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Review article

Investigating landfill leachate toxicity *in vitro*: A review of cell models and endpoints

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ABSTRACT

Landfill leachate is a complex mixture characterized by high toxicity and able to contaminate soils and waters surrounding the dumpsite, especially in developing countries where engineered landfills are still rare. Leachate pollution can severely damage natural ecosystems and harm human health. Traditionally, the hazard assessment of leachate is based on physicochemical characterization but the toxicity is not considered. In the last few decades, different bioassays have been used to assess the toxicity of this complex matrix, including human-related *in vitro* models. This article reviews the cell bioassays successfully used for the risk assessment of leachate and to evaluate the efficiency of toxicity removal of several processes for detoxification of this wastewater. Articles from 2003 to 2018 are covered, focusing mainly on studies that used human cell lines, highlighting the usefulness and adequacy of *in vitro* models for assessing the hazard involved with exposure to leachate, particularly as an integrative supporting tool for chemical-based risk assessment. Leachate is generally toxic, mutagenic, genotoxic and estrogenic *in vitro*, and these effects can be measured in the cells exposed to already low concentrations, confirming the serious hazard of this wastewater for human health.

1. Introduction

Waste management is a crucial point in developed and developing countries in view of general population growth, rapid urbanization and also the increased *per capita* waste generation (Renou et al., 2008; Omar and Rohani, 2015; USEPA, 2018). Landfilling, one of the oldest disposal approaches, is still the most widespread option worldwide (Clarke et al., 2015; Mukherjee et al., 2015; Torretta et al., 2017). Once stored in landfills, the waste undergoes several physicochemical and biological degradation processes and transformation, generating highly contaminated wastewater known as leachate (Kamaruddin et al., 2015; Ghosh et al., 2017; Yao, 2017). Although the composition of this dark mixture depends mainly on the types of waste in the landfill and its residual moisture content, water infiltration, stage of degradation and landfilling technology, Christensen et al. (2001) reported that most leachates contain those common main pollutant categories: dissolved organic matter, inorganic macro components, heavy metals and organic xenobiotics. Landfill leachate is considered a source of environmental concern because the pollutant mixture can have adverse effects on ecosystems and public health when leachate reaches soil, surface and groundwater arounding the landfill (Davoli et al., 2010; Baderna et al., 2011; Ghosh et al., 2014, 2017; Khalil et al., 2018). This kind of contamination is particularly common in developing countries where engineered landfills with liners and leachate treatment plants are still lacking (Alimba et al., 2016; Kumari et al., 2016; Swati et al., 2017; Khalil et al., 2018).

Chemical characterization is traditionally used to assess the hazard and the risk of landfill leachate, focusing in particular on heavy metals and organic compounds with toxic, estrogenic and carcinogenic potential even at trace levels (Kjeldsen et al., 2002; Benfenati et al., 2007; Andrews et al., 2011; Clarke et al., 2015; Qi et al., 2018). Recently, integrated assessment approaches have been proposed in which chemical analyses are supported by a toxicological evaluation using biological assays with model organisms, and also *in vitro* systems (Thomas et al., 2009; Ghosh et al., 2017). The main advantage of bioassays is their ability to respond to all the chemical and biological agents present, providing a measure of the overall toxicity of the investigated matrix (Farre and Barcelo, 2003; Ghosh et al., 2017). If the model is

Abbreviations: EROD, 7-ethoxy-resorufin-O-deethylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS, 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; PAHs, Polycyclic aromatic hydrocarbons

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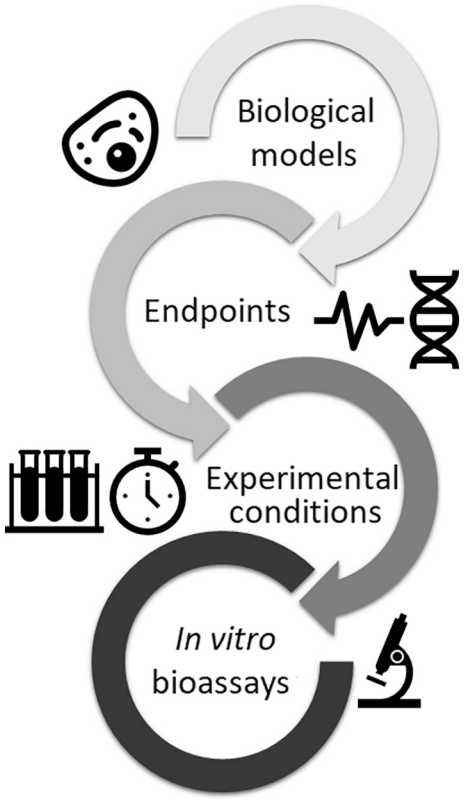

Fig. 1. Components of *in vitro* model according to Frazier (1993).

chosen correctly, the biological response will take account of both the bioavailability and the interactions of all the agents leading to additive, synergistic or antagonist effects in the selected model (Farre and Barcelo, 2003).

Bioassays have been used in environmental toxicology and their use as screening tools has significantly increased in the last 30 years also for hazard assessment of landfill leachate (Andersen and Krewski, 2009; Thomas et al., 2009; Ghosh et al., 2017; Poteser, 2017). Ghosh and collaborators have reviewed the assays available for the eco- and toxicological evaluation of leachate, particularly analysing the use of ecotoxicological assays and models including bacteria, plants and aquatic organisms (Ghosh et al., 2017).

This review examines cell-based bioassays used to assess the potential risks of leachate for human health. More than 20 papers published since 2003 were analysed, focusing mainly on human-derived models.

2. Background

According to Frazier (1993), an *in vitro* model has three basic components: the biological model, the endpoint and the protocol (Fig. 1). The biological model is the system in which the effects of substances are studied. The endpoint is the effect determined by the substance or mixture in the biological system after exposure of the cell model. The third component is the protocol as the set of experimental conditions (temperature, time, reagents, equipment) necessary for reproducible measurement of the selected endpoint in the model.

Eleven cell types are used to assess leachate toxicity in the articles reviewed. Most of the models are human cell lines. Fig. 2 shows the cell models and their frequencies. Cell lines were selected as models of target organs potentially affected after accidental exposure to leachate or to leachate-contaminated environmental matrices, or to assess specific effects induced by the wastewater such as estrogenicity.

Each model is discussed with its main endpoints and protocols.

3. Basal toxicity and genotoxicity with HepG2 cells

The liver is one of the organs affected by leachate components after accidental ingestion of this wastewater or soil and water contaminated by leachate. Increased levels of protein oxidation, lipid peroxidation, DNA-protein crosslinks and a global alteration of antioxidative defences were found in mice treated with leachate (Bakare et al., 2003; Li et al., 2006, 2010; Ghosh et al., 2017). The risk assessment guidelines for contaminated environmental matrices describe ingestion as the main route of exposure to contaminants in soil and water (USEPA, 1989; Health Canada, 2010, 2012, 2017). All this evidence makes the liver a very interesting target organ for studying the adverse effects of leachate in humans.

Human hepatoma cell line HepG2 is the most widely used cell model for the investigation of leachate toxicity. Table 1 lists papers in which this cell line was used.

HepG2 cells are a well-known *in vitro* model of liver, retaining several morphological characteristics of liver parenchymal cells and expressing xenobiotic metabolizing enzymes involved in the bioactivation and detoxification of various xenobiotics (Knowles and Aden, 1983; Valentin-Severin et al., 2003; Mersch-Sundermann et al., 2004; Castell et al., 2006; Costantini et al., 2013; Morgado et al., 2017; Ramirez et al., 2018). HepG2 are commonly used in drug metabolism and hepatotoxicity studies and as alternatives to human primary hepatocytes (Wilkening et al., 2003; Donato et al., 2013, 2015). This cell line was also successfully used in the toxicological evaluation of environmental matrices such as water, soils and sediments (Vidic et al., 2009; Baderna et al., 2013; Zhou et al., 2013; Baderna et al., 2014; Costa et al., 2014; Pinto et al., 2014).

HepG2 cells were used to investigate the basal toxicity of landfill leachate for the first time in 2011 (Baderna et al., 2011). Cells were exposed for 72 h to leachate from a controlled landfill for non-hazardous industrial waste and municipal solid waste in Northern Italy. Raw leachate, dichloromethane-extracted organic phase and aqueous residual phase were tested in concentrations ranging from 0 to 30% v/v. Toxicity was evaluated daily as effects on cell proliferation and cytotoxicity using respectively the MTS assay and adenylate kinase release. Leachate inhibited cell proliferation at low doses (2.5–5% v/v), causing cytotoxic events after prolonged exposure or higher treatment concentrations (from 10% v/v) (Baderna et al., 2011). No significant differences were found in cells treated with the whole leachate or its aqueous phase, while the organic phase did not induce significant toxicity. These findings suggest that hydrophilic components are the main agents responsible for leachate toxicity. Chemical characterization of the aqueous phase indicated the presence of heavy metals and ammonia, known to cause oxidative stress and cell cycle block in the HepG2 model (Eckers et al., 2009; Patlolla et al., 2009).

Four Indian functioning landfill sites were investigated in 2015, focusing on cytotoxic and genotoxic effects induced by raw leachate in the liver *in vitro* model after 24 h exposure (Ghosh et al., 2015). MTT and alkaline comet assays were used in cells exposed to different samples dilutions (0 to 20% v/v). The bioassays highlighted that the leachates from the investigated sites contained different loads of cytotoxic and genotoxic compounds. These results were in accordance with those obtained by chemical characterization of the leachates.

HepG2 cells have been efficiently used to evaluate the efficiency of several leachate treatment processes for toxicity removal.

Ghosh et al. (2014) used the cells to assess the detoxification by *Pseudomonas* sp. ISTDF1 bacterial strain on leachate collected from an unlined landfill in India. Cells were treated with raw or bacteria-treated leachates for 24 h (from 0 to 20% v/v) and toxicological changes were measured in terms of cell viability, genotoxicity and cytochrome P4501A induction, respectively with MTT, comet and EROD assays. Bacterial treatment significantly reduced the leachate toxicity, measured with the MTT assay, and the efficiency of the process was enhanced on prolonging the time of bacterial detoxification. A similar

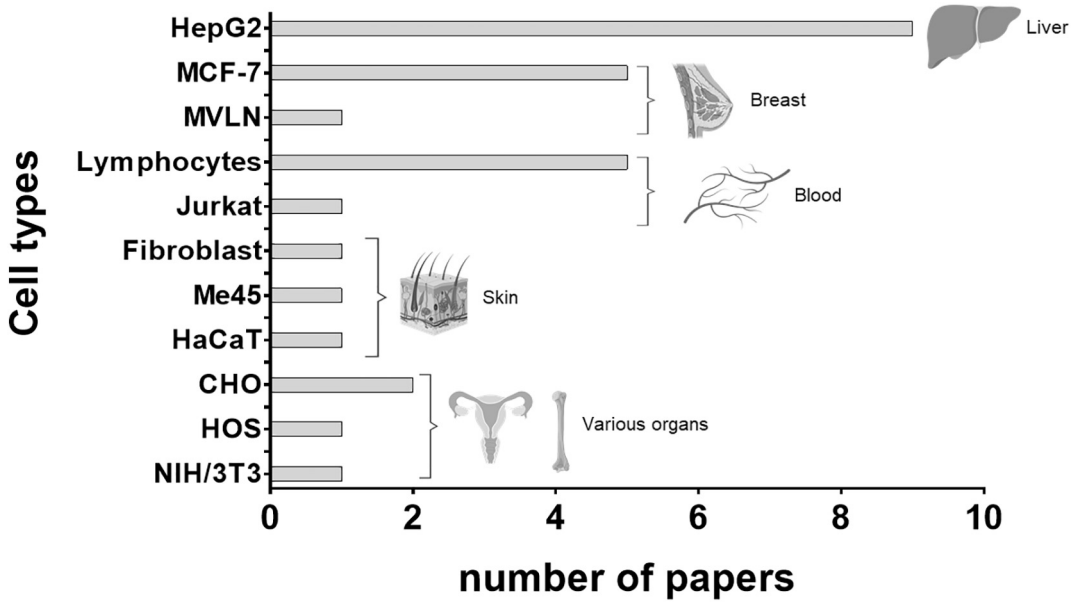

Fig. 2. Cell models and frequency in the selected papers.

Table 1
List of papers involving HepG2 cells.

Reference	Endpoint	Assay	Treatment time	Landfill type/source	Leachate details
Baderna et al., 2011	Proliferation Cytotoxicity	MTS assay Adenylate kinase release	24–48-72 h	Non-hazardous industrial waste + municipal solid waste landfill (Italy)	1. Raw leachate 2. Concentrated organic phase obtained with dichloromethane 3. Aqueous phase
Ghosh et al., 2014	Viability Genotoxicity Metabolic activity	MTT assay Comet assay EROD[a] assay	24 h 24 h 6 h	Unlined landfill receiving municipal solid waste, construction materials and biomedical waste (India)	1. Acidified raw leachate 2. Bacteria-remediated leachate
Ghosh et al., 2015	Proliferation Genotoxicity	MTT assay Comet assay	24 h	4 landfills (India)	Raw leachate
Alimba et al., 2016	Viability Cell morphology Genotoxicity	MTT assay Morphology changes Comet assay	24 h	4 landfills (3 India, 1 Nigeria)	Simulated landfill soil leachate
Kumari et al., 2016	Viability Genotoxicity	MTT assay Comet assay	24 h	Unengineered landfill (India)	Lysimeter-derived leachate from waste
Wang et al., 2016a	Viability Genotoxicity	MTT assay Comet assay CBMN[b] assay	24 h	Landfill (China)	1. Raw membrane concentrate from leachate treatment plant 2. UV-Fenton treated membrane concentrate
Cheng et al., 2017	Proliferation Genotoxicity Metabolic activity	MTT assay Comet assay γH2AX EROD assay	24 h	Landfill (China)	1. Raw leachate 2. Nitration-UV treated leachate 3. nitration/ultrafiltration/reverse osmosis treated leachate
Hong et al., 2017	Genotoxicity	Comet assay CBMN assay	24 h	Landfill (China)	1. Raw membrane concentrate 2. UV-Fenton treated membrane concentrate 3. Fenton treated membrane concentrate 4. Activated carbon treated membrane concentrate
Swati et al., 2017	Viability Genotoxicity	MTT assay Comet assay	24 h	Municipal solid waste landfills (India)	DCM/Acetone extract from soils

[a] 7-ethoxy-resorufin-O-deethylase.
[b] Cytokinesis-Block MicroNuclei assay.

pattern was confirmed by the comet assay, showing a lower percentage of DNA in tail and Olive tail moment (OTM) in cells exposed to treated leachate to than in cells treated with raw leachate (both at 4% v/v). Finally, the evaluation of cytochrome induction revealed effective detoxification by bacteria, testifying to their ability to mineralize compounds that induce EROD, including the dioxin-like compounds identified by chemical analysis.

Kumari et al. (2016) investigated the efficiency of bacterial, algal and bacto-algal co-culture. MTT and comet assays were used to measure changes in the toxic potential of biologically treated leachate in 24 h treated cells (0–20% v/v for cell viability, 4% v/v for comet assay). *Paenibacillus* sp. ISTP10 bacterial strain and *Scenedesmus* sp. ISTGA1 microalgae both successfully reduced leachate toxicity and microalgae were more efficient as biological treatment. The co-culture enhanced the bioremediation efficiency by increasing the reduction of both the toxic and genotoxic potential of the treated leachate.

Table 2
List of papers with lymphocytes.

Reference	Endpoint	Assay	Treatment time	Landfill type/source	Leachate details
Bakare et al., 2007	Viability Genotoxicity	Trypan blue exclusion assay Comet assay	3 h	Solid wastes from polyfiber industry, aeronautical industry and municipal sludge	Artificial leachates following ASTM D3987-85 (1992)
Amahdar et al., 2009	Proliferation Genotoxicity	Cell proliferation index CBMN assay	72 h	Uncontrolled landfill (Morocco)	Raw leachate
Gajski et al., 2012	Cytotoxicity Genotoxicity	Trypan blue exclusion assay Comet assay CBMN assay	6–24 h	Sanitary landfill (Croatia)	0.45 μm-filtered raw leachate
Garaj-Vrhovac et al., 2013	Cytotoxicity Genotoxicity	Acridine orange/ethidium bromide dual staining Comet assay CBMN assay	4–24 h	Sanitary landfill (Croatia)	1. Raw leachate 2. Chemically-treated leachate 3. Electrochemically-treated leachate
Toufexi et al., 2013	Genotoxicity	CBMN assay	72 h	Active landfill (Greece)	Raw leachate

Fenton and UV-Fenton reagents are two advanced oxidation processes used in leachate treatment (Mukherjee et al., 2015). Wang et al. (2016a) applied these techniques to membrane-concentrated leachate from a Chinese landfill, assessing the toxic and genotoxic potential of treated and untreated wastewaters with MTT, cytokinesis-block micronucleus (CBMN) and comet assays after 24 h exposure of HepG2 cells to samples from 0 to 30% v/v. Both chemical treatments almost completely reduced the toxicity of membrane concentrate and the UV-Fenton reagent eliminated wastewater genotoxicity as demonstrated by the comet and micronucleus assays also in cells exposed to the highest concentrations of treated leachate.

A similar approach was used by Cheng et al. (2017) to study the detoxification efficiency of membrane treatment in a dumpsite in China. Cells were treated for 24 h with raw leachate, nitration/ultrafiltration-treated leachate or nitration/ultrafiltration/reverse osmosis treated leachate (treatment concentrations from 0 to 30% v/v). Membrane-treated leachates had significantly lower overall toxicity than raw wastewater. The reverse osmosis markedly enhanced detoxification, reducing leachate genotoxicity, as seen in the comet assay and also the γH2AX assay used to measure double-strand breakage.

Hong et al. (2017) compared the genotoxicity of untreated membrane concentrate and concentrates treated with UV-Fenton, Fenton or activated carbon adsorption processes. Comet and CBMN assays were applied on HepG2 cells treated for 24 h with the different leachates selecting the percentage of DNA (% DNA in tail) as comet parameter and micronucleus frequency and cytokinesis-block proliferation index (CBPI) for the CBMN assay. Untreated wastewater induced the concentration-dependent appearance of micronuclei, even at lower concentrations. Micronuclei frequency in cells exposed to UV-Fenton treated leachate was comparable to that in control cells, while leachate treated with the Fenton process or activated carbon adsorption still showed genotoxic potential even if the effects were lower than those induced by the untreated leachate. The results of the comet assay were consistent with those from the micronucleus assay, confirming that the UV-Fenton process is more efficient in removing compounds with genotoxic potential.

MTT and comet assays were also applied for the toxicity evaluation of simulated leachate obtained from landfill soil (Alimba et al., 2016; Swati et al., 2017). Three landfills in Nigeria and one in India were investigated by Alimba et al. (2016), generating artificial leachates from soils collected in each dumpsite and following the toxicity characteristic leaching procedure proposed by the United States Environmental Protection Agency (USEPA, 1992) with acidified water and rotary shaking. Toxic and genotoxic effects and morphological alterations were evaluated in HepG2 cells after 24 h exposure to simulated leachates (0 to 100% leachate/medium, v/v). Significant effects on cell proliferation were seen in all treated cells, even at low concentrations (6.25 and 12.5% v/v). Morphological assessment indicated cytoplasmic vacuolization, loss of substrate adhesion and reduced cell size as an early signal of cell death. Treating cells with sublethal concentrations of leachate resulted in significant genotoxicity and tail length seemed the most sensitive parameter in a comet assay applied to the leachate toxicological profile.

Swati et al. (2017) exposed hepatoma cells to the organic fraction of soil collected in three landfill sites in India. Soils were extracted with a mixture of dichloromethane and acetone according to US EPA method 3500C (2007). Extracts were then evaporated to dryness and resuspended in DMSO for the *in vitro* test. Inhibition of cell proliferation was concentration-dependent in all the leachate-treated cells (0.01 to 100 g SedEq L^{-1}), with significant differences in the toxic potential of the tested leachates. Differences were also found in terms of induced DNA damage measured by comet assay using the OTM and tail moment as parameters. Toxicological rankings based on the MTT or comet assay were comparable and in accordance with those from the chemical-based risk assessment of soils focused, in particular, on the effects of PAHs levels found in the soils.

4. Genotoxicity in human peripheral blood cells

Tewari et al. (2006) showed that municipal waste leachates could cause DNA damage in bone marrow and blood of Swiss albino mice treated *in vivo* with daily doses of leachate by oral gavage. This suggests that blood cells are affected by leachate after ingestion, making the circulatory system an important potential target for humans. Moreover, lymphocytes are considered sentinel cells for early warning effects associated with adverse health outcomes (Faust et al., 2004a).

Human peripheral blood cells were used in several studies to investigate the genotoxicity of landfill leachate (Bakare et al., 2007; Gajski et al., 2012; Garaj-Vrhovac et al., 2013; Toufexi et al., 2013; Alimba et al., 2016) (Table 2). Lymphocytes from whole blood of healthy, non-smoking donors were exposed *in vitro* to different percentages of leachate in their culture medium and the alkaline comet assay or CBMN assay were used.

The Comet assay, also known as single cell gel electrophoresis (SCGE) assay, is a well-known method to assess genetic damage *in vivo* and *in vitro* and can be applied to a wide variety of cells (Faust et al., 2004b; Møller, 2005; Frenzilli et al., 2009; Speit et al., 2009; Martins and Costa, 2015; Zare Sakhvidi et al., 2016). It is a rapid, simple and sensitive method for detecting DNA single-strand breaks, incomplete excision repair sites and alkali-labile sites in single cells (Singh et al., 1988; Sunjog et al., 2013; Glei et al., 2016).

Micronuclei (MNi) are biomarkers of genome stability because they originate from chromosome breakage and whole chromosome loss during nuclear division (Fenech, 2000, 2007; Bonassi et al., 2007). Like the comet assay, the micronucleus assay has been widely used to assess the genotoxicity of compounds, mixtures and ionizing radiations *in vitro* and *in vivo*. An increase in micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans (Bonassi et al., 2007, 2011). The CBMN assay is the gold standard method for measuring MNi in human and animal cells (OECD, 2016a, 2016b; Fenech, 2007).

Bakare et al. (2007) investigated the DNA damage induced by artificial leachates from different solid waste applying the comet assay on Histopaque-isolated lymphocytes exposed *in vitro* for 3 h to several concentrations of each leachate (0–20% v/v). The percentage tail DNA, tail length (migration distance of the DNA from the nucleus) and Olive tail moments (the product of the tail length and the fraction of total DNA in the tail, OTM) were scored in 50 cells for each treatment (Olive et al., 1990). Parameters were scored only in the treatment that did not lower cell viability (determined by the trypan blue exclusion assay) by more than 30% compared to untreated control cells. Leachates induced DNA damage in a concentration-dependent manner. OTM and % tail DNA were the best parameters to describe the genotoxic potential of leachates. The importance of these parameters for interpreting DNA damages was previously seen in human biomonitoring studies (Lee et al., 2004; Kumaravel and Jha, 2006; Ursini et al., 2006).

The CBMN assay with lymphocytes was used to investigate the genotoxicity of landfill leachates collected in Morocco (Amahdar et al., 2009) and in Greece (Toufexi et al., 2013). Lymphocytes were exposed *in vitro* to increasing concentrations of leachate for 72 h directly diluting increasing volume of samples to the cultures (100–1500 μL). Cytochalasin-B to block cytokinesis after one cellular division. The proliferation index and MNi frequency in binucleated cells were scored as indexes of cytotoxicity and genotoxicity respectively. A concentration-dependent significant increase in MNi frequency was found in leachate-treated lymphocytes, confirming the genotoxic risk due to the leachate pollutants.

Gajski et al. (2012) applied both the comet and micronuclei assays as an integrated approach for genotoxic evaluation of leachate from a sanitary landfill in Croatia. Lymphocytes were treated for 6 and 24 h with whole raw leachate from two monthly samplings (100 μL of samples added into 900 μL of blood solution, corresponding to a 10% v/v treatment). Tail length, tail intensity and tail moment were selected as comet parameters while micronuclei, nucleoplasmic bridges and nuclear buds were scored in binucleated cells in the CBMN assay. Cytotoxic effects were evaluated with the trypan blue exclusion assay and the cytokinesis-block proliferation index. No significant toxicity was induced by leachates in the exposed lymphocytes and the effects were comparable in the two samples. The alkaline comet assay revealed significant differences in treated cells compared to the control group. Differences in tail length were found in cells exposed both to 6 and 24 h while significant changes in tail intensity and tail moment were found only in cells treated for 24 h. Micronuclei frequency was increased in cells treated with leachates and there were also differences between exposure periods. Comparable results were found by scoring nucleoplasmic bridges. Correlation studies among the results of the two tests indicated that the total number of micronuclei and the total number of nucleoplasmic bridges best correlated with the comet parameters.

The combination of comet and micronuclei assays was applied to evaluate the efficiency of chemical and electrochemical treatments of leachate from a sanitary landfill in Croatia (Garaj-Vrhovac et al., 2013). Lymphocytes were exposed for 4 and 24 h to untreated and treated leachates (100 μL of samples added into 900 μL of blood solution, corresponding to a 10% v/v treatment). Both treatments efficiently lowered the toxic and genotoxic potential of leachate. No significant effects on cell viability were found in cells exposed to treated leachates compared to the control groups but marked toxicity was seen in cells treated with the original leachate. Exposure to the untreated leachate resulted in significant increases in the comet assay (tail length and tail moment) and CBMN assay parameters (MNi, nucleoplasmic bridges and nuclear buds) but no significant differences were found between control cells and cells exposed to the treated leachates.

5. Estrogenic potential in breast cancer cells

Endocrine-disrupting chemicals (EDCs) are frequently found in landfill leachate as part of the xenobiotic organic compounds representing one of the fractions identified in the common basal composition of leachate (Benfenati et al., 1999, 2007; Coors et al., 2003; Bertanza and Pedrazzani, 2008; Baderna et al., 2011; Bertanza et al., 2013; Yi et al., 2017; Qi et al., 2018). EDCs can interfere with natural hormone actions, altering reproductive and immune functions, raising the incidence of breast cancer and causing neurodevelopmental delays and abnormal growth patterns in children (Diamanti-Kandarakis et al., 2009; WHO/UNEP, 2013; Monneret, 2017).

Several *in vitro* models are available to measure the endocrine-disrupting bio-activity of compounds and mixtures, especially for evaluation of their estrogenic potential (Sonneveld et al., 2005; Avberšek et al., 2011; Alvarez et al., 2013; Cooper et al., 2013; Mertl et al., 2014). The *E*-Screen bioassay is the most widely used method in environmental biomonitoring and for analysis of the estrogenic bioactivity of wastewater before and after purification in wastewater treatment plants (Soto et al., 1995; Alvarez et al., 2013; Bertanza et al., 2013; Leusch et al., 2014; Liu et al., 2018). The *E*-Screen assay assesses the estrogenicity of chemicals by the enhanced proliferative effect of estrogens or estrogen-like compounds on the human breast cancer cell line MCF-7 that endogenously expresses the estrogen receptor alpha (ERα) (Soto et al., 1995). Cells are treated with a steroid-free medium containing different concentrations of the sample under investigation and, generally after six days, the cell number is compared to those in cells exposed to 17β-estradiol as reference positive control.

Studies with breast cancer lines are shown in Table 3.

Talorete et al. (2008) investigated the stress response of MCF-7 cells exposed to several leachate samples from landfills in Tunisia. The estrogenic potential of samples was evaluated with *E*-Screen assay, treating the cells for six days (0 to 20% v/v). Cell number was obtained with MTT and neutral red assays. DNA fragmentation and LDH assays were also used to study the cytotoxicity of the samples, coupled with proteomic analysis of treated cells to evaluate the expression of stress-related proteins induced by leachate. All the leachates were toxic at concentrations higher than 5% v/v. Estrogenic activity, measured only in cells treated with 5% v/v, indicated that they have estrogenic potential. The cytotoxicity tests showed that leachates induced necrosis in a dose-dependent manner. Proteomic analysis revealed the increased expression of the heterogeneous nuclear ribonucleoprotein E1 (hnRNP-E1), the phosphoglycerate mutase 1 and the nuclear matrix protein 200 (NMP 200), three proteins involved in the stress response.

The *E*-screen assay was also used to evaluate the removal efficiency of advanced oxidation treatments of leachate (Wang et al., 2016b; Hong et al., 2017; Hou et al., 2017).

This assay was integrated with other bioassays with freshwater organisms (algae and daphnids) to evaluate the toxicity and estrogenicity of a concentrated leachate collected during UV-Fenton and Fenton treatments (Wang et al., 2016b). MCF-7 BUS cells, a special clone characterized by the highest proliferative response to 17β-estradiol (Villalobos et al., 1995), were treated for 5 days with organic extracts from raw and treated leachates. The estrogenic potential of treated concentrated leachate expressed as 17β-estradiol equivalent concentrations (EEQ), was significantly lowered than the untreated wastewater (EEQ = 104 ± 24.6 ng/L) since after 30 min of UV-Fenton treatment (1.53 ± 0.82 ng/L) while Fenton oxidation required longer time to achieve the same results (EEQ 2.29 ± 1.56 ng/L after 120 min).

Similar results were obtained by Hong et al. (2017) comparing the estrogenic activity of untreated membrane concentrate and those treated with UV-Fenton, Fenton or activated carbon adsorption. The *E*-screen assays showed that the UV-Fenton was the most efficient treatment, completely reducing the estrogenicity of treated leachate. This is consistent with the results obtained with comet and micronuclei assays with HepG2 cells used as markers of genotoxicity.

Contrasting results were reported by Hou et al. (2017), who used the E-screen assay to evaluate the estrogenic effect of membrane concentrates from raw and UV-Fenton treated leachates, comparing these

Table 3
List of papers with breast cancer cells.

Reference	Cell model	Endpoint	Assay	Treatment time	Landfill type/source	Leachate details
Coors et al., 2003	MVLN	Estrogenicity	Luciferase gene reporter assay	72 h	Municipal landfill (Germany)	Concentrated organic phase obtained with SPE[a] and acetone from 1. raw leachate 2. leachate after biological treatment and ultrafiltration 3. leachate after adsorption to activated carbon 4. effluent of reverse osmosis treatment
Talorete et al., 2008	MCF-7	Cytotoxicity Apoptosys/necrosis Estrogenicity Proteomics	LDH DNA fragmentation assay *E*-Screen Mass spectrometry	15–30 min 24–48 h 6 days 24 h	Solid waste landfill sites (Tunisia)	0.45 μm-filtered raw leachate
Suo et al., 2016	MCF-7	Viability Cell migration	MTT assay Cell scratch damage	72 h	–	1. Raw leachate 2. Phenol extract
Wang et al., 2016b	MCF-7 BUS	Estrogenicity	E-Screen	5 days	Municipal landfill (China)	1. Untreated membrane-concentrated leachate 2. UV-Fenton treated membrane concentrate
Hou et al., 2017	MCF-7	Estrogenicity	E-Screen	48 h	Landfill (China)	1. Untreated membrane-concentrated leachate 2. UV-Fenton treated membrane concentrate
Hong et al., 2017	MCF-7	Estrogenicity	E-Screen	48 h	Landfill (China)	1. Raw membrane concentrate 2. UV-Fenton treated membrane concentrate 3. Fenton treated membrane concentrate 4. Activated carbon treated membrane concentrate

[a] Solid Phase Extraction.

effects with those induced by estrogen simulation solutions enriched with different phthalic acid esters (PAEs). These compounds are considered responsible for the estrogenicity of wastewater. Compared to the usual protocol, cells were exposed only for 48 h (0–30% v/v). Leachate treated with UV-Fenton had more estrogenic effect than the untreated leachate. This might be explained by the presence of process intermediates of phthalic acid esters in the UV-Fenton treated leachate, that has higher estrogenicity than parental compounds as demonstrated by the study of estrogen simulation solutions with the different PAEs.

MCF-7 cells are also used as *in vitro* models for basal toxicity, with protocols different from the *E*-screen. Suo et al. (2016) found that interior micro-electrolysis (IME) and Fenton oxidation-coagulation (FOC) processes drastically reduced the proliferative effects of leachate on MCF-7 cells. They used the MTT assay and cell scratch damage to study the effects of leachate phenol extracts and the cells exposed to treated leachate had less proliferation and slower migration rate than the cells exposed to raw leachate. They also proposed the combination of proliferation and cell scratch damage assays as promising bioanalytical tools to detect phenols at concentrations lower than 10^{-15} g/L.

Several other *in vitro* models are available for the measurement of estrogenic activity and some of them are derived from breast cancer cells such as the MLVN, MELN, MELP and T47D-KBluc cell lines. In these models, cells are transfected with an estrogen response element-luciferase promoter reporter gene construct (Demirpence et al., 1993; Gagne et al., 1994; Balaguer et al., 2001; Alvarez et al., 2013; Freyberger and Schmuck, 2005; Buteau-Lozano et al., 2008; Schilirò et al., 2013).

MLVN cells are a bioluminescent MCF-7-derived cell line in which the luciferase gene is under the control of the estrogen-responsive element (Pons et al., 1990; Demirpence et al., 1993). These cells were used to detect the estrogenic activity of leachate treated with different purification processes and the efficiency of these treatments to remove estrogenicity (Coors et al., 2003). In these studies, extract from raw leachate showed dose-dependent estrogenic activity in the MLVN assay. Leachate treatment processes were able to significantly reduced the estrogenicity of leachate: with the combination of aerobic biological treatment, ultrafiltration, and adsorption on activated carbon there was no significant estrogenic activity and the removal efficiency was more than 98.5% expressed as estradiol equivalent (EEQ). The reverse osmosis achieved slightly lower efficiency (97.2% of the EEQ).

6. Studies with other cell models

Lymphocytes, HepG2 and MCF-7 cells are the most widely used *in vitro* models for the risk assessment of landfill leachates, but other cell lines and assays too have been used less frequently (Table 4).

According to risk assessment guidelines (USEPA, 1989; Health Canada, 2010, 2012, 2017), the skin could be a secondary target organ for landfill leachate, considering skin deposition and dermal absorption of soil particles contaminated with the wastewater, but also after accidental exposure in an uncontrolled dumpsite.

Normal human dermal fibroblasts (NHDF) and Me45 human melanoma cell lines were used to investigate the genotoxicity of leachate from a solid waste landfill in Poland (Widziewicz et al., 2012), comparing the effects of raw and treated leachates from a biological anaerobic, anoxic aerobic (A2/O) purification system. Skin cells were exposed for 15 min to three leachate concentration (0.1, 1 and 10%) collected before and after the A2O treatment. Comet assay was selected as the genotoxicity method and the Olive tail moment as comet parameter. Genotoxicity was higher in cells exposed to untreated leachates than in those obtained with treated leachate, as demonstrated by the fact that the median OTM scored in raw leachate-treated cells were significantly lower than in cells exposed to treated wastewater. The genotoxic investigation was repeated also after removing the treatments and leaving cells in normal medium for recovery. Cells treated with raw leachate had slower DNA repair and more residual unrepaired damages even after 180 min of recovery than the cells exposed to biologically treated leachate. This suggests that raw leachate caused irreversible damage, probably affecting DNA damage repair enzymes and mechanisms.

Human immortalized keratinocyte HaCaT cells were selected as a skin model for the risk evaluation of leachates from regulated and unregulated municipal landfills in Lebanon (Khalil et al., 2018). Cells were treated with raw leachates for 2 and 24 h (0.3 to 80% v/v), assessing the toxicity with MTS and Comet assays. Concentration-dependent changes in cell viability were found in cells exposed to increasing

Table 4
Papers in which other *in vitro* models were used.

Reference	Cell model	Tissue	Endpoint	Assay	Treatment Time	Landfill type/Source	Leachate details
Talorete et al., 2008	CHO	Ovary (hamster)	Stress response	HSP 47 assay	3 h	Solid waste landfill sites (Tunisia)	0.45 μm-filtered raw leachate
Widziewicz et al., 2012	Me45 Fibroblast	Skin	Genotoxicity	Comet assay	15 min	Solid waste landfill (Poland)	1. Raw leachate 2. Biological A2/O-treated leachate
Alabi et al., 2013	NIH/3 T3	Embryo (mouse)	Proliferation	MTT assay	24 h	Soil from dumpsite of electrical and electronic market (Nigeria)	Artificial leachate following ASTM D3987-85 (1992)
			Mitochondrial membrane potential	JC-1 assay			
			Oxidative stress	DCFH-DA[a]			
			Cell cycle	Flow cytometry of PI[b]-stained cells			
			Apoptosys	Acridine orange/ethidium bromide dual staining			
Alimba et al., 2016	HOS and jurkat	Bones and Blood respectively	Viability	MTT assay	24 h	4 landfills (3 India, 1 Nigeria)	Simulated landfill soil leachate
			Cell morphology	Morphology changes			
			Genotoxicity	Comet assay			
Morozesk et al., 2016	CHO-k1	Ovary (hamster)	Proliferation	MTT assay	12 h (1 cell cycle)	Active landfill (Brazil)	1. Raw leachate 2. Electrocoagulate leachate
			Viability	Trypan blue exclusion assay			
			Genotoxicity	CBMN assay			
Khalil et al., 2018	HaCaT	Skin	Proliferation	MTS assay	2–24 h	Regulated and unregulated municipal dumpsites (5 sites) (Lebanon)	Raw leachate
			Genotoxicity	Comet assay			

[a] Dichloro-dihydro-fluorescein diacetate.
[b] Propidium Iodide.

amounts of leachate, with significant among samples from different dumpsites. The mutagenic potential of wastewaters was assessed by the comet assay, evaluating the tail moment index: most of the leachates induced significant DNA damage comparable to that with the positive control. Genotoxic results were consistent with the effects measured with in MTS assay.

To complete the overview of human cell lines used for the risk assessment of leachate, Alimba et al. (2016) investigated the toxicity of simulated leachate obtained from soil samples collected in Nigerian and Indian landfills using human osteosarcoma HOS and lymphoma Jurkat cell lines. MTT and comet assay protocols were used to measure the cytotoxicity and genotoxicity of the wastewaters (0 to 100% v/v). The same evaluations were also done on the HepG2 cell line and the results have already been described in Section 3. Leachates induced both cytotoxic and genotoxic effects on the different models, but toxicological rankings of the samples were comparable. Jurkat cells were the most sensitive for both MTT and comet assays while HOS cells were the least sensitive.

The toxicity of landfill leachate was also evaluated in non-human mammal cells (Talorete et al., 2008; Alabi et al., 2013; Morozesk et al., 2016).

The CHO cell line and its subclone CHO-K1 are epithelial cell lines derived from the ovary of the *Cricetulus griseus* (Chinese hamster) (Puck et al., 1958; Lewis et al., 2013). They are validated models for the *in vitro* mammalian cell micronucleus test (OECD, 2016b).

CHO cells were transfected with murine heat shock protein 47 (HSP47) promoter gene ligated upstream of the β-galactosidase coding sequence to evaluate the stress-response induced by two landfills in Tunisia (Talorete et al., 2008). HSP 47 is a collagen-specific stress glycoprotein involved in the synthesis and assembly of collagens as a molecular chaperone. HSP 47 is synthethyzed in response to stresses such as toxic agents, radiation and heat shock (Dafforn et al., 2001; Talorete et al., 2008; Ben Fredj et al., 2010; Etteieb et al., 2016). The study showed that leachates induced stress responses after 3 h of incubation, measured as increased β-galactosidase activity in treated cells.

Morozesk et al. (2016) assessed the toxicity removal efficiency of electrocoagulation (EC) leachate treatment. CHO-K1 cells were exposed to leachate before and after the purification process and the effects on cell proliferation, viability and genotoxicity were measured. Five milliliters of leachate samples were directly added to the medium (15 mL). EC reduced the cytotoxicity of leachate but was not efficient in genotoxicity removal, as shown by the increased numbers of chromosomal and nuclear alterations such as nucleoplasmic bridges, buds and micronuclei in cells exposed to treated leachate. The researchers hypothesized that the genotoxic potential in treated leachate could be due to byproducts produced during the EC process.

Finally, Alabi et al. (2013) investigated the noxious effects on the NIH/3 T3mouse fibroblast cell line induced of some artificial leachates obtained from soils collected in a dumpsite of electronic waste in Nigeria. Cell cycle analysis, cytotoxicity, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) generation assays were done on exposed cells (leachate concentration ranging from 0 to 70% v/v). *E*-waste simulated leachates induced concentration-dependent negative effects on cell viability and mitochondrial health, detected by MTT and JC-1 assays. Cells exposed to wastewater at concentrations higher than 10% v/v had higher levels of oxidative stress measured by the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) protocol. Cell cycle analysis revealed that leachate can damage DNA, increasing the cell population in the Sub/G1 phase associated with apoptotic events. Induction of apoptotic DNA fragmentation was confirmed by acridine orange/ethidium bromide dual staining of cells exposed to the lowest concentration of leachate, while necrosis was found after treatment with higher concentrations (20 and 40%). This evidence supports the hypothesis that the apoptotic pathway might be responsible for the cytogenotoxicity induced by e-waste.

7. Conclusions

Landfill leachate is a highly toxic by-product of waste disposal in dumpsites. This wastewater can contaminate soils and groundwater not only in the proximity of the landfill but also at some distance, causing environmental and human health problems. The hazard of this matrix increases when landfills are not controlled, are illegal dumpsites, or do not have adequate treatment and purification systems for leachate. Following regulatory approaches, the risk assessment of leachate is based on physicochemical characterization without considering its inner toxicity. With the spread of rapid, sensitive and reliable methods, several studies have been published in the last few years focusing on the use bioassays in leachate risk assessment. The most common methods are based on small model organisms such as bacteria, algae and aquatic micro-invertebrates but also more complex organisms such as mussels, fish and plants are used too. The use of *in vitro* models for assessing the hazards associated with leachate exposure is more recent, but since 2003 there has been growing interest in this field. We analysed more than 20 papers published from 2003 to 2018, highlighting the usefulness and adequacy of *in vitro* models for assessing the hazard associated with exposure to leachate, particularly as an integrative and supporting tool for chemical-based risk assessment. Selected cell models reflect possible human organs that could be affected by the xenobiotics after accidental exposure to leachate (*e.g.* liver cells) or are used to measure specific mechanisms of actions (such as breast cancer cells for estrogenicity or lymphocytes for genotoxicity). Leachate was generally toxic, mutagenic, genotoxic and estrogenic in the reviewed papers. These effects can be measured even in cells treated with low concentrations, confirming the severe hazard of this wastewater for human health.

The most important limitation of the cell-based approach is that the results cannot be directly used in human risk assessment due to the absence of detoxification, defence or repair mechanisms acting *in vivo* in whole organisms (Ghosh et al., 2017). On the other hand, these human-related systems are definitely more helpful than chemistry in defining the overall bioactivity and potential hazards, especially when applied on environmental matrices because they react to the additive, synergistic or antagonistic effects that could arise in complex mixtures. Moreover, still focusing on multi-component mixtures, the *in vitro* models offer the most rapid and economical source of data linkable to potential effects on human health if compared to animal models. The technological innovations introduced in recent years has led increasingly complex cellular systems and more performing and efficient instruments than the ones described in this review for the evaluation of the most relevant endpoints: for example the use of probes and dyes such as MTS or WST-8 allows to measure cell proliferation faster and more accurately than the MTT while the use of an advanced flow cytometry platform could allow the evaluation of different endpoints on the same cell sample, especially for the studies focusing on genotoxicity and cell cycle alteration.

In addition, alongside the use in human risk assessment, the published studies indicate that cell-based bioassays are efficient for evaluating the performance of state-of-the-art detoxification and purification treatments. These studies, in fact, show how the processes are often able to reduce not only the chemical load of pollutants but also the leachate toxicity by removing the most dangerous compounds or leading to the formation of less toxic process residues.

As for the traditional bioassays with organisms, a complete investigation of the biological activity of leachate requires a battery of cell tests in order to consider organ-related toxicity due to the different metabolic activity of each cell or to the presence of specific molecular pathways. The use of different cellular models, through an integrated approach with different models and endpoints interconnected to create a more complex system could serve as an innovative strategy for future studies not only on landfill leachate but also on many other environmental matrices or complex mixtures.

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References

Alabi, O.A., Bakare, A.A., Filippin-Monteiro, F.B., Sierra, J.A., Creczynski-Pasa, T.B., 2013. Electronic waste leachate-mediated DNA fragmentation and cell death by apoptosis in mouse fibroblast (NIH/3T3) cell line. Ecotoxicol. Environ. Saf. 94, 87–93.

Alimba, C.G., Gandhi, D., Sivanesan, S., Bhanarkar, M.D., Naoghare, P.K., Bakare, A.A., 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.

Alvarez, D.A., Shappell, N.W., Billey, L.O., Bermudez, D.S., Wilson, V.S., Kolpin, D.W., Perkins, S.D., Evans, N., Foreman, W.T., Gray, J.L., Shipitalo, M.J., Meyer, M.T., 2013. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res. 47, 3347–3363.

Amahdar, L., Anouar, A., Ababou, B., Verschaeve, L., Hilali, A., 2009. In vitro genotoxicity of Settat town landfill leachate, Morocco. Arh. Hig. Rada Toksikol. 60, 179–184.

American Society for Testing and Materials (ASTM), 1992. Standard Test Method for Shake Extraction of Solid Waste with Water, ASTM Designation: D3987-85. ASTM, West Conshohocken, PA, pp. 4.

Andersen, M.E., Krewski, D., 2009. Toxicity testing in the 21st century: bringing the vision to life. Toxicol. Sci. 107, 324–330.

Andrews, W.J., Masoner, J.R., Cozzarelli, I.M., 2011. Emerging contaminants at a closed and an operating landfill in Oklahoma. Ground Water Monit. Remidiat. 32, 120–130.

Avberšek, M., Žegura, B., Filipič, M., Heath, E., 2011. Integration of GC-MSD and ER-Calux® assay into a single protocol for determining steroid estrogens in environmental samples. Sci. Total Environ. 409, 5069–5075.

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.

Baderna, D., Colombo, A., Amodei, G., Cantù, S., Teoldi, F., Cambria, F., Rotella, G., Natolino, F., Lodi, M., Benfenati, E., 2013. Chemical-based risk assessment and in vitro models of human health effects induced by organic pollutants in soils from the Olona valley. Sci. Total Environ. 463–464, 790–801.

Baderna, D., Colombo, A., Romeo, M., Cambria, F., Teoldi, F., Lodi, M., Diomede, L., Benfenati, E., 2014. Soil quality in the Lomellina area using in vitro models and ecotoxicological assays. Environ. Res. 133, 220–231.

Bakare, A.A., Mosuro, A.A., Osibanjo, O., 2003. Landfill leachate-induced toxicity in mice. J. Environ. Biol. 24, 429–435.

Bakare, A.A., Pandey, A.K., Bajpayee, M., Bhargav, D., Chowdhuri, D.K., Singh, K.P., Murthy, R.C., Dhawan, A., 2007. DNA damage induced in human peripheral blood lymphocytes by industrial solid waste and municipal sludge leachates. Environ. Mol. Mutagen. 48, 30–37.

Balaguer, P., Boussioux, A.M., Demirpence, E., Nicolas, J.C., 2001. Reporter cell lines are useful tools for monitoring biological activity of nuclear receptor ligands. Luminescence 16, 153–158.

Ben Fredj, F., Irie, M., Han, J., Yamada, P., Limam, A., Ghrabi, A., Morio, T., Isoda, H., 2010. Stress response of heavy metal mixture present in wastewater and leachate on heat-shock protein 47-transfected cells. Environ. Toxicol. Chem. 29, 1637–1647.

Benfenati, E., Pierucci, P., Fanelli, R., Preiss, A., Godejohann, M., Astratov, M., Levsen, K., Barceló, D., 1999. Comparative studies of the leachate of an industrial landfill by gas chromatography–mass spectrometry, liquid chromatography–nuclear magnetic resonance and liquid chromatography–mass spectrometry. J. Chromatogr. A 831, 243–256.

Benfenati, E., Maggioni, S., Campagnola, G., Senese, V., Lodi, M., Testa, S., Schramm, K.W., 2007. A protocol to evaluate organic compounds present in a landfill. In: Velinni, A.A. (Ed.), Landfill Research Trends. Nova Science, pp. 141–166.

Bertanza, G., Pedrazzani, R., 2008. Presence of EDCs (endocrine disruptin compounds) in landfill leachate and municipal wastewaters. In: Pawlowska, M., Pawlowski, L. (Eds.), Management of Pollutant Emission from Landfills and Sludge. Taylor and Francis group, London.

Bertanza, G., Papa, M., Pedrazzani, R., Repice, C., Mazzoleni, G., Steimberg, N., Feretti, D., Ceretti, E., Zerbini, I., 2013. EDCs, estrogenicity and genotoxicity reduction in a mixed (domestic + textile) secondary effluent by means of ozonation: a full-scale experience. Sci. Total Environ. 458–460, 160–168.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28, 625–631.

Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26, 93–100.

Buteau-Lozano, H., Velasco, G., Cristofari, M., Balaguer, P., Perrot-Applanat, M., 2008. Xenoestrogens modulate vascular endothelial growth factor secretion in breast cancer cells through an estrogen receptor-dependent mechanism. J. Endocrinol. 196, 399–412.

Castell, J.V., Jover, R., Martínez-Jiménez, C.P., Gómez-Lechón, M.J., 2006. Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies. Expert Opin. Drug Metab. Toxicol. 2, 183–212.
Cheng, R., Zhao, L., Yin, P., 2017. Genotoxic effects of old landfill leachate on HepG2 cells after nitration/ultrafiltration/reverse osmosis membrane treatment process. J. Appl. Toxicol. 37, 1455–1463.
Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Jensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H.-J., Heron, G., 2001. Biogeochemistry of landfill leachate plumes. Appl. Geochem. 16 (7–8), 659–718.
Clarke, B.O., Anumol, T., Barlaz, M., Snyder, S.A., 2015. Investigating landfill leachate as a source of trace organic pollutants. Chemosphere 127, 269–275.
Cooper, E.R., McGrath, K.C.Y., Heather, A.K., 2013. In vitro androgen bioassays as a detection method for designer androgens. Sensors (Basel) 13, 2148–2163.
Coors, A., Jones, P.D., Giesy, J.P., Ratte, H.T., 2003. Removal of estrogenic activity from municipal waste landfill leachate assessed with a bioassay based on reporter gene expression. Environ. Sci. Technol. 37, 3430–3434.
Costa, P.M., Pinto, M., Vicente, A.M., Gonçalves, C., Rodrigo, A.P., Louro, H., Costa, M.H., Caeiro, S., Silva, M.J., 2014. An integrative assessment to determine the genotoxic hazard of estuarine sediments: combining cell and whole-organism responses. Front. Genet. 5.
Costantini, S., Di Bernardo, G., Cammarota, M., Castello, G., Colonna, G., 2013. Gene expression signature of human HepG2 cell line. Gene 518, 335–345.
Dafforn, T.R., Della, M., Miller, A.D., 2001. The molecular interactions of heat shock protein 47 (Hsp47) and their implications for collagen biosynthesis. J. Biol. Chem. 276, 49310–49319.
Davoli, E., Fattore, E., Paiano, V., Colombo, A., Palmiotto, M., Rossi, A.N., Il Grande, M., Fanelli, R., 2010. Waste management health risk assessment: a case study of a solid waste landfill in South Italy. Waste Manag. 30, 1608–1613.
Demirpence, E., Duchesne, M.J., Badia, E., Gagne, D., Pons, M., 1993. MVLN cells: a bioluminescent MCE-7-derived cell line to study the modulation of estrogenic activity. J. Steroid Biochem. Mol. Biol. 46, 355–364.
Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr. Rev. 30, 293–342.
Donato, M.T., Jover, R., Gómez-Lechón, M.J., 2013. Hepatic cell lines for drug hepatotoxicity testing: limitations and strategies to upgrade their metabolic competence by gene engineering. Curr. Drug Metab. 14, 946–968.
Donato, M.T., Tolosa, L., Gómez-Lechón, M.J., 2015. Culture and functional characterization of human hepatoma HepG2 cells. Methods Mol. Biol. 1250, 77–93.
Eckers, A., Reimann, K., Klotz, L.O., 2009. Nickel and copper ion-induced stress signaling in human hepatoma cells: analysis of phosphoinositide 30-kinase/Akt signaling. Biometals 22, 307–316.
Etteieb, S., Kawachi, A., Han, J., Elayni, F., Tarhouni, J., Isoda, H., 2016. Assessment of organic micropollutants occurrence in treated wastewater using heat shock protein 47 stress responses in Chinese hamster ovary cells and GC/MS-based non-target screening. Water Sci. Technol. 74, 2407–2416.
Farre, M., Barcelo, D., 2003. Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. Trends Anal. Chem. 22, 299–310.
Faust, F., Kassie, F., Knasmüller, S., Kevekordes, S., Mersch-Sundermann, V., 2004a. Use of primary blood cells for the assessment of exposure to occupational genotoxicants in human biomonitoring studies. In: Toxicology, Toxicology in the New Century, Opportunities and Challenges - Proceedings of the 5th Congress of Toxicology in Developing Countries. 198. pp. 341–350.
Faust, F., Kassie, F., Knasmüller, S., Boedecker, R.H., Mann, M., Mersch-Sundermann, V., 2004b. The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. Mutat. Res. Rev. Mutat. Res. 566, 209–229.
Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. Fundam. Mol. Mech. Mutagen. 455, 81–95.
Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat. Protoc. 2, 1084–1104.
Frazier, J.M., 1993. In vitro models for toxicological research and testing. Toxicol. Lett. 68 (1–2), 73–90.
Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. 681, 80–92.
Freyberger, A., Schmuck, G., 2005. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol. Lett. 155, 1–13.
Gagne, D., Balaguer, P., Demirpence, E., Chabret, C., Trousse, F., Nicolas, J.C., Pons, M., 1994. Stable luciferase transfected cells for studying steroid receptor biological activity. J. Biolumin. Chemilumin. 9, 201–209.
Gajski, G., Oreščanin, V., Garaj-Vrhovac, V., 2012. Chemical composition and genotoxicity assessment of sanitary landfill leachate from Rovinj, Croatia. Ecotoxicol. Environ. Saf. 78, 253–259.
Garaj-Vrhovac, V., Oreščanin, V., Gajski, G., Gerić, M., Ruk, D., Kollar, R., Radić Brkanac, S., Cvjetko, P., 2013. Toxicological characterization of the landfill leachate prior/after chemical and electrochemical treatment: a study on human and plant cells. Chemosphere 93, 939–945.
Ghosh, P., Das, M.T., Thakur, I.S., 2014. 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., Indu Thakur, I., 2015. Combined chemical and toxicological evaluation of leachate from municipal solid waste landfill sites of Delhi, India. Environ. Sci. Pollut. Res. 22.
Ghosh, P., Thakur, I.S., Kaushik, A., 2017. Bioassays for toxicological risk assessment of landfill leachate: a review. Ecotoxicol. Environ. Saf. 141, 259–270.
Glei, M., Schneider, T., Schlörmann, W., 2016. Comet assay: an essential tool in toxicological research. Arch. Toxicol. 90, 2315–2336.
Health Canada/Santè Canada, 2010. Federal Contaminated Site Risk Assessment in Canada, Part V: Guidance on Human Health Detailed Quantitative Risk Assessment for Chemicals. 978-1-100-17926-1.
Health Canada/Santè Canada, 2012. Federal Contaminated Site Risk Assessment in Canada, Part I: Guidance on Human Health Preliminary Quantitative Risk Assessment (PQRA), Version 2.0. 978-1-100-17671-0.
Health Canada/Santè Canada, 2017. Federal Contaminated Site Risk Assessment in Canada: Supplemental Guidance on Human Health Risk Assessment of Oral Bioavailability of Substances in Soil and Soil-Like Media. 978-0-660-09285-0.
Hong, M., Lu, G., Hou, C., She, S., Zhu, L., 2017. Advanced treatment of landfill leachate membrane concentrates: performance comparison, biosafety and toxic residue analysis. Water Sci. Technol. 76, 2949–2958. https://doi.org/10.2166/wst.2017.469.
Hou, C., Lu, G., Zhao, L., Yin, P., Zhu, L., 2017. Estrogenicity assessment of membrane concentrates from landfill leachate treated by the UV-Fenton process using a human breast carcinoma cell line. Chemosphere 180, 192–200.
Kamaruddin, M.A., Yusoff, M.S., Aziz, H.A., Hung, Y.T., 2015. Sustainable treatment of landfill leachate. Appl Water Sci 5, 113–126.
Khalil, C., Al Hageh, C., Korfali, S., Khnayzer, R.S., 2018. Municipal leachates health risks: chemical and cytotoxicity assessment from regulated and unregulated municipal dumpsites in Lebanon. Chemosphere 208, 1–13.
Kjeldsen, P., Barlaz, M.A., Rooker, A.P., Baun, A., Ledin, A., Christensen, T.H., 2002. Present and long-term composition of MSW landfill leachate: a review. Crit. Rev. Environ. Sci. Technol. 32, 297–336.
Knowles BB, Aden DP., 1983. Human hepatoma derived cell line, process for preparation thereof, and uses therefor. US Patent 4,393,133 dated Jul 12 1983.
Kumaravel, T.S., Jha, A.N., 2006. Reliable comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat. Res. 605, 7–16.
Kumari, M., Ghosh, P., Thakur, I.S., 2016. Landfill leachate treatment using bacto-algal co-culture: an integrated approach using chemical analyses and toxicological assessment. Ecotoxicol. Environ. Saf. 128, 44–51.
Lee, E., Oh, E., Lee, Joohyun, Sul, D., Lee, Juneyoung, 2004. Use of the tail moment of the lymphocytes to evaluate DNA damage in human biomonitoring studies. Toxicol. Sci. 81, 121–132.
Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H., Reitsema, T., 2014. Assessment of wastewater and recycled water quality: a comparison of lines of evidence from in vitro, in vivo and chemical analyses. Water Res. 50, 420–431.
Lewis, N.E., Liu, X., Li, Y., Nagarajan, H., Yerganian, G., O'Brien, E., Bordbar, A., Roth, A.M., Rosenbloom, J., Bian, C., Xie, M., Chen, W., Li, N., Baycin-Hizal, D., Latif, H., Forster, J., Betenbaugh, M.J., Famili, I., Xu, X., Wang, J., Palsson, B.O., 2013. Genomic landscapes of Chinese hamster ovary cell lines as revealed by the *Cricetulus griseus* draft genome. Nat. Biotechnol. 31, 759–765.
Li, G., Sang, N., Wang, Q., 2006. Oxidative damage induced in brains and livers of mice by landfill leachate. Ecotoxicol. Environ. Saf. 65, 134–139.
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.
Liu, Y.-Y., Lin, Y.-S., Yen, C.-H., Miaw, C.-L., Chen, T.-C., Wu, M.-C., Hsieh, C.-Y., 2018. Identification, contribution, and estrogenic activity of potential EDCs in a river receiving concentrated livestock effluent in Southern Taiwan. Sci. Total Environ. 636, 464–476.
Martins, M., Costa, P.M., 2015. The comet assay in environmental risk assessment of marine pollutants: applications, assets and handicaps of surveying genotoxicity in non-model organisms. Mutagenesis 30, 89–106.
Mersch-Sundermann, V., Knasmüller, S., Wu, X.-J., Darroudi, F., Kassie, F., 2004. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology 198, 329–340.
Mertl, J., Kirchnawy, C., Osorio, V., Grininger, A., Richter, A., Bergmair, J., Pyerin, M., Washüttl, M., Tacker, M., 2014. Characterization of Estrogen and Androgen Activity of Food Contact Materials by Different In Vitro Bioassays (YES, YAS, ERα and AR CALUX) and Chromatographic Analysis (GC-MS, HPLC-MS).
Møller, P., 2005. Genotoxicity of environmental agents assessed by the alkaline comet assay. Basic Clin. Pharmacol. Toxicol. 96 (Suppl. 1), 1–42.
Monneret, C., 2017. What is an endocrine disruptor? C. R. Biol. 340, 403–405 Endocrine disruptors/Les perturbateurs endocriniens.
Morgado, P.I., Jose, S., Wanke, R., Antunes, A.M.M., Cardoso, A.S., Jordao, L., 2017. Integration of cellular and molecular endpoints to assess the toxicity of polycyclic aromatic hydrocarbons in HepG2 cell line. Environ. Toxicol. Chem. 36, 3404–3414.
Morozesk, M., Bonomo, M.M., Rocha, L.D., Duarte, I.D., Zanezi, E.R.L., Jesus, H.C., Fernandes, M.N., Matsumoto, S.T., 2016. Landfill leachate sludge use as soil additive prior and after electrocoagulation treatment: a cytological assessment using CHO-k1 cells. Chemosphere 158, 66–71.
Mukherjee, S., Mukhopadhyay, S., Hashim, M.A., Gupta, B.S., 2015. Contemporary environmental issues of landfill leachate: assessment and remedies. Crit. Rev. Environ. Sci. Technol. 45 (5), 472–590.
OECD, 2016a. Test No. 474: mammalian erythrocyte micronucleus test. In: OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264264762-en.
OECD, 2016b. Test No. 487: in vitro mammalian cell micronucleus test. In: OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264264861-en.
Olive, P.L., Banáth, J.P., Durand, R.E., 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “comet” assay. Radiat. Res. 122, 86–94.
Omar, H., Rohani, S., 2015. Treatment of landfill waste, leachate and landfill gas: a

review. Front. Chem. Sci. Eng. 9 (1), 15–32.
Patlolla, A.K., Barnes, C., Hackett, D., Tchounwou, P.B., 2009. Potassium dichromate induced cytotoxicity, genotoxicity and oxidative stress in human liver carcinoma (HepG2) cells. Int. J. Environ. Res. Public Health 6, 643–653.
Pinto, M., Costa, P.M., Louro, H., Costa, M.H., Lavinha, J., Caeiro, S., Silva, M.J., 2014. Determining oxidative and non-oxidative genotoxic effects driven by estuarine sediment contaminants on a human hepatoma cell line. Sci. Total Environ. 478, 25–35.
Pons, M., Gagne, D., Nicolas, J.C., Mehtali, M., 1990. A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. BioTechniques 9 (4), 450–459.
Poteser, M., 2017. Cell-based in vitro models in environmental toxicology: a review. Biomonitoring 4, 11–26.
Puck, T.T., Cieciura, S.J., Robinson, A., 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J. Exp. Med. 108, 945–956.
Qi, C., Huang, J., Wang, B., Deng, S., Wang, Y., Yu, G., 2018. Contaminants of emerging concern in landfill leachate in China: A review. Emerg. Contam. https://doi.org/10.1016/j.emcon.2018.06.001. ISSN 2405-6650.
Ramirez, T., Strigun, A., Verlohner, A., Huener, H.-A., Peter, E., Herold, M., Bordag, N., Mellert, W., Walk, T., Spitzer, M., Jiang, X., Sperber, S., Hofmann, T., Hartung, T., Kamp, H., van Ravenzwaay, B., 2018. Prediction of liver toxicity and mode of action using metabolomics in vitro in HepG2 cells. Arch. Toxicol. 92, 893–906.
Renou, S., Givaudan, J.G., Poulain, S., Dirassouyan, F., Moulin, P., 2008. Landfill leachate treatment: review and opportunity. J. Hazard. Mater. 150, 468–493.
Schilirò, T., Porfido, A., Longo, A., Coluccia, S., Gilli, G., 2013. The E-screen test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues. Food Chem. Toxicol. 62, 82–90.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., van der Burg, B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 83, 136–148.
Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103 (Suppl. 7), 113–122.
Speit, G., Vasquez, M., Hartmann, A., 2009. The comet assay as an indicator test for germ cell genotoxicity. Mutat. Res. 681, 3–12.
Sunjog, K., Kolarević, S., Héberger, K., Gačić, Z., Knežević-Vukčević, J., Vuković-Gačić, B., Lenhardt, M., 2013. Comparison of comet assay parameters for estimation of genotoxicity by sum of ranking differences. Anal. Bioanal. Chem. 405, 4879–4885.
Suo, J., Zhao, L., Yin, P., Lu, G., Liu, P., 2016. Estrogen effect of MCF-7 cells on phenol extracts after IME-Fenton treatment of aged landfill leachate. Acta Sci. Circumst. 36 (3), 1121–1128.
Swati, Null, Ghosh, P., Thakur, I.S., 2017. An integrated approach to study the risk from landfill soil of Delhi: chemical analyses, in vitro assays and human risk assessment. Ecotoxicol. Environ. Saf. 143, 120–128.
Talorete, T., Limam, A., Kawano, M., Jenhani, A.B.R., Ghrabi, A., Isoda, H., 2008. Stress response of mammalian cells incubated with landfill leachate. Environ. Toxicol. Chem. 27, 1084–1092.
Tewari, A., Dhawan, A., Gupta, S.K., 2006. DNA damage in bone marrow and blood cells of mice exposed to municipal sludge leachates. Environ. Mol. Mutagen. 47, 271–276.
Thomas, D.J.L., Tyrrel, S.F., Smith, R., Farrow, S., 2009. Bioassays for the evaluation of landfill leachate toxicity. J. Toxicol. Environ. Health B Crit. Rev. 12, 83–105.
Torretta, V., Ferronato, N., Katsoyiannis, I.A., Tolkou, A.K., Airoldi, M., 2017. Novel and conventional technologies for landfill leachates treatment: a review. Sustainability 9 (1), 9. https://doi.org/10.3390/su9010009.
Toufexi, E., Tsarpali, V., Efthimiou, I., Vidali, M.-S., 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.
United States Environmental Protection Agency (USEPA), 1989. Risk Assessment Guidance for Superfund. Volume 1. EPA/540/1-89/002. Office of Emergency and Remedial Response. U.S. Environmental Protection Agency, Washington, D.C.
Ursini, C.L., Cavallo, D., Colombi, A., Giglio, M., Marinaccio, A., Iavicoli, S., 2006. Evaluation of early DNA damage in healthcare workers handling antineoplastic drugs. Int. Arch. Occup. Environ. Health 80, 134–140.
USEPA, 1992. Test Methods for Evaluating Solid Waste, Toxicity Characteristic Leaching Procedure (TCLP). Method 1311, SW-846. US EPA, Washington, DC.
USEPA, 2007. SW-846 Test Method 3500C: Organic Extraction and Sample Preparation. US EPA, Washington, DC.
USEPA, 2018. Advancing sustainable materials management: 2015 fact sheet. Assessing trends in material generation, recycling, composting, combustion with energy recovery and landfilling in the United States. Available at. https://www.epa.gov/sites/production/files/2018-07/documents/2015_smm_msw_factsheet_07242018_fnl_508_002.pdf, Accessed date: 8 December 2018.
Valentin-Severin, I., Le Hegarat, L., Lhuguenot, J.C., Le Bon, A.M., Chagnon, M.C., 2003. Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays. Mutat. Res. 536, 79–90.
Vidic, T., Lah, B., Berden-Zrimec, M., Marinsek-Logar, R., 2009. Bioassays for evaluating the water-extractable genotoxic and toxic potential of soils polluted by metal smelters. Environ. Toxicol. 24 (5), 472–483.
Villalobos, M., Olea, N., Brotons, J.A., Olea-Serrano, M.F., Ruiz De Almodovar, J.M., Pedraza, V., 1995. The E-screen assay: a comparison of different MCF7 cell stocks. Environ. Health Perspect. 103, 844–850.
Wang, G., Lu, G., Yin, P., Zhao, L., Jimmy Yu, Q., 2016a. 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, G., Lu, G., Zhao, J., Yin, P., Zhao, L., 2016b. Evaluation of toxicity and estrogenicity of the landfill-concentrated leachate during advanced oxidation treatment: chemical analyses and bioanalytical tools. Environ. Sci. Pollut. Res. Int. 23, 16015–16024.
WHO/UNEP, 2013. State of the science of endocrine disrupting chemicals 2012. In: United Nations Environment Programme and the World Health Organization, (ISBN: 978-92-807-3274-0 (UNEP) and 978 92 4 150503 1 (WHO)).
Widziewicz, K., Kalka, J., Skonieczna, M., Madej, P., 2012. The comet assay for the evaluation of genotoxic potential of landfill leachate. Sci. World J. 2012, 1–8.
Wilkening, S., Stahl, F., Bader, A., 2003. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metab. Dispos. 31, 1035–1042.
Yao, P., 2017. Perspectives on technology for landfill leachate treatment. Arab. J. Chem. 10, S2567–S2574.
Yi, X., Tran, N.H., Yin, T., He, Y., Gin, K.Y.-H., 2017. Removal of selected PPCPs, EDCs, and antibiotic resistance genes in landfill leachate by a full-scale constructed wetlands system. Water Res. 121, 46–60.
Zare Sakhvidi, M.J., Hajaghazadeh, M., Mostaghaci, M., Mehrparvar, A.H., Zare Sakhvidi, F., Naghshineh, E., 2016. Applicability of the comet assay in evaluation of DNA damage in healthcare providers' working with antineoplastic drugs: a systematic review and meta-analysis. Int. J. Occup. Environ. Health 22, 52–67.
Zhou, X., Xiang, L., Wu, F., Peng, X., Xie, H., Wang, J., Yang, K., Lu, W., Wu, Z., 2013. Comparison of extracts and toxicities of organic compounds in drinking water concentrated by single and composite XAD resins. J. Water Health 11, 692–699.