring regimes employing the analytical techniques like HPLC and GC have been developed, there is a growing demand for studying the harmful effect of TCE pollution on biota. Such studies are expected to provide an insight as to what is happening at the gross biochemical level. Thus biomarker utilization in monitoring programmes is increasingly gaining popularity. In contrast to chemical analysis of contaminants, biomarkers can offer more and biologically relevant information on the potential impact of toxic pollutants on the health of organisms. They can be used as early warning signals for general or particular stress.

TCE, heavy metals and phenolics are the major water pollutants of this region. This study was conducted with an aim to develop simple and cost effective biomarkers of toxicity for TCE in view of its contamination especially in wastewaters. For this purpose, the toxicity testing systems of both the animal and plant origin as well as various genotoxicity tests were employed.

In the first chapter of the thesis, a comprehensive overview has been presented on the available literature related with the pollution, toxicity and biomarkers of TCE.

Second chapter describes the general materials and methods like composition of media, buffers and incubation mixtures, bacterial strains and the protocol for the standard toxicity bioassays.

The use of enzymatic and non-enzymatic antioxidants as the potential biomarkers of TCE in the natural milieu, is the matter of discussion in the third chapter.

Fourth chapter incorporates the data on the role of antioxidants and activities of marker enzymes of tissue damage in the TCE exposed rats.

Fifth chapter of this thesis deals with the use of antioxidant/detoxification enzymes of Allium cepa as biomarkers of TCE pollution.

The study on certain isozymes of CYP450 of rat and A. cepa for the selection of toxicity biomarkers in the test system has been embodied in the sixth chapter.

Seventh chapter has been devoted for the in vitro genotoxicity testings of the test water samples as well as of TCE under natural milieu employing Ames plate
incorporation assay, Ames fluctuation test, Allium cepa genotoxicity test and plasmid nicking assay.

The last chapter is dedicated for general discussion. The bibliography and summary are presented in the end.
Chapter I:
Introduction, Review of Literature and Objectives
1. Introduction

Our environment contains innumerable kinds of obnoxious physical and chemical agents and the number has been continuously increased due to various industrial and socio-cultural activities. The biosphere is constantly exposed to these natural and man made hazardous substances, posing enormous threat to the very survival of living organisms and jeopardizing the ecological balance.

Environmental pollution implies any alterations in the surroundings but is restricted in use especially to mean any deterioration in the physical, chemical or biological quality of the environment (Gleick, 2001). All types of pollution directly or indirectly affect human health. The pollutants fall under the broad classification of xenobiotic compounds and are released into the environment by the action of man and occur in concentrations higher than “natural levels”.

1.1. Water Pollution

Water contaminants pose a high potential risk for the health of populations (Novelli et al. 1998), for this reason their toxic effects should be urgently established. Organic compounds, pesticides, oils, solvents, and heavily used industrial products (Wegman, 1992) reach streams and rivers via run off from unregulated waste disposal. The rate of urbanization has also undergone a rapid increase, including an increase in wastewater discharge, so that exposure of humans to water pollutants is rarely limited to a single chemical or organic residue (Heindel et al. 1995).

1.2. Categories of water pollution

Surface water and groundwater have often been studied and managed as separate resources, although they are interrelated (USGS, 1998) Sources of surface water pollution are generally grouped into two categories based on their origin namely point source and non-point source.

1.3. Groundwater vs surface water pollution

Interactions between groundwater and surface water are complex (USGS, 1998). By its very nature, groundwater aquifers are susceptible to contamination from sources that may not directly affect surface water bodies, and the distinction of point vs. non point source may be irrelevant. A spill of a chemical contaminant on soil, located
away from a surface water body, may not necessarily create point source or non-point source pollution, but nonetheless may contaminate the aquifer below.

1.4. Water pollution: national scenario

During the past few decades Indian industries have registered a quantum jump, which has contributed to high economic growth but simultaneously it has also given rise to severe environmental pollution. Consequently, ambient air and water quality is seriously affected which is far lower in comparison to the international standards. The problem is worse in the case of water pollution. It is found that one-third of the total water pollution comes in the form of effluent discharge, solid wastes and other hazardous wastes. The surface water is the main source of industries for waste disposal. It is found that almost all rivers are polluted in most of the stretches by some industry or the other. Although all industries function under the strict guidelines of the Central Pollution Control Board (CPCB) but still the environmental situation is far from satisfactory. Different norms and guidelines are given for all the industries depending upon their pollution potentials. In India there are sufficient evidences available related with the mismanagement of industrial wastes (Mishra, 2008). Consequently, at the end of each time period the pollution problem becomes of menacing concern. So far no clear-cut estimations have been made to determine the overall effects of the industrial pollution, especially industrial water pollution. In very few instances the problem has been identified partially. However, many studies have listed heavy metals and pesticides as the major water pollutants in India (Malik and Ahmad, 1995; Rehana et al. 1995; 1996; Datta, 1999; Singh et al. 2005a; 2005b; Fatima and Ahmad, 2005; 2006).

1.5. Global picture of water pollution

Water pollution is also a major problem in the global context. It has been suggested that it is the leading worldwide cause of deaths and diseases (Pink and Daniel, 2006; West and Larry, 2006) and accounts for the deaths of more than 14,000 people daily (West and Larry, 2006). In addition to the acute problems of water pollution in developing countries, industrialized countries also continue to struggle with these problems.
1.6. Water pollution with special reference to TCE contamination

TCE, a chlorinated solvent has been produced commercially since 1920s in many countries by chlorination of ethylene or acetylene. Its use in metal degreasing began in 1920s. In the 1930s, it was introduced for use in dry cleaning, but it has had limited use in that way since 1950s. Currently, 80-90% of TCE worldwide is used for degreasing metals. Use for all applications in Western Europe, Japan and the United States in 1990 was about 225 thousand tonnes. Thus, the primary sources of releasing TCE into the environment are metal cleaning and degreasing operations (USEPA, 1985). TCE has been detected in air, water, soil, food and animal tissues. The most heavily exposed people are those working in the degreasing of metals, which are exposed by inhalation.

TCE was first detected in groundwater in 1977, and has been one of the most frequently detected contaminants in groundwater in the United States of America. TCE was identified in 34% of drinking water supplies in one nationwide survey (Westrick et al. 1984), though United States Environment Protection Agency (USEPA, 2001) had reported that most water supplies were in compliance with the maximum contaminant level of 5μg/L. TCE has been found at 50% of the 1,300 hazardous waste disposal sites proposed for inclusion on the National Priorities List (HAZDAT, 1995). Spillage and leakage from storage tanks and chemical waste sites are often responsible for contamination of groundwater by TCE and other volatile organic chemicals (ATSDR, 1997). In addition, a growing concern in recent years at sites with TCE contamination in soil or groundwater has been vapour intrusion in buildings, which has resulted in indoor air exposures. TCE has been detected in 852 superfund sites across the United States, according to the Agency for Toxic Substances and Disease Registry (ATSDR, 1997). TCE has also been the most frequently found chemical contaminant of groundwater in the proximity of hazardous waste sites in the USA (Fay and Mumtaz, 1996).

2. Alcoholics vis-à-vis industrial pollution

Alcohol, by its virtue of generating free radicals, causes severe damage to the membrane and affects almost all organs of the human body. Alcohol-related disorders are one of the challenging current health problems with far reaching medical, social and economic consequences. Long-term alcohol use potentially results in serious illnesses, including alcoholic fatty liver, hypertriglyceridemia, cirrhosis,
cardiovascular disease and inflammation of the pancreas. One of the factors that play a central role in many pathways of alcohol-induced damage is oxidative stress (Devipriya et al. 2007). Due to excessive ethanol (EtOH) consumption by people who may be exposed to different xenobiotics environmentally and occupationally (Schioeler, 1991; Meyer et al. 2000) interactions between different xenobiotics and EtOH are important for epidemiological and medical perspectives.

3. Toxicity with particular reference to genotoxicity

Among the various types of toxicities, organ toxicity like hepato/ cardio/ neuro/nephro-toxicity is limited to animal kingdom, phytotoxicity restricts itself to plant kingdom and other toxicities though covering the different trophic levels but limit themselves to the exposed organisms only. However, it is the genotoxicity which encompasses the whole range of biota and all living organisms. Moreover, such harmful effects extend up to the future generation of living organisms.

Genotoxicity refers to the harmful effects of an agent on the genetic material (Pratt and Barron, 2003). It has been found to be directly associated with the neoplastic transformation (i.e. cancer) in humans and animals (Zeiger, 2001). Any measure directed at prevention of this as yet incurable disease would be highly desirable. Thus genotoxicity testing not only addresses the problem of toxicity at the very root level since targeting the genetic material is just like targeting the king of the country or the heart in the body, it also provides a clue to the cancer- causing potential of an agent at the individual level as well as incidence of genetic diseases in the exposed population. A wide variety of genotoxicity tests have been developed for biomonitoring purposes. These include use of micronuclei counts (Spies et al. 1990), DNA adducts (Varanasi et al. 1987), strand breakage (Stamato and Denko, 1990), his+ reversion (Kummarow et al. 2003; Umbuzeiro et al. 2004) etc. A combination of these assays provides a powerful method for assessing short and long term genotoxicity.

There are many assays for detecting the genotoxicity of surface waters but the utilization of bioassays with bacteria has proven to be very effective for monitoring because these assays are sensitive, inexpensive, reliable and can be performed in a short period of time with relatively low cost. Some representative examples of mutagenicity assays at different trophic levels are described as under:
3.1. Ames *Salmonella* mutagenicity test

Among the microbial bioassays, the *Salmonella* mutagenicity test has been the most widely used for detecting mutagenicity/genotoxicity in surface waters. The different responses of the *Salmonella typhimurium* strains can provide information on the classes of mutagens present in the water samples. This test has been proposed by the USEPA for clean water compliance monitoring (USEPA, 1989).

Rehana et al. (1995, 1996) used five different *Salmonella typhimurium* strains to compare the mutagenic activity of water samples from four sites of Ganga River, India. Samples always showed extreme mutagenic activity for TA98 and TA100 strains, both with and without S9 fraction. They also found a similar pattern in the responsiveness of the tester strains for a mixture of pesticides suggesting that the mutagenicity of the water extracts may be attributable to the pesticides used in the upstream region. Aleem and Malik (2003) and Siddiqui and Ahmad (2003) further reported that the mutagenic potential of the XAD-concentrated water samples from the river Yamuna, India was also extremely high for TA98 strains both with and without S9 mix. They also suggested the presence of oxidative mutagens in the Indian riverine system. Fatima and Ahmad (2006) compared genotoxic potential of wastewater samples from two different stations namely Aligarh and Ghaziabad. Both the test water samples were found to be highly mutagenic by this test and the best sensitivity was recorded in case of TA102 and TA98 strains. An extremely high mutagenic potential of the water samples from a river in Brazil was suggested by Vargas et al. (1993) employing TA98 strain and S9 fraction. Numerous other studies have also employed the *Salmonella* mutagenicity test for the evaluation of water pollution (Kummrow et al. 2003; Umbuzeiro et al. 2004; Kutlu et al. 2007; Gana et al. 2008).

3.2. SOS chromotest/umu-test

Although the *Salmonella* microsome test has been widely used for the detection of mutagenicity in environmental samples, a variety of other assays also exist for investigating complex environmental mixtures. The SOS-chromotest and the umu test were developed as alternatives to the Ames test by Quillardet et al. (1982) and Oda et al. (1985) respectively. These are widely used for the routine monitoring of water samples as the results are available in a single day with minimal advance preparation. The microplate version of the SOS chromotest and umu test was developed as a rapid
and sensitive screening tool for the detection of genotoxins in surface waters (Langevin et al. 1992; White et al. 1996). The application of a fluorometric umu-test system has been developed in order to increase the sensitivity of the test for the detection of genotoxic compounds in surface water (Reifferscheid and Zipperle, 2000).

The Mutatox test, employing a dark mutant strain of luminescent Photobacterium phosphoreum, the phage induction test and the DNA repair assays with E. coli mutants have also been widely used for screening surface water samples for genotoxic activity and have been promoted as candidates for a battery of screening assays (Helma et al. 1996; Vahl et al. 1997; De Maagd and Tonkes, 2000).

3.3. Escherichia coli lacZ reversion mutagenicity assay

The Escherichia coli lacZ reversion mutation assay was introduced by Cupples and Miller (1988). The lacZ assay uses a set of E. coli lacZ strains. Each strain carries a lacZ allele which codes for an inactive β-galactosidase protein. The use of lactose as a carbon source by E. coli requires the activity of β-galactosidase which catalyzes hydrolysis of lactose to glucose and galactose. Therefore, reversion to lacZ results in a colony which can grow on lactose minimal medium. The lacZ alleles used in the Cupples and Miller (1988) system were rationally designed so that only a single DNA sequence change generates a selectable mutant from each allele. This means that the lacZ assay can be used directly to test the mutational specificity of a particular mutagen without need for DNA sequencing. This test has been used in the detection of mutagenicity of effluent from dye industry (Chung et al. 1998; 2000).

3.4. Single cell gel electrophoresis/ comet assay

In recent years the comet assay has gained broad attention, because the test is relatively easy to handle and can be applied with cells from different organisms and tissues. The alkaline version of the comet assay has been developed by Singh et al. (1988). Several studies have employed the comet assay for assessing the level of DNA strand breakage in cells from aquatic organisms treated with surface water samples in vivo and in vitro (Klobucar et al. 2003; Russo et al. 2004; Woo et al. 2006). Advantages of the test are the possibility to choose a broad range of test organisms and tissues, the use of even non-proliferating cells, and that results can be obtained within one day. On the other hand there are still no standard test protocols
and a certain degree of handling skills is a necessary prerequisite to routinely performing the test (Angerer et al. 2007).

3.5. *Saccharomyces cerevisiae* gene mutation assay

The test performance of the gene mutation assay with unicellular yeast (*Saccharomyces cerevisiae*) is more comparable with the bacterial assays than with other eukaryotic tests. The test principle is the detection of forward or reverse mutations (Zimmermann, 1984).

3.6. Chromosome aberration assay

Chromosome aberrations include structural aberrations such as fragments or intercalations and numerical aberrations. Cytogenetic effects can be studied either in whole animals or in cells grown in culture. Generally the cell culture is exposed to the test substance and then treated with a metaphase-arresting substance. Following suitable staining the metaphase cells are analysed microscopically for the presence of chromosomal abnormalities (Fučić et al. 2007).

3.7. Micronucleus induction assay

The micronucleus assay is a widely used cytogenetic assay for the assessment of *in vivo* or *in vitro* chromosomal damage. There are several reports on micronucleus induction in aquatic organisms, plants and cultured cells treated with surface water (Campana et al. 2001; Dixon et al. 2002). Micronucleus formation along with the sister chromatid exchanges and chromosome aberration assays is considered as a clastogenic endpoint. In principle flow cytometric measurement of micronuclei is possible (Kohlpoth et al. 1999; Sánchez et al. 2000) but the costs of equipment are high.

3.8. Other genotoxicity assessment methods

Sister chromatid exchange (SCE) assay, UDS assay, DNA adduct formation and the Tradescantia stamen hair mutation assay have also been widely used for the detection of aquatic pollution (Müllerschön, 1989; Duan et al. 1999; Grummt, 2000; Ericson and Larsson, 2000; Stephenson et al. 2000; Bakare et al. 2003).
3.9. The *Allium cepa* test: a sensitive tool in environmental monitoring

The *Allium cepa* test was introduced in 1938 by Levan. Since then, it has been widely used for environmental monitoring. This test has been widely used for the toxicity assessment of heavy metals, pesticides and industrial wastewaters (Chauhan et al. 1986; Fiskesjo, 1988; Grover and Kaur, 1999; Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006). Kovalchuk et al. (1998) used this assay to evaluate the genotoxic potential of waste water. Our group (Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006) have also used this test for the genotoxicity assessment of wastewater from Aligarh region. The *Allium cepa* test has also been used by other investigators for detecting the genotoxicity of surface water (Monarca et al. 2003; Leme and Morales, 2009) and the clastogenicity of atrazine (Bolle et al. 2004).

The *Allium cepa* anaphase-telophase chromosomal aberration test has been used to study the mutagenic effects of N-methyl-N-nitrosourea, maleic hydrazide, sodium azide and ethyl methane sulphonate (Rank and Nielsen, 1997). Both versions of the *Allium cepa* test viz. root length inhibition assay as well as genotoxicity assay are cost-effective, easy to perform and sensitive enough to respond to low concentrations of toxicants.

4. Hazard assessment: Toxicity Bioassays

The widespread use and release of natural and synthetic chemicals into the environment, singly or as complex domestic and industrial effluents, has necessitated the development of rapid and cost effective toxicity tests to protect humans and other biota (Sherry et al. 1997; Dalzell et al. 2002). Thus both the short-term and long-term bioassays exist utilising all trophic levels including microorganisms, invertebrates, higher plants and animals. The general purpose of toxicity testing is to establish the potential impact of chemicals on biota in the environment. The information gained can then be used to manage the treatment or release of chemicals. No single toxicity test can determine the effect of toxicants on all biota because of differences in response by organisms at different trophic levels (Kaiser, 1993). With respect to biological testing, it is known that organisms within the same (Codina et al. 1993) and in different (Ribo, 1997; Shoji et al. 2000) trophic levels respond differently to a range of toxicants, either as single or complex mixtures, and hence there is a need to develop toxicity bioassays using a battery of susceptible organisms. This is important
because of the complexity in terms of organisms diversity present in natural environments and differences in physiological status (Chapman, 2000).

5. Metabolism and toxicity of trichloroethylene

Like many other chlorinated hydrocarbons, TCE has become an important environmental pollutant because of its toxic properties and widespread occurrence as a soil, air and water contaminant (Candura and Faustman, 1991). Knowledge of trichloroethylene metabolism is critical for determining susceptibility, target organ specificity, and gender and species differences and for extrapolating animal data to humans. Liver is the major site of trichloroethylene metabolism; it undergoes metabolism by two major pathways (Fig. 1), cytochrome P450 dependent oxidation and conjugation with glutathione. Most of the focus on the oxidative pathway has been on the liver, which has the highest activities of the various isoforms of cytochrome P450s (CYPs). CYP-mediated metabolites of trichloroethylene have been directly associated with liver injury. Trichloroethylene is metabolized primarily by CYP2E1 to a trichloroethylene oxide intermediate, which spontaneously rearranges to chloral. Chloral is further metabolized to trichloroethanol, trichloroethanol glucuronide and trichloroacetic acid. Minor metabolites include carbon dioxide, dichloroacetic acid, oxalic acid and N-(hydroxyacetyl)aminoethanol. The glutathione-dependent pathway yields glutathione conjugate, S-(1,2-dichlorovinyl)glutathione, which occurs predominantly in the liver and can also occur in extrahepatic tissues, but additional biotransformation of S-(1,2-dichlorovinyl)glutathione takes place in the kidney (Miller and Guengerich, 1983; Davidson and Beliles, 1991; Goeptar et al. 1995; Lash, 1999; Lash et al. 2000).
Fig. 1: Metabolism of trichloroethylene. Arrows with broken lines indicate other possible steps in forming DCA.

Abbreviations: CYP, Cytochrome P450; DCA, dichloroacetic acid; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl) glutathione; DCVT, S-(1,2-dichlorovinyl) thiol; GSH, glutathione; GST, glutathione S-transferase; NAcDCVC, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; TCA, trichloroacetic acid; TCE, trichloroethylene; TCE-O-CYP, trichloroethyleneoxide-cytochrome P450 complex; TCOH, trichloroethanol; TCOG, trichloroethanol glucuronide.

[Adopted from Lash et al. (2001) Environmental Health Perspectives, 297: 155-164]
5.1. Hepatotoxicity of trichloroethylene

It is well documented that trichloroethylene induces hepatotoxicity in experimental animals and humans (ATSDR, 1997; USEPA, 2001). One report suggested that TCE exposure through inhalation route induced hepatotoxicity in terms of marked necrosis and fatty changes by modulating the lysosomal enzymes (Kumar et al. 2001a). Moreover, TCE was found to produce liver enlargement as well as diversified types of biochemical and histological changes (Kjellstrand et al. 1981; Tucker et al. 1982; Nomiyama et al. 1986). S-(1,2-dichlorovinyl)-L-cysteine, a metabolite of TCE metabolism did not target the liver in vivo, but it was found to be a very potent hepatotoxicant in vitro (Lash et al. 2000).

5.2. Nephrotoxicity of trichloroethylene

Metabolites derived from the GSH conjugate of TCE, in contrast, have been associated with the kidney as target organ. Specifically, metabolism of cysteine conjugate of TCE by the cysteine conjugate β-lyase generates a reactive metabolite that is nephrotoxic and may be nephrocarcinogenic also (Dekant et al. 1999; Pähler et al. 1999; Lash et al. 2000). S-(1,2-dichlorovinyl)-L-cysteine (DCVC), a common metabolite of TCE was found to be a selective proximal nephrotoxicant (Vaidya et al. 2003). TCE causes kidney damage at exposure concentration of more than 250 ppm (Green et al. 2004). Nephrotoxicity of TCE in humans has been reported in those individuals who ingested 70mL of TCE (Bruning et al. 1997).

5.3. Genotoxicity/Mutagenicity of trichloroethylene

Genotoxic potential of TCE is often conflicting, in part because of the presence of impurities or mutagenic stabilizers in the test material (McGregor et al. 1989). In fact, the information from many of the early studies may not be adequate for complete evaluation of the genotoxic potential of TCE.

5.3.1. Prokaryotic vs eukaryotic systems

Studies on the mutagenicity of TCE have been performed in several living systems including bacteria, fungi, yeast and cultured mammalian cells.

TCE was not mutagenic in a bacterial mutagenicity assay using *Salmonella typhimurium* TA100 (Crebelli et al. 1982) nor were his^+^ revertants detected in *S. typhimurium* TA100 in the presence of rat kidney S9 fraction (Simmon et al. 1977).
Chloral hydrate the presumed product of TCE epoxide decomposition was directly mutagenic in *S. typhimurium* TA100 but ineffective in TA1535, TA1537 and TA98 strains (Waskell, 1978; Bignami et al. 1980; Haworth et al. 1983). It is conceivable, however, that the high reactivity and low stability of TCE epoxide in aqueous solution (Half life 1.5 min) could hinder appreciating its mutagenicity in bacterial reversion assay where plenty of nucleophilic targets other than DNA are available for chemical interaction. The mutation spectra of volatile metabolites of TCE i.e. dichloroacetyl chloride (DCAC) and dichloroacetic acid (DCA) obtained at the base-substitution allele *hisG46* of *Salmonella typhimurium* strain TA100 demonstrated GC → AT transition (Demarini et al. 1994). However, the microscreen prophage-induction assay showed the genotoxicity of DCA only in the presence of S9 fraction (Demarini et al. 1994).

Both in the presence and absence of S9 activation, multi-complex mixture of CHCl3, CCl4 and TCE (85:8:7) was mutagenic for a narrow range of doses in the tester strains, TA1535, TA1537, TA98 and TA100 of *S. typhimurium* (Varma et al. 1988).

TCE was weakly mutagenic in the mold *Aspergillus nidulans* that too in the growing phase only (Crebelli at al. 1985). TCE free of epoxides, has been assayed for its ability to induce gene mutations and mitotic segregation in the mould *Aspergillus nidulans*. No increase in the spontaneous frequency of methionine suppressors was observed when conidia of a haploid strain were plated on selective medium and exposed to TCE vapours. A weak but statistically significant increase in methionine suppressors was detected however, when conidia of cultures grown and conidiated in the presence of TCE vapours were plated on to selective media (Crebelli at al. 1985). In cultured mammalian cells, TCE did not induce sister chromatid exchange in Chinese hamster ovary cells (White et al. 1979) nor was able to induce DNA repair in primary culture of rat hepatocytes (Shimanda et al. 1985).

### 5.3.2. Genotoxicity of TCE and its metabolites

In one review on mutagenicity of TCE and its metabolites, Moore and Harrington-Brock (2000) concluded that TCE and its metabolites namely chloral hydrate (CH), dichloroacetic acid (DCA) and trichloroacetic acid (TCA) require very high doses to become genotoxic. Moreover, TCA and DCA are not direct mitogens in hepatocyte cultures (Walgren et al. 2005). Tao et al. (2000) have reported that DCA, TCA and
TCE decreases methylation of DNA at the promoter region of proto oncogenes *c-myc* and *c-jun* after 5 days of exposure in mouse liver. Increased level of mRNA and protein for *c-myc* and *c-jun* were also reported after DCA and TCA treatment. Metabolic studies of potential aneugens in genetically engineered human lymphoblastoid cells demonstrated detoxification of aneugenic activity of CH and the activation of 2,3-dichlorobutane, trichloroethane and TCE by phase 1 biotransforming enzymes (Parry et al. 1996).

Various TCE metabolites bind to DNA and proteins in a dose dependent manner in liver (Kautiainen et al. 1997). Significant covalent binding confirmed by Sephadex column chromatography was observed after incubation of $^{14}$C-TCE with calf thymus DNA in the presence of hepatic microsomes from phenobarbital induced rats (DiRenzo et al. 1982). Cysteine conjugated β-lyase bioactivated DCVC to a reactive intermediate which is capable of binding cellular macromolecules (DuTeaux et al. 2003).

In general, TCE, TCA and DCA have all been shown to cause DNA strand breaks in rodent liver cells in vivo and in culture at higher concentrations as either the parent molecule or its metabolites (Bull, 2000). However, the results of some studies appear to contradict these findings (Styles et al. 1991; Chang et al. 1992).

5.3.3. In vivo vs in vitro genotoxicity

Studies in lymphocytes of workers exposed to TCE did not provide any evidence of chromosomal damage at TCE exposure levels of up to 30 ppm (Nagaya et al. 1989). Metal workers exposed to TCE showed no difference between exposed and unexposed persons with respect to sperm count and its morphology. In contrast, there was a highly significant increase in frequency of structural aberrations and hyper diploid cells in cultured lymphocytes from TCE degreasers (Rasmussen et al. 1988). TCE caused a statistically significant increase in micronuclei at different concentrations, inducing an approximately four fold increase over control levels at 5000 ppm in mice (Kligerman et al. 1994). It also showed positive C-mitotic effects accompanied with increases of mitotic index and decreased frequencies of anaphases at higher doses in mouse bone marrow cells (Sujata and Hegde, 1998). Significant dose-dependent increases in the frequency of DNA single-strand breaks and alkali-labile sites as measured by the comet assay and in micronuclei frequency were obtained in primary kidney cells from both male rats and humans with the
concentrations of 1 to 4 $\mu$M of TCE (Robbiano et al. 2004).

Some studies indicated a weak mutagenic activity of TCE both \textit{in vitro} where liver microsomes produced electrophilic TCE metabolites and also \textit{in vivo} in mouse bone marrow where high rates of micronuclei but no structural chromosome aberrations were found (Crebelli and Carere, 1989; Fahrig et al. 1995). Furthermore, TCA and CH were also found to be genotoxic \textit{in vivo} inducing structural and numerical chromosome abnormalities respectively (Crebelli and Carere, 1989). TCE was also observed to induce increased DNA synthesis and mitosis in mouse liver (Dees and Travis, 1993). A single high dose of TCE can induce an increase in oxidative DNA damage in rat liver (Toraason et al. 1999).

Despite the apparent lack of typical genetic toxicity, TCE could be involved in the expression of carcinogen-induced mutations due to its potential to induce recombination and aneuploidy (Fahrig et al. 1995). Moreover, while the genotoxicity data are not fully conclusive, there appears to be evidence to show that TCE has a weak, likely indirect, genotoxic effect at high doses. Therefore, the mutagenic potential for this compound cannot be disregarded.

7. Relevance of biomarkers in environmental toxicology

Limitations in understanding the relationship between occupational and environmental exposures and diseases present ample opportunities for using biological markers to fill the gaps in knowledge. The most compelling reason for using biomarkers is that they can give information on the biological effects of pollutants rather than a mere quantification of their environmental levels. Biomarkers may provide insight into the potential mechanisms of contaminant effects (Altenburger et al. 2003). The practical goal of biomarker research and application is to prevent disease by reducing exposures to hazardous agents through the early identification of exposure and response (Silbergeld and Davis, 1994). Potential utility of biomarkers for monitoring of environmental quality has received increasing attention during the last few years (Zhang et al. 2004). Identification of early and sensitive biomarkers of exposure allows the development of strategies to prevent cell damage that results in persistent or irreversible injury (Brooks, 2001).
7.1. Biomarker concept

Typically, biomarkers are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure and/or doses of xenobiotic substances that lead to biological effects. Although not explicitly contained in most definitions, the use of the term ‘biomarkers’ or ‘biomarker response’ is often restricted to cellular, biochemical or molecular or physiological change that are measured in cells, body fluids, tissues or organs within an organism and are indicative of xenobiotic exposure and/or effect (Oikari and Jimenez, 1992; Lam and Gray, 2003).

Changes that occur at the organismic, population and assemblage levels are usually referred to as ‘bioindicators’. One possible reason for limiting the term ‘biomarkers’ to sub-organismic changes is that one of the functions of biomarkers is supposedly to provide early warning signals of biological effects and that it is generally believed that sub-organismic (molecular, biochemical and physiological) responses tend to precede those at the organismic or higher levels (Jimenez and Stegeman, 1990; Lam and Gray, 2003). Biomarkers could also be used to test the effectiveness of environmental controls. The utility of a biomarker is expected to influence by the degree to which it is validated (Schulte, 1995; Molitoris et al. 2008; Trepicchio and Mulligan, 2008).

According to the National Research Council of Canada (NRC, 1985) and WHO (1993), biomarkers can be subdivided into three classes:

I. Biomarkers of exposure, which cover the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism.

II. Biomarkers of effect, which include measurable biochemical, physiological or other changes within tissues of body fluids of an organism that can be recognized as associated with an established or possible health hazard.

III. Biomarkers of susceptibility, which include the inherent or acquired ability of an organism to respond to the challenge of exposure to specific xenobiotic substances including genetic factors.
At a practical and operational level, there are four desirable characteristics of the biomarker assay: sensitivity, specificity, simplicity and stability. The assay should be sensitive enough to detect early stage of the toxicity while specificity is desirable because it can provide evidence of the harmful effect of a particular type of pollutant. Simplicity is desirable to make an assay available to non-experts in a cost-effective way. Stability is also important in the sense that unstable and short-lived responses are difficult to measure and interpret in field studies (Walker, 1998; van der Oost et al. 2003). Use of biomarker is regarded as ethically acceptable. Good biomarkers are sensitive indices of both pollutant bioavailability and early biological responses.

The selection of the appropriate markers for the study of the effect of contaminants is frequently a controversial issue especially when information on the mechanism of action of the contaminant is insufficient (Lauwerys et al. 1995). In reality, no biomarker assay exists that has all of the aforementioned attributes and it is unlikely that there ever will be such a biomarker. This limitation can be overcome by using a combination of biomarkers (Lagadic et al. 1997; 1998). By screening multiple biomarker responses, important information will be obtained about organism toxicant exposure and stress. A pollutant stress situation normally triggers a cascade of biological responses, each of which may serve as a biomarker at least theoretically (Besten and Munawar, 2005).

7.2. Biomarkers vis-à-vis xenobiotic biotransformation

Xenobiotics are usually biotransformed in the liver according to the simplified mechanism, which can be subdivided into phases I, II and III. Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original foreign molecule, which can then be conjugated in phase II and catabolized in phase III (van der Oost et al. 2003). The enzymes of phase III (e.g. peptidases, hydrolases and β-lyase) catalyze the catabolism of conjugated metabolites to form easily excretable products.

7.2.1. Phase I of biotransformation

Phase I is the predominant biotransformation pathway. It generally involves the addition or exposure of functional groups on the xenobiotic, e.g. by oxidation or hydrolysis (Goeptar et al. 1995). The most extensively examined system, from the point of view of biomarkers, is the mixed function oxidase system (MFO) which
involves oxidation by a variety of isozymes of cytochrome P450 (Lewis, 2001). Since the MFO system is sensitive to certain environmental pollutants, its activity may serve as a biological monitor for exposure to certain classes of xenobiotic chemicals (Bucheli and Fent, 1995; van der Oost et al. 2003).

Cytochrome P450 was discovered as a pigment that in a complex with CO could absorb light at 450 nm (Schenkman and Jansson, 1998). Its inactive form has an absorption maximum of 420 nm. Cytochromes P450, comprising a large and still expanding family of heme proteins, are membrane-bound proteins which predominantly are located in the endoplasmic reticulum of the liver (Jia et al. 2009). Its activity depends on the presence of NADPH-cytochrome P450 reductase and phospholipid membrane function. All these components constitute a monooxygenase system (Kvanickova, 1995).

7.2.1.1. Biochemistry of cytochrome P450

In a simplified form, cytochrome P450 function is shown by the following formula:

\[
\text{NADPH} + \text{H}^+ + \text{RH} + \text{O}_2 \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ 
\]

This is a monooxygenase reaction in which one molecule becomes more planar by the insertion of an oxygen atom.

For the detection of pollution in aquatic environments, the CYP1A1 family members have so far been proved to be the most sensitive indicators (Machala et al. 2000; Schelnk and DiGiulio, 2002). They respond to water contamination at levels too low to be detected by other laboratory methods.

7.2.2. Phase II enzymes

Enzymes active in Phase II are located in the cytoplasm and endoplasmic reticulum and promote conjugations of Phase I products with endogenous ligands such as glutathione (Talalay, 2000) to inactivate the xenobiotics by making it more water-soluble (polar), which is important for elimination and excretion. The function of Phase II enzymes can be defined by the following properties (Talalay, 2000).

- Regulation by mechanisms that are very similar and may involve common promoter elements (e.g. Antioxidant Responsive Element, ARE).
• Catalysis of a wide variety of reactions that serve to protect cells against toxicities of electrophiles and reactive oxygen species by converting them to less toxic products.

• The induction of Phase II enzymes is effective and sufficient to accomplish cellular protection against toxic and neoplastic effects of electrophiles and reactive oxygen species (ROS).

Numerous endogenous sources of oxyradical production exist, but of more immediate interest with respect to environmental biomarkers is the ability of a number of structurally diverse compounds to enhance intracellular oxyradical production through the process of redox oxidant-mediated effects with a potential suitability as biomarkers. These include either adaptive responses, such as increased activities of antioxidant enzymes and concentrations of non-enzymatic compounds, or manifestations of oxidant-mediated toxicity in terms of oxidations of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Filho, 1996; Howcroft et al. 2009). Defense systems that tend to inhibit oxyradical formation include the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD, CAT, GST and GPx are critically important in the detoxification of radicals to nonreactive molecules. Numerous low-molecular-weight antioxidants, such as GSH, β-carotene (vitamin B), ascorbate (vitamin C), α-tocopherol (vitamin E) and ubiquinol 10 have also been found to play a vital role (Lopez-Torres et al. 1993; Griffiths et al. 2002).

7.2.2.1. Glutathione-S-transferases (GST)

The family of glutathione-S-transferase (GST) enzymes is of physiological importance because its members provide protection against electrophilic xenobiotics, such as heavy metals, pesticides, carcinogens, etc. by conjugating them to glutathione (GSH) (Mannervik and Danielson, 1988). GSTs are also essential components of the cellular antioxidant defence system, since they catalyze the conjugation of GSH to several dangerous compounds produced by lipid peroxidation (Arrigo, 1999; Rahaman et al. 1999).
A remarkable sensitivity of glutathione S-transferases to a large variety of pollution conditions, either organic or inorganic is identified by several workers (Zakharov and Clarke, 1993; Fatima and Ahmad, 2005).

As regards with CYP1A, the mechanism of induction for most GSTs in mammals is regulated via the Ah-receptor (George, 1994; Kirby and Ottea, 1995). An additional form of GST induction which functions independently of the Ah-receptor has been elucidated. It requires metabolism of the compound before transcriptional activation of the respective subunit gene takes place (Rushmore and Pickett, 1990). Due to the role that GSTs play in conjugating reactive epoxide species and other electrophiles, induction of these enzymes must be considered to be beneficial, although metabolic activation of halogenated xenobiotics by GST is also well recognized (Armstrong, 1990; Commandeur et al. 1995).

7.2.2.2. Superoxide dismutase (SOD)

The superoxide dismutase comprises a family of multi functional enzymes with broadly overlapping substrate specificities, which play important roles in detoxification of superoxide radical (Pereira et al. 1998). Similar to other antioxidant enzymes, SOD is known to be influenced by a large number of toxic compounds (Novelli et al. 1997).

SOD is considered as the first line of defense against oxygen toxicity and the central regulators of ROS levels by catalyzing the decomposition of superoxide, the first but most abundant ROS, into hydrogen peroxide and water.

SOD can be activated to scavenge excessive superoxide in the presence of moderate oxidative stress with compensation (Landis and Tower, 2005). Hence, biphasic fluxes of SOD activity are common, and an increase or decrease may relate to the presence of excessive superoxide.

7.2.2.3. Catalase (CAT)

Catalases are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H2O2), which is metabolized to molecular oxygen (O2) and water. Unlike some peroxidases that can reduce various lipid peroxides as well as H2O2, CATs can only reduce H2O2 (Filho, 1996; Menone et al. 2008). It was demonstrated that peroxisome-proliferating compounds (a class of non-genotoxic carcinogens) induce both the activities of H2O2-generating fatty acid oxidases and CAT in rodents (Reddy
and Lalwani, 1983; Halliwell and Gutteridge, 1999). Since CATs are localized in the peroxisomes of most cells and are involved in fatty acid metabolism, changes in activities may often be difficult to interpret (Stegeman et al. 1992; van der Oost et al. 2003). Therefore, CAT activities in erythrocytes may be a more appropriate marker for oxidant exposures in vertebrates.

7.2.2.4. Glutathione peroxidase (GPx)

Peroxidases are enzymes that reduce a variety of peroxides. While CAT employs one molecule of H2O2 as donor in the reduction of another H2O2 molecule, peroxidases employ other reductants. GPx catalyzes the metabolism of H2O2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG). GPx is considered to play an especially important role in protecting membranes from damage due to lipid peroxidation (LPO). This observation led to the view that the major detoxification function of GPx is the termination of radical chain propagation by quick reduction to yield further radicals (Lauterburg et al. 1983). The use of peroxidases in plants may be used to detect early oxidant responses, but the use of GPx received relatively little attention as a biomarker in animals (Stegeman et al. 1992). The GPx/glutathione system is considered to be a major defense in low-level oxidative stress (Wassmann et al. 2004).

7.2.2.5. Glutathione reductase (GR)

Glutathione reductase perhaps is not involved in antioxidant defense in the same way as described for the aforementioned enzymes. However, GR merits attention because of its importance in maintaining GSH/GSSG homeostasis under oxidative stress conditions (Winston and DiGiulio, 1991). GR catalyzes the transformation of the oxidized disulfide forms of glutathione (GSSG) to the reduced form (GSH), with the concomitant oxidation of NADPH to NADP⁺.

7.2.2.6. Monodehydroascorbate reductase (MDHAR)

Monodehydroascorbate reductase belongs to the family of oxidoreductases. In plants, the MDHAR is an enzymatic component of the glutathione-ascorbate cycle that is one of the major antioxidant systems of plant cells for the protection against the damages produced by ROS. This is found in several cell compartments, such as chloroplasts, cytosol, mitochondria, glyoxysomes, and leaf peroxisomes (Leterrier et al. 2005).
MDHAR could account for the regeneration of ascorbate from monodehydroascorbate (MDA) produced by ascorbate peroxidase (APx) activity. In the absence of MDHAR, MDA disproportionated to ascorbate (ASC) and dehydroascorbate (DHA). The DHA is reduced to ASC by dehydroascorbate reductase (DHAR) in chloroplasts (Chen and Gallie, 2006).

7.2.2.7. Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase is an enzyme that is critical for maintenance of an appropriate level of ascorbate in plant cells (Kato et al. 1997). It is responsible for regenerating ASC from an oxidized state and regulates the cellular ASC redox state which in turn affects cell responsiveness and tolerance to environmental ROS (Chen and Gallie, 2006).

7.2.2.8. Reduced and oxidized glutathione (GSH and GSSG)

Reduced GSH, a tripeptide consisting of glutamine, cysteine and glycine, can be conjugated in the initial step of mercapturic acid formation (Commandeur et al. 1995; Mutlib et al. 2000). Among its functions are two contrasting roles in detoxifications, as a key conjugate of electrophilic intermediates, principally via GST activities in phase II metabolism, and as an important antioxidant (Stegeman et al. 1992; Commandeur et al. 1995).

Perhaps the most obvious direct effect of certain pollutants is a decrease in thiol status, i.e. the ratio of reduced to oxidized glutathione (GSH: GSSG) (Stegeman et al. 1992; Otto and Moon, 1995). Alternatively, normal GSH: GSSG ratios can be maintained due to increased activities of GR or increased GSH synthesis. In mammals, GSH synthesis is considered to be tightly regulated via feedback inhibition by GSH on a rate-limiting synthetic enzyme (Suh al. 2004). In the healthy cell, GSH: GSSG ratios are typically very high i.e usually greater than 10 (Stegeman et al. 1992; Harwood et al. 2009).

There are strong indications that the tissue thiol status modulates Ah receptor inducible CYP1A gene expression and catalytic activity, indicating a 'cross-talk' between the GSH and cytochrome P450 systems (van der Oost et al. 2003).
7.2.2.9. Ascorbic acid (ASC)

Ascorbate is a major metabolite in plants. It is an antioxidant and, in association with other components of the antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis and a range of pollutants (Smirnoff, 1996).

7.3. Oxidative stress parameters

Many environmental contaminants (or their metabolites) have been shown to exert toxic effects related to oxidative stress (Winston and DiGiulio, 1991). Oxygen toxicity is defined as injurious effects due to cytotoxic reactive oxygen species (ROS), also referred to as reactive oxygen intermediates (ROIs), oxygen free radicals or oxyradicals (DiGiulio et al. 1989). Of particular interest are the reduction products of molecular oxygen which may react with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and ultimately cell death (Winston and DiGiulio, 1991). The activities of the antioxidant enzymes, which defend the organisms against ROS, are critically important in the detoxification of radicals to non-reactive molecules.

7.3.1. Lipid peroxidation (LPO)

The oxidation of polyunsaturated fatty acids is a very important consequence of oxidative stress and has been investigated extensively (Hageman et al. 1992; Stegeman et al. 1992). The process of LPO proceeds by a chain reaction and, as in the case of redox cycling, demonstrates the ability of a single radical species to propagate a number of deleterious biochemical reactions. The actual chemistry of LPO and associated production of various free-radical species is extremely complex (Kappus, 1987).

LPO appears to have considerable potential as a biomarker for environmental risk assessment (ERA) (Stegeman et al. 1992; Hai et al. 1995), although it can occur as a consequence of cellular damage due to a variety of insults other than exposure to xenobiotics causing oxidative stress (Mlambo et al. 2004).

7.4. Aminotransaminases

The aminotransferases, alanine transaminase (ALT or GPT) and aspartate transaminase (AST or GOT), constitute a group of enzymes that catalyze the
interconversion of amino acids and α-ketoacids by transfer of amino groups. The α-ketoglutarate/L-glutamate couple serves as an amino group acceptor and donor pair in amino-transfer reactions (Moss et al. 1986). ALT catalyzes the transfer of amino group from alanine to α-ketoglutarate to form glutamate and pyruvate, while AST catalyzes the transfer of amino group from aspartate to α-ketoglutarate to form glutamate and oxaloacetate (Moss et al. 1986).

An increase of aminotransferases activity in the extracellular fluid or plasma is a sensitive indicator of even minor cellular damage since the levels of aminotransferases within the cell exceed those in the extracellular fluids by more than three orders of magnitude (Moss et al. 1986). The measurement of enzyme activities in the serum is, therefore, frequently used as a diagnostic tool in human medicine (Goetz, 1980). Most research on the use of serum transaminase activities as an indicator of tissue damage has, therefore, been performed on humans, since both ALT and AST activities are of great clinical significance (Moss et al. 1986). Although the serum levels of both AST and ALT become elevated whenever disease processes affect liver cell integrity, but ALT is considered more specific enzyme for the liver damage.

7.5. Ethoxy resorufin-o-deethylase (EROD)/ CYP1A1

EROD activity describes the rate of the CYP1A mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin. The catalytic activity towards this substrate is an indication of the amount of enzyme present and is measured as the concentration of resorufin produced per mg protein per minute (mol/mg/min) (Kennedy and Jones, 1994). Because metabolism is generally highest in hepatic tissue, the assay is typically conducted using liver. For this reason, EROD is often termed an "early warning system" (Payne et al. 1987). EROD is a highly sensitive indicator of contaminant uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependant monooxygenases (the CYP1A subfamily specifically) by xenobiotic chemicals. The most useful aspect of CYP1A for biomonitoring purposes is the enzyme’s tendency to increase in concentration upon chemical exposure. Induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR). Induction of EROD is an extremely sensitive indicator of environmental alterations and is usually one of the first detectable, quantifiable responses to exposure (Stegeman, 1992). Receptor
binding is followed by a series of molecular events leading to the expression of several genes (including CYP1A) known as the “Ah-gene battery” (Nebert et al. 1993).

![Structural formulae of 7-ethoxyresorufin and resorufin. Deethylation of the substrate is mediated by CYP1A (7-ethoxyresorufin-o-deethylase) to yield the fluorescent product resorufin.](image)

**Fig. 2:** Structural formulae of 7-ethoxyresorufin and resorufin. Deethylation of the substrate is mediated by CYP1A (7-ethoxyresorufin-o-deethylase) to yield the fluorescent product resorufin.

### 7.6. N-nitrosodimethylamine demethylase (NDMA-d)/ CYP 2E1

Cytochrome (CYP) P450 2E1 is clinically and toxicologically important and it is constitutively expressed in the liver and many other tissues. Because rodent and human CYP2E1 enzymes catalyze similar reactions, the rat and mouse are good models for screening for substrates of this enzyme. It is constitutively expressed in the liver under the normal condition but it can also be induced in the liver and other tissues by acetone, ethanol, isoniazid and other compounds, many of which are the substrates of this enzyme (Johansson et al. 1986; Nakajima et al. 1989).

### 7.7. Limitations of biomarkers

Biomarker responses are powerful because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure to, as well as effects of, environmental contaminants. However, these factors may also complicate interpretation of the significance of the biomarker responses in ways that may not always be anticipated (McCarthy, 1990). Many non-pollution-related variables may have an additional impact on the various enzyme systems, and may thus interfere with biomarker responses when experimental conditions are not thoroughly analyzed or controlled. Examples of such ‘confounding’ or ‘modifying’ factors are the organisms’ health, condition, sex, age, nutritional status, metabolic activity, reproductive and developmental status, and population density, as well as
factors like season, ambient temperature, heterogeneity of the environmental pollution, etc. Unfortunately, most available toxicity data rarely quantify the potency that confounding factors are likely to exhibit in natural environments (De Kruijf, 1991). Moreover, estimates of confounding factor interactions are scarce, as evidenced by the extensive use of uncertainty factors in risk assessment to address unknowns (Power and McCarty, 1997).

7.8. Toxicity biomarkers of trichloroethylene

As far as the TCE biomarkers in animal models are concerned, several studies suggested the significant changes in the oxidative machinery with the generation of P450 dependent reactive intermediates (Lash et al. 2000; DuTeaux et al. 2004). A potential biomarker for TCE exposure has been identified in mouse studies namely a chloral-protein adduct that has been detected in tissues of TCE treated mice (Griffin et al. 2000). Extensive binding of TCE metabolites was observed with liver proteins and to lesser extent with lung and kidney proteins in mice (Bergman, 1983) and rats (Bolts and Filser, 1977) exposed to inhalation to $^{14}$C-TCE. TCE exposure induces the activities of peroxisomal enzyme, catalase and also leads to palmityl CoA oxidation in mice but not in rats (Elcombe et al. 1985). A time and concentration dependent release of LDH was observed in one study after normal human epidermal keratinocyte cells were exposed to different doses of TCE. It also caused an increased level of malonaldehyde, while an inhibition of superoxide dismutase (SOD) activity was obtained in a concentration dependent manner (Zhu et al. 2005). TCE also induced significant changes in the activities of antioxidative enzymes such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glucose-6-phosphate dehydrogenase (Watanabe and Fukui, 2000). Higher concentration of TCA causes a greater excretion of urinary formic acid. The dose dependent increase in urinary methylmalonic acid concentration and urinary glutathione S-transferase alpha activity was also observed in rats exposed to TCE (Green et al. 2004). Elevated level of acid phosphatase and catalase and decreased activity of delta-aminolevulinic acid dehydratase as a result of TCE exposure to mice was also reported in support of the susceptibility of liver and kidney as target tissues in its toxicity (Goel et al. 1992). Inhalations of TCE by male rats for 12 to 24 weeks brought about significant reduction in absolute testicular weight and altered activities of marker testicular enzymes associated with spermatogenesis and germ cell maturation along with
marked histopathological changes showing depletion of germ cell and spermatogenic arrest (Kumar et al. 2001b). Significant decrease in total epididymal sperm count, sperm motility, specific activities of enzymes glucose 6-phosphate dehydrogenase and 17 β-hydroxy steroid dehydrogenase with concomitant decrease in serum testosterone concentrations in TCE inhaled rats, was recorded by Kumar et al. (2000). High and long term exposure of TCE in rats resulted in an increase in concentration of N-acetyl-β-D-glucosaminidase (NAG) and low molecular weight proteins in urine. A histological alteration was also observed in glomeruli and globuli of TCE exposed rats (Mensing et al. 2002). Such a high and long exposure of TCE to humans resulted in an increase in the level of α1-microglobulin excretion, which is a potential biomarker of renal toxicity (Hermann et al. 2004). The specific content of CYP3A in liver microsomes was found to be increased more than 2 fold by the administration of TCE (Koop et al. 1985).

TCE exposure was also found to up-regulate the expression of numerous stress response genes and homeostatic genes (Collier et al. 2003). Moreover, DNA arrays analysis also showed highly selective TCE induced gene induction. However, at a very high dose, only Hsp 25, Hsp 86 and Cyp2a were found to be up regulated (Bartosiewicz et al. 2001).
Objectives of the study

In view of the literature presented in the preceding pages as well as the surveys conducted by Dua (2003) and Chiya Nivaran Samithi (2009) that reported various ailments probably associated with TCE exposure in Aligarh city, an attempt was made (i) to estimate the genotoxicity of TCE in natural milieu, and (ii) drinking system to identify certain biomarkers of TCE alone as well as of wastewater containing TCE. The detailed objectives of the study are listed below:

1. Since *Allium cepa* test is a simple, sensitive and cost effective indicator of toxicity, it was thought that its efficacy would be greatly increased if biomarker studies were carried out in the same onion bulbs employed for the TCE toxicity bioassay.

2. Various enzymes of the detoxification machinery like GST, GR, SOD, CAT, APx, GPx and MDHAR have been shown to get modulated in response to pollutant exposure (Panda, 2003; Fatima and Ahmad, 2005; Metwally and Fouad, 2008; Sinha et al. 2009).

3. EROD, PROD and NDMA-d, the representative markers of the phase I detoxification enzymatic machinery have also been proposed as biomarkers of pollution (Fernandes et al. 2002; Ferrat et al. 2003; Lee et al. 2008). It seemed also reasonable to study the potential of these enzymes of *Allium cepa* as well as of animal system as biomarkers of TCE pollution.

4. The Ames plate incorporation test being a rapid and sensitive indicator of genotoxicity is routinely employed for testing the mutagenicity/genotoxicity of water samples. The Ames fluctuation test, on the other hand, has the potential to be automated and a large number of samples/compounds could be screened by this test. It was worth studying to compare the suitability of these well established tests along with the *Allium cepa* genotoxicity test. The pattern of response of the various Ames tester strains towards the test water sample could serve as a biomarker of a particular genotoxicant present in water samples.

5. The toxicity of water pollutants has been reported to be mediated by reactive oxygen species (DiGiulio et al. 1995; Livingstone, 2001; Hassoun et al. 2004; Wenhua, 2005). The generation of ROS can provide an indication about the
presence of specific pollutants and can thus be utilized as a biomarker for their presence. The involvement of ROS in general and individual species in particular in the toxicity/genotoxicity of the test water samples was proposed to be studied.

6. The water samples to be used for the biomarker and genotoxicity studies were proposed to be collected from the industrial areas of Aligarh and Saharanpur cities of northern India. These samples in earlier studies were found to contain TCE, heavy metals and organic xenobiotics especially phenolics, due to the strategic positions of these cities.
Chapter II:

General Materials and Methods
The chemicals used in the present study along with their sources are listed below:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
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<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
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<tr>
<td>7-ethoxy resorufin</td>
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<td>7-pentoxy resorufin</td>
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</tr>
<tr>
<td>Oxidized glutathione</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>pBR 322 plasmid DNA</td>
<td>Bangalore Genei, India</td>
</tr>
<tr>
<td>Phenylmethysulphonyl fluoride (PMSF)</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>Para-nitrophenyl phosphate</td>
<td>Sigma chemical Co, USA</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>Resorufin</td>
<td>Sigma chemical Co, USA</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>Sodium ascorbate</td>
<td>Sisco research laboratories, India</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Sigma chemical Co, USA</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Hi media, India, Ltd</td>
</tr>
<tr>
<td>Thiobarbituric acid</td>
<td>Sigma chemical Co, USA</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>Qualigens fine chemicals, India</td>
</tr>
</tbody>
</table>
1. Materials

1.1. Experimental animals
Swiss albino rats weighing 100-150 g were housed under standard laboratory conditions of temperature (25 ± 5 °C) and natural 12-h light/dark cycle with free access of standard pellet diet (Aashirwad Industries, Chandigarh, India) and water ad libitum. All animals were kept under conditions that prevented them from experiencing unnecessary pain and discomfort according to guidelines approved by the institutional ethical committee.

1.2. Experimental protocol
After one week of acclimatization, animals were randomly selected into eight groups of six animals each. Dosing solutions were prepared daily to minimize possible instability of the chemicals in the mixture. The toxicants taken in single or as mixture as the case may be were administered via oral route for 15 consecutive days. Corn oil served as control for TCE whereas distilled water was the control for EtOH and heavy metal mixture (H.M.M).

The treatment schedule for the study embodied in chapter III and VI were as follows:

Group I: Distilled water only (10 ml/ kg/day)
Group II: Corn oil only (10 ml/ kg/day)
Group III: TCE only (1000 mg/kg/day)
Group IV: Ethyl alcohol only (1000 mg/kg/day)
Group V: Heavy metal mixture only (Ionic forms of cadmium, copper, lead and iron each at 2.5 mg/kg/day)
Group VI: TCE and ethyl alcohol simultaneously (1000 mg/kg/day each)
Group VII: TCE and heavy metal mixture concurrently (TCE at 1000 mg/kg/day and cadmium, copper, lead and iron each at 2.5 mg/kg/day)
Group VIII: TCE, heavy metal mixture and ethyl alcohol simultaneously (1000 mg/kg/day each for TCE and EtOH, and ionic forms of cadmium, copper, lead and iron at 2.5 mg/kg/day each)
The treatment schedule for the study embodied in chapter IV was as follows:

Group I: Mineral water of standard quality, Aquaguard water (10 ml/kg/day)
Group II: Aligarh wastewater (10 ml/kg/day)
Group III: Saharanpur wastewater (10 ml/kg/day)

This treatment schedule via oral route was followed daily during the experimental period of 15 days in the chapters III, IV and VII, at the end of which animals were sacrificed under light ether anaesthesia. Liver and kidney were removed for biochemical studies.

Important note: The sampling sites chosen for the study at Aligarh and Saharanpur were presumed to contain trichloroethylene (TCE) in test water samples in the light of available literatures (Suntio et al. 1988; Dua, 2003; McLean et al. 2006; Chiya Nivaran Samithi, 2009) as well as from our sample analysis by gas liquid chromatography (Table 2; Chapter IV).

1.3. Preparation of animal tissue homogenates

The 10% (w/v) liver and kidney homogenates was prepared in ice cold 10 mM Tris-HCl buffer, pH 7.5. The homogenate was then centrifuged under cold at 9000g for 20 min and supernatant was stored at -20 °C until further analysis. On the day of experiment the homogenates (liver and kidney) was again centrifuged at 3000g for 15 min at 4 °C, and the supernatant was used for assaying the test enzymes.

1.4. Preparation of animal tissue microsomes

Hepatic and renal tissues were washed with ice-cold normal saline and homogenized in 4 volumes of ice-cold 0.25 M potassium phosphate buffer, pH 7.25, containing 0.15 M KCl, 0.25 mM PMSF, 0.01 M EDTA, and 0.1 mM DTT. The subcellular fractionation of the liver and kidney homogenate was performed by the method of Das et al. (1981).

2. Requirements of the Allium cepa test

- Locally available Allium cepa (pinkish red variety) onion bulbs of size around 10 cm in circumference.
- Test tubes/boiling tubes (60 ml capacity).
- Test tube stands.
- Different concentration of TCE solution.
2.1. Moderate exposure of *Allium cepa* to TCE: The basic protocol of Fiskesjo (1985) was followed with slight modifications. Using a sharp knife, the yellowish brown scales and the bottom plates of the onion bulbs were removed. Test tubes were filled with different concentrations of TCE and one *A. cepa* bulb was placed on the top of each test tube. Aquaguard purified water which had the organic and inorganic impurities within the permissible limit served as the negative control in all the experiments. The treatment was continued for 48 hrs in the absence of light in a dark chamber at 20 °C. After 48 hrs, the onion bulbs were taken out and used for enzymatic studies.

2.2. Enzyme extraction from onion bulbs: Treated as well as untreated onion bulbs were cut into small pieces and homogenized with chilled sodium phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA. The homogenate was squeezed through muslin cloth and the extract thus obtained was centrifuged at 9000g for 20 min at 4 °C. The clear supernatant was stored at 0-4 °C in 5 ml vials and was properly thawed prior to enzyme analysis.

2.3. Preparation of onion microsomes homogenate

Trichloroethylene treated as well as untreated *Allium cepa* bulbs were homogenized with ice-cold 50 mM Tris-0.25 M sucrose (pH 8.0). The homogenate was then centrifuged under cold at 9000 g for 20 minutes. The supernatant fraction was recentrifuged at 100000 g for 1 hour at 4 °C. The pelleted microsomes were resuspended in 100 µl of 50 mM Tris-0.25 M sucrose, aliquoted in 1 ml fractions and finally frozen at -80 °C until further analysis.

2.4. Inhibitor studies: Some experiments on *Allium cepa* bulb treated with TCE were done in the presence of 50 µg/ml cycloheximide (CHX), which is an inhibitor of eukaryotic protein synthesis involving 80S ribosomal particles. These studies were carried out to find out whether the increase in the activity of the test enzymes was at the synthesis or the activity level. Chloramphenicol (50 µg/ml), another protein synthesis inhibitor acting on the ribosomal system of mitochondria and chloroplasts, was also included in parallel experiments to ascertain the location of these enzymes. For these studies the concentration of TCE taken was same at which maximum induction of the test enzyme had been observed.
3. Enzyme assays in animal and plant tissue homogenates

a. Superoxide dismutase (SOD): SOD activity in the animal/onion tissues homogenate was assayed by measuring its ability to inhibit the autooxidation of pyrogallol (Marklund and Marklund, 1974).

b. Catalase (CAT): CAT activity was measured as the decrease in H2O2 concentration by recording the absorbance at 240 nm (Aebi, 1984).

c. Glutathione reductase (GR): Oxidized glutathione (GSSG) was used as the substrate. Activity was measured as the decrease in NADPH concentration by recording the absorbance at 340 nm (Carlberg and Mannervik, 1975).

d. Glutathione-S-transferase (GST): CDNB (1-chloro-2,4-dinitrobenzene) was used as the substrate and activity was measured as the amount of CDNB conjugate formed by recording the absorbance at 340 nm (Habig et al. 1974).

e. Glutathione peroxidase (GPx): GPx activity was assayed according to the method of Mohandas et al. (1984). The assay mixture consisted of phosphate buffer, EDTA, sodium azide, glutathione reductase (3 units), GSH, NADPH and H2O2. Oxidation of NADPH was recorded at 340 nm.

f. Ascorbate peroxidase (APx): APx activity was measured by the method of Nakano and Asada (1981). The reaction mixture contained phosphate buffer, sodium ascorbate and H2O2. The reaction was followed at 290 nm.

g. Monodehydroascorbate reductase (MDHAR): MDHAR activity was assayed by the method of Hossain et al. (1984). The reaction mixture contained Tris–HCl, NADH, ascorbate and 0.15 units of ascorbate oxidase. The reaction was monitored at 340 nm.

h. Dehydroascorbate reductase (DAHR): DHAR was assayed by the method of Hossain and Asada (1984). The reduction of DHA to ASC was followed at 265 nm.

i. Alanine transaminase (ALT): ALT activity was carried out by commercially available kit, Span diagnostic Ltd. India.

j. Aspartate transaminase (AST): AST activity was analyzed by commercially available kit, Span diagnostic Ltd. India.

k. Alkaline phosphatase (ALP): ALP activity was determined by the formation of paranitrophenol from para-nitrophenyl phosphate (PNPP) as substrate at 405 nm (Principato et al. 1985).
1. Ethoxyresorufin/ Pentoxyresorufin-O-dealkylase (EROD/PROD): Activities of P450 isozymes EROD and PROD were measured according to the method of Burke and Mayer (1974) and Rutten et al. (1992) respectively. The reaction mixture in 1.25 ml contained 0.05 M Tris, pH 7.5, 0.025 M MgCl₂, 5 µM pentoxy or ethoxyresorufin, 500 µM NADPH, and a suitable amount of liver/kidney/onion microsomes. The reaction was started with NADPH and allowed to run for 10 minutes (Liver microsomes) or 30 minutes (kidney/onion microsomes). The reactions were stopped with 2 ml methanol and resorufin production was monitored at excitation wavelength of 550 nm and emission wavelength of 585 nm.

m. N-nitrosodimethylamine demethylase (NDMA-d): NDMA-d activity was estimated in liver/kidney/onion microsomes by a slight modification of the method of Castonguay et al. (1991). The assay mixture contained a suitable amount of liver/kidney/onion microsomes, 70.0 mM Tris-HCl, pH 7.4, 10 mM semicarbazide, 14 mM MgCl₂, 215 mM KCl, 1 mM NADPH and 4 mM NDMA in 1.0 ml final volume. The reaction mixture was incubated at 37 °C for 30 minutes and the reaction was stopped by the addition of 0.1 ml of 25% zinc sulphate and 0.1 ml of saturated solution of barium hydroxide. After centrifugation at 2000g for 10 minutes, 0.7 ml of the supernatant was mixed with an equal amount of Nash reagent. The tubes were then incubated at 70 °C for 20 min and the formaldehyde (HCHO) production was measured at 415 nm.

3.1. Determination of the oxidative stress marker, LPO and important metabolites

a. Lipid peroxidation (LPO): Tissue samples of experimental rats were analyzed for the level of malonaldehyde (MDA) according to the method of Beuge and Aust (1978).

b. Hydrogen peroxide (H₂O₂): The amount of H₂O₂ was estimated by the horse radish peroxidase method (Pick and Keisari, 1980).

c. Ascorbate (ASC): ASC content was estimated using the bipyridyl method as described by Knorzer et al. (1996).

d. Glutathione (GSH): GSH levels were estimated by the method of Jollow et al. (1974).
3.2. Determination of protein concentration: Total protein was estimated by the method of Lowry et al. (1951).

3.3. Statistical evaluation
Data was expressed as Mean ± S.D of six values and analyzed by one-way ANOVA. Differences among controls and treatment groups were determined using Student’s t-test. $P$ values of less than 0.05 were considered statistically significant. All comparisons were made with untreated control.

4. Maintenance and growth of bacteria
Each strain of *Salmonella typhimurium* was streaked over master plate. A single colony was picked up, grown in minimal medium and repurified by streaking over fresh master plate.

The *Salmonella typhimurium* strains were tested on the basis of associated genetic markers after raising it from the single colony from the master plate. Having satisfied with the tester strains the culture was raised and streaked over minimal and nutrient agar slants. It was then allowed to grow O/N at 37 °C and stored at 4 °C. Every month the cultures were transferred over fresh slants with TA102 as an exception. It was transferred after every fifteen days. Stabs were prepared for longer storage. For longer storage glycerol cultures of the tester strains were prepared and stored at –80 °C.

5. Media for Ames strains
5.1. Medium for master plates and slants: The composition of the medium for Ames tester strains to prepare master plates and slants was as under:

Sterile Agar | 15g/910 ml
---|---
Sterile 50X VB Salts | 20 ml
Sterile 40% Glucose | 50 ml
Sterile histidine.HCl.H2O (2g/400 ml H2O) | 10 ml
Filter sterile 0.5mM biotin solution (30.9 mg per 250 ml H2O) | 6 ml
Filter sterile ampicillin solution (8 mg/ml 0.02N NaOH) | 3.15 ml
Filter sterile tetracycline solution (8 mg/ml 0.02N HCl) | 0.25 ml

Tetracycline solution was added only for use with the TA102 strain which is tetracycline resistant.
The thermo resistant ingredients were autoclaved at 121 °C for 20 minutes. Sterile glucose, 50X VB salts and histidine solutions were added to the hot solution. It was mixed and allowed to cool approximately to 50 °C. After that filter sterile biotin, ampicillin and tetracycline solutions were added aseptically.

5.2. Stock solution of 50X VB salts

Stock solution of VB salts (50X) was prepared using the following ingredients:
1. MgSO4.7H2O 10.0 g/l
2. Citric acid monohydrate 100.0 g/l
3. K2HPO4 (anhydrous) 500.0 g/l
4. NaHNNH4PO4.4H2O 175.0 g/l

The salts were added in the order indicated as above in a 2-litre beaker or flask containing 670 ml of water. Each salt was allowed to dissolve completely before adding the next. The volume was then made upto to 1 litre and distributed into two 1-litre glass bottles. This stock solution was autoclaved, loosely capped for 20 min at 121 °C. After the solutions cooled down, the caps were tightened and stored at 4 °C.

5.3. Minimal glucose plates for mutagenicity assay

Sterile 50X VB Salts 20 ml
Sterile 40% Glucose 50 ml
Sterile Agar 15 g/930 ml distilled water

The above components were mixed with the molten agar and then approx 30 ml was poured over each plate.

5.4. Top agar for mutagenicity assay: The top agar contained 0.6% Agar powder and 0.5% NaCl, 10 ml of 0.5 mM histidine. Histidine- HCl/biotin was added to 100 ml of the molten agar and mixed thoroughly by swirling.

5.5. 0.5 mM histidine.HCl/biotin for mutagenicity assay

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 250 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Biotin (F.W. 247.3)</td>
<td>30.9 mg</td>
</tr>
<tr>
<td>L-Histidine.HCl (F.W. 191.7)</td>
<td>24.0 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>250 ml</td>
</tr>
</tbody>
</table>

Biotin was dissolved by heating the water to boiling point and then histidine was mixed to it and sterilized at 121 °C for 20 minute.
5.6. Preparation of liver homogenate S9 fraction from rats: For the preparation of liver homogenate S9 fractions, the methods of Green et al. (1977) and Maron and Ames (1983) was followed.

Male Sprague Dawley rats weighing around 150 g were administered Aroclor-1254 at 500 mg/kg of rat 5 days before preparation of S9 liver fraction (Green et al. 1977). After this the animals were sacrificed by cervical dislocation ensuring the complete removal of blood before the excision of the liver.

All the steps of the procedure were carried out aseptically and at temperatures between 0-4 °C using cold, sterile solutions and glasswares. The freshly excised livers were placed in pre weighed beakers containing approximately 1 ml of chilled 0.15 M KCl per gram of wet liver. One rat liver weighed approximately 10-15 g. After weighing, the livers were washed several times in fresh chilled KCl. The washed livers were transferred to a beaker containing 3 volume of 0.15 M KCl (3 ml/g) wet liver and were minced with sterile scissors, and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle or with a Polytron homogenizer. The homogenate was centrifuged for 10 min at 9000 g and the supernatant (the S9 fraction) was decanted and saved. The sterility of the preparation was tested by plating 0.1 ml sample on minimal agar plate containing histidine and biotin. The freshly prepared S9 fraction was distributed in portions of 1 ml in eppendorfs, frozen quickly in a bed of crushed dry ice, and stored at -80 °C (Maron and Ames, 1983).

5.7. Recipe for preparing various components of S9 mix

5.7.1. MgCl2-KCl Salt solution

**Ingredients**

- Potassium chloride
- Magnesium chloride (MgCl2.6H2O)
- Distilled water

**Per 500 ml**

- 61.5 g
- 40.7 g

Dissolve ingredients in water. Autoclave for 20 minutes at 121 °C and store at 4 °C.

5.7.2. 0.2 M phosphate buffer, pH 7.4

**Ingredients**

- 0.2 M disodium hydrogen phosphate
- 0.2 M sodium dihydrogen phosphate

**Per 500 ml**

- 14.2 g
- 13.8 g
The pH was finally adjusted by pH meter using one of the ingredients only. This buffer was sterilized by autoclaving at 121 °C for 20 minute.

5.7.3. 0.1 M NADP solution (nicotinamide adenine dinucleotide phosphate)

**Ingredients**

<table>
<thead>
<tr>
<th></th>
<th>Per 5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP (F.W. 765.4)</td>
<td>383 mg</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

5.7.4. 1 M Glucose-6-Phosphate

**Ingredients**

<table>
<thead>
<tr>
<th></th>
<th>Per 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-Phosphate</td>
<td>2.82 g</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

5.7.4. S9 mix (rat liver microsomal enzymes plus cofactors)

**Ingredients**

<table>
<thead>
<tr>
<th></th>
<th>Standard S9 mix</th>
<th>High S9 mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>2.0 ml (4%)</td>
<td>5.0 ml (10%)</td>
</tr>
<tr>
<td>MgCl2-KCl</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1 M Glucose-6-phosphate</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>0.1 M NADP</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>0.2 M phosphate buffer (pH 7.4)</td>
<td>25.0 ml</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>19.8 ml</td>
<td>16.8 ml</td>
</tr>
</tbody>
</table>

The ingredients should be added in the reverse order indicated above so that the liver should be added to the buffered solution. The solutions must be prepared fresh and kept on ice.

6. Ames fluctuation test

We followed the method of Venitt et al. (1984) in a 96 well microplate format. The components required for the Ames fluctuation test do not differ much from the components required by the *Salmonella typhimurium* strains for the mutagenicity testing by the plate incorporation test.

The requirements of the Ames fluctuation test were as follows:

a. 50X VB salts
b. 20% Glucose
c. 0.1% histidine (100 mg histidine is dissolved in 100 ml distilled water, sterilized by autoclaving at 121 °C for 20 min)
d. 0.1% biotin (100 mg biotin dissolved in 100 ml distilled water, sterilized by autoclaving at 121 °C for 20 min).
e. Bromocresol purple, 10 mg/ml ethanol.
### Final concentrations of constituents for Ames fluctuation test

<table>
<thead>
<tr>
<th>Incubation mixture/constituents</th>
<th>Volume (ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>45</td>
<td>9</td>
</tr>
<tr>
<td>50X VB salts</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>20% glucose</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>0.1% biotin</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>0.1% histidine for TA98 strain</td>
<td>0.075</td>
<td>0.015</td>
</tr>
<tr>
<td>0.1% histidine for TA97a, TA100, TA102, TA104 strains</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Bacterial culture (µl)</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>

### Composition of the selective medium for Ames fluctuation test

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume (ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>959.5</td>
<td>479.75</td>
</tr>
<tr>
<td>Sterile 20% Glucose</td>
<td>40.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Bromocresol purple (10 mg/ml)</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>
7. Sample collection

Wastewater samples were collected from the industrial estates of Aligarh city and Saharanpur city, India, in sterile glass bottles as described in APHA (1998). It was stored at 4 °C in plastic jar cans. Prior to use, the particulate matter was removed by means of filtration using Whatman No. 1 filter paper. The test samples collected from the two sites were analyzed for some physicochemical characteristics such as pH, dissolved oxygen (DO), biochemical oxygen demand (BOD) and chemical oxygen demand (COD), using procedures described in APHA (1998). TOC was measured on Shimadzu TOC analyzer (TOC-V-CSN). Inductive conductivity plasma mass spectrometry (ICP-MS, PerkinElmer) was used for metal analysis of water samples from both the locations.
Table 1: Typical characteristics of *Salmonella typhimurium* strains used in this study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant genetic markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td><em>uvrB, hisD6610, bio, rfa,</em> R-factor plasmid-pKM101, frame shift mutation at G-C site</td>
<td>Prof. Takehiko Nohmi</td>
</tr>
<tr>
<td>TA98</td>
<td><em>uvrB, hisD3052, bio, rfa,</em> R-factor plasmid-pKM101, frame shift mutation at G-C site</td>
<td>Prof. Takehiko Nohmi</td>
</tr>
<tr>
<td>TA100</td>
<td><em>uvrB, hisG46, bio, rfa,</em> R-factor plasmid-pKM101, base pair substitution mutation at G-C site</td>
<td>Prof. Takehiko Nohmi</td>
</tr>
<tr>
<td>TA102</td>
<td><em>rfa, uvrB,</em> R-factor plasmid-pKM101, multicopy plasmid-pAQ1 containing <em>hisG428</em> auxotrophic marker and <em>tet</em> transition mutation at A-T site</td>
<td>Prof. Takehiko Nohmi</td>
</tr>
<tr>
<td>TA104</td>
<td><em>uvrB, hisG428, rfa,</em> R-factor plasmid-pKM101, transition mutation at A-T site</td>
<td>Prof. Takehiko Nohmi</td>
</tr>
</tbody>
</table>
Chapter III:

Some enzymatic/non enzymatic antioxidants as potential stress biomarkers of trichloroethylene, heavy metal mixture and ethyl alcohol in rat tissues
1. Introduction

Increasing rate of urbanization coupled with industrialization has resulted in greater amount of wastewater discharge as well as higher level of exposure to xenobiotics. Human and animal exposure to environmental chemicals is rarely limited to a single compound. Rather, they are exposed concurrently or sequentially to multiple chemicals from a variety of sources (Jadhav et al. 2007a). It is well known fact that toxic effects of a xenobiotic can be modified by the presence of other substances (Brus et al. 1999; Gupta and Gill, 2000). As simultaneous exposure to two or more xenobiotics can take place in the environment and/or under occupational conditions, the investigation of interactions between toxic substances is an important problem in modern toxicology (Jurczuk et al. 2004).

Trichloroethylene (TCE) is a common metal degreasing agent and has been used indiscriminately in the house based lock manufacturing and electroplating units in particular but probably in several small scale industries of India in general. Like many other chlorinated hydrocarbons, TCE has become an important environmental pollutant of the global dimension because of its toxic properties and widespread occurrence as a soil, air and water contaminant (Candura and Faustman, 1991). The United States environmental protection agency (USEPA) has set a maximum contaminated level of 5μg/l of TCE in drinking water (ACGIH, 1999).

Heavy metals are also important environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary and environmental reasons (Nagajyoti et al. 2008). Heavy metals cannot be destroyed through biological degradation and the ability to accumulate in the environment make these toxicants deleterious to the environment and consequently to humans.

Because of excessive ethanol (EtOH) consumption by some people who may be exposed to different xenobiotics environmentally and occupationally, undesirable interaction between xenobiotics and EtOH in the exposed population becomes pertinent on the epidemiological and medical perspectives (Meyer et al. 2000).

According to estimates of the Aligarh office of Uttar Pradesh Pollution Control Board, there were at least 125 units using TCE in Aligarh (Dua, 2003). The two surveys conducted by Dua (2003) and Chiya Nivaran Samithi (2009) have suggested the prevalence of various ailments probably associated with TCE exposure in Aligarh city. In view of the strategic position of Aligarh city which houses several
small electroplating industries one should not expect that its population is exposed only to TCE, exposure of some people to heavy metals along with TCE also can not be ruled out and some of them would certainly be alcoholics. Thus, there was a need to study the effect of these toxicants to find out some sensitive biomarkers in the animal system. In the previous study of our lab colleagues (Fatima and Ahmad, 2005) it was reported a very high concentration of heavy metals in wastewater of Aligarh city. Therefore, four heavy metals namely cadmium, copper, lead and iron were selected for this study because of their relatively high concentrations in Aligarh wastewater.

The present study embodied in this chapter was undertaken to assess the oxidative stress and antioxidants status in liver and kidney of rats orally administered with TCE, test heavy metals and EtOH individually or in combination. This could also provide a valuable lead towards suitable toxicity biomarkers. As our knowledge goes, there is no available report on the effect of TCE in the presence of heavy metals especially in alcoholics.

3. Results

3.1. Effect of TCE administration alone in rats on certain enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

SOD response to TCE in liver showed an increase of 86% whereas no significant change was observed in kidney. Activity of GR showed around 75% increase in liver as well as in kidney in TCE treated animals. Increase in GST activity was a mere 50% in liver but an astonishing rise of 218% was observed in kidney (Fig 4a,b). The increase in enzymatic activity of GPx was quite high in liver (127%) compared with kidney (50%). A noteworthy decrease of around 38% in CAT activity was also observed in both the tissues (Fig 2a,b). Furthermore, GSH level too showed a considerable decrease of around 40% both in liver (P < 0.001) and kidney (P < 0.01) due to TCE treatment. Level of MDA, an indicator of lipid peroxidation, showed a remarkable enhancement i.e. 187% in liver (Fig 7a) than in kidney (50%, Fig 7b) in response to TCE treatment. H2O2 content showed significant decrease of roughly 35% (P < 0.01) in both the tissues as a result of TCE treatment.
3.2. Effect of EtOH administration alone in rats on the enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

In this treatment system, SOD activity displayed an increase of 45% in liver over the untreated control (Fig 1a) and it remained unchanged in kidney (Fig 1b). Interestingly, catalase did not exhibit any change in its activity while in liver a slight ($P < 0.01$) decrease was seen in kidney. Moreover, an increase of around 24% ($P < 0.05$) was observed in GR activity in liver as well as in kidney (Fig 3a,b). GST activity showed a meager increase of 13% ($P < 0.05$) in liver but no significant change was observed in kidney after the treatment of alcohol. GPx activity was higher both in liver and kidney by 42% and 27% respectively (Fig 5a,b). A small decrease of around 25% in the level of GSH was also observed in both the tissues compared to control. The level of MDA was appreciably higher in liver (+58%, Fig 7a) but kidney did not show any significant change. A drop of around 30% ($P < 0.01$) in H$_2$O$_2$ level upon alcohol treatment was also observed in liver as well as in kidney.

3.3. Effect of heavy metals (H.M.M) administration in rats on the enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

SOD profile in this treatment system showed much enhanced activities in response to H.M.M ingestion of rats in liver (125%, Fig 1a) but the increase was only 76% in kidney. Contrary to 79% enhancement in CAT activity in liver a significant decrease of around 50% was observed in kidney of H.M.M treated animals (Fig 2a,b). GR activity in liver and kidney were increased by 69% and 93% respectively. A substantial rise (~83%) in GST activity was also observed in these tissues. Activity of GPx showed a considerable dip in H.M.M treated group in liver and an increase by 90% in kidney. GSH level showed a considerable decline of around 55% in both the tissues as a result of H.M.M treatment (Fig 6a,b). MDA level rose up in liver and kidney by 229% and 100% respectively compared with control animals. A significant drop of approximately 55% in the H$_2$O$_2$ level was noticed in both the tissues after H.M.M treatment (Fig 8a,b).

3.4. Effect of TCE + EtOH administration in rats on the enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

Enzymatic activity of SOD showed a rise of 90% in liver but the change was insignificant in kidney of combined treatment group of TCE and alcohol (Fig 1a,b).
CAT activity showed an increase of 108% in liver but in kidney it showed decline of 38% \((P < 0.01)\). Increase in the GR activity of liver was 106% and in kidney it was found to be 71%. GST showed much higher increase of 171% in kidney (Fig 4b) contrary to liver, which exhibited a meagre enhancement only \((+31\%, \ P < 0.01)\). Elevation in GPx activity was found to be 100% in liver and 72% in kidney. A decrease to the extent of around 50% in the level of GSH was observed in both the tissues (Fig 6a,b). Rise of 200% was observed in MDA level in the liver of combined treated animals but only 63% increase was found in kidney compared to control animals. A considerable decrease of 50% in H2O2 level was observed in liver (Fig 8a) as well as in kidney (Fig 8b) upon treatment.

3.5. Effect of TCE + H.M.M administration in rats on the enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

An increase in the SOD activity by 165% over and above the control value was observed upon combined treatment of TCE and H.M.M in liver (Fig 1a) whereas an increase of 100% was observed in kidney (Fig 1b). CAT activity was greatly reduced in liver as well as in kidney. GR activity showed an increase of 157% in liver and 117% in kidney compared to control animals. An astonishing rise of 258% in GST activity was observed in kidney (Fig 4b) as compared to 111% in liver (Fig 4a) upon this combined treatment. GPx activity showed an increase of 92% in liver and 136% in kidney. Considerable decrease of about 60% in the level of GSH was recorded in this combination groups compared to control. Level of MDA was enormously increased i.e. 300% in liver (Fig 7a) but only 130% rise was observed in kidney (Fig 7b). Decrease of around 70% in H2O2 level was observed in liver as well as in kidney. The changes in all the measured parameters were highly significant \((P < 0.001)\) in the entire treatment groups.

3.6. Effect of TCE + EtOH + H.M.M administration in rats on the enzymatic/non-enzymatic antioxidants levels in liver and kidney tissues

Activity of SOD was increased by 202% in liver (Fig 1a) compared to 164% in kidney (Fig 1a) over untreated controls. A similar pattern of decrease in CAT activity was recorded in liver and kidney tissues. GR activity showed a remarkable increase of around 220% in both tissues. GST activity showed a remarkable degree of elevation in kidney \((+341\%, \ Fig 4b)\) contrary to that in liver \((+163\%, \ Fig 4a)\). GPx showed an
increase of 135% in liver and 163% in kidney. However, a significant drop of roughly 75% in the level of GSH was observed in both the tissues. MDA levels were exorbitantly high in liver (+382%) as compared to kidney (+160%) over the respective control values. An amazing drop of around 85% in H2O2 level was observed in both the tissues in this combined treatment group (Fig 8a,b). The change was highly significant ($P < 0.001$) in the whole treatment groups in each and every parameter.

3. Discussion
To the best of our knowledge, it is the first report on the response of enzymatic/non-enzymatic defense system in rat after the treatment of TCE in the presence of heavy metals and ethanol. Another striking feature of this data is the higher susceptibility of liver rather than kidney of the rats following oral administration of TCE combined with EtOH and/or H.M.M.

Increase in MDA level is frequently observed in various mammalian tissues during oxidative stress and has generally been used as the marker of oxidative damage (Husain et al. 2001; Jadhav et al. 2007a,b). Lipid peroxidation measured in terms of MDA levels, showed around 3 fold induction in liver whereas only a nominal increase in MDA was recorded in the kidney of TCE exposed animals (Fig 7b). These findings are consistent with the previous reports (Toraason et al. 1999; Watanabe and Fukui, 2000; Zhu et al. 2005). Ethanol exposed animals did not show any significant change in MDA level in kidney. However, a small rise in MDA level compared with control value was recorded in the livers of alcohol administered rats (Fig 7a). Extensive researches have shown an increase in MDA equivalents resulting from ethanol ingestion (Jurczuk et al. 2004; Yao et al. 2006; Lu and Cederbaum, 2008). Exposure of rats to metals is characterized by their accumulation particularly in the liver and kidney cortex (Novelli et al. 1997). We obtained a rise in MDA level of more than 3 fold in liver and 2 fold in kidney as a result of H.M.M intake by rats (Fig 7 a,b). Heavy metal intake brought about a similar pattern of MDA in earlier studies also (Ozcelik et al. 2003; Jurczuk et al. 2004; Jadhav et al. 2007a,b).

Primary biochemical components of the oxidative stress response include elevation of LPO, suppression of GSH and alteration of the activities of antioxidant enzymes. The perturbation of antioxidant defense system is manifested by marked increase in LPO and activities of SOD, GPx, GR and GST with concomitant decrease
in CAT activity. The increased tissue MDA level indicates an enhanced pro-oxidant status in the tissues largely brought about by higher generation of free radicals. The capacity to scavenge the free radicals and repair of oxidatively modified macromolecules may also decreases in such cases (Holland et al. 1982; Sies, 1997). The accumulation of ROS results in significant functional alterations in lipid, protein and DNA molecules. The oxidative lipid damage (LPO) produces a gradual loss of cell membrane fluidity and reduces the membrane electromotive potential and increases the permeability of ions like Ca²⁺ (Jacob and Burri, 1996). The increased SOD activity is an adaptive tissue response to detoxify the superoxide radicals thus giving rise to H₂O₂ which is in turn handled by increased GPx activity. GPx is a selenium containing enzyme which seems to scavenge H₂O₂ and other peroxides in the present study. It also appears that such increase in LPO level might be caused by the enhanced SOD activity concomitant with reduced level of CAT which in turn could be due to CAT inactivation by the free radicals generated due to oxidative stress. The increased GPx activity to some extent can be attributed to the fall in H₂O₂ levels. As GPx functions in conjugation with reduced glutathione and is known to scavenge H₂O₂ and lipid peroxide (Bhattacharya et al. 2003). Under severe oxidative stress GSH levels are suppressed due to the loss of compensatory response and oxidative conversion of GSH to its oxidized form (Chen and Lin, 1977).

GSH is considered one of the most important antioxidants involved in protection against ROS/free radicals (Meister, 1989). GSH needs to be recycled back as it is the key non-enzymatic antioxidant of the cell and also serves as the substrate for the chief H₂O₂ scavenging enzyme namely GPx. Decrease in GSH level as a result of TCE, EtOH or heavy metal exposure has been reported by many investigators (Jadhav et al. 2007a,b; Lu and Cederbaum, 2008). Our findings are consistent with these workers since we also recorded a significant decline in GSH level in both the tissues compared to untreated animals (Fig 6 a,b). Our group has also reported a dose dependent depletion in GSH levels in Allium cepa exposed to heavy metal mixture (Fatima and Ahmad, 2005). However, Goel et al. (1992) had reported an increase in GSH level as a result of TCE intake in mice.

A major cellular defense against ROS is provided by SOD and catalase, which together convert superoxide radicals first to H₂O₂ and then to water and molecular oxygen. Other enzymes, such as glutathione peroxidase, use the thiol reducing power of glutathione for the reduction of oxidized lipids and protein targets of ROS.
Superoxide is considered as the central component of the signal transduction which triggers the genes responsible for antioxidant enzymes (Alavarez and Lamb, 1997). Since SOD is induced by its own substrate, its activation in the test tissues may be an indication that the animals were experiencing the pollutant induced superoxide radical stress (Allen and Tresini, 2000). In the present investigation, the level of SOD increased around 2 fold in liver but there was no change in the kidney upon TCE treatment (Fig 1a,b). Such an increase in the level of SOD by TCE exposure may be due to the formation of ROS and especially the superoxide radical. Watanabe and Fukui (2000) also reported an increase in SOD activity in mouse liver after TCE treatment. Husain et al. (2001) also found an enhanced level of hepatic SOD as a result of ethanol treatment which is in concurrency with our result. However, Devipriya et al. (2007) reported a decline in SOD after ethanol treatment. Moreover, a significant increase in the activity of SOD in liver and kidney as a result of heavy metal intake is in good agreement with the results reported by other investigators (Ozcelik et al. 2003; Jadhav et al. 2007a). Jadhav et al. (2007a) reported an increase in SOD activity after 30 days subchronic exposure to a mixture of eight metals in rats erythrocytes.

Catalase and peroxidases are the major enzymes involved in H2O2 detoxification. Similar reduction in CAT activity was found in both the tissues of TCE treated animals (Fig 2a,b). This result is in conformity with the earlier findings of Elcombe et al. (1985) who reported such a decline as a result of TCE treatment in rats. However, an increase in CAT activity as a result of TCE intake in both liver and kidney of mice was reported by several workers (Elcombe et al. 1985; Goel et al. 1992, Watanabe and Fukui, 2000). The difference in species may be the reason behind the opposing results (Elcombe et al. 1985). Ethanol intake on the other hand brought about a small decrease in CAT activity in renal tissue concomitant with an insignificant change in liver (Fig 2a,b). However, Ashakumary and Vijayammal (1996) found a decrease in CAT activity in liver as well as in kidney of rats after ethanol administration.

The role of GST in detoxification is well documented (Rees 1993; Fernandes et al. 2002; Ferrat et al. 2003). The fact that GST is ubiquitous also makes it a more suitable stress biomarker (Stenersen et al. 1987). Our results are consistent with the previous report of Stajn et al. (1997). Dose dependent increase in GST levels as a result of heavy metal mixture exposure has also been reported by our group in Allium
cepa system (Fatima and Ahmad, 2005). However, El-Demerdash et al. (2004) found a decrease in GST activity in liver and plasma of rats after cadmium chloride intake.

TCE as well as heavy metal exposed animals showed almost similar rise in GR level in both the tissues. Not so significant change in GR activity was observed in both the tissues of ethanol exposed animals. All these findings are consistent with earlier reports (Ashakumary and Vijayammal, 1996; Watanabe and Fukui 2000, Jadav et al. 2007a). Different variety of toxicants in combination brought about a prominent change in GR activity (Fig 3a,b).

Increase in GPx activity was recorded in both the tissues of almost all treatment groups. However, the rats treated with heavy metals alone as well as with TCE and H.M.M combination showed a decrease in GPx activity in liver tissue. These results are in good agreement with the previous report of Boccio et al. (1990). It is known that GPx has higher affinity for H2O2 than CAT and thus it is effective in decomposing H2O2 (Halliwell, 1974; Wassmann et al. 2004). However, Watanabe and Fukui (2000) and Yao et al. (2006) found a decrease in GPx activity as a result of TCE and alcohol treatment respectively. An appreciable fall in H2O2 level was recorded by us in every treatment group.

From our result, it is quite clear that the level of MDA the end product of lipid peroxidation can be used as the best biomarker for these test toxicants in both the tissues of rats. Moreover, GST can act as a biomarker for TCE in kidney of treated animals. However, SOD and GR can act as a potential biomarker of H.M.M intoxication in hepatic and renal tissue respectively. Overall, present results demonstrate appreciable changes in the levels of antioxidant enzymes and glutathione system as well as alterations in the LPO and H2O2 levels as a result of TCE or heavy metal mixture intake in rat tissues. Solitary dose of alcohol in general did not show a significant change. This may be due to the comparatively low dose of alcohol selected in this study. The changes were much more prominent when these toxicants were given in combination rather than alone. The rise in the activities of antioxidant enzymes may be a compensatory response to these test toxicants.
Fig 1: Effect of TCE and other test toxicants on SOD activity in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. ***: $P < 0.001$ compared with control by ANOVA.

**Important note:** Group I animals received distilled water at a dose of only 10 ml/kg/day as a solvent control. However, all the animal groups were kept with free access of mineral water *ad libitum.*
Fig 2: Effect of TCE and other test toxicants on CAT activity in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. **, ***: $P < 0.01, P < 0.001$ respectively compared with control by ANOVA.
Fig 3: Effect of TCE and other test toxicants on GR activity in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***: $P < 0.05, P < 0.01, P < 0.001$ respectively compared with control by ANOVA.
Fig 4: Effect of TCE and other test toxicants on GST activity in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. ***: $P < 0.001$ compared with control by ANOVA.
Fig 5: Effect of TCE and other test toxicants on GPx activity in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 6: Effect of TCE and other test toxicants on GSH level in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. **, ***: $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 7: Effect of TCE and other test toxicants on MDA level in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 8: Effect of TCE and other test toxicants on H2O2 level in liver (panel a) and kidney (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Chapter IV:

Effect of wastewater intake on antioxidants and marker enzymes of tissue damage in rat tissues: Implications for the use of biochemical markers
1. Introduction

Water pollution is one of the most critical environmental issues these days and thus the hazardous effects of water pollutants on human health are of special concern. Wastewater may also percolate into the ground water through the unregulated waste discharge. It enters the aquatic environment and is taken up into the tissues of living organisms (Walsh and O'Halloran, 1998; Stecko and Bendell-Young, 2000). The rate of urbanization has also undergone a rapid increase, including an increase in wastewater discharge, so that exposure of humans to water pollutants is rarely limited to a single chemical or organic residue (Heindel et al. 1995).

The two sampling sites chosen for this study have different types of industries. Aligarh city is famous for lock manufacturing and electroplating industries. The effluents of these industries contain quite a large amount of metals like Cd, Cu, Cr and Zn. Aligarh also houses the lead battery and thermometer manufacturing units which accounts for the presence of high levels of Pb and Hg in the water sample. These industries as well as brass industries of Aligarh also use trichloroethylene as cleansing agents, another toxicant and suspected carcinogen. Trichloroethylene in the presence of heavy metals might be causing serious health problems to human population in Aligarh (Dua, 2003; Fatima and Ahmad, 2006).

Saharanpur is famous for paper and pulp industries. Paper mill effluent, which is a complex mixture of heavy metals, phenols, dioxins, furans, lignocellulose components, guaichol, catechols, polyaromatic hydrocarbons, trichloroethylene, fatty acids and resin acid, is reported to induce oxidative stress (Hamm et al. 1986, Suntio et al. 1988; Mather-Miyaich and DiGiulio, 1991; Fatima et al. 2000; McLean et al. 2006). The role of heavy metals, the common constituents of paper mill effluent in oxidative stress, is well-documented (Hamm et al. 1986).

Enzymatic and non-enzymatic antioxidants serve as an important biological defense against environmental oxidative stress (Ahmad et al. 2000). Both enzymatic and non-enzymatic processes counter the impact of ROS (DiGiulio et al. 1989; Lopez-Torres et al. 1993; Filho, 1996). Induction of antioxidant enzymes represents a cellular defense mechanism to counteract toxicity of ROS and they have extensively used in several field studies. The purpose of the work embodied in this chapter was to investigate the pollution load in the wastewaters and compare their toxic effects from Aligarh and Saharanpur samples in laboratory rats.
2. Results

2.1. Physicochemical parameters of water sample

Some physicochemical characteristics of test water samples are given in Table 1. The level of DO at Saharanpur site was found to be 1.1 mg/l, whereas at Aligarh it was found to be 2.8 mg/l. The mean values of BOD, COD and TOC in AWW were recorded to be 82 mg/l, 283 mg/l and 553 mg/l respectively. However, at Saharanpur site the mean values of BOD, COD and TOC were found to be 378 mg/l, 1259 mg/l and 2932 mg/l respectively (Table 1). The concentration of TCE in AWW was found to be 28.4 mg/l while SWW contains 8.97 mg/l of TCE (Table 2).

2.2. Effect of oral AWW administration in rats on the levels of enzymatic/non-enzymatic antioxidants and tissue injury marker enzymes in liver

Figs 1-11(a) depict the levels of antioxidant enzymes/non-enzymatic and tissue injury marker enzymes as a result of AWW intake. Under the experimental conditions specified in this chapter in general and mentioned above in particular, an astonishing increase by 325% in MDA level, an indicator of lipid peroxidation was observed in the liver of treated animals (Fig 7a). GR activity recorded in the experimental rats exhibited an increase by 179% in liver over control values following AWW ingestion (Fig 3a). Moreover, ALT activity rose up to around 175% in liver in this experimental condition (Fig 9a). An enhancement by 144% in SOD activity in hepatic tissue compared to that in control group of animals was obtained (Fig 1a). Activity profile of GST also showed an increase by 94% (Fig 4a). A rise of 33% \((P < 0.01)\) in GPx activity (Fig 5a) was also observed in experimental animals fed with AWW. A meagre rise of 13% and 4% \((P < 0.05)\) was recorded in the CAT and AST activity respectively (Fig 2a, 10a). However, liver of AWW ingested rats showed a reduction of around 28% \((P < 0.01)\) in GSH and ALP content (Fig 6a, 11a). On the other hand, H2O2 content also underwent a significant reduction by 23% \((P < 0.05)\) in liver of AWW administered animals (Fig 8a).

2.3. Effect of oral AWW administration in rats on the levels of enzymatic/non-enzymatic antioxidants and tissue injury marker enzymes in kidney

Figs 1-11(b) illustrate the alteration in antioxidant enzymes and marker enzymes of kidney damage as a result of AWW administration. A significant enhancement of 102% and 91% was recorded in renal SOD and CAT activity respectively in Aligarh
wastewater ingested experimental animals (Fig 1b and 2b). GPx activity also evinced an increase by 81% compared with untreated animals (Fig 5b). ALP level exhibited an amazing rise of around 220% in the kidney (Fig 11b) of AWW treated animals. However, activity of ALT and AST rose up only in the range of 45-50%. GSH content exhibited roughly 110% rise as a result of AWW intake in renal tissue. The level of MDA also displayed similar rise i.e. around 110%. The enzymatic activity in renal GST and GR exhibited a fall by 74% and 19% \((P < 0.05)\) in AWW exposed experimental animals respectively (Figs 3b and 4b). 

2.4. Effect of oral SWW administration in rats on enzymatic/non-enzymatic antioxidants and tissue injury marker enzymes levels in liver

Treatment procedure specified under this head resulted in the tremendous increase in MDA level to the extent of 405% (Fig 7a). Similarly, an appreciable rise by 275% in GR activity was also observed in the liver of rats kept on Saharanpur wastewater intake (Fig 3a). Level of ALT also displayed a rise of around 220% (Fig 9a). Enzymatic activity of SOD signalled a significant increase of 181% over and above the control value (Fig 1a) whereas only around 21% \((P < 0.01)\) rise in CAT activity was recorded in hepatic tissue of the treated animals (Fig 2a). GST and GPx activity also exhibited a significant increase of around 100% and 66% respectively in livers of treated rats (Fig 4a and 5a). GSH content also showed a rise of 31% in this tissue (Fig 6a). However, ALP enzymatic activity showed only around 11% \((P < 0.05)\) rise under the said experimental conditions (Fig 11a). SWW administration resulted a considerable fall of 45% \((P < 0.01)\) in liver H2O2 level (Fig 8a). However, treated rats displayed a small drop in AST activity in the liver and regaining about 87% activity to that in control group (Fig 10a).

2.5. Effect of oral SWW administration in rats on the levels of enzymatic/non-enzymatic antioxidants and tissue injury marker enzymes in kidney

Present treatment system demonstrates a remarkable rise of around 240% and 140% in renal ALP and CAT enzymatic activity respectively (Fig 2b and 11b). MDA level also showed a significant increase by roughly 160% in SWW fed animals (Fig 7b). Fig (5b) depicts the changes in renal GPx level as a result of SWW exposure where the activity of GPx rose up to 110% compared with control group. GST activity
exhibited an increase by 74% (Fig 4b) whereas 56% increase in SOD level was recorded in kidney of the experimental group of rats (Fig 1b). A quite similar enhancement in ALT and AST activity i.e. around 65% and 50% was recorded in the renal tissues of treated rats respectively (Fig 9b and 10b). GSH content also showed a rise of 73% ($P < 0.01$) in renal tissue (Fig 6b). However, a significant decrease of around 60% ($P < 0.001$) was observed in GR activity of rats exposed to Saharanpur wastewater (Fig 3b). SWW administration also caused a 60% reduction in H$_2$O$_2$ levels of kidney cells of experimental rats (Fig 8b).

3. Discussion

The physicochemical analysis has long been employed to assess the quality of water. DO, BOD, COD and TOC are often used as indices of pollution load in wastewater and natural waters. The levels of BOD, COD and TOC were found to be relatively higher in Saharanpur wastewater suggesting the presence of comparatively greater quantities of pollutants especially of organic origin at that sampling site. The data of physicochemical and oxidative stress parameters support each other to a large extent. Most of the parameters when compared between the two sites showed high level of upregulation in antioxidant enzyme activities in the rats fed with Saharanpur wastewater, which by all account was a comparatively more polluted site than Aligarh site. Saharanpur is famous for paper and pulp industry whereas Aligarh city houses several small scale electroplating and lock manufacturing industries spilling out the metals at very high concentrations which was confirmed by ICP analysis (Table 3). This type of industrial profile could also provide explanation to some extent to the higher BOD, COD and TOC levels found at Saharanpur.

To the best of our knowledge, no body has done the biomarker study in such system selecting for enzymatic/non-enzymatic defence system as well as the activity of tissue injury marker enzyme in rats orally administered with wastewater. Effluents collected from Saharanpur industrial area were found to be more toxic than that of Aligarh. Moreover, liver rather than kidney was found to be more affected tissue of the rats as a result of these wastewater intakes. From the available literature, it is quite clear that lot of toxicological work had been performed on fish collected from polluted water, though only one group (Silva et al. 1999) reported the effect of wastewater intake on the serum of rats. However, investigating the effect of
wastewater intake on different tissues of rat is probably the first attempt in biomarker studies.

Oxidative stress refers to the cytological consequences of a mismatch by the production of free radicals and the ability of the cell to defend against them. The extent of oxidative stress appears to be different in different organs which are a reflection of differences in metabolism, function, ROS production and the distribution of endogenous antioxidant defenses (Xi and Chen, 2000). Liver is the major site of metabolism and detoxification, stronger oxidative defenses are developed in liver to cope with free radical generation (Radi and Matkovics, 1988). In the present study, liver appears to be more oxidatively challenged organ compared with kidney.

There is no universal marker for oxidative stress. A single standard biomarker for oxidative stress that can be used in case of environmental samples will be insufficient because information on the mechanism of action of the contaminants present in these samples is insufficient (Lauwerys et al. 1995). From our result, it is clear that the use of a multiple biomarker approach in biomonitoring of environmental samples is the need of the hour.

Lipid peroxidation (LPO) is a free radical-mediated chain reaction and once initiated it is self-perpetuating. The length of the chain propagation depends upon chain-breaking antioxidant enzymes (Harris, 1992). Our results indicate a significant elevation in MDA level, a marker of LPO in both the tissues as a result of wastewater administration. The level of MDA was recorded to attain around 4 fold amplification in liver and 2 fold rise in kidney as a result of AWW intake. On the other hand, SWW administered animals displayed a 5 fold and 2.5 fold induction of MDA levels in hepatic and renal tissues over untreated controls. These findings are consistent with the previous studies by several authors (Silva et al. 1999; Ahmad et al. 2000; Pandey et al. 2003; Farombi et al. 2007). Especially, Silva et al. (1999) found an increasing level of lipoperoxide production up to extent of 5 fold during 30 days intake of Tiete river water of Brazil in the serum of rat.

GR is the crucial enzyme for the regeneration of GSH from GSSG, hence GR activities have been proposed as a useful biomarker of redox cycling compounds (Stephensen et al. 2002). A remarkable increase by 3 to 4 fold in GR activity was recorded in the liver of wastewater treated rats. Our results are in good agreement with those of Stephensen et al. (2000) and Farombi et al. (2007). Stephensen et al. (2000) demonstrated that fishes from polluted sites have high GR activity due to
higher peroxidative components in the polluted water. On the contrary, GR level was reduced up to 57% by the intake of these water samples in kidney. In an earlier investigation, our group has also reported an increase in GST, GR, SOD and CAT activity in *Allium cepa* exposed to AWW (Fatima and Ahmad, 2005).

Superoxide dismutase comprises a family of multi-functional enzymes with broadly overlapping substrate specificities, which play important role in detoxification of superoxide radical (Pereira et al. 1998). Increase in the activity of CAT and SOD is usually observed in the face of environmental pollutants (Dimitrova et al. 1994; McCord, 1996; Dautremepuits et al. 2004) since SOD-CAT system represents the first line of defence against oxidative stress caused by ROS (Hassan and Scandalios, 1990; McCord, 1996). In the present study this relationship was also observed in both the samples as well as in test tissues of rats. SOD level was found to be enhanced up to 3 fold in the liver by the intake of these wastewater samples while only 2 fold induction was recorded in kidney. Rise in CAT activity was not as prominent as SOD. However, the increase ranged from 1.2 to 2.5 fold in the test tissues by the test wastewater samples. The activity of SOD reported to be higher in fish from polluted sites by several authors (Roberts et al. 1987; Rodriguez-Ariza et al. 1992; Avci et al. 2005; Farombi et al. 2007) suggesting there by a high production of superoxide radicals. However, Silva et al. (1999) reported a decrease in SOD level in rat serum after the intake of contaminated drinking water. Ahmad et al. (2000) also found an increase in CAT activity in liver of fish exposed to 1% paper mill effluent. The lowering of CAT activity could be attributed to high production of superoxide anion radical which has been reported to inhibit CAT activity in case of excess production of O$_2^{-}$ (Kono and Fridovich, 1982).

Glutathione S-transferase acts as the catalyst of a very wide variety of conjugation reactions of glutathione with xenobiotic compounds containing electrophilic centres (Regoli and Principato, 1995). A remarkable sensitivity of GST to a large variety of pollutants, either organic or inorganic is identified by several authors (Sheenan et al. 1991; Zakharov and Clarke, 1993; Livingstone, 1998). GST activity rose up to 2 fold in the liver of AWW and SWW exposed rats (Fig 4a). The studies of Ahmad et al. (2000) and Farombi et al. (2007) also support this trend. Contrary to 74% enhancement in GST activity in SWW administered rats, a reduction of equal extent was observed in kidney of AWW treated animals (Fig 4b).
Glutathione peroxidase activity has been reported to be induced by certain environmental pollutants (Radi et al. 1985). Around 1.3 and 1.7 fold induction in GPx activity was recorded in the liver of AWW and SWW exposed rats respectively in our case. However, the kidneys of the wastewater administered rats displayed around 2 fold enhancements in GPx activity (Fig 5b). Pandey et al. (2003) also reported a high level of GPx level in the liver and kidney of fish collected from polluted water of Agra and Panipat.

Glutathione is responsible for maintaining the redox status of cells and plays an important role in quenching oxyradicals (Ross, 1988). Its level might have increased (Fig 6b) probably due to an adaptive and protective role in response to slight oxidative stress through an increase in its synthesis as reflected in the kidney tissue. Similar to these observations, Pandey et al. (2003) and Farombi et al. (2007) also found an increase in GSH level in liver and kidney tissues of fish collected from polluted river water. However, GSH levels are supposed to be suppressed under severe oxidative stress due to the loss of compensatory responses and oxidative conversion of GSH to its oxidised form (Chen and Lin, 1977). Thus, the reduced levels of GSH in liver in AWW treated animals would be handled by increased GR activities (Fig 3a) which would regenerate the depleting GSH. Whereas, kidney tissue does not seem to require a high GR activity as GSH synthesis apparently is adaptively improved (Fig 3b).

The level of ALT can act as a biomarker of hepatotoxicity. It has also been associated with other organ toxicities thus indicating that the enzyme has specificities beyond liver. ALT plays an important role in amino acid metabolism and gluconeogenesis. ALT and AST catalyze the reductive transfer of an amino group from alanine or aspartate respectively to alpha-ketoglutarate to yield glutamate and pyruvate or oxaloacetate respectively (Ozer et al. 2008). Our results showed a remarkable increase of ALT level roughly by 3 fold in the liver as a result of these wastewater ingestion (Fig 9a). Moreover, a significant rise of around 1.5 fold in ALT activity was also observed in the kidneys of wastewater exposed animals (Fig 9b). Silva et al. (1999) also found an increase in serum ALT activity in rats exposed to polluted river water. AST level in the kidney of the wastewater treated rats was found to be 1.5 fold higher than that in control animals (Fig 10b). These results are consistent with Avancini et al. (2007) who found an increase in ALT and AST level in
mice fed with wastewater. However, AST activity in liver did not show a significant change as a result of intake of the wastewater samples.

Alkaline phosphatase is associated with cell membranes in multiple tissues. It hydrolyzes mono phosphatases at an alkaline pH (Ozer et al. 2008). ALP has been proposed as a good biomarker in ecotoxicology (Boge et al. 1992). Its activity increased more than 3 fold in kidney of ingested rats under our experimental conditions. However, a meager induction of around 10% in ALP activity was found in the liver of SWW exposed rat. Contrary to this, roughly 30% reduction in the ALP level was recorded in the livers of AWW administered rats. Muthuviveganandavel et al. (2008) also recorded a decrease in ALP activity in rat liver after the treatment with α-cypermethrin.

Our results suggest that the test wastewater from both sites could be able to induce a significant degree of oxidative stress in rats by disregulation in the antioxidant system. Although the pattern was not similar in liver and kidney of rats yet the alterations were quite prominent. Hence, the oxidative change observed in these tissues may indicate a potential health hazard to humans posed by the Aligarh as well as Saharanpur industries. Regarding the plausible mechanism of antioxidant induced gene expression, an extensive research will be required on the molecular aspects of signal transduction originating from antioxidants and xenobiotics.
Fig 1: Effect of wastewater intake by rats on SOD activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. **, ***: $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
**Fig 2:** Effect of wastewater intake by rats on CAT activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 3: Effect of wastewater intake by rats on GR activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, ***, $P < 0.05$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 4: Effect of wastewater intake by rats on GST activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. ***: $P < 0.001$ compared with control by ANOVA.
Fig 5: Effect of wastewater intake by rats on GPx activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05, P < 0.01, P < 0.001$ respectively compared with control by ANOVA.
Fig 6: Effect of wastewater intake by rats on GSH level in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 7: Effect of wastewater intake by rats on MDA level in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. ***: $P < 0.001$ compared with control by ANOVA.
Fig 8: Effect of wastewater intake by rats on H2O2 level in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 9: Effect of wastewater intake by rats on ALT activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 10: Effect of wastewater intake by rats on AST activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 11: Effect of wastewater intake by rats on ALP activity in liver (panel a) and kidney (panel b). Animals in the treatment group received the test waters for 15 consecutive days. ***: $P < 0.001$ compared with control by ANOVA.
Table 1: Physicochemical parameters of the test wastewater samples
(expressed as mean value ± S.D)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Aqua guard Water</th>
<th>AWW</th>
<th>SWW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pH</td>
<td>6.6 ± 0.05</td>
<td>7.5 ± 0.1</td>
<td>8.2 ± 0.17</td>
</tr>
<tr>
<td>2.</td>
<td>BOD (mg/l)</td>
<td>0.8 ± 0.03</td>
<td>82 ± 7.4</td>
<td>378 ± 14</td>
</tr>
<tr>
<td>3.</td>
<td>COD (mg/l)</td>
<td>3.2 ± 0.1</td>
<td>283 ± 9.2</td>
<td>1259 ± 36</td>
</tr>
<tr>
<td>4.</td>
<td>DO (mg/l)</td>
<td>7.9 ± 0.3</td>
<td>2.8 ± 0.5</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>5.</td>
<td>TC (mg/l)</td>
<td>4.9 ± 0.1</td>
<td>787 ± 22</td>
<td>3089 ± 52</td>
</tr>
<tr>
<td>6.</td>
<td>TIC (mg/l)</td>
<td>0.9 ± 0.02</td>
<td>234 ± 7</td>
<td>157 ± 6</td>
</tr>
<tr>
<td>7.</td>
<td>TOC (mg/l)</td>
<td>4.0 ± 0.1</td>
<td>553 ± 12</td>
<td>2932 ± 46</td>
</tr>
<tr>
<td>8.</td>
<td>Total Phenol (mg/l)</td>
<td>-</td>
<td>1.3 ± 0.2</td>
<td>15.5 ± 1.4</td>
</tr>
</tbody>
</table>

BOD: Biological oxygen demand  
COD: Chemical oxygen demand  
TC: Total carbon  
TIC: Total inorganic carbon  
TOC: Total organic carbon

Table 2: Concentration of TCE in the test wastewater samples detected by gas liquid chromatography (GLC)

<table>
<thead>
<tr>
<th>Test water samples</th>
<th>Trichloroethylene concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWW</td>
<td>8.97</td>
</tr>
<tr>
<td>AWW</td>
<td>28.4</td>
</tr>
</tbody>
</table>
Table 3: Concentration of metals in the test wastewater samples by inductive conductivity plasma (ICP-MS) spectrometry (expressed as mean value ± S.D)

<table>
<thead>
<tr>
<th>Metals</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AWW</td>
</tr>
<tr>
<td>Arsenic (As)</td>
<td>—</td>
</tr>
<tr>
<td>Barium (Ba)</td>
<td>27 ± 0.6</td>
</tr>
<tr>
<td>Boron (B)</td>
<td>18 ± 0.4</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>672 ± 2.4</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>74 ± 0.8</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>—</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>376 ± 1.5</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>104 ± 1.0</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>216 ± 0.7</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>9 ± 0.1</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>0.4 ± 0.08</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>11 ± 0.4</td>
</tr>
<tr>
<td>Silicon (Si)</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>Vanadium (V)</td>
<td>4 ± 0.2</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>233 ± 2.1</td>
</tr>
</tbody>
</table>
Chapter V:

Antioxidant system in *Allium cepa* as toxicity biomarker of trichloroethylene
1. Introduction

An increase in industrialization and urbanization over the last half century in India has witnessed an ever growing demand of chemicals, which eventually caused deterioration of environment. With particular reference to TCE vis-a-vis Aligarh, India, a small survey through Mitra Nagar in this city revealed that several ailments in local population could be attributed to TCE exposure (Dua, 2003; Chiya Nivaran Samithi, 2009). This chemical is used as a cleansing agent in city’s lock and brass industry. According to the estimates of Aligarh Office of Uttar Pradesh Pollution Control Board (UPPCB), there were at least 125 units using this chemical in Aligarh (Dua, 2003). TCE has shown to exhibit various types of toxic response in animal and plant kingdoms. Several investigators have especially reported the hazardous effects of TCE on microsomal mixed function oxidase system in animal models (Kawamoto et al. 1988; Bloemen et al. 2001; Kumar et al. 2001a; Vidal et al. 2001).

Allium cepa is the common onion and is widely used in all parts of the world as flavouring vegetable. A. cepa root length inhibition bioassay has been recommended for the routine monitoring of water pollution since it is quite sensitive and valid indicator of toxicity (Fiskesjo, 1985). The primary aim of the study embodied in this chapter was to evaluate the potential of the antioxidant/detoxification enzymes of A. cepa for use as biomarkers of trichloroethylene (TCE) pollution in water. It was envisaged that this would further increase the efficacy of the A. cepa test in routine water monitoring studies. Moreover, biomarker studies on TCE exposure, in strict sense of term are quite insufficient especially in the plant system despite the fact that plant based biomarker studies are simple, cost effective and sometimes more sensitive too compared with the animal system (Fatima and Ahmad, 2005).

3. Results

Exposure of Allium cepa to TCE induced the xenobiotic metabolizing enzymes supposedly involved in phyto transformation of TCE. Fig 1 (a) depicts the level of SOD observed in A. cepa bulb after treatment with TCE. SOD activity showed an increase of up to 79% \((P < 0.001)\) at 250 ppm of TCE exposure. However, at 300 ppm exposure a significant fall was observed. Fig 1 (b) presents the induction in CAT activity in A. cepa as a result of TCE exposure. A rise of around 120% \((P < 0.001)\) in
CAT level was observed at 150 ppm of TCE treatment. However, higher doses of TCE exposure showed a sharp decline in CAT activity. The induction in GR activity was witnessed until 200 ppm (Fig 2a). GR activity was recorded to be maximum at 200 ppm and was 25 U/min/mg proteins contrary to the control which exhibited a value of 8.7 U/min/mg proteins. Further increase in TCE concentration led to the decrease in GR activity with maximum inhibition at 300 ppm. Fig 2 (b) illustrates GST activity in A. cepa bulbs exposed to different concentration of TCE. The GST activity was also found to be maximum at 200 ppm and was 269 U/min/mg protein compared with control which was 88.7 U/min/mg protein. GST level was found to be inhibited at higher concentration of TCE exposure. Fig 3 (a) presents the changes recorded in GPx activity consequent upon TCE treatment in A. cepa bulbs. The level of GPx showed continuous rise up to the maximum experimental concentration. At 300 ppm GPx activity rose up to 110% \( (P < 0.001) \) in comparison with control. APx activity showed roughly 180% \( (P < 0.001) \) induction at 300 ppm of TCE exposure compared to control (Fig 3b). Similar sort of enhancement was also witnessed in the activity profile of MDHAR (Fig 4a), which displayed an induction by 175% at 300 ppm of TCE exposure. DHAR too showed a gradual increase in activity but the rise was mere 60% compared to control (Fig 4b). Fig 5 (a) shows the levels of hydrogen peroxide recorded in A. cepa bulb after treatment with different concentrations of trichloroethylene. H2O2 level showed a maximum decline of up to 70% compared to control value. Fig 5 (b) and (c) depict the changes in ASC and GSH levels respectively in A. cepa treated with various concentrations of TCE. Both ASC and GSH levels also moderately dropped upon TCE exposure up to the extent of 50–55%. Fig (6) presents the changes recorded in the test antioxidant enzymes of A. cepa bulbs exposed to TCE in the presence of cycloheximide (50 μg/ml). It is evident from Fig (6) that activities of all the test antioxidant enzymes returned to the values equal to those of untreated controls. Fig (7) shows the levels of APx, GPx and MDHAR of A. cepa in the presence of cycloheximide before and after TCE treatment. It is apparent from this figure that cycloheximide brought down the enzyme activity back to the level of untreated controls.

4. Discussion

Several enzymes of detoxification machinery have been used as the biomarkers of xenobiotic pollution (Oesh and Arand, 1999).
SOD is an endogenous enzymatic scavenger and constitutes the first line of defense against oxygen derived free radicals converting the superoxide anion (O$_{2}^{-}$) into H$_2$O$_2$ (Briganti and Picardo, 2003; Sezer et al. 2007). Three classes of SOD have been identified in plants i.e. cytosolic and chloroplastic Cu-Zn/SODs, a chloroplastic Fe/SOD and a mitochondrial Mn/SOD, of which the former two classes have been shown to be regulated by oxidative stress (Azevedo et al. 1998; Kliebenstein et al. 1998). In the present investigation, the rise in the level of SOD in A. cepa tissue homogenate was not exceeded to 80% over and above the control. Watanabe and Fukui (2000) on the other hand reported a continuously increasing SOD activity in mouse liver after TCE treatment. However, a concentration dependent inhibition in SOD activity in human epidermal keratinocytes was obtained by Zhu et al. (2005) as a result of TCE exposure.

Catalase and peroxidases are the major enzymes involved in H$_2$O$_2$ detoxification. CAT exhibited a concentration dependent increase up to 200 ppm of TCE treatment (More than 2 fold) in A. cepa system. After that the activity dropped drastically and attained the level close to that of control at 300 ppm. Vidal et al. (2001) found an increase in the CAT level in fresh water clams exposed to TCE in aquarium for 5 days. An increase in CAT activity in the livers of mice as a result of TCE intake was also reported by several workers (Elcombe et al. 1985; Goel et al. 1992; Watanabe and Fukui, 2000). A decrease in CAT activity as a result of TCE treatment in rats was also reported by Elcombe et al. (1985).

The GR and GST activity in the TCE exposed A. cepa bulbs have been shown in Figs 3 and 4. GR participates not only in H$_2$O$_2$ scavenging, but also favours a high GSH/GSSG ratio to maintain a proper cellular redox (Srivastava et al. 2004). The role of GST in xenobiotic detoxification is well documented (Rees, 1993; Fernandes et al. 2002; Ferrat et al. 2003). Interestingly, both the GR and GST activity attained a peak at 200 ppm of TCE treatment followed by decline at higher doses. Such pattern of xenobiotic metabolizing enzymes is expected in view of typical tolerance and toxicity of this pollutant. Watanabe and Fukui (2000) also reported a rise in GR level as a result of TCE intake in mice. The increasing trend of the GR and GST activity up to 200 ppm of TCE could serve as biomarkers of TCE pollution. The dose dependent activity of these enzymes seems to be a better index of TCE pollution provided other pollutants are ruled out to enhance this activity. These findings also lead us to suggest that TCE might exert a sort of oxidative stress on A. cepa system. A gradual rise of
more than 2 fold in GPx level was recorded in the test plant system. However, Watanabe and Fukui (2000) found a decline in GPx level following TCE treatment in mice.

The ascorbate-glutathione cycle also plays an important role in maintaining ASC level (Noctor and Foyer, 1998; Hancock and Viola, 2005). In this cycle, ASC is oxidized to monodehydroascorbate (MDHA) radical while APx is using ASC as electron donor to scavenge hydrogen peroxide. The MDHA can reconstitute itself non-enzymatically to ascorbate and dehydroascorbate (DHA) or be enzymatically reduced to ASC by MDHAR. The DHA can also be reduced to ascorbate in a reaction mediated by DHAR, using GSH as the reducing substrate. The resulting oxidized-glutathione is then reduced back to GSH by GR (Noctor and Foyer, 1998; Davey et al. 2000). Ascorbate and GSH are recycled via this route when H2O2 is scavenged in the cells.

Ascorbate plays a role as primary cellular antioxidant (Alscher et al. 1997). Ascorbate and glutathione are considered among the most important antioxidants involved in protection against ROS/free radicals through the ascorbate/GSH cycle (Gossett et al. 1996; Kuzniak and Maria, 2001; Drazkiewicz et al. 2003).

MDHAR and APx activities were found to be approximately 3 fold enhanced at 300 ppm of TCE exposure compared with control. Though DHAR also displayed a gradual rise in activity but that could only reached to 160%. Both ASC as well as GSH levels dropped upon exposure to TCE which was found to be in the range of 50–55%. Our group has also reported a dose dependent depletion in GSH levels in Allium cepa exposed to heavy metal mixture in an earlier study (Fatima and Ahmad, 2005). However, Goel et al. (1992) had found an increase in GSH level as a result of TCE intake in mice. In the present study, H2O2 level was also dropped 30% in A. cepa system at 300 ppm of TCE treatment which is well justified by the induction of APx and GPx (Figs 3 and 5).

In the presence of cycloheximide, all the test enzymes displayed the levels as those of untreated controls while the enzymatic activities remained unchanged in the presence of chloramphenicol, there by suggesting for de novo synthesis of the cytosolic components of the antioxidant enzymes as a result of trichloroethylene exposure.

In view of the present findings, it is suggested that variations in the antioxidant enzymes of A. cepa can serve as useful biomarkers for the detection of TCE pollution.
in water. In fact, *A. cepa* root inhibition test is a cost effective toxicity bioassay routinely used in water monitoring studies (Fiskesjö, 1985). Therefore, it is our contention that its efficacy would be greatly increased if enzymatic studies are also carried out in the same onion bulbs exposed to the toxicant for the standard *A. cepa* bioassay.
Fig. 1: Activity profiles of SOD (panel a) and CAT (panel b) in onion bulbs exposed to increasing concentrations of TCE for 48 hours. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 2: Activity profiles of GR (panel a) and GST (panel b) in onion bulbs exposed to increasing concentrations of TCE for 48 hours. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 3: Activity profiles of GPx (panel a) and APx (panel b) in onion bulbs exposed to increasing concentrations of TCE for 48 hours. **, ***: $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 4: Activity profiles of MDHAR (panel a) and DHAR (panel b) in onion bulbs exposed to increasing concentrations of TCE for 48 hours. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 5: Patterns of H2O2 (panel a), ascorbate (panel b) and GSH (panel c) levels in *A. cepa* bulbs as a result of 48 hour exposure to increasing concentrations of TCE. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 6: Effect of cycloheximide on CAT, GR and GST activities in *A. cepa* bulb as a result of 48 hour exposure to TCE.

Fig. 7: Effect of cycloheximide on GPx, MDHAR and APx activities in *A. cepa* bulb as a result of 48 hour exposure to TCE.
Chapter VI:

Some isozymes of cytochrome P450 system as potential biomarkers of certain toxicants: Comparison between plant and animal models
1. Introduction

Biochemical markers are biochemical responses induced in the presence of a specific group of contaminants that have the same mechanism of toxic activity. These are measurable responses to the exposure of an organism to xenobiotics. They usually respond to the mechanism of toxic activity and not to the presence of a specific xenobiotic and therefore, may react to a group of either similar or very heterogeneous xenobiotics. Biochemical markers detect the type of toxicity; in some of them, the magnitude of their response correlates with the level of pollution. The great advantage of biochemical markers is providing evidence of the state of pollution in a comprehensive way based on the synergistic and antagonistic effects of all contaminants involved (Siroka and Drastichova, 2004). In recent years there has been great interest in developing assays that can be used as a biomarker of effect caused by exposure to toxic agents (Shah et al. 2009).

One of the most intensively studied biomarkers, in both laboratory and field conditions, is cytochrome P450. The cytochromes P450 are a diverse multigene family of heme-containing proteins that oxidize or hydrolyze compounds through the insertion of an atom of atmospheric oxygen to the substrate during the reaction cycle (Nebert et al. 1993; Nelson et al. 1996). CYP450 exhibit broad substrate specificity, reflecting the existence of various isozymes. Various compounds of diverse structures can be metabolized by P450 enzymes.

Trichloroethylene is a volatile organic compound that has found a wide variety of industrial applications, as a metal degreaser, dry cleaning agents etc (Shang and Gordon, 2002). It is a well known air, soil and water pollutant (Bruckner et al. 1989), and thus has been found in underground water sources and many surface waters in the proximity of its manufacture, application and disposal sites. Its exposure has been linked to neurological dysfunction as well as several types of cancers in animals (Brown et al. 2003). Because of its widespread commercial use, TCE has become one of the most abundant organic contaminants at many of the superfund sites of USA (Westrick et al. 1984; Griffin et al. 1998). TCE is being used in developing countries like India as an industrial solvent in small factories where degreasing metal parts is carried out at a low cost. The indiscriminate use of this toxicant in India especially in Aligarh is causing health hazard in human population (Dua, 2003; Chiya Nivaran Samiti, 2009).
Heavy metals are industrial and environmental pollutants and are toxic to several tissues such as the liver, kidney, testis etc (Rikans and Yamano, 2000; Liu et al. 2000; Hao et al. 2002). Major industrial and environmental pollutants in the class of heavy metals include mercury, cadmium, lead, copper and iron. The harmful effects of heavy metals manifest in several ways (Trivedi and Gundap, 1992; ATSDR, 2001). Some of them like lead are suspected to be a human carcinogen (Fracasso et al. 2002).

The aim of this study was to identify, some isozymes of CYP450 system that can acts as a biomarker of exposure to the test toxicants in the animal and plant systems. The interaction between TCE and ethanol in the exposed population may be important because the workers who are exposed to TCE in their occupational environment might also have the habit of drinking alcoholic beverages. Since liver is the primary site both for TCE and ethanol metabolism, their interaction especially in the same tissue is of concern to humans. Keeping this in mind, co-exposure to TCE and alcohol in the plant and animal systems was planned for the biomarker studies. Similarly, one can not rule out the co-exposure of heavy metals and TCE also because of their strategic association in Indian scenario. Hence the latter was also included in the treatment protocol for the study targeting the CYP450 system.

2. Results

2.1. Effect of TCE administration alone in rats on the selected CYP450 isozymes in liver and kidney tissues

Oral administration of TCE to rats resulted in the amplification in NDMA-d activity by around 280% in liver tissue compared with the control group (Fig 3a). However, only 120% rise was observed in case of renal tissues (Fig 3b). PROD activity rose up to around 130% in hepatic cells (Fig 2a) and roughly 90% ($P < 0.001$) in renal cells (Fig 2b). The enzymatic activity of CYP1A1 (EROD) displayed an enhancement of around 30% ($P < 0.01$) in both the tissues as a result of TCE intake (Fig 1a,b).

2.2. Effect of EtOH administration alone in rats on the selected CYP450 isozymes in liver and kidney tissues

Ethyl alcohol ingestion in rats caused a remarkable induction of around 4.2 fold in the NDMA-d activity of liver tissue and 1.33 fold in the kidney over and above the activity in control group of animals (Fig 3a,b). EROD activity showed a rise of 90%
(liver) and 55% (kidney) as a result of ethyl alcohol administration compared with control animals (Fig 1a,b). PROD activity exhibited a small rise of around 23% ($P < 0.01$) in liver (Fig 2a). However, the same enzyme did not show any significant change in renal cells (Fig 2b).

2.3. Effect of heavy metals (H.M.M) administration alone in rats on the selected CYP450 isozymes in liver and kidney tissues

EROD activity profiles under this treatment condition, displayed an inhibition of around 25% in both the tissues compared with that in untreated control animals (Fig 1a,b). PROD and NDMA-d both exhibited quite a similar decline in activity of around 10% in both tissues (Fig 2-3a,b).

2.4. Combined effect of TCE and EtOH administration in rats on the selected CYP450 isozymes in liver and kidney tissues

In the present experimental condition, NDMA-d activity exhibited a significant decrease of around 60% in liver (Fig 3a) and 30% in kidney (Fig 3b) compared with untreated control. The activity profiles of PROD showed a 55% and 75% decrease in liver and kidney tissues respectively compared with the control values (Fig 2a,b). EROD activity also displayed a small but significant decrease of around 15% in both the tissues (Fig 1a,b).

2.5. Combined effect of TCE + H.M.M administration in rats on the selected CYP450 isozymes in liver and kidney tissues

NDMA-d activity displayed a remarkable induction of around 240% over and above the control in livers as a result of combined exposure of TCE and heavy metals (Fig 3a). However, the same enzyme exhibited an increase of around 115% (Fig 3b) in the renal tissue of treated rats. PROD activity also rose up to 97% in liver (Fig 2a) and 72% in kidney (Fig 2b) of treated animals. However, EROD activity showed only a small increase of around 10% in both the tissues (Fig 1a,b).

2.6. Combined effect of TCE + EtOH + H.M.M administration in rats on the selected CYP450 isozymes in liver and kidney tissues

Fig 1-3(a,b) illustrate the change in activities of CYP450 isozymes (EROD, PROD and NDMA-d) in the hepatic and renal tissues of TCE, ethyl alcohol and heavy metals treated rats. NDMA-d activity exhibited an increase of around 180% in liver (Fig 3a)
and 90% in kidney (Fig 3b) compared with untreated control rats. The activity profiles of PROD displayed a rise of around 65% and 35% in the liver and kidney of TCE, ethyl alcohol and heavy metals ingested rats respectively over and above the control (Fig 2a,b). However, EROD activity showed a decline of around 45% in liver (Fig 1a) and 35% in kidney (Fig 1b).

2.7. Effect of increasing concentration of TCE exposure to onion bulbs on the test CYP450 isozymes activities

Fig 4(b) depicts the activity of PROD in onion bulb after the treatment with increasing concentration of TCE. A continuous and tremendous amplification of up to 22 fold in PROD activity was observed in Allium cepa bulb as a result of 20 ppm TCE exposure. The dose dependent increase was also observed in EROD and NDMA-d enzymatic activities. The activity profile of NDMA-d displayed around 11 fold induction (Fig 4c) and EROD showed around 9 fold enhancement (Fig 4a) at 20 ppm TCE exposure in onion bulbs compared with untreated controls.

2.8. Effect of TCE exposure to Allium cepa on the activities of EROD, PROD and NDMA-d in the presence of cycloheximide

Fig (5) displayed the effect of cycloheximide on the de novo synthesis of the selected test enzymes. Cycloheximide brought down the activities of all the test enzymes equal to the untreated control values.

3. Discussion

To the best of our knowledge, the studies on the effect of TCE ingestion in rats along with the heavy metals and ethanol on the test CYP450 isozymes as well as on the effect of TCE treatment in the same isozymes of Allium cepa are being reported for the first time.

The first step in TCE metabolism is either conjugation with glutathione or oxidation by cytochrome P450. The oxidative pathway is the major pathway for TCE metabolism (Lash et al. 2001a). TCE is mainly metabolized by CYP2E1 (NDMA-d) or CYP2B1/2 (PROD) in the mouse and rat in vitro (Nakajima et al. 1992). NDMA-d and PROD activity was recorded to be amplified up to 280% (Fig 3a) and 130% (Fig 2a) in liver of TCE ingested rats respectively. Koop et al. (1982) also reported around 3 fold induction of CYP2E1 by TCE in rabbit liver. However, a 2.4 fold rise in PROD...
activity as well as an increase of around 20% NDMA-d activity in rats microsomes was reported by Wang et al. (1996). In the present study, TCE showed higher preference to CYP2E1 than CYP2B1/2.

Induction of EROD (CYP1A1) is an extremely sensitive indicator of environmental alterations and is usually one of the first detectable, quantifiable responses to exposure to xenobiotics (Stegeman et al. 1992). Previous study from our laboratory has proposed EROD as a biomarker of pesticide pollution in A. cepa system (Fatima and Ahmad, 2006). EROD activity in rats was found to be induced by roughly 30% in both tissues in TCE treated animals (Fig 1a,b). Our results are consistent with the previous investigation of Wang et al. (1996) and Hanioka et al. (1997).

NDMA-d (CYP2E1) is the key enzyme of the microsomal pathway that catalyzes the oxidation of numerous xenobiotics, including ethanol, acetaminophen, benzene, and halogenated solvents (Cederbaum, 2009). CYP2E1 is mainly expressed in the liver (Waziers et al. 1989) and to a lesser extent, in the kidney (Hong et al. 1987; Thomas et al. 1990). NDMA-d is strongly induced by chronic ethanol intake, which was also found in our case. Approx 4 fold enhancement in NDMA-d activity in liver of rats was also reported by Nakajima (1998) and Dey et al. (2002) by ethyl alcohol intake.

Our study exhibited an inhibition of the test isozymes in general but relatively higher inhibition was recorded in EROD activity as a result of heavy metals intoxication. These results are consistent with the report where in unexpectedly low levels of EROD activity was demonstrated with a relatively higher content of heavy metals (Romeo et al. 1994). An inhibition in EROD activity brought about by metals has also been reported in several studies on fish (Stien et al. 1997; Ghosh et al. 2001; Bozcaarmutlu and Arinc, 2004; Sanchez et al. 2005). In an earlier study, this group has also reported an inhibition in EROD activity as a result of heavy metal exposure in Allium cepa system (Fatima and Ahmad, 2006).

Simultaneous exposure of trichloroethylene and ethyl alcohol to rats showed an inhibition in the enzymatic activities of the test CYP450 isozymes. Several investigators have reported an inhibition in metabolism of TCE, when ethyl alcohol is co-administered in the experimental animals because both TCE and ethyl alcohol compete for the same CYP450 isozyme (Müller et al. 1975; Ikatsu et al. 1998; Watanabe et al. 1998). However, combined treatment to animals with TCE, heavy
metals and ethyl alcohol resulted in an induction of the test isozymes. Most probably the heavy metals might be nullifying the inhibition caused by TCE and ethyl alcohol co-exposure. It is quite strange but one process is antagonising another by unknown mechanism. There is one such report in which two drugs namely ketoconazole and saquinavir when co-administered in humans resulted in the inhibition of CYP450 isozymes however, administration of these drugs along with ritonavir (another inhibitor) brought about an induction of CYP450 system (Sagir et al. 2003).

The assay of test isozymes in *Allium cepa* system showed a very high rise in activity as a result of TCE exposure. The rise was as high as 22 fold in PROD, 11 fold in NDMA-d and 9 fold in EROD activity (Fig 4a-c). We did not come across with any literature on the effect of TCE on plant system especially in *Allium cepa*. Fig (5) presents the effect of cycloheximide, a *de novo* protein synthesis inhibitor on the test isozymes activity in *Allium cepa*. Cycloheximide brought the activity back to control levels in TCE treated *Allium cepa* bulbs suggesting that the test isozymes in *Allium cepa* belong to an inducible system whose expression is enhanced by TCE exposure. This is supported by earlier studies wherein it has shown to be induced in response to xenobiotic exposure (Jimenez and Stegeman, 1990; Fouchecart et al. 1999; Lagueux et al. 1999; Fernandes et al. 2002).

The wide range between the basal and the induced level of the test CYP450 isozymes could serve as the better biomarkers for the detection of TCE pollution. In view of this study, we recommend the assay of EROD, PROD and NDMA-d enzymes in the *Allium cepa* system as a presumptive test for the detection of TCE before using the analytical techniques like HPLC or GC.
Fig 1: Activity profile of EROD following oral administration of the test toxicants to rats in the liver (panel a) and kidney tissues (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 2: Activity profile of PROD following oral administration of the test toxicants to rats in the liver (panel a) and kidney tissues (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively compared with control by ANOVA.
Fig 3: Activity profile of NDMA-d following oral administration of the test toxicants to rats in the liver (panel a) and kidney tissues (panel b). Animals in the treatment group received above mentioned toxicants for 15 consecutive days. *, ***, $P < 0.05$, $P < 0.001$ respectively compared with control by ANOVA.
Fig. 4: Activity profiles of EROD (panel a), PROD (panel b) and NDMA-d (panel c) in onion bulbs exposed to increasing concentrations of TCE for 48 hours. ***: $P < 0.001$ compared with control by ANOVA.
Fig 5: Response of *Allium cepa* test isozymes in the presence of cycloheximide.
Chapter VII:

Mutagenicity of TCE containing industrial wastewaters collected from two different stations in northern India
1. Introduction

Human and industrial activities are the origin of the discharge of multiple chemical substances in the environment and are the main causes of environmental pollution (White and Rasmussen, 1998). The contamination of water resources by genotoxic compounds is a worldwide problem (Vargas et al. 1995; Claxton et al. 1998; Kong, 1998; Ohe et al. 2003; Buschini et al. 2004). With rapid strides in industrialization, there has been an alarming increase in the pollution of various water bodies in India during the past few decades (ISGE, 1990; Rehana et al. 1995; 1996; SOER, 2001; Aleem and Malik, 2003).

Genotoxicity test of surface waters or industrial effluents using a variety of bioassays demonstrates that these mixtures contain many unidentified and unregulated toxicants that may pose risks and carcinogenicity of unknown magnitude (Lerda and Prosperi, 1996; Magliola et al. 1997; Magdaleno et al. 2001; Ohe et al. 2004). Most of the toxicity testing systems rely on small mammals such as rats or mice and hence are time consuming, very expensive and attract considerable ethical criticism (Tsuda et al. 2001). For these reasons a number of in vitro tests have been developed which employ bacteria or plant cells (Ames, 1984; Wilcox and Denny, 1985; LeCurieux et al. 1995; Vargas et al. 1995; Liu et al. 1999). Among the tests that are routinely advocated for the genotoxic evaluation of water, Ames plate incorporation test, Ames fluctuation test and Allium cepa test, occupy a prominent position (Fiskesjo, 1985; Rank and Nielsen, 1993; Claxton et al. 1998; Kong, 1998; Siddiqui and Ahmad, 2003). Ames test is a rapid test for the detection of environmental mutagens (Ames, 1984). This test is based on the ability of chemicals to induce reverse mutations in certain histidine requiring strains of Salmonella typhimurium. On the other hand, Allium cepa genotoxicity test is an excellent plant-based test for studying anaphase aberrations (Fiskesjo, 1979, 1985; Rank and Nielsen, 1993). Many tests that evaluate the in vitro DNA damage are also used in routine water monitoring studies. Among them, the plasmid nicking assay has been widely used and is a valid indicator of genotoxicity (Khan et al. 2003).

From our study (chapter IV) as well as from the available literature, it is well known that water samples from Aligarh city have been loaded with heavy metals and trichloroethylene (Dua, 2003; Fatima and Ahmad, 2005; Chiya Nivaran Samithi, 2009) as this city houses various lock, electroplating, brass and lead battery
manufacturing units while Saharanpur city has a large number of sugar as well as paper and pulp industries obviously releasing a plethora of organic waste chemicals namely chloroform, chlorinated hydrocarbons like trichloroethylene, phenols, catechols etc (Suntio et al. 1988; Peck and Daley, 1994; Freire et al. 2003; McLean et al. 2006). Although the effluent treatment facilities are legally binding to be installed by all industries, yet a significant amount of toxicants was present in these wastewater samples (Fatima and Ahmad, 2005).

Various water pollutants including heavy metals and phenolics are known to produce reactive oxygen species like superoxide and hydroxyl radicals which in turn may account for their genotoxicity (Tapley et al. 1999; Chandra and Khuda-Buksh, 2004).

The work embodied in this chapter was carried out to evaluate and compare the genotoxicity of wastewater samples collected from Aligarh and Saharanpur cities of northern India employing four bioassays viz. Ames plate incorporation test, Ames fluctuation test and Allium cepa genotoxicity test as well as plasmid nicking assay. This work could help understand the role of natural milieu to address the basic question as to whether TCE is genotoxic or promotes genotoxicity under certain environmental conditions. Thus, the genotoxicity of the trichloroethylene in the natural milieu was determined by Ames fluctuation test and Allium cepa genotoxicity test.

2. Materials and methods

2.1. Water sampling

Wastewater samples were collected from the industrial areas of Aligarh and Saharanpur cities of northern India, in sterile glass bottles strictly according to the method described in APHA (1998). Prior to the mutagenicity assay, the test samples were filter sterilized by passing through 0.45 μm filters. The S. typhimurium strains employed for this study were obtained from Prof. Takehiko Nohmi, National Institute of Health Sciences, Japan. S9 fraction was prepared from liver of Sprague-Dawley male rats using Aroclor-1254 as the inducer. The composition of S9 mix was as follows: Rat liver S9 fraction (4%), magnesium chloride (1 M), glucose-6-phosphate (1 M) and NADP (0.1 M). The revertant colonies were screened using an electronic colony counter supplied by Mac India Ltd.
2.2. Ames plate incorporation test

The method of Maron and Ames (1983) was essentially followed. Different volumes (2.5-20 \( \mu l \)) of the test samples were added to phosphate buffer or S9 mixture. Revertant colonies were obtained after subtracting the spontaneous mutants from induced mutants. Methyl methane sulphonate (MMS) served as the positive control to ensure that responses of the tester strains were within the recommended limits. Each experiment was repeated thrice and two plates were taken for each dose of the test sample.

2.3. Ames fluctuation test

Ames fluctuation test was carried out according to the method of Venitt et al. (1984) in a 96 well microplate format. Filter sterilized wastewater samples at different doses (2.0-20 \( \mu l \)) as well as different doses of test water samples containing 50 ppm or 100 ppm trichloroethylene were added to the incubation mixture containing the minimal medium. Presence of revertants was ensured by a drop in the pH of the medium and thus changes in the colour and turbidity caused by the luxuriant growth of \( \text{his}^+ \) revertants.

2.4. \textit{Allium cepa} genotoxicity test

The basic protocol of Fiskesjo (1979) was followed. Small bulbs of \textit{Allium cepa} were exposed to the test water samples as well as the test water samples containing 50 ppm or 100 ppm of trichloroethylene for 48 h such that their roots primordia were exposed. The dividing root cells were arrested at the metaphase stage by the use of colchicine. After this the cells were fixed in 95\% alcohol-glacial acetic acid (3:1) for 30 min. The slides were then stained with the Feulgen reagent as described by Darlington and Lacour (1976). The chromosomal aberrations were scored using a CH20i Olympus microscope (India) as described by Grant (1982).

2.5. ROS generation in the test water samples

Superoxide radicals and hydrogen peroxide were assayed by the method of Nakayama et al. (1983). Quantification of the hydroxyl radical was done according to the method of Richmond et al. (1981). These assays were conducted using varying amounts of the test water samples under \textit{in vitro} conditions and measuring the respective free radicals over a time frame of 1 h for hydroxyl radical and \( \text{H}_2\text{O}_2 \), whereas the superoxide
radical generation was evaluated over a time frame of 3 h. A separate reaction was also run in the presence of specific scavenger for authentication (Richmond et al. 1981; Nakayama et al. 1983).

2.6. Plasmid nicking assay

This assay was carried out as described by Rahman et al. (1990). 0.5 µg of covalently closed circular pBR322 DNA was treated with the test samples in a total volume of 20 µl for 3 hours. After the treatment, 5 µl of 5x tracking dye (40 mM EDTA, 0.05% bromophenol blue and 50% (v/v) glycerol) was added and loaded on 1% agarose gel. The gel was run at 50 mA for 2 hours and stained with ethidium bromide (0.5 µg/l) for 30 minutes at room temperature. After washing, the gel was visualized on photodyne UV-transilluminator (USA) and photographed.

2.7. Statistical analysis

2.7.1. Ames plate incorporation test

Mean values of the revertant colonies as well as the standard deviation were obtained for this purpose. Mutagenic potential of the test samples was based on the initial linear portion of the dose-response curve with various strains. The slope \( m \) was obtained by the least square regression method using the initial linear portion of the curve.

2.7.2. Ames fluctuation test

Statistical significance of the data was calculated using Chi-square method as described by Green et al. (1977). The formula used was as under:

\[
\chi^2 = 2n \left( t - c \right)^2 / (t + c)(2n - t - c)
\]

Where \( n \) is the total number of wells, \( c \) the number of positive wells with solvent (control) and \( t \) is the number of positive wells with the test sample.

The mean number of induced mutants per well ‘\( M_i \)’ was obtained by the following expression:

\[
M_i = \ln \left( \left( n - c \right) / (n - t) \right)
\]

Where \( n \) is the total number of wells, \( c \) the number of positive wells with control and \( t \) is the total number of positive wells with the test sample. This equation has been used by earlier researchers for evaluating the mutagenic potential of various toxicants (Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006).
2.7.3. *Allium cepa* genotoxicity test

Various parameters of genotoxicity were expressed as the number of specific anaphase aberrations per 100 cells or the total number of aberrations per 100 cells (Grant, 1982). Mitotic index (MI) is defined as the number of dividing cells per 1000 cells.

3. Results

3.1. Ames testing of the Saharanpur wastewater (SWW) samples in the absence and presence of S9 fraction

SWW sample was recorded to be highly mutagenic for all the tester strains in terms of the slopes ($m$) of the initial “concentration-response curves” (Table 1). TA100 and TA98 strains were the best responders, both in the absence and presence of S9 fraction. The values of $m$ for TA100 and TA98 strains were calculated to be 86 and 51 respectively (Table 1).

Potency of the test sample with S9 supplementation in order of decreasing sensitivity of strain is given as under:

TA100> TA98> TA102> TA97a> TA104

Pattern of sensitivity of tester strains incubated with SWW but without S9 supplementation is as follows:

TA100> TA98> TA102> TA104> TA97a

3.2. Ames testing of the wastewater samples from Aligarh (AWW) in the absence and presence of S9 fraction

The reversion data of the Ames tester strains with AWW is presented in Table 2. TA102 strain exhibited the maximum mutagenic response with AWW in terms of the slope ($m$) in the presence of S9. However, the maximum potency in the absence of S9 was displayed by TA98 strain. The highest ‘$m$’ value of 43 was recorded for TA102 strain (Table 2).

Potency of AWW samples on various tester strains with metabolic activation by S9 fraction is sequenced as under:

TA102> TA100> TA98> TA97a> TA104

Potency of the sample without S9 supplementation in the decreasing order is as given below:

TA98> TA102> TA97a> TA100> TA104
3.3. Ames fluctuation test conducted on Saharanpur wastewater with and without metabolic activation of the sample

Table 3 enlists the data of the Ames fluctuation test carried out with the test samples from Saharanpur, both in the presence and absence of S9 fraction. While the strain TA100 was estimated to be the most sensitive strain against the genotoxic effect of SWW in the absence of S9, TA98 was the best responder among all tester strains after metabolic activation. The highest Mi value was recorded to be 3.0 for TA100 in the absence of the S9 fraction. However, metabolic activation resulted in the decrease of Mi value from 3 to 1.8 for this wastewater sample (Table 3). The order of sensitivity of the strains in terms of Mi was evaluated to be as under:

TA98 > TA100 > TA102 > TA97 with S9 supplementation
TA100 > TA98 > TA102 > TA97 without S9 supplementation

3.4. Ames fluctuation test conducted on Aligarh wastewater in the presence and absence of S9 fraction

In the fluctuation test, all the strains responded significantly and more or less equally when exposed to AWW (Table 4). Interestingly, TA100 strain exhibited the highest sensitivity under both the experimental conditions. Mi value of 1.1 was recorded for TA100 without S9 supplementation. However, in the presence of S9, Mi was increased from 1.1 to 1.9 suggesting a relatively higher mutagenic response compared with the experiments carried out without metabolic activation.

3.5. Ames fluctuation test conducted on Saharanpur wastewater sample supplemented with different concentrations of trichloroethylene in the presence and absence of S9 fraction

Supplementation of TCE at 50 ppm in this water samples does not seem to result in a significant increase in its mutagenicity (Tables 5 and 6). However, addition of 100 ppm of TCE in the test water samples exhibited a significant increase in the genotoxicity, where Mi value of 4.04 was recorded compared with 2.69 for TA100 strain in the absence of the S9 fraction. Moreover, a rise in Mi value from 1.74 to 2.46 was also recorded in the test water sample in the presence of S9 fraction. The corresponding rise of around 50% and 41% in Mi values in the absence and presence of S9 fraction, respectively, was obviously due to TCE supplementation (Table 6). As stated earlier, the metabolic activation of SWW resulted in reduction in Mi value.
particularly in this case from 2.69 to 1.74 (36%) in the absence of additional TCE. However, metabolic activation in TCE supplemented SWW resulted in the reduction in Mi value from 4.04 to 2.46 a decrease of 39%.

3.6. Ames fluctuation test on Aligarh wastewater sample with different concentrations of trichloroethylene in the presence and absence of S9 fraction

Tables 7 and 8 present the results of Ames fluctuation test with AWW in the presence of different concentration of TCE. In this system also addition of 50 ppm TCE seems to produce an insignificant change in genotoxicity of the test water samples even with S9 supplementation (Tables 7 and 8). Supplementation of 100 ppm TCE caused a slight increase in mutagenicity where a rise in Mi value from 1.17 to 1.47 (26%) and 1.96 to 2.43 (24%) in TA100 strain in the absence and presence of S9 fraction was noticed respectively. This rise in Mi value of around 25% was obviously due to additional exposure of 100 ppm TCE (Table 8). The presence of S9 fraction caused an increase in Mi value from 1.17 to 1.96 (67%) in AWW sample alone. Moreover, 100 ppm of TCE supplemented test water sample resulted a rise in Mi value from 1.47 to 2.43 (65%).

3.7. *Allium cepa* genotoxicity test with SWW and AWW in the presence of different concentration of trichloroethylene

Various parameters of the *Allium cepa* test have been presented in Table 9. A decrease in mitotic index (MI) was recorded with both the samples as well as in the presence of TCE also. However, a higher reduction in MI was observed in case of SWW (28±7.2) than that of AWW (39±3.1) compared with control (53±6.2). The total number of anaphase aberrations was also relatively higher in SWW (12±6.4) than that of AWW (8.6±4.3). Additional exposure of 50 ppm TCE did not result in any significant change in total chromosomal aberration by both the test water samples. However, a slight increase in aberration frequency was observed in the presence of 100 ppm of TCE by both the test water samples exhibiting 34% rise in case of AWW and 15% increase in TCE supplemented SWW samples.
3.8. ROS generation

3.8.1. Generation of superoxide radicals

Fig 1a presents the dose-dependent pattern of the superoxide radicals generated by the test samples. A 100 µl SWW sample produced the superoxide radicals in much higher amounts (0.41 µmol) than AWW (0.11 µmol) could generate with equal amount of samples. Presence of SOD used as scavenger in the reaction mixture caused a drop in superoxide radical generation from 0.41 to 0.04 µmol in case of 100 µl sample of SWW.

3.8.2. Generation of H2O2

Fig 1b depicts the profile of H2O2 generated by the increasing concentrations of test water samples. H2O2 production by 100 µl of SWW was higher (1.9 µmol) than that in AWW (1.2 µmol). Presence of CAT to confirm the specificity of the reaction essentially resulted in a decline in H2O2 production from 1.9 to 0.12 µmol by a 100 µl sample of SWW and 1.2 to 0.08 µmol in case of AWW.

3.8.3. Generation of hydroxyl radicals

Fig 1c shows the profile of hydroxyl radicals generated by the test samples. A 100 µl of AWW sample produced hydroxyl radicals in significantly higher amounts (2.5 µmol) compared with SWW (0.47 µmol). Presence of mannitol in the reaction mixture led to a steep decline in the hydroxyl radical generation from 2.5 to 0.18 µmol in case of AWW and 0.47 to 0.07 µmol for SWW at the maximum experimental concentration of the samples (100 µl).

3.9. Plasmid nicking assay

3.9.1. Plasmid nicking assay with SWW and AWW samples

Fig 2 presents the DNA band profiles of the plasmid nicking assay with SWW in panel ‘A’ and AWW in panel ‘B’. Only 0.24X of SWW sample resulted in the conversion of the supercoiled pBR322 DNA into relaxed form (lane c, 2A) and 0.48X of this sample (lane e, 2A) caused extensive linearization of the plasmid with complete loss of supercoiled form. 0.72X of AWW resulted in the conversion of the plasmid DNA into the linear form (lane e, 2B)
4. Discussion

Plate incorporation method of Ames Salmonella bioassay is a widely used short-term testing system and has been recommended by various workers as a valid indicator of mutagenicity of various substances present in the environment (Ames, 1984; Cerna et al. 1991). However, Ames fluctuation test, the liquid medium version, could not gain such popularity despite its high sensitivity (Monarca et al. 1985; Wilcox and Denny, 1985), ability of automation (Dutka, 1996), possibility of using hepatocytes for metabolic activation (Green et al. 1977) and its better sensitivity for aqueous samples containing low levels of mutagens (Bridges, 1980). It is thus obvious that both the tests have some merits and demerits in their own right. We have, therefore, used both the tests to evaluate the mutagenicity of the water samples.

It is quite clear from the results that the water samples from both stations were highly genotoxic as estimated by both the version of Ames test (Table 1-4). To evaluate the mutagenic potential of the test water samples by Ames plate incorporation method, the slopes of the initial portion of dose-response relationship were obtained. The maximum slope of 86 was recorded for Saharanpur sample compared with 43 for Aligarh sample even in the presence of S9 fraction with the best responder strains (Table 1-2). Several investigators have used single tester strain employing either TA98 or TA100 strain for the mutagenic assessment of water samples (Gartiser and Brinker, 1996; Gartiser et al. 1997; Kinae et al. 2000). Keeping this in mind, we compared the ‘m’ values with these strains also, which were again comparatively greater for SWW. Ames fluctuation test also demonstrated a higher mutagenicity of SWW than that of AWW. These findings suggest that Saharanpur wastewater was more genotoxic than that of Aligarh. However, the response of TA102 strain which is usually recommended for the detection of ROS generating mutagens (Islam and Ahmad, 1991; Qadri et al. 1992; Fatima and Ahmad, 2006) was not as susceptible strain for Saharanpur sample as with Aligarh sample. Moreover, the ‘m’ value of 35 with TA102 for Saharanpur sample contrary to 43 for Aligarh sample was obtained in this study (Table 1-2).

Previous investigations undertaken in our lab have shown the strains TA102 and TA104 to be the best responders in the genotoxic evaluation of industrial wastewater samples especially by Ames plate incorporation method (Malik and Ahmad, 1995; Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006). However,
present study exhibited a slightly different sensitivity pattern compared to earlier findings obtained within the same region, suggesting for the corresponding change in the nature of the toxicants during the passage of time.

The sensitivity patterns of the various Ames tester strains were quite different for the two wastewaters suggesting for a significant difference in nature and concentrations of mutagens in the two samples. This idea gains further support by the distinguishable patterns of reactive oxygen species generated by SWW and AWW samples (Fig 1). A distinct qualitative difference in the mutagens present in SWW and AWW was also discernable by the fact that S9 supplementation resulted in the opposite responses with the two samples (Tables 1-4).

The efficacy of the tester strains, TA97a, TA98 and TA102 to detect the genotoxicity of industrial wastewater has been demonstrated in several studies (Miadokova et al. 1999; Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006). However, the mutagenic behaviour of the sample depends on the way the results are interpreted in the two tests (Siddiqui and Ahmad, 2003). For instance, in the plate incorporation test, the sensitivity of the strains is gauged on the basis of the slope \( m \) of the initial dose-response curve (Malik and Ahmad, 1995; Rehana et al. 1995; 1996; Cantor, 1997), whereas 'Mi' is the indicator of sensitivity in the fluctuation assay (Venitt et al. 1984). Both the tests make use of common strains and the endpoints and hence a similar trend in sensitivity is expected for the same sample in both the tests. However, the patterns of sensitivity as well as the best responders were found to be sometimes different in Ames plate incorporation and fluctuation tests. This disparity was reported by our group in earlier investigations (Siddiqui and Ahmad, 2003). However, such problem was not encountered in the present work (Tables 1-4).

Present study again clearly suggests the complementary nature of the two Ames testing systems for the genotoxicity assessment of complex water samples. While Ames plate incorporation assay was able to discern the nature of mutagens in the samples more precisely, Ames fluctuation could be used to evaluate the mutagenic potency of the samples in a better way.

Presence of S9 fraction appeared to reduce the mutagenic effects of SWW sample. Metabolism of active mutagenic substances present in SWW into less genotoxic compounds by S9 fraction might be the main cause of this lowering effect. Similar results were obtained by several authors in genotoxicity assessment of pulp
mill effluents (Nestmann et al. 1980; Kamra et al. 1983; Perez-Alzola and Santos, 1997).

We have detected high concentrations of various heavy metal ions like Cd$$^{2+}$$, Fe$$^{2+}$$, Cu$$^{2+}$$ and Cr (VI) in AWW samples (chapter IV) as well as in earlier studies (Fatima and Ahmad, 2005). A high concentration of heavy metals in AWW would lead to the generation of hydroxyl radicals as these heavy metals are known to produce this species (Okamato and Colepicolo, 1998). Contrary to the above, phenolics are known to generate superoxide radicals and hydrogen peroxide (Li and Trush, 1994). Thus relatively higher levels of phenolics in SWW (Table 1 of chapter IV) might have led to more generation of superoxide radicals and H$$\text{2}$$O$$\text{2}$$.

The mutagenicity of the test water samples seemed to be partly mediated by reactive oxygen species (Fig 1). Higher amounts of heavy metals in AWW would lead to the generation of hydroxyl radical as these heavy metals are known to produce this species (Okamato and Colepicolo, 1998). Hydroxyl radical is the ultimate ROS that interacts with DNA and promotes genetic damage (Hajjouji et al. al. 2007). Higher level of hydroxyl radicals in AWW samples might have given rise to relatively higher response to TA102 strain in AWW compared with SWW.

Phenolics display their genotoxicity by different mechanisms for instance pyrogallol exhibits its mutagenicity completely via ROS mediation (Silva et al. 2003) while ROS generation was not the main mechanism involved in the genotoxicity of catechol (Silva et al. 2003). The complex nature of genotoxicants in the form of phenolics in SWW might be the reason behind lower susceptibility of TA102 strain. Relatively lower sensitivity of TA102 strain in SWW sample might also be reflecting a mechanistic feature of genotoxicants especially the specific phenolics present in Saharanpur wastewater (Table 1; Chapter IV).

Use of multiple strains instead of a single strain for mutagenicity testing of complex samples in a way is well justified, since single strain would neither reflect the real potency nor would be able to discern the multiplicity of mutagens in the wastewater samples containing a plethora of hazardous substances. Our findings are also suggestive of the presence of multiple mutagens in the test samples.

TCE alone was found to be non-mutagenic by all the genotoxicity testing systems employed in the present work including A. cepa genotoxicity test (Data not shown). Although, TCE was not found to be genotoxic in the absence of other toxicants but in the natural milieu, its higher concentrations could be able to increase
the genotoxicity of the test water sample (Table 5-9). It is interesting to note here, that the presence of S9 fraction also would not be playing a significant role in the promotion of genotoxicity though the small contribution of S9 fraction in certain cases can not be ruled out (Table 7-8). This aspect will be critically evaluated in general discussion.

Saharanpur samples brought about a remarkable amount of chromosome bridges, which could be attributed to chromosome breaks, stickiness and breakage and reunion of the broken ends (Liu et al. 1996; Gari et al. 1998). However, chromosomal fragmentation was the predominant effect of AWW, which could be attributed to the failure of the broken chromosomes to recombine at metaphase (Nielsen and Rank, 1994). An insignificant change in total chromosomal aberration was observed by both the test water samples supplemented with 50 ppm of TCE. However, slight increase in aberration frequency was obtained with both the test water samples exposed to 100 ppm of TCE (Table 9).

It is quite clear from the results of all the testing systems selected by us that SWW samples were relatively more genotoxic than AWW since not only the Mi(p), Mi(f) and aberration frequency for SWW were higher than those for AWW, the MI value of SWW in the Allium cepa test was also found to be significantly smaller than that of AWW.

Plasmid nicking assay also revealed a clear quantitative difference in the genotoxicity of SWW and AWW with the same conclusion as above since the doses of water samples were quite different to bring about the similar damage in the plasmid DNA.

In view of the results presented in this chapter and chemical analysis of the test samples conducted by us (chapter IV) as well as by others in earlier studies (Freire et al. 2003; Fatima and Ahmad, 2005), it can be concluded that the genotoxicity of the test water samples may be largely attributed to phenolics in SWW and heavy metals in AWW. However, this is only a simplistic representation of the complex system.

It may also be emphasized that the results presented in this chapter actually reflect the genotoxicity of the dissolved portion of the genotoxicants only and do not include the toxic effects of the compounds forming particulate material or those adsorbed on to the suspended particles. The cumulative effect of dissolved and adsorbed portions would undoubtedly be much higher.
Table 1: Reversion of Ames tester strains induced by Saharanpur wastewater (SWW) sample

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9 Fraction</th>
<th>Control</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>15.0</th>
<th>20.0</th>
<th>m*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>78 ±15</td>
<td>104 ±15</td>
<td>154 ±22</td>
<td>245 ±32</td>
<td>314 ±36</td>
<td>216 ±19</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>89 ±14</td>
<td>97 ±18</td>
<td>133 ±17</td>
<td>196 ±28</td>
<td>257 ±28</td>
<td>163 ±16</td>
<td>12.2</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>47 ±8</td>
<td>241 ±27</td>
<td>432 ±35</td>
<td>603 ±36</td>
<td>841 ±54</td>
<td>618 ±35</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>55 ±8</td>
<td>187 ±22</td>
<td>274 ±33</td>
<td>413 ±42</td>
<td>588 ±47</td>
<td>397 ±28</td>
<td>34.0</td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>183 ±19</td>
<td>462 ±32</td>
<td>712 ±27</td>
<td>1143 ±33</td>
<td>1514 ±54</td>
<td>1197 ±42</td>
<td>86.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>185 ±25</td>
<td>347 ±38</td>
<td>554 ±39</td>
<td>723 ±64</td>
<td>1025 ±81</td>
<td>791 ±56</td>
<td>53.9</td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>254 ±25</td>
<td>363 ±29</td>
<td>457 ±43</td>
<td>605 ±52</td>
<td>265 ±26</td>
<td>243 ±28</td>
<td>34.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>263 ±19</td>
<td>302 ±22</td>
<td>353 ±35</td>
<td>448 ±42</td>
<td>363 ±23</td>
<td>302 ±18</td>
<td>18.7</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>266 ±22</td>
<td>292 ±29</td>
<td>333 ±37</td>
<td>421 ±40</td>
<td>536 ±42</td>
<td>414 ±38</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>272 ±18</td>
<td>287 ±25</td>
<td>301 ±32</td>
<td>364 ±32</td>
<td>428 ±37</td>
<td>294 ±23</td>
<td>11.0</td>
</tr>
</tbody>
</table>

m* = Slope of the initial linear dose – response curve as determined by linear regression analysis up to the increasing response of strains by increasing concentrations of genotoxicants/sample

+ S9: Aroclor-1254 induced S9 fraction
Table 2: Reversion of Ames tester strains induced by Aligarh wastewater (AWW) sample

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9 Fraction</th>
<th>Control</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>15.0</th>
<th>20.0</th>
<th>m*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>85 ±13</td>
<td>127 ±15</td>
<td>263 ±17</td>
<td>306 ±45</td>
<td>356 ±28</td>
<td>283 ±19</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>94 ±17</td>
<td>154 ±21</td>
<td>301 ±22</td>
<td>349 ±38</td>
<td>512 ±36</td>
<td>337 ±16</td>
<td>26.8</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>43 ±6</td>
<td>64 ±9</td>
<td>167 ±17</td>
<td>331 ±11</td>
<td>380 ±14</td>
<td>297 ±12</td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>51 ±8</td>
<td>73 ±11</td>
<td>202 ±13</td>
<td>394 ±23</td>
<td>492 ±29</td>
<td>343 ±17</td>
<td>31.9</td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>175 ±20</td>
<td>205 ±19</td>
<td>265 ±17</td>
<td>343 ±37</td>
<td>247 ±42</td>
<td>211 ±39</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>181 ±23</td>
<td>234 ±16</td>
<td>349 ±23</td>
<td>505 ±44</td>
<td>313 ±34</td>
<td>258 ±35</td>
<td>33.4</td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>247 ±19</td>
<td>286 ±29</td>
<td>343 ±43</td>
<td>305 ±19</td>
<td>265 ±16</td>
<td>243 ±17</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>262 ±21</td>
<td>359 ±38</td>
<td>476 ±38</td>
<td>351 ±27</td>
<td>289 ±21</td>
<td>271 ±24</td>
<td>42.8</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>254 ±17</td>
<td>291 ±17</td>
<td>323 ±22</td>
<td>371 ±24</td>
<td>449 ±48</td>
<td>363 ±13</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>268 ±19</td>
<td>345 ±24</td>
<td>402 ±27</td>
<td>489 ±27</td>
<td>613 ±48</td>
<td>443 ±28</td>
<td>22.0</td>
</tr>
</tbody>
</table>

m* = Slope of the initial linear dose – response curve as determined by linear regression analysis up to the increasing response of strains by increasing concentrations of genotoxicants/sample

+ S9: Aroclor-1254 induced S9 fraction
Table 3: Ames fluctuation test conducted on Saharanpur wastewater sample

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of sample (μl/ml)</th>
<th>Chi Square*</th>
<th>Mi(f)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>TA97a</td>
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</tr>
<tr>
<td>2.0</td>
<td>5.5</td>
<td>4.5</td>
<td>0.62</td>
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<td>7.3</td>
<td>6.1</td>
<td>0.80</td>
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<td>9.3</td>
<td>7.9</td>
<td>1.30</td>
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<tr>
<td>15.0</td>
<td>11.5</td>
<td>9.9</td>
<td>1.30</td>
</tr>
<tr>
<td>20.0</td>
<td>11.5</td>
<td>9.9</td>
<td>1.30</td>
</tr>
<tr>
<td>TA98</td>
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<td></td>
<td></td>
</tr>
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<td>2.0</td>
<td>5.5</td>
<td>3.7</td>
<td>0.50</td>
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<td>11.6</td>
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<td>1.1</td>
</tr>
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<td>10.0</td>
<td>17.3</td>
<td>9.3</td>
<td>1.8</td>
</tr>
<tr>
<td>15.0</td>
<td>23.6</td>
<td>12.6</td>
<td>2.4</td>
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<tr>
<td>20.0</td>
<td>23.6</td>
<td>14.3</td>
<td>2.4</td>
</tr>
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<td>TA100</td>
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<td>3.9</td>
<td>0.45</td>
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<td>0.71</td>
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<td>10.1</td>
<td>1.59</td>
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<td>20.9</td>
<td>12.4</td>
<td>2.26</td>
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<td>20.0</td>
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<td>8.7</td>
<td>1.0</td>
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<td>10.0</td>
<td>12.0</td>
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<td>1.52</td>
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<td>15.0</td>
<td>16.0</td>
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<td>2.2</td>
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<tr>
<td>20.0</td>
<td>16.0</td>
<td>14.9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Induction factor
* p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 4: Ames fluctuation test conducted on Aligarh wastewater sample

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of sample (μl/ml)</th>
<th>Chi Square*</th>
<th>Mi(f)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-S9</td>
<td>+S9</td>
</tr>
<tr>
<td>TA97a</td>
<td>2.0</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.0</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4.8</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>5.5</td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>5.5</td>
<td>8.3</td>
</tr>
<tr>
<td>TA98</td>
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<td>3.6</td>
<td>2.8</td>
</tr>
<tr>
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</tr>
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<td>10.5</td>
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<td>3.7</td>
</tr>
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<td>12.6</td>
</tr>
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<td>20.0</td>
<td>9.6</td>
<td>15.4</td>
</tr>
<tr>
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<td>3.4</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>8.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

**: Induction factor
*: p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 5: Ames fluctuation test conducted on Saharanpur wastewater (SWW) samples employing TA98 strain in the presence of different concentrations of trichloroethylene

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration of sample (μl/ml)</th>
<th>Chi Square*</th>
<th>Mi(f)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>SWW sample alone</td>
<td>2.0</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
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</tr>
<tr>
<td></td>
<td>10.0</td>
<td>15.2</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>18.2</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>18.2</td>
<td>15.6</td>
</tr>
<tr>
<td>SWW sample + 50 ppm TCE</td>
<td>2.0</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>8.0</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>13.6</td>
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<td>15.0</td>
<td>18.9</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>18.9</td>
<td>16.1</td>
</tr>
<tr>
<td>SWW sample + 100 ppm TCE</td>
<td>2.0</td>
<td>5.3</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>11.3</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>17.0</td>
<td>9.3</td>
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<td>15.0</td>
<td>20.2</td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.2</td>
<td>17.5</td>
</tr>
</tbody>
</table>

**: Induction factor
*: p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 6: Ames fluctuation test conducted on Saharanpur wastewater (SWW) samples employing TA100 strain in the presence of different concentrations of trichloroethylene

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration of sample (μl/ml)</th>
<th>Chi Square*</th>
<th>Mi(f)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-S9</td>
<td>+S9</td>
</tr>
<tr>
<td>SWW sample alone</td>
<td>2.0</td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.2</td>
<td>14.9</td>
</tr>
<tr>
<td>SWW sample + 50 ppm TCE</td>
<td>2.0</td>
<td>4.5</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>20.0</td>
<td>19.4</td>
<td>16.7</td>
</tr>
<tr>
<td>SWW sample + 100 ppm TCE</td>
<td>2.0</td>
<td>5.1</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>9.7</td>
<td>7.3</td>
</tr>
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<td></td>
<td>10.0</td>
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<tr>
<td></td>
<td>20.0</td>
<td>21.7</td>
<td>19.5</td>
</tr>
</tbody>
</table>

**: Induction factor
*: p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 7: Ames fluctuation test conducted on Aligarh wastewater (AWW) samples employing TA98 strain in the presence of different concentrations of trichloroethylene

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration of sample (μl/ml)</th>
<th>Chi Square*</th>
<th>Mi(f)**</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
<td>+S9</td>
</tr>
<tr>
<td>AWW sample alone</td>
<td>2.0</td>
<td>3.6</td>
<td>4.6</td>
<td>0.36</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.7</td>
<td>6.0</td>
<td>0.52</td>
<td>0.59</td>
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<td>10.0</td>
<td>7.3</td>
<td>9.3</td>
<td>0.67</td>
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<td>12.0</td>
<td>0.90</td>
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<td>3.6</td>
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<td>0.37</td>
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<td>TCE</td>
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<td>7.4</td>
<td>0.55</td>
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</tr>
<tr>
<td></td>
<td>15.0</td>
<td>10.0</td>
<td>13.4</td>
<td>0.81</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>10.0</td>
<td>13.4</td>
<td>0.94</td>
<td>1.42</td>
</tr>
<tr>
<td>AWW sample + 100 ppm</td>
<td>2.0</td>
<td>3.9</td>
<td>3.9</td>
<td>0.36</td>
<td>0.39</td>
</tr>
<tr>
<td>TCE</td>
<td>5.0</td>
<td>6.0</td>
<td>7.1</td>
<td>0.55</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>7.7</td>
<td>11.0</td>
<td>0.71</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>10.4</td>
<td>15.6</td>
<td>0.98</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>14.2</td>
<td>15.6</td>
<td>1.36</td>
<td>1.75</td>
</tr>
</tbody>
</table>

**: Induction factor
*: p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 8: Ames fluctuation test conducted on Aligarh wastewater (AWW) samples employing TA100 strain in the presence of different concentrations of trichloroethylene

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration of water sample (μl/ml)</th>
<th>Chi Square*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td></td>
</tr>
<tr>
<td>AWW sample alone</td>
<td>2.0</td>
<td>3.9</td>
<td>3.9</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.0</td>
<td>6.8</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>7.8</td>
<td>9.1</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>9.7</td>
<td>12.8</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>11.5</td>
<td>16.1</td>
<td>1.17</td>
</tr>
<tr>
<td>AWW sample + 50 ppm TCE</td>
<td>2.0</td>
<td>3.6</td>
<td>3.6</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.7</td>
<td>7.5</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>8.9</td>
<td>11.8</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>9.3</td>
<td>16.1</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>11.0</td>
<td>16.1</td>
<td>1.11</td>
</tr>
<tr>
<td>AWW sample + 100 ppm TCE</td>
<td>2.0</td>
<td>3.4</td>
<td>4.2</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
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<td>6.4</td>
<td>8.6</td>
<td>0.67</td>
</tr>
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<td></td>
<td>10.0</td>
<td>10.8</td>
<td>13.4</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>13.3</td>
<td>18.8</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>13.3</td>
<td>18.8</td>
<td>1.47</td>
</tr>
</tbody>
</table>

**: Induction factor
*: p < 0.05 for $\chi^2 > 3.84$, p < 0.01 for $\chi^2 > 6.63$

+S9: Aroclor-1254 induced S9 fraction
Table 9: *Allium cepa* genotoxicity test conducted on SWW and AWW samples alone and in the presence of different concentrations of trichloroethylene

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mitotic Index (MI)</th>
<th>No. of chromosomal aberrations in the observed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stickiness</td>
</tr>
<tr>
<td>Negative Control *</td>
<td>53 ± 6.2</td>
<td>-</td>
</tr>
<tr>
<td>Positive control**</td>
<td>45.7 ± 9.1</td>
<td>8</td>
</tr>
<tr>
<td>SWW</td>
<td>28 ± 7.2</td>
<td>4</td>
</tr>
<tr>
<td>SWW + 50 ppm TCE</td>
<td>27 ± 6.4</td>
<td>3</td>
</tr>
<tr>
<td>SWW + 100 ppm TCE</td>
<td>25 ± 5.5</td>
<td>4</td>
</tr>
<tr>
<td>AWW</td>
<td>39 ± 3.1</td>
<td>5</td>
</tr>
<tr>
<td>AWW + 50 ppm TCE</td>
<td>37 ± 3.8</td>
<td>5</td>
</tr>
<tr>
<td>AWW + 100 ppm TCE</td>
<td>34 ± 3.6</td>
<td>7</td>
</tr>
</tbody>
</table>

*: Mineral water

**: MMS (10mg/l)
Fig 1: Typical patterns of the different ROS generated by increasing concentrations of the test water samples: (a) superoxide radical (b) hydrogen peroxide (c) hydroxyl radical
Fig 2: Plasmid nicking assay conducted on the test water samples

A) Lane a: pBR322 DNA alone  
    Lane b: pBR322 DNA + M.M.S  
    Lane c-e: pBR322 DNA + 0.24X, 0.48X and 0.72X SWW sample respectively

B) Lane a: pBR322 DNA alone  
    Lane b: pBR322 DNA + M.M.S  
    Lane c-e: pBR322 DNA + 0.24X, 0.48X and 0.72X AWW sample respectively
Chapter VIII:

General Discussion
Trichloroethylene is a common industrial solvent and contaminant of hazardous waste sites, groundwater, and drinking water (Candura and Faustman, 1991). Because of the wide spread use of large quantities of trichloroethylene, it has become a common environmental contaminant. The discharge of TCE by industries and its leakage from industrial setting into the general environment, particularly into ground water are the main concern for human population. Its indiscriminate use as metal degreasing agent in electroplating industries especially calls for assessing its hazards as an essential companion of heavy metals in the wastewaters. Moreover, risk assessment studies should also take into consideration of TCE exposure in the alcoholic people.

The ability of various pollutants to mutually affect their toxic actions complicates the risk assessment based solely on environmental levels (Calabrese, 1991). This has initiated the researches to establish early-warning signals, or biomarkers, reflecting the adverse biological responses towards anthropogenic environmental pollutants (Bucheli and Fent, 1995). In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed among tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990). By screening multiple biomarker responses, important information can be obtained about organisms toxicant exposure and stress. A pollutant stress situation normally triggers a cascade of biological responses, each of which may, theoretically, serve as a biomarker (McCarthy et al. 1991).

Comparing with chemical residue analysis, biomarkers have the advantage of being more relevant biologically (Rees, 1993). Studies on the identification of biomarkers of TCE have been carried out extensively in animal system (Goel et al. 1992; Vidal et al. 2001). Though not performed on TCE, earlier biomarker studies have also been conducted in many plants exposed to various environmental toxicants (Ferrat et al. 2003; Vitoria et al. 2001).

In the present study, an attempt has been made to identify some simple and sensitive toxicity biomarkers for TCE pollution in plant and animal systems especially in view of the typical conditions of its exposure via oral route. Hence, wastewaters collected from two sampling sites viz. Saharanpur and Aligarh were used for biomarker studies in animal system to compare the toxicity biomarkers of TCE with those of wastewaters containing TCE. The mutagenic potentials of the TCE laden...
SWW and AWW samples were also estimated by different genotoxicity assays in the absence and presence of additional concentrations of TCE.

Fig 1 depicts the interplay of various biomarkers that have been identified in this study. For biomarker studies in animal system, rats were used and after acclimatization for a week, different toxicants as well as wastewaters were fed orally.

Plant based biomarker studies were conducted on *Allium cepa* (onion) bulbs. *Allium cepa* toxicity bioassay has been widely recommended for the routine monitoring of water pollution since it is a quite sensitive and valid indicator of toxicity (Fiskesjo, 1985; Rank and Nielsen, 1997). Moreover, it can also be extended for the evaluation of genotoxicity of toxicants.

A single genotoxicity testing system does not necessarily reflect the actual behaviour of the test sample. Therefore, a battery of tests is recommended for the toxicity evaluation and risk assessment studies (Malik and Ahmad, 1995; Rehana et al. 1995; Fatima and Ahmad, 2006). The bioassays used in the present study included Ames plate incorporation tests, Ames fluctuation test and *Allium cepa* genotoxicity test. In support of these tests involving the living organisms, *in vitro* DNA damage caused by the test water samples was also estimated by the plasmid nicking assay.

The invariable increase in antioxidant enzymes like GST, GR, SOD, and the oxidative stress marker, LPO was recorded following TCE and heavy metals ingestion by rats in liver and kidney tissues especially in combined treatment animal groups (Fig 1, 3, 4 and 7; chapter III). Many researchers have reported the utility of these enzymes as markers of TCE and heavy metal exposure in animal system (Stajn et al. 1997; Watanabe and Fukui, 2000; Zhu et al. 2005; Jadhav et al. 2007a, b).

As far as the biomarkers for TCE laden wastewaters intake in rats were concerned, GR and SOD proved to be suitable toxicity biomarkers in liver tissue. Both the antioxidant enzymes exhibited a remarkable induction in hepatic tissues of the rats as a result of oral administration of wastewaters (Fig 1a and 3a; chapter IV). Comparing the two treatment systems in chapter III and chapter IV (model water vs raw water), it is clear that SOD, GR and GST followed similar pattern. However, raw sample fed animals exhibited relatively higher induction in enzymatic activities than in the model water which can be explained by the presence of higher levels of organics and heavy metals in the test raw water samples compared with model water (Table 1 and 3; chapter IV).
A remarkable increase in the activities of antioxidant/detoxification enzymes like GST, GR was obtained in the TCE exposed *Allium cepa* bulbs (Fig 3 and 4; chapter V). Moreover, this rise in the activities of antioxidant enzymes was found to be at the level of synthesis in *Allium cepa* system. Induction of various antioxidant enzymes at higher doses of TCE administration was reported by several investigators in rats and mice (Goel et al. 1992; Watanabe and Fukui, 2000). However, a remarkable induction in GR and GST levels of *A. cepa* (Fig 3 and 4; chapter V) upon exposure to only 200 ppm TCE compared with such a rise in GR level in mice fed with 1000 ppm of TCE (Watanabe and Fukui, 2000) clearly suggests that *Allium cepa* system is a better candidate to monitor the changes in GR as a result of TCE exposure.

As far as isozymes of CYP450 system were concerned, a remarkable increase in enzymatic activities of PROD and NDMA-d to the extent of 22 and 11 folds respectively was recorded upon quite a low concentration (20 ppm) of TCE exposure in *Allium cepa* system (Fig 4b,c; chapter VI). Moreover, the increase in these CYP450 enzymatic activities of *A. cepa* was found to be at the synthesis level. We, therefore, recommend the inclusion of PROD and NDMA-d also among the toxicity biomarkers of TCE in the plant system. Animal system, on the other hand, exhibited quite a different result, where NDMA-d displayed only around 3.5 fold induction in the liver of TCE administered rats. Simultaneous exposure of trichloroethylene and ethyl alcohol to rats showed an inhibition in the enzymatic activities of the test CYP450 isozymes where both TCE and ethyl alcohol seem to compete for the same enzyme. However, combined treatment to animals with TCE, heavy metals and ethyl alcohol resulted in an induction of the test isozymes. Most probably the heavy metals might be antagonising the repression caused by TCE and ethyl alcohol co-exposure. It is quite strange that one toxicant is antagonising other evidently by an unknown mechanism as yet. Moreover, our studies also indicate that TCE might be using two different pathways for its metabolism in animal and plant system i.e. CYP2E1 mediated in rat (Fig 3a,b; chapter VI) and involving CYP2B1 in *A. cepa* (Fig 4b; chapter VI).

Comparison between the toxicity biomarker responses against the TCE exposure in animal and plant systems shows that every antioxidant enzymes studied by us exhibited similar pattern except for CAT which displayed opposing trend. Opposing responses of CAT in the plant and animal systems suggest the existence of
compensatory mechanisms were operating in response to increased oxidative stress by TCE in rats where GPx might be doing major job of H2O2 detoxification process. It is well known that GPx has higher affinity for H2O2 than CAT and thus it is quite effective in decomposing H2O2 (Halliwell, 1974). Our results clearly indicate the better susceptibility of Allium cepa system compared with the animal system because the former exhibited a remarkable change in enzymatic activities and the response was also within the range of environmental concentrations of TCE. Therefore, we recommend Allium cepa system for the biomarker based detection of TCE pollution due to its high sensitivity towards TCE exposure.

Cytochrome P450 system is involved in the oxidative metabolism of a wide variety of drugs and xenobiotics/toxicants (Lewis, 1996; Nelson et al. 1996). Similarly, antioxidant enzymes also operate on the oxidative stress caused by the xenobiotics/toxicants (Farombi, 2007). Present study clearly revealed the role of cytochrome P450 isozymes in the metabolism of the test water pollutants. This enzymatic complex showed relatively higher induction compared to that of antioxidant enzymes. However, for risk assessment studies, it seems more appropriate to use both biomarker systems i.e. susceptible cytochrome P450 and antioxidant enzymes.

As far as the genotoxicity of Sharanpur and Aligarh water samples is concerned, both the test water samples were found to be highly genotoxic in both the Ames Salmonella tests (Tables 1-4; chapter VII). TA98 and TA100 were the most sensitive strains in both the testing systems. In fact, TA98 detects those mutagens which cause frame shift mutation while the strain TA100 is acted upon by the mutagens that lead to base pair substitution at G-C site. Earlier studies in our laboratory have established the utility of these strains in detecting the genotoxic impact of industrial wastewater (Malik and Ahmad, 1995; Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006).

Surprisingly, previous investigations undertaken in our lab have shown the strains TA102 and TA104 to be the best responders in the genotoxic evaluation of industrial wastewaters of the region under study especially by Ames plate incorporation method (Malik and Ahmad, 1995; Siddiqui and Ahmad, 2003; Fatima and Ahmad, 2006). However, present investigation showed a slightly different sensitivity pattern compared with earlier findings obtained within the same region,
suggesting for the corresponding change in the nature of the toxicants during the passage of time.

The mutagenicity of the two test water samples under study, by the Ames plate incorporation assay as well as by the Ames fluctuation test, showed the opposite effect upon metabolic activation (Table 1-4; chapter VII). AWW exhibited an invariable increase in mutagenicity in the presence of S9 fraction, clearly suggesting for the presence of some indirectly acting mutagens which would presumably be metabolized in the liver to become more potent mutagens and/or carcinogen. On the other hand, SWW displayed a drop in mutagenic potential upon metabolic activation. Conversion of active mutagenic substances present in SWW into less genotoxic compounds by S9 fraction might be the main cause of this lowering effect. Similar results were also reported by several authors in genotoxicity assessment of pulp mill effluents (Nestmann et al. 1980; Kamra et al. 1983; Perez-Alzola and Santos, 1997). It is also noteworthy that SWW supplemented with 100 ppm of TCE resulted in a rise of around 37% in Mi value with TA100 strain both in the presence and absence of S9 fraction. This clearly suggests the proactive role of TCE under certain experimental conditions like those in SWW, regardless of its own metabolic state.

As far as the genotoxicity of TCE alone was concerned, it was found to be non-mutagenic by all the selected genotoxicity testing systems employed in the present work including *A. cepa* genotoxicity test (Data not shown). Several workers also reported non-genotoxic nature of TCE (Simmon et al. 1977; Crebelli et al. 1982; Fahrig et al. 1995). The non-genotoxic effect of TCE might be due to its high reactivity and low stability character which could hinder the mutagenicity where plenty of nucleophilic targets other than DNA are available for chemical interaction (Crebelli et al. 1982). But in the natural milieu, it was found to enhance genotoxicity especially at an additional exposure of 100 ppm TCE with test water samples as observed in Ames testing with TA100 strain (Table 6 and 8; chapter VII). In view of these findings we can further say without contradicting our previous opinion that TCE might also serve as a promutagen under certain environmental conditions such as defined by AWW, and obviously in the presence of S9 fraction.

The matter of generation of ROS in the test water samples was also of interest. AWW led to OH radical production (Fig 1c; chapter VII) while SWW produced O$_2^-$ radical predominantly (Fig 1a; chapter VII). Continuous generation of high level of ROS in test water samples (Fig 1a,b,c; chapter VII) would be the most plausible
reason behind the observed oxidative stress in terms of increased MDA levels (LPO) in the wastewater fed rats (Fig 7a,b; chapter IV). The mutagenicity involving reactive oxygen species and lipid peroxidation was also reported by several investigators (Bohr et al. 1999; Bunout and Cambiazo, 1999; Kang et al. 1999; Valko et al. 2006).

*Allium cepa* genotoxicity test was found to be highly capable of differentiating the two samples obtained from different cities. It is eukaryotic system and thus obviously more close to the humans and other higher organisms in comparison with bacteria. Moreover, it has its own detoxification machinery also like humans. Furthermore, this test directly targets mitotic chromosomes and evaluates chromosomal aberration. Interestingly, *Allium cepa* genotoxicity test displayed an increase in total chromosomal aberration by SWW in contrast to the Ames test result where a decrease in mutagenic potential was observed. *A. cepa* system has its own detoxification machinery (S9), hence this response might be due to the difference in prokaryotic and eukaryotic systems. The basic function of detoxification machinery is to reduce the harmful effect of xenobiotics on the living system. This was actually the case with Saharanpur sample where decrease in genotoxicity was observed in the presence of S9 fraction.

Aforementioned enzymatic system in *Allium cepa* is thus proposed as valuable biomarkers in TCE pollution monitoring and is likely to be most effective when used to complement well established chemical and biological monitoring techniques. Non-specific biomarkers are recommended in routine pollution monitoring as these are easier to measure (Cheung et al. 2004). Most biomarkers respond to a range of contaminants and various environmental factors. However, in some cases single specific biomarker is used when the presence of a particular pollutant is suspected (Lam and Gray, 2003). It is, therefore, recommended to use cost effective and rapid non specific biomarkers in the first stage of monitoring. The samples which show positive results can be further analyzed using specific biomarkers or costly analytical techniques. Another reason to use a combination of biomarkers in the initial stages of monitoring is that living organism will simultaneously be exposed to a range of contaminants acting antagonistically or synergistically. Specific biomarkers may not detect the effects of certain chemicals whereas non specific biomarkers are expected to respond to the complex mixture of chemicals present in the environment (Nicholson and Lam, 2005).
Fig 1: The role and interplay of various biomarkers used in this study
1 Mild increase with respect to control
2 Moderate increase
3 Remarkable increase
4 Decrease
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LIST OF PUBLICATIONS AND PRESENTATIONS


5. **Shams Tabrez, Masood Ahmad** (2009) Antioxidant system in *Allium cepa* as the toxicity biomarkers of trichloroethylene (TCE). (Communicated)

6. **Shams Tabrez, Masood Ahmad** (2010) Some isozymes of Cytochrome P450 system as potential biomarkers of certain toxicants: Comparison between plant and animal models. (Communicated)

7. **Shams Tabrez, Masood Ahmad** (2010) Mutagenicity of industrial wastewaters collected from two different stations in northern India. (Communicated)

8. **Shams Tabrez, Masood Ahmad** (2008) Some enzymatic/non enzymatic antioxidants as potential stress biomarkers of trichloroethylene, heavy metal mixture and ethyl alcohol in rat tissues. Proceedings of National symposium on Advances in Clinical Biochemistry - Biomarkers, Molecular Diagnosis and Quality Assurances & 1st UP Chapter of Association of Clinical Biochemists of India, November 15-16, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India.

9. **Shams Tabrez, Masood Ahmad** (2008) Some isozymes of Cytochrome P450 enzymes as a potential biomarker of trichloroethylene (TCE) pollution in *Allium cepa*. National symposium on Recent Advances in Biochemistry and Allied Sciences, UGC-DRS II Programme, March 25, Department of Biochemistry, Aligarh Muslim University, Aligarh, India.
10. **Shams Tabrez, Masood Ahmad** (2009) Effect of waste water intake on antioxidant enzymes and marker enzymes of tissue damage in rat tissues: Implications for the use of biochemical markers. International SFRR Satellite Symposium on Free Radicals in Health and Disease, March 17-18, Department of Biochemistry, Aligarh Muslim University, Aligarh, India.

11. **Shams Tabrez, Masood Ahmad** (2009) Antioxidant system in *Allium cepa* as the toxicity biomarkers of trichloroethylene (TCE). International SFRR Satellite Symposium on Free Radicals in Health and Disease, March 17-18, Department of Biochemistry, Aligarh Muslim University, Aligarh, India.
Toxicity, Biomarkers, Genotoxicity, and Carcinogenicity of Trichloroethylene and Its Metabolites: A Review

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Toxicity, Biomarkers, Genotoxicity, and Carcinogenicity of Trichloroethylene and Its Metabolites: A Review

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Trichloroethylene (TCE) is a prevalent occupational and environmental contaminant that has been reported to cause a variety of toxic effects. This article reviews toxicity, mutagenicity, and carcinogenicity caused by the exposure of TCE and its metabolites in the living system as well as on their (TCE and its metabolites) toxicity biomarkers.

Key Words: Trichloroethylene; biomarker; toxicity; cancer; genotoxicity

INTRODUCTION

Trichloroethylene (TCE), a chlorinated solvent, has been produced commercially since the 1920s in many countries by chlorination of ethylene or acetylene. Its use in vapor degreasing began in 1920s. In the 1930s, it was introduced for use in dry cleaning, but it has had limited use in that way since the 1950s. Currently, 80–90% of TCE worldwide is used for degreasing metals. Use for all applications in Western Europe, Japan, and the United States in 1990 was about 225,000 tonnes. Thus, the primary sources of releasing TCE into the environment are metal cleaning and degreasing operations (1). TCE has been detected in air, water, soil, food, and animal tissues. The most heavily exposed people are those working in the degreasing of metals, mainly through inhalation of vapor.

TCE was first detected in groundwater in 1977 and has been one of the most frequently detected contaminants in groundwater in the United States.
Some Enzymatic/Nonenzymatic Antioxidants as Potential Stress Biomarkers of Trichloroethylene, Heavy Metal Mixture, and Ethyl Alcohol in Rat Tissues

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ABSTRACT: Enzymatic and nonenzymatic antioxidants serve as an important biological defense against environmental pollutants. Various enzymatic and nonenzymatic antioxidants as a stress biomarker in liver and kidney of rat were investigated. The antioxidant enzymes that were analyzed included superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase. Levels of lipid peroxidation (LPO), reduced glutathione (GSH), as well as hydrogen peroxide ($H_2O_2$) were also measured in homogenates of the liver and kidney of the treated animals to determine oxidative stress induced by trichloroethylene (TCE), ethyl alcohol, and heavy metal mixture (H.M.M) individually and in different combinations. An increase up to the extent of 382% in malonaldehyde, a marker of LPO, was recorded in almost all the treatment groups in both the tissues. Similarly, a rise of 218% in GST activity was also recorded in kidney of TCE-treated animals. Although H.M.M ingestion resulted in significant change of 125% in SOD activity of hepatic tissue, the level of GR was increased by 93% in the renal tissue of the exposed rats. Solitary dose of alcohol in general did not show a significant change. Moreover, the changes in the levels of antioxidants were much more prominent when these toxicants were given in combination rather than alone. Overall, these results demonstrate the changes in the levels of antioxidant enzymes and GSH system, as well as alterations in the LPO and $H_2O_2$ levels as a result of test toxicants.

INTRODUCTION

Increasing rate of urbanization coupled with industrialization has resulted in greater amount of wastewater discharge as well as higher level of exposure to xenobiotics. Human and animal exposure to environmental chemicals is rarely limited to a single compound. Rather, they are exposed concurrently or sequentially to multiple chemicals from a variety of sources (Jadhav et al., 2007a). It is a well known fact that toxic effects of a xenobiotic can be modified by the presence of other substances (Brus et al., 1999; Gupta and Gill, 2000). As simultaneous exposure to two or more xenobiotics can take place in the environment and/or under occupational conditions, the investigation of interactions between toxic substances is an important problem in modern toxicology (Jurczuk et al., 2004).

Trichloroethylene (TCE) is a common metal degreasing agent and has been used indiscriminately in the house-based lock manufacturing and electroplating units. Like many other chlorinated hydrocarbons, TCE has become an
Effect of wastewater intake on antioxidant and marker enzymes of tissue damage in rat tissues: Implications for the use of biochemical markers

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ABSTRACT

In the present study, alteration in antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) and marker enzymes of tissue damage alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) with laboratory exposure to wastewaters from Aligarh (AWW) and Saharanpur (SWW) were investigated in rat liver and kidney. Levels of malondialdehyde (MDA), reduced glutathione (GSH) and hydrogen peroxide (H₂O₂) were also determined.

A profound enhancement of 5 and 2.5-folds in MDA level was recorded in the liver and kidney respectively as a result of oral administration of SWW to the rats. Exposure to both AWW and SWW resulted in 3-4-fold increase in CR activity and 3-fold increase in SOD and ALT activity in the hepatic tissue compared to control values. Ingestion of AWW and SWW resulted in 3.5-fold rise in renal AST levels whereas AWW caused 75% decline in GST activity in kidney of treated rats.

Results indicate that wastewater (AWW/SWW) caused severe damage to renal and hepatic tissues and the effect seems in part to be mediated by suppression of antioxidant system with CR and SOD as potential candidates for hepatic toxicity biomarkers of wastewaters.

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1. Introduction

Water pollution is one of the most critical environmental issues these days. Use of industrial effluent and sewage sludge on agricultural land has become a common practice in India as a result of which these effluents have hazardous effects on human’s health. Wastewater may also percolate into the ground water through the unregulated waste discharge. It enters the aquatic environment and is taken up into the tissues of living organisms (Walsh and O’Halloran, 1998; Stecko and Bendell-Young, 2000). The rate of urbanization has also undergone a rapid increase, including an increase in wastewater discharge, so that exposure of humans to water pollutants is rarely limited to a single chemical or organic residue (Heindel et al., 1995).

The two study sites chosen for this sampling have different types of industries. Aligarh city is famous for lock manufacturing and electroplating industries. The effluents of these industries contain quite a large amount of metals like Cd, Cu, Cr and Zn. Aligarh also houses the lead battery and thermometer manufacturing industries which accounts for the presence of high levels of Pb and Hg in the water sample.

Saharanpur is famous for paper and pulp industries. Paper mill effluent, which is a complex mixture of heavy metals, phenols, dioxins, furans, lignocellulose components, guaichols, catechols, polyaromatic hydrocarbons, fatty acids and resin acid is reported to induce oxidative stress (Hamm et al., 1986; Suntio et al., 1988; Mather-Mihalic and DiGiulio, 1991; Fatima et al., 2000). The role of heavy metals, the common constituents of paper mill effluent in oxidative stress, is well-documented (Hamm et al., 1986). Enzymatic and non-enzymatic antioxidants serve as an important biological defense against environmental oxidative stress (Ahmad et al., 2000). Both enzymatic and non-enzymatic processes counter the impact of ROS (DiGiulio et al., 1989; Lopez-Torres et al., 1993; Filho, 1996). Induction of antioxidant enzymes represents a cellular defense mechanism to counteract toxicity of ROS and they have extensively used in several field studies. The purpose of this study was to investigate pollution load in wastewater and compare the toxic effect of water contaminants from Aligarh and Saharanpur in laboratory rats.

2. Materials and methods

Wastewater samples were collected from the industrial estates of Aligarh city and Saharanpur city, India, in sterile glass bottles as per the method described by APHA (1998). It was stored at 4 °C in plastic jar cans. Prior to use, the particulate
Summary
SUMMARY

Due to the ever increasing pollution of water bodies by TCE and other xenobiotics, there is a growing demand for robust and cost effective monitoring strategies. In addition to the routine analytical techniques, biomarkers are being increasingly used for the detection of aquatic pollution (Galloway et al. 2002; Ferrat et al. 2003; Nazar et al. 2008). Biomarkers have the advantage that they can predict a change much before it occurs at higher levels of biological organization (Jimenez and Stegeman, 1990; Pretti and Cognetti-Varriale, 2001).

The work presented in this thesis was carried out to study the utility of the different enzymes of rat tissues and Allium cepa as biomarkers of TCE pollution and different toxicants. Most of the toxicity testing systems rely on small mammals such as rats or mice, and hence are time consuming, expensive and attract considerable ethical criticism (Tsuda et al. 2001; Fatima and Ahmad, 2006). For these reasons, plant based toxicity biomarker studies was also under taken to compare the efficacy of both the system. Allium cepa system was selected since the Allium cepa test is a cost effective toxicity bioassay routinely used in water monitoring studies (Fiskesjo, 1985; Rank and Nielsen, 1993). It was thought that the efficacy of this test would increase greatly if enzymatic studies are carried out in the same onion bulbs used in the toxicity bioassay (Fatima and Ahmad, 2005). Moreover, the Allium cepa test can also be used to study the genotoxicity of TCE containing test samples.

Trichloroethylene, heavy metals and phenolics are considered to be the major water pollutants present in northern India especially of the region under study (Malik and Ahmad, 1995; Datta, 1999; Dua, 2003; Fatima and Ahmad, 2005; Chiya Nivaran Samithi, 2009). Test water samples were collected from the industrial areas of Aligarh and Saharanpur cities of northern India. These samples were especially found to contain TCE, heavy metals and phenolics. Keeping in mind the contradictory reports on the genotoxicity of TCE, It was our contention to clear this issue once and for all whether TCE is genotoxic or promotes genotoxicity under certain environmental conditions. For this purpose we have supplemented different concentration of TCE into the test water sample and performed genotoxicity assays employing Ames fluctuation test and Allium cepa genotoxicity test.
The genotoxicity/mutagenicity of the test water samples was estimated using Ames plate incorporation test, Ames fluctuation test, *Allium cepa* test and the plasmid nicking assay. Moreover, the quantitation of various ROS in the test wastes was also carried out.

The significant findings along with their possible explanations are summarized as under:

(I) **Enzymatic and non-enzymatic antioxidants as biomarker of TCE, ethyl alcohol and heavy metals toxicity in rat tissues**

1. MDA levels seemed to be the best biomarker for the test toxicants in both renal and hepatic tissues.
2. SOD can be considered as a prominent biomarker of heavy metals induced hepatotoxicity.
3. GST and GR can serve as potential biomarkers of TCE and heavy metals induced renal toxicity.

(II) **Antioxidants and the tissue marker enzymes as the biomarkers of TCE contaminated wastewaters from Aligarh and Saharanpur in rat tissues**

It is to emphasize here that the water samples were not supplemented with any additional amount of TCE rather it was present as an essential contaminant of the wastewaters.

1. The most striking effect of the test wastewaters intake in rats was also found to be on MDA level.
2. A remarkable rise to the extent of 5 and 2.5 fold in MDA level was recorded in the liver and kidney respectively as a result of oral administration of the test wastewaters to the rats.
3. SOD and GR can act as a potential biomarker of toxicity for the test water samples in the rat liver.
4. Relatively higher levels of ALT in hepatic and AST in renal tissues of treated rats also suggests that intake of the test water samples might have caused injury to liver and kidney tissues.
(III) Antioxidant enzymes of *Allium cepa* as biomarkers for the detection of TCE pollution in water samples

1. GST and GR in *Allium cepa* system can serve as the appropriate biomarkers of TCE contamination in water. Other enzymes like APx and CAT could also serve as sensitive biomarkers of TCE pollution.

2. The increase in the activities of all the test antioxidant enzymes was at the level of synthesis.

3. All the test enzymes displaying variation in the activities consequent upon exposure to TCE are synthesized in the cytosolic compartment of the cell.

These findings suggest that the *Allium cepa* system can be used for monitoring the changes in antioxidant enzymes in case of suspected TCE pollution in water bodies.

(IV) Some CYP450 isozymes of plant and animal origin as the potential biomarker of TCE in natural milieu

1. PROD and NDMA-d can act as potent biomarkers in *A. cepa* system for assessing the TCE pollution and its hazard.

2. NDMA-d can serve as a potential biomarker of TCE and/or alcohol intake in rats both in the liver and kidney tissues.

(V) Genotoxicity testing by Ames plate incorporation test, Ames fluctuation test and *Allium cepa* test as well as plasmid nicking assay for *in vitro* DNA damage conducted on the wastewater sample

1. The test water samples were found to be highly mutagenic by both Salmonella mutagenicity assay namely Ames plate test as well as Ames fluctuation test.

2. Both the Ames tests were able to differentiate the two test samples qualitatively and quantitatively.

3. TA100 was found to be the most sensitive strain for SWW sample by both Ames plate incorporation and Ames fluctuation tests in the presence or absence of S9. However, TA98 strain was found to be the most
responsive strain in the absence of metabolic activation towards AWW while TA102 was recorded to be the most sensitive strain in the presence of S9 by Ames plate test.

4. The test water samples collected from Aligarh exhibited an increase in mutagenicity upon metabolic activation evaluated by both tests. However, Saharanpur waste water invariably displayed a decrease in mutagenicity upon metabolic activation.

5. A slight increase in mutagenicity of the test water samples supplemented with 100 ppm of TCE was observed in TA100 strain by Ames fluctuation test.

6. Both the test samples induced a mitodepressive effect in the root cells of Allium cepa, though the intensity of depression was quite different.

7. Fragmentation of chromosomes was the predominant effect of AWW while SWW led to the bridge formation to a large extent.

8. The plasmid nicking assay was also quite effective in detecting the genotoxicity of the test samples.

9. Addition of 100 ppm of TCE in these test water samples resulted in significant increase in total aberration frequency of chromosomes in A. cepa system. However, supplementation of 50 ppm of TCE in the water samples did not exhibit any significant change in the total chromosomal aberration.

10. As far as the reactive species in the test water samples are concerned, hydroxyl radicals were the prominent species in Aligarh sample and superoxide radicals were the major species in paper mill effluents.

The test water samples were highly genotoxic and could induce mutations in the exposed organisms. The antioxidant/ detoxification enzymes of Allium cepa showed significant induction upon exposure to TCE. Therefore, these enzymes would act as suitable biomarkers of TCE pollution. Based on the various findings obtained in this study, it is quite evident that biomarker studies in Allium cepa are very useful in assessing TCE pollution. They can complement well established analytical techniques like GC, HPLC and at the same time provide valuable information about the effects of toxicants on the living organisms.
The various biomarkers that could be utilized for the effective monitoring of TCE and heavy metal pollution in water samples are:

(i) *Allium cepa* GST and GR can be utilized as the biomarkers of TCE pollution.
(ii) SOD can also be used as a biomarker of heavy metal stress in rats with special reference to liver.
(iii) PROD and NDMA-d activity analysis may be successfully used for the detection of the TCE pollution in *A. cepa* system.
(iv) The generation of different ROS may also be utilized as an indicator of a particular contaminant. As far as the typical Indian surface waters are concerned, hydroxyl radical generation is an indicator of heavy metal pollution and superoxide radical can be a marker of paper mill contaminants.

Investigation of enzymatic biomarkers of genotoxicity was not included in our plan of work because the genotoxicity in quantitative and qualitative terms as examined by the *Allium cepa* test as well as by other genotoxicity tests, was quite efficiently determined and differentiated for the test water samples.

The results from our studies on toxicity and genotoxicity of TCE especially in the natural milieu as well as test wastewater samples demand that necessary action should be taken without delay to prevent unregulated discharge of TCE and other organic and inorganic xenobiotics into water bodies from the industries flourishing in these areas.