

# Mammalian cell line-based bioassays for toxicological evaluation of landfill leachate treated by *Pseudomonas* sp. ISTDF1

Pooja Ghosh · Mihir Tanay Das · Indu Shekhar Thakur

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**Abstract** Landfill leachate has become a serious environmental concern because of the presence of many hazardous compounds which even at trace levels are a threat to human health and environment. Therefore, it is important to assess the toxicity of leachate generated and discharge it conforming to the safety standards. The present work examined the efficiency of an earlier reported *Pseudomonas* sp. strain ISTDF1 for detoxification of leachate collected from Okhla landfill site (New Delhi, India). GC-MS analysis performed after treatment showed the removal of compounds like alpha-limonene diepoxide, brominated dioxin-2-one, Bisphenol A, nitromusk, phthalate derivative, and nitrobenzene originally found in untreated leachate. ICP-AES analysis for heavy metals also showed reduction in concentrations of Zn, Cd, Cr, Fe, Ni, and Pb bringing them within the limit of safety discharge. Methyl tetrazolium (MTT) assay for cytotoxicity, alkaline comet assay for genotoxicity, and 7-ethoxyresorufin-*O*-deethylase (EROD) assay for dioxin-like behavior were carried out in human hepato-carcinoma cell line HepG2 to evaluate the toxic potential of treated and untreated leachates. The bacterium reduced toxicity as shown by 2.5-fold reduction of MTT EC<sub>50</sub> value, 7-fold reduction in Olive Tail Moment, and 2.8-fold reduction in EROD induction after 240 h of bacterial treatment.

**Keywords** Landfill leachate · Bioremediation · *Pseudomonas* sp. · Toxicity reduction · HepG2 cell line

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P. Ghosh · M. T. Das · I. S. Thakur (✉)  
School of Environmental Sciences, Jawaharlal Nehru University,  
New Delhi 110067, India  
e-mail: isthakur@hotmail.com

P. Ghosh  
e-mail: pooja.ghosh9@gmail.com

## Introduction

Landfills are the final depositories for a wide range of solid waste. This commonly consists of waste from residential, as well as commercial sources and products such as pharmaceuticals, food additives, soaps, detergents, solvents, perfumes, fire retardants, and plasticizers. The landfill leachate thus produced contains a significant number of xenobiotic compounds, either found in the disposal site or formed due to chemical and biological transformations within the landfill (Banar et al. 2006). Many of these organic micropollutants, such as halogenated aliphatic and aromatic compounds, nitrogen containing compounds, phenols, phthalate esters, and pesticides found in the leachate, are highly toxic, estrogenic, and carcinogenic even at trace levels (Cozzarelli et al. 2011; Andrews et al. 2011). Leaching of these organic chemicals and heavy metals from unlined landfills is a major threat to the groundwater resources and can cause deleterious effects to human health through their synergistic and additive actions (Baderna et al. 2011). Adverse effects of landfill leachate have been confirmed by recent investigations using aquatic animal bioassays (Deguchi et al. 2007), plant bioassays (Li et al. 2008; Sang et al. 2006, 2010), and mammals (Li et al. 2004a; 2006a, b), suggesting that the mixture of contaminants has the potential to cause harmful effect to public health through seepage into the ground or surface waters.

Depending on the factors like hydrogeology, waste composition, amount of rainfall, and age of the landfill, the characteristics of landfill leachate vary widely from one place to another (Bean et al. 1995). Therefore, it does not necessarily guarantee that the treatment methods employed in one location can be effective as an efficient treatment elsewhere. Hence, there is a growing concern on site-specific monitoring, identification, and risk assessment of the micropollutants CBT present in landfill leachate and finding a sustainable option to treat them effectively before being discharged into the environment. The use of microbes for biodegradation of persistent

organic pollutants as well as biosorption of heavy metals is an efficient and cost-effective tool for the treatment of landfill leachate as compared to various physicochemical methods such as adsorption, membrane filtration, chemical precipitation, and ion exchange which have the limitation of high operating cost and limited versatility (Kapoor and Viraraghavan 1995). White-rot fungi have been exploited in the past for the treatment of landfill leachate by Tigrini et al. (2013) and Razarinah et al. (2011). A number of bacteria particularly *Burkholderia* sp., *Pseudomonas* sp., *Mycobacterium* sp., *Rhodococcus* sp., and *Sphingomonas* sp. which have been reported to degrade PAHs, PCBs, pesticides, and other persistent organic pollutants are promising candidates for the treatment of landfill leachate (Bamforth and Singleton 2005; Egorova et al. 2010; Kim et al. 2003; Kanaly and Harayama 2000; Seo et al. 2009). In spite of their great potential, sparse studies have been conducted on their use for the treatment of leachate.

Characterization of the organic compounds present in pre- and post-treated leachate has a limitation of not all of them being identified because of their low concentrations underestimating their potential biological effects. Also, bioremediation looks like a promising tool for the removal of pollutants from the contaminated sites, but the removal of parent contaminants may not always correspond to a reduction in health risk (Lemieux et al. 2009). There may also be substantial increase in toxicity following bioremediation as documented by Hughes et al. (1998) and Gillespie et al. (2007). This may be either because of the formation of toxic intermediary metabolites like oxy-PAH's (Lundstedt et al. 2003) or increased bioavailability of native toxins over the course of bioremediation (Andersson et al. 2009). Thus, it is important to ensure that the contaminated material is suitably detoxified at the end of the treatment. Thus, it is best to characterize the compounds not only by chemical means but also by the use of mammalian cells and in vitro bioassays which are rapid, simple, sensitive, as well as cost-effective (Talorete et al. 2008). Hepatic carcinoma cell lines such as HepG2 has been shown to be very promising for detoxification studies because of their ability to synthesize xenobiotic metabolizing enzymes cytochrome P450 (CYP) 1A1 (Chaloupka et al. 1994). In the present investigation, Methyltetrazolium (MTT), alkaline comet, and 7-ethoxyresorufin-*O*-deethylase (EROD) assays were carried out using HepG2 cell line for comparative toxicity evaluation of landfill leachate before and after bacterial treatment. The MTT assay is an overall indicator of cytotoxicity and is based on the ability of metabolically active cells to reduce yellow tetrazolium MTT to purple formazan by the action of mitochondrial succinate dehydrogenase (Mosmann 1983). Alkaline comet assay is a sensitive technique for measuring different types of DNA damage like DNA double-strand breaks (DSB), single-strand breaks (SSB), alkali-labile sites (ALS),

DNA-DNA and DNA-protein crosslinks, and SSB associated with incomplete excision repair (Žegura and Filipič 2004; Singh et al. 1988). The EROD assay monitors the induction of the xenobiotic metabolizing enzyme cytochrome P-450 (CYP) 1A1 and is widely used as a biomarker for substances that bind the aryl hydrocarbon (Ah) receptor and is sensitive to the presence of a diverse group of chemicals like dioxins, furans, polychlorinated biphenyls (PCBs), and polyaromatic hydrocarbons (PAHs) (Tillitt et al. 1991). It involves the oxidative deethylation of 7-ethoxyresorufin (7-ER) to resorufin, catalyzed by CYP 1A1 (John et al. 2001). Since landfill leachate is heavily contaminated with these persistent organic pollutants, the EROD assay is an effective tool to monitor their presence.

The present investigation was designed to evaluate the potential of *Pseudomonas* sp. ISTDF1 for the treatment and detoxification of Okhla landfill leachate. The strain ISTDF1 was earlier isolated from the sludge and sediment sample of a pulp and paper mill and was shown to degrade dibenzofuran as a sole source of carbon in minimal salt medium (MSM). The presence of diverse dioxygenation and aromatic ring oxidation enzymes allows it to utilize various recalcitrant organic compounds, and its alkalotolerant nature makes it suitable for the treatment of highly alkaline wastewater (Jaiswal et al. 2011). Further, the strain has also shown its degradation efficiency in a mixture of carbon source in the form of paper mill sludge sediment extract (Das et al. 2012). Present study exploits the degradation potential of the strain to detoxify landfill leachate. The study simultaneously aimed to reveal whether the MTT, EROD, and alkaline comet assays, using HepG2 cell line, can be used to evaluate detoxification efficiency.

## Materials and methods

### Chemicals

All chemicals and cell culture-related reagents were procured from Sigma-Aldrich (St. Louis, MO, USA). All solvents were purchased from Merck (Darmstadt, Germany) and were of HPLC grade.

### Sampling, sampling site, and preliminary analysis

Leachate samples were collected from three sampling points across Okhla landfill site (28° 30' 48"N, 77° 17' 4"E), Delhi, an unlined landfill site established in 1994. It receives an average of 1,500 t of municipal solid waste per day consisting of household garbage, street sweeping, demolition, and biomedical wastes. Leachate samples were collected in glass bottles presoaked with 1 M HNO<sub>3</sub> for 24 h and rinsed with distilled water. The samples collected were preserved by the addition of concentrated HNO<sub>3</sub> (1 mL L<sup>-1</sup> of leachate sample)

for heavy metal analysis. In order to obtain a homogeneous sample, the effluent was combined and parameters like electrical conductivity (EC), pH, and total dissolved solids (TDS) were measured using Cyberscan PC 510 meter, COD by open reflux method and color using platinum-cobalt method (APHA 2005) without delay and sample was stored at 4 °C until complete analysis.

#### Microorganism and culture conditions

An already reported dibenzofuran degrading alkalotolerant bacterial strain *Pseudomonas* sp. ISTDF1 (gene bank accession number- EU834943) isolated from sludge and sediment sample from Century Pulp and Paper Mill, Lalkuan, Nainital, Uttarakhand, India (Jaiswal et al. 2011) was used for bioremediation of landfill leachate. The bacterial culture was maintained in chemostat with Minimal salt medium (MSM) containing ( $\text{g L}^{-1}$ ),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 7.8;  $\text{KH}_2\text{PO}_4$ , 6.8;  $\text{MgSO}_4$ , 0.2;  $\text{NaNO}_3$ , 0.085;  $\text{NH}_4(\text{CH}_3\text{COO})_3\text{Fe}$ , 0.05; and  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.05 with dibenzofuran (1 mM) as carbon source at 30 °C.

#### Treatment of leachate by the bacterial strain

Bacterial strain was inoculated in 500 mL MSM broth (pH=8.3, without  $\text{NH}_4(\text{CH}_3\text{COO})_3\text{Fe}$ ) containing 20 % v/v leachate and incubated up to 240 h in different shaking flasks under aerobic conditions at 30 °C and 150 rpm. Inoculum's size at 0 h was  $5 \times 10^4$  CFU  $\text{mL}^{-1}$ , and further growth was monitored at every 24-h interval up to 240 h by measuring turbidity ( $A_{600}$ ). The samples (300 mL) were removed after 0, 24, 48, 120, and 240 h. All the collected samples (denoted as; UT, T24, T48, T120, and T240 for 0 h untreated, 24, 48, 120, and 240 h samples, respectively) were centrifuged at 7,000 rpm for 10 min to remove bacterial biomass, and the supernatants were processed for GC-MS analysis, ICP-AES analysis, and in vitro bioassays for the identification of organic compounds, analysis of heavy metals, and toxicological evaluation, respectively.

#### Extraction of organic contaminants and GC-MS analysis

Classical solvent extraction technique was used to extract the organic compounds present in the samples. For this, 100 mL 1:1 v/v dichloromethane (DCM) and acetone was added to 250 mL sample (Das et al. 2012). Extraction process was repeated thrice. The extracted organic fraction was filtered through Whatman No. 54 filter paper and then evaporated to dryness at room temperature using a vacuum rotator evaporator. After evaporation, it was dissolved in 2 mL DCM (Crude organic extracts) for GC-MS analysis. The analysis was done using a Shimadzu GC-MS-QP 2010 Plus equipped with a capillary column Rtx-5 (dimensions; 0.25- $\mu\text{m}$  film thickness, 0.25-mm internal diameter, and 30 m in length). One microliter

of extract was analyzed by GC at conditions: splitless mode with a split ratio of 10:0; initial temperature 60 °C for 1.0 min; and temperature increased from 60–320 °C at a rate of 22 °C  $\text{min}^{-1}$ ). Data were compared with the inbuilt standard mass spectra library system (NIST-05 and Wiley-8) of GC-MS.

#### Chemical analysis of heavy metals

For the analysis of heavy metals, the samples were acid digested according to Ogundiran et al. (2008). Briefly, 50 mL of sample was taken in a digestion tube along with 10 mL of  $\text{HNO}_3$  (69 %). This was then evaporated to around 10 mL, cooled at room temperature, filtered, and finally diluted with 50 mL double distilled water. Samples were digested and analyzed using Jobin Yvon ICP-OES (Ultima 2) in triplicates.

#### Toxicological analysis

The effect of the bacterial treatment on the toxicity of the leachate was evaluated by MTT, Comet, and EROD assays using HepG2 cell line. HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % antibiotic antimycotic solution in 5 %  $\text{CO}_2$  at 37 °C. The test samples for toxicity assays were prepared from the bacterial-treated leachate samples after the removal of bacterial biomass by centrifugation followed by filter sterilization using 0.22  $\mu\text{m}$  syringe filter. In MTT assay, 50  $\mu\text{M}$  BaP (positive control), 0.5 % v/v Milli-Q (negative control), and test samples were added to the cell culture in different dilutions to work out the dose response relationship. In the rest of the toxicity assays, 20 % v/v of test samples was used. In biodegradation study, the leachate concentration was 20 % v/v (crude leachate/MSM). But in toxicity assays, 20 % of the bacterial-treated samples (test samples) were added to the cell culture media. Thus, the effective leachate concentration for treatment (except MTT assay) in cell culture media was 4 %.

#### Cell viability

Viability was assessed using MTT assay according to Nwagbara et al. (2007) with a few modifications. At 90 % confluency, cells were treated with 0.5 % v/v Milli-Q, 50  $\mu\text{M}$  BaP, and different doses of test samples (UT, T24, T48, T120, and T240) for 24 h. All experiments were carried out in triplicates. After 24 h of treatment, medium was removed and replaced by fresh medium containing MTT at a final concentration of 0.5  $\text{mg mL}^{-1}$  and further incubated for 2 h. Then, MTT solubilization solution (DMSO) was added into each well and incubated at room temperature for 1 h for proper solubilization. Absorbance was read at 570 nm and background absorbance at 650 nm was later on subtracted.

*Alkaline single-cell gel electrophoresis (comet assay)*

Comet assay was used to evaluate the genotoxic potential and done according to Žegura et al. (2004) with slight modification (15 min electrophoresis at 25 V). HepG2 cells were treated with 0.5 % v/v Milli-Q (negative control), 1 mM Benzo (α) pyrene (positive control), and with 20 % v/v test samples for 24 h. The slides were stained with ethidium bromide (2 μg mL<sup>-1</sup>, 100 μL per slide). The comets were visualized with fluorescent microscope at excitation and emission setting of 518/605 nm. The percentage of DNA in tail, tail moment, and olive tail moment (OTM) of 40 randomly selected cells were analyzed from each slide by using CometScore Freeware Software ([www.tritekcorp.com](http://www.tritekcorp.com)). The comets were divided into five classes on the basis of DNA in the tail; Class I, less than 1 % DNA in tail (intact nucleus); Class II, 1–20 % DNA in tail; Class III, 20–50 % DNA in tail; Class IV, 50–75 % DNA in tail; and Class V, more than 75 % DNA in tail (Miyamae et al. 1998).

*EROD assay*

EROD activity in cells was assessed as previously described by Laville et al. (2004) with some modifications. In short, cells were seeded in a 96 well plate, and after 24 h at 90 % confluency, cells were treated with 0.5 % v/v Milli-Q (negative control), 1 mM Benzo (α) pyrene (positive control), and with 20 % v/v test samples for 6 h. Thereafter, medium was removed from the culture plate and replaced by 100 μL of fresh medium containing 5 μM 7-ethoxyresorufin and 10 μM dicumarol. Culture plates were incubated at 37 °C for 30 min and then 130 μL of methanol was added to stop the reaction. Resorufin production was measured at 530 and 590 nm excitation and emission wavelengths, respectively, using a multi-well fluorescence plate reader (SpectraMax M2, Molecular devices). EROD activity induced by the positive control (1 mM BaP) was taken as 100 %, and the corresponding activity of the samples were expressed relative to the positive control.

*Statistical analysis*

All experimental data were expressed as means±standard deviation of three replicates. All statistical analyses including global curve fitting were performed with sigma plot 11 statistical package (Systat Software, San Jose, CA). Statistical differences between the control and treated cells were examined with the aid of ANOVA followed by multiple comparisons (Dunnett’s Method). A value of *P*<0.05 was used to determine the significance in statistical analyses.

**Results and discussion**

*Physicochemical analyses*

Physicochemical characteristics of Okhla landfill leachate is presented in Table 1. It is characterized by dark brown color, unpleasant odor, and high values of EC (35,700 μS cm<sup>-1</sup>) and TDS (31,469 mg L<sup>-1</sup>). Unfortunately, no standard maximum allowable discharge limit for landfill leachate is there in India, so the limits set by developed countries such as Germany (Kurniawan et al. 2006) is here used as guideline. Heavy metals Fe (6.51±0.01 mg L<sup>-1</sup>), Zn (2.10±0.01 mg L<sup>-1</sup>), Cr (1.32±0.03 mg L<sup>-1</sup>), and Cd (1.26±0.01 mg L<sup>-1</sup>) were found to exceed the German permissible discharge limit. Although within the discharge limit, presence of Pb and Ni cannot be neglected because of their known toxicity even at trace levels (Caicedo et al. 2008; Flora et al. 2012). The concentrations of heavy metals found in the present study are similar to the study conducted on Okhla landfill leachate by Singh and Mittal (2009) with the exception of Cd and Zn whose concentrations were found to be higher. The permissible limit for Cd in drinking water as suggested by EPA agencies is 0.005 ppm. The presence of such high concentrations of Cd along with the close proximity of the landfill site to river Yamuna and the absence of a liner system make leachate percolation and ground water pollution a serious problem.

Organic micropollutants detected in the leachate (Table 2) included alpha-limonene diepoxide commonly used in the

**Table 1** Physicochemical characteristics of the leachate

Parameter	Concentrations *	MAV
pH	8.3	–
EC	35,700	–
TDS	31,469	–
COD	29,020	–
Color (PtCo)	8,120	–
Ca	64.27±1.48	–
Mg	76.60±4.02	–
K	1,745.63±0.1	–
Na	1,198.77±0.08	–
Zn	2.10±0.01	2.0
Cd	1.26±0.01	0.1
Cr	1.32±0.03	0.5
Fe	6.51±0.01	2.0
Ni	0.53±0.01	1.0
Pb	0.11±0.01	0.5

*EC* electrical conductivity; *MAV* maximum allowed value; *TDS* total dissolved solids.\*All in milligram per liter except pH, EC (μS cm<sup>-1</sup>), and color

**Table 2** Identification of metabolites formed at different stages of bacterial degradation

Peak retention time (min)	Present in sample					Name of identified compound
	UT	T24	T48	T120	T240	
4.371	–	–	–	+	–	3-Hexen-2-one
8.207	–	–	–	–	+	1-Decanol
8.708	–	–	–	–	+	Octadecane
9.068	+	+	–	–	–	alpha-Limonene diepoxide
9.875	–	–	–	+	–	Methylcyclohexanol
9.894	+	+	+	–	–	1-(Dodecyloxy)-2-Nitrobenzene
9.966	+	+	–	–	–	1-(Hydroxymethyl)-2,5,5,8A-Tetramethyldecahydro-2-Naphthalenol
10.06	–	–	+	+	–	Phthalic acid
10.100	+	+	–	–	–	7,8-Dibromo-4,4,7-trimethyl-hexahydro-benzo[1,3]dioxin-2-one
10.489	–	–	–	+	–	Hexadecanoic acid
10.634	–	–	–	+	–	Nonane
11.208	+	–	–	–	–	2,2-dichloro-1-methyl-cyclohexanol
11.281	–	–	+	+	–	9-Octadecanoic Acid
11.474	+	–	–	–	–	2-Naphthol
11.643	+	+	–	–	–	2,7-Naphthalenediol
12.217	+	+	–	–	–	4,6-Dinitro-1,1,3,3,5-pentamethylindane (Nitro Musk)
13.155	+	+	–	–	–	4,4'-isopropylidene diphenol (Bisphenol A)
14.212	+	+	+	–	–	Bis-(2-Ethylhexyl)Phthalate (DEHP)
17.358	+	+	–	–	–	1-Methylundecylcyclohexane

flavor industry; nitromusks used in perfumery industries; brominated dioxin formed by burning of PVC-based plastics and materials containing brominated flame retardants; and Bisphenol A and bis-(2-ethylhexyl) phthalate (DEHP) commonly used as plasticizers. Along with these, products like 2-Naphthol, 2,7-Naphthalenediol, and Naphthalenol were also detected in the leachate organic extract indicating the process of degradation going on in the landfill site. The detected organic compounds are similar to the ones detected in landfill leachate in Oklahoma (Andrews et al. 2011) and Sweden (Oman and Junestedt 2008). Bisphenol A, well known endocrine disrupter, detected in present study, has been frequently detected in other landfill leachate studies (Baderna et al. 2011; Oman and Junestedt 2008). After 55 monitoring campaigns done in 11 years, Baderna et al. (2011) reported Bisphenol A to be the most recurrent compound in the leachate of an industrial landfill in northern Italy. A large number of other compounds can also be expected to be present in the leachate at a concentration below the detection limit of analytical methods used in the present study. But, low concentration does not eliminate the threat posed to the health of humans and aquatic and terrestrial animals. Also, the toxicity of the mixture is not always the sum of the expected effects from each component as there may be synergistic or antagonistic interactions between the mixture components (Hu et al. 2012).

In vitro microbial degradation of organic contaminants present in landfill leachate

The untreated (UT) sample and bacterial-treated samples were collected at intervals of 24 (T24), 48 (T48), 120 (T120), and 240 h (T240) for COD and GC-MS analysis. The results of COD analysis showed 31.12 % decrease in the values after 240 h of bacterial treatment as shown in Fig. 1. The compounds present in the crude organic extracts were identified on the basis of standard GC-MS database of the authentic compounds documented in NIST-05 and Wiley-8 libraries (Table 2). Results of the study indicated that there was hardly any degradation of the compounds observed in the T24 sample. However, in the T48 sample, increase in the number of peaks of early retention times was observed and the removal of alpha-limonene diepoxide (RT=9.068), brominated dioxin-2-one (RT=10.10), Bisphenol A (RT=13.155), and nitromusk (RT=12.217) was also seen to occur. Phthalate derivative DEHP (RT=14.212) and nitrobenzene (RT=9.894) were also removed after 120-h bacterial treatment. With the ongoing degradation process, formation of phthalic acid (RT=10.06), hexadecanoic acid (RT=10.489), and 9-Octadecanoic acid (RT=11.281) was observed in the treated samples.

Formation of intermediary products like Hexadecanoic acid (RT=10.489) and 9-Octadecanoic acid (RT=11.281) suggests the oxidation process involved in the process of microbial degradation of variety of organic compounds by

the bacterial strain. Diverse oxidation enzymes (monooxygenases and dioxygenases) are involved in the process of aerobic degradation of aromatic compounds like dioxins and PAH's (Seo et al. 2009). The presence of diverse dioxygenation and aromatic ring oxidation enzymes has already been postulated in this strain by Jaiswal et al. (2011). Dehalogenation of brominated (RT=10.10) and chlorinated compound (RT=11.208) present in the leachate was also found to occur. Previous study by Das et al. (2012) using *Pseudomonas* sp. strain ISTDF1 for the treatment of paper mill sewage sediment has shown the dechlorination ability of the strain even in the presence of mixed carbon source. Previous studies have also reported the formation of phthalic acid as an intermediate metabolite during degradation of phenanthrene (Arias et al. 2008), pyrene (Vila et al. 2001), and fluoranthene (López et al. 2006) by *Mycobacterium* sp. strain AP1 and during degradation of fluorene (Grifoll et al. 1994) and phenanthrene (Samanta et al. 1999) by *Pseudomonas* sp.

Reduction in EC, TDS, and heavy metal concentration after bacterial treatment

The values of EC and TDS were found to reduce from 8,180  $\mu\text{S cm}^{-1}$  (UT) to 5,326  $\mu\text{S cm}^{-1}$  (T240) and 6,132  $\text{mg L}^{-1}$  (UT) to 4,164  $\text{mg L}^{-1}$ , respectively. Thus, a reduction of 34.89 % of EC and 32.09 % of TDS was observed after 240-h bacterial treatment as shown in Fig. 1. High value of EC in leachate can be attributed to high levels of cations and anions present in it. TDS mainly comprises of inorganic salts and dissolved organics. Post treatment reduction in the amount of TDS clearly reflected the extent of mineralization by the bacterial strain, which is further supported by reduction in COD values.

The acid digested samples collected at different day's intervals were analyzed with inductively coupled plasma-atomic emission spectrometer (ICP-AES) to evaluate the removal of

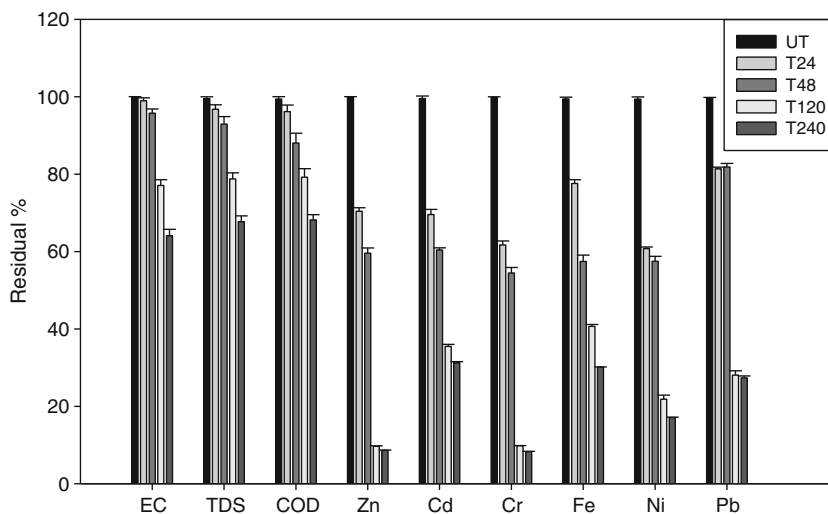
Zn, Cd, Cr, Fe, Ni, and Pb from leachate. Results of the study are also shown in Fig. 1. In the T240 sample, reductions of 91.43, 69.05, 91.67, 69.89, 83.02, and 72.73 % for Zn, Cd, Cr, Fe, Ni, and Pb, respectively, were observed. As mentioned in the previous section, the crude leachate contained Cr, Cd, Fe, and Zn beyond the permissible limit of discharge. Reduction in the heavy metal concentration can be attributed to biosorption potency of the bacterium. Biosorption capacity of *Pseudomonas* sp. for treating contaminated effluents containing Ni, Cu, Pb, and Cr has been reported earlier by Leung et al. (2001) and Uzel and Ozdemir (2009). The present study further shows the ability of the bacterium for the removal of high concentration of heavy metals.

Cell viability

The effects of untreated and treated leachate samples on viability of HepG2 cells were evaluated using MTT assay. The positive control and test samples were added in different dilutions to work out the dose response relationships. Cell viability was expressed as percentage of the corresponding control (0.5 % v/v Milli-Q). The sigmoid dose response curves for the samples were plotted, and the EC<sub>50</sub> values were derived from the global curve fitting analysis with four parameter logistic curve equation (Das et al. 2012). The cell viability in terms of MTT assay derived EC<sub>50</sub> values (Table 3) and the dose response curves (Fig. 2a) showed that the level of toxicity decreased by approximately 2.5 times in 240 h. The lowest EC<sub>50</sub> value was found for the UT sample (19.9607 %) and the highest for T240 (49.8112 %).

Overall, results of MTT assay suggest that the landfill leachate contained a significant load of toxicants that were reduced after 240-h bacterial treatment. Disturbance in cellular proliferation has been reported for nitrobenzene (O'Brien et al. 1990), dioxin (Aly and Khafagy 2011), phthalate esters (Erkekoglu et al. 2010), and bisphenol A (Nakagawa and

**Fig. 1** Percent residual EC, TDS, COD and heavy metals present in landfill leachate after treatment with *Pseudomonas* sp. ISTDF1 for different time intervals. Values are mean±SD of triplicates

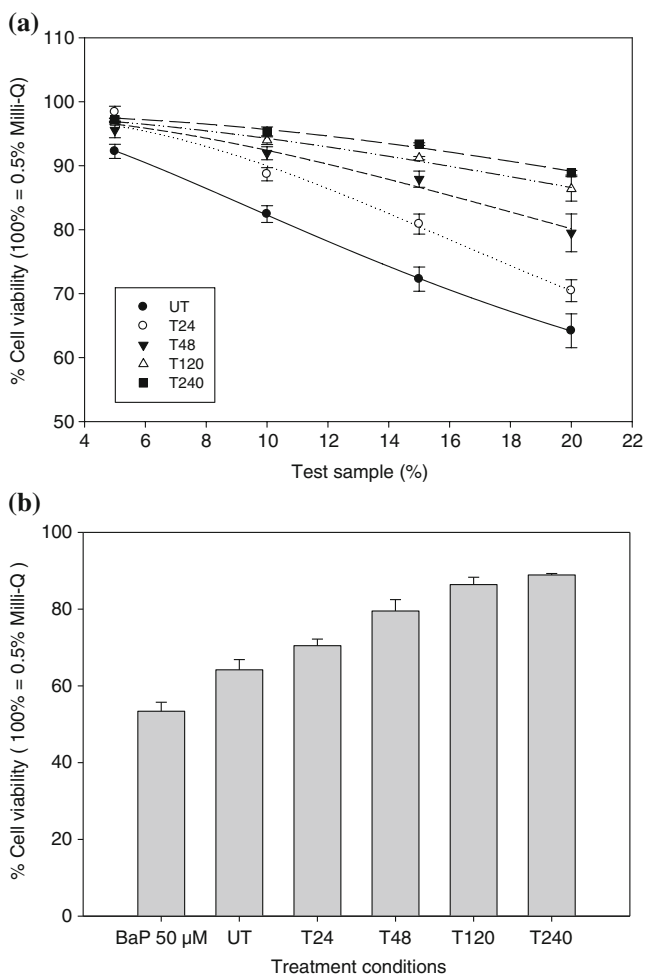


**Table 3** MTT EC<sub>50</sub> values of untreated and treated samples along with the corresponding %DNA in tail and comet OTM values

Treatments <sup>a</sup>	MTT EC <sub>50</sub> <sup>b</sup>	R <sup>2</sup> (EC <sub>50</sub> )	Comet assay	
			% DNA in tail	OTM
UT	19.9607	0.9800	80.22091±14.55534	147.6991±66.41559
T24	23.4261	0.9737	68.25595±20.42574	128.0851±69.99407
T48	33.4589	0.9228	47.36427±11.94373	62.49799±31.87236
T120	48.5276	0.9479	30.72695±13.69436	26.60099±11.72544
T240	49.8112	0.9698	14.11317±8.077487	20.64553±17.09153

<sup>a</sup> HepG2 cell lines were treated with different test samples (dilutions, 5 to 20 % v/v test samples) for 24 h in MTT assay. For comet assay, only single concentration (20 % v/v test samples) was considered

<sup>b</sup> EC<sub>50</sub> was derived using global curve fitting model with four parameters logistic nonlinear regression equation, expressed in terms of percent test samples



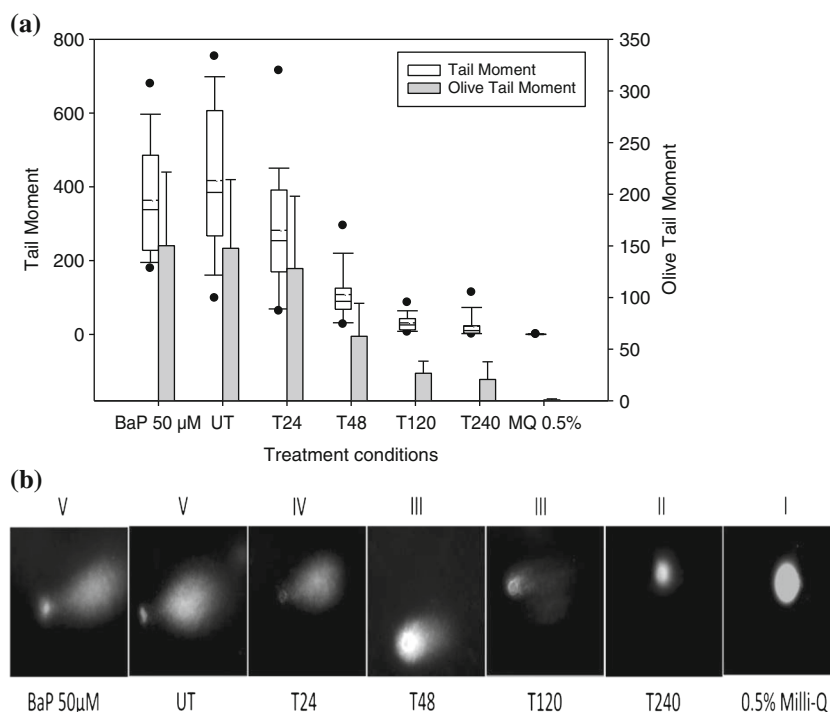
**Fig. 2** Evaluation of cytotoxicity of contaminants prior and after treatment by *Pseudomonas* sp. strain ISTDF1. Acronyms correspond to the different samples to which the HepG2 cells were exposed. BaP Benzo (a) Pyrene (Positive control), UT Untreated leachate, T24 24 h treated leachate, T48 48 h treated leachate, T120 120 h treated leachate, T240 240 h treated leachate. Values represent the mean±SD, n=3. **a** Cell viability measured after 24-h exposure period. 100 % cell viability was considered for 0.5 % v/v Milli-Q treatment. Global goodness of fit R<sup>2</sup>=0.9796. **b** Cell viability after treatment with BaP and 20 % v/v test sample at different stages of treatment

Tayama 2000) found in the untreated leachate of the present study. The removal of these classes of compounds in the 240 h sample explains the increase in EC<sub>50</sub> value. In contrast to above mentioned compounds, aliphatic alkane (octadecane) and aliphatic alcohols (1-decanol) found in the 240 h bacterial-treated samples pose no human health risk of concern (USEPA 2007). Similar to the organic contaminants, heavy metals like Cr (Naik et al. 2014), Cd (Koizumi et al. 1996), and Ni (Ermolli et al. 2001) have also been reported to have cytotoxic effects. Landfill leachate induced cytotoxicity has been reported earlier in HepG2 cells through inhibition of cell proliferation (Baderna et al. 2011) and also in MCF-7 breast cancer cells by necrosis (Talorete et al. 2008). The individual synergistic or antagonistic effects of the leachate constituents may be responsible for the cell disturbances causing cell death as assessed in our study and suggest that the exposure of human populations to these leachates may lead to adverse health effects. However, for the comparative analysis of the bacterial treatment of leachate, no earlier reports based on MTT assay are available.

#### Comet assay

Results of the comet assay for untreated and treated leachate samples are shown in Fig. 3a and b. The comets were divided into five classes on the basis of amount of DNA in tail. HepG2 cells treated with the untreated (UT) sample resulted in 37.5 and 62.5 % comets that fell under classes IV and V, respectively. Whereas in 24 h treatment (T24), percentage of comets lying in class V was reduced to 42.5 %. In the 240 h treated sample (T240), 85 and 15 % of the comets were found to belong to classes II and III, respectively. Tail moment and olive tail moment (OTM) of 40 randomly selected comets are presented as quantile box plots. Treatment of leachate with the bacteria resulted in decrease in genotoxicity as indicated by the %DNA in tail and OTM values (Table 3). The quantile box

**Fig. 3** Genotoxicity of the contaminants prior and after treatment with *Pseudomonas* sp. strain ISTDF1. **a** The tail moment and the olive tail moment plotted against different samples. Tail moments of 40 randomly selected comets are presented as quantile box plots. The edges of the box represent the 25th and the 75th percentiles; a *solid line* in the box represents the median value while *dotted line* represents mean value. *Error bars* indicate 90th and 10th percentiles and the *black circles* indicate outlying points beyond 5th and 95th percentiles. Olive tail moments of the same 40 comets are shown as the mean±standard deviation. **b** Representative images of different classes of comets seen under fluorescent microscope after ethidium bromide staining

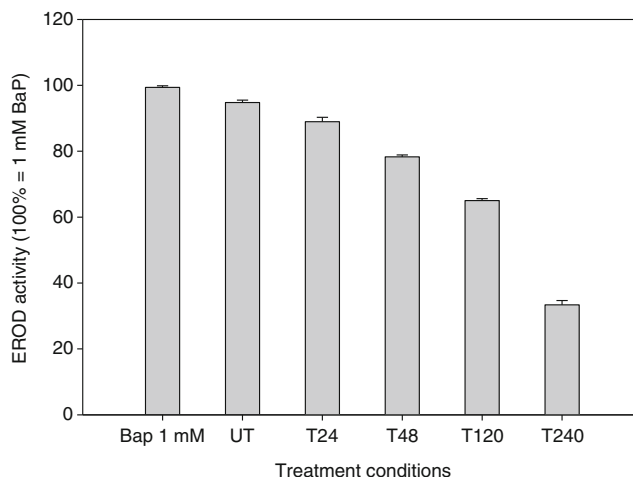


plot also shows that the distribution of comets became more homogenous with lower tail moment ( $21.9281 \pm 30.78685$ ) in the T240 sample compared to the UT sample (tail moment =  $417.111 \pm 195.2496$ ). Although statistically significant DNA damage (Dunnett’s Method  $p < 0.05$ ) was noticed in all the samples with respect to negative control (0.5 % v/v Milli-Q), T240 sample showed significant reduction of DNA damage compared to the untreated (UT) leachate. The olive tail moment data also showed a decreasing trend with increasing duration of bacterial treatment. T240 sample resulted in a 7-fold decrease in DNA migration (OTM =  $20.64553 \pm 17.09153$  compared to the UT sample (OTM =  $147.6991 \pm 66.41559$ ).

Higher values of %DNA tail, tail moment, and OTM of untreated (UT) leachate confirmed its high genotoxic nature. Genotoxicity can be induced by the formation of free radicals, either via autoxidation or by enzyme-catalyzed oxidation of organic compounds in leachate as shown by Li et al. (2004). Present study is in agreement with a series of similar studies where leachate has been found to impact genome (Gajski et al. 2012; Bortolotto et al. 2009). Genotoxic effects have been reported for organic contaminants like nitrobenzene (Yuan et al. 2011), phthalate esters (Kleinsasser et al. 2000), Bisphenol A (Lee et al. 2013), and heavy metal contaminants like Cr (Quievryn et al. 2003) found in the untreated leachate of the present study. The removal of these classes of compounds in the 240 h sample explains the decrease in the OTM value in the present study. The treated sample was less toxic but still exhibited genotoxicity to some extent.

EROD assay

The EROD induction by different test and control samples are shown in Fig. 4. As shown in the figure, the untreated (UT) sample elicited high EROD activity, comparable with positive control (1 mM BaP). This clearly indicated the contamination of untreated landfill leachate with dioxin-like and other EROD-inducing chemicals. GC-MS analysis also confirmed the presence of compounds like 7, 8-Dibromo-4,4,7-trimethyl-hexahydro-benzo[1,3]dioxin-2-one (RT=10.10) which was mineralized during the course of bacterial



**Fig. 4** EROD induction measured after 6-h exposure period with 1 mM BaP and 20 % v/v test sample at different stages of treatment; 100 % EROD induction was considered for 1 mM BaP treatment. Values represent the mean±SD, n=3



treatment. After 240 h of bacterial treatment, the EROD activity reduced to 33 from 95 %, which indicated steady mineralization of EROD-inducing chemicals.

EROD induction by dioxin-like compounds in liver cell lines has been effectively utilized to monitor the concentration of such compounds in environmental samples (Brack et al. 2000; Kaisarevic et al. 2009; Kinani et al. 2010; Louiz et al. 2008). The presence of 7, 8-Dibromo-4,4,7-trimethyl-hexahydro-benzo[1,3]dioxin-2-one (RT=10.10) in the untreated leachate explains the high EROD activity. Previous study by Osaki et al. (2006) employed a model fish Medaka to study the toxicity of landfill leachate through the induction of CYP1A revealing a very high EROD activity on exposure to leachate solution diluted to even 1 %. Moreover, Das et al. (2012) have effectively employed EROD assay using hepatocarcinoma (HuH7) cell line to study biodegradation and detoxification efficiency. The present report further establishes the effectiveness of EROD assay in monitoring biodegradation efficiency of dioxin like compounds.

## Conclusions

The chemical and toxicological analysis of the Okhla landfill leachate raised potential threat to the ecological health including humans. The bacterium *Pseudomonas* sp. strain ISTDF1 was not only found to reduce the level of organic and metal contaminants but also reduce toxicity of the leachate effectively. The results from the study highlight the importance of MTT, EROD, and comet assays using HepG2 cells in evaluating treatment efficiency. These bioassays can serve as reliable biomarkers to monitor pollution load in leachate before and after treatment. Combining a battery of biotests with chemical analyses is the best approach for the assessment of risk posed by a complex mixture of organic and inorganic constituents which need to be carried out before their disposal.

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