

Genotoxicity of sludges, wastewater and effluents from three different industries

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Abstract Many surface waters in Europe, Asia and South America have been reported to be contaminated with genotoxic substances. Therefore, it is important to establish strategies for identification of the most critical sources. In this study, we used a battery of four genotoxicity assays namely chromosomal aberration, DNA strand break, DNA laddering and P53 accumulation tests in mononuclear blood cells. Before cleaning of wastewater high levels of genotoxic contamination could be observed. For instance, we observed an increase in chromosomal aberrations from 2.6 ± 1.1 (aberrant cells in %; control), to 33.6 ± 6.6 in a petrochemical plant, 29.4 ± 3.3 in a petroleum refinery and 14.4 ± 1.8 in a coke plant of steel industry. A good correlation between the four assays was found. The most sensitive and reproducible results were obtained with the chromosomal aberration assay. Interestingly, clear differences in the efficiency of wastewater cleaning in three different

treatment plants were observed. The first and second treatment plants in petrochemical industry and coke plant of steel industry completely eliminated genotoxicity of the wastewater. However, the third plant in petroleum refinery could achieve a reduction in genotoxicity but significant genotoxic contaminations were still present. In conclusion, our battery of genotoxicity tests allows the identification of critical sources contributing to contamination of surface waters.

Keywords Effluent treatment plant · Wastewater · Effluent extract · Genotoxicity · Polycyclic aromatic hydrocarbons · Wastewater extract

Introduction

Thousands of anthropogenic chemicals are released into air, land, groundwater and surface water through industrial and other activities (Bunger et al. 2007; Devi et al. 2007; Kim et al. 2007; Periyakaruppan et al. 2007; Roos et al. 2008). Therefore, it is important to identify the most relevant sources causing environmental pollution (Bolt et al. 2004; Hengstler et al. 2003, 2006; Krishnamurthi and Devi 2007; Lilienblum et al. 2008). A large number of different assays have been recommended to assess the presence of genotoxic contaminants in wastewater (Dizer et al. 2002; Lah et al. 2004; Ohe et al. 2004). The most frequently applied techniques in wastewater monitoring include the umu-test (Schmitt et al. 2005) Ames test (Ohe et al. 2004), comet or single cell gel electrophoresis assay (Lah et al. 2004), Microtox bioluminescence test (Dizer et al. 2002) and micronucleus as well as anaphase aberration assays (Grover and Kaur 1999). In the present study we used a battery of cost effective short-term tests for chromosomal aberration,

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DNA strand break, DNA laddering and P53 protein accumulation. For evaluation of our battery of assays we tested wastewater before and after processing in wastewater treatment systems. A good correlation was observed between the results obtained with all four assays. Interestingly, we observed a clear difference in the cleaning efficiency between the two treatment systems in petrochemical and petroleum refinery plants located in India.

Materials and methods

Chemicals and media

Dulbecco's Modified Eagle's medium (DMEM) culture media for human lymphocytes cells, benzo[*a*]pyrene and polycyclic aromatic hydrocarbon (PAH) standard mixture were purchased from Sigma (St Louis, MO, USA). Trypsin-EDTA, fetal bovine serum (FBS), penicillin and streptomycin were procured from Gibco Technologies, UK; agarose, ethidium bromide, sodium sulfate, colchicine and D-Glucose from Hi-Media laboratories, India; Tris-Cl, dimethyl sulfoxide (DMSO), sodium hydroxide, potassium chloride were obtained from Sisco Research Laboratories, India; methylene chloride, benzene, methanol and acetic acid were purchased from E-Merck, India. Lysing solution for P53 protein induction and apoptosis were purchased from Amersham International Plc, UK. Phenobarbitone and hydrocortisone were received as a gift from Prof. I.S. Grover, Punjab University. All other chemicals and solvents were of the highest analytical grade available. The S-9 liver fraction was prepared from the liver of male albino rats, which had been injected with Phenobarbital (75 mg/kg body weight) and hydrocortisone (50 mg/kg body weight) every 8 h for 2 days to induce cytochrome P-450 oxidase and reductase (Wang and Lin 1995).

Collection and extraction of wastewater

Wastewater was collected from three different plants: (1) a petrochemical industry plant located in western part, Gujarat State, (2) a petroleum refinery located in Eastern part, West Bengal State, (3) a coke plant of steel industry located in Northeast part, Jharkhand State. All plants are located in India. Wastewater or sludge before and after processing in the respective plants (1–3) was collected. The schematics of effluent treatment plants (ETPs) are given in Supplementary Figure 1a, b (NEERI Report 1995, 1998)

Extraction and analyses

Five liters of each wastewater sample were extracted using liquid–liquid extraction with 150 ml of dichloromethane

thrice according to a method described by EPA (1986). The extracts were pooled and passed through anhydrous sodium sulfate to remove the moisture. One part of the dichloromethane extract was concentrated and redissolved in dichloromethane for Gas Chromatography–Mass Spectrometer (GC–MS) analysis. The other part of dichloromethane fraction was flash evaporated and the concentrate was exchanged with DMSO for genotoxicity studies. Chemical analysis of the extracts were carried out using a GC–MS (Saturn Model-3, Walnut Creek, CA, USA) with a fused silica column (15 m × 0.3 mm inner diameter) and helium as carrier gas. The injector and transfer line temperatures were 50 and 250°C, respectively. The program was hold 1 min, rise to 150°C at 25°C/min, hold 2 min, then to 320°C at 5°C/min. The individual PAH's were identified by computer search of the National Bureau of Standard Library of Mass Spectra on the basis of retention time and partly quantified using external standards obtained from Sigma Co. USA.

Isolation and incubation of human peripheral mononuclear blood cells

Human peripheral mononuclear blood cells (PBMC) were isolated from venous blood as described by Hengstler et al. (1992). PBMC were cultured in DMEM containing 100 IU/ml penicillin, 100 g/ml streptomycin and 20% FBS at 37°C in a humidified 5% CO₂ and 95% O₂ atmosphere. The duration of cell culture varied between 24 and 48 h. The same type of culture was used to cultivate lymphocytes for analysis of chromosomal aberration, P53 protein expression and DNA fragmentation. PBMC with a cell density of 6×10^6 /ml were cultivated for 24 h and subsequently exposed to different concentrations of DMSO extracts of wastewater and from coke oven (10, 25 and 50 µl) as well as petrochemical and petroleum refinery plants (50, 100 and 200 µl) for a period of 3 h, except for the DNA unwinding assay where an incubation period of 1 h was chosen. Incubations were performed in duplicate by S-9 mix. Trypan blue exclusion was determined as described (Carmo et al. 2005, 2007).

DNA unwinding assay

Human PBMC were exposed as above. After the incubation period of 1-h ice-cold saline (0.9% sodium chloride) was added. The mixture was centrifuged at 400 × g for 10 min at 4°C and the cell pellets were suspended in 1.0 ml of balanced salt solution. Aliquots of this suspension was subjected to fluorimetric analysis of DNA unwinding (FADU) as described by Birnboim and Jevcak (1981) with little modifications reported elsewhere Krishnamurthi et al. (2003).

Chromosomal aberration test

Chromosomal aberrations were analyzed as described by Lovreglio et al. (2006). Briefly, PBMC were incubated with test substances as described above. Subsequently, cells were incubated with colchicine (1 µg/ml) 3 h prior to harvest, the cultures were lysed with hypotonic KCl (0.56%) and fixed with Carnoy's Fixative, slides were prepared and analyzed according to the method of Api and San (1999).

Immunoblot analysis

Peripheral mononuclear blood cells were incubated with test substances as described above. After incubation with test substances were lysed as described (Spangenberg et al. 2006). The protein samples (20 mg) were fractionated on 10% (w/v) polyacrylamide gels (Hermes et al. 2008). For electrophoresis the protein was transferred to hybond ECL membrane (Amersham International, UK) using an electroblotting apparatus (Hausherr et al. 2006). The membranes were incubated with an anti P53 mutant monoclonal antibody Ab-3 (Amersham International, UK). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Hohme et al. 2007; Schormann et al. 2008; Trost et al. 2005). Immunodetection was carried out using the ECL Western Blotting Analysis System and the bands developed were photographed.

DNA fragmentation assay

Peripheral mononuclear blood cells were incubated with test substances as described above. Subsequently, the cells were washed with PBS; the cellular DNA was isolated and analyzed according to the method of Martikainen et al. (1991). The cell pellets were resuspended in 1.0 ml of lysis buffer and digested with proteinase-K (300 mg/ml, Sigma Laboratories, USA) for 18 h at 37°C. After digestion, the DNA was sequentially extracted with phenol, phenol:chloroform (1:1) and chloroform and then precipitated with ethanol. The precipitate was dissolved in TE buffer and incubated treated with 100 mg/ml of RNase (Sigma Laboratories, USA) at 60°C for 1 h. DNA was separated electrophoretically on 1.5% agarose gels, stained with ethidium bromide (0.5 g/ml), and the gels were visualized and photographed under ultraviolet illumination (Brulport et al. 2007).

Statistical analysis

The results were statistically analyzed using the ANOVA one way test with "Analyze it Software" and expressed as *P* values which were considered non-significant when *P* > 0.05 (Tanner et al. 2006).

Results

Evaluation of genotoxicity markers: the influence of wastewater treatment in a petrochemical plant

In order to evaluate our battery of genotoxicity markers we tested wastewater and sludge of a petrochemical plant before and after cleaning in an effluent treatment system. Significantly increased levels of chromosomal aberrations (Table 1), DNA strand breaks (Table 2), DNA laddering (Supplementary Figure 2) and P53 protein accumulation (Supplementary Figure 3) were observed before wastewater treatment. In contrast, after processing of wastewater in a cleaning system no genotoxicity could be observed (Tables 1, 2, Supplementary Figures 4, 5). Therefore, our battery of genotoxicity markers clearly indicates an effective processing.

Table 1 Chromosomal aberrations induced by extracts of wastewater and sludge collected from a petrochemical industry plant

Compounds	Aberrant cells (%)	
	Before treatment plant (sludge)	After treatment plant (effluent water)
Negative control (0.1% DMSO)	2.6 ± 1.14	2.2 ± 0.45
Extract 50 µl	23.0 ± 4.85**	2.8 ± 1.30
Extract 100 µl	33.6 ± 6.58**	3.0 ± 0.84
Extract 200 µl	NT	4.1 ± 1.58

Data are given before and after processing of wastewater in a treatment plant. Mean ± standard deviation of five independent experiments. Benzo[*a*]pyrene (30 µg/ml) was used as a positive control resulting in 45.8 ± 5.12** aberrant cells

NT not tested

** *P* < 0.01

Table 2 Level of DNA strand breaks (relative units) induced by extracts of wastewater and sludge collected from a petrochemical plant

Compounds	Relative units	
	Before treatment plant (sludge)	After treatment plant (effluent water)
Negative control (0.1% DMSO)	22 ± 1.12	20 ± 1.15
Extract 50 µl	45 ± 2.1*	27 ± 0.85
Extract 100 µl	48 ± 1.05*	29 ± 0.91
Extract 200 µl	NT	31 ± 1.10

Data are given before and after processing of wastewater in a treatment plant. Mean ± standard deviation of five independent experiments. Benzo[*a*]pyrene (10 µg/ml) was used as a positive control resulting in 42%**

NT not tested

* *P* < 0.05, ** *P* < 0.01



Evaluation of the efficiency of wastewater cleaning in a petroleum refinery plant

As a second example we evaluated the wastewater cleaning efficiency in a petroleum refinery plant using the same genotoxicity markers. The sludge from a refinery site (sludge before treatment) induced a significant increase in genotoxicity markers, including chromosomal aberrations (Table 3), DNA strand breaks (Table 4), DNA laddering (Supplementary Figure 6) and, P53 accumulation (Supplementary Figure 7). After cleaning the wastewater in an ETP genotoxicity was strongly reduced but not completely negative. Chromosomal aberrations were negative using 50 or 100 μl sludge or wastewater extract after cleaning (Table 3). However, 200 μl of wastewater extract still resulted in a weak, but significant increase in chromosomal aberrations (Table 3). It should be considered that due to

Table 3 Chromosomal aberrations induced by extracts of wastewater and sludge collected from a petroleum refinery plant

Compounds	Aberrant cells (%)		
	Before treatment plant (sludge)	After treatment plant (sludge)	After treatment plant (effluent water)
Negative control (0.1% DMSO)	2.2 \pm 0.84	3.2 \pm 0.84	2.8 \pm 0.84
Extract 50 μl	17.6 \pm 0.90**	4.6 \pm 1.82	2.4 \pm 1.14
Extract 100 μl	29.4 \pm 3.30**	5.0 \pm 2.35	2.6 \pm 1.34
Extract 200 μl	NT	4.6 \pm 1.52	5.4 \pm 1.75*

Data are given before and after processing of wastewater in a treatment plant. Mean \pm standard deviation of five independent experiments. Benzo[*a*]pyrene (30 $\mu\text{g}/\text{ml}$) was used as a positive control resulting in 45.8 \pm 5.12** aberrant cells

NT not tested

* $P < 0.05$, ** $P < 0.01$

Table 4 DNA strand breaks (relative units) induced by extracts of wastewater and sludge collected from a petroleum refinery plant

Compounds	Before treatment plant (sludge)	After treatment plant (sludge)	After treatment plant (effluent water)
Negative control (0.1% DMSO)	24 \pm 0.72	18 \pm 0.58	20 \pm 0.56
Extract 50 μl	36 \pm 0.80*	22 \pm 0.92	21 \pm 0.78
Extract 100 μl	41 \pm 0.56*	25 \pm 1.01	24 \pm 0.85
Extract 200 μl	NT	41 \pm 1.05*	37 \pm 0.95*

Data are given before and after processing of wastewater in a treatment plant. Mean \pm standard deviation of five independent experiments. Benzo[*a*]pyrene (10 $\mu\text{g}/\text{ml}$) was used as a positive control resulting in 42%**

NT not tested

* $P < 0.05$, ** $P < 0.01$

too high cytotoxicity 200 μl sludge extract before processing in the treatment plant could not be tested for genotoxicity. In agreement with the chromosomal aberration data, levels of DNA strand breaks were also not increased for 50 and 100 μl sludge and wastewater extracts, but an increase in DNA strand breaks was seen for 200 μl (Table 4). Analysis of DNA laddering (Supplementary Figure 8) and P53 protein accumulation (Supplementary Figure 9) in extracts after processing in a treatment plant resulted in negative data up to the highest dose levels tested (200 μl of sludge or wastewater extracts). The results suggest that chromosomal aberrations and DNA strand breaks are more sensitive for detection of genotoxic contaminants in wastewater compared to DNA laddering and P53 accumulation.

GC–MS analysis of wastewater before and after cleaning

Next we evaluated the wastewater of a coke plant from steel industry. A clear increase in chromosomal aberrations (Table 5), DNA strand breaks (Table 6), DNA laddering (Supplementary Figure 10) and P53 accumulation was observed (Supplementary Figure 11) when wastewater was analyzed before treatment. After treatment all the data were negative (Tables 5, 6 and Supplementary Figures 12, 13) except DNA strand breaks at the highest dose (50 μl) that caused a slightly increased damage (Table 6). Wastewater and sludge of the three industry plants before and after cleaning in a treatment system were analyzed by GC–MS (Table 7). A clear decrease in number and quantity of contaminants was observed after cleaning (Table 7). However, removal of contaminants was not complete. Interestingly, some substances that have not been identified before treatment were detected after the cleaning process. Obviously, they have been formed during the cleaning process. Examples for newly formed substances are 1,2-ethyl-7-methyl benzo[*b*]thiophene and 1,4-dimethyl naphthalene. How-

Table 5 Chromosomal aberrations induced by extracts of wastewater collected from a coke plant of steel industry

Compounds	Before treatment plant (wastewater)	After treatment (effluent water)
Negative control (0.1% DMSO)	1.6 \pm 0.55	1.6 \pm 0.55
Extract 10 μl	8.4 \pm 1.34**	2.6 \pm 0.34
Extract 25 μl	14.4 \pm 1.82**	3.9 \pm 0.24
Extract 50 μl	NT	4.5 \pm 0.71

Data are given before and after processing of wastewater in a treatment plant. Mean \pm standard deviation of five independent experiments. Benzo[*a*]pyrene (30 $\mu\text{g}/\text{ml}$) was used as a positive control resulting in 45.8 \pm 5.12**

NT not tested

* $P < 0.05$, ** $P < 0.01$

Table 6 DNA strand breaks (relative units) induced by extracts of wastewater collected from coke plant of a steel industry

Compounds	Before treatment plant (wastewater)	After treatment plant (effluent water)
Negative control (0.1% DMSO)	20 ± 0.65	20 ± 0.44
Extract 10 µl	36 ± 0.95*	23 ± 0.58
Extract 25 µl	45 ± 0.88**	29 ± 1.08
Extract 50 µl	57 ± 0.91**	37 ± 1.05*

Data are given before and after processing of wastewater in a treatment plant. Mean ± standard deviation of five independent experiments. Benzo[*a*]pyrene (10 µg/ml) was used as a positive control resulting in 42%**

* $P < 0.05$, ** $P < 0.01$

Table 7 Compounds identified in wastewater and sludge of three plants before and after cleaning in treatment systems by GC–MS analysis

	Before cleaning	After cleaning		
Petrolchemical industry	Anthracene	27.0	Naphthalene	NQ
	Fluoranthene	31.0		
	Benzo[<i>a</i>]anthracene	19.0		
	9H-fluorene, 2-methyl	11.0		
	1,2,5,6-Tetramethyl acenaphthylene	30.8		
	Phenanthrene 2,5-dimethyl	NQ		
	Pyrene 1,3-dimethyl	NQ		
Petroleum refinery plant	1,3,6-Trimethyl naphthalene	NQ		
	Phenanthrene	27.0	1,2-ethyl-7-methyl benzo(b) thiophene	NQ
	Fluoranthene	31.0	1,4-dimethyl naphthalene	NQ
	Enzo(a) pyrene	19.0		
	Chrysene	11.0		
	Anthracene	30.8		
	Naphthalene-1,7-dimethyl	NQ		
	Acenaphthene	NQ		
	1,3,6-Trimethyl naphthalene	NQ		
	Fluorene	NQ		
	9H-fluorene,3-methyl	NQ		
	Dibenzothiophene	NQ		
	1-m\Methyl anthracene	NQ		
	1,2,5,6-Tetramethyl acenaphthylene	NQ		
2,3-Dimethyl phenanthrene	NQ			
Coke plant of a steel industry	Benz[<i>a</i>]anthracene	23.0		
	Benz[<i>e</i>]acephenanthralene	11.0		
	Benzo[<i>a</i>]pyrene	48.0		
	<i>p</i> -Tolyl isocyanide	NQ		
	2,6-Dimethyl phenol	NQ		
	2,3-dihydro-benzofuran	NQ		
	1H-indole-6-methyl,2-naphthalenol	NQ		
	3-Biphenylol	NQ		
	Naphthalene-1-(2-naphthalenyloxy)	NQ		

NQ not quantified

ever, the majority of PAH compounds clearly decreased during the treatment process.

Cytotoxicity of waste and effluent water and sludges

The focus of this study is analysis of genotoxic contaminants. Nevertheless, cytotoxicity data are required for a comprehensive interpretation. Therefore, we incubated human PBMC for 2, 4 and 6 h with wastewater and sludge extracts and measured trypan blue exclusion. Although the wastewater cleaning process clearly diminished cytotoxicity, toxic effects could still be detected also after treatment (Supplementary Figure 14).

In conclusion, the wastewater cleaning system in the evaluated plants decreased the level of genotoxic contami-



nants, but obviously a complete removal was not observed. The physicochemical parameters of treated and untreated wastewaters are shown in Supplementary Table 1.

Discussion

In several studies contamination of surface water with genotoxic and mutagenic contaminants has been reported (Ohe et al. 2004). Many rivers in Europe, Asia and South America have been shown to be contaminated with potent direct acting and indirect acting mutagens. Among all surface water data 15% of the analyzed samples have been reported to be mutagenic, with 3–5% classified as highly mutagenic with more than 5,000 revertants per liter (Ohe et al. 2004). This situation underlines the necessity to identify and eliminate the most important sources contributing to genotoxic contamination of surface water. Industrial effluents cause a severe contribution to contamination of surface waters. Therefore, it is important to establish sensitive and cost effective strategies to identify wastewaters with contamination. For this purpose we used a battery of four genotoxicity assays, namely the chromosomal aberration test, DNA strand break analyses by the DNA unwinding assay, DNA laddering as an indicator for apoptosis and P53 protein accumulation. Similar results were obtained with the four assays, whereby chromosomal aberrations and DNA strand break analysis allowed a more sensitive and accurate detection of genotoxic contaminants than DNA laddering and P53 accumulation. Genotoxic contaminations could be detected in untreated wastewater of three different petrochemical industries, petroleum refinery and steel industry.

We compared the efficiency of the wastewater cleaning process by two treatment plants. The first treatment plant in a petrochemical ETP completely eliminated genotoxicity of the wastewater. This underlines the efficiency of the cleaning process, since genotoxicity of the wastewater was high before treatment. However, it should be considered that GC–MS analysis nevertheless identified some contaminants. Also the cytotoxicity test still indicated cytotoxicity of the wastewater even after cleaning in treatment plants. As per the Bureau of Indian Standards (1982) the treated wastewater in this plant has delivered the quality for irrigation purpose.

A different result was obtained in a second treatment plant in petroleum refinery. Although the cleaning process strongly reduced toxicity, genotoxic contaminants were still present, such as 1,2-ethyl-7-methyl benzo[*b*]thiophene and 1,4-dimethyl naphthalene. Therefore, a more efficient cleaning should be achieved in this particular case. Similar to our observations, several investigators detected genotoxic contaminants in wastewaters despite of cleaning in

treatment plants (Houk 1992; Schaeffer and Kerster 1985; Van Hoof and Manteleers 1983). For instance, Grover and Kaur (1999) reported that effluents from industries showed positive responses for micronuclei and anaphase aberration assays. Lah et al. (2004) have tested the genotoxic potential of influent and effluent of a municipal wastewater treatment plant and observed that genotoxicity was substantially reduced in the effluent in comparison to the influent sample.

In conclusion, we have shown that the battery of genotoxicity tests used in the present study allows the evaluation of industrial wastewaters and effluents and also helps to identify critical sources of surface water contamination.

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