Biodegradation of chlorophenols by the bacterial strains from pulp and paper mill effluents and genotoxicity testing of wastewater and soil

THESIS
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IN
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BY
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Samira Umme Aiman
Dated……………………

CERTIFICATE
This is to certify that the research work embodied in this thesis entitled “Biodegradation of chlorophenols by the bacterial strains from pulp and paper mill effluents and genotoxicity testing of wastewater and soil” submitted to Aligarh Muslim University, Aligarh for the degree of Doctor of Philosophy in Agricultural Microbiology has been carried out by Miss Samira Umme Aiman under my supervision. This is an original work and no part of the thesis has been submitted for any other degree. The thesis is suitable to be considered for the award of the degree of Doctor of Philosophy.

Prof. Abdul Malik
(Research Supervisor)
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<td>λ</td>
<td>Lambda</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>2,4-Dichlorophenol</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>2,4,6-Trichlorophenol</td>
</tr>
<tr>
<td>4 AAP</td>
<td>4 Amino 2,3 dimethyl-1-phenyl-3-pyrazolin-5-on</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectrophotometer</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One way analysis of variance</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological Oxygen Demand</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CA</td>
<td>Chromosome aberration</td>
</tr>
<tr>
<td>Cd</td>
<td>Cadmium</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CPs</td>
<td>Chlorophenols</td>
</tr>
<tr>
<td>Cr</td>
<td>Chromium</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMRT</td>
<td>Duncan multiple range test</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mono Nucleotide</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pair</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kg t&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Kilograms per metric tonne</td>
</tr>
<tr>
<td>km</td>
<td>Kilometer</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MI</td>
<td>Mitotic index</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MS</td>
<td>Minimal Salt</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (Reduced)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Ni</td>
<td>Nickel</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NSS</td>
<td>Normal Saline Solution</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Pb</td>
<td>Lead</td>
</tr>
<tr>
<td>PCP</td>
<td>Pentachlorophenol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA</td>
</tr>
<tr>
<td>TDS</td>
<td>Total Dissolved Solids</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/ volume</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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3. Different types of chromosomal aberrations induced by the pulp and paper mill effluent in *Allium cepa* root-tips

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1. Survival of *E. coli* K-12 strains treated with hexane extract of soil (a) site I (b) site II; dichloromethane extract of soil (a’) site I (b’) site II

2. Survival of bacteriophage λ treated with hexane extract of soil (a) site I (b) site II; dichloromethane extract of soil (a’) site I (b’) site II

3. Different types of chromosomal aberrations induced by the soil extracts (contaminated agricultural soil) in *Allium cepa* root-tips
Introduction

Water and its governance has become top global agenda and have attracted increased attention as a policy concern in recent years (Woodhouse and Muller, 2017). With the growing industrialization and urbanization the increased demand of water resources and its misuse has increased the levels of environmental pollution, both from urban and industrial waste (Islam and Tanaka, 2004; Afroz et al., 2014). The water ecosystem is clearly impaired by uncontrolled waste disposal from industrialized sectors and urbanized municipal waste, and presents great concern about the pollution sources of public water supply (Garcia et al., 2017b). In this scenario, the main aggravating factor to increase environmental imbalance is determined by the chemical composition of industrial effluents (Lemos et al., 2008; Rotter et al., 2015).

The pulp and paper industry is one of the most important industries and its future is economically important for many developing nations. Pulp and paper industry is water and energy intensive industry; therefore it has been associated with various environmental issues including high levels of wastewater generation (Soucy et al., 2014; Adhikari and Bhattacharyya, 2015). The wastewater generated from pulp and paper industry contains variety of adsorbable organic halides along with dioxins and furans which have been suspected for their mutagenic and endocrine disrupting property (Kamali and Khodaparast, 2015; Balabanic et al., 2017). Chlorinated phenols are other primary concerned pollutants from the effluent of pulp and paper industries due to its toxic and recalcitrant nature (Haq et al., 2016).

Halogenated aromatic compounds are extensively used in the preparation of herbicides, pesticides and general biocides (Colombo et al., 2015). These are among the most persistent environmental pollutants because of their recalcitrant nature (Manzetti et al., 2014; Cagnetta et al., 2016). Their widespread use has attracted several criticisms for causing damages to the environment and living organisms (Lu et al., 2011; He et al., 2012; Gong et al., 2016). Currently, the ecological risk from chlorinated phenols has received great attention due to their ability to bioaccumulate in aquatic life through food chain along with having toxic, organoleptic and persistence characteristics (Qin et al., 2014a; Zhao et al., 2017). Their treatment, disposal, and general management have become a serious challenge and therefore it is imperative to restrict and assess the fate of chlorophenols into the ecosystem with a
view to protect the environment and preserve the public health communities (Igbinosa et al., 2013).

Kashipur city located at 29°22' N and 78°95' E in Uttarakhand (India) is becoming an emerging industrial centre. The city is well known for the presence of several pulp and paper manufacturing industries which discharge large amount of effluent. There are reports on scarcity of fresh water supply which has lead towards the utilization of wastewater from industrial and municipal sewage sources for crop irrigation (Kaur et al., 2012; Dey et al., 2015).

The physico-chemical analysis of wastewater and soil allows critical assessment of their pollution level. The toxic pollutant present in wastewater or soil may seriously affect the microbial community structure by decreasing their number, reducing their biochemical activities and affecting diversity (Torsvik and Ovreas, 2002). Generally the microbial diversity reduces with increased pollution because the sensitive microorganisms abruptly decline by the effect of pollutants, whereas the tolerant microorganisms become predominant (Cao et al., 2017).

The treatment of wastewater can be accomplished by physico-chemical or biological treatment or combination of both. The biological treatment have proven to be better for being efficient, cost effective and environmental-friendly than conventional processes that are generally associated with high chemical and operational costs as well as complex sludge generation (Nawaz and Ahsan, 2014; Zhao et al., 2015; Toczyłowska-Mamińska, 2017).

Microorganisms have shown to play crucial role in nutrient recycling and converting toxic organic compounds to harmless products. Extensive studies have been carried out exploring microbial diversity, particularly of contaminated areas in search of microbes that could degrade a wide range of pollutants at high pollution load (Gargouri et al., 2014; Coelho et al., 2015). Biodegradation studies have confirmed that chlorophenols can be effectively degraded by bacteria and fungi (Chen et al., 2014; Chen et al., 2015). During the last decade, there has been a growing interest in the development of more efficient strategies for treatment of wastewater with chlorinated compounds (Diaz et al., 2016; Garbou et al., 2017). It has been found that various microbial species isolated from nature, such as Pseudomonas sp., Sphingomonas sp., Alcaligenes sp., Rhodococcus sp., Ralstonia sp., Arthrobacter sp., and Herbaspirillum sp., utilize chlorophenols as substrates (Farrell and Quilty, 1999; Olaniran and Igbinosa, 2011; Arora and Bae, 2014). Biological treatment of
chlorophenols by the bacteria has shown great promise for bioremediation (Li and Loh, 2007; Arora and Bae, 2014; Hassan et al., 2016). Moreover chlorophenol degrading bacterial strains have shown to contain cluster of genes responsible for encoding enzymes that can catalyze the degradation of chlorophenols (Nordin et al., 2005; Arora and Bae, 2014). These genes can be cloned and the corresponding enzymes can be overexpressed and purified which could be used for mass production of enzymes in vitro and can be optimized for facilitating biodegradation of organic contaminants by catalyzing the initial biodegradation steps (Kang et al., 2017).

In view of the environmental hazards associated with pulp and paper industry wastewater, the present work was conducted to analyse the physico-chemical and microbial status of pulp and paper industry effluent and contaminated soil. For cost effective biological removal of toxic chlorophenols, indigenous bacteria were isolated and screened for their chlorophenol degrading ability. Furthermore genotoxicity of wastewater and contaminated soil was carried out to predict the putative hazards of industrial effluent. The genotoxicity tests were carried out using set of short term assays beside namely Ames Salmonella/mammalian microsome test, Escherichia coli DNA repair defective mutation assay, bacteriophage λ system and Allium cepa chromosomal aberration assay, which are helpful in evaluating ecological risk assessment of contaminated soil and wastewater, as these tests involve different genetic end points which provide more comprehensive information about genotoxicity and mutagenicity of the test samples.

Chapter I gives a brief account of working mechanism of pulp and paper industries and associated environmental pollution hazard contributed from its wastewater. The bioremediation approaches, chlorophenol toxicity and its degradation, genotoxicity of wastewater and soil have been reviewed.

Chapter II describes the general materials and methods like sampling sites, bacterial strains, composition of media and buffers etc.

Chapter III includes physicochemical analysis of pulp and paper industry wastewater and soil irrigated with the wastewater as well as microbial diversity of wastewater and soil viz. aerobic heterotrophic bacteria, fungi, actinomycetes and asymbiotic nitrogen fixers.

Chapter IV focuses on isolation, screening and identification of chlorophenol degrading bacteria. The effect of pH, temperature, aeration, concentration and addition of co-substrate on chlorophenol degradation are studied.
Genotoxicity of pulp and paper industry wastewater and contaminated soil are presented in Chapters V and VI respectively.

The references and summary is documented at the end.
Water is an important key resource required to sustain the life on this planet. In the present scenario, water pollution is a serious and emerging problem in the majority of developing countries. Due to rapid industrialization and fast urbanization, there has been an increase in the amount of effluent being discharged into natural water bodies. Industrial effluents and sewage containing plethora of toxic compounds endangers aquatic biota and deteriorates water quality (Paul, 2017). Major industrial pollutants found in water include toxic metals, suspended solids and recalcitrant organic compounds (Wu et al., 2016a; Yang et al., 2017). The heavy metals and persistent organic pollutants are of special concern due to their toxicity, persistent and bioavailability (Wu et al., 2016b). Various more emerging contaminants are likely to be identified in the future as scientists are discovering new pollutants with potential health hazards (Xiao et al., 2017). Among various industries, Pulp and paper industries play important role to the regional economies of several industrialized countries (Hussain and Bernard, 2017). The pulp and paper industry are important sectors in world trade and is helpful in creating jobs and raising standards of living. Nonetheless, Pulp and paper industries are also great contributor to environmental pollution because of their water intensive nature and production of significant pollutants some of which are emitted to the air and others are discharged to the wastewaters (Mahmood and Elliot, 2006; Ince et al., 2011; Zhang et al., 2012). Unfortunately, the wastewater treatment procedures applied in many developing countries are not capable of removing these pollutants efficiently and does not meet the international standards for agricultural use developed by WHO, FAO and UNEP with potential consequences for ecosystem health and functions (Corcoran et al., 2010; Gupta and Thakur, 2015; Rodriguez et al., 2017).

**Paper industry**

The pulp and paper industry is one of the largest industries in the world, with very high capital investments. The world’s total paper production amounted to 403 million tons in 2013 (Bajpai, 2015). The writing and printing segment accounted for 32 percent of this consumption while packaging, tissue and sanitary and newsprint for 50 percent, 6 percent and 12 percent respectively. The global consumption of paper and paperboard increased 5.9% CAGR from 171 million tonnes per annum in 1980 to 350 million tonnes per annum in 2003, driven in the later years largely by higher Asian economic growth (7-10 percent annum) (CPPRI, 2007). North America, Europe and Asia account today for more than 90% of total paper and paperboard...
consumption (360 million tonnes in 2004), with almost equal shares amongst them. Oceania, Africa and Latin America together account for less than 8%. World paper and paperboard demand is expected to grow with growth rate that covers high variation amongst the countries: in the developing regions it is expected to exceed 4%, while in the mature markets (North America, EU, Japan) this rate is expected to be around 0.5–1%. This means that developing Asia (mainly China) will play a crucial role in the future paper market (Szabo et al., 2009).

According to the recent statistics published by FAO (2013), U.S. is the leading producer of wood pulp, accounting for almost 30 percent of total world production, followed by Canada which accounts for almost 11 percent of the global production. In case of paper and paperboard production both U.S. and Canada are among the top five producers in the world and they jointly account for 22 percent of total world production. In both type of products, they are among the top five exporting countries (Hussain and Bernard, 2017).

World paper and paperboard demand and production are increasing; according to the report of IEA (2009) annual paper and paperboard production is expected to grow from approximately 365 million tonnes (Mt) in 2006 to between 700 Mt (low estimate) and 900 Mt (high estimate) in 2050. The largest share of this growth will take place in China, India, and other developing Asian countries (Kong et al., 2015).

**Status of pulp and paper industry in India**

The Indian paper industry comprises of more than 600 mills with capacities ranging from less than 1000 tonne per annum to over 1,00,000 tonne per annum. Majority of the mills are operating at small level, only 60 mills have a capacity of over 33,000 tonnes per annum. The global share for India accounts for less than 2% with the total production of 10.11 million tonnes in the year 2010-11, for paper production (Kulkarni, 2013). Per capita consumption of paper in India is around 10 kg, which is significantly lower than world average of around 58 kg (even the average consumption in Asia is 21 kg). However, an increasing trend of paper consumption has been observed in the recent past (from 7.3 kg in 2008 to 10-11 kg in 2012) (Tripathi, 2014). It is estimated that the Indian paper industry is growing at almost 6 percent annual growth rate, three times the annual global growth rate (CPPRI, 2005; Sharma et al., 2016).

In India pulp and paper industry is among the core industrial sectors generating more than 0.3 million direct and around 1 million indirect employments.
through agricultural activities (Roy, 2007). At present, there are 666 pulp and paper mills in India, of which 632 units are agro-residue and recycled fiber based units (CPPRI, 2005; Tewari et al., 2009). There are about 540 small and medium size units which account for 60 percent of the total capacity and the rest of the supply comes from large industry. The production in 2005-2006 was 5.9 million tonnes paper and paperboard, against an installed capacity of 7.6 million tonnes (MoCI, 2007). However, the demand is ever increasing with rise in literacy, education and income level, and overall economic growth.

Even though the growth of the Indian paper industry has been commensurate with the GDP growth, the country’s per capita consumption continues to be low compared to other countries. It is dismally low when compared with World Average, developed countries and some of the Asian countries (CPPRI, 2005).

**Pulp and paper industry manufacturing process**

Pulp and paper mills exist as integrated operations. The raw materials used in paper manufacturing contain cellulosic fibres generally wood, recycled paper, and agricultural residues. In developing countries, about 60% of cellulose fibres are derived from non-wood raw materials such as bagasse, cereal straw, bamboo, reeds, esparto grass, jute, flax, and sisal (Gullichsen, 2000; Abou-Yousef et al., 2005; Ogunsile and Quintana, 2010). The paper industry working mechanism is described in Fig. 1. The main steps in pulp and paper manufacturing are:

**Raw material preparation and handling**

Wood is converted into chips or logs suitable for pulping in a series of steps which may include debarking, sawing, chipping and screening. However, the available wood supply in many countries cannot meet the growing demand (Jahan et al., 2007) therefore the use of non-wood fibres such as bagasse fibre are playing an important role (Khristova et al., 2006; Jimenez et al., 2008).

**Pulping**

Pulping is the process by which the bonds within the wood structure are ruptured either mechanically or chemically. Chemical pulps can be produced by either alkaline (i.e., sulphate or kraft) or acidic (i.e., sulphite) processes (Ogunsile and Quintana, 2010). The highest proportion of pulp is produced by the sulphate method, followed by mechanical (including semi-chemical, thermomechanical and mechanical) and sulphite methods. Soda-oxygen pulping is advancement over conventional technology
due to its higher delignification capabilities and more recyclable use (Dumitrescu et al., 2008; Yue et al., 2016).

**Pulp washing and screening**

The pulp is screened to remove uncooked wood, washed to remove the spent cooking mixture (now black liquor), and sent either to the bleach plant or to the pulp machine room. Uncooked wood is either returned to the digester or sent to the power boiler to be burned (Gavrilescu, 2004; Gong, 2005).

**Chemical recovery**

The digesting chemicals are recovered in most chemical pulping operations today. The principal objectives are to recover and reconstitute digestion chemicals from the spent cooking liquor for its reuse (Klugman et al., 2007; Monte et al., 2009).

**Bleaching**

Bleaching is a multi-stage process that refines and brightens raw pulp. The general bleaching agents used are Chlorine (Cl₂), Sodium hydroxide (NaOH), Chlorine dioxide (ClO₂), Sodium hypochlorite (NaOCl), Oxygen (O₂), Hydrogen peroxide (H₂O₂), Ozone (O₃), Acid washing (SO₂) and Sodium dithionite (NaS₂O₄) (Bajpai, 2012b).

**Paper and paperboard making**

Pulp slurry is usually screened once more and its consistency adjusted. The pulp is then spread onto a travelling metal screen or plastic mesh. The pulp sheet is passed through a series of rotating rolls (“presses”) that squeeze out water and the sheet is then floated through a multi-storey sequence of hot-air dryers until the consistency is 90 to 95%. Finally, the continuous pulp sheet is cut into pieces and stacked into bales. The pulp bales are compressed, wrapped and packaged into bundles for storage and transport (Ban et al., 2014).
The pulp and paper making industry is one of the most polluting industries in India (Mishra et al., 2013). In most of the Asian countries, effluents from pulp and paper mill are discharged without proper treatment directly into water streams which irrigate agricultural lands (Iqbal et al., 2013; Mishra et al., 2013). Pulp and paper production employ wood as raw material along with consumption of large scale chemicals such as chlorine and sodium hydroxide, etc (Pokhrel and Viraraghvan, 2004). These industries consume a large amount of fresh water and discharge a large quantity of effluents with a high organic and inorganic pollution load. The most energy and chemicals consuming stage in pulp and paper technology is pulp making. This is also environmentally most important step due to its association with harmful emissions (Beschkov, 2006).

The air pollution from pulp and paper industries include highly malodorous emissions of reduced sulfur compounds, measured as total reduced sulfur (TRS), volatile organic compounds (VOCs), sulfur oxides, nitrogen oxides and fly ash.
(Cheremisinoff and Rosenfeld, 2007; Ince et al., 2011). Total reduced sulfur compounds including hydrogen sulfide, methyl mercaptan, dimethyl sulfide, and dimethyl disulfide, are emitted, typically at a rate of 0.3-3 kilograms per metric tonne (kg t\(^{-1}\)) of air-dried pulp (ADP). Particulate matter emitted at a rate of 75-150 (kg t\(^{-1}\)); sulfur oxides at a rate of 0.5-30 (kg t\(^{-1}\)); nitrogen oxides at a rate of 1-3(kg t\(^{-1}\)); and volatile organic compounds (VOCs) at a rate of 15 (kg t\(^{-1}\)) from black liquor oxidation. In the sulfite pulping process, sulfur oxides are emitted at rates ranging from 15 to over 30 (kg t\(^{-1}\)). Other pulping processes, such as mechanical and thermomechanical methods generate significantly lower quantities of air emissions. Steam- and electricity-generating units using coal or fuel oil emit fly ash, sulphur oxides, and nitrogen oxides. Coal burning can emit fly ash at the rate of 100 (kg t\(^{-1}\)) of ADP (Cheremisinoff and Rosenfeld, 2007).

During paper making variety of processing materials such as additives, fluorescent brighteners, biocides, surfactants, defoamers, azo dyes, starch and waxes are used for providing strength, opacity, brightness, water resistance, coloration and controlling slime formation to the pulp. The environmental characteristics of these raw materials show that they are either poorly biodegraded or non-biodegradable and possess toxicity along with affecting oxygenation of water (Thompson et al., 2001; Lacorte et al., 2003; Cadotte et al., 2007). The chemicals used during pulping include chlorine, hypochlorite, chlorine dioxide, oxygen, hydrogen peroxide, hydrosulphite, sodium hydroxide for oxidizing, solubilising lignin and brightening of the pulp. Though their usage is advantageous in terms of effective and economical but generate large quantity of polluting organic load especially with chlorine and hypochlorite where organochlorines along with dioxins and furans are formed (Solomon, 1996; Suess, 2010, Kumar et al., 2015; Fang et al., 2017). Pulp and paper industries generally produce large amount of solid waste in the form of sludge, ash, wood waste, screening rejects, centricleaner rejects, lime sludge, printing ink sludge (loaded with heavy metals) indicating significant environmental concerns (Mathur et al., 2004; Badar and Farooqui, 2012).

**Physico-chemical characteristics of wastewater from pulp and paper mills**

Agro-based pulp and paper mills in India are one of the most polluting industries. Untreated pulp and paper mill wastewater contained a high concentration of pollutants with a high fluctuate value. Studies have shown that the raw wastewater contains a high range of color, BOD, COD, TDS, TSS and AOX levels (Tewari et al.,
Several other inorganic ions such as Ca$^{2+}$, Mg$^{2+}$, Na$^+$, K$^+$, HCO$_3^-$, Cl$^-$, SO$_4^{2-}$ have been found in high amount (Singh et al., 2012). Pulp and paper mills are also found to be contaminated with heavy metals such as Cu, Cr, Fe, Ni, Zn, Cd, Pb, Mn which might be due to bioaccumulation of these metals by plants, which are used as raw material as well as from various chemicals used during the pulping process (Su et al., 2011; Chandra et al., 2017). Yadav and Chandra (2015) found very high amount of Fe, Ni and Zn from pulp mill when compared to the permissible limit as recommended by EPA (USEPA, 2000). Luce et al. (2017) demonstrated high concentration of heavy metals predicted by visible near infrared reflectance spectroscopy in agricultural soils that were amended with paper mill biosolids and liming by-products. Reza et al. (2013) studied the spatial distribution and hazard assessment of heavy metals in the paper mill contaminated area and found high concentrations of Cr, Ni and Pb.

Chanworrawoot and Hunsom (2012) demonstrated that raw wastewater collected from pulp and paper mill possess the following in the range: COD 129,600-137,600 (mg L$^{-1}$), BOD 90,000-112,000 (mg L$^{-1}$), TDS 154,833-178,540 (mg L$^{-1}$), TSS 683-1020 (mg L$^{-1}$), colour 1,030,650-1,505,000 (Pt-Co unit) and electrical conductivity 193.3-199.5 (µS). Besides this through their FTIR spectra, demonstrated presence of hydroxyl groups, carbonyl groups, aromatic skeletal, syringyl and guaiacyl ring indicating the presence of lignin in the wastewater. The characteristic absorption bands of organic contaminants of the original wastewater from the pulp and paper mill have been previously studied (Sun et al., 1999; Jin et al., 2005; Guo et al., 2008). Ashrafi et al. (2015) and Kamali et al. (2016) illustrated the physicochemical parameters of the wastewater generated from pulp and paper industries depending on the types of applied processes using virgin or recovered fibres. The high levels of TSS, BOD and COD have been reported at different operational units of pulp production such as during wood yard chipping, thermo-mechanical pulping, chemical thermo-mechanical pulping, kraft cooking section, pulping process, bleaching, paper machine, integrated pulp and paper mill and recycled paper mill (Wang et al., 2007; Avsar and Demirer, 2008; Kansal et al., 2008; Liu et al., 2011; Ince et al., 2011; Qu et al., 2012; Zwain et al., 2013; Kamali and Khodaparast, 2015).
Adsorbable organic halides (AOX)

The total content of organic halogens in water can be expressed in terms of the parameter referred to as Adsorbable Organic Halides (AOX). Due to the increased use of halogenated organic compounds in industries, agriculture and household, substantial amounts of these compounds are now present in the environment, particularly in wastewaters and contaminated soil (Kummerer, 2001; Goi, 2009; Bajpai, 2015; Kinani et al., 2016). Organic compounds of halogens are often hazardous substances, some of the compounds are toxic and show the tendency of biological accumulation because of their lipophilic nature (Noma et al., 2001; Lopez, 2003; Dann and Hontela, 2011, Xie et al., 2017). The AOX contains more than 300 different organochlorines (Mckague, 1988). Many of them are persistent and have a tendency to bioaccumulate (Ong et al., 1996; Jaacks and Staimez, 2015, Fang et al., 2016).

One of the main sources of AOX discharge world-wide is the pulp and paper industry. When using chlorine and chlorine chemicals to bleach fibres, some of the chlorine reacts with organic matter to form chlorinated organic substances which may then be discharged with the treated effluent and detected as AOXs (Savant et al., 2006). Even during elemental chlorine free (ECF) bleaching technology, chlorine dioxide is used instead of chlorine gas, for delignification but absorbable organic halogens (AOX) will still be produced (Nie et al., 2015b). The low molecular weight chloroorganic compounds (mol wt.<1000) such as chlorophenols, chlorocatechols, chloroguaiacols, chlorobenzenes, chlorinated dibenzodioxins and dibenzofurans are major contributors to mutagenicity and bioaccumulation due to their hydrophobicity and excellent ability to penetrate cell membranes (Pang et al., 2007; Hameed et al., 2009, Lindholm-Lehto et al., 2015a).

Due to the serious toxic effects of AOXs, many countries have imposed discharge limits for AOX from pulp and paper mills. According to PARCOM (Paris Convention for Prevention of Marine Pollution for Land Based Sources and Rivers), twelve European countries signed for a general AOX emission limit of 1 kg t\(^{-1}\) for bleached chemical pulp in 1995 and the discharge limits were gradually lowered upto 0.3–0.5 kg t\(^{-1}\) (Savant et al., 2006). Germany has totally banned production of pulp using chlorine containing chemicals and has also banned consumption of total chlorine free (TCF) pulps. In India, the Central Pollution Control Board has set discharge limit of 1.5 kg t\(^{-1}\) pulp for AOX. The USA has proposed to restrict the
discharge limit at 2.5–5.0 ppb for polychlorinated phenolics (Bajpai, 2000; Savant et al., 2006). Generally the Kappa no. index, used by the pulp and paper industry to express the lignin content of a pulp affects the amount of AOX release. Higher the lignin content more is the kappa no. Pulp and paper industries have incorporated various measures to reduce the kappa no. and also to minimize the carry-over of organic matter along with pulp as it governs the bleach chemical demand during the bleaching process. Some of these measures include oxygen delignification, extended delignification, improved pulp washing, substitution of elemental chlorine with chlorine dioxide, oxidative alkali extraction stage bleaching etc. (CPCB, 2007). The production of absorbable organic halogen (AOX) is greatly reduced when the elemental chlorine free (ECF) process is employed for pulp bleaching (Singh et al., 2008; Nie et al., 2015a; Sharma et al., 2014).

Cleaner technologies in pulp and paper mills

The amount, type and analysing the characteristics of the wastes generated from pulp and paper industries are important to provide the best treatment for managing the pulp and paper mill pollution load. The waste generated differs in their physical characteristics and therefore different technologies are adopted specifically for concerned waste (Mahmood and Elliott, 2006; Ince et al., 2011; Das and Mazumdar, 2016).

Disposal strategy of solid wastes generated from pulp and paper industry is varied which include thermal application such as combustion or anaerobic digestion to obtain energy or landfills deposit (Valdez-Vazquez et al., 2005; Gavrilescu, 2008; Ince et al., 2011; Lin et al., 2013). However, the solid wastes should be monitored after landfill deposition because of toxic characteristics of the compounds (Monte et al., 2009). The gaseous pollutants are other environmental problems generated from these industries and can be minimized by several physico-chemical methods such as adsorption to activated coal, filters absorption, thermal oxidation, catalytic oxidation and condensation have been widely used (Eweis et al., 1998; Ince et al., 2011)

As large quantity of effluents are discharged from pulp and paper mill effluents with a high organic and inorganic pollution loading different physico-chemical approaches are being used for the treatment of the effluents (Amat et al., 2005; Wang et al., 2007; Ko et al., 2009; Li and Zhang, 2011; Wang et al., 2011, Hubbe et al., 2016). Physico-chemical processes are used to remove suspended solids, colloidal particles, toxic compounds, floating matters, and colors from
wastewaters (Bhatnagar et al., 2013, Mukherjee et al., 2014, Coimbra et al., 2015). Generally the main treatment processes followed at pulp and paper mill plants include the primary clarification (sedimentation or flotation), secondary treatment (activated sludge process or anaerobic digestion) and/or tertiary processes (membrane processes, such as ultrafiltration) (Ksibi et al., 2003). Variety of different processes used for wastewater treatment are sedimentation, ultra-filtration (Bhattacharjee et al., 2007), flotation (Hogenkamp, 1999), screening (El-Ashtoukhy et al., 2009), coagulation, floculation (Wong et al., 2006, Khandegar and Saroha, 2013), ozonation and electrolysis (Kishimoto et al., 2010; Chanworrawoot and Hunsom, 2012).

The other clean technologies generally adopted for reduction of pollution include use of elemental chlorine-free (ECF) bleaching where chlorine dioxide is used in lieu of elemental chlorine to bleach pulp, total chlorine-free (TCF) bleaching where bleaching is done without chlorine containing chemicals such as oxygen and hydrogen peroxide, extended delignification, improved pulp washing, alternative pulping technologies for reducing the use of traditional chemicals such as sulfur-containing compounds, use of nonwood fibre sources, black liquor gasification and minimizing water use by adopting closed systems for reduction of effluent from the manufacturing process (EC, 2015).

Degradation of AOX

Various physical, chemical and biological methods have been developed for the degradation of AOX (Nie et al., 2015a; Virkutyte, 2016; Yao et al., 2017; Farooqi and Basheer, 2017). Yao et al. (1994) reported the use of ultrafiltration through which 99% reduction in AOX was achieved. Nancy et al. (1996) used fly ash as an adsorbing medium to remove chlorinated organics and colour efficiently. Shawwa et al. (2001) reported the use of petroleum coke for the removal of AOX from bleaching wastewater at a concentration higher than 15000 mg L\(^{-1}\). Photocatalytic treatment for the removal of colour TOC, AOX and COD in the effluent has been reported previously (Torrades et al., 2001; Moiseev et al., 2004, Hermosilla et al., 2015). Seiss et al. (2001) reported use of nano-filtration in combination with electrodialysis at a pilot scale, for removal of 95% of the contaminating toxic organic halides, salts and colourants leaving the treated effluents suitable for process reuse. Moebius and Helble (2004) described a combination of ozonation followed by biodegradation in a biofilm reactor to achieve elimination of AOX, colour and other disturbing substances. Singh and Thakur (2006) conducted sequential treatment with the combination of aerobic
and anaerobic process for reduction of COD, AOX, lignin and phenol. The treatment using an activated carbon is increasingly becoming popular due to high adsorption capacities of activated carbons which is related to its high-surface area, pore volume, and porosity (Dias et al., 2007; Margot et al., 2013; Streicher et al., 2016; Gholizadeh et al., 2016). In addition, activated carbon, which has a coarse texture and a large surface area, is suitable for biofilm attachment (Ahmaruzzaman, 2008). The activated carbon can be generated from various types of resources including those originating from agricultural waste, such as coconut shells, coconut husks, rice husks, apricot shells and bamboo (Anirudhan et al., 2009; Hameed et al., 2009; Santana et al., 2017).

Nie et al. (2015b) used sodium sulphide for (AOX) reduction in elemental chlorine free (ECF) bleaching of bagasse pulp. Ozone and ozone/UV post-treatment reached removal efficiencies of organochlorine compounds near 40% in a study conducted by Chaparro and Pires, (2015). Yao et al. (2017) studied the influence of lignin on AOX formation which was eliminated by the control of hot water extraction, AOX formation was decreased by 33.27% under optimal hot water extraction conditions. However, these techniques require pretreatment and are uneconomical when applied alone at field scale operations. This necessitates consideration of developing economical and eco-friendly methods for removal of AOX compounds (Savant et al., 2006).

Biological oxidation is the most widely used technique to remove BOD, consisting complete oxidation of organic compound to CO₂ and water (Virkutyte, 2016; Bajpai, 2017; Ahmed et al., 2017). Most commonly used aerobic reactors for the treatment of pulp and paper industry wastewater are aerated lagoon, aerated stabilization basin, activated sludge and sequential batch reactors (Kargi and Uygur, 2004; Sahinkaya and Dilek, 2007; Muhamad et al., 2015). Advanced aerobic reactors can bring about 80% AOX reduction (Savant et al., 2006; Singh and Thakur, 2006). Activated sludge (AS) to remove COD and AOX from different pulp and paper wastewaters in a laboratory-scale plant has also been used efficiently (Norris et al., 2000; Thompson and Forster, 2003; Mahmood and Elliott, 2006; Lerner et al., 2007; Bengtsson et al., 2008). Aerated stabilization basins (ASB) is another alternative aerobic treatment process to remove BOD, COD as well as AOX from wastewaters (Achoka, 2002; Mahmood and Paice, 2006; Ghoreishi and Haghighi, 2007; Bryant, 2010). Osman et al. (2013) showed efficient removal of AOX and COD from real
recycled paper wastewater using GAC-SBBR. Sequential batch reactors have advantages of low operating costs, no sludge settler required, no recycling pumps required, good control over filamentous bulking, tolerance to shock loads and peak flow denitrification during anoxic fill and settle stage. Farooqi and Basheer (2017) used Sequential Batch Reactor for the treatment of pulp and paper mill wastewater having AOX 15-20 mg L\(^{-1}\) and achieved significant degradation where AOX concentration reached below 5 mg/L.

Use of bacteria to remove organochlorine removal from bleached kraft pulp and paper mill effluents has been shown previously (Alvarez et al., 2012; Abraham et al., 2014). Fulthorpe and Allen (1995) used bacteria such as *Pseudomonas, Ancylobacter aquaticus* and *Methylobacterium* for AOX degradation in softwood effluents. Chandra and Abhishek (2011) carried out biodegradation of pulping by-product using *Citrobacter freundii* and *Citrobacter* sp. which was able to reduce 82% COD, 79% AOX and 60% lignin after 144 h of incubation period. Similar experiments with other micro-organisms showed that AOX removal efficiency can be increased with use of specific dechlorinating bacteria (Garg et al., 2012; Garg et al., 2013; Tripathi and Garg, 2013).

The aerobic treatments are applicable where sufficient molecular oxygen is available. The rate of degradation is proportional to dissolved oxygen and therefore the process demands large inputs of energy, making it expensive therefore anaerobic treatment system has also been found helpful as an alternative technology in removing AOX from wastewater (Badiei et al., 2011; Faraghaly et al., 2015). Among the high rate anaerobic reactors, anaerobic filters, upflow anaerobic sludge bed reactors (UASBs) and anaerobic membrane bioreactors (AnMBR) have been mainly applied to treat the pulp and paper mill effluent (Kamali et al., 2016). Among the several types of anaerobic reactors, UASB has been commonly adopted by pulp and paper industry due to its advantages when compared with other types of high-rate anaerobic reactors, such as low investment requirements (Buzzini et al., 2005; Buzzini & Pires, 2007; Chong et al., 2012). Buzzini and Pires (2002) treated diluted black liquor from a kraft pulp mill by using an UASB reactor and reached 80% on average removal of COD. Ortega-Clemente et al. (2007) used packed bed reactor (PBR) and fluidized bed reactor (FBR) for the treatment of anaerobically pretreated weak black liquor from kraft pulping wastewater. Deshmukh et al. (2009) used up flow anaerobic filter (UAF) to remove BOD, COD, and AOX from bleaching wastewater and obtained removal
efficiencies of 70%, 50%, and 50%, respectively. AnMBRs which combine the advantages of anaerobic digestion process and membrane separation mechanisms have received considerable attention due to their advantages for wastewater treatment such as lower sludge production and energy requirements over conventional anaerobic treatment methods (Jeison and Vanlier, 2007). By adopting anaerobic membrane technologies, it is possible to achieve complete solid-liquid phase separation and as a result complete biomass retention (Gao et al., 2011).

**Bioremediation of paper mill effluent**

Bioremediation technologies are energy efficient, affordable and environment-friendly. Several physical and chemical methods (electrocoagulation, ozonation, and ultrafiltration) or combination of different methods in series are available for the treatment of effluent, but they are more energy intensive and suffer from residual effect and hence are less desirable than biological process which is cost-effective (Yang et al., 2008).

The major environmental pollutants discharged from pulp and paper industry include lignin and chlorinated phenols. Lignin is responsible for darkening of effluent colour which inhibits the growth of phototrophic organisms by decreasing the transmission of sunlight in water (Karrasch et al., 2006). Chlorinated phenolics cause toxicity to both flora and fauna. Chlorinated organic compounds, also generate dioxins and furans, which are suspected to cause genetic mutations in exposed organisms (Nestmann, 1985; Easton et al., 1997; Theodorakis et al., 2006; Malik et al., 2009).

In addition, many developing countries including India due to the non-availability of alternative sources of irrigation, farmers irrigated their crop plants with industrial effluents containing high level of several toxic compounds including persistent organic pollutant and heavy metals. This may cause adverse effect on human through food chain (Yadav and Chandra, 2015).

Microorganisms (fungi and bacteria) play a crucial role in recycling toxic organic compounds and converting it harmless products. Extensive research has been carried out to explore the microbial diversity, particularly of contaminated areas in search of organisms that can degrade a wide range of pollutants at high pollution load (Thavamani et al., 2012, Leal et al., 2017). Intensive studies on white rot fungi had been done which are found to possess good ligninolytic activity because of production of enzymes like LiP, MnP and laccase (Minussi et al., 2007). However, the
requirement of narrow pH 4-5 for the growth and enzyme production of fungi constrain the effluent treatment process as pH values of pulp and paper mill are high (pH 7-9) and the requirement to reduce the pH prior to the application of fungal system adds additional cost. In contrast to fungi, bacteria growing at neutral to alkaline pH (pH 7- pH 9) may play an important role in decolourization of effluents and degradation of toxic compounds of pulp and paper mill without any need for pH adjustment. Bacterial laccases are highly active and have shown more stability at high temperatures and high-pH values (Sharma et al., 2007). Several species of bacteria have been evaluated for lignin degradation (Chandra et al., 2007; EL-Hanafy et al., 2007, 2008; Abd-Elsalam and EL-Hanafy, 2009) and for pulp and paper mill effluent treatment (Thakur, 2004; Raj et al., 2007; Singh et al., 2011; Raj et al., 2014). The immense environmental adaptability and biochemical versatility has made bacteria more effective than fungi for the bioremediation of environmental pollutants. Several bacteria viz. Aeromonas, Bacillus sp., Bacillus subtilis, Flavobacterium, Pseudomonas, Aneurinibacillus, Azotobacter, Serratia marcescens, Critobacter sp., Nocardia, Novosphingobium sp. B-7, Paenibacillus sp., Pandoraea Achromobacter and Xanthomonas are reported to utilize lignocellulosic and chloro-organic components of pulp-paper effluent (Vora et al., 1988; Morii et al., 1995; Jain et al., 1997; Gupta et al., 2001; Chandra et al., 2007; Raj et al., 2007; Bandounas et al., 2011; Bugg et al., 2011; Chandra et al., 2011; Chen et al., 2012; Raj et al., 2014; Yadav and Chandra, 2015; Bramhachari et al., 2016).

Optimization of factors such pH, temperature, aeration, O₂ supply and co-substrate addition plays an important role in stable performance and increase in efficiency during biological removal have been carried out by researchers (Sahoo and Gupta, 2005; Tiku et al., 2010; Khan and Hall, 2013; Mathews et al., 2014). It has been reported that the particular consortium of bacteria have the ability to substantially degrade the pulp mill effluent compounds as well as effectively reduce the toxicity, but not all the bacteria can effectively carry out biodegradation of mixtures of recalcitrant compound instead a well-formulated consortia of bacteria may be highly effective in serving the above purpose (Li et al., 2011; Liang et al., 2015). The microbial diversity and their catabolic properties can be successfully exploited for interventions into environmental processes for providing efficient and effective bioremediation of xenobiotics (Jain et al., 2005; Devpura et al., 2017).
Chlorophenols

Chlorophenols are a group of aromatic compounds in which chlorine atom (between one and five) have been added to phenol. Phenol is derived from benzene, the simplest aromatic hydrocarbon, by adding a hydroxy group to a carbon to replace hydrogen. There are five basic types of chlorophenols: monochlorophenols, dichlorophenols, trichlorophenols, tetrachlorophenols, and pentachlorophenols. In all, there are 19 different chlorophenols. Except for 2-chlorophenol, which is a liquid at room temperature, all of the chlorophenols are solids. The chlorophenols have a strong medicinal taste and odor; small amounts (even at the levels of parts per billion concentrations) can be tasted in water. The isomers of chlorophenols possess both acute and chronic toxicity which varies with the number of chlorines present. As many of the isomers typically co-occur in the environment and have qualitatively (but not quantitatively) similar toxicological effects (US EPA and ATSDR, 1999).

Chlorophenols are generated from a number of important industrial manufacturing processes, such as pesticide, biocide, paint, solvent, colorant, antiseptics, pulp and paper industries. Additionally, wastewater from other industries such as polymeric resin, oil refineries, petrochemicals and coking plants also contain chlorophenols (Quan et al., 2003, Quintana and Ramos, 2008). These compounds have become one of the major kinds of emerging contaminants in water, air and soil due to their carcinogenicity and non-biodegradability (Chandra et al., 2009). Chlorophenols have been of particular public concern due to their high toxicity to living organisms, organoleptic character, bioaccumulation, and persistence in the environment; these chlorinated aromatic compounds pose severe problems to the environment, because they may lead to acute toxicity, histopathological changes, mutagenicity, and cancer (ATSDR, 1999; Cooper and Jones, 2008; Qin et al., 2014a). The serious health and environmental issues contributed by the toxicity of chlorophenols makes it imperative to restrict and assess the fate of chlorophenols into the ecosystem with a view to protect the environment and preserve the public health communities (Igbinosa et al., 2013).

Biodegradation of Chlorophenols

Chlorinated aromatic compounds such as chlorinated phenols are major environmental pollutants released from pulp and paper manufacturing process. They are often released in substantial quantities, are toxic, resistant to degradation and accumulate in sediment and biota. Chlorinated phenols and their derivatives are
widely used in the environment during manufacturing of pesticides and herbicides formed and also as a result of chlorination of natural organic matter (Zouari et al., 2002; Matafonova et al., 2006). The toxicity of these compounds tends to increase with the degree of chlorination. The chlorinated phenolics are contaminated with chlorinated dibenzofuran and dioxins which are highly toxic compounds produced as contaminants during the manufacture of pesticides and during incineration. Moreover, chorophenols are considered as possibly carcinogenic to humans and show cytotoxicity (IARC, 1999; Vlastos et al., 2016). Due to their toxicity, a high concentration of chlorophenols in industrial wastewater or landfill leachate can cause upsets in the operation of the activated sludge process, or make a microbial treatment impossible (Ren and Frymier, 2002).

In treating chlorophenols, despite its toxicity and persistence, the biological method has attracted more attention than physical and chemical methods because of relative inexpensive cost and less secondary pollution (Kargi and Konya, 2006). The different physical, and chemical methods such as adsorption, ion exchange, activated carbon adsorption, coagulation, solvent extraction, liquid membrane permeation, electrokinetics are used for removal of chlorinated aromatics from wastewater (Singh and Srivastava, 2002; Jain et al., 2004; Gill et al., 2014; Kusmierek, 2016). Chemical oxidation methods are fast, but the main disadvantages include too expensive processing and also formation of undesirable by products (Demirak et al., 2011). Moreover, many different types of microorganisms utilizing CP’s as their sole carbon and energy source were isolated, such as Sphingomonas chlorophenolica (Steiert et al., 1987), Arthrobacter chlorophenolicus (Backman and Jansson, 2004), Bacillus sp. (Matafonova et al., 2006) Rhizobium sp. (Yang and Lee, 2008), Rhodococcus rhodochrous (Pamukoglu and Kargi, 2008), Pseudomonas sp., Stenotrophomonas sp. (Gallego et al., 2009; Garg et al., 2012).

Pentachlorophenol (PCP) is a general biocide used primarily as preservative of wood, leather, textile and related commercial products. Microbial degradation is a useful strategy to eliminate these compounds and detoxify wastewaters and polluted environments (Singh et al., 2009). PCP degradation has been studied by both pure culture and mixed culture (Chandra et al., 2009; Singh et al., 2009; Tripathi et al., 2011; Garg et al., 2013; Tripathi and Garg, 2013). The capacity to transform PCP into less toxic product depends on environmental conditions including, temperature (Valo et al., 1985), pH level and the organic matter (Cea et al., 2005), humic substances
(Ruttimann-Johnson and Lamar, 1997), oxygen and electron acceptors (D’Angelo and Reddy, 2000) and concentration of PCP (Yang and Lee, 2007). PCP degradation can be achieved through three ways: oxygenolysis, hydroxylation or reductive dehalogenation (McAllister et al., 1996; Field and Sierra-Alvarez, 2008).

Under anaerobic conditions, reductive dehalogenation by bacteria transform PCP, where the chlorine atoms are sequentially replaced by hydrogen atoms until complete removal of chlorine which convert PCP into phenol, benzoate, acetate, carbon dioxide and methane (Mohn and Tiedje, 1992). Reductive dechlorination has been observed in many soils, sediments and sewage sludge (D’Angelo and Reddy, 2000; Xu et al., 2017).

During aerobic condition, PCP transformation occurs by incorporating one or two oxygen atoms from the diatomic O₂ through the enzyme catalysed oxidative dechlorination reaction. Further aromatic ring gets cleaved and the intermediates formed later get transformed to CO₂ though a slow aerobic transformation (Reddy and Gold, 2000). Since aromatic ring is deficient in electrons and less susceptible to electrophilic attack by O₂ it can get transformed by means of hydroxylation reactions which convert PCP into other compounds such as tetrachlorohydroquinone (TCHQ) (Xun and Orser, 1991; Crawford et al., 2007; Xun et al., 2010) by replacing the chlorine atom with a hydroxide, converting the PCP into intermediate products (Vijay et al., 2000; Crawford et al., 2007; Xun et al., 2010).

The process of biodegradation of chlorophenols with the help of microorganisms that can tolerate, degrade and mineralize such pollutants represent the first step to realize an excellent bioremediation processes. The molecular aspect of chlorophenol degradation has received little attention (Juwarkar et al., 2010; Villemur, 2013; Carvalho et al., 2013; Copley et al., 2013). Moreover the genetic understanding and its manipulation can offer a means of engineering microorganism to deal with chlorophenol degradation that may be present in a contaminated site (Villemur, 2013). Therefore the thorough study of chlorophenol degradation along with the determination of its intermediates and metabolic and genetic processes would extend our knowledge for better bioremediation approach (Bosso and Cristinzio, 2014).

**Genotoxicity of wastewater**

Globally, pulp and paper industry is considered as one of the most polluting industry generating toxic organic pollutants into the environment (Dey et al., 2013;
Toczyłowska-Mamińska, 2017). Most of chemicals used and manufactured in the pulp and paper industry are recalcitrant to degradation and creates toxicity problems upon disposal of these effluents into the waterbodies. The exposure to sulphur gases and organochlorinated compound mixtures from these industries have adverse impacts on environment and human as some of these may act as potent carcinogen (Monge-Corella et al., 2008; Zhao et al., 2013; Zehra et al., 2015). Due to water scarcity all over the world, there is an increasing utilization of these wastewaters for irrigation of agricultural lands (Thawale et al., 2011).

Industrial pulping and bleaching processes releases phenols from plant cells due to its solubilisation and this cause serious environmental problem. Phenol and its derivatives contribute to genotoxicity, carcinogenicity, immunotoxicity, hematological, and physiological effects in fishes in receiving waters and have a high bioaccumulation rate along the food chain due to their lipophilicity (Tsutsui et al., 1997; Abdelkareem et al., 2013; Wang et al., 2016). Apart from this, sulphate ions, heavy metals and persistent organic pollutant generated from the pulp and paper mill effluent contribute to genotoxicity and toxicity (Karrasch et al., 2006; Chamorro et al., 2009; Singhal and Thakur, 2009; Raj et al., 2014; Shrivastava, 2015; Haq et al., 2016).

The impact of complex mixtures of pollutants in water on environmental safety and public health has raised concerned globally (Schwarzenbach et al., 2006). Despite the advancement of rapid detection technique in environmental chemistry, it remains infeasible to track every pollutant potentially present in water because of limitations in analytical capacity, cost, and time. The identification of many environmental chemicals may not happen because of their trace concentration, unknown structure or lack of sample preparation and detection methods (Jia et al., 2015). Moreover, conventional chemical monitoring cannot provide information on cumulative effects of these complex mixtures of environmental contaminants and also about health impacts. Bioassays can predict potential toxicity of individual chemicals or chemical mixtures and can also evaluate the genotoxic potential of unknown toxic chemicals present in complex mixtures and have therefore been increasingly used in water quality assessment over several years (Escher and Leusch, 2012; Liu and Zhang, 2014; Yang and Zhang, 2014).
The assessment of cytotoxic and genotoxic potential of refinery waste effluent using plant, animal and bacterial systems was carried out by Gupta and Ahmad (2012) and they found that exposure to the refinery wastewater resulted in the formation of micronuclei and bridges in chromosomes of *A. cepa* cells. A significant decline occurred in survival of DNA repair defective mutants of *E. coli* K12 exposed to refinery wastewater. Also calf thymus DNA–EtBr fluorescence intensity decreased and percent hemolysis of human blood cells increased upon incubation with refinery wastewater. Genotoxic effects of hospital laundry wastewaters treated with photocatalytic ozonation were detected in *Allium cepa* test by Kern et al. (2013). They observed normalization of the mitotic index and reduction of micronucleated cells in *A. cepa* after the treatments.

Jia *et al.* (2015) conducted 36 different bioassays for mutagenicity and genotoxicity determination of wastewater from treatment plant. They also included Ames test for determination of mutation using three different strains of *Salmonella typhimurium* namely TA98, TA100 and TAmix (a 6-strain mixture from TA7001 to TA7006) and found mutagenic responses were prevalently found in both of TA100 and TAmix strains, which target different location of basepair substitution. It was observed that TA100 has a higher sensitivity than TAmix (a 6-strain mixture from TA7001 to TA7006, indicating different location of base-pair substitution) in detecting mutagenicity for environmental samples, and this may be possibly due to a diluted strain density of TAmix compared with pure TA100 strain. They also found TA98 with S9 activation was more mutagenic than without S9; suggesting some of the compounds have higher toxicity after metabolic activation.

Iqbal (2015) evaluated cytotoxicity and mutagenicity of textile effluents, treated by gamma radiation in combination with hydrogen peroxide using *Allium cepa*, heamolytic, brine shrimp bioassays and Ames test and observed mutagenicity of gamma radiation treated effluents reduced up to 59% and 54% in case of TA98 and TA100, respectively. Lutterbeck *et al.* (2015) observed genotoxicity and cytotoxicity of anticancer drugs present in wastewater using *Allium cepa* assays and observed significant differences in the Mitotic indexes (MI) and chromosomal aberrations indicating potential cytotoxic activity of the drugs. Xiao *et al.* (2017) evaluated mutagenicity and estrogenicity of raw and drinking water in an industrialized city in the Yangtze River Delta. The mutagenicity was found to increase as demonstrated by more than two folds increase in the mutation ratio of many samples of XAD extracted
finished water as compared to raw water which may be due to mutagenic by-products formed during chlorination. Haq *et al.* (2017) evaluated genotoxicity of pulp and paper mill effluent using *Alium cepa* and observed that the root tip cells grown in untreated effluent of pulp and paper mill showed a significant decrease in MI from 69% (control) to 32%, 27%, 22% and 11% at 25%, 50%, 75% and 100% effluent concentration, respectively. They also showed cytotoxic nature of effluent by observing different chromosomal and nuclear aberrations.

**Genotoxicity of Soil**

The hazard and risk assessment of soil contaminated with variety of xenobiotic compounds such as heavy metals, adsorbable organic halides, petroleum hydrocarbons, polynuclear hydrocarbons (such as naphthalene and benzopyrene), solvents and pesticides due to industrial disposal and anthropogenic activities is needed for acquiring information about the ecotoxicological effects (White and Claxton, 2004; Feng *et al*., 2013; Floehr *et al*., 2015). Chemical analyses in combination with the biological effects imparted by mutagens are helpful to assess the risks of soils as physical-chemical analytical techniques alone cannot evaluate the potential toxic hazards (Vargas *et al*., 2008; Umbuzeiro *et al*., 2008; Cappi da Costa *et al*., 2012; Biruk *et al*., 2017).

Kwasniewska *et al.* (2012) observed mutagenicity of two soil samples from municipal solid waste landfill sites and found 4-fold increase in the number of revertants in the assay performed with both the TA98 and TA100 strains. Similarly genotoxicity of sewage sludge and composted sewage sludge was demonstrated by Kapanen *et al.* (2013) using Vitotox™ test. In addition, endocrine-disrupting and dioxin-like activity was also studied using yeast-cell-based assays and detected acute toxicity, genotoxicity, and elevated endocrine disruption due to the presence of potentially harmful substances in complex mixture. Anjum and Krakat (2015) carried out genotoxicity assessments of alluvial soil irrigated with wastewater from a pesticide manufacturing industry. They employed DNA repair (SOS)-defective *Escherichia coli* K-12 mutant assay and the bacteriophage lambda system to estimate the genotoxicity of soils. They observed maximum decline in hexane extract, where survival was found to be 15.2% in polA mutants of DNA repair-defective *E. coli* K-12 strains.

Man *et al.* (2013) studied mutagenicity and genotoxicity of Hong Kong soils contaminated by polycyclic aromatic hydrocarbons and dioxins/furans using Ames
test and SOS chromotest. They demonstrated significant mutagenicity of the soil samples from 12 different land use types (contaminated with Poly Aromatic Hydrocarbons and dioxins/furans) carried out by S. typhimurium strains TA98 and TA100 with S9 mix and found high mutagenic potency of upto 13.8 and 7.43. Medunic et al. (2016) reported cytotoxicity and genotoxicity of contaminated soil and ash with coal combustion. Swati et al. (2017) analyzed heavy metal concentration, organic contaminants and toxicity study of landfill soil of three municipal solid waste landfill sites of Delhi, India and found it to be contaminated with heavy metals and persistent organic contaminants like phthalates, benzene derivatives, halogenated aliphatic compounds and PAHs derivatives, the soil were found to be toxic enough to affect human health due to the synergistic or additive actions of present contaminants. Ghosh et al. (2017) reviewed the hazards posed by landfill leachates and emphasized the importance of battery of bioassays for ecotoxicological characterization.

**Bioassays for Genotoxicity**

The natural and xenobiotic substances are known to affect living organisms which can be evaluated by means of ecotoxicological studies. These studies are often helpful in evaluating risk assessment of pure chemicals or environmental samples which may not be evaluated by physical or chemical means alone (Pessala et al., 2004). The harmful effects can be measured by different endpoints used in the bioassays. Traditionally reproduction, growth and survival were used as endpoints in the bioassays. Even though the types of responses were visible to eye but the major drawbacks involved long term durations especially if they are to cover the organism’s full life cycle (from several days to months or even years). Several short term bioassays that are based on cells, bacteria or plants are reported to be used more and more as they have the advantage of being simple, reproducible and rapid (Buitrago et al., 2013; Biruk et al., 2017). Development of such new methods has driven minimized use of vertebrate tests. In addition to these, the short term bioassays are cost effective and require small amount of test sample to be examined. Thus, besides the ethical considerations, short term bioassays are also economically more feasible and thus possess more potential for routine control and assessment use. They are often relatively sensitive and thus also reduce the risk of the biological effects being underestimated (Blaise, 1998; Jia et al., 2015).

Among the microbial bioassays, the *Salmonella* mutagenicity test is undoubtedly the most popular bioassay. This test determines the mutagenicity of
complex environmental mixtures by measuring the mutagenic activity in histidine deficient strains of *Salmonella typhimurium* with or without additional activating enzymes (Umbuzeiro *et al*., 2001; Wegrzyn and Czyz, 2003; Chakraborty and Mukherjee, 2009; Pereira *et al*., 2009; Valente-Campos *et al*., 2009; Zhao *et al*., 2010; Anjum and Malik, 2013; Masood and Malik 2013 a,b).

The Ames test is being able to demonstrate nuclear DNA effects of gene mutation, such as transition/transversion, frameshift mutation and base pair substitution after exposure to environmental contaminants in bacterial DNA. The different responses of the *Salmonella* strains can provide information on the classes of mutagens present in the test samples. The most popular tester strains TA98 and TA100 carry hisD3052 and hisG46 alleles, respectively. TA98 is a -1 frameshift mutation which affects the reading frame of a nearby repetitive –C–G–C–G–C–G–C–G– sequence, various frameshift mutagens such as aromatic nitroso derivatives of amine induce its reversion to wild type. TA100 results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC) and mutagens that cause base-pair substitution mutations primarily at one of the GC pairs revert it to wild type. TA97a, a frameshift strain that carries a +1 frameshift at a run of cytosines (i.e., hisD6610) while the strains TA102 and TA104 with an AT-rich mutation target (i.e., hisG428) are effective at detecting cross-linking agents such as mitomycin C (Mortelmans and Zeiger, 2000). All strains are histidine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations such as *uvrB-bio* genes mutation, *rfa* mutation, insertion of pKM101 have been incorporated which made the tester strains more sensitive to chemical mutagens (Tejs, 2008). These strains have been extensively employed for the detection of environmental mutagens including poly aromatic hydrocarbons (Kim Oanh *et al*., 2002; Piekarska, 2010), aromatic amines (e.g., N containing heterocyclics) (Leong *et al*., 2010; Roemer *et al*., 2016), phthalic acid esters and bisphenol A (Al Saleh *et al*., 2017) and adsorbable organic halides (Zeng *et al*., 2015; Xie *et al*., 2017).

Another widely used bacterial genotoxicity tests, includes DNA repair assays. These repair assays usually measures the extent of DNA damage to determine the genotoxic/mutagenic potential of a chemical or test sample. The induction of SOS repair system in bacteria following exposure to DNA damaging agents is critical parameter for evaluating genotoxicity. It is comprehensively studied and is known for its sensitivity, reproducibility and rapidity (Ruiz and Marzin, 1997; Ohe *et al*., 2004;
Escobar et al., 2013; Sanchez-Vicente et al., 2016). The regulon having two proteins inducer recA and repressor lexA, playing key roles in regulation of the SOS response (Little and Gellert, 1983). After exposure to DNA damaging agents, recA binds to single-stranded DNA formed in response to damage and gets activated and act as a co-protease, thus stimulating the cleavage of lexA and triggering the transcription of SOS genes (Hori et al., 1981; Michel, 2005; Zhang et al., 2010). The elevated expression of these genes increases the capacity of cells for DNA repair, damage tolerance, DNA replication, and mutagenesis (Renzette et al., 2005). Since mutant strains do not permit induction of the SOS response, the lack of SOS repair renders such strains extremely sensitive to DNA-damaging agents (Kuzminov, 1999).

Cytological analysis with respect to either mitotic or meiotic stages study is considered to be one of the most dependable indices to estimate the potency of mutagens. Higher plants offer excellent genetic models to assess environmental pollutants, being frequently used in monitoring studies. It not only provides great sensitivity to detect mutagens in different environments but also the possibility of assessing several genetic endpoints, which range from point mutations to chromosomal aberrations (CA) in cells of different organs and tissues, such as leaves, roots and pollen (Patil and Bhat, 1992; Grant, 1994; Nefic et al., 2013). Variety of higher plants species are frequently used to evaluate environmental contamination, the most common ones are Allium cepa, Vicia faba, Zea mays, Tradescantia, Nicotiana tabacum, Crepis capillaris and Hordeum vulgare (Grant, 1994; Amin, 2011; Christofoletti et al., 2013; Loubna et al., 2015; Martins et al., 2016). However, among these species, A. cepa has been regarded as one of the best model plant system for the assessment of chromosomal damages and disturbances in the mitotic cycle due to the presence of good chromosome conditions, such as large chromosomes and in a reduced number (2n=16) (Fiskesjo, 1985). The A. cepa test was introduced by Levan (1938), since then, it has been routinely used for the toxicity assessment of heavy metals, pesticides, and industrial wastewaters (Sik et al., 2009; Srivastava and Mishra, 2009; Yildiz et al., 2009; Barbosa et al., 2010). Allium cepa is an efficient test system and provide high sensitivity and good correlation with mammalian test system to evaluate the genotoxic potential of chemicals in the environment (Chauhan et al., 1999). Many researchers have suggested that A. cepa test as low cost, easy handling and reliable assay for cytological analysis which present some advantages over other short-term tests employed in environmental monitoring (Rank and Nielsen, 1993,
1994; Rank, 2003; Masood and Malik, 2013; Haq et al., 2016). This test can be directly used where prior exposure of complex mixtures without previous treatment of the test sample is not needed (Kwasniewska et al., 2012; Bhat et al., 2016; Garcia et al., 2017; Haq et al., 2017).
## Chemicals/ reagents/ kits/enzymes and suppliers

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4 Dichlorophenol</td>
<td>Hi Media, India</td>
</tr>
<tr>
<td>2, 4, 6 Trichlorophenol</td>
<td>Hi Media, India</td>
</tr>
<tr>
<td>Acetic acid (glacial)</td>
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</tr>
<tr>
<td>Acetone</td>
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<tr>
<td>Acetocarmine</td>
<td>Hi Media, India</td>
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<td>Agar</td>
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<tr>
<td>Agarose</td>
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<td>Aminoantipyrine</td>
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<td>Biotin</td>
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<td>Histidine hydrochloride</td>
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<tr>
<td>XAD-8</td>
<td>Serva GmbH, Germany</td>
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</tbody>
</table>

**Kits**

- Bacterial Genomic Miniprep Kit: Sigma, USA

**Enzymes/DNA marker**

- DNase: Fermentas, USA
- Lysozyme: Hi Media, India
- Taq DNA polymerase: Fermentas, USA
- 1 kb DNA ladder: Fermentas, USA

**Media used in the study**

**Jensens medium**

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</tr>
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<tr>
<td>Magnesium sulfate</td>
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<td>Sodium chloride</td>
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<tr>
<td>Agar</td>
<td>15</td>
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<tr>
<td>Distilled water</td>
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**KenKnight and Munaier’s medium**

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<tr>
<td>Dextrose</td>
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<td>Monopotassium dihydrogen phosphate</td>
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<td>Sodium nitrate</td>
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<td>Potassium chloride</td>
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<td>Magnesium sulfate</td>
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</tbody>
</table>
Agar : 15.0
Distilled water : 1000 mL

**Luria Bertani broth (pH 7.5 ± 0.2)**

- Tryptone : 10.0
- Yeast extract : 5.0
- NaCl : 10.0
- Distilled water : 1000 mL

**Medium for master plates**

- 50X VB salt : 20.0 mL
- 40% glucose : 50.0 mL
- Histidine HCl.H₂O (2 g/400 mL) : 10.0 mL
- 0.5 mM Biotin : 6.0 mL
- Ampicillin solution (8 mg/mL 0.02M NaOH) : 3.15 mL
- Tetracycline solution (8 mg/l 0.02 N HCl) : 0.25 mL
- Agar : 15 g
- Distilled water : 910.0 mL

**Minimal glucose plates for mutagenicity assay**

- 50X VB salts : 20 mL
- 40% glucose : 50 mL
- Agar : 15 g
- Distilled water : 930 mL

**Minimal medium (pH 7.2±0.2)**

- Ammonium sulphate : 1.0 g
- Dipotassium hydrogen orthophosphate : 1.0 g
- Disodium hydrogen orthophosphate : 1.0 g
- Calcium chloride : 0.1 g
- Magnesium sulphate : 0.01 g
- Copper sulphate : 0.04 g
- Ferric chloride : 0.001 g
- Sodium molybdate : 0.002 g
- Distilled water : 1000 mL

**Nutrient agar (pH 7.4)**

- Peptone : 5.0
- Beef extract : 1.5
| **Yeast extract** | : 1.5 |
| **Sodium chloride** | : 5.00 |
| **Agar** | : 15 |
| **Distilled water** | : 1000 mL |

**Nutrient broth (pH 7.4)**

- Peptone : 5.0
- Beef extract : 1.5
- Yeast extract : 1.5
- Sodium chloride : 5.0
- Distilled water : 1000 mL

**Rose Bengal agar (pH 7.2)**

- Papaic digest of soybean meal : 5.00
- Dextrose : 10.00
- Monosodium phosphate : 2.00
- Magnesium sulphate : 0.50
- Rose Bengal : 0.50
- Agar : 15.0
- Distilled water : 1000 mL

**Soft agar (pH 7.4)**

- Peptone : 5.0
- Beef extract : 1.5
- Yeast extract : 1.5
- Sodium chloride : 5.0
- Agar : 7.0
- Distilled water : 1000 mL

**S9 mix (rat liver microsomal enzymes + cofactors)**

- Rat liver S9 (aroclor 1254 induced) : 2 mL
- MgCl₂-KCl salts : 1.0 mL
- 1 M glucose-6-phosphate : 0.25 mL
- 0.1 M NADP : 2.0 mL
- 0.2 M phosphate buffer, pH 7.4 : 25.0 mL
- Distilled water : 19.75 mL
Top agar for mutagenicity assay

Sodium chloride : 0.50 g  
Agar : 0.60 g  
Distilled water : 100 mL

0.5 mM histidine/ biotin solution (10 mL) was added to 100 mL top agar.

Staining reagents

Crystal violet

Solution A

Crystal violet (90% dye content) : 2.0 g  
Ethyl alcohol (95%) : 20.0 mL

Solution B

Ammonium oxalate : 0.8 g  
Distilled water : 80 mL

Gram’s Iodine

Iodine : 1.0 g  
Potassium iodide : 2.0 g  
Distilled water : 300 mL

Safranin

Safranin (2.5% solution in 95% ethyl alcohol) : 10 mL  
Distilled water : 100 mL

All the media were sterilized by autoclaving at 121 °C at 15 p.s.i for 20 min. Heat labile components were sterilized by filtration through 0.22 µm membrane filter and added to the respective media only after cooling them to ≈ 55 °C.

Preparation of important buffers and solutions

Glucose solution (40%)  

Glucose : 40 g  
Distilled water : 100 mL

Histidine/ Biotin solution for mutagenicity assay (0.5 mM)

D- Biotin : 30.0 mg  
L- Histidine HCl : 24.0 mg  
Distilled water : 250 mL
Magnesium sulfate solution (0.01 M, pH 8.5)
Magnesium sulfate : 2.4 g
Distilled water : 1000 mL

Marker dye
Glycerol : 50%
Bromophenol blue : 0.05%
EDTA : 40 mM

Nicotinamide dinucleotide solution (10 mM)
Nicotinamide dinucleotide : 7.09 mg
Distilled water : 1 mL

Normal saline solution (NSS)
Sodium chloride : 0.85 g
Distilled water : 100 mL

Potassium chloride solution (0.15 M)
Potassium chloride : 5.59 g
Distilled water : 500 mL

Potassium ferricyanide solution (2.4% w/v)
Potassium ferricyanide : 2.4 g
Distilled water : 100 mL

Sodium bicarbonate solution (0.5 N)
Sodium bicarbonate : 4.2 g
Distilled water : 100 mL

4 Aminoantipyrine (4-AAP) solution (0.6% w/v)
4 Aminoantipyrine : 0.6 g
Distilled water : 100 mL

TAE buffer (50X, pH 8.0)
Tris Base : 242 g
Acetic acid (glacial) : 57.1 mL
0.5 M EDTA : 100 mL
Total volume : 1000 mL

TE (pH 8.0)
Tris.HCl : 10 mM
EDTA : 1 mM
Bacterial strains used in the study

The characteristics of *Salmonella typhimurium* and *Escherichia coli* K-12 strains are given in Table 1. The *Salmonella typhimurium* strains were kindly provided by Prof. T. Nohmi (National Institute of Hygienic Sciences, Division of Genetics and Mutagenesis, Japan). The strains were maintained in frozen stocks and grown as described by Maron and Ames (1983). Each strain was tested on the basis of associated genetic markers raising it from a single colony from the master plate (Maron and Ames, 1983). *Escherichia coli* K-12 strains were obtained from B. J. Barbara (*E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, USA). The bacterial strains of *E. coli* K-12 were always plated on nutrient broth containing 1.5% (w/v) agar.

Collection of wastewater and soil samples

Kashipur located at 29°22' N and 78°95' E in Uttarakhand (India) is becoming an emerging industrial centre with many large industries along with several pulp and paper mills. A total of 12 samples were collected from August 2012 to July 2015. Wastewater samples were collected as described in Standard Methods (APHA, 2005) from discharging sites of pulp and paper mills, Kashipur (Uttarakhand). Composite soil samples were also collected according to the method of Aleem and Malik (2003). Two sites were selected for collection of samples, one site was the agricultural soil irrigated with ground water (site I), while other soil was irrigated with wastewater from pulp and paper industries (site II).

Preparation of S9 (mammalian liver microsomal fraction)

Rat liver microsomal fraction was prepared as described by Garner *et al.* (1972) and Maron and Ames (1983).
Table 1. Characteristics of *Salmonella typhimurium* and *Escherichia coli* K-12 strains used in the study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant Genetic Markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ames Tester Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA97a</td>
<td><em>uvrB, hisD661, bio, rfa</em>, R-factor plasmid-pKM101, frame shift mutation at G-C site</td>
<td>T. Nohmi, National Institute of Hygienic Sciences, Division of Genetics and Mutagenesis, Tokyo, Japan</td>
</tr>
<tr>
<td>TA98</td>
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</tr>
<tr>
<td>TA102</td>
<td><em>rfa</em>, R-factor plasmid-pKM101, multicopy plasmid paQ1 containing hisG428 auxotrophic marker and Tet', transition mutation at A-T site</td>
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</tr>
<tr>
<td>TA104</td>
<td><em>uvrB, hisG428, rfa</em>, R-factor plasmid-pKM101, transition mutation at A-T site</td>
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</tr>
<tr>
<td><strong>Escherichia coli K-12 strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB1157</td>
<td><em>thi-1, argE3, thr-1, leuB6, proA2, hisG4, lacY1, F', Str', λ</em></td>
<td><em>E. coli</em> Genetic Stock Center, MCD Biology Department, Yale University, New Haven, USA</td>
</tr>
<tr>
<td>AB2463</td>
<td><em>recA13, thi-1, argE3, thr-1, leuB6, proA2, hisG4, F', Str', λ</em></td>
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<tr>
<td>KL403</td>
<td><em>polA1, ara-14, lex86, lac2236, proC32, hisF860, ThyA54, RspE2115, rsp2109, malA38, xyl-5, mtl-1</em></td>
<td></td>
</tr>
</tbody>
</table>
Introduction

Pulp and paper industries employ variety of raw materials ranging from forest based wood to agricultural residues including bagasse, wheat and rice straw. These are lignocellulosic material and lignin degradation during pulp and paper making generate both chlorinated and non-chlorinated phenolic compounds (Mckague, 1981; Tiku et al., 2010; Otero et al., 2013). These lignin derived macromolecular compounds are responsible for the hazardous effects of untreated discharged effluents from the pulp and paper industry in receiving waters. A large amount of chemicals is used during pulping and bleaching of paper and pulp manufacture, especially sodium hydroxide, solvents and chlorine compounds, when released along with effluent result in high BOD, COD and coloured water. The discharge consists of potentially toxic chlorinated compounds, degraded lignin compounds, tannins, resin acids and sulphur compounds present both in dissolved form and as suspended solids (Pokhrel and Viraraghavan, 2004). Toxicity of paper mill effluent and its potential adverse effects on aquatic biota have been widely studied (Ali and Srikrishnan, 2001; Lammer et al., 2009; Mench and Bes, 2009; Orrego et al., 2011; Oakes et al., 2013). A reduced reproductive activity and occurrence of intersex is well documented with fishes exposed to toxicants of pulp and paper mill effluent (Munkittrick et al., 1994; Pollock et al., 2010; Chiang et al., 2011, 2015; Costigan et al., 2012).

Water pollution all over the world has become a very serious issue as all the human activities are directly or indirectly related to water (Hu and Cheng, 2013; Kumar and Saroj, 2014). It supports every form of life, and human being are dependant entirely on water for household, recreation, agriculture, fishing and other commercial activities. More than 600 organic and inorganic pollutants from the industrial discharge have been reported to deteriorate water quality. Contamination by heavy metals has worsened the condition; therefore, there is ever increasing demand for improving the water quality (Ahmaruzzaman, 2011; Abdel-Satar et al., 2017, Saha et al., 2017). On the other hand, soil is a key component of terrestrial ecosystem acting as important factor of terrestrial biota and serve as nutrient reservoir supporting almost every life form by nutrient recycling. Anthropogenic activities have led to the contamination of soils with xenobiotics and heavy metals (Singh et al., 2002; Orta-Garcia et al., 2016) so periodic monitoring of the quality of the soils, good practices
for organic residues application and clean water resources near the agricultural area are therefore essentially required (Doran and Zeiss, 2000; Zehra et al., 2015).

The discharge of improperly treated paper and pulp industry wastewater into the natural water has caused great concern over several decades because of the presence of harmful organochlorines which have proven to be recalcitrant and toxic in nature (Butler and Pont, 1992; Mahmood et al., 2014; Kafilzadeh, 2015). Chlorophenolics are among such group which are formed during chlorination of pulp during bleaching where phenolic units of lignin reacts with chlorine atom and their long term exposure have been reported to cause significant damage to flora and fauna (Michaleowicz, 2005; Manderville and Pfohl-Leszkowicz, 2006; Lindholm-Lehto et al., 2015a).

This chapter deals with the physico-chemical analysis of wastewater and soil in the vicinity of pulp and paper industries. Quantitative determination of heavy metals by atomic absorption spectrophotometry and organic compounds were identified by GC-MS. Further microbial diversity of wastewater and soil samples were determined in terms of total aerobic heterotrophs, fungi, actinomycetes and asymbiotic nitrogen fixers.

Materials and Methods

Sample collection

Wastewater and soil samples were collected as described earlier (Chapter II: General materials and methods).

Physicochemical analysis of wastewater and soil

Physicochemical analysis of wastewater and soil samples were carried out as described by Gupta (2000).

Heavy metal analysis of wastewater and soil samples

Twenty-five mL of wastewater were digested with nitric acid: perchloric acid as described in Standard Methods (APHA, 2005). The soil samples were oven dried (40 °C), finely ground (< 0.1 mm), and burned into ashes in a crucible. Ashes of 1 g of soil were taken in a conical flask. The samples were moistened with 1 ml of double-distilled water. Then concentrated HCl and HNO₃ were successively added in a 3:1 ratio. The flask was heated gently on a heating plate until the sample was digested, indicated by the formation of clear solution above the soil residue. The mixture was reduced to a volume of 1 ml. Double-distilled water was added to make up the volume of the filtrate to 100 ml and filtered through Whatman filter paper nos.
1 and 42. Digested samples were analyzed for heavy metals by atomic absorption spectrophotometer (GBC 932 Plus, Australia).

**Identification of organic compounds by GC-MS**

Wastewater samples were extracted separately with two different organic solvents, namely dichloromethane (DCM) and n-hexane (HPLC grades, SRL, India.) as described in Standard Methods (APHA, 2005). Extraction of the wastewater with a solvent was done with 500 ml of effluent, which was shaken vigorously with 25 ml of the extraction solvent in a separating funnel. The process was repeated thrice, each time using 25 ml fresh solvent. When solvent and water layers were separated, the solvent layer was collected in a beaker. The extracted organic phase was evaporated at room temperature under reduced pressure with the help of vacuum pump and made up to 5 ml. These extracts were filtered through 0.22 μm membrane filter before they were used for GC-MS analysis.

Extraction of soil with different solvents (hexane and DCM) was done according to the method of Knize et al. (1987). Ten grams of soil was extracted with 10 ml of the extraction solvent. The extracts were centrifuged at 7000 rpm for 10 min. The extracts were evaporated to dryness and then re-dissolved in 1 ml of DMSO (SRL, India). These extracts were filtered sterilized through 0.22 μm filter and stored at -20 °C until testing was completed.

**Enumeration of microbial population**

Microbial counts i.e. total aerobic heterotrophs, fungi, actinomycetes and asymbiotic nitrogen fixers were determined by plating on different selective media. Serial dilutions of wastewater and soil samples were made in normal saline solution. The standard spread plate technique was followed according to Adesemoye et al. (2006). Aerobic heterotrophic bacteria were counted after 24 h of incubation at 30 °C on nutrient agar medium. Fungi were grown at 28 °C for 5 days on Rose Bengal agar medium. Actinomycetes and asymbiotic nitrogen fixers were grown at 30 °C for 2-4 days on Kenknight & Munaier’s medium and Jensen’s medium, respectively. Microbial counts were also determined on pentachlorophenol (PCP) amended plates in the concentration ranging from 25 to 100 μg mL⁻¹ as described by Tripathi et al. (2011b).

**Results**

Physico-chemical analyses of wastewater and soil samples are presented in Table 1. The wastewater was found to be yellowish brown in colour and suspended solids were found to be 2384 ± 70.0 mg L⁻¹, and total dissolved solids were 528.6 ±
50.0 mg L\(^{-1}\). The COD of the effluent was found to be 1312 ± 55.4 mg L\(^{-1}\) (Table 1). The atomic absorption spectrophotometric (AAS) analysis of wastewater revealed the presence of several heavy metals such as Cr, Cd, Cu, Zn, Ni and Pb at the concentration of 1.28, 0.29, 0.9, 17.8, 3.0 and 5.0 mg L\(^{-1}\) respectively. Agricultural soils irrigated with wastewater also contained several heavy metals. The concentration of Cr, Cd, Cu, Zn, Ni and Pb was found to be 22.36, 12.53, 15.3, 37.26, 2.23 and 5.3 mg Kg\(^{-1}\) in contaminated soil (Table 1). Agricultural soil irrigated with ground water contained low concentration of heavy metals as compared to contaminated soil and concentration of Cr, Cd, Cu, Zn, Ni and Pb was found to be 2.36, 1.53, 5.3, 17.6, 1.4 and 1.4 mg Kg\(^{-1}\) respectively.

The GC-MS analysis of the wastewater and soil samples are presented in Table 2-3. All the extracts showed the presence of phthalic acid and its ester. The Hexane and DCM extracts of wastewater revealed the presence of various long chain and aromatic hydrocarbons i.e. bis-phthalate, diphenyl ether, nonanediol, ethyl propyl disulphide, phenyl methyl carbamate, heneicosane, tetracosane and tetracontane etc. (Table 2). Similarly the GC-MS analysis of hexane and DCM fraction of soil samples revealed the presence of aliphatic and aromatic hydrocarbons including phthalates, diphenyl ether, decanoic acid, nonyl phenol diethoxylate, hexadecanoic acid, dodecanoic acid 1,2,3-propanetriylester, tetracontane, pentatriacontane and phenyl methyl carbamate (Table 3).

The microbial counts of wastewater and soil samples is shown in Table 4. The wastewater exhibited rich diversity of aerobic heterotrophic bacteria (12.4×10\(^5\) CFU/mL), fungi (9.3×10\(^3\) CFU/mL), actinomycetes (13.2×10\(^4\) CFU/mL) and asymbiotic N\(_2\) fixers (17.4×10\(^4\) CFU/mL). The microbial count of wastewater and soil samples was found to be decreased with increasing concentration of PCP (0-100 μg mL\(^{-1}\)) and a gradual decline in their colony forming units have been observed. At 100 μg mL\(^{-1}\) of PCP concentration, the count of aerobic heterotrophic bacteria (1.6×10\(^5\) CFU/mL), fungi (1.7×10\(^3\) CFU/mL), actinomycetes (6.6×10\(^4\) CFU/mL) and asymbiotic N\(_2\) fixers (6.6×10\(^4\) CFU/mL) were observed in wastewater (Table 4).

The microbial counts of soil irrigated with ground water were found to be high than the contaminated soil and wastewater. In case of ground water irrigated soil, count of aerobic heterotrophic bacteria, fungi, actinomycetes and asymbiotic N\(_2\) fixers was found to be 4.1×10\(^6\), 3.5×10\(^4\), 16.4×10\(^5\) and 8.2×10\(^5\) colony forming units per gram of soil respectively, whereas the microbial count of contaminated soil for
aerobic heterotrophic bacteria, fungi, actinomycetes and asymbiotic N\textsubscript{2} fixers was $1.4 \times 10^6$, $1.8 \times 10^4$, $6.5 \times 10^5$ and $5.2 \times 10^5$ colony forming units per gram of soil, respectively. A gradual decline of microbial count was also found in soil microbial flora with the increasing concentration of PCP. At 100 $\mu$g mL$^{-1}$ of the PCP concentration, aerobic heterotrophic bacteria, fungi, actinomycetes and asymbiotic nitrogen fixers and were reduced to $0.6 \times 10^6$, $0.2 \times 10^4$, $1.6 \times 10^5$ and $2.6 \times 10^5$ CFU/g of soil respectively in contaminated soil (Table 4).

**Discussion**

Physico-chemical parameters of wastewater such as COD, TDS and SS were analysed and found to be high as compared to maximum permissible limits as listed in Minimum National Standards (MINAS), and Central Pollution Control Board (CPCB, 1993). Various heavy metals have also been detected in wastewater and soil samples which were beyond the maximum permissible limit (EPA, 2002). The elevated concentrations of heavy metals may be attributed to the adoption of irrational agricultural practices like irrigation of agricultural fields with industrial discharges, excessive application of pesticides etc. (Gimeno-Garcia et al., 1996; Alloway, 2013; Chen et al., 2015). The concentration of Cr, Cd, Cu, Zn, Ni and Pb in test wastewater samples was found to be 1.28, 0.29, 0.9, 17.8, 3.0 and 5.0 mg L$^{-1}$ respectively. Raj et al. (2014) reported Cu, Ni and Zn to be 0.09, 5.03 and 9.83 mg L$^{-1}$ respectively in the pulp mill effluent. Thippeswamy et al. (2012) reported the concentration of Cu, Cr, Ni, Zn, Pb and Cd to be 8.01, 63.15, 3.76, 3.64, 1.92 and 1.72 mg L$^{-1}$ respectively from untreated effluent of Mysore paper industry. In the present study the concentration of Cr, Cd, Cu, Zn, Ni and Pb was found to be 22.36, 12.53, 15.3, 37.26, 2.23 and 5.3 mg Kg$^{-1}$ respectively in contaminated soil. Khan et al. (2013) found maximum concentrations of Cd Cr, Cu, Mn, Ni and Zn to be 0.14, 0.81, 0.61, 10.05, 0.77 and 0.59 mg Kg$^{-1}$ respectively in contaminated agricultural soil. Pandey et al. (2016) found heavy metal concentration to be in the range of 26.0 – 71.98, 0.01 – 0.26, 21.45 – 50.99, 28.38 – 68.76, 31.62 – 40.21, and 0.01 – 1.30 mg Kg$^{-1}$ of Cr, Cd, Cu, Zn, Ni, and Pb respectively in agricultural soil irrigated with paper mill effluent. Chandra et al. (2017) reported the concentration of Zn, Cu, Cr, Cd, Ni and Pb to be 13.90, 2.15, 2.30, 0.25, 3.30 and 1.50 mg Kg$^{-1}$ respectively in the sludge of pulp mill, whereas the concentration of Zn, Cu, Cr, Cd and Ni was found to be 0.27, 0.09, 0.11, 0.02 and 0.19 mg L$^{-1}$ in water extractable leachates. Our results are similar to those of previous findings, where excessive metal concentrations were
detected in soil and wastewaters contaminated with industrial discharges (Singh et al., 2008; Hazarika et al., 2017; Carolin et al., 2017; Oyetibo et al., 2017; Islam et al., 2017). Pulp and paper industry fall under the category of those industries which release heavy metals in their effluents as they utilize variety of complexing agents like ethylenediamine tetra acetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA) which show excellent ability to form stable and water-soluble complexes with many metal ions. Transition metals such as manganese, iron and copper are also utilized in these industries at high concentrations as they catalyze the decomposition of oxygen based bleaching agents and their excessive use cause mobilization of heavy metals in the natural waters and eventually its retention in soil (Metsarinne et al., 2005).

GC-MS have been extensively used previously to determine organic contaminants in wastewater and soil (Rogers, 1996; Lacorte et al., 2003; Latorre et al., 2005; Harrison et al., 2006; Manamsa et al., 2016; Haleyur et al., 2016; Chandra et al., 2017). The persistent organic pollutant generated from pulp and paper industry wastewater and contaminated soil revealed the presence of several endocrine disrupting constituents such as phthalate, diphenyl ether, nonanediol, ethyl propyl disulphide, phenyl methyl carbamate, nonyl phenol diethoxyilate, hexadecanoic acid, dodecanoic acid 1,2,3-propanetriylester, tetracountane, pentatracontane heneicosane etc. (Table 2-3). Swati et al. (2008) reported the presence of higher alkanes such as nonanes and decanes, fatty acids and esters, phenolic compounds, phathalates, chlorinated compounds and plasticizers by GC-MS in the municipal solid waste and leachates. Gupta and Thakur (2015) detected the presence of a diverse group of chemical compounds such as pharmaceuticals, personal care products, sterols, surfactants and plasticizers in wastewater from sewage treatment plant. They have also identified several contaminants such as aliphatic and alicyclic hydrocarbons like undecane, dodecane, cyclohexane, 1,54-dibromotetrapentacontane, propanoic acid, 3,3’-thiobis-, didodecyl ester, fatty acid esters such as 9-octadecenoic acid, 1,2,3-propanetriyl ester, 9-hexadecenoic acid, eicosyl ester, hexadecyl ester oleic acid, eicosyl ester, octadecanoic acid, octadecyl ester, and the presence of bisphenol, haloalkanes such as 1-chlorohexadecane, 1-bromodocosane, pentadec-7-ene, 7-bromomethyl and chloroacetate such as dichloroacetic acid, 4-hexadecyl ester is indicative of their recalcitrant and persistence nature. Al Saleh et al. (2017) identified bisphenol A (BPA) and six phthalate esters (PAEs), namely dimethyl phthalate
(DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), bis (2-ethylhexyl) phthalate (DEHP) and dioctyl phthalate (DOP), in secondary- and tertiary-treated wastewater collected from water treatment plants using headspace SPME followed by GC-MS.

The European Union and the United States Environmental Protection Agency (US EPA) have considered many phthalates in the list of priority toxic substances (Xu et al., 2010). Exposure to phthalates has shown alarming damaging effect on male reproductive system of both humans and animals. The detrimental effects include decreased sperm count, infertility, cryptorchidism, hypospadias, testicular dysgenesis syndrome, and other disorders (Ema and Miyawaki, 2002; Lin et al., 2011). Other compounds such as tetracosane, carbamate, diphenyl ether etc have shown to be persistent organic pollutant, toxic and/or carcinogenic for humans (Balaji et al., 2006; Pietron and Malagocki, 2017).

The microbial population of soil vary with environmental conditions such as nutrient availability, soil texture, and type of vegetation cover (Nannipieri et al., 2003; Allen and Schlesinger, 2004). Soil microorganisms contribute to soil structure and are important in maintaining soil fertility (Yao et al., 2000; Kirk et al., 2004). Therefore the study of microbial community and their catabolic function is an important aspect in assessing soil health. The microbial structural diversity of soil helps to describe a soils capacity to recover from perturbations and functional assessments give insight into the carbon and nutrient cycling capacities (Griffiths et al., 2001; Tripathi et al., 2011a). Heavy metals and persistent organic pollutants may pose adverse impacts on soil microbial community structure, biomass, and activities (Rajapaksha et al., 2004; Lenart-Boron and Boron, 2014; Klimek et al., 2016, Xie et al., 2016). The microbial diversity of the soil irrigated with ground water showed the highest number of colony forming units of aerobic heterotrophic bacteria, fungi, actinomycetes, and asymbiotic N₂ fixers as compared to the contaminated soil (irrigated with the wastewater) (Table 4). The probable reason for the decline in the microbial population may be associated with the detrimental effect of toxic contaminants present in the effluent. The effects of discharge of pulp mill effluent directly into the surrounding water sources and its influence on bacterial community have been discussed previously (Fulthorpe et al., 1993; Tripathi et al., 2014; Chiellini et al., 2014; Guo et al., 2016). Tripathi et al. (2014) reported diverse population structure and function for the bacterial communities from soil irrigated with effluent in comparison to fields irrigated with
well water, this may be due to increase in organic matter content in soil. Several workers have reported that the wastewaters or sludge generated from paper making processes could be effective incubators of a wide variety of resistant adapted bacteria due to the presence of lignin, cellulose, hemicellulose, lipids, in addition to some potentially toxic compounds such as chlorinated organics, resin acids, heavy metals and others (Raj et al., 2007b; Karn et al., 2010; Maki et al., 2011; Karn et al., 2013). Ghribi et al. (2016) studied microbial diversity in various types of paper mill sludge, and isolated 41 strains encompassing 11 different genera, both biodiversity and enzymatic activities were correlated with sludge composition, and microbial diversity found in press sludge was greater than the diversity of secondary sludge. Guo et al. (2016) screened the microbial communities using denaturing gradient gel electrophoresis and identified bacterial taxa greatly affected by paper mill pollution using next generation sequencing data. They indicated that bacterial communities in downstream sediments were similar to those in paper mill discharge sites, indicating obvious effects of pollution, while bacterial communities in downstream water samples showed similar profiles to those in upstream sites, both being quite different from the bacterial communities in paper mill discharge sites that was possibly because of the short contact period.

Chlorphenols are widely used by the chemical industry as intermediate products for the production of herbicide, fungicide, and insecticide. Owing to their biocide properties, these compounds are widely used for timber protection in pulp and paper industries, and also formed during chlorination, bleaching and pulping of lignocellulosic compounds and pose serious ecological problems as environmental pollutants (Sharma et al., 1997; Garg et al., 2013). Chlorophenols show lipophilic character and this tendency increase with increase in chlorine number and thus they tend to bioaccumulate in living system and exhibit toxicity to biological systems, particularly pentachlorophenol (PCP) and are included in the US EPA list of priority contaminants (Bock et al., 1996; Xu et al., 2012).

Present findings indicated that the pulp and paper industry wastewater and contaminated soil contained several heavy metals (i.e. Ni, Cu, Cr, Pb, Cd and Zn) and a wide variety of organic compounds (aliphatic and aromatic hydrocarbons, organic acids, phthalic acids and its esters). Our findings are suggestive of the idea that physico-chemical status of the system influenced microbial diversity through rendering them properties such as tolerance to undesirable agents. Moreover, the
presence of toxic metals and persistent organic pollutants in the wastewater and soil may cause deleterious effect on the indigenous microbes and lead to survival of resistant microbial population. Furthermore, many sensitive microbial species might have been eliminated by the pollutants and their place is taken by resistant species which have different ecological properties.
Table 1. Physico-chemical analysis of pulp and paper mill wastewater and soil

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ground water irrigated soil (Uncontaminated soil) (mg kg(^{-1}))</th>
<th>Wastewater irrigated soil (Contaminated soil) (mg kg(^{-1}))</th>
<th>Wastewater (mg L(^{-1}))</th>
<th>Permissible limits (EPA(^*))</th>
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<tbody>
<tr>
<td>pH</td>
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<td>6.8 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>5.0-9.0</td>
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<td>Texture</td>
<td>Loamy</td>
<td>Loamy</td>
<td>-</td>
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<tr>
<td>COD</td>
<td>N.D</td>
<td>N.D</td>
<td>1312 ± 55.4</td>
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<td>N.D</td>
<td>2384 ± 70.0</td>
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<td>TDS</td>
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<td>N.D</td>
<td>528.6 ± 50.0</td>
<td>-</td>
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<td>EC (µS/cm)</td>
<td>N.D</td>
<td>N.D</td>
<td>474 ± 4.0</td>
<td>300</td>
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<tr>
<td>Cr</td>
<td>2.36 ± 0.25</td>
<td>22.36 ± 0.30</td>
<td>1.28 ± 0.004</td>
<td>0.05</td>
</tr>
<tr>
<td>Cd</td>
<td>1.53 ± 0.2</td>
<td>12.53 ± 0.56</td>
<td>0.29 ± 0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Cu</td>
<td>5.3 ± 0.05</td>
<td>15.3 ± 0.11</td>
<td>0.9 ± 0.002</td>
<td>0.5</td>
</tr>
<tr>
<td>Zn</td>
<td>17.6 ± 1.1</td>
<td>37.26 ± 1.70</td>
<td>17.8 ± 0.005</td>
<td>2</td>
</tr>
<tr>
<td>Ni</td>
<td>1.4 ± 0.1</td>
<td>2.23 ± 0.20</td>
<td>3.0 ± 0.004</td>
<td>0.1</td>
</tr>
<tr>
<td>Pb</td>
<td>1.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.0 ± 0.006</td>
<td>0.05</td>
</tr>
</tbody>
</table>

COD- Chemical oxygen demand, SS- Suspended solids, TDS-Total dissolved solids, EC- Electrical conductivity, N.D- Not done.

*values specified by USEPA, 2002 (Chandra et al., 2017), applicable only to wastewater.
### Table 2. Compounds identified in pulp and paper mill wastewater using GC-MS

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Compounds identified</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hexane extract</strong></td>
<td>Cyclopropaneoctanoic acid</td>
</tr>
<tr>
<td></td>
<td>Bis (2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td></td>
<td>Diphenylether</td>
</tr>
<tr>
<td></td>
<td>1,9-Nonanediol</td>
</tr>
<tr>
<td></td>
<td>Pyrido[2,3-d] pyrimidine-2, 4 (1H, 3H)</td>
</tr>
<tr>
<td></td>
<td>Phenol,2,5-bis (1,1dimethylethyl)</td>
</tr>
<tr>
<td><strong>DCM extract (acidic)</strong></td>
<td>Hexadecen-1-ol, trans-9-</td>
</tr>
<tr>
<td></td>
<td>Dotriacontane</td>
</tr>
<tr>
<td></td>
<td>9-methylnonadecane</td>
</tr>
<tr>
<td></td>
<td>1-Octadecanol</td>
</tr>
<tr>
<td></td>
<td>Di-n-octyl phthalate</td>
</tr>
<tr>
<td></td>
<td>Tetracosane</td>
</tr>
<tr>
<td></td>
<td>Phathalic acid, isobutoyloactylester</td>
</tr>
<tr>
<td><strong>DCM extract (basic)</strong></td>
<td>Phenol, 2,4- bis (1,1-dimethylethyl)-</td>
</tr>
<tr>
<td></td>
<td>Di-n-octyl phthalate</td>
</tr>
<tr>
<td></td>
<td>Pheny l N-methylcarbamate</td>
</tr>
<tr>
<td></td>
<td>1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester</td>
</tr>
<tr>
<td></td>
<td>Nonadecane</td>
</tr>
</tbody>
</table>
Table 3. Compounds identified in soil samples using GC-MS

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Compounds identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane extract</td>
<td>1,2. Benzene dicarboxylic acid iso octyl ester</td>
</tr>
<tr>
<td></td>
<td>Bis (2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td></td>
<td>2-(3-acetooxy 4,4,1trimethylandrost,8-ene-17-yl)</td>
</tr>
<tr>
<td></td>
<td>Propanoic Acid</td>
</tr>
<tr>
<td></td>
<td>1,2. Benzene dicarboxylic acid bis (2-methylpropyl) ester</td>
</tr>
<tr>
<td></td>
<td>Phthalic Acid diisobutyl ester</td>
</tr>
<tr>
<td></td>
<td>Diphenylether</td>
</tr>
<tr>
<td></td>
<td>Decanoic acid</td>
</tr>
<tr>
<td></td>
<td>1,2. Benzene dicarboxylic acid dioctylester</td>
</tr>
<tr>
<td></td>
<td>NonylPhenol diethoxylate</td>
</tr>
<tr>
<td></td>
<td>Phenol 2,4 bis-(1,1-dimethylethyl)</td>
</tr>
<tr>
<td>DCM fraction</td>
<td>1,2. Benzene dicarboxylic Acid bis (2-methylpropyl) ester</td>
</tr>
<tr>
<td></td>
<td>Dodecanio Acid 1,2,3-propanetriylester</td>
</tr>
<tr>
<td></td>
<td>Phthalic acid diisobutyl ester</td>
</tr>
<tr>
<td></td>
<td>Tetracontane</td>
</tr>
<tr>
<td></td>
<td>9-(2’, 2’-Dimethylpropanoilhydrazono-3,6 dichloro-2,4 bis-(diethyl amino)-ethoxy fluorine</td>
</tr>
<tr>
<td></td>
<td>17 Pentatriaconatene</td>
</tr>
<tr>
<td></td>
<td>Phenyl N-methylcarbamate</td>
</tr>
<tr>
<td></td>
<td>Hexadecanoic Acid</td>
</tr>
</tbody>
</table>
Table 4. Microbiological characteristics of wastewater and soil

<table>
<thead>
<tr>
<th>Samples</th>
<th>Microbial population (CFU/g of soil or CFU/mL of wastewater)</th>
<th>Pentachlorophenol concentration (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Agricultural soil irrigated with ground water</td>
<td>Bacteria (×10⁶)</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Fungi (×10⁴)</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Actinomycetes (×10⁵)</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Asymbiotic N₂ fixers (×10⁵)</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>Agricultural soil irrigated with wastewater</td>
<td>Bacteria (×10⁶)</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Fungi (×10⁴)</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Actinomycetes (×10⁵)</td>
<td>6.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Asymbiotic N₂ fixers (×10⁵)</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Pulp and paper mill wastewater</td>
<td>Bacteria (×10⁵)</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Fungi (×10³)</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Actinomycetes (×10⁴)</td>
<td>13.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Asymbiotic N₂ fixers (×10⁴)</td>
<td>17.4 ± 1.7</td>
</tr>
</tbody>
</table>

± Standard deviation.
Introduction

Halogenated aromatic compounds are extensively used in the preparation of herbicides, pesticides and general biocides (Zouari et al., 2002; Colombo et al., 2015). Of these, 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol (2,4,5-TCP) and pentachlorophenol (PCP) are listed as priority contaminants in the European Union (EU) and US Environmental Protection Agency (US EPA) list (EPA, 1999; 2000; European Environment Agency, 2007). Their widespread use has attracted several criticisms for causing damages to the environment and to living organisms (Igbinosa et al., 2013). The physicochemical characteristics of these aromatic compounds show high mobility, high boiling and melting point and possess organoleptic properties thereby cause severe odour and taste problems even at very low concentrations. These compounds are known for their toxic, carcinogenic and recalcitrant nature (Haggblom, 1992; Ikehata and Nicell, 2000; Marsolek et al., 2007; Kotresha and Vidyasagar, 2008; Singh et al., 2009; Ge et al., 2017), and formed during bleaching of pulp with the chlorine in paper making (Annachhatre and Gheewala, 1996; Czaplicka, 2004; Chandra et al., 2009), and as breakdown products of herbicides such as 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic thereby yielding 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) (Wang and Chu, 2012; Conte et al., 2017).

Various physicochemical methods have been applied for the removal of these harmful contaminants such as photo-fenton reaction precipitation/coagulation, osmosis, ion-exchange, ultrafiltration, electrodialysis, electrochemical degradation, flotation (Gernjak et al., 2003; Herath et al., 2011; Watson et al., 2012; Hussain et al., 2013; Gill et al., 2014) quick adsorption on the activated sludge (Zhao et al., 2008), adsorption on carbon blacks (Dominguez-Vargas et al., 2009), adsorption on powdered activated carbon and pyrolysed rice husk (Loo et al., 2010). The physicochemical treatment processes are generally expensive, tedious and not efficient; biological treatment have proven to be better for being efficient, cost effective and environment friendly in removing or reducing the hazardous pollutants from environmental matrices (Jarvinen and Puhakka, 1994; Lovley, 1995; Agamuthu and Fauziah, 2013; Nawaz and Ahsan, 2014; Zhao et al., 2014; 2015).

Varieties of microorganisms are known to utilize chlorophenols as their sole carbon or energy source (Zilouei et al., 2006; Yang and Lee, 2007; Karn et al., 2011). The environmental parameters such as temperature, pH, oxygen, and nutrient
amendments are important factors which influence the degradation capabilities and survival of microorganisms. These parameters directly influence the extent of mineralization, growth and activity of micro-organisms (Providenti et al., 1993). For successful bioremediation metabolic intermediates formed during chlorophenol degradation must not retard by accumulating as toxic and non-degradable products (Cho et al., 2000). The determination of optimal conditions and monitoring of intermediates are therefore requisite choice for efficient biodegradation of chlorophenols. Many bacterial species have been employed to completely mineralize polychlorinated phenols and have been successfully applied to bioremediate chlorophenol contaminated sites (Barbeau et al., 1997; Mrozik and Seget, 2010; Caliz et al., 2011).

Present chapter describes the isolation and characterization of chlorophenol degrading bacteria from pulp and paper industry wastewater and agricultural soil irrigated with wastewater. The influence of various physico-chemical parameters were studied in order to determine the optimal conditions for chlorophenol degradation. Further, the possible degradation products of chlorophenol were determined on the basis of Gas chromatography-Mass spectrometry (GC-MS) analysis.

Materials and methods

Sample collection

Wastewater and soil samples were collected as described earlier (Chapter II: General materials and methods).

Chlorophenols and chemicals

Pentachlorophenol (PCP) used in this study was purchased from Sigma-Aldrich (USA), while dichlorophenol (2,4-DCP) and trichlorophenol (2,4,6-TCP) were purchased from Hi media, Mumbai (India). All the other chemicals were of analytical grade.

Tolerance of bacteria to chlorophenols

Enrichment culture technique was used for the isolation of chlorophenol tolerating bacteria using minimal salt medium (MSM) as described by Singh et al. (2011). The minimal salt broth (100 mL) amended with 100 µg mL\(^{-1}\) of PCP was inoculated with 10 mL of wastewater and 10 g of soil samples separately and cultured under shaking condition (200 rpm) at 35 °C for 4 days. 100 µl of the suspension was plated on the minimal salt agar plates containing 100 µg mL\(^{-1}\) of PCP. Following incubation the colonies with different morphology appearing on PCP amended plates
were purified. These bacterial isolates were further screened for their ability to tolerate different chlorophenols (i.e. DCP, TCP and PCP) in the concentration range of 100-1000 µg mL⁻¹ in minimal salt agar medium.

**Amplification and sequencing of 16S rDNA**

Bacterial isolates were identified by sequencing of 16S rDNA. Total DNA was extracted from overnight grown cultures using Bacterial Genomic Miniprep Kit (Sigma, USA) as per manufacturer’s instructions. PCR amplification of 16S rDNA was performed using the universal primers 27f and 1492r (Weisburg *et al*., 1991). PCR amplifications were carried out in a 25 µL reaction volume containing 100 ng of template DNA; 0.5 µM each primers; 10 mM Tris-Cl (pH 8.3); 3.0 mM MgCl₂; dNTP mix (Fermentas, USA) 0.6 mM; and 1.25 U of Taq DNA polymerase (Fermentas, USA). The reaction mixture was heated at 95 °C for 2 min followed by amplification in DNA thermal cycler (BioRad, Germany) programmed for 30 cycles at 95 °C for 30 s, 57 °C for 60 s and 72 °C for 2 min followed by final extension at 72 °C for 10 min. An aliquot (5µL) of each reaction mixture was analyzed by agarose gel electrophoresis (1.5% agarose). The electrophoresed products were stained with ethidium bromide and visualized under UV light. The remaining amplified product was purified (QIAGEN Inc., Valencia, USA) and sequenced bi-directionally (Big Dye, Applied Bio Systems). The identity of the sequence obtained was determined by BLAST (Altschul *et al*., 1990). Sequences were aligned, verified and edited using BioEdit 7.2.0 (Hall, 1999) and Molecular Evolutionary Genetics Analysis (MEGA v6.0) (Tamura *et al*., 2013) software programs. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

**Screening of chlorophenol degrading bacterial isolates**

The bacterial isolates exhibiting high MIC for chlorophenols were tested for their PCP (100 µg mL⁻¹) degrading ability in minimal salt medium with (0.1% w/v) and without glucose at 35 °C for 5 days under shaking condition (200 rpm). After every 24 h, the culture was withdrawn up to 120 h and centrifuged at 8000×g for 20 min. The supernatant obtained was estimated for the residual chlorophenol by aminoantipyrine method as described in Standard Methods (APHA, 2005). The percent degradation was calculated spectrophotometrically by determining the initial absorbance (before degradation) and final absorbance (after degradation).
Effect of different parameters on chlorophenol degradation

In order to determine the effect of different parameters on chlorophenol degradation, the overnight grown culture of bacterial isolates SM1, N1, N6 and W1 were inoculated in 250 ml Erlenmeyer flasks containing 100 ml of MS medium supplemented with 100 µg mL⁻¹ of CP (except for when using different CP concentrations) at 35 °C (except for when at different temperatures), pH 7.0 (except for when at different pH) with continuous shaking at 200 rpm up to 120 h. For degradation studies, each experiment was performed in triplicate. The chlorophenol degradation was performed at different parameters, such as pH (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (25, 30 and 35 °C), shaking speed (0 rpm, 100 rpm and 200 rpm) and concentration (25, 50, 100, 200, 300, 400 and 500 µg mL⁻¹) both in the presence (0.1% w/v) and absence of glucose.

Growth of the bacterial isolates was determined by measuring the optical density at 600 nm and chlorophenol degradation was analysed by reduction in absorbance at 460 nm at different time interval using UV–Vis spectrophotometer (double beam UV-Vis Spectrophotometer UV 5704SS).

Analysis of the degradation products

For biodegradation analysis, minimal salt medium amended with 100 µg mL⁻¹ of the chlorophenols (PCP, 2,4 DCP and 2,4,6 TCP) with (0.1% w/v) and without glucose was incubated with bacterial isolates SM-1, N1, N6 and W1 separately, under shaking condition (200 rpm) at 35 °C. After 120 h of incubation, the culture supernatant was initially acidified to pH 2.0 using 1 N HCl and extracted thrice with ethyl acetate (HPLC grade) as described by Chandra et al. (2009). The extracted upper organic layer containing residual CP was filtered through 0.22 µm filter (Axiva, India) and reduced to 5 mL at room temperature and analyzed by GC-MS.

The analysis of all organic extracts was done using GC-MS (Shimadzu QP2010 Plus). 1 µL of each sample was analyzed by GC-MS at conditions: split-less mode; initial temperature 50 °C held for 6 min; temperature increased from 50 °C to 280 °C at a rate of 5 °C min⁻¹ held for 25 min on reaching 280 °C; The head pressure of the helium carrier gas was 69 kPa; helium flow rate 1.0 ml min⁻¹. The compounds were identified on the basis of mass spectra using the NIST library (National Institute of Standards and Technology). Control samples extracted from minimal salt broth amended with chlorophenols only (i.e. without bacterial isolates) were also analyzed simultaneously.
Results

A total of 40 bacterial strains (25 from agricultural soil irrigated with wastewater and 15 from pulp and paper mill effluent) were isolated from PCP amended minimal agar plates. These bacterial isolates tolerated high concentration of DCP, TCP and PCP (Table 1). Out of 40 isolates tested, 14 exhibited highest minimum inhibitory concentration (MIC) in the range of 400 to 800 µg mL$^{-1}$ were selected for degradation studies. To determine the phylogenetic relationship among the bacterial isolates, gene corresponding to 16S rDNA was successfully amplified and sequenced in all the fourteen bacterial isolates. The partial sequences were aligned with the 16S rDNA sequences of the corresponding sequences in GenBank (Accession no. DQ002384, FJ959366, EF432791, DQ002385, HQ259593) and compared using Bioedit (Version 7.2.0). It was observed that the obtained sequences showed 98-100% identity with the reported sequences on BLAST analysis. Of the 14 isolates tested, 11 were found to be most similar to *Pseudomonas* sp. and 3 with *Klebsiella* sp. A tree representing the phylogenetic relationship among the 16S rDNA was constructed using Neighbor-joining method (Fig. 1) by MEGA (version 6.0). The accession numbers of the partial 16S rDNA sequences (KX353799-KX353811, KF764698) obtained in this study are available at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

All the 14 bacterial isolates were further tested for their ability to degrade PCP in minimal salt broth amended with 100 µg mL$^{-1}$ of PCP, both in the absence and presence of glucose (0.1% w/v) (Fig. 2). The bacterial isolates exhibited different degree of PCP degradation ranging from 55-100% after 120 h of incubation (Fig. 2). Based on the extent of PCP degradation, four bacterial isolates i.e., SM1, N1, N6 and W1 displayed maximum degradation potential were identified as *P. aeruginosa* SM1, *K. oxytoca* N1, *K. oxytoca* N6 and *P. aeruginosa* W1 based on the BLAST analysis of 16S rDNA sequence (Fig. 1).

Time course of growth of the bacterial isolates in minimal salt broth amended with different concentrations of CPs (PCP with *P. aeruginosa* SM1 and *K. oxytoca* N6), TCP with *P. aeruginosa* W1 and DCP with *K. oxytoca* N1 were plotted both in the absence and presence of glucose (0.1% w/v) (Fig. 3 and 4). Isolates grew well in the medium containing different chlorophenols, although the growth of the isolates with glucose showed significantly faster as compared to those without glucose in the minimal medium.
Effect of various physico-chemical parameters on chlorophenol degradation were studied with PCP (*P. aeruginosa* SM1 and *K. oxytoca* N6), TCP (*P. aeruginosa* W1) and DCP (*K. oxytoca* N1). *P. aeruginosa* SM1 showed efficient PCP degradation in the presence of glucose and it was observed that maximum degradation was at pH 7.0. Degradation was found to be 68%, 83%, 100%, 74% and 65% at pH 5.0, 6.0, 7.0, 8.0 and pH 9.0 respectively (Fig. 5a). The optimal temperature for the degradation of PCP was found to be 35 °C (100%) while at room temperature (25 °C) only 56% degradation was observed (Fig. 5b). Degradation process was higher under shaking condition (100% at 200 rpm) compared to static condition (66%) (Fig. 5c). The isolate also grew in the absence of glucose and showed significant amount of degradation (73% at 100 µg mL⁻¹), however co-metabolism was found to enhance the PCP degradation (100% at 100 µg mL⁻¹). It was observed that PCP degradation decreased gradually with increase in the concentration of PCP (100% 100 µg mL⁻¹, 47% at 300 µg mL⁻¹, 30% at 400 µg mL⁻¹ and only 13% at 500 µg mL⁻¹) (Fig. 5d).

*K. oxytoca* N6 exhibited significant PCP degradation in the pH range of 5-9 and temperature range of 25-35 °C. The isolate showed 98% degradation at shaking speed of 200 rpm. Further, the bacterium was found to tolerate high concentration of PCP and significant degradation (53%) was observed at 500 µg·mL⁻¹ of PCP (Fig. 6).

*P. aeruginosa* W1 was selected for the degradation study of 2,4,6 TCP and carried out in the pH range of 5.0-9.0. The optimal pH was found to be 7.0 at which 100% degradation occurred in the presence of glucose (Fig. 7a). The optimal temperature was found to be 30 °C (Fig. 7b). Under static condition degradation was observed to be 64% but increased with shaking condition (72% at 100 rpm, 100% at 200 rpm) (Fig. 7c). Degradation was observed to be 64% in the absence of glucose (100 µg mL⁻¹) which was increased in the presence of glucose as a co-substrate. Further rate of degradation was observed to be inhibited by the increase in the concentration of 2,4,6 TCP (Fig. 7d).

100% degradation was achieved for 2,4 DCP inoculated with the bacterial strain *K. oxytoca* N1 at pH 7.0 and temperature of 35 °C at 200 rpm (Fig. 8). The addition of glucose was favorable and significantly enhanced degradation rate. Complete degradation of 2,4 DCP at 25, 50 and 100 µg mL⁻¹ was recorded, while degradation was found to be 93%, 86%, 74%, and 62% at 200, 300, 400 and 500 µg mL⁻¹ respectively (Fig. 8d).
In order to determine the degradation products of chlorophenols, the ethyl acetate extracted samples were analyzed by GC-MS and the gas chromatograms displayed different peaks as compared to the control samples. Gas chromatogram of the control sample (extracted from minimal salt broth amended with PCP only and without bacteria) showed a single peak with retention time of 15.817 (m/z 265.84) (Fig. 9a), whereas the samples containing the PCP degraded products using *P. aeruginosa* SM1 revealed the presence of several peaks with retention time of 9.53, 10.94, 11.05, 11.97, 12.30 and 20.442 (without glucose) (Fig. 9b) and 5.83, 6.55, 9.53, 11.49, 11.75 and 20.37 (with glucose) (Fig. 9c). From the fragmentation pattern and m/z values obtained by the mass spectral analysis, several intermediate compounds were identified as pentachloroanisole, 2,5-dichloro-1,4-dimethoxybenzene, 3-hydroxy-4-methoxy benzoic acid (in the absence of glucose) and pentachloroanisole, tetrachlorohydroquinone, tetrachloroanisole, 2,5-dichloro-1,4dimethoxy benzene, maleylacetate and 2-hydroxy-muconic-semialdehyde in the presence of glucose (Fig. 10-12).

The GC-MS of culture extracts of the MS medium with PCP and *K. oxytoca* N6 showed different peaks with retention time of 10.01, 10.07, 13.77, 19.16, 19.43 and 23.54, were recorded after 120 h of inoculation (Fig. 13b). The co-metabolism of PCP by the bacterial strain *K. oxytoca* N6 showed more peaks with retention time of 10.01, 10.11, 11.04, 11.81, 12.58, 13.86, 14.98, 15.29, 16.18, 19.23, 20.21, 21.59, 22.69 23.95, 24.84, 25.43, and 26.01 after 120 h of incubation under the same experimental condition (Fig. 13c). The intermediate compounds released from PCP degradation by *K. oxytoca* N6 after dechlorination due to degradation process were identified as dichlorophenol, trichlorophenol, tetrachlorophenol, tetrachlorohydroquinone and some low molecular weight compounds like maleylacetate, 2-hydroxy-muconic-semialdehyde were identified by mass spectra of the compounds (Fig. 14-16).

The intermediate compounds obtained from 2,4,6 TCP degradation after dechlorination with *P. aeruginosa* W1 were identified as 2,4 dichlorophenol, 2,6-dichloro-1,4-benzenediol, 1,2 benzene diol and 2-hydroxy-muconic-semialdehyde (Fig. 17-20), whereas the intermediates released from 2,4 DCP degradation with *K. oxytoca* N1 degradation process were identified as dichlorobenzoquinone, 2 chlorophenol, 5 chloro-2-hydroxymuconic semialdehyde and 5-chloro-2-oxopent-4-enoic acid (Fig. 21-24).
Discussion

The pulp and paper industries and their products have become an important part of modern lifestyle around the world. Pulp and paper manufacturing processes use a variety of chemicals which are constantly being added to the surrounding environments through the release of wastewater. Due to their toxicity and recalcitrance, many of these chemicals are known to be hazardous to animals and humans and are listed as priority contaminants by the US Environmental Protection Agency (EPA, 2000; Zhao et al., 2014; Ge et al., 2017). Chlorinated aromatic derivatives such as chlorophenols are produced during bleaching of wood pulp with chlorine and also as disinfection by-products during chlorination of wastewater and drinking water. They are also used extensively as biocides, wood preservatives and intermediates to the manufacture of herbicides, pesticides, higher chlorinated congeners and dyes and represent serious environmental problem because of their toxicity, persistence and accumulation potential in plants and animal tissues (Igbinosa et al., 2013; Zhao et al., 2014).

In the present study, bacterial strains were isolated from the pulp and paper mill effluents and contaminated soils (Fig.1) and screened for their ability to degrade the chlorophenols. Among chlorophenols, pentachlorophenol (PCP) degradation is the most difficult because of its stable aromatic ring system and the highest chloride content (Okeke et al., 1997). The isolation and screening of chlorophenol degrading microorganisms from sludge samples collected from the pulp and paper mill have been reported previously (Okeke et al., 1997; Mannisto et al., 1999; Louie et al., 2002; Chandra et al., 2009; Singh et al., 2009; Garg et al., 2013; Hayes et al., 2014; Hou et al., 2016) indicating natural adaptation of these microorganisms to survive in the presence of toxic compounds. 16S rDNA sequence analysis, showed 98-100% similarity with the reported sequences on BLAST analysis and were found to belong to two different bacterial genera namely Pseudomonas and Klebsiella, indicating the chlorophenol degrading enzymes distribution across these bacterial species/genera. On the basis of chlorophenol degradation studies, four bacterial isolates viz. P. aeruginosa SM1, K. Oxytoca N1, K. oxytoca N6 and P. aeruginosa W1 were found to be efficient degraders (Fig. 2).

The PCP degradation efficiency of P. aeruginosa SM1 and K. oxytoca N6 is comparable to those reported by Karn et al. (2011) in Kocuria sp. CL2, they showed 95% PCP degradation up to a concentration of 0.37 mM. Similarly, Tewari and
Shukla (2012) demonstrated PCP degradation by various bacterial species and observed that Flavobacterium degraded up to 55% PCP within 42 h of incubation at 20 μg mL\(^{-1}\) of PCP concentration. Effect of nutritional parameters such as carbon (viz., glucose, maltose, sucrose) have previously been tested for bioremediation of chlorophenols (Yang and Lee, 2008; Tripathi and Garg, 2013). However, the maximum simultaneous remediation was evident when glucose was employed as an additional carbon and energy source as it is most easily metabolizable sugar. Tripathi and Garg (2013) have shown 57% PCP removal within 60 h of incubation using Bacillus cereus isolate with 0.4% glucose, whereas Wang et al. (2000) reported 65% removal of 2,4,6 TCP in the presence of phenol as a co-substrate, similarly complete biodegradation of 2,4,6 TCP in the presence of phenol and glycerol as a co-substrate was reported by Andreoni et al. (1998) and Snyder et al. (2006). In our study, efficient removal of 2,4 DCP was observed by K. oxytoca N1 strain which is comparable to those reported earlier (Kargi and Eker, 2004; Matafonova et al., 2006; Sahinkaya and Dilek, 2006a; 2006b). Kargi and Eker (2004) reported 100% DCP removal at the concentration of 30 mg L\(^{-1}\) which dropped to 26 and 12% with the increasing DCP concentrations of 160 and 300 mg L\(^{-1}\) respectively. Matafonova et al. (2007) reported 56% removal of 200 μM of 2,4 DCP by B. cereus within 48 h.

A number of limiting factors have been recognized that affect the degradation of chlorophenols such as pH, temperature, chlorophenol concentration, addition of co-substrates (Liu et al., 2007; Nowak et al., 2016; El-Naas et al., 2017). pH plays an important role in bacterial growth and CP degradation. The optimal pH for CP degradation was found to be 7 and at acidic/alkaline pH, CP degradation was significantly decreased. It has also been observed that pH near neutral range is optimal for PCP degradation for many bacterial species like Arthrobacter, Pseudomonas sp. and Acinetobacter sp. (Edgehill 1994; Wolski et al., 2006; Sharma et al., 2009). Louie et al. (2002) demonstrated 2,4,6-TCP degradation by Ralstonia eutropha at pH 7.

Temperature is another important environmental factor that influence the rate of pollutants degradation (Barbeau et al., 1997) as bacteria require an optimal temperature for their growth and enzyme production. The optimal temperature for the PCP degradation was recorded to be 30 °C in case of P. aeruginosa SM-1 and K. oxytoca N6, the degradation decreased at both higher and lower temperatures after 120 h of incubation (Fig 5b and Fig 6b). The optimum temperature for 2,4,6 TCP
degradation was observed to be 30 °C, while for 2,4 DCP, it was 35 °C. Ren et al. (2016) demonstrated high 2,4-DCP removal ability (>80% removal) between 20 and 35 °C but it was decreased significantly when the temperature was >40 °C. They suggested that as the reaction temperature increased, the chance of a collision between the enzyme and substrate molecules increased and this phenomenon may help to form enzyme–substrate complexes and cause an increase in enzyme activity which could result in increased removal rate of 2,4-DCP. However at very high or low temperature degradation process gets inhibited due to enzyme deactivation.

Degradation was found to be decreased with increasing concentration of CP as a sole carbon source, as high CP concentration does not sustain bacterial cell growth (Leung et al., 1997). This might be due to toxicity of the CP to bacteria that inhibit the metabolic activities at higher concentration and tend to destroy membrane function of microorganisms (Copley, 2000; Scelza et al., 2008). Similar results have also been reported by Sharma et al. (2009) who found that PCP degradation decreases with increasing concentration in Acinetobacter sp. Our isolates grew well in high concentration of 2,4 DCP; 2,4,6 TCP and PCP and utilized it as a sole source of carbon and energy showing significant CP degradation potential (Fig. 5-8).

Chlorophenol degradation is greatly influenced by the presence of co-substrate such as glucose, acetate, glutamate, pyruvate etc as they may generate NADH which is required by oxygenases for aromatic compound degradation (Wang and Loh, 1999). Several workers have also reported that co-metabolism greatly increases the process of CP degradation (Singh et al., 2008; Tripathi and Garg, 2013; Khessairi et al., 2014). In the present study it was observed that supplementation of glucose 0.1% (w/v) greatly enhanced the degradation process with an effective rate (100% degradation at 25, 50, 100 µg mL⁻¹ of CP concentration).

Present results indicated that K. oxytoca N6, P. aeruginosa SM1, P. aeruginosa W1 and K. oxytoca N1 can efficiently degrade PCP, 2,4,6 TCP and 2,4-DCP under co-metabolic condition to simpler compounds (Figs. 9-24). The intermediate compounds as a result of PCP degradation by P. aeruginosa SM1 revealed the presence of pentachloroanisole, 2,5-dichloro-1,4-dimethoxybenzene, 3-hydroxy-4-methoxy benzoic acid while during co-metabolism pentachloroanisole, tetrachlorohydroquinone, tetrachloroanisole, 2,5-dichloro-1,4-dimethoxybenzene, and some low molecular weight compounds like maleylacetate, 2-hydroxy-muconic-semialdehyde were observed (Fig. 11-12). There are few reports on the methylation of
chlorinated phenols by bacterial strains (Neilson et al., 1983; Coque et al., 2003; Ozhan et al., 2007), while fungi have been reported for methylation and biotransformation of pentachlorophenol to pentachloroanisole and its further degradation (Okeke et al., 1997; Rubilar et al., 2007; Feltrer et al., 2010).

The degradation intermediates of PCP by K. oxytoca N6 after GC-MS analysis were identified as 2,6 dichlorophenol, 2,4,6 trichlorophenol, 2,3,4,6 tetrachlorophenol, tetrachlorohydroquinone and some low molecular weight compounds like maleylacetate, 2-hydroxy-muconic-semialdehyde were also observed (Fig. 15-16). Singh et al. (2009) reported formation of 6-chlorohydroxyquinol, tetrachloro-p-hydroquinone and 2,3,4,6- tetrachlorophenol after degradation of PCP in the extracts of degraded samples by bacterial consortium whereas 2,3,5,6-tetrachlorohydroquinone and 2-chloro-1,4 benzenediol were found as intermediate products of PCP degradation by Acinetobacter sp. (Sharma et al., 2009). Garg et al. (2013) identified tetrachloro-p-hydroquinone, 2,4,6-trichlorophenol, and 2,6-dichlorophenol as PCP dechlorination products by Pseudomonas putida.

Present study showed the biodegradation products of 2,4,6 TCP by P. aeruginosa W1 as identified by GC-MS are 2,6 dichlorobenzoquinone, 2,4 dichlorophenol, 2 chlorophenol, catechol and 2-chloro-hex-2-ene-1,6-dioic acid or 2-chloro-maleylacetic acid (Fig. 19-20). These intermediates were also identified and reported previously by many researchers with different bacterial strains (Ma et al., 2001; Louie et al., 2002; Snyder et al., 2006; Khan et al., 2011; Aifie et al., 2015). Hatta et al. (1999) have illustrated the metabolic pathway for 2,4,6 TCP degradation and demonstrated the role of hydroxyquinol 1,2-dioxygenase in converting hydroxiquinol into maleylacteate. Further they have also studied the molecular mechanism and metabolic pathway for 2,4,6 TCP degradation in Ralstonia pickettii DTP0602 (Hatta et al., 2012).

GC-MS analysis of degraded samples of 2,4 DCP by pure cultures of K. oxytoca N1 showed that as compared to control sample different intermediates were identified using the NIST mass spectral database. During co metabolism, complete degradation was observed indicating formation of metabolic intermediates such as 2 chlorophenol, 2 chloro 1,4-benzoquinone, 5 chloro-2-hydroxymuconic semialdehyde, and maleyl acetate (Fig. 23-24). Valli and Gold (1991) reported degradation of 2,4-dichlorophenol via oxidative dechlorination to yield 2,4 dichlorobenzoquinone and 2 chlorobenzoquinone which was later reduced to 2 chloro 1,4 dihydroxy benzoquinone.
by the Fungus *Phanerochaete chrysosporium*. Tomeja *et al.* (2014) reported effective removal of CPs with 2,4-DCP hydroxylase through hydroxylation in the oxidative degradation pathway; the obtained products are less toxic and easily degradable during the subsequent enzymatic reaction.

Chlorophenols are among the most stable, toxic and persistent molecules (Haggblom, 1992; Olaniran and Igbinosa, 2011; Arora and Bae, 2014). Role of dehalogenases and oxygenases are sought for the elimination of chlorine atoms from aromatic compounds (Arora and Bae, 2014), whereas several other enzymes belonging to oxidoreductases, such as 2,4-dichlorophenol hydroxylase (2,4-DCP hydroxylase), peroxidase, and laccase have been utilized to remove CPs through hydroxylation (Vergara-Dominguez *et al*., 2013; Ren *et al*., 2016), while chlorophenol-O-methyltransferase is responsible for methylation of chlorophenols (Coque *et al*., 2003) leading to less toxic and easily degradable products during the subsequent enzymatic reaction.

In the present study, the indigenous bacterial strains having significant ability to degrade chlorophenols were isolated and identified as *P. aeruginosa* and *K. oxytoca* on the basis of 16S rDNA sequencing. Present findings indicated that biodegradation was greatly enhanced by the addition of glucose (0.1% w/v) as a co-substrate. These bacterial strains degraded chlorophenols in a wide range of pH (5-9) and temperature range of 30-35 °C. Further high shaking speed was found to be effective in the biodegradation of chlorophenols. GC-MS analysis revealed the presence of several low molecular weight intermediates as compared to the control samples which confirm the mineralization of chlorinated organic compounds by the bacterial isolates. Our results indicate the remarkable chlorophenol degradation potential by the selected bacterial isolates which could be exploited for the bioremediation of chlorophenols.
Table 1. Minimum inhibitory concentration (MIC) of bacterial isolates from soil and wastewater against chlorophenols

<table>
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<tr>
<th>Bacterial isolates</th>
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Fig. 1. 16S rDNA sequence based phylogenetic tree of bacterial isolates used in the study and related sequences obtained through BLAST analysis.
**Bacterial isolates**

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- with glucose
- without glucose

**Fig. 2.** PCP reduction in the presence and absence of glucose by bacterial isolates
Fig. 3. Time course of growth of the bacterial isolates in the presence of different concentration of chlorophenols CP’s: (a) PCP with Pseudomonas aeruginosa SM1 (b) PCP with Klebsiella oxytoca N6 (c) 2,4,6 TCP with Pseudomonas aeruginosa W1 (d) 2,4 DCP with Klebsiella oxytoca N1.
Fig. 4. Time course of growth of the bacterial isolates in the presence of glucose (0.1% w/v) and different concentration of chlorophenols CP’s: (a) PCP with Pseudomonas aeruginosa SM1 (b) PCP with Klebsiella oxytoca N6 (c) 2,4,6 TCP with Pseudomonas aeruginosa W1 (d) 2,4 DCP with Klebsiella oxytoca N1.
Fig. 5. Effect of different parameters on PCP degradation (a) pH (b) temperature (c) shaking speed and (d) initial PCP concentration by *Pseudomonas aeruginosa* SM1.
Fig. 6. Effect of different parameters on PCP degradation (a) pH (b) temperature (c) shaking speed and (d) initial PCP concentration by *Klebsiella oxytoca* N6.
Fig. 7. Effect of different parameters on 2,4,6 TCP degradation (a) pH (b) temperature (c) shaking speed and (d) initial 2,4,6 TCP concentration by *Pseudomonas aeruginosa* W1.
Fig. 8. Effect of different parameters on 2,4 DCP degradation (a) pH (b) temperature (c) shaking speed and (d) initial 2,4 DCP concentration by *Klebsiella oxytoca* N1.
Fig. 9. Gas chromatograms: (a) pentachlorophenol only.
Fig. 9. Gas chromatograms: (b) degradation products of pentachlorophenol by *Pseudomonas aeruginosa* SM1 after 120h of incubation.
Fig. 9. Gas chromatograms: (c) degradation products of pentachlorophenol in the presence of glucose (0.1% w/v) by Pseudomonas aeruginosa SM-1 after 120h of incubation.
Fig. 10. Mass spectra of the peaks detected in gas chromatogram of pentachlorophenol.
Fig. 11. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol by *Pseudomonas aeruginosa* SM1.
Fig. 12. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol in the presence of glucose (0.1% w/v) by Pseudomonas aeruginosa SM1.
Fig. 13. Gas chromatograms: (a) pentachlorophenol only.
**Fig. 13.** Gas chromatograms: (b) degradation products of pentachlorophenol by *Klebsiella oxytoca* N6 after 120h of incubation.
Fig. 13. Gas chromatograms: (c) degradation products of pentachlorophenol by *Klebsiella oxytoca* N6 in the presence of glucose (0.1% w/v) after 120h of incubation.
Fig. 14. Mass spectra of the peaks detected in gas chromatogram of pentachlorophenol.
Fig. 15. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol by *Klebsiella oxytoca* N6.
Fig. 16. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol in the presence of glucose (0.1% w/v) by *Klebsiella oxytoca* N6 (Fig. 16. Continues on next page).
Fig. 16. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol in the presence of glucose (0.1% w/v) by *Klebsiella oxytoca* N6 (Fig. 16. Continues on next page).
Fig. 16. Mass spectra of the peaks detected in gas chromatogram of the degraded products of pentachlorophenol in the presence of glucose (0.1% w/v) by *Klebsiella oxytoca* N6.
Fig. 17. Gas chromatograms: (a) 2,4,6 trichlorophenol only.
Fig. 17. Gas chromatograms: (b) degradation products of 2,4,6 trichlorophenol by *Pseudomonas aeruginosa* W1 after 120h of incubation.
Fig. 17. Gas chromatograms: (c) degradation products of 2,4,6 trichlorophenol in the presence of glucose (0.1% w/v) by *Pseudomonas aeruginosa* W1 after 120h of incubation.
Fig. 18. Mass spectra of the peaks detected in gas chromatogram of 2,4,6 trichlorophenol.
Fig. 19. Mass spectra of the peaks detected in gas chromatogram of the degraded products of 2,4,6 trichlorophenol by *Pseudomonas aeruginosa* W1.
Fig. 20. Mass spectra of the peaks detected in gas chromatogram of the degraded products of 2,4,6 trichlorophenol in the presence of glucose (0.1% w/v) by *Pseudomonas aeruginosa* W1.
Fig. 21. Gas chromatograms: (a) 2,4 dichlorophenol only.
Fig. 21. Gas chromatograms: (b) degradation products of 2,4 dichlorophenol by *Klebsiella oxytoca* N1 after 120h of incubation.
Fig. 21. Gas chromatograms: (c) degradation products of 2,4 dichlorophenol by *Klebsiella oxytoca* N1 in the presence of glucose (0.1% w/v) after 120h of incubation.
Fig. 22. Mass spectra of the peaks detected in gas chromatogram of 2,4 dichlorophenol.
Fig. 23. Mass spectra of the peaks detected in gas chromatogram of the degraded products of 2,4 dichlorophenol by *Klebsiella oxytoca* N1.
Fig. 24. Mass spectra of the peaks detected in gas chromatogram of the degraded products of 2,4 dichlorophenol in presence of glucose (0.1% w/v) by *Klebsiella oxytoca* N1.
Introduction

Water is fundamental need required for quality of life, economic growth and for environment. With world facing booming economy and growing population, urbanization has lead towards increased industrial activity and thus contamination of aquatic environments via atmospheric deposition and effluents discharge. Pulp and paper industry ranks sixth among largest polluter (after oil, cement, leather, textile, and steel industries) thereby discharging variety of gaseous, liquid, and solid wastes into the environment (Haahtela et al., 1992; Ali and Sreekrishnan, 2001; Ebrahimi et al., 2015).

During paper making process, raw materials such as wood chips, bamboo, grasses, straw, reeds and bagasse are mechanically and chemically pulped by cooking in a mixture of water, sodium hydroxide, sodium sulphate and sodium carbonate (Puitel et al., 2015; Gonzalo et al., 2017). The pulp is then bleached using chlorine or similar oxidising agents (Bajpai, 2012a; Li et al., 2015). When these effluents are discharged untreated they can cause considerable damage since they possess high biochemical oxygen demand (BOD), chemical oxygen demand (COD) and release chlorinated compounds (measured as adsorbable organic halides, AOX), sulfur and sulphur compounds, suspended solids mainly fibers, fatty acids, tannins, resin acids, lignin and its derivatives (Peck and Daley, 1994; Kumar et al., 2014). Apart from these naturally occurring polluting wood extractives; there are some other xenobiotic compounds formed during the process of pulping and paper making like chlorinated phenols, lignins, polychlorinated dioxins and furans which are highly persistent and recalcitrant to biodegradation thereby maximizing the contamination of pulp and paper mill effluents (Ince et al., 2011; Badar and Farooqui, 2012; Kamali et al., 2016). These dioxins and furans are toxic substances which can cause a number of health effects like cancer, alteration in hormone levels, change in foetal development, suppressed immune system and decreased reproducibility (Kulkarni et al., 2011; Shen et al., 2012; Sun et al., 2017). The indiscriminate use of untreated or inefficiently treated industrial wastewaters is major source of ground, surface water pollution and piling up of xenobiotic contaminants and heavy metals in the food chain leading to concerning health related issues (Chou et al., 2014; Richardson and Kimura, 2017).

Use of prokaryotes in test system enables the detection of primary DNA damages and agents responsible for inducing gene mutation, while eukaryotic test
system can do a more comprehensive study ranging from gene mutations, chromosome damages, aneuploidies, growth inhibition and cytotoxicity (Masood and Malik, 2013b; Mazzeo et al., 2016). These bioassays having different principles and biological end points indicate that these experimental models are not alternative but complementary, thus suggesting that they should be conveniently combined in battery of short-term tests for ecotoxicological assessment (Aleem and Malik, 2003; Anjum and Malik, 2013; Ghosh et al., 2017).

The Ames test employs histidine deficient strains of *Salmonella typhimururium* and measures the mutagenic potential of complex mixtures in environmental samples. It is widely used for determining mutagenicity (Rao et al., 1995a; Iqbal, 2015; Balabanic et al., 2017). The survival pattern of *Escherichia coli* K-12 mutants and bacteriophage λ system are also helpful in predicting genotoxicity of environmental samples (Aleem and Malik, 2003).

*Allium cepa* bioassay is considered as best studied standard model plant for cytotoxicological analysis in environmental monitoring (Fiskesjo, 1985; Fatima and Ahmad, 2006; Nefic et al., 2013; Pandey et al., 2014). Use of *A. Cepa* test is accepted by regulatory bodies as an alternative first-tier assay system for identifying genotoxic potential caused by complex contaminants present in waste discharged into the environment (Grant, 1994; Bhat et al., 2016).

In order to study the potential risk of wastewater generated from pulp and paper industry; several short term bioassays were performed to assess the genotoxicity of wastewater employing Ames *Salmonella*/mammalian microsome test, DNA repair defective *Escherichia coli* K-12 mutants, bacteriophage λ system and *A. cepa* chromosomal aberration assay.

**Materials and methods**

**Sample collection**

Wastewater samples were collected as described earlier (Chapter II: General materials and methods).

**XAD extraction**

Wastewater samples were filtered through two membrane filters with pore sizes of 11 and 0.45 μm before the concentration of organic constituents. 1 L of wastewater sample was taken to carry out the adsorption of organic constituents on the XAD resins as described by Kool et al. (1982). An equal mixture of XAD-4/8 was used as described by Santos et al. (2001). The adsorbed organic material was then
eluted with 20 ml of acetone (HPLC grade). This eluate was evaporated to dryness and reconstituted in dimethyl sulfoxide (DMSO) (HPLC grade) such that 5 ml of extract was equivalent to 1 L of original water. Extracts were filter sterilized through 0.22 μm filter and stored at -20 °C until further testing.

**Liquid–liquid extraction**

Filtered wastewater samples were extracted separately with two different organic solvents, namely dichloromethane (DCM) and n-hexane (HPLC grades) as described in Standard Methods (APHA, 2005). Extraction of the wastewater with a solvent was done with 500 ml of paper mill effluent, which was shaken vigorously with 25 ml of the extraction solvent in a separatory funnel. This process was repeated thrice, each time using 25 ml fresh solvent. When solvent and water layers were separated, the solvent layer was collected in a beaker. The extracted organic phase was evaporated and reduced to 5 ml at room temperature under reduced pressure with the help of vacuum pump. These extracts were filtered through 0.22 μm membrane filter before they were used for genotoxicity testing.

**Salmonella mutagenicity assay**

The Ames *Salmonella* mutagenicity assay was performed as described by Maron and Ames (1983) with some modifications (Pagano and Zeiger, 1992). Five doses of each water extract, i.e., 1, 5, 10, 20 and 40 μl/plate (0.2, 1, 2, 4, and 8 ml-equivalent wastewater/plate, respectively) were plated in triplicate with 0.1 ml of the bacterial culture. The test sample and bacterial culture were incubated for 30 min at 37 °C, 2.0 ml top agar containing traces of histidine and biotin was added and uniformly mixed, and the contents were poured on minimal glucose agar plates. Plates were incubated at 37 °C for 48-72 h. All mutagenicity assay were also included negative and positive controls. The negative control plates contained solvent (DMSO) and bacteria but no test sample. Methyl methane sulfonate and sodium azide served as positive controls. These test extracts were also tested in the presence of 20 μl of S9 liver homogenate mix per plate. The criteria to classify the results for positive mutagenic activity were according to Vargas *et al.* (1993, 1995): number of revertants double the spontaneous yields accompanied by a reproducible dose–response curve.

**Treatment of *Escherichia coli* K-12 strains with XAD-concentrated and liquid–liquid-extracted wastewater samples**

The SOS defective recA, lexA, and polA mutants of *E. coli* K-12 and their isogenic wild-type counterparts were harvested by centrifugation from an
exponentially growing culture (1–3×10⁸ viable counts/ml). The pellets so obtained were suspended in 0.01 M MgSO₄ solution and treated with 20 μl of XAD-concentrated and liquid–liquid-extracted (dichloromethane and hexane) wastewater samples separately. Samples were withdrawn at regular intervals, suitably diluted, and plated to assay the colony-forming ability. Plates were incubated overnight at 37 °C. Solvent control was also run simultaneously.

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

Purified bacteriophage λ (10¹⁰ PFU/ml) was treated with 20μl/ml of XAD-concentrated and liquid–liquid-extracted (dichloromethane and hexane) samples. Aliquots of 0.1ml were withdrawn at regular intervals, suitably diluted in 0.01M MgSO₄ solution of pH 8.0 and allowed to adsorb on DNA repair defective and wild type hosts of *E. coli* K-12 strains at 37 °C. Plating was done on nutrient agar by double layer method. Plaques were counted after overnight incubation at 37 °C.

**Allium cepa anaphase-telophase chromosomal aberration assay**

To detect the cytotoxic and genotoxic potential of wastewater samples, meristematic cells of *A. cepa* root tips was used as described by Ma *et al.* (1995). Small and healthy bulbs of *A.capa* (2n=16) were taken and its outer scales was removed without destroying the root primordial. Prior to the treatment, the onion bulbs were grown in small beakers with their basal ends dipped in distilled water at room temperature (25±2 °C). When the newly germinated roots were approximately 2 cm in length, they were treated with the different concentration of wastewater i.e., 5, 10, 25, 50, and 100 % for 72 h. Double distilled water and methyl methane sulfonate (10 mg L⁻¹) were used as negative and positive controls, respectively. After the exposure of 72h, the roots of each bulb was removed and fixed in freshly prepared mixture of 3:1 ethanol/glacial acetic acid and stored at 4 °C.

For the preparation of root tip chromosome slides aceto-carmine squash technique was used (Fiskesjo, 1985). The root tips were hydrolysed in 1N hydrochloric acid (HCl) at 60 °C for 4-5 min to dissolve the cell wall and then the roots were transferred to distilled water and left for a few minutes. All the slides were examined microscopically to calculate the mitotic index (MI) and scored chromosomal aberrations (CA) under the light microscope (Olympus BX60). MI was determined by scoring approximately 6000 cells (2000 cells per slide). MI was calculated as: MI = A/B x 100%, where A: total number of dividing cells and B: total counted cells per concentration. The number of chromosomal aberrations was
recorded in approximately 300 dividing cells (preferably 100 per slide) for every treatment.

**Statistical analysis**

**Mutagenic index**

The number of $his^+$ revertants in the sample was compared to the negative control by its mutagenic index value.

$$\text{Mutagenic index} = \frac{\text{Number of } his^+ \text{ revertants induced in the sample}}{\text{Number of } his^+ \text{ revertants induced in the negative control}}$$

**Mutagenic potential (m)**

The mutagenic potential of the test samples was calculated by the initial linear portion of the dose-response curve with tester strains. The slope (m) was obtained by the least square regression of the initial linear portion of the curve of initial dose-response.

**Induction factor (Mi)**

The induction factor for various test strains for different water concentrates was evaluated as follows:

$$\text{Mi} = \ln \frac{n}{c}$$

Where $n$ is the number of revertant colonies in the sample and $c$ is the number of revertants in solvent control. The induction factor was calculated to determine the difference between two samples if the sensitivity pattern based on the slope (m) was similar.

**ANOVA**

To determine the significance of the number of $his^+$ revertants in the sample as compared to the control, one-way analysis of variance (ANOVA) was done at $P \leq 0.05$. The data of MI and CA were expressed as percents and the levels of significance in different treatment groups were analyzed by Duncan multiple range test.

**Results**

The mutagenicity of XAD-concentrated and liquid-liquid extracted wastewater samples from pulp and paper mill was evaluated using Ames *Salmonella* strains. All the test samples showed significant response in terms of mutagenic index, induction factor (Mi) and slope (m) of the initial linear dose-response curve as determined by linear regression analysis up to the increasing doses. The reversion of *Salmonella typhimurium* strains with XAD-concentrated water samples is summarized in Table 1. It was found that the test samples showed maximum response with TA98
strain in the presence as well as in the absence of S9 fraction. There was a dose related increase in the reversion frequency up to 20 µl/plate and decline was observed at a dose of 40 µl/plate. Among all the strains tested, TA98 shows maximum mutagenic index of 13.64 (without S9) and 13.67 (with S9 fraction) for XAD-concentrated samples. TA98 also showed maximum response in terms of induction factor (Mi 2.53 and 2.54 without and with S9 fraction, respectively) and slope (m 6.75 and 7.68 without and with S9 fraction, respectively) of the initial linear dose-response curve for XAD-concentrated wastewater. The order of responsiveness based on the mutagenic index and induction factor in the presence and absence of S9 fraction for XAD-concentrated wastewater was in the following order:

TA98 > TA97a > TA100 > TA102 > TA104

The response of different strains in terms of slope (m) of the initial linear dose response curve was obtained by the least square regression analysis. It was found that TA98 shows maximum value of the slope for XAD, hexane and acidic fraction of dichloromethane extracts, except for basic fraction of dichloromethane TA100 showed maximum value of the slope without S9 fraction.

The order of responsiveness of different strains in terms of slope for XAD concentrates with and without S9 fraction was as follows: TA98 > TA100 > TA102 > TA 97a > TA104 (Table 1), while hexane, acidic and basic fractions of dichloromethane extracts exhibited variable trend in responsiveness of the different strains with and without S9 fraction in terms of slope of the initial dose response curve.

The significance of the reversion of tester strains with increasing doses was determined by one-way ANOVA. The analysis shows that reversion of all the strains increases significantly for each test sample in comparison to the negative control with increasing doses.

Reversion of the Ames tester strains with hexane extracted wastewater sample is shown in Table 2. The revertants increases with the increasing dose up to 20 µl/plate and declined at 40 µl/plate. The trend in increase was similar as with XAD-concentrated samples; undoubtedly, the number of revertants was lower. TA98 exhibited maximum response in terms of mutagenic index (10.1 without S9 and 10.7 with S9 fraction); induction factor (2.21 without S9 and 2.27 with S9 fraction); and mutagenic potential (5.00 without S9 and 6.82 with S9 fraction) (Table 2). For hexane
extract the dose-dependent response in terms of slope \((m)\) without S9 fraction was as follows: \(TA98 > TA102 > TA100 > TA97a > TA104\).

TA98 showed maximum response in terms of mutagenic index (11.0 and 11.1 without and with S9 fraction), induction factor (2.31 with and without S9 fraction) and mutagenic potential (5.98 and 6.50 without and with S9 fraction) when treated with acidic fraction of dichloromethane extract (Table 3). The order of responsiveness of different strains in terms of slope for acidic fraction of dichloromethane extract without S9 fraction was as under: \(TA98 > TA100 > TA102 > TA97a > TA104\) (Table 3), whereas basic fraction of dichloromethane extract displayed maximum response of 7.4 (without S9 fraction) and 9.7 (with S9 fraction) in terms of mutagenic index; 1.85 (without S9) and 2.16 (with S9) in terms of induction factor; and 3.13 (without S9) and 4.23 (with S9) in terms of mutagenic potential for TA98 strain (Table 4).

The mutagenic index for XAD concentrated and liquid liquid extracted wastewater samples were significantly higher in comparison to the control in all the tester strains suggesting dose-dependent mutagenicity. The net revertants per liter for the most responsive strains TA97a, TA98 and TA100 are presented in Table 5. The XAD concentrated sample showed maximum number of net revertants per liter for TA100 in the absence (116500 revertants per liter) as well as in the presence of S9 fraction (130250 revertants per liter). The mutagenic activity was slightly enhanced in the presence of S9 fraction when treated with XAD-concentrated wastewater samples.

The survival pattern of \(recA\), \(lexA\) and \(polA\) mutants of \(E. coli\) K-12 and their isogenic wild-type counterparts with XAD-concentrated and liquid-liquid extracted wastewater samples is shown in Fig. 1. The XAD-concentrated sample showed maximum damage to the cells at a dose of 20µl/ml of culture as compared to liquid liquid extracted samples. Among all the mutants, \(polA\) exhibited maximum decline in survival with the XAD concentrate as well as liquid-liquid extracted wastewater samples after 6 h of treatment. The survival was 21% in \(polA\), 24% in \(lexA\) and 38% in \(recA\) mutants when treated with XAD concentrated sample, while the survival was found to be 24% in \(polA\), 28% in \(lexA\) and 41% in \(recA\) mutants with acidic fraction of dichloromethane. Survival was observed to be 32% in \(polA\), 38% in \(lexA\) and 45% in \(recA\) mutants with hexane extract of wastewater samples.

Extracellular treatment of bacteriophage \(\lambda\) with the XAD-concentrated and liquid-liquid extracted wastewater samples is shown in Fig 2. XAD-concentrated
wastewater sample showed maximum decline in plaque forming units as compared to liquid-liquid extracted wastewater samples. Among the DNA repair defective mutants of *E.coli* K 12, *lexA* showed maximum decline in plaque forming units. The XAD-concentrated wastewater sample showed a decrease in survival up to 16% in *lexA* and 36% in *recA* mutants, while acidic fraction of dichloromethane exhibited decline of 18% in *lexA* and 42% in *recA* mutants after 6 h of treatment.

The effect of pulp and paper mill effluent on mitotic index (MI) and changes in the mitotic phases of *A. cepa* root meristem cells are presented in Table 6. The MI of bulbs grown in the negative control (tap water) was recorded to be 58.73%; whereas the MI of the bulbs rooted in the positive control (10 mg/L MMS) was 7.95%. When the bulbs were rooted in different concentration of effluent, the reduction of MI was concentration dependent (Table 6). The lowest MI (11.44%) was recorded in 100% effluent. The mitotic phases declined significantly with increasing concentration of wastewater. There was a decrease in metaphase and anaphase stages with the increase in the dose of effluent indicating antiproliferative activity. The chromosomal aberrations observed in different treatment in the mitotic phases of root tip cells of *A. cepa* are recorded in Table 7. It was apparent that negative control exhibited very less abnormalities during mitosis as compared to positive control. The total aberrant cells were recorded to be (1.6%) for the negative control, while it was found to be (27.5%) for the positive control. The aberration in the cell was found to be increased with increase in the dose of wastewater i.e. 15.2% were observed with the 100% of wastewater. The chromosomal aberrations observed were c-mitosis, anaphase bridge, stickiness, laggard, disturbed anaphase-telophase, vagrant chromosomes, disturbed metaphase and polyploidy (Fig. 3). Based on the statistical analysis using the Duncan multiple range test (P<0.05), significant results were found for the wastewater samples.

**Discussion**

The pulp and paper industry is among the largest water intensive industry and discharge a wide variety of trace elements, organic and inorganic compounds, some of these compounds are most persistent, ubiquitous, mutagenic and toxic. Toxicity concerns arise from the presence of chlorinated organic compounds such as chlorinated biphenyls, dioxins, furans, and others (collectively referred to as adsorbable organic halides or AOX) in wastewaters after the chlorination/extraction process (Ince *et al.*, 2011; Hermosilla *et al.*, 2015; Yao *et al.*, 2015).
The wastewater samples collected from the industrial discharge of pulp and paper industry were concentrated using XAD resins. The tandem XAD-4/XAD-8 resins allows the extraction of most hydrophobic macromolecular organic solutes within the macropores of the XAD-8 resin, while the low molecular sizes and hydrophilic molecules are retained in the XAD-4 resin. These methods have been employed for the isolation of dissolved organic matter from natural waters (Boerschke et al., 1996; Santos and Duarte, 1998; Santos et al., 2001; Duarte and Duarte, 2005). The liquid liquid extraction of wastewater was carried out using solvents such as dichloromethane and hexane which are helpful in extraction of several organic compounds such as herbicides, pesticides and poly aromatic hydrocarbons (Potter et al., 1991; Fernandez et al., 1996; Haleyur et al., 2016).

The genotoxic effect of the XAD and liquid liquid extract of effluents of pulp and paper mill was evaluated by a battery of short term tests which are routinely recommended for evaluation of mutagenicity and cytotoxicity of wastewater and soil for environmental monitoring (Rao et al., 1995b; Pandey et al., 2014; Iqbal, 2015).

In the present study, Ames plate incorporation assay with the wastewater indicated significant mutagenicity with all the tester strains. However, TA98 and TA97a were the most responsive with the test samples in terms of the mutagenic index and induction factor both with and without metabolic activation (Tables 1-4). It was found that TA98 and TA100 were the most sensitive strains in terms of mutagenic potential both in the presence and absence of S9 fraction. Use of multiple strains is helpful in predicting the mutagenicity response of complex organic pollutants which might be due to the presence of multiple mutagens in the test samples. The enhancement of revertant frequency in the presence of S9 mix indicated that promutagen can be converted to more hazardous mutagens after metabolic activation. Previous studies of chronic exposures of pulp and paper industry effluent have shown to induce mutagenicity (Holmbom et al., 1984; Rao et al., 1995b; Ard and McDonough, 1996).

In the present study, XAD concentrated samples showed significant mutagenicity as compared to the hexane, acidic and basic fraction of dichloromethane extracts of wastewater. This may be due to the fact that XAD resins can effectively concentrate broad class of mutagenic compounds, including polycyclic aromatic hydrocarbons, aryl amines, nitro compounds, quinolines, anthraquinones, etc. (Junk et al., 1974; Aleem and Malik, 2005).
Rao et al. (1995a) conducted Ames fluctuation assay on raw and biologically treated pulp mill wastewater using bacterial strain TA100 and found XAD extract contained direct acting mutagens causing mainly base-pair substitution mutations. Lindstrom-Seppa et al. (1998) found mutagenic activity during chlorination step in pulp and paper mill effluents and reported that mutagenic activity was greatly reduced after biological and chemical treatment processes. The mutagenic activity of pulp and paper mill wastewater due to the refractory organic pollutants is also reported by Lindholm-Lehto et al. (2015a). Xiao et al. (2017) evaluated mutagenicity of raw and drinking water in an industrialized city in the Yangtze River Delta using Salmonella strain TA98. They demonstrated more than two folds increase in the mutation ratio of many samples of XAD extracted finished water as compared to raw water. Balbanic et al. (2017) used Ames MPF™ 98/100 Aqua assay, a modification of a classical Ames assay (Maron and Ames, 1983) designed for mutagenicity testing of native, non-concentrated water samples. They found that raw wastewater from paper mill were mutagenic for strain TA100 in the presence of metabolic activation, whereas the wastewater samples collected after aerobic and anaerobic biological treatment did not induce mutagenic response.

E. coli survival assay has been established as valid test system in the genotoxicity determination of complex environmental mixtures (Aleem and Malik, 2005; Ansari and Malik, 2009b). The XAD concentrated and the solvents extracted samples exhibited significant decline in survival of DNA repair defective mutants as compared to their isogenic wild-type counterparts suggesting damage to the DNA of exposed cells (Fig. 1). This test also indicates the role of $\text{recA}^+$, $\text{lexA}^+$, and $\text{polA}^+$ genes in coping up with the hazardous effect of the pollutants. The role of these genes in the SOS repair of E. coli K-12 is well documented (Courcelle and Hanawalt, 2001; Masood and Malik, 2013b). Siddiqui et al. (2011a) reported that isogenic wildtype AB1157 strain and the $\text{lexA}$ mutant exhibited 40% and 4.2% survival, respectively exposed to industrial wastewater. In another study on genotoxic nature of Mathura refinery wastewater was investigated by Gupta and Ahmad (2012) employing E. coli survival assay and found that AB2480 ($\text{recA uvrA}$) double mutant was the most sensitive to killing by the test sample with the percent survival reducing to zero (approximately) after 6 h of exposure. AB2463 ($\text{recA}$) and AB 2494 ($\text{lexA}$) showed survival of 3% and 19% with the test samples.
Extracellular treatment of bacteriophage λ with test samples resulted in significant loss of plaque-forming units (Fig. 2). Prophage induction is useful for screening genotoxic compound present in environmental samples (Vargas et al., 2001; Casjens and Hendrix, 2015). Anjum and Malik (2013) demonstrated that lexA mutant was found to be most sensitive strain when treated with industrial wastewater samples and survival declined to 15% when treated with XAD-concentrated sample while it was 35% after treatment with liquid liquid extracts.

Mitotic index provide choicest method for biomonitoring the harmful effect of persistent pollutant on cell division. The proportion of cell in mitotic phases is indicated by its mitotic index and its decline can be interpreted as inhibition of the cell cycle. Chromosomal aberrations are characterized by changes in the structure and total number of chromosomes which can occur both spontaneously and as a result of the exposure to physical or chemical agents. Both the MI and chromosomal aberration analysis in meristematic root tip cells of A. cepa is considered an excellent method to investigate the genotoxic potential of chemical agents, sewage, and industrial wastewaters (Lutterbeck et al., 2015; Martins et al., 2016; Haq et al., 2017).

Iqbal et al. (2017) evaluated cytotoxicity of petroleum refinery wastewater (PRW) by A. cepa assay and reported that raw PRW was cytotoxic in nature and after treatment with UV/H$_2$O$_2$/TiO$_2$, the cytotoxicity was reduced significantly. They found that MI of petroleum refinery wastewater before treatment was lower (8.0) than the negative control (11.3). In a study conducted by Haq et al. (2017) the root tip cells grown in untreated effluent of pulp and paper mill showed a significant decrease in MI from 69% (control) to 32%, 27%, 22% and 11% at 25%, 50%, 75% and 100% effluent concentration, respectively. They observed different types of chromosomal and nuclear aberrations such as c-mitosis, stickiness, chromosome loss, chromosome break, anaphase bridge, multipolar anaphase, vagrant chromosomes, micronucleated and binucleated cells.

The impact of different concentrations of pulp and paper mill wastewater has been investigated in meristematic cells of A. cepa and it was observed that mitotic index decreased significantly with increase in concentration of the wastewater (Table 5). Decrease in MI reflects the inhibition of cell division and thus toxicity of wastewater (Chaparro and Pires, 2011; Bhat et al., 2016; Haq et al., 2016). The wastewater was able to induce significant chromosomal aberrations and more
pronounced effect was induced with the increase in dose of the wastewater this might be due to the presence of genotoxins.

Garcia et al. (2017b) compared the genotoxicity of sugarcane and orange vinasse in effluent and found chromosomal aberrations were induced by both the test samples at a dilution of 2.5% and 5% and alterations reported were nuclear buds, anaphasic bridges, micronucleus, chromosomal loss and chromosomal break.

Present findings indicated that paper industry wastewater exhibited considerable antiproliferative activity which depends on its concentration. Moreover the constituents are able to induce various cytotoxic effects such as breaks, stickiness, chromosome bridge, laggardness, c-mitosis and vagrant chromosome. Stickiness results due to adhesion in the proteins of the chromosome which may lead to the formation of chromosomal bridges and thus cause difficulty in free movement and separation of chromatids (Patil and Bhat, 1992; Radic et al., 2010). Chromosome bridge can lead to chromosome breakage and thus show clastogenic effect. Laggards result due to the loose attachment of chromosomes to the spindle fibre and thus inefficient movement of the chromosomes to the poles (Turkoglu, 2007), while vagrant chromosomes arise due to unequal distribution of chromosomes because of nondisjunction of chromatids in anaphase (Sondhi et al., 2008; Firbas and Amon, 2014).

Studies have shown that both bleached and unbleached kraft pulp mills effluent exhibited the evidences of genotoxicity, cytotoxicity and presence of endocrine disrupting compounds (Ali and Sreekrishnan, 2001; Fraser et al., 2009; Balbanic et al., 2017; Haq et al., 2017). Report of delay, retard and decline in the germination of rice, mustard and pea seeds and seedling growth have been recorded with the treatment of paper mill effluent (Baruah and Das, 1997, Medhi et al., 2011).

Our study confirm the mutagenicity of wastewater from pulp and paper mill as analysed by the bacterial reverse mutation assay in Ames Salmonella strains TA97a, TA98, TA100, TA102 and TA104 in the presence and absence of metabolic activation system, DNA repair defective E. coli K-12 mutants and bacteriophage λ system. XAD extracts of the wastewater samples were found to be more mutagenic as compared to those of liquid-liquid extracts (hexane and dichloromethane extracts). This study not only confirms the idea of the presence of mutagens in the wastewater but also suggest the mutagenic hazards of the pollutants in the wastewater. Allium cepa chromosomal aberration assay also confirms that the raw wastewater contain
mixtures of genotoxins which significantly decrease the mitotic index and induce several chromosomal aberrations such as c-mitosis, anaphase bridges, polyploidy, binucleate cells, stickiness, disturbed metaphase, disturbed anaphase, laggards and breaks in the test samples. The present results indicated potential mutagenic/genotoxic activity of the pulp and paper mill effluent possessing complex assortment of chemicals which is exerting toxic effect to the receiving water bodies and need continuous monitoring to provide basis for improvement.
Table 1. Reversion of *Salmonella* tester strains in the presence of XAD-concentrated wastewater samples

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>1 (µl/plate)</th>
<th>5 (µl/plate)</th>
<th>10 (µl/plate)</th>
<th>20 (µl/plate)</th>
<th>40 (µl/plate)</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>97 ± 2</td>
<td>269 ± 8 (2.7)</td>
<td>300 ± 5 (3.1)</td>
<td>339 ± 13 (3.5)</td>
<td>417 ± 8 (4.3)</td>
<td>343 ± 6 (4.6)</td>
<td>1.19</td>
<td>4.17</td>
<td>13.24</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>96 ± 2</td>
<td>301 ± 8 (3.1)</td>
<td>345 ± 5 (3.6)</td>
<td>412 ± 4 (4.3)</td>
<td>472 ± 8 (4.9)</td>
<td>417 ± 7 (4.3)</td>
<td>1.36</td>
<td>5.49</td>
<td>7.27</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>31 ± 6</td>
<td>185 ± 5 (5.9)</td>
<td>233 ± 8 (7.5)</td>
<td>282 ± 7 (9.0)</td>
<td>423 ± 12 (13.6)</td>
<td>360 ± 10 (11.6)</td>
<td>2.53</td>
<td>6.75</td>
<td>16.57</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>34 ± 2</td>
<td>221 ± 12 (6.5)</td>
<td>266 ± 11 (7.8)</td>
<td>318 ± 10 (9.3)</td>
<td>465 ± 5 (13.6)</td>
<td>418 ± 24 (12.2)</td>
<td>2.54</td>
<td>7.68</td>
<td>18.46</td>
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<tr>
<td>TA100</td>
<td>-</td>
<td>140 ± 5</td>
<td>261 ± 10 (1.8)</td>
<td>331 ± 10 (2.3)</td>
<td>374 ± 6 (2.6)</td>
<td>466 ± 4 (3.3)</td>
<td>407 ± 6 (2.9)</td>
<td>0.84</td>
<td>5.31</td>
<td>9.62</td>
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<tr>
<td></td>
<td>+</td>
<td>143 ± 3</td>
<td>289 ± 13 (2.1)</td>
<td>365 ± 15 (2.6)</td>
<td>427 ± 14 (3.1)</td>
<td>521 ± 10 (3.8)</td>
<td>470 ± 10 (3.4)</td>
<td>0.97</td>
<td>6.48</td>
<td>19.84</td>
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<tr>
<td>TA102</td>
<td>-</td>
<td>246 ± 7</td>
<td>365 ± 5 (1.4)</td>
<td>400 ± 9 (1.6)</td>
<td>484 ± 8 (1.9)</td>
<td>531 ± 10 (2.1)</td>
<td>473 ± 8 (1.9)</td>
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<td></td>
<td>+</td>
<td>238 ± 8</td>
<td>393 ± 8 (1.6)</td>
<td>441 ± 14 (1.8)</td>
<td>522 ± 9 (2.1)</td>
<td>596 ± 16 (2.5)</td>
<td>542 ± 10 (2.2)</td>
<td>0.40</td>
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<tr>
<td>TA104</td>
<td>-</td>
<td>306 ± 7</td>
<td>426 ± 11 (1.3)</td>
<td>473 ± 7 (1.5)</td>
<td>532 ± 10 (1.7)</td>
<td>593 ± 15 (1.9)</td>
<td>520 ± 10 (1.6)</td>
<td>-0.06</td>
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<td></td>
<td>+</td>
<td>322 ± 10</td>
<td>457 ± 18 (1.4)</td>
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<td>580 ± 6 (1.8)</td>
<td>642 ± 10 (1.9)</td>
<td>551 ± 12 (1.7)</td>
<td>-0.06</td>
<td>4.23</td>
<td>22.35</td>
</tr>
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</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 2. Reversion of *Salmonella* tester strains in the presence of hexane extracted wastewater samples

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Wastewater extract (µl/plate)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
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</thead>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA97a</td>
<td>-</td>
<td>94 ± 4</td>
<td>162 ± 10 (1.7)</td>
<td>211 ± 10 (2.2)</td>
<td>272 ± 7 (2.8)</td>
<td>311 ± 10 (3.3)</td>
<td>252 ± 8 (1.5)</td>
<td>0.83</td>
<td>3.21</td>
<td>14.66</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>99 ± 2</td>
<td>209 ± 14 (2.1)</td>
<td>263 ± 13 (2.6)</td>
<td>319 ± 10 (3.2)</td>
<td>360 ± 11 (3.6)</td>
<td>285 ± 15 (2.8)</td>
<td>0.96</td>
<td>3.32</td>
<td>20.42</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>32 ± 2</td>
<td>129 ± 9 (4.0)</td>
<td>158 ± 11 (4.9)</td>
<td>225 ± 5 (7.0)</td>
<td>325 ± 15 (10.1)</td>
<td>262 ± 13 (8.1)</td>
<td>2.21</td>
<td>5.00</td>
<td>18.92</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>37 ± 2</td>
<td>162 ± 8 (4.3)</td>
<td>197 ± 13 (5.3)</td>
<td>275 ± 6 (7.4)</td>
<td>398 ± 18 (10.7)</td>
<td>352 ± 13 (9.5)</td>
<td>2.27</td>
<td>6.82</td>
<td>19.92</td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>133 ± 3</td>
<td>182 ± 8 (1.3)</td>
<td>236 ± 10 (1.7)</td>
<td>283 ± 8 (2.1)</td>
<td>353 ± 14 (2.6)</td>
<td>292 ± 8 (2.1)</td>
<td>0.50</td>
<td>3.60</td>
<td>16.96</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>139 ± 2</td>
<td>244 ± 11 (1.7)</td>
<td>273 ± 11 (1.9)</td>
<td>352 ± 9 (2.5)</td>
<td>437 ± 13 (3.1)</td>
<td>386 ± 10 (2.7)</td>
<td>0.76</td>
<td>5.25</td>
<td>8.53</td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>234 ± 3</td>
<td>281 ± 11 (1.2)</td>
<td>344 ± 11 (1.4)</td>
<td>391 ± 10 (1.6)</td>
<td>469 ± 10 (2.0)</td>
<td>400 ± 10 (1.7)</td>
<td>0.00</td>
<td>3.82</td>
<td>10.91</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>250 ± 8</td>
<td>332 ± 8 (1.3)</td>
<td>392 ± 11 (1.5)</td>
<td>452 ± 13 (1.8)</td>
<td>510 ± 10 (2.0)</td>
<td>454 ± 10 (1.8)</td>
<td>0.03</td>
<td>4.21</td>
<td>10.12</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>335 ± 5</td>
<td>382 ± 8 (1.1)</td>
<td>437 ± 9 (1.3)</td>
<td>485 ± 5 (1.4)</td>
<td>532 ± 8 (1.5)</td>
<td>470 ± 10 (1.4)</td>
<td>-0.53</td>
<td>2.92</td>
<td>14.66</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>348 ± 7</td>
<td>419 ± 12 (1.2)</td>
<td>475 ± 8 (1.3)</td>
<td>523 ± 12 (1.5)</td>
<td>570 ± 10 (1.4)</td>
<td>532 ± 8 (1.5)</td>
<td>-0.44</td>
<td>3.77</td>
<td>17.33</td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 3. Reversion of *Salmonella* tester strains in the presence of acidic fraction of dichloromethane extracted wastewater samples

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Wastewater extract (µl/plate)</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>92 ± 2</td>
<td>169 ± 9 (1.8)</td>
<td>222 ± 9 (2.4)</td>
<td>286 ± 9 (3.1)</td>
<td>344 ± 12 (3.7)</td>
<td>290 ± 10 (3.1)</td>
<td>1.00</td>
<td>4.16</td>
<td>17.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>98 ± 2</td>
<td>225 ± 15 (2.2)</td>
<td>281 ± 8 (2.8)</td>
<td>341 ± 10 (3.4)</td>
<td>394 ± 6 (4.0)</td>
<td>352 ± 13(3.5)</td>
<td>1.10</td>
<td>4.79</td>
<td>11.79</td>
<td></td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>32 ± 2</td>
<td>145 ± 5 (4.5)</td>
<td>188 ± 8 (5.8)</td>
<td>261 ± 13 (8.1)</td>
<td>355 ± 14 (11.0)</td>
<td>311 ± 10 (9.7)</td>
<td>2.31</td>
<td>5.98</td>
<td>19.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>38 ± 2</td>
<td>197 ± 13 (5.1)</td>
<td>234 ± 11 (6.1)</td>
<td>310 ± 12 (8.1)</td>
<td>422 ± 9 (11.1)</td>
<td>361 ± 10 (9.5)</td>
<td>2.31</td>
<td>6.50</td>
<td>16.06</td>
<td></td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>132 ± 3</td>
<td>192 ± 8 (1.4)</td>
<td>273 ± 11 (2.0)</td>
<td>319 ± 10 (2.4)</td>
<td>401 ± 11 (3.0)</td>
<td>352 ± 13 (2.6)</td>
<td>0.71</td>
<td>4.90</td>
<td>16.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>145 ± 5</td>
<td>262 ± 9 (1.8)</td>
<td>333 ± 11 (2.2)</td>
<td>405 ± 11 (2.7)</td>
<td>474 ± 8 (3.2)</td>
<td>382 ± 10 (2.6)</td>
<td>0.81</td>
<td>4.63</td>
<td>13.85</td>
<td></td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>218 ± 8</td>
<td>258 ± 13 (1.1)</td>
<td>364 ± 13 (1.6)</td>
<td>431 ± 11 (1.9)</td>
<td>482 ± 10 (2.2)</td>
<td>429 ± 16 (1.9)</td>
<td>0.19</td>
<td>4.77</td>
<td>14.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>237 ± 8</td>
<td>373 ± 8 (1.5)</td>
<td>405 ± 15 (1.7)</td>
<td>476 ± 13 (2.0)</td>
<td>544 ± 6 (2.2)</td>
<td>486 ± 10 (2.0)</td>
<td>0.25</td>
<td>4.76</td>
<td>7.27</td>
<td></td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>315 ± 5</td>
<td>383 ± 8 (1.2)</td>
<td>442 ± 11 (1.4)</td>
<td>496 ± 12 (1.5)</td>
<td>552 ± 10 (1.7)</td>
<td>472 ± 10 (1.4)</td>
<td>-0.28</td>
<td>3.24</td>
<td>12.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>339 ± 8</td>
<td>439 ± 9 (1.2)</td>
<td>482 ± 10 (1.4)</td>
<td>552 ± 20 (1.6)</td>
<td>599 ± 11 (1.7)</td>
<td>560 ± 12 (1.6)</td>
<td>-0.26</td>
<td>4.39</td>
<td>20.09</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 4. Reversion of *Salmonella* tester strains in the presence of basic fraction of dichloromethane extracted wastewater samples

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>91 ± 1</td>
<td>151 ± 8 (1.6)</td>
<td>201 ± 12 (2.2)</td>
<td>251 ± 10 (2.7)</td>
<td>292 ± 10 (3.2)</td>
<td>249 ± 8 (2.7)</td>
<td>0.79</td>
<td>3.28</td>
<td>10.29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>97 ± 3</td>
<td>221 ± 19 (2.2)</td>
<td>257 ± 13 (2.6)</td>
<td>351 ± 11 (3.6)</td>
<td>378 ± 12 (3.8)</td>
<td>330 ± 10 (3.4)</td>
<td>1.06</td>
<td>4.36</td>
<td>21.83</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>30 ± 2</td>
<td>110 ± 11 (3.6)</td>
<td>144 ± 13 (4.8)</td>
<td>173 ± 9 (5.7)</td>
<td>222 ± 12 (7.4)</td>
<td>192 ± 8 (6.4)</td>
<td>1.85</td>
<td>3.13</td>
<td>18.96</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>35 ± 4</td>
<td>148 ± 7 (4.2)</td>
<td>215 ± 8 (6.1)</td>
<td>281 ± 10 (8.0)</td>
<td>341 ± 8 (9.7)</td>
<td>256 ± 18 (7.3)</td>
<td>2.16</td>
<td>4.23</td>
<td>19.16</td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>133 ± 6</td>
<td>194 ± 14 (1.4)</td>
<td>245 ± 6 (1.8)</td>
<td>296 ± 12 (2.2)</td>
<td>351 ± 10 (2.6)</td>
<td>297 ± 16 (2.2)</td>
<td>0.49</td>
<td>3.50</td>
<td>18.55</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>142 ± 8</td>
<td>249 ± 8 (1.7)</td>
<td>291 ± 10 (2.0)</td>
<td>371 ± 8 (2.6)</td>
<td>441 ± 6 (3.1)</td>
<td>381 ± 9 (2.6)</td>
<td>0.74</td>
<td>4.92</td>
<td>7.50</td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>235 ± 5</td>
<td>251 ± 13 (1.0)</td>
<td>311 ± 11 (1.3)</td>
<td>376 ± 12 (1.6)</td>
<td>442 ± 11 (1.8)</td>
<td>372 ± 10 (1.5)</td>
<td>-0.12</td>
<td>3.53</td>
<td>18.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>248 ± 7</td>
<td>333 ± 10 (1.3)</td>
<td>389 ± 8 (1.5)</td>
<td>452 ± 12 (1.8)</td>
<td>501 ± 11 (2.0)</td>
<td>454 ± 10 (1.8)</td>
<td>0.01</td>
<td>4.18</td>
<td>16.37</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>322 ± 13</td>
<td>378 ± 8 (1.1)</td>
<td>409 ± 8 (1.2)</td>
<td>482 ± 10 (1.4)</td>
<td>532 ± 13 (1.6)</td>
<td>464 ± 14 (1.4)</td>
<td>-0.42</td>
<td>3.15</td>
<td>13.85</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>342 ± 8</td>
<td>415 ± 12 (1.2)</td>
<td>463 ± 12 (1.3)</td>
<td>533 ± 8 (1.5)</td>
<td>571 ± 10 (1.6)</td>
<td>532 ± 18 (1.5)</td>
<td>-0.40</td>
<td>3.94</td>
<td>17.06</td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 5. Mutagenicity of wastewater samples in terms of net revertants/liter for the most responsive TA97a, TA98 and TA100 strains

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Strain</th>
<th>S9 fraction</th>
<th>XAD-concentrated water sample</th>
<th>Dichloromethane extract (acidic)</th>
<th>Hexane extract</th>
<th>Dichloromethane extract (basic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater</td>
<td>TA97a</td>
<td>-</td>
<td>104250</td>
<td>86000</td>
<td>77750</td>
<td>73000</td>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>118000</td>
<td>98500</td>
<td>90000</td>
<td>94500</td>
</tr>
<tr>
<td></td>
<td>TA98</td>
<td>-</td>
<td>105750</td>
<td>93500</td>
<td>81250</td>
<td>55500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>116250</td>
<td>105500</td>
<td>99500</td>
<td>85250</td>
</tr>
<tr>
<td></td>
<td>TA100</td>
<td>-</td>
<td>116500</td>
<td>100250</td>
<td>88250</td>
<td>87750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>130250</td>
<td>118500</td>
<td>109250</td>
<td>110250</td>
</tr>
</tbody>
</table>
Table 6. Effect of different concentrations of wastewater on mitotic index and mitotic phase of *Allium cepa* root meristematic cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (% v/v)</th>
<th>Mitotic Phases (%)</th>
<th>Mitotic index (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prophase</td>
<td>Metaphase</td>
</tr>
<tr>
<td>Site I</td>
<td>5</td>
<td>38.88</td>
<td>33.34</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>43.75</td>
<td>31.25</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>46.67</td>
<td>28.67</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>51.85</td>
<td>27.40</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>57.63</td>
<td>23.73</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>60.90</td>
<td>25.45</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>35.20</td>
<td>34.40</td>
</tr>
</tbody>
</table>

Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test); ±: Standard deviation.
Table 7. Chromosomal aberrations in the root meristematic cells of *Allium cepa* exposed to different concentrations of wastewater for 72 h

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (%)</th>
<th>Types of aberrations</th>
<th>Total aberrant cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM</td>
<td>AB</td>
</tr>
<tr>
<td>Site I</td>
<td>5</td>
<td>2.6±0.5</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.6±1.5</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2.6±0.5</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.6±1.5</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.6±0.5</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>14.3±0.5</td>
<td>9.3±5.5</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>1.6±1.1</td>
<td>1.0±0.0</td>
</tr>
</tbody>
</table>

CM: c-mitosis, AB: anaphase bridge, S: stickiness, L: laggard, DAT: disturbed anaphase-telophase, VC: vagrant chromosomes, DM: disturbed metaphase, P: polyploidy. Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test).
Fig. 1. Survival of *E. coli* K-12 strains treated with (a) XAD concentrated wastewater (b) acidic fraction of dichloromethane extract (c) hexane extracted wastewater (d) basic fraction of dichloromethane extract.
**Fig. 2.** Survival of bacteriophage λ treated with (a) XAD concentrated wastewater (b) acidic fraction of dichloromethane extract (c) hexane extracted wastewater (d) basic fraction of dichloromethane extract.
Fig. 3. Different types of chromosomal aberrations induced by the pulp and paper mill effluent in Allium cepa root-tips (a) diploid (b) disturbed metaphase (c) disturbed anaphase with chromosome stickiness (d) disturbed anaphase with chromosome break (e) c-mitosis (f) disturbed metaphase with chromosome loss and break (g) anaphase bridge (h) laggard (i) stickiness (j) bipolar anaphase (k) disturbed anaphase (l) disturbed anaphase with chromosome stickiness.
Introduction

Persistent organic pollutants (POPs) and heavy metals are known as a major source of contamination discharged by various industrial activities and are potentially harmful to animals as well as humans due to its tendency to accumulate in the food chain (Singh and Prasad, 2015). The increase in agricultural and industrial activities to support growing population would result in water and soil stress (Lamastra et al., 2016). The generation of hazardous industrial waste has led to steady increase in the deposition of thousands of chemicals into the soils which are resistant to degradation. Humans and ecosystems are exposed to highly variable and unknown combination of pesticides, heavy metals and recalcitrant contaminants which cause deleterious effect by bioaccumulation and toxicity (Matamoros et al., 2012; Ghattas et al., 2017; Wang et al., 2017).

Pulp and paper mills along with other industries serve as major source of production of polyhalogenated compounds which may act as persistent organic pollutants (POPs) in soil environment. There are 23 distinct substances which have been listed as POP including polychlorinated biphenyls, polychlorinated phenols, polychlorinated dibenzo-p-dioxins and dibenzofurans (Xu et al., 2013; Cariou et al., 2016). These substances though appear at very low concentrations but still act as genotoxins or carcinogens. The interactions between the contaminants which are discharged as complex mixtures from industries can lead to unpredictable genotoxicity responses. The hazardous metabolite can bind to DNA molecules and trigger a damaging change in cellular processes, such as impaired metabolism, enzyme function, growth inhibition, reproductive dysfunctioning, cytotoxicity, immunotoxicity and carcinogenesis (Ohe et al., 2004). Previous studies have shown that several POPs have posed potential risk by being endocrine disrupting contaminants and may further lead to the development of cancers (Siddiqui et al., 2016; Archer et al., 2017).

The industrial effluents are flooded with emerging contaminants which may have synthetic origin or can be derived from natural source and their environmental and public health risks are yet to be established. Lack of information on fate and their potential toxicities of such contaminants make difficult management of these chemicals by regulatory agencies which may be attributed due to limited financial resources and the lack of analytical techniques for their detection (Naidu et al., 2016). Combination of several short term bioassays allow the detection of genotoxic and
cytotoxic potential of complex mixtures of these contaminants and thus determination of their potential environmental or public health risk which would otherwise be difficult to measure at sublethal concentrations (Jha, 2008; Kwasniewska et al., 2012; Man et al., 2013).

Although hazard caused and the associated risk to human health and ecosystem by polluted soil and aquifers can be assessed by means of physical and chemical measurements, but these analysis may not be enough due to limited extent of extractable compounds and presence of broader classes of chemical compounds, which show high heterogeneity of toxic properties for environmental targets. Also, the bioavailability of organic and inorganic pollutants can vary among different organisms. Thus, alternative methods which include biological tests (bioassays) provide a better determination of hazard identification by exposing biological organisms to polluted materials (Bispo et al., 1999; Pandey et al., 2014; Radic et al., 2014; Bhat et al., 2016; Kumari et al., 2016).

The present study focuses on the genotoxicity of agricultural soil irrigated with wastewater from pulp and paper industries, using Ames Salmonella/mammalian microsome test, DNA repair defective E.coli K-12 mutants and Allium cepa chromosomal aberration assay.

Materials and Methods

Sample collection

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

Extraction of soil samples with different solvents

The extraction of different soil samples with the solvents (hexane and dichloromethane; HPLC-grade) was carried out according to the method of Knize et al. (1987). 10 g of soil was extracted with 10 ml of the extraction solvent. The extracts were centrifuged at 8000 rpm for 10 min. The extracts were evaporated to dryness and then re-dissolved in 1 ml of DMSO (SRL, India). These extracts were filtered sterilized through 0.22 µm filter and stored at -20 ºC until genotoxicity testing was completed.

Salmonella mutagenicity assay

The Ames Salmonella mutagenicity assay was performed as described by Maron and Ames (1983) with some modifications (Pagano and Zeiger, 1992). Five doses of each soil extract i.e. 1, 5, 10, 20 and 40 µl/plate (10, 50, 100, 200 and 400 mg
equivalent soil per plate) were plated in triplicate with 0.1 ml of the bacterial culture. After incubating the test sample and bacterial culture for 30 min at 37 °C, 2.0 ml top agar containing traces of histidine and biotin was added and the contents were poured on minimal glucose agar plates. Plates were incubated at 37 °C for 48-72 h. Negative and positive controls were included in each assay. The negative control plates contained bacteria and solvent (DMSO) but no test sample. Methyl methane sulphonate and sodium azide served as positive controls (Maron and Ames, 1983; Masood and Malik, 2013a). All the soil extracts were also tested in the presence of (+S9) microsomal fraction, to which 20 µl of S9 liver homogenate mix per plate was added. The criterion used to classify the results as positive was similar to those of Vargas et al. (1993; 1995).

**Treatment of Escherichia coli K-12 strains with soil samples**

The SOS defective recA, lexA and polA mutants of *Escherichia coli* K-12 strains as well as their isogenic wild type strains were harvested by centrifugation from an exponentially growing culture (1-3 x 10^8 viable counts/ml). The pellets so obtained were suspended in 0.01 M MgSO_4 solution and treated with 20 µl of each soil extracts separately. Samples were withdrawn at regular intervals, suitably diluted, and plated to assay the colony-forming ability. Plates were incubated overnight at 37 °C. Solvent control was also run simultaneously.

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

Purified bacteriophage λ (10^{10} PFU/ml) was treated with 20µl/ml of test soil samples and incubated at 37 °C. Aliquots of 0.1ml were withdrawn at regular intervals, suitably diluted in 0.01M MgSO_4 solution of pH 8.0 and allowed to adsorb on DNA repair defective and wild type hosts of *E. coli* K-12 strains at 37 °C. The infective centers were plated on nutrient agar by the double layer method. Plaques were counted after overnight incubation at 37 °C.

**Allium cepa anaphase-telophase test**

Small bulbs (1.5-2.0 cm in diameter) of the common onion, *A. cepa*, (2n=16) were purchased from a local supermarket. Prior to initiating the test, the outer scales of the bulbs were removed without destroying the root primordia. Bulbs of *A. cepa* were placed in beakers with their basal ends dipping in distilled water and germinated at room temperature (25±2 °C). The soil extracts were prepared by suspending soil in distilled water in ratio of 1:10 (w/v) for 24 h at room temperature (Cotelle *et al.*, 1999). When the newly emerged roots were approximately 2 cm in length, they were
ready for the treatment with the different concentrations (5, 10, 25, 50 and 100%) of extracts. Distilled water and methyl methane sulphonate (10 mg L\(^{-1}\)) were used as negative and positive controls. After 72 h of exposure, several root tips were removed from the bulbs, fixed in 3:1 ethanol:glacial acetic acid and stored overnight at 4 °C. The next day, they were rinsed in tap water and stained with aceto-carmine. The squash technique was applied for the study of mitotic index (MI) and chromosomal aberrations as described earlier (chapter V: Materials and methods section of Genotoxicity of wastewater).

Statistical analysis

Statistical analysis of the results of mutagenicity testing for the different parameters; mutagenic index (MI), mutagenic potential (m), induction factor and analysis of variance (ANOVA) were carried out according to Masood and Malik, (2013a).

Mutagenic index

The number of his\(^+\) revertants in the sample was compared to the negative control by its mutagenic index value.

\[ \text{Mutagenic index} = \frac{\text{Number of his}^+\text{ revertants induced in the sample}}{\text{Number of his}^+\text{ revertants induced in the negative control}} \]

Mutagenic potential (m)

The mutagenic potential of the test samples was calculated by the initial linear portion of the dose-response curve with tester strains. The slope (m) was obtained by the least square regression of the initial linear portion of the curve of initial dose-response.

Induction factor (Mi)

The induction factor for various test strains for different soil extracts was evaluated as follows:

\[ \text{Mi} = \ln \frac{n}{c} \]

Where \(n\) is the number of revertant colonies in the sample and \(c\) is the number of revertants in solvent control. The induction factor was calculated to determine the difference between two samples if the sensitivity pattern based on the slope (m) was similar.

ANOVA

To determine the significance of the number of his\(^+\) revertants in the sample as compared to the control, one-way analysis of variance (ANOVA) was done at \(P \leq\)
The data of MI and CA were expressed as percents and the levels of significance in different treatment groups were analyzed by Duncan multiple range test.

Results

Soil samples were assayed for mutagenicity using Ames Salmonella test. The reversion of Salmonella typhimurium strains with different extracts of soil samples are summarized in Table 1-4. Site I soil is irrigated with ground water, while site II soil is irrigated with pulp and paper mill effluent. All the samples exhibited significant mutagenicity with TA97a, TA98, and TA100, strains in the presence as well as in the absence of S9 fraction. There was an increase in the reversion of tester strains with increasing doses up to 20 µl/plate and a decline was observed at the dose of 40 µl/plate.

The order of responsiveness based on the mutagenic index and induction factor in the presence and absence of S9 fraction for dichloromethane and hexane extracts of ground water irrigated soil (Site I) and contaminated soil (Site II) were as follows: TA98 > TA97a > TA100 > TA102 > TA104.

Maximum number of revertant colonies were observed for TA98 strain in DCM extract of contaminated soil, which was also the most responsive strain in terms of mutagenic index (12.81 and 11.81 with and without S9 fraction), induction factor (2.47 with and 2.38 without S9 fraction) and mutagenic potential/slope of the initial linear dose response curve (7.87 and 7.51 with and without S9 fraction).

The response of different strains in terms of slope (m) of the initial linear dose response curve was obtained by the least square regression analysis. It was found that TA98 shows maximum value of the slope followed by TA97a for dichloromethane extracts of contaminated soil (Site II). The order of responsiveness of different strains in terms of slope for dichloromethane extracts of contaminated soil (Site II) was as follows: TA98 > TA97a > TA100 > TA102 > TA104 (with S9 fraction) (Table 4).

The dichloromethane extracts of ground water irrigated soil (Site I) and hexane extracts of both the sites, have shown the maximum value of slope for TA97a followed by TA98. For dichloromethane extracts of ground water irrigated soil (Site I), the pattern was in the following order: TA97a > TA98 > TA102 > TA100 > TA104 (with and without S9 fraction) (Table 3).

The hexane extract of contaminated soil showed mutagenic index of 8.9 and 5.8 (with and without S9 fraction); induction factor of 2.06 and 1.58 (with and
without S9 fraction); and mutagenic potential of 4.91 and 2.58 (with and without S9 fraction) with TA98 strain (Table 2). The response of the tester strains was significantly higher when treated with contaminated soil (Site II) extracts as compared to ground water irrigated soil (Site I) extracts. DCM extract of wastewater irrigated soil was found to be more mutagenic than hexane extracted sample in terms of mutagenic index, induction factor and mutagenic potential (Table 3-4).

The survival pattern of recA, lexA and polA mutants of E. coli K-12 with soil extracts is shown in Fig. 1. The dichloromethane extracts of the soil exhibited maximum damage to the cell at a dose of 20 µl of soil extracts/ml of culture. Among all the mutants, polA exhibited maximum decline in survival. Soil extracts of contaminated soil (Site II) displayed maximum decline in survival as compared to ground water irrigated soil (Site I). The survival was 31% in polA, 38% in lexA and 46% in recA mutants when treated with dichloromethane extract of ground water irrigated soil (Site I), while survival was 25% in polA, 30% in lexA and 37% in recA mutants after 6 h of treatment of contaminated soil (Site II). Similar trend in survival was found with hexane extracts of soil samples, the survival was 42% in polA, 46% in lexA and 57% in recA mutants when treated with hexane extract of ground water irrigated soil (Site I), while the survival was 34% in polA, 39% in lexA and 45% in recA mutants when treated with hexane extract of contaminated soil (Site II) after 6 h of treatment.

Extracellular treatment of bacteriophage ƛ with the soil extracts of dichloromethane and hexane is shown in Fig. 2. Soil extracts of wastewater irrigated soil (Site II) displayed maximum decline in plaque forming units as compared to ground water irrigated soil (Site I). Among all the strains lexA mutant shows maximum decline in PFUs. The dichloromethane extracts of wastewater irrigated soil exhibited decrease in survival up to 18% in lexA and 27% in recA mutants, while dichloromethane extracts of groundwater irrigated soil showed decrease in survival up to 30% in lexA and 41% in recA mutants after 6 h of treatment, whereas the hexane extracts of wastewater irrigated soil showed a decrease in survival up to 24% in lexA and 43% in recA mutants and hexane extracts of groundwater irrigated soil showed a decline of 34% in lexA and 45% in recA mutants after 6 h of treatment.

The effect of aqueous extracts of soil samples on the mitotic index (MI) of A. cepa root tip meristem cells exposed for 72 h is presented in Table 5. The MI significantly decreased in a dose dependent manner with each concentration of soil
extracts. It was observed that decline of MI was more prevalent in wastewater irrigated soil (Site II) than ground water irrigated soil (Site I) treated A. cepa cells. MI was observed to be 15.94% (P<0.05) at 100% concentration for ground water irrigated soil (Site I), whereas it was 10.04% (P<0.05) at 100% concentration for wastewater irrigated soil (Site II). There were significant differences between 5% and 100% treated groups in MI for both the sites. The mitotic index (MI) in the negative control was higher compared to the soil extracts.

Statistically significant (P<0.05) frequencies of chromosomal abnormalities were recorded with 5-100% soil extracts of wastewater irrigated soil (Site II) compared to ground water irrigated soil (Site I) (Table 6). The soil samples caused several types of aberrations in the A. cepa root tips, such as breakage, anaphase bridges, c-mitosis, laggards, binucleated cells, stickiness, unequal distributions of chromosomes at anaphase stage of cell division (Fig. 3). However, the wastewater irrigated soil sample displayed greater frequency of aberrations as compared to ground water irrigated soil. It was 15.1% (P<0.05) at 100% concentration for ground water irrigated soil (Site I), whereas it was 20.9% (P<0.05) for wastewater irrigated soil (Site II) respectively at 100% concentrations.

**Discussion**

Analysis of the test soils revealed the presence of various organic compounds as discussed previously in Chapter II. The assessment of toxic chemical constituents in soil is not an easy task despite the recent advances in the sensitive techniques available for the detection and quantification of these contaminants (Gupta and Thakur, 2015; Haleyur et al., 2016). The use of bioassays are best advocated for predicting the toxicity of complex chemical mixtures (Ansari and Malik, 2009a; Cigerci et al., 2015; Kumari et al., 2016). Moreover, bioassay-directed chemical analyses can subsequently be employed in an effort to identify the putative mutagens as it integrates the effects of all mixture components, whether or not they are known and identified (White and Claxton, 2004; Man et al., 2013; Kirkland et al., 2016).

In the present study soil irrigated with industrial wastewater showed considerable mutagenicity with an increase in the number of revertants with increasing the dose. All the soil extracts gave positive results and dichloromethane extract of wastewater irrigated soil (Site II) displayed maximum mutagenicity with TA98 and TA97a strains in the presence as well as in the absence of S9 fraction. The strain TA98 was the most responsive strain among all the strains tested in terms of
mutagenic index and induction factor (Table 1-4). Our results indicated that the TA98, TA97a and TA100 gave best response with all the extracts of the soil samples. Katnoria et al. (2011) demonstrated mutagenic activity of industrial waste contaminated soil by TA100 and reported dose-related increase in the number of revertants per plate. Vincent-Hubert et al. (2011) reported mutagenicity of suspended particulate matter (SPM) in river receiving effluents from chemical dyes industry, petroleum refineries and pulp and paper mills by S. typhimurium strain TA98 with metabolic activation and found responses were 2-21 times greater than negative control indicating the presence of frame shift mutagens in the SPM extracts. Kwasniewska et al. (2012) observed mutagenicity of two soil samples from municipal solid waste land fill sites and found 4-fold increase in the number of revertants in the assay performed with both TA98 and TA100 strains. In another study, Man et al. (2013) demonstrated significant mutagenicity of the soil from 12 different land use types (contaminated with Poly Aromatic Hydrocarbons and dioxins/furans) carried out by S. typhimurium strains and found high mutagenic potency of upto 13.8 and 7.43 on both strains of TA98 and TA100 with S9 mix; similarly Ansari and Malik, (2009a) and Masood and Malik, (2013a) reported TA98 and TA100 to be the most sensitive strains tested with different organic extracts of agricultural soil irrigated with wastewater.

Genotoxicity tests involving bacterial DNA repair assay is comprehensively studied due to its sensitivity, reproducibility and rapidity (Ruiz and Marzin, 1997; Ohe et al., 2004; Escobar et al., 2013; Sanchez-Vicente et al., 2016). These assays evaluate genotoxicity based on activation of SOS response in bacteria exposed to DNA-damaging agents. The regulon having two proteins inducer recA and lexA repressor, playing key roles in regulation of the SOS response (Little and Gillert, 1983). During normal condition lexA act as repressor and suppress the action of SOS genes promoters by binding to specific sequence in the SOS box (Thliveris et al., 1991). After exposure to DNA damaging agents, recA binds to single-stranded DNA formed in response of damage due to mutations and gets activated and act as a co-protease, thus stimulating the cleavage of lexA and triggering the transcription of SOS genes (Horii et al., 1981; Michel, 2005; Zhang et al., 2010). In the present study the recA, lexA and polA mutants were found to be highly sensitive to soil extracts (Fig. 1) suggesting thereby, the damage to the DNA of the exposed cells as well as the role of recA+, lexA+ and polA+ genes in response to the exposure to mutagenic substances
present in soil extracts. Siddiqui et al. (2011a) and Masood and Malik (2013a) reported significant decline in the survival of DNA repair defective *E. coli* K-12 mutants as compared to their isogenic wild type counterparts upon treatment with different soil extracts of wastewater and groundwater irrigated agricultural soil respectively. Prophage induction by mutagens, radiation and chemicals has been described previously to be one among the many SOS responses (Cabrera, 2000; Vargas et al., 2001; Ansari and Malik, 2009a). Anjum and Malik (2013) reported significant loss of plaque forming units (PFUs) at the dose 40 µl/ml of culture (XAD concentrated) and 80 µl/ml of culture (liquid–liquid extract and pesticide mixture). The *lexA* mutants were found to be most sensitive strains in the presence of test samples as compared to their isogenic wild-type counterparts. In another study done by Anjum and Krakat (2015) genotoxicity assessments of alluvial soil irrigated with wastewater from a pesticide manufacturing industry was assessed by the bacteriophage λ test system and found that hexane extracts of soil irrigated with wastewater exhibited a maximum decline of plaque forming units for *lexA* mutants of *E. coli* K-12 pointing to an elevated genotoxic potential.

In the present study, test soil exhibited influence on mitotic index (MI) and were also able to induce chromosomal aberrations in *A. cepa* root meristem cells (Fig. 3). Wastewater from pulp and paper industries contain several persistent organic pollutants and heavy metals that may affect growth and cell division which may appear as significant decline in MI (Table 5). The chemical agents present in soil may cause major alteration to the genetic material of eukaryotic cells by its direct manifestation of damage on DNA/protein synthesis. Genotoxic potential of the test soil can be elucidated by significant and concentration dependent induction of chromosomal aberrations and increased or decrease of MI in the exposed root tip cells (Fernandes et al., 2007). Increased MI above those of negative control indicates increased cell division which may lead to uncontrolled proliferation and even tumor formation, while decreased MI than the negative control indicates the detrimental effect on growth and development of exposed organisms (Cigerci et al., 2016; Martins et al., 2016).

Present results of *A. cepa* test showed an increase in anaphase aberration frequencies in contaminated soil and ground water irrigated soil sample as compared to negative control (Table 6). Kong and Ma (1999) studied genotoxic effect of pesticide contaminated soil and found greater chromosomal aberration frequencies
compared to the well irrigated soil. Several types of chromosomal aberrations were observed during cell division such as chromosomal breaks, bridges, chromosomal losses, laggards, adherence, multipolarity, diploidy and c-mitosis, which are indicators of clastogenic and aneugenic effects (Leme et al., 2008, Firbas and Amon, 2014). The breakage in chromosomal fragments results due to breaks during anaphase bridge by cohesive chromosomal translocation (Fiskesjo and Levan, 1993). Present data showed significant anaphase bridge value in A. cepa meristematic cells exposed to the soil extracts of pulp and paper mill (Table 6). Marcano et al. (2004) showed that the chromosomal adherence can result in the formation of chromosomal bridges and can lead to chromosomal breaks. According to Fiskesjo and Levan (1993) and Turkoglu (2007), chromosomal adherence poses irreversible deleterious effect on genetic material, triggering the cell death process. The aneugenic effect such as inactivation of spindle fibres results in the formation c-metaphases which may lead to multinuclear cells (Fernandes et al., 2007; Leme et al., 2008). Stickiness was also observed which is likely the result of cross linkage of chromo-proteins (Radic et al., 2010). Another common sign of dysfunction of the mitotic spindle results unbalanced chromosome distribution, heading them for more than two poles onto the cells, opposite to what occurs in the normal division cycle (Rank and Nielsen, 1998; Pathiratne et al., 2015).

Our study revealed that pulp and paper mill effluents contain several recalcitrant agents capable of inducing mutations. Use of battery of short term assays are helpful in evaluating the toxic and genotoxic impacts of the complex matrices which could have been underestimated and indicate the complementarity of these approaches. In the present study, TA98 was found to be the most responsive strain in terms of mutagenic index, mutagenic potential and induction factor, indicating the presence of frame shift mutagens in the soil samples. Cytotoxicity and genotoxicity caused by the test soil samples was evident by decreased mitotic index and induction of several chromosomal aberrations in the plant root meristematic cells. Our findings suggest the potential genotoxic effect of wastewater irrigated soil from industrial sources which may enter into the food chain and cause health hazards to humans. Therefore the common practice of application of untreated wastewater to agricultural land should be strictly prohibited so that human beings could be protected from the toxicological effects of these xenobiotics.
### Table 1. Reversion of *Salmonella* tester strains in the presence of hexane extract of agricultural soil irrigated with ground water (Site I)

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Soil extract (µl/plate)</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>TA97a -</td>
<td>94 ± 2</td>
<td>128 ± 17 (1.3)</td>
<td>211 ± 19 (2.2)</td>
<td>246 ± 5 (2.6)</td>
<td>292 ± 5 (3.1)</td>
<td>253 ± 9 (2.6)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>96 ± 2</td>
<td>161 ± 11 (1.6)</td>
<td>221 ± 12 (2.3)</td>
<td>278 ± 12 (2.8)</td>
<td>328 ± 20 (3.4)</td>
</tr>
<tr>
<td>TA98  -</td>
<td>35 ± 3</td>
<td>83 ± 12 (2.3)</td>
<td>101 ± 14 (2.8)</td>
<td>121 ± 10 (3.4)</td>
<td>175 ± 10 (5.0)</td>
<td>151 ± 9 (4.3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>36 ± 2</td>
<td>112 ± 13 (3.1)</td>
<td>163 ± 9 (4.5)</td>
<td>227 ± 3 (6.3)</td>
<td>275 ± 3 (7.6)</td>
</tr>
<tr>
<td>TA100 -</td>
<td>145 ± 5</td>
<td>178 ± 13 (1.2)</td>
<td>211 ± 12 (1.4)</td>
<td>264 ± 7 (1.8)</td>
<td>299 ± 10 (2.0)</td>
<td>269 ± 10 (1.8)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>148 ± 3</td>
<td>200 ± 10 (1.3)</td>
<td>243 ± 8 (1.6)</td>
<td>275 ± 10 (1.8)</td>
<td>337 ± 15 (2.2)</td>
</tr>
<tr>
<td>TA102 -</td>
<td>241 ± 3</td>
<td>253 ± 13 (1.0)</td>
<td>282 ± 13 (1.1)</td>
<td>335 ± 11 (1.3)</td>
<td>363 ± 8 (1.5)</td>
<td>320 ± 4 (1.3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>255 ± 5</td>
<td>293 ± 7 (1.1)</td>
<td>353 ± 11 (1.3)</td>
<td>382 ± 8 (1.4)</td>
<td>445 ± 16 (1.7)</td>
</tr>
<tr>
<td>TA104 -</td>
<td>339 ± 18</td>
<td>355 ± 15 (1.0)</td>
<td>391 ± 10 (1.1)</td>
<td>438 ± 12 (1.2)</td>
<td>465 ± 9 (1.3)</td>
<td>413 ± 12 (1.2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>357 ± 9</td>
<td>392 ± 10 (1.0)</td>
<td>455 ± 6 (1.2)</td>
<td>505 ± 14 (1.4)</td>
<td>554 ± 14 (1.5)</td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 2. Reversion of *Salmonella* tester strains in the presence of hexane extract of contaminated soil (Site II)

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Soil extract (µl/plate)</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
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<tbody>
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<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
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<tr>
<td>TA97a</td>
<td>-</td>
<td>97 ± 3</td>
<td>166 ± 11 (1.7)</td>
<td>214 ± 14 (2.2)</td>
<td>322 ± 22 (3.3)</td>
<td>403 ± 8 (4.1)</td>
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<tr>
<td></td>
<td>+</td>
<td>98 ± 2</td>
<td>197 ± 16 (2.0)</td>
<td>251 ± 12 (2.5)</td>
<td>383 ± 7 (3.9)</td>
<td>441 ± 17 (4.5)</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>35 ± 5</td>
<td>101 ± 12 (2.8)</td>
<td>139 ± 9 (3.9)</td>
<td>183 ± 12 (5.2)</td>
<td>205 ± 10 (5.8)</td>
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<tr>
<td></td>
<td>+</td>
<td>35 ± 3</td>
<td>133 ± 8 (3.8)</td>
<td>171 ± 10 (4.8)</td>
<td>251 ± 11 (7.1)</td>
<td>312 ± 11 (8.9)</td>
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<tr>
<td>TA100</td>
<td>-</td>
<td>151 ± 9</td>
<td>184 ± 11 (1.2)</td>
<td>229 ± 18 (1.5)</td>
<td>270 ± 11 (1.7)</td>
<td>325 ± 8 (2.1)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>154 ± 4</td>
<td>241 ± 12 (1.5)</td>
<td>293 ± 11 (1.9)</td>
<td>356 ± 6 (2.3)</td>
<td>441 ± 12 (2.9)</td>
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<tr>
<td>TA102</td>
<td>-</td>
<td>247 ± 7</td>
<td>277 ± 8 (1.1)</td>
<td>318 ± 9 (1.2)</td>
<td>360 ± 35 (1.4)</td>
<td>423 ± 6 (1.7)</td>
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<tr>
<td></td>
<td>+</td>
<td>251 ± 9</td>
<td>281 ± 10 (1.1)</td>
<td>327 ± 7 (1.3)</td>
<td>373 ± 8 (1.4)</td>
<td>426 ± 14 (1.6)</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>349 ± 8</td>
<td>353 ± 12 (1.0)</td>
<td>411 ± 19 (1.1)</td>
<td>478 ± 15 (1.3)</td>
<td>523 ± 11 (1.4)</td>
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<tr>
<td></td>
<td>+</td>
<td>355 ± 5</td>
<td>411 ± 11 (1.1)</td>
<td>461 ± 10 (1.2)</td>
<td>487 ± 21 (1.3)</td>
<td>552 ± 11 (1.5)</td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 3. Reversion of *Salmonella* tester strains in the presence of dichloromethane extract of agricultural soil irrigated with ground water (Site I)

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Soil extract (µl/plate)</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>TA97a</td>
<td>-</td>
<td>96 ± 3</td>
<td>180 ± 17 (1.8)</td>
<td>229 ± 17 (2.3)</td>
<td>330 ± 26 (3.4)</td>
<td>406 ± 11 (4.2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>99 ± 5</td>
<td>208 ± 8 (2.1)</td>
<td>255 ± 11 (2.5)</td>
<td>383 ± 8 (3.8)</td>
<td>469 ± 13 (4.7)</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>33 ± 3</td>
<td>98 ± 7 (2.9)</td>
<td>168 ± 13 (5.0)</td>
<td>253 ± 15 (7.6)</td>
<td>331 ± 18 (10.0)</td>
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<tr>
<td></td>
<td>+</td>
<td>34 ± 4</td>
<td>149 ± 8 (4.1)</td>
<td>214 ± 23 (5.9)</td>
<td>278 ± 13 (7.7)</td>
<td>355 ± 13 (9.8)</td>
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<td>TA100</td>
<td>-</td>
<td>141 ± 12</td>
<td>193 ± 16 (1.3)</td>
<td>233 ± 13 (1.6)</td>
<td>279 ± 13 (1.9)</td>
<td>353 ± 12 (2.5)</td>
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<td></td>
<td>+</td>
<td>152 ± 11</td>
<td>252 ± 11 (1.6)</td>
<td>301 ± 11 (1.9)</td>
<td>370 ± 11 (2.4)</td>
<td>413 ± 17 (2.7)</td>
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<tr>
<td>TA102</td>
<td>-</td>
<td>252 ± 12</td>
<td>383 ± 8 (1.1)</td>
<td>315 ± 11 (1.2)</td>
<td>389 ± 9 (1.5)</td>
<td>472 ± 20 (1.8)</td>
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<td>275 ± 7</td>
<td>323 ± 13 (1.1)</td>
<td>381 ± 10 (1.3)</td>
<td>451 ± 11 (1.6)</td>
<td>515 ± 15 (1.8)</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>342 ± 7</td>
<td>384 ± 5 (1.1)</td>
<td>426 ± 23 (1.2)</td>
<td>468 ± 24 (1.3)</td>
<td>544 ± 24 (1.5)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>351 ± 10</td>
<td>425 ± 12 (1.2)</td>
<td>476 ± 11 (1.3)</td>
<td>554 ± 13 (1.5)</td>
<td>589 ± 9 (1.6)</td>
</tr>
</tbody>
</table>

Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.
Table 4. Reversion of *Salmonella* tester strains in the presence of dichloromethane extract of contaminated soil (Site II)

<table>
<thead>
<tr>
<th>Strain</th>
<th>S9</th>
<th>Control</th>
<th>Soil extract (µl/plate)</th>
<th>Mi</th>
<th>m</th>
<th>LSD P≤0.05</th>
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</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>-</td>
<td>91 ± 1</td>
<td>206 ± 6 (2.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>101 ± 10</td>
<td>225 ± 10 (2.2)</td>
<td>1.28</td>
<td>6.66</td>
<td>21.13</td>
</tr>
<tr>
<td>TA98</td>
<td>-</td>
<td>37 ± 1</td>
<td>128 ± 8 (3.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>37 ± 1</td>
<td>173 ± 8 (4.6)</td>
<td>2.38</td>
<td>7.51</td>
<td>23.79</td>
</tr>
<tr>
<td>TA100</td>
<td>-</td>
<td>149 ± 1</td>
<td>210 ± 15 (1.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>158 ± 7</td>
<td>282 ± 8 (1.7)</td>
<td>0.51</td>
<td>4.01</td>
<td>22.86</td>
</tr>
<tr>
<td>TA102</td>
<td>-</td>
<td>247 ± 7</td>
<td>305 ± 13 (1.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>282 ± 7</td>
<td>349 ± 8 (1.2)</td>
<td>-0.01</td>
<td>4.53</td>
<td>22.64</td>
</tr>
<tr>
<td>TA104</td>
<td>-</td>
<td>354 ± 4</td>
<td>405 ± 14 (1.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>368 ± 15</td>
<td>441 ± 11 (1.1)</td>
<td>-0.44</td>
<td>3.34</td>
<td>17.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TA97a: Values in parentheses are mutagenic index; Mi = induction factor; m = mutagenic potential; LSD = least significant difference.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Effect of different concentrations of soil extract on mitotic index and mitotic phase of *Allium cepa* root meristematic cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (%)</th>
<th>Mitotic Phases (%)</th>
<th>Mitotic index (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prophase</td>
<td>Metaphase</td>
</tr>
<tr>
<td>Site I</td>
<td>5</td>
<td>36.35</td>
<td>38.53</td>
</tr>
<tr>
<td>(Agricultural soil irrigated</td>
<td>10</td>
<td>38.85</td>
<td>33.58</td>
</tr>
<tr>
<td>with ground water)</td>
<td>25</td>
<td>39.38</td>
<td>38.14</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>44.76</td>
<td>34.74</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>49.23</td>
<td>26.15</td>
</tr>
<tr>
<td>Site II</td>
<td>5</td>
<td>38.29</td>
<td>32.32</td>
</tr>
<tr>
<td>(Contaminated soil)</td>
<td>10</td>
<td>42.87</td>
<td>29.01</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>45.20</td>
<td>28.40</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52.60</td>
<td>24.60</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>56.75</td>
<td>24.32</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>63.50</td>
<td>23.90</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>34.25</td>
<td>35.45</td>
</tr>
</tbody>
</table>

Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test); ±: Standard deviation.
Table 6. Chromosomal aberrations in the root meristematic cells of *Allium cepa* exposed to different concentrations of soil extract for 72 h

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (%)</th>
<th>Types of aberrations</th>
<th>Total aberrant cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM</td>
<td>AB</td>
</tr>
<tr>
<td>Site I</td>
<td>5</td>
<td>2.6 ± 0.5</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td>(Agricultural soil irrigated with ground water)</td>
<td>10</td>
<td>2.9 ± 0.6</td>
<td>2.5±0.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.2 ± 0.3</td>
<td>3.3±0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.8 ± 0.5</td>
<td>4.6±0.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.5 ± 1.4</td>
<td>4.8±1.0</td>
</tr>
<tr>
<td>Site II</td>
<td>5</td>
<td>4.1 ± 1.6</td>
<td>1.5±0.5</td>
</tr>
<tr>
<td>(Contaminated soil)</td>
<td>10</td>
<td>6.4 ± 2.1</td>
<td>2.9±0.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7.5 ± 1.8</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.7 ± 2.8</td>
<td>5.6±0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9.8 ± 1.4</td>
<td>6.5±1.0</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>14.3±0.5</td>
<td>9.3±5.5</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td>1.6 ± 1.1</td>
<td>1.0±0.0</td>
</tr>
</tbody>
</table>

CM: c-mitosis, AB: anaphase bridge, S: stickiness, L: laggard, DAT: disturbed anaphase-telophase, VC: vagrant chromosomes, DM: disturbed metaphase, P: polyploidy. Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test).
Fig. 1. Survival of *E. coli* K-12 strains treated with hexane extract of soil (a) site I (b) site II; dichloromethane extract of soil (a’) site I (b’) site II.
Fig. 2. Survival of bacteriophage λ treated with hexane extract of soil (a) site I (b) site II; dichloromethane extract of soil (a') site I (b') site II.
**Fig. 3.** Different types of chromosomal aberrations induced by the soil extracts (contaminated agricultural soil) in *Allium cepa* root-tips (a) anapahse bridge (b) disturbed metaphase (c) disturbed anaphase with chromosome stickiness (d) chromosome breaks (e) disturbed metaphase (f) disturbed metaphase with chromosome break (g) bipolar anaphase (h) disturbed anaphase with vagrant chromosome (i) vagrant chromosomes (j) disturbed metaphase with chromosome break (k) stickiness (l) chromosome breaks and loss.
Growing population and rapid economic development over recent decades has created adverse environmental impacts, including serious water pollution. Also the fast urbanization and industrialization has led to an increase pollution of surrounding agricultural soils (Liu et al., 2013). Water pollution has caused shortage of availability of fresh water resources and increases pressure on demand. Use of treated waste water for irrigation of agricultural lands is now a common practice in arid and semi-arid region (Azouzi et al., 2017).

Among various industries, Pulp and paper industry play important role to the regional economies of several industrialized countries (Hussain and Bernard, 2017). Accelerated population growth combined with the economic growth observed in several countries has greatly contributed to an increased demand for paper. However pulp and paper industry is water and energy intensive industry; therefore it has been associated with various environmental issues including high levels of wastewater generation (Soucy et al., 2014; Adhikari and Bhattacharyya, 2015). The wastewater generated from pulp and paper industry contains variety of adsorbable organic halides along with dioxins and furans which have been suspected for their mutagenic and endocrine disrupting property (Kamali and Khodaparast, 2015; Balabanic et al., 2017).

The physico-chemical analysis of wastewater and soil allows critical assessment of their pollution level. The toxic pollutant present in wastewater or soil may seriously affect the microbial community structure by decreasing their number, reducing their biochemical activities and affecting diversity (Torsvik and Ovreas, 2002). Generally the microbial diversity reduces with increased pollution because the sensitive microorganisms abruptly decline by the effect of pollutants whereas the tolerant microorganisms become predominant (Cao et al., 2017).

Chlorinated aromatic compounds such as chlorophenols are common environmental pollutant and are recalcitrant to degradation (Tuhina and Verma, 2012). Different physico-chemical methods have been assessed to facilitate the removal of chlorophenols (CPs). However, biological methods such as microbial biodegradation or transformation of CPs, are regarded as key remediation and environmentally friendly options that can be utilized to solve environmental pollution problems caused by such toxic compounds (Olaniran and Igbinosa, 2011; Ren et al., 2016). Many microbial species isolated from natural sources viz. *Pseudomonas* sp., *Sphingomonas* sp., *Alcaligenes* sp., and *Rhodococcus* sp. have been reported to utilize
chlorophenols as substrates. Nonetheless aerobic, co-metabolic degradation is regarded as an effective strategy for chlorophenols removal (Huang et al., 2013; Tripathi and Garg, 2013; Kang et al., 2017).

Despite the advancement of rapid detection technique in environmental chemistry, it remains infeasible to track every pollutant potentially present in wastewater and the hazard caused by the cumulative effects of the complex mixtures of environmental contaminants (Jia et al., 2015). A set of bioassays i.e., Ames Salmonella/mammalian microsome test, Escherichia coli DNA repair defective mutation assay, bacteriophage λ test system and Allium cepa chromosomal aberration assay are helpful in predicting genotoxic potential of unknown toxic chemicals present in complex mixtures and have therefore been increasingly used in water quality assessment over several years.

In view of the environmental hazards associated with pulp and paper industries wastewater and chlorophenol generation, the present work was conducted to analyse the physico-chemical and microbial assessment of pulp and paper industries effluent and contaminated soil. For cost effective biological removal of toxic chlorophenols, indigenous bacteria were isolated and screened for their chlorophenol degrading activity. Furthermore the genotoxicity of wastewater and contaminated soil was carried out to predict the putative hazards of industrial effluent.

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

I. Physico-chemical analysis and microbial diversity of pulp and paper industry wastewater and soil

- The wastewater was yellowish brown in colour and found to possess high concentration of suspended solids (2384 ± 70.0 mg L⁻¹), total dissolved solids (528.6 ± 50.0 mg L⁻¹) and chemical oxygen demand (1312 ± 55.4 mg L⁻¹).
- The atomic absorption spectrophotometric analysis of wastewater and soil samples revealed the presence of several heavy metals. The concentration of Cr, Cd, Cu, Zn, Ni and Pb in the wastewater was found to be 1.28, 0.29, 0.9, 17.8, 3.0 and 5.0 mg L⁻¹ respectively. The concentration of Cr, Cd, Cu, Zn, Ni and Pb was found to be 22.36, 12.53, 15.3, 37.26, 2.23 and 5.3 mg Kg⁻¹ in agricultural soil irrigated with wastewater.
- GC-MS analysis of the wastewater and soil samples revealed the presence of a large number both aliphatic and aromatic organic compounds like phthalic acid and its
ester. Various other compounds such as heneicosane, tetracosane, tetracontane, diphenyl ether, decanoic acid, dodecanoic acid 1,2,3-propanetriylester etc. were also detected.

- The wastewater from pulp and paper industry exhibited rich diversity of aerobic heterotrophic bacteria (12.4×10^5 CFU/mL), fungi (9.3×10^3 CFU/mL), actinomycetes (13.2×10^4 CFU/mL) and asymbiotic N2 fixers (17.4×10^4 CFU/mL). The microbial counts of soil irrigated with ground water were found to be higher than the contaminated soil and wastewater.

Present findings indicated that the pulp and paper industry wastewater and contaminated soil contained several heavy metals (i.e. Ni, Cu, Cr, Pb, Cd and Zn) and a wide variety of organic compounds (aliphatic and aromatic hydrocarbons like phthalic acids and its esters). Our findings are suggestive of the idea that physico-chemical status of the system influenced microbial diversity through rendering them properties such as tolerance to undesirable agents. Moreover, the presence of toxic metals and persistent organic pollutants in the wastewater and soil may cause deleterious effect on the indigenous microbes and lead to survival of resistant microbial population. Furthermore, many sensitive microbial species might have been eliminated by the pollutants and their place is taken by resistant species which have different ecological properties.

II. Degradation of chlorophenols by the bacterial isolates

- A total of 40 bacteria (15 and 25 each from pulp and paper mill effluent and contaminated soil) were isolated from PCP amended minimal agar plates. Out of the 40 isolates, 14 bacteria were selected for degradation studies on the basis of minimal inhibitory concentration (MIC) for 2,4 DCP, 2,4,6 TCP and PCP.

- 16S rDNA sequence analysis revealed that out of the 14 isolates tested, 11 were found to be most similar to Pseudomonas sp. and 3 with Klebsiella sp. The accession numbers of the partial 16S rDNA sequences (KX353799-KX353811, KF764698) obtained in this study are available at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

- These bacterial isolates exhibited different degree of PCP degradation ranging from 55-100% after 120 h of incubation. Based on the extent of PCP degradation, four bacterial isolates i.e., SM1, N1, N6 and W1 displayed maximum degradation potential
were identified as *P. aeruginosa* SM1, *K. oxytoca* N1, *K. oxytoca* N6 and *P. aeruginosa* W1 based on the BLAST analysis of 16S rDNA sequence.

- The bacterial isolates *P. aeruginosa* SM1, *K. oxytoca* N1, *K. oxytoca* N6 and *P. aeruginosa* W1 grew well in the minimal salt broth, amended with the chlorophenols as observed by the time course of growth of the bacterial isolates. The isolates exhibited a high chlorophenol degradation efficiency at a wide range of pH (5-9) and temperatures (25-35 °C).

- *P. aeruginosa* SM1 completely degraded PCP (100 µg-mL\(^{-1}\) concentration) at pH 7.0 and temperature of 35 °C, whereas *K. oxytoca* N6 exhibited 98% PCP degradation at pH 7.0 and temperature of 30 °C. *P. aeruginosa* W1 exhibited 100% degradation of 2,4,6 TCP at pH 7.0 and temperature of 30 °C. Complete degradation of 2,4 DCP was achieved using *K. oxytoca* N1 bacterial isolates at pH 7.0 and temperature of 35 °C.

- Gas chromatogram of the degradation products of chlorophenols displayed several peaks as compared to the control samples. The intermediate compounds formed from PCP degradation by *P. aeruginosa* SM1 were identified as pentachloroanisole, tetrachlorohydroquinone, tetrachloroanisole, 2,5-dichloro-1,4dimethoxy benzene and some low molecular weight compounds like maleylacetate and 2-hydroxy-muconic-semialdehyde; whereas dichlorophenol, trichlorophenol, tetrachlorochlorophenol, tetrachlorohydroquinone, maleylacetate and 2-hydroxy-muconic-semialdehyde intermediates were detected after PCP degradation by *K. oxytoca* N6.

- 2,4,6 TCP degradation intermediates were 2,4 dichlorophenol, 2,6-dichloro-1,4-benzenediol, 1,2 benzene diol and 2-hydroxy-muconic-semialdehyde as indicated by the gas chromatogram of TCP degradation by *P. aeruginosa* W1, whereas the intermediates released from 2,4 DCP degradation by *K. oxytoca* N1 were dichlorobenzoquinone, 2 chlorophenol, 5 chloro-2-hydroxymuconic semialdehyde and 5-chloro-2-oxopent-4-enoic acid.

In the present study, the indigenous bacterial strains having significant ability to degrade chlorophenols were isolated and identified as *P. aeruginosa* and *K. oxytoca* on the basis of 16S rDNA sequencing. Present findings indicated that biodegradation was greatly enhanced by the addition of glucose (0.1% w/v) as a co-substrate. These bacterial strains degraded chlorophenols at a wide range of pH (5-9) and temperature.
range of 30-35 °C. GC-MS analysis revealed the presence of several low molecular weight intermediates as compared to the control samples that confirmed the mineralization of chlorinated organic compounds by the bacterial isolates. Our results indicate the remarkable chlorophenol degradation potential by the selected bacterial isolates which could be exploited for the bioremediation of chlorophenols.

III. Genotoxicity of pulp and paper industry wastewater

- Genotoxicity of wastewater was evaluated employing Ames Salmonella/mammalian microsome test, DNA repair defective Escherichia coli K-12 mutants, bacteriophage λ system and A. cepa chromosomal aberration assay.
  - The XAD concentrated sample was found to be more mutagenic than the liquid-liquid extracted samples (hexane and dichloromethane extracts).
  - For all the samples tested, there was a dose related increase in the reversion frequency up to 20 µl/plate and decline was observed at a dose of 40 µl/plate. It was found that the test samples showed maximum response with TA98 strain in the presence as well as in the absence of S9 fraction.
  - The order of responsiveness based on the mutagenic index and induction factor in the presence and absence of S9 fraction for XAD-concentrated wastewater samples was in the following order: TA98 > TA97a > TA100 > TA102 > TA104.
  - For hexane extracted wastewater sample, TA98 exhibited maximum response in terms of mutagenic index (10.1 without S9 and 10.7 with S9 fraction); induction factor (2.21 without S9 and 2.27 with S9 fraction); and mutagenic potential (5.0 without S9 and 6.82 with S9 fraction).
  - TA98 also showed maximum response in terms of mutagenic index (11.0 and 11.1 without and with S9 fraction), induction factor (2.31 without and with S9 fraction) and mutagenic potential (5.98 and 6.50 without and with S9 fraction) when treated with acidic fraction of dichloromethane extract.
  - Basic fraction of dichloromethane extract displayed maximum response of 7.4 (without S9 fraction) and 9.7 (with S9 fraction) in terms of mutagenic index; 1.85
(without S9) and 2.16 (with S9) in terms of induction factor; and 3.13 (without S9) and 4.23 (with S9) in terms of mutagenic potential for TA98 strain.

- The survival pattern of recA, lexA and polA mutants of E. coli K-12 and their isogenic wild-type counterparts demonstrated that XAD-concentrated sample showed maximum damage to the cells at a dose of 20 µl/ml of culture as compared to liquid liquid extracted samples.
- Among all the mutants, polA exhibited maximum decline in survival with the XAD concentrated as well as liquid-liquid extracted wastewater samples after 6 h of treatment. The survival was 21% in polA, 24% in lexA and 38% in recA mutants when treated with XAD concentrated sample.
- The survival was found to be 24% in polA, 28% in lexA and 41% in recA mutants with acidic fraction of dichloromethane, whereas survival was observed to be 32% in polA, 38% in lexA and 45% in recA mutants with hexane extract, at a dose of 20 µL/ mL of culture.
- Extracellular treatment of bacteriophage λ demonstrated that XAD-concentrated wastewater sample showed maximum decline in plaque forming units as compared to liquid-liquid extracted wastewater samples. Among the DNA repair defective mutants of E.coli K 12, lexA showed maximum decline in plaque forming units.
- The XAD-concentrated wastewater sample showed a decrease in survival up to 16% in lexA and 36% in recA mutants, while acidic fraction of dichloromethane exhibited decline of 18% in lexA and 42% in recA mutants after 6 h of treatment.
- *Allium cepa* root meristematic cells, treated with wastewater significantly affected the mitotic phases and different chromosomal aberrations were observed e-mitosis, anaphase bridge, stickiness, laggard, disturbed anaphase-telophase, vagrant chromosomes, disturbed metaphase and polyploidy. Further decline in mitotic index was observed in a dose dependent manner.

Our study confirm the mutagenicity of wastewater from pulp and paper mill as analysed by the bacterial reverse mutation assay in Ames *Salmonella* strains TA97a, TA98, TA100, TA102 and TA104 in the presence and absence of metabolic activation system, DNA repair defective *E. coli* K-12 mutants and bacteriophage λ system. XAD extracts of the wastewater samples were found to be more mutagenic as compared to those of liquid-liquid extracts (hexane and dichloromethane extracts). This study not
only confirms the idea of the presence of mutagens in the wastewater but also suggest the mutagenic hazards of the pollutants in the wastewater. *Allium cepa* chromosomal aberration assay also suggested that the raw wastewater contain mixtures of genotoxins which significantly decrease the mitotic index and induce several chromosomal aberrations such as c-mitosis, anaphase bridges, polyploidy, binucleate cells, stickiness, disturbed metaphase, disturbed anaphase, laggards and breaks with the test samples. The present results indicated potential mutagenic/genotoxic activity of the pulp and paper mill effluent possessing complex assortment of chemicals which is affecting the receiving water bodies and need continuous monitoring to provide basis for improvement.

**IV. Genotoxicity of soil**

Genotoxicity of agricultural soil irrigated with wastewater from pulp and paper industries was evaluated using a combination of test systems i.e. Ames *Salmonella*/mammalian microsome test, DNA repair defective *E.coli* K-12 mutants, bacteriophage λ system and *Allium cepa* chromosomal aberration assay.

- DCM extract of wastewater irrigated soil was found to be more mutagenic than hexane extracted soil in terms of mutagenic index, induction factor and mutagenic potential. All the samples exhibited significant mutagenicity with TA97a, TA98, and TA100 strains in the presence as well as in the absence of S9 fraction.
- Maximum number of revertant colonies were observed for TA98 strain in case of DCM extract of contaminated soil, which was also the most responsive strain in terms of mutagenic index (12.81 and 11.81 with and without S9 fraction), induction factor (2.47 with and 2.38 without S9 fraction) and mutagenic potential/ slope of the initial linear dose response curve (7.87 and 7.51 with and without S9 fraction). The following trend was found for the tester strains based on mutagenic index and induction factor: TA98 > TA97a > TA100 > TA102 > TA104 (with and without S9 fraction), whereas the order of responsiveness in terms of mutagenic potential/ slope was TA98 > TA97a > TA100 > TA102 > TA104 (with S9 fraction).
- Hexane extract of contaminated soil showed mutagenic index of 8.9 and 5.8 (with and without S9 fraction); induction factor of 2.06 and 1.58 (with and without S9 fraction); and mutagenic potential of 4.91 and 2.58 (with and without S9 fraction).
- The response of the tester strains were significantly higher when treated with contaminated soil (site II) extracts as compared to uncontaminated soil (site I).
SOS defective recA, lexA and polA mutants as well as their isogenic wild type counterparts exhibited maximum decline in colony forming units when treated with dichloromethane extract of contaminated soil. The survival was 31% in polA, 38% in lexA and 46% in recA mutants when treated with dichloromethane extract of ground water irrigated soil (site I), while survival was 25% in polA, 30% in lexA and 37% in recA mutants when treated with dichloromethane extract of contaminated soil (site II) after 6 h of treatment.

The survival was 42% in polA, 46% in lexA and 57% in recA mutants when treated with hexane extract of ground water irrigated soil (site I), while the survival was 34% in polA, 39% in lexA and 45% in recA mutants when treated with hexane extract of contaminated soil (site II) after 6 h of treatment.

Extracellular treatment of bacteriophage λ demonstrated that contaminated soil (site II) displayed maximum decline in plaque forming units as compared to ground water irrigated soil (site I). Among all the mutants lexA exhibited maximum decline in PFUs.

The dichloromethane extracts of wastewater irrigated soil (site II) exhibited decrease in survival (PFUs) up to 18% in lexA and 27% in recA mutants, while groundwater irrigated soil (site I) showed decrease in survival up to 30% in lexA and 41% in recA mutants after 6 h of treatment.

The hexane extracts of wastewater irrigated soil (site II) showed a decrease in survival (PFUs) up to 24% in lexA and 43% in recA mutants whereas hexane extracts of groundwater irrigated soil (site I) showed a decline of 34% in lexA and 45% in recA mutants after 6 h of treatment.

Allium cepa root tip cells treated with the different concentrations of the extracts of the wastewater and ground water irrigated soil exhibited significant decline in mitotic index (MI) in a dose dependent manner with increasing concentration of soil extracts. It was observed that decline of MI was more prevalent in wastewater irrigated soil (site II) than ground water irrigated soil (site I). MI was observed to be 15.94% (P<0.05) at 100% concentration for ground water irrigated soil (site I), whereas it was 10.04 % (P<0.05) at 100% concentration for wastewater irrigated soil (site II).

Statistically significant (P<0.05) frequencies of chromosomal abnormalities were recorded with 5-100% soil extracts of wastewater irrigated soil sample (site II)
compared to ground water irrigated soil (site I). The soil samples caused several types of aberrations in the A. cepa root tips, such as chromosomes breaks, anaphase bridges, c-mitosis, laggards, binucleated cells, stickiness and unequal distributions of chromosomes at anaphase stage of cell division.

Our study revealed that pulp and paper mill effluents contain several recalcitrant agents capable of inducing mutations. Use of battery of short term assays are helpful in evaluating the toxic and genotoxic impacts of the complex matrices which could have been underestimated and indicate the complementarity of these approaches. In the present study, TA98 was found to be the most responsive strain in terms of mutagenic index, mutagenic potential and induction factor, indicating the presence of frame shift mutagens in the soil samples. Cytotoxicity and genotoxicity caused by the test soil samples was evident by decreased mitotic index and induction of several chromosomal aberrations in the plant root meristematic cells. Our findings suggest the potential genotoxic effect of wastewater irrigated soil from industrial sources which may enter into the food chain and cause health hazards to humans. Therefore the common practice of application of untreated wastewater to agricultural land should be strictly prohibited so that human beings could be protected from the toxicological effects of these xenobiotics.


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