Research article

Journal of Applied Toxicology

Received: 13 March 2017, Revised: 12 April 2017, Accepted: 14 April 2017 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jat.3490

Genotoxic effects of old landfill leachate on HepG2 cells after nitration/ultrafiltration/ reverse osmosis membrane treatment process

Rong Cheng[a], Ling Zhao[b]* and Pinghe Yin[c]*

ABSTRACT: **Toxicity assessment of nitration/ultrafiltration/reverse osmosis (nitration/UF/RO) project, which has recently been widely used as an efficient process with applications in practical leachate treatment, was very limited. In the present study, DNA damage of leachates was investigated before and after the nitration/UF/RO process by a battery of assays with human hepatoma cells. Methyletrazolium assay showed a high cytotoxicity of 97.1% after being exposed to the highest concentration of raw leachate for 24 h, and a cytotoxicity of 26% in effluent at a concentration of 30% (*v*/v). Both comet assay (24 h) and γH2AX flow cytometer assay (3 h) showed increased levels of DNA damage in cells exposed to raw leachate and after nitration/UF-treated leachate followed by a significant increase of 7-ethoxyresorufin-*O*-deethylase activity. However, the effluent after nitration/UF/RO treatment showed no significant difference compared to negative control for γH2AX flow cytometer assay but slight DNA damage at concentrations of 20% and 30% (*v*/v) as well as increase of 7-ethoxyresorufin-*O*-deethylase. Analysis showed that nitration/UF/RO process exhibited high removal of physicochemical indexes and significant reduction of toxic and genotoxic effects of leachate, but still demands an improvement to reduce all possible negative risks to the environment and humans.** **Copyright © 2017 John Wiley & Sons, Ltd.**

Keywords: DNA damage assessment; landfill leachate; nitration/UF/RO process; comet assay; γH2AX flow cytometry (FCM) assay; EROD assay

Introduction

Landfill leachate is well known as a heavily polluted liquid characterized by four basal groups of pollutants: dissolved organic, inorganic salts, heavy metals and xenobiotic organic compounds (Christensen *et al.,* 2009). In addition, pathogens in leachate can also pose a risk to the environment (Baun *et al.,* 2000; Svenson *et al.,* 2003). It has been reported that small amounts of leachate can pollute large volumes of groundwater, which could induce the potential risk for biota and the human path through the food chains (Baderna *et al.,* 2011; Gajski *et al.,* 2012; Li *et al.,* 2008). Therefore, leachate must be treated before discharging into the environment.

The treatment methods of leachate have been reported including physical, chemical and biological processes (Abu Amr and Aziz, 2012; Abu Amr *et al.,* 2013; Bashir *et al.,* 2011; Hagman *et al.,* 2008). Recently, membrane technology has been widely used for wastewater treatment, which has advantages of simple treatment process, high efficient, no secondary pollution and economic space occupation compared with traditional methods (Dai *et al.,* 2015). Membrane technologies are also used for leachate treatment, including microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and forward osmosis (FO) (Surampalli *et al.,* 2016). Owing to the complexity of leachate components, single treatment technology cannot meet the demands. Hence, the method combined multiple membrane technologies to treat leachate has been widely come into use as relatively advanced and useful, particularly the method that combined UF, NF and RO. According to statistical data, nearly 100 research institutes and universities were engaged in membrane technology in 2010. However, relevant studies on the toxicological assessment of UF/RO-treated effluents in practice were less reported. Although the UF/RO process had been found to be effective concerning the basic physicochemical characteristics of final effluent, many of the compounds, 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) and negative effects are usually induced by multiple and synergistic effects (Oman and Junestedt, 2008). Therefore, it does not guarantee that the effluent of UF/RO treatment process can be harmless; the potential risk of the treatment must be evaluated by using toxicological assessments.

Landfill leachate ability to cause genotoxic and toxic effects on various organisms, including aquatic biota (Oliveira *et al.,* 2014;

*Correspondence to: Ling Zhao, Key Laboratory of Water/Soil Toxic Pollutants Control and Bioremediation of Guangdong Higher Education Institutes, Department of Environmental Engineering, Jinan University, No. 601, Huangpu Da Dao Xi, Guangzhou 510632, People's Republic of China.
E-mail: zhaoling@jnu.edu.cn

Pinghe Yin, Department of Chemistry, Jinan University, No. 601, Huangpu Da Dao Xi, Guangzhou 510632, People's Republic of China.
E-mail: tyinph@jnu.edu.cn

[a]School of Environment, Jinan University, Guangzhou 510632, People's Republic of China

[b]Key Laboratory of Water/Soil Toxic Pollutants Control and Bioremediation of Guangdong Higher Education Institutes, Department of Environmental Engineering, Jinan University, Guangzhou 510632, People's Republic of China

[c]Research Center of Analysis and Test, Jinan University, Guangzhou 510632, People's Republic of China

Toufexi *et al.,* 2013; Tsarpali and Dailianis, 2012), plants (Kalčíková *et al.,* 2012; Kwasniewska *et al.,* 2012; Sang *et al.,* 2006) and mammalian cell line (Alimba *et al.,* 2016; Gajski *et al.,* 2012; Ghosh *et al.,* 2014a) have been assessed. However, to our knowledge, reports on genotoxic effects of pre- and post-UF/RO technology treated leachates were not found. Regarding *in vitro* tests, human hepatoma (HepG2) cells are often used as a useful tool for assessment of genotoxicity (Baderna *et al.,* 2011; Ghosh and Swati, 2014b; Wang *et al.,* 2016, 2017) due to their ability to synthesize antioxidant and xenobiotic metabolizing enzymes cytochrome P450 (CYP) 1A1, which can be induced or inhibited by dietary and non-dietary agent (Chaloupka *et al.,* 1994). Potential genotoxicity of leachate before and after treated by UF/RO technology was evaluated by the γH2AX flow cytometry (FCM) assay and alkaline comet assay. In addition, 7-ethoxyresorufin-*O*-deethylase (EROD) assays were carried out for comparative toxicity evaluation of landfill leachates, pre- and post-treated. Alkaline comet assay is a sensitive technique for measuring different types of DNA damage such as DNA double-strand breaks (DSB), single-strand breaks, alkali-labile sites, DNA–DNA and DNA-protein crosslinks, and single-strand breaks associated with incomplete excision repair (Singh *et al.,* 1988; Žegura and Filipič, 2004). Among them, the DSB was considered as one of the most serious types of DNA damage. The γH2AX FCM assay recently has received extensive concern (Darzynkiewicz *et al.,* 2011) because of the corresponding relationship between the formation of γH2AX and DSB (Kuo and Yang, 2008). The EROD assay monitors the induction of P450 (CYP) 1A1 and is an effective tool to monitor the presence of persistent organic chemicals such as dioxins, furans, polychlorinated biphenyls and polyaromatic hydrocarbons (Baderna *et al.,* 2011; Ghosh *et al.,* 2014a; Tillitt *et al.,* 1991) that could impair DNA integrity and increase EROD.

The aim of the present study was to investigate the removal efficiency of physicochemical indices and to assess the potential DNA damaging effects and toxicity of old landfill leachate before/after treatment using the nitration/UF/RO process. Meanwhile, this study exploits the results of assays to provide a theoretical foundation for the potential risk of nitration/UF/RO as a widely used and practical leachate treatment process.

Materials and methods

Leachate collection

Samples of leachate were collected every 10 days in November 2015 from the landfill leachate treatment expansion project of Xingfeng, Guangzhou, which was started since 2002. The daily treated leachate of the project was 1149 m^3 day^{-1}, and schematic diagram of the treating project is shown in Fig. 1. Selected samples contained raw leachate, nitration/UF-treated leachate and nitration/UF/RO-treated leachate. All the samples were mixed respectively in amber glass bottles presoaked with methanol and purified water, and stored at 4°C.

Physicochemical characterization

Physical and chemical analyses of leachate were performed as soon as possible after being transported to the laboratory. Concentrations of the pH and color were determined by a GDYS-201 M water quality multiparameter monitor (Little Swan Instruments Co., Ltd., Changchun Jilin University, China). Heavy metals were measured by atomic absorption spectroscopy. Organic component (COD), total nitrogen and ammonia nitrogen were determined by ultraviolet and visible spectrophotometer. Phthalic acid esters (PAEs) containing dimethyl phthalate, diethyl phthalate, di-n-butyl phthalate, n-butyl benzyl phthalate, di-2-ethyl hexyl phthalate, di-n-octyl phthalate were analyzed according to the literature (Li *et al.,* 2015).

Toxicological analysis

Cell cultures and treatments. HepG2 cells (purchased from the biochemistry laboratory, the First Affiliated Hospital of Jinan University) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were maintained in an incubator at 37°C in a humidified atmosphere with 95% air and 5% CO_2. All the samples for toxicity analysis were filtered with a 0.22 μm sterile Millex-GS filter (Millipore, USA).

Cell viability assay. The viability of HepG2 cells was studied using the methyletrazolium assay according to a previous method (Nwagbara *et al.,* 2007). Cells were seeded at 1×10^4 cells per well into 96-well culture plates, and then treated with culture medium (negative controls) and different concentrations (1.25, 2.5, 5, 10, 20 and 30%, *v*/v) of raw leachate, leachate treated by nitration/UF and nitration/UF/RO after the cells filled with 90%. All experiments were performed in triplicate. After 24 h of treatment, medium was removed, cells were washed with phosphate-buffered saline (PBS) and fresh medium containing methyletrazolium at a final concentration of 0.5 mg ml^{-1} was added into each well and further incubated for 4 h. Then, the solubilization solution (DMSO) was added into each well and incubated at room temperature on a microtiter plate shaker for 10 min. Absorbance was read using automatic microplate reader at 490 nm.

Ethoxyresorufin-O-deethylase assay. EROD activity in cells was estimated by measuring fluorometrically the conversion of resorufin from 7-ethoxyresorufin, which was described previously (Ghosh *et al.,* 2014a). Briefly, cells were seeded in six-well plates

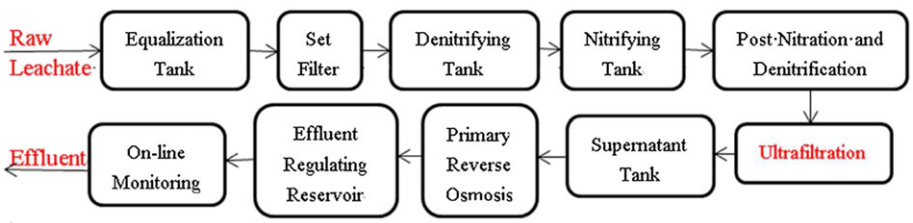

Figure 1. Schematic diagram of the nitration/ultrafiltration/reverse osmosis progress for landfill leachate treatment. Words in red represent the selected samples. [Colour figure can be viewed at wileyonlinelibrary.com]

for adherent and then cells were exposed to culture medium (negative control) and different concentrations of leachates for 24 h. After exposure, cells were harvested and resuspended in PBS and sonicated at 100 W and 45% duty cycle in SCIENTZ-IID ultrasonic cell disruptor. The suspensions were centrifuged for 10 min at 600 × g, thereafter, supernatant was added to the reaction mixture (5 μM 7-ethoxyresorufin and 10 μM dicumarol) in 96-well plate and incubated at 37°C for 30 min. Kinetics of resorufin production were measured instantly using a multiwall fluorescence plate reader at 530 and 590 nm excitation and emission wavelengths, respectively, at 37°C. EROD activity was expressed in pmol resorufin min^{-1} mg $protein^{-1}$, and the protein concentration was determined according to the method (Bradford, 1976) with Coomassie Brilliant Blue G-250 dye.

Alkaline single-cell gel electrophoresis (comet assay) method. DNA damage in HepG2 cells was evaluated by the comet assay according to the study (Danellakis *et al.,* 2011). As references (Ghosh *et al.,* 2015; Toufexi *et al.,* 2013; Wang *et al.,* 2016) investigated 24 and 72 h exposure times, we explored 24 and 48 h exposure times as pre-experiment, the lowest cell viability of raw leachate was 9% for 48 h exposure that cannot continue DNA damage assay, and there were no big difference between 24 and 48 h treated of other two leachate samples. HepG2 cells were seeded in six-well plates at a density of 2×10^5 cells per well and after attachment, then treated with leachates and negative (culture medium only) for 24 h and positive (200 μM H_2O_2) for the last 30 min. After exposure, cells were harvested and resuspended in PBS (1×10^6 cells ml^{-1}), then suspension was mixed with 75 μl of 0.7% low melting agarose and added to slides pre-coated with 100 μl of 0.5% normal melting agarose and 75 μl 0.7% low melting agarose was finally added. Slides were covered with coverslips and solidified on ice for 20 min and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% Triton X-100, 10% DMSO, pH 10) at 4°C for 2 h in the dark with coverslips removed. Slides were washed with cold distilled water three times, unwinded in horizontal gel electrophoresis tray with cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min and subjected to the same fresh electrophoresis at 25 V for 30 min. After electrophoresis, cells were rinsed in neutralization buffer (0.4 mM Tris–HCl, pH 7.5) for 10 min at 4°C. Then slides were stained with 20 μl propidium iodide and visualized with a fluorescent microscope (×200 magnifications) at excitation settings of 515–560 nm. One hundred randomly selected cells from each slide (three slides for each concentration) were analyzed by using Comet Assay Software Project image analysis Software (CASP; Computer Automated Stowage Planning from Total Soft Bank Co., Ltd, www.tritekcorp.com). Results are expressed as the percentage of DNA in comet tail (% DNA in tail) and olive tail moment (OTM), which are considered the most reliable parameters for detecting single-strand DNA breaks (Toufexi *et al.,* 2013). Comets were classified into five classes based on % DNA in the tail according to a previous report (Miyamae *et al.,* 1998), class I, less than 1%, intact nucleus; class II, 1–20%; class III, 20–50%; class IV, 50–75%; and class V, more than 75% . Background levels of DNA damage in control cells showed low variability, thus ranging with similar levels in HepG2 cells and other cellular types previously reported (Ghosh *et al.,* 2015; Toufexi *et al.,* 2013; Tran *et al.,* 2007).

γH2AX flow cytometry assay. The γH2AX analysis was performed according to previously described assay (Li *et al.,* 2006). Briefly, HepG2 cells were seeded in six-well plates at 5×10^5 cells ml^{-1} and incubated at 37°C for 24 h and then treated with DNA DSB inducer (1: 200 dilution of 20 mM etoposide and culture medium), culture medium only (negative control) and different concentrations for 3 h. After centrifugation (10 min, 600 × g and aspiration of supernatants, cells were fixed in 3.7% formaldehyde/PBS at 4°C for 15 min and postfixed in ice-cold methanol (90%) for 10 min at 4°C. Cells were centrifuged, washed with PBS and incubated in blocking buffer (1% bovine serum albumin/PBS) at room temperature for 30 min. After another centrifugation, cells were resuspended in 100 μl antiphospho-histon H2AX (diluted 1: 100; Millipore) and incubated at 37°C for 1 h. Then the pellet was washed with PBS/Tween 20, centrifuged and resuspended in 100 μl fluorescein isothiocyanate conjugated secondary antibody (diluted 1: 100; Millipore) for 1 h under subdued light. Subsequently, samples were rinsed with PBS/Tween 20, centrifuged and resuspended with 200 μl PBS and measured with an FCM (Backman Coulter, USA). At minimum, 10 000 cells were counted per sample.

Statistical analysis

All the data obtained were expressed as mean ± standard deviation of replicates and tested for normality and homogeneity of variance. Statistical differences in all cases were examined by one-way analysis of variance and Newman–Keuls test. $P < 0.05$ was determined statistically significant in all analyses.

Results

Physicochemical analysis

Values of physicochemical characteristics of collected leachate samples are presented in Table 1. Results showed that the raw leachate was characterized with slightly alkaline properties, dark brown color, unpleasant odor, high concentration of COD (10 320 ± 145 mg l^{-1}) and nitrogen species. After the nitration/UF/RO process, all the examined indexes decreased significantly compared to raw leachate except the heavy metal of total Pb with slight decrease, resulting in a clear, colorless and odorless effluent.

Cytotoxic effects of treated and untreated old landfill leachates

The cell viability of cells exposed to leachate samples were presented in Fig. 2. A significant inhibition of cellular viability was observed between untreated and treated cells after 24 h of exposure at a dose of 2.5–30%. Specifically, HepG2 cells treated with raw leachate at concentrations of 20% and 30% showed high reductions of 78.5% and 90.4%, respectively. Previous studies reported that cell viability was almost 80%, which is considered appropriate for DNA damage analysis (Tice *et al.,* 2000). However, in the present study, the cell viability was higher than 50%, which was in accordance with reported studies (Ghosh *et al.,* 2014a; Toufexi *et al.,* 2013; Wang *et al.,* 2016). As a result, the rest of the tested parameters treated with raw leachate in HepG2 were investigated at concentrations of 1.25, 2.5, 5 and 10%, eliminating the interference of cell death.

7-Ethoxyresorufin-*O*-deethylase activity of leachate samples

The results of EROD activity detected in cells exposed to leachate samples are shown in Fig. 3. According to the results, cells showed

Table 1. Physicochemical characteristics of leachates prior and after nitration/UF/RO process

Characteristics	Raw leachate	Ni/UF treated leachate	Ni/UF/RO effluent
pH	7.97 ± 0.21	8.66 ± 0.33	6.51 ± 0.25
Color (PCU)	3660 ± 340	1000 ± 134	5 ± 1
COD (mg l^{-1})	10 320 ± 145	1170 ± 32	28 ± 4
Fe (mg l^{-1})	0.93 ± 0.11	0.42 ± 0.05	0.29 ± 0.05
Mn (mg l^{-1})	6.20 ± 0.31	4.75 ± 0.15	0.44 ± 0.02
Ni (mg l^{-1})	1.72 ± 0.29	0.10 ± 0.02	0.01 ± 0.01
Cr(VI) (mg l^{-1})	0.40 ± 0.11	0.26 ± 0.07	0.02 ± 0.01
NH_3-N (mg l^{-1})	5672.6 ± 930.1	28.4 ± 6.8	2.5 ± 1
N-tot (mg l^{-1})	10 418.7 ± 1324.5	125.4 ± 23.7	43.1 ± 5.9
Cd-tot (μg l^{-1})	3.64 ± 0.72	1.82 ± 0.38	0.09 ± 0.02
Cr-tot (mg l^{-1})	2.32 ± 0.51	0.39 ± 0.03	0.04 ± 0.01
Pb-tot (mg l^{-1})	0.49 ± 0.12	0.38 ± 0.04	0.35 ± 0.02
PAEs (mg l^{-1})	12.78 ± 2.07	10.34 ± 1.58	0.15 ± 0.01

Cd-tot, total cadmium; Cr-tot, total chromium; NH_3-N, ammonia nitrogen; Ni/UF, nitration/ultrafiltration; Ni/UF/RO, nitration/ultrafiltration/reverse osmosis; N-tot, total nitrogen; Pb-tot, total lead.
Values are expressed as mean ± standard deviation.

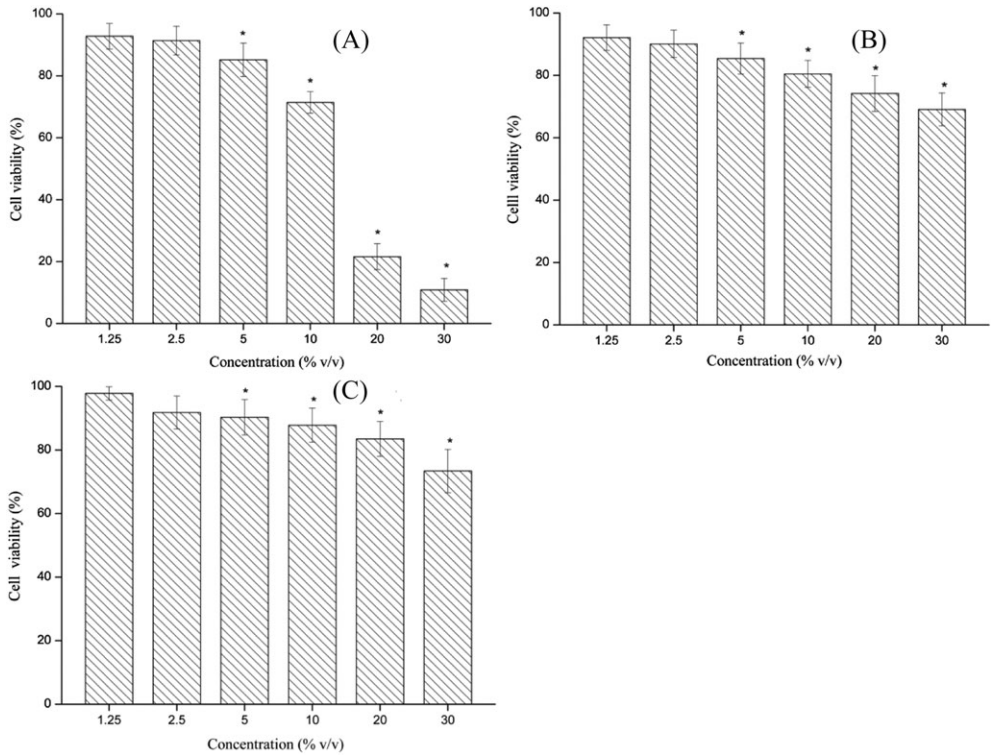

Figure 2. HepG2 cell viability after 24 h exposure to leachate samples: (a) raw leachate, (b) nitration/ultrafiltration treated, (c) nitration/ultrafiltration/reverse osmosis treated. Values represent the mean ± SD, $n = 3$. Significantly different from control: $^*P < 0.05$.

increased EROD activity after exposure to different samples with increasing concentration. The raw leachate elicited high EROD activity compared to negative control. Significant reduction of EROD activity was found in HepG2 cells exposed to all leachate concentrations accompanied with the treatment of UF and RO. There was no significant difference between control and effluent after treatment with nitration/UF/RO. Effluent showed a minimal EROD induction of 1.2 ± 0.3 pmol mg^{-1} min^{-1} at a concentration of 1.25% and the highest EROD activity was observed as 2.9 ± 1.1 pmol mg^{-1} min^{-1} at 30% concentration.

DNA damage effects of leachates pre- and post-treatment with nitration/UF/RO process

The typical comet images of leachate samples are shown in Fig. 4. Results of the comet assay parameters are shown in Table 2 and Figs 5 and 6. According to the results, HepG2 cells exposed in raw leachate for 24 h showed a statistical difference of % DNA in the tail and OTM, the lowest concentration sample caused a significant increase of 12.98% (% DNA in tail) and 4.50 (OTM), the highest reached 66.84% (% DNA in tail) and 31.36 (OTM), compared to

Journal of Applied Toxicology

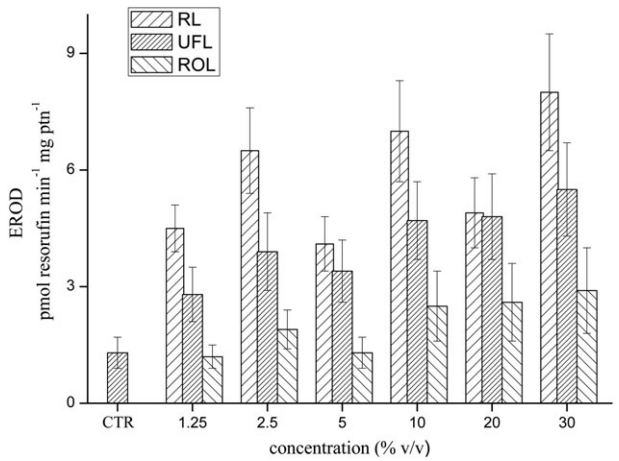

Figure 3. Activities of EROD in cells exposed for 24 h to different concentrations of leachates. Values are presented as mean ± SD, $n = 3$. CTR, control; EROD, 7-ethoxyresorufin-*O*-deetilase; RL, raw leachate; ROL, nitration/ultrafiltration/reverse osmosis-treated leachate; UFL, nitration/ultrafiltration-treated leachate.

the control cells (1.72% of DNA in tail, OTM 0.43). Cells exposed to raw leachate showed a significant dose–response increase in DNA damage compared to control cells (Figs 5a and 6a). The treated leachate exhibited a significant decrease in % DNA in the tail and OTM compared to raw leachate. However, samples after UF treatment still showed a significant difference in contrast with negative controls for % DNA in the tail and OTM (Figs 5b and 6b). There was no significant difference between negative control and effluent exposure from 1.25% to 5% for both parameters (Figs 5c and 6c) and 18.95% of DNA in the tail and 7.28% of the OTM was examined in the highest concentration of nitration/UF/RO treated leachate. As a whole, cells showed almost a dose-dependent decrease in DNA damage with the progressive treatment.

Double strand break damage effects of leachates treated with the nitration/ultrafiltration/reverse osmosis process

The γH2AX levels and corresponding DSB damage determined by FCM in HepG2 cells after treatment with tested samples for 3 h are shown in Fig. 7. Marked concentration-dependent increases in mean intensity of γH2AX were found in cells treated with raw leachate compared to control (mean intensity: 1.01; 14.34 per million of DNA damage rate). In addition, the highest mean intensity reached 2.24 at 10% corresponding to 17.75 per million of the DNA damage rate. In contrast, HepG2 cells showed a general concentration-dependent increase in γH2AX mean intensity at all concentrations of UF-treated leachate, and there were no significant differences between samples and controls except for treatment of 30%. However, exposure to 10, 20 and 30% effluent for 3 h also induced the formation of γH2AX, but no significant differences were obtained in all cells.

Discussion

Owing to a huge number of harmful contaminants and the potential risk to environmental receptors and humans, treatment of landfill leachate was one of the most essential issues in current environment technology. According to Table 1, concentrations of heavy metals such as Ni, Pb, Cr, Cd, Mn and Fe and general characterization of raw leachate were observed to be consistent with previous research (Christensen *et al.,* 2001; Kjeldsen *et al.,* 2002), and all of the examined results were found to be higher than the maximum allowed values for wastewater suitable for discharge into the environment (Gajski *et al.,* 2012) in raw leachate, indicating a serious threat to the environment. After treatment of the nitration/UF/RO process, though the total nitrogen concentration of effluent was slightly higher than the discharge limits (GB 18918–2002), the present study showed a high removal of more than 99% COD, ammonia nitrogen and total nitrogen, and 98% removal of PAEs. The selected parameters were under the discharge limits in effluent, but still cannot be neglected as their known toxicity even at trace levels (Caicedo *et al.,* 2008; Flora *et al.,* 2012). It appears that the nitration/UF/RO process could effectively reduce the general characterization of leachate.

Although the process showed satisfactory results related to physicochemical parameters of effluent, the toxic assays still revealed the potential genotoxicity. The present study showed leachate ability to disturb DNA integrity, which indicated by the increased levels of DNA damage (% DNA in the tail and OTM), exposed both in raw leachate and effluent at concentrations of 20% and 30%. In addition, the enhanced γH2AX focus formation (mean intensity of γH2AX), in combination with the increased EROD and cell proliferation inhibition, clearly revealed leachate DNA damage and cytotoxic potency. Leachate dose-dependent

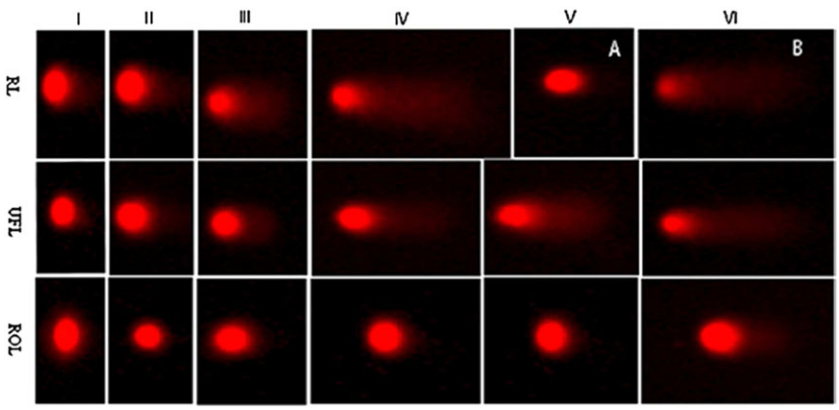

Figure 4. Representative images of different classes of comets. A and B represents negative control and positive control (200 μM H_2O_2), respectively. I–VI represent different sample concentrations of 1.25, 2.5, 5, 10, 20 and 30% (*v*/v). RL, raw leachate; ROL, nitration/ultrafiltration/reverse osmosis-treated leachate; UFL, nitration/ultrafiltration-treated leachate.

Table 2. Parameters of comet assay exposed in leachate samples on HepG2 cells

		Leachate (% *v*/v)							
		0	1.25	2.5	5	10	20	30	H_2O_2
%DNA in tail	RL	1.72 ± 0.27	12.98 ± 0.86	24.46 ± 2.5	41.54 ± 2.31	66.84 ± 2.94			
	UFL		11.03 ± 0.56	15.39 ± 0.83	22.37 ± 1.69	32.27 ± 1.60	41.56 ± 1.98	51.61 ± 3.05	69.84 ± 4.04
	ROL		2.24 ± 0.14	3.57 ± 0.33	4.58 ± 0.37	7.28 ± 0.66	12.71 ± 0.91	18.95 ± 1.02	
OTM	RL		4.50 ± 0.39	8.84 ± 1.02	17.91 ± 1.23	31.36 ± 3.97			
	UFL	0.43 ± 0.1	3.93 ± 0.29	5.51 ± 0.78	9.25 ± 0.84	14.86 ± 1.73	23.92 ± 2.06	34.76 ± 3.5	61.35 ± 4.53
	ROL		0.55 ± 0.08	0.97 ± 0.19	1.24 ± 0.26	1.93 ± 0.23	3.42 ± 0.44	7.28 ± 0.65	

RL, raw leachate; ROL, nitration/ultrafiltration/reverse osmosis-treated leachate; UFL, nitration/ultrafiltration-treated leachate.
Results are mean ± SD of median from three independent measurements.

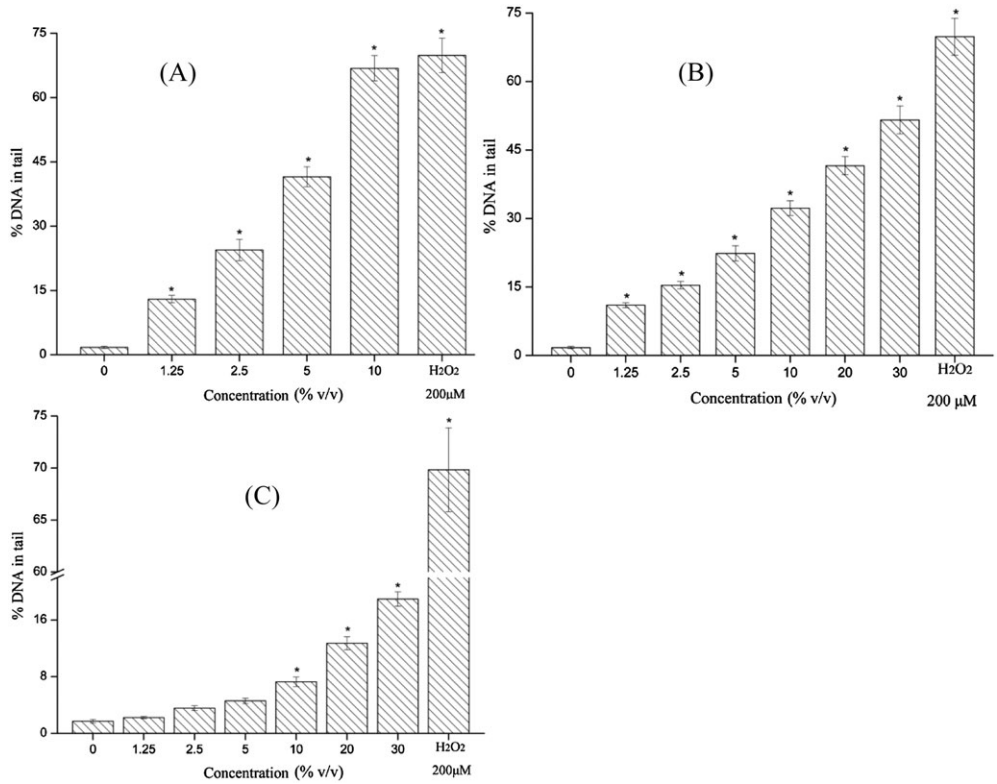

Figure 5. Percentages of DNA in tail after exposure to different concentrations of untreated and treated leachates for 24 h. (a) Raw leachate, (b) nitration/ultrafiltration treated, and (c) nitration/ultrafiltration/reverse osmosis treated. Results are mean ± SD of median from three independent measurements. Significantly different from control: $^*P < 0.05$.

genotoxicity could be induced by the relatively high concentrations of heavy metals (Ni, Pb, Cr, Cd, Mn), high concentration of organic biorefractory compounds (COD, color) and/or nitrogen species (Adamsson *et al.,* 1998; Gajski *et al.,* 2012; Rodrigues *et al.,* 2007). Some studies have reported that the presence of dioxins, polychlorinated biphenyls, polyaromatic hydrocarbons (not measured in the present study) and PAEs could also impair DNA integrity (Ernst *et al.,* 1994; Kleinsasser *et al.,* 2000; Toufexi *et al.,* 2013), and the possible mechanism of leachate-induced DNA damage might be related with its ability to enhance oxidative stress caused by several xenobiotics (Bertoldi *et al.,* 2012; Eckers *et al.,* 2009; Patlolla *et al.,* 2009; Shim *et al.,* 2017). On the other hand, leachate absorption into HepG2 cells could change the pH outside and within cells, which might change the structures of DNA (Li *et al.,* 2010).

The combined nitration/UF/RO treatment process significantly decreased leachate DNA damage proved by significantly less formation of γH2AX focus, lower % DNA in tail and OTM in HepG2 cells exposed to treated leachate compared to raw leachate. It reported that different treatment technology exhibited different removal efficiencies of leachate genotoxicity. Morozesk *et al.* (2016) reported that the electrocoagulation process has high removal efficiency of genotoxic effects of landfill leachate. Wang *et al.* (2016) reported that the Fenton reagent and ultraviolet-

Journal of AppliedToxicology

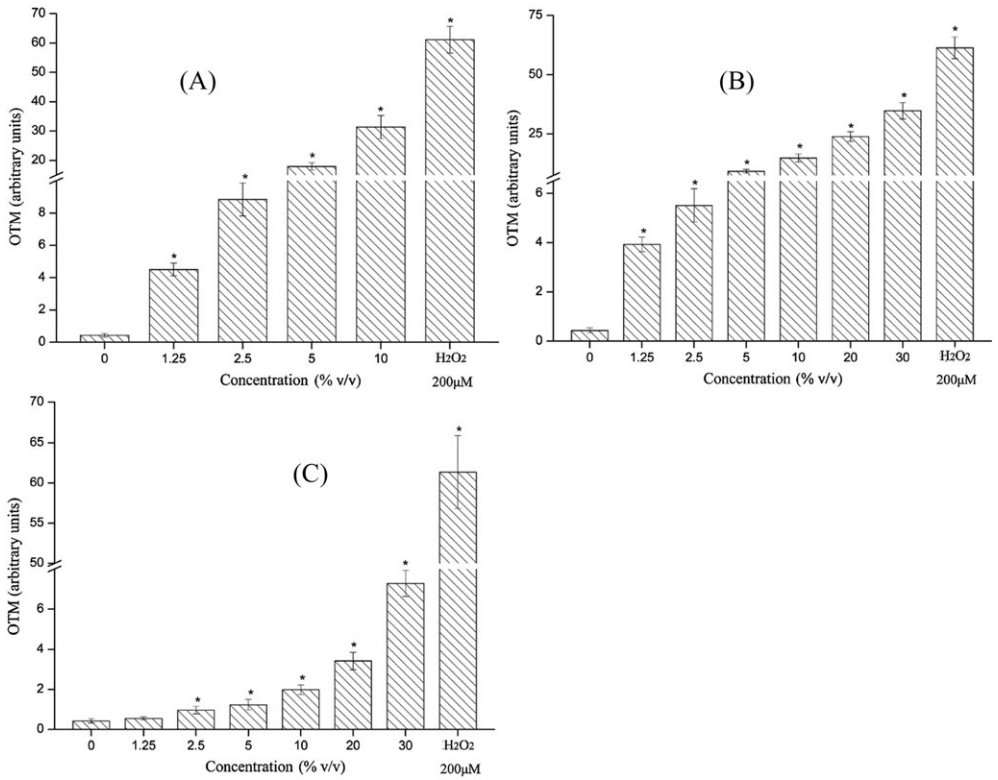

Figure 6. Comet OTM values after 24 h exposure to different concentrations of untreated and treated leachates. (a) Raw leachate; (b) nitration/ultrafiltration treated; and (c) nitration/ultrafiltration/reverse osmosis treated. Results are mean ± SD of median from three independent measurements. Significantly different from control: $^*P < 0.05$. OTM, olive tail moment.

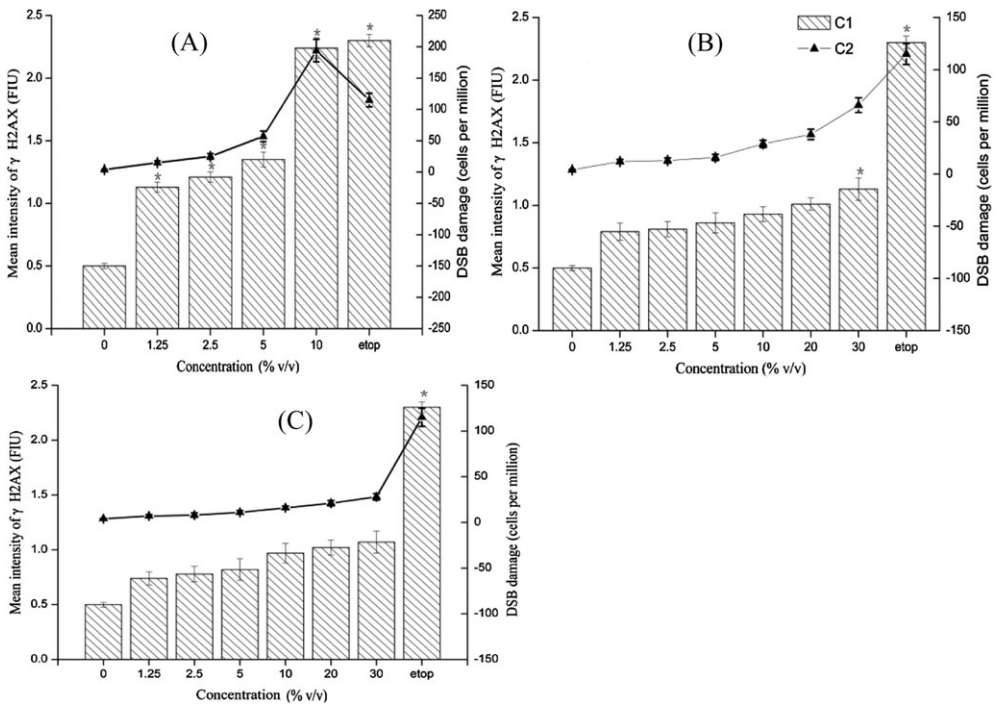

Figure 7. Dose–response of H2AX phosphorylation and DSB damage in HepG2 cells treated with different concentrations. (a) Raw leachate; (b) nitration/ultrafiltration treated; and (c) nitration/ultrafiltration/reverse osmosis treated. (C1) Mean intensity, (C2) DSB damage. Results are mean ± SD, $n = 3$. Significantly different from control: $^*P < 0.05$. DSB, DNA double-strand breaks.

Fenton reagent were efficient in reducing genotoxic effects of leachate concentrates. Gajski *et al.* (2011) reported that genotoxic effects of sludge leachate after treatment with calcium oxide-based solidification reduced significantly. In comparison, the nitration/UF/RO process was efficient in reducing DNA damage of leachate, but the effluent still exhibited slight DNA damage at concentrations of 20% and 30% as Ghosh *et al.* (2014a) reported that despite less toxic samples treated with *Pseudomonas* sp. ISTDF1 still showed genotoxicity to some extent; this might be indirectly shown by the increased EROD, which was related to potential toxic components. However, the effluent showed no DSB damage, which was considered as the most serious DNA damage. As leachate contained high concentrations of refractory organic pollutants and inorganic salts, RO has been extensively used for treatment of landfill leachate due to removal efficiency of organic matter, ammoniacal nitrogen and metals (Kurniawan *et al.,* 2006; Renou *et al.,* 2008), as well as in the desalination process (Madaeni and Eslamifard, 2010), and UF is often used as a pretreatment process for RO to remove further the suspended substances (Huang *et al.,* 2011). Furthermore, the slight DNA damage showed in effluent after nitration/UF/RO treatment might be related to membrane fouling caused by pollutants, which could decline the flux and therefore reduce treatment efficiency.

Conclusions

The present study showed clear DNA strand breaks and cytotoxic effects after exposure to relevant concentrations of leachates by comet assay and γH2AX FCM assay in HepG2 cells. The process of the UF/RO membrane combining nitration pretreatment proved to be effective due to its high removal efficiency of all measured parameters. The comet assay and γH2AX FCM assay indexes revealed that the process has a capacity to induce DNA damage, but HepG2 cells exposed to effluent still showed slight DNA damage to some extent, which was not reflected by physicochemical analysis. Thus, the individual, synergistic or antagonistic effect on DNA damage of leachate contaminants should be applied in parallel with various toxicity tests to evaluate more potential threats even at trace. Moreover, the investigated nitration/UF/RO process was suitable and efficient for treating old landfill leachate, but demands an improvement to reduce all possible negative risks to the environment and humans.

Acknowledgments

This work was supported by the Joint Fund of Science and Technology Program of Guangdong, China (2014A020217007), Pearl River S&T Nova Program of Guangzhou (201605131205366), NSFC-Guangdong Province Joint Key Project (U1301235) and Industry-university-research combination project of Guangdong Province (2013B090600009).

Conflict of interest

The authors did not report any conflict of interest.

References

Abu Amr SS, Aziz HA. 2012. New treatment of stabilized leachate by ozone/Fenton in the advanced oxidation process. *Waste Manag.* **32**: 1693–1698.

Abu Amr SS, Aziz HA, Adlan MN. 2013. Optimization of stabilized leachate treatment using ozone/persulfate in the advanced oxidation process. *Waste Manag.* **33**: 1434–1441.

Adamsson M, Dave G, Forsberg L, Guterstam B. 1998. Toxicity identification evaluation of ammonia, nitrite and heavy metals at the Stensund Wastewater Aquaculture plant, Sweden. *Water Sci. Technol.* **38**: 151–157.

Alimba CG, Gandhi D, Sivanesan S, Bhanarkar MD, Naoghare PK, Bakare AA, Krishnamurthi K. 2016. Chemical characterization of simulated landfill soil leachates from Nigeria and India and their cytotoxicity and DNA damage inductions on three human cell lines. *Chemosphere* **164**: 469–479.

Baderna D, Maggioni S, Boriani E, Gemma S, Molteni M, Lombardo A, Colombo A, Bordonali S, Rotella G, Lodi M, Benfenati E. 2011. A combined approach to investigate the toxicity of an industrial landfill's leachate: chemical analyses, risk assessment and in vitro assays. *Environ. Res.* **111**: 603–613.

Bashir MJ, Aziz HA, Yusoff MS. 2011. New sequential treatment for mature landfill leachate by cationic/anionic and anionic/cationic processes: optimization and comparative study. *J. Hazard. Mater.* **186**: 92–102.

Baun A, Jensen SD, Bjerg PL, Christensen TH, Nyholm N. 2000. Toxicity of organic chemical pollution in groundwater downgradient of a landfill (Grindsted, Denmark). *Environ. Sci. Technol.* **34**: 1647–1652.

Bertoldi K, Spindler C, dos Santos MF, Vanzella C, Lovatel GA, Elsner VR, Rodrigues MA, Siqueira IR. 2012. Effect of landfill leachate on oxidative stress of brain structures and liver from rodents: modulation by photoelectrooxidation process. *Ecotoxicol. Environ. Saf.* **84**: 319–324.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. *Anal. Biochem.* **72**: 248–254.

Caicedo M, Jacobs JJ, Reddy A, Hallab NJ. 2008. Analysis of metal ion-induced DNA damage, apoptosis, and necrosis in human (Jurkat) T-cells demonstrates Ni^{2+} and V^{3+} are more toxic than other metals: Al^{3+}, Be^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Mo^{5+}, Nb^{5+}, Zr^{2+}. *J. Biomed. Mater. Res. A* **86**: 905–913.

Chaloupka K, Santostefano M, Goldfarb IS, Liu G, Myers MJ, Tsyrolv IB, Gelboin HV, Krishnan V, Safe S. 1994. Aryl hydrocarbon (Ah) receptor-independent induction of Cypla2 gene expression by acenaphthylene and related compounds in B6C3F1 mice. *Carcinogenesis* **15**: 2835–2840.

Christensen TH, Kjeldsen P, Albrechtsen H, Jr, Heron G, Nielsen PH, Bjerg PL, Holm PE. 2009. Attenuation of landfill leachate pollutants in aquifers. *Crit. Rev. Environ. Sci. Technol.* **24**: 119–202.

Christensen TH, Kjeldsen P, Bjerg PL, Jensen DL, Christensen JB, Baun A, Albrechtsen H-J, Heron G. 2001. Biogeochemistry of landfill leachate plumes. *Appl. Geochem.* **16**: 659–718.

Cozzarelli IM, Bohlke JK, Masoner J, Breit GN, Lorah MM, Tuttle ML, Jaeschke JB. 2011. Biogeochemical evolution of a landfill leachate plume, Norman, Oklahoma. *Ground Water* **49**: 663–687.

Dai Y, Song Y, Gao H, Wang S, Yuan Y. 2015. Bibliometric analysis of research progress in membrane water treatment technology from 1985 to 2013. *Scientometrics* **105**: 577–591.

Danellakis D, Ntaikou I, Kornaros M, Dailianis S. 2011. Olive oil mill wastewater toxicity in the marine environment: alterations of stress indices in tissues of mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.* **101**: 358–366.

Darzynkiewicz Z, Traganos F, Zhao H, Halicka HD, Skommer J, Wlodkowic D. 2011. Analysis of individual molecular events of DNA damage response by flow and image assisted cytometry. *Methods Cell Biol.* **103**: 115–147.

Eckers A, Reimann K, Klotz L-O. 2009. Nickel and copper ion-induced stress signaling in human hepatoma cells: analysis of phosphoinositide 3′-kinase/Akt signaling. *Biometals* **22**: 307–316.

Ernst W, Hennigar P, Doe K, Wade S, Julien G. 1994. Characterization of the chemical constituents and toxicity to aquatic organisms of a municipal landfill leachate. *Water Q. Res. J. Canada* **29**: 89–101.

Flora G, Gupta D, Tiwari A. 2012. Toxicity of lead: A review with recent updates. *Interdiscip. Toxicol.* **5**: 47–58.

Gajski G, Oreščanin V, Garaj-Vrhovac V. 2011. Cytogenotoxicity of sewage sludge leachate before and after calcium oxide-based solidification in human lymphocytes. *Ecotoxicol. Environ. Saf.* **74**: 1408–1415.

Gajski G, Orescanin V, Garaj-Vrhovac V. 2012. Chemical composition and genotoxicity assessment of sanitary landfill leachate from Rovinj, Croatia. *Ecotoxicol. Environ. Safe.* **78**: 253–259.

Ghosh P, Das MT, Thakur IS. 2014a. Mammalian cell line-based bioassays for toxicological evaluation of landfill leachate treated by *Pseudomonas* sp. ISTDF1. *Environ. Sci. Pollut. Res. Int.* **21**: 8084–8094.

Ghosh P, Gupta A, Thakur IS. 2015. Combined chemical and toxicological evaluation of leachate from municipal solid waste landfill sites of Delhi, India. *Environ. Sci. Pollut. Res. Int.* **22**: 9148–9158.

Ghosh P, Swati TIS. 2014b. Enhanced removal of COD and color from landfill leachate in a sequential bioreactor. *Bioresour. Technol.* **170**: 10–19.

Hagman M, Heander E, Jansen JC. 2008. Advanced oxidation of refractory organics in leachate–potential methods and evaluation of biodegradability of the remaining substrate. *Environ. Technol.* **29**: 941–946.

Huang X-F, Ling J, Xu J-C, Feng Y, Li G-M. 2011. Advanced treatment of wastewater from an iron and steel enterprise by a constructed wetland/ultrafiltration/reverse osmosis process. *Desalination* **269**: 41–49.

Kalčíková G, Zagorc-Končan J, Zupančič M, Gotvajn AŽ. 2012. Variation of landfill leachate phytotoxicity due to landfill ageing. *J. Chem. Technol. Biotechnol.* **87**: 1349–1353.

Kjeldsen P, Barlaz MA, Rooker AP, Baun A, Ledin A, Christensen TH. 2002. Present and long-term composition of MSW landfill leachate: a review. *Crit. Rev. Environ. Sci. Technol.* **32**: 297–336.

Kleinsasser NH, Kastenbauer ER, Weissacher H, Muenzenrieder RK, Harréus UA. 2000. Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. *Environ. Mol. Mutagen.* **35**: 9–12.

Kuo LJ, Yang LX. 2008. γ-H2AX-a novel biomarker for DNA double-strand breaks. *In Vivo* **22**: 305–309.

Kurniawan TA, Lo WH, Chan GY. 2006. Physico-chemical treatments for removal of recalcitrant contaminants from landfill leachate. *J. Hazard. Mater.* **129**: 80–100.

Kwasniewska J, Nalecz-Jawecki G, Skrzypczak A, Plaza GA, Matejczyk M. 2012. An assessment of the genotoxic effects of landfill leachates using bacterial and plant tests. *Ecotoxicol. Eviron. Safe.* **75**: 55–62.

Li G, Yun Y, Li H, Sang N. 2008. Effect of landfill leachate on cell cycle, micronucleus, and sister chromatid exchange in *Triticum aestivum*. *J. Hazard. Mater.* **155**: 10–16.

Li H, Han M, Hou L, Li G, Sang N. 2010. Landfill leachate ingestion induces protein oxidation and DNA–protein crosslinks in mouse viscera. *J. Hazard. Mater.* **174**: 54–58.

Li T, Yin PH, Zhao L, Wang GF, Yu QJ, Li HY, Duan SS. 2015. Spatial-temporal distribution of phthalate esters from riverine outlets of Pearl River Delta in China. *Water Sci. Technol.* **71**: 183–190.

Li Z, Yang J, Huang H. 2006. Oxidative stress induces H2AX phosphorylation in human spermatozoa. *FEBS Lett.* **580**: 6161–6168.

Madaeni S, Eslamifard M. 2010. Recycle unit wastewater treatment in petrochemical complex using reverse osmosis process. *J. Hazard. Mater.* **174**: 404–409.

Miyamae Y, Yamamoto M, Sasaki YF, Kobayashi H, Igarashi-Soga M, Shimoi K, Hayashi M. 1998. Evaluation of a tissue homogenization technique that isolates nuclei for the in vivo single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories. *Mutat. Res.* **418**: 131–140.

Morozesk M, Bonomo MM, Rocha LD, Duarte ID, Zanezi ER, Jesus HC, Fernandes MN, Matsumoto ST. 2016. Landfill leachate sludge use as soil additive prior and after electrocoagulation treatment: A cytological assessment using CHO-k1 cells. *Chemosphere* **158**: 66–71.

Nwagbara O, Darling-Reed SF, Tucker A, Harris C, Abazinge M, Thomas RD, Gragg RD. 2007. Induction of cell death, DNA strand breaks, and cell cycle arrest in DU145 human prostate carcinoma cell line by benzo[a] pyrene and benzo[a]pyrene-7, 8-diol-9, 10-epoxide. *Int. J. Environ. Res. Public Health* **4**: 10–14.

Oliveira LF, Silva SM, Martinez CB. 2014. Assessment of domestic landfill leachate toxicity to the Asian clam *Corbicula fluminea* via biomarkers. *Ecotoxicol. Environ. Saf.* **103**: 17–23.

Oman CB, Junestedt C. 2008. Chemical characterization of landfill leachates – 400 parameters and compounds. *Waste Manag.* **28**: 1876–1891.

Patlolla AK, Barnes C, Hackett D, Tchounwou PB. 2009. Potassium dichromate induced cytotoxicity, genotoxicity and oxidative stress in human liver carcinoma (HepG2) cells. *Int. J. Environ. Res. Public Health* **6**: 643–653.

Renou S, Givaudan J, Poulain S, Dirassouyan F, Moulin P. 2008. Landfill leachate treatment: review and opportunity. *J. Hazard. Mater.* **150**: 468–493.

Rodrigues RV, Schwarz MH, Delbos BC. 2007. Acute toxicity and sublethal effects of ammonia and nitrite for juvenile cobia *Rachycentron canadum*. *Aquaculture* **271**: 553–557.

Sang N, Li G, Xin X. 2006. Municipal landfill leachate induces cytogenetic damage in root tips of *Hordeum vulgare*. *Ecotoxicol. Environ. Saf.* **63**: 469–473.

Shim I, Choi K, Hirano S. 2017. Oxidative stress and cytotoxic effects of silver ion in mouse lung macrophages J774.1 cells *J. Appl. Toxicol.* **37**: 471–478.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**: 184–191.

Surampalli RY, Zhang TC, Tyagi RD, Selvam A. 2016. Landfill leachate collection and treament in Sustainable Solied Waste Management. *Am. Soc. Civ. Eng.* 605–631.

Svenson A, Allard A-S, Ek M. 2003. Removal of estrogenicity in Swedish municipal sewage treatment plants. *Water Res.* **37**: 4433–4443.

Tice R, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **35**: 206–221.

Tillitt DE, Giesy JP, Ankley GT. 1991. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ. Sci. Technol.* **25**: 87–92.

Toufexi E, Tsarpali V, Efthimiou I, Vidali MS, Vlastos D, Dailianis S. 2013. Environmental and human risk assessment of landfill leachate: an integrated approach with the use of cytotoxic and genotoxic stress indices in mussel and human cells. *J. Hazard. Mater.* **260**: 593–601.

Tran D, Moody AJ, Fisher AS, Foulkes ME, Jha AN. 2007. Protective effects of selenium on mercury-induced DNA damage in mussel haemocytes. *Aquat. Toxicol.* **84**: 11–18.

Tsarpali V, Dailianis S. 2012. Investigation of landfill leachate toxic potency: an integrated approach with the use of stress indices in tissues of mussels. *Aquat. Toxicol.* **124–125**: 58–65.

Wang G, Lu G, Yin P, Zhao L, Yu QJ. 2016. Genotoxicity assessment of membrane concentrates of landfill leachate treated with Fenton reagent and UV-Fenton reagent using human hepatoma cell line. *J. Hazard. Mater.* **307**: 154–162.

Wang J, Che B, Zhang LW, Dong G, Luo Q, Xin L. 2017. Comparative genotoxicity of silver nanoparticles in human liver HepG2 and lung epithelial A549 cells. *J. Appl. Toxicol.* **37**: 495–501.

Žegura B, Filipič M. 2004. *Application of* In Vitro *Comet Assay for Genotoxicity Testing*. Humana Press; 301–313.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.