Food Chemistry 205 (2016) 81–88

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Biodetection of potential genotoxic pollutants entering the human food chain through ashes used in livestock diets

Laura Sanchez-Vicente [a], Elisa Herraez [a,c], Oscar Briz [a,c], Rogelio Nogales [b], Eduarda Molina-Alcaide [b], Jose J.G. Marin [a,c,*]

[a] Laboratory of Experimental Hepatology and Drug Targeting (HEVEFARM), IBSAL, University of Salamanca, Salamanca, Spain
[b] Estación Experimental del Zaidín (CSIC), Granada, Spain
[c] Centre for the Study of Liver and Gastrointestinal Diseases (CIBERehd), Carlos III National Institute of Health, Madrid, Spain

ARTICLE INFO

Article history:
Received 17 March 2015
Received in revised form 21 February 2016
Accepted 28 February 2016
Available online 3 March 2016

Chemical compounds:
Cadmium dichloride (PubChem CID: 24947)
cis-Diamminedichloroplatinum(II) (PubChem CID: 2767)
Lead nitrate (PubChem CID: 24924)
Potassium dichromate (PubChem CID: 24502)

Keywords:
By-products
Cancer
Carcinogenesis
Cisplatin
DNA damage
Olive cake
RecA
Recycled wood
SOS response

ABSTRACT

Ash derived from energy generation is used as a source of minerals in livestock feeds. The microbial biosensor recApr–Luc2 was built to detect genotoxic hazard in recycled ash. *Escherichia coli* SOS gene (*recA*, *lexA*, *dinI* and *umuC*) expression in response to cisplatin-induced DNA damage led to the selection of the *recA* promoter. The biosensor required functional RecA expression to respond to genotoxic heavy metals (Cr > Cd ≈ Pb), and polluted ash induced a strong recApr–Luc2 response. In human liver and intestinal cells, heavy metals induced acute toxicity (Cr > Cd > Pb) at concentrations sufficient to activate recApr–Luc2. Cytostatic effects, including genotoxicity, were cell- and metal-dependent, apart from Cr. In agreement with the recApr–Luc2 bioassay, Cr had the strongest effect in all cells. In conclusion, recApr–Luc2 could be useful for evaluating the genotoxic risk of pollutants present in ash that might be concentrated in animal products and, thus, entering the human food chain.

1. Introduction

Although the recycling of by-products is a desirable goal of modern society, safety controls must be implemented to prevent the carry-over of noxious pollutants. These include genotoxic compounds that might be present in ash from renewable energy production that uses residual biomass. The source of this hazard is diverse; for instance, heavy metals are present in painted wood, fertilizers and pesticides, which together with soil pollution might contaminate agricultural by-products. These, together with wood from industrial waste or recycled materials, are commonly a source of ash used in the preparation of livestock feeds. This was the case for ash used in the present study, which was produced by combustion of wood and olive cake, a by-product from olive oil extraction. It is, therefore, important to detect genotoxic compounds that, otherwise, might be incorporated in the human food chain. Similar risks, due to the accumulation of heavy metals in raw cows' milk (Arianejad, Alizadeh, Bahrami, & Arefhoseini, 2015), hens' eggs (Bautista, Puschner, & Poppenga, 2014) and in the flesh of fish (El-Sadaawy, El-Said, & Sallam, 2013) have been reported previously.

The main threats to human health from heavy metals, such as chromium (Cr), cadmium (Cd) and lead (Pb), are associated with exposure through contact with products containing these metals or contaminated soil, air, water and food as a result of industrial processes or environmental pollution (Wasi, Tabrez, & Ahmad,

* Corresponding author at: Department of Physiology and Pharmacology, Campus Miguel de Unamuno ED-231, 37007 Salamanca, Spain.
E-mail address: jjgmarin@usal.es (J.J.G. Marin).

http://dx.doi.org/10.1016/j.foodchem.2016.02.160
0308-8146/

2013; Chowdhury & Chandra, 1987; Glombitza & Reichel, 2014; Jarup, 2003; Nriagu & Pacyna, 1988; Tchounwou, Yedjou, Patlolla, & Sutton, 2012). The use of contaminated by-products in livestock feeds might lead to accumulation in dairy products and meat (Adetunji, Famakin, & Chen, 2014; Rychen, Jurjanz, Fournier, Toussaint, & Feidt, 2014). Exposure to heavy metals is an important problem because they have been classified as carcinogens by regulatory agencies including the WHO International Agency for Research on Cancer (IARC, 1990, 1993, 2006).

More specifically, the toxicity of heavy metals is due to interference with functional groups in biologically important molecules, such as proteins and nucleic acids, but the exact mechanisms of action for each metal differ and, in most cases, are not fully understood (Beyersmann & Hartwig, 2008). For example, DNA damage leading to carcinogenic changes might arise via several mechanisms, including chemical modification of nucleotides, strand breakage, crosslinking, micronucleus formation and aneuploidy induction. Many assays *in vitro* and *in vivo* have been used to evaluate genotoxic hazards by measuring their capacity to induce DNA damage (Kang, Kwon, Lee, & Seo, 2013). These include: (i) Ames test, which assesses mutagenic capacity based on whether the effects can be reversed (Ames, Durston, Yamasaki, & Lee, 1973), (ii) Comet assay, which detects DNA fragmentation, specifically single- and double strand breaks (Ostling & Johanson, 1984), (iii) tests based on the bacterial SOS response including the *umu*-test (Oda, Nakamura, Oki, Kato, & Shinagawa, 1985) and the SOS-Chromotest (Quillardet, Huisman, D'Ari, & Hofnung, 1982), which are two of the most commonly used; and (iv) assays based on the observation of structural DNA alterations, such as the chromosome aberration test, micronucleus test, and sister chromatid exchange assay (Perry & Evans, 1975).

In the present study, an *in vitro* method was developed to evaluate genotoxicity based on SOS response in bacteria exposed to DNA-damaging agents. This method was employed to measure genotoxicity risks resulting from heavy metal pollutants in ash used as a supplement in livestock feeds. The bacterial DNA repair system is activated by expression of SOS genes that form part of a regulon (Michel, 2005). Two proteins, inducer RecA and repressor LexA, have key roles in regulation of the SOS response (Little, 1982). During normal growth, LexA forms a dimer that binds to a specific sequence in the SOS box of the SOS genes promoters, suppressing the expression of these genes (Thliveris, Little, & Mount, 1991). In response to DNA damage, RecA binds to single-stranded DNA (ssDNA) to form a nucleoprotein filament (Zhang, Pigli, & Rice, 2010). Once bound to DNA, the RecA protein is activated and acts as a co-protease, triggering the self-cleaving activity of LexA (Horii et al., 1981). The cleaved repressor then dissociates from DNA, allowing the transcription of SOS genes (Michel, 2005).

Although it would be useful to develop a functional test that could detect any toxic effect related to heavy metals in a single assay (as opposed to individual assays for the different metals), this is not possible. Instead, in the present study, we developed a method able to detect pollutants with genotoxic activity. To validate this approach, we selected four model sources of ash from renewable energy production using residual wood and olive cake biomass.

2. Materials and methods

2.1. Chemicals and ashes

Ampicillin, $CdCl_2$, cisplatin or cis-diamminedichloroplatinum(II), dimethyl sulfoxide (DMSO), MEM, sodium bicarbonate, sodium pyruvate and thiazolyl blue tetrazolium bromide were obtained from Sigma-Aldrich Quimica (Madrid, Spain). The antibiotic–antimycotic solution and fetal calf serum were obtained from Gibco (Thermo Fisher Scientific, Madrid, Spain). Agar, tryptone and yeast extract were obtained from Pronadisa (Laboratorios Conda, Madrid, Spain). $K_2Cr_2O_7$ and $Pb(NO_3)_2$ were obtained from Panreac Quimica (Barcelona, Spain). Ashes were obtained from combustion of wet (OK1: Baena, Cordoba, Spain) and dry (OK2: La Loma, Jaen, Spain) olive cake, and from recycled/residual wood (RW1: Tradema, Linares, Jaen, Spain; RW2: Ence, Huelva, Spain), which were provided by the Estación Experimental del Zaidín (Granada, Spain). Ashes (0.5 g/ml) and heavy metal salts (up to 3 mM depending on the metal and the experimental setting) were prepared freshly in ultrapure H_2O and filtered (0.22 μm-pore size filter) immediately prior to use. There was no need to adjust the pH after the addition of heavy metal salts or aqueous extracts of ashes to bacterial or mammalian cell cultures.

2.2. Chemical composition of ashes

The components of ashes, except for Cr, were analyzed by inductively coupled plasma mass spectrometry after samples were digested in concentrated aqua regia, as previously reported (McGrath & Cunliffe, 1985). In brief, 0.5 g of ash samples were treated with $HCl:HNO_3$ (4:1 v/v) at room temperature for 24 h and heated at 150 °C until they were dry. Cr content was determined by flameless atomic absorption spectrophotometry (Z-8100 Polarized Zeeman; Hitachi, Tokyo, Japan), after the samples were treated with 37% HCl at room temperature for 24 h and then heated at 150 °C until they were dry. Then, to precipitate Fe, and prevent interference with the Cr measurements, the samples were dissolved in 20 mM HCl at 60 °C for 4 h and mixed with NH_4Cl (20 g/l). All samples were filtered (Whatman no. 42 filters) and diluted to 100 ml with deionized, distilled water prior to analysis. Blank solutions were also prepared without addition of ash.

2.3. Bacteria strains, growth conditions and toxicity tests

DH5α and BL21(DE3) strains of *Escherichia coli* were donated by the Department of Microbiology and Genetics, University of Salamanca (Spain). Recombinant *E. coli* cells were selected on Luria Agar (LA) plates containing 100 μg/ml of ampicillin and used to inoculate Luria Broth (LB) media supplemented with the same antibiotic. Non-genetically modified *E. coli* cells were grown without this antibiotic. Bacterial cultures in LB medium were incubated with shaking (220 rpm) at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.3 (Spectrophotometer Hitachi U-2000 with 1 cm path-length cuvettes). Bacterial cultures were then considered to be in the early exponential phase of growth and were placed in vented test tubes for the different assays.

To perform the toxicity assays, 5 ml of *E. coli* BL21(DE3) culture at OD_{600} = 0.3 were placed in test tubes containing 10–50 μl of different heavy-metal salt solutions in ultrapure H_2O at the desired final concentration (0–2 mM). Bacterial growth was analyzed using OD_{600}.

2.4. Cloning of the expression vector pcDNA6.2–recApr–Luc2

A 110-bp sequence located in the 5′-flanking region of the *recA* gene, which was previously identified as the proximal *recA* promoter (recApr) (Horii, Ogawa, & Ogawa, 1980), was selected for cloning using genomic DNA from *E. coli* BL21(DE3). The recApr sequence was amplified by PCR using high-fidelity AccuPrime *Pfx* DNA polymerase (Invitrogen; Thermo Fisher Scientific, Madrid, Spain) and specific oligonucleotide primers (Supplementary Table 1), to which *att*B sites were added to obtain cDNA adapted for Multisite Gateway® cloning (Invitrogen). The pGL4.10[Luc2] plasmid (Promega, Madrid, Spain) was used as a template with

appropriate primers (Supplementary Table 1) to amplify the open reading frame of the firefly luciferase gene (Luc2) by PCR. The PCR products were recombined with the corresponding pDONR vectors (Invitrogen) to generate entry plasmids, which were further recombined with a promoter-less destination vector (pcDNA6.2pl-DEST) (Invitrogen) to obtain the expression vector p cDNA6.2–recApr–Luc2 (Supplementary Fig. 1). The exact nucleotide sequences of constructs were confirmed using gel-electrophoresis-based sequencing.

2.5. SOS gene expression analysis

E. coli strain BL21(DE3) samples (0.5 ml) were diluted to OD_{600} = 0.09 and mixed with 1 ml RNAprotect Bacteria Reagent (Qiagen, Izasa Scientific, Barcelona, Spain). After 10 min, the cells were lysed using lysozyme (Qiagen) and proteinase K (Invitrogen), and the total RNA isolated according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). cDNA was synthesized by retrotranscription (RT) and used for quantitative PCR (QPCR), as previously described (Briz et al., 2003). Primer oligonucleotide sequences and thermal cycling conditions for measuring the mRNA abundance of *dinI*, *lexA*, *recA* and *umuC* as well as 16S rRNA (used as a normalizer) are shown in Supplementary Table 2. Specific and non-specific PCR products were detected as previously reported (Herraez et al., 2012).

2.6. Use of the microbial biosensor in genotoxicity assays

E. coli strains DH5α and BL21(DE3) were transformed with pc DNA6.2–recApr–Luc2 plasmid by heat shock. The selected colonies were grown up to OD_{600} = 0.3, and 5 ml cultures were placed in vented test tubes containing 10–50 µl of cisplatin, $Pb(NO_3)_2$, $CdCl_2$ or $K_2Cr_2O_7$ at the desired concentrations or 20 µl-aqueous extracts from different samples of ash. Cisplatin was dissolved in dimethyl sulfoxide (DMSO) and immediately diluted with ultrapure H_2O (⩽0.2% DMSO in final solutions), whereas the heavy-metal salts were dissolved directly in ultrapure H_2O. All solutions were prepared freshly for each experiment to prevent chemical modifications. To detect SOS response activation by DNA-damaging compounds, the following conditions were optimized previously: (i) exposure temperature and time; (ii) method of cell lysis; (iii) volume of the microbial biosensor sample to be processed before measuring the bioluminescence; and (iv) incubation time of the microbial biosensor sample with the luciferase reagent. The results (data not shown) led to the following method: bacteria were incubated with the agent or sample extract by shaking at 37 °C for 2.5 h. Aliquots (100 µl) were collected on ice, lysed by sonication in an ultrasonic bath, and diluted (1:1) in ultrapure H_2O. A 50-µl aliquot of lysed bacteria was mixed (1:1) with reagents from the Steady-Glo Luciferase Assay System (Promega). After incubation for 10 min, luciferase activity was measured in a LAS-4000 image reader (FujiFilm, TDI, Madrid, Spain).

2.7. Toxicity assays in human cell lines

Human cell lines (liver – HepG2 and Alexander or PLC/PRF/5 and colon – Caco-2 and LS174T) were obtained through LGC Standards S.L.U. (Barcelona, Spain), and used as previously reported (Blazquez et al., 2013; Herraez et al., 2012). Cells were cultured with MEM supplemented with 1 mM sodium pyruvate, 26.2 mM sodium bicarbonate, 10% fetal calf serum and 1% antibiotic–antimycotic solution (10,000 U/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B) (Gibco, Thermo Fisher) in a humidified CO_2:air (5:95%) atmosphere at 37 °C. Cells were seeded in 96-well plates (Corning, Thermo Fisher Scientific) at different densities, depending on the cell type and experimental design (Supplementary Table 3). After 24 h, the cells were incubated in the presence of heavy metal salts dissolved in the culture medium. The MTT test (Sigma-Aldrich) was used to evaluate changes in cell viability both short- (6 h) and long-term (72 h).

2.8. Statistical analyses

The data are shown as the means ± SD. To calculate the statistical significance of the differences paired or unpaired *t*-tests or the Bonferroni method of multiple-range testing were used.

3. Results

3.1. Chemical composition of ashes

A comparative analytical study was carried out on several samples of ash to assess carcinogenic capacity. This included the analysis of some genotoxic heavy metals, such as Pb, Cd and Cr (Table 1). Based on the results, we selected four model samples from different origins that matched the aims of the present study. Cd was barely detected in any of the ashes. The concentration of Pb was lower in OK1 and RW2 than in OK2 and RW1. The concentrations of Cr were negligible in OK1 and low in RW2, but markedly higher in OK2 and RW1. Accordingly, the order of potential genotoxic risk, based on Pb/Cd/Cr content was RW1 > OK2 > RW2 > OK1. The concentrations of several other elements determined in the ashes also supported this order of metal pollution (Supplementary Table 4).

3.2. Analysis of Pb/Cd/Cr toxicity in bacteria

Because the effects of Pb, Cd and Cr on bacterial growth could artefactually reduce the number of cells in the microbial biosensor assay and lead to inaccurate results, the effect of these three heavy metals on growth of *E. coli* BL21(DE3) was analyzed. Although oxides are likely to be produced during the generation of ash, we determined the effects of salts, which are 10,000–100,000-fold more soluble in aqueous biological media. At least under the concentrations assayed here (up to 3 mM for 4 h), $Pb(NO_3)_2$ was not associated with a reduction in cell growth (Fig. 1A). $CdCl_2$ induced a marked reduction in cell viability, but only at concentrations greater than 1 mM (Fig. 1B). Exposure to $K_2Cr_2O_7$ resulted in a concentration-dependent decrease in cell growth that was statistically significant at concentrations greater than 0.5 mM (Fig. 1C). These results allowed us to establish the maximum non-cytotoxic concentrations for Pb, Cd and Cr to be used in subsequent assays for genotoxic activity (400–500 µM).

3.3. Validation of the microbial biosensor recApr–Luc2

In the recApr–Luc2 microbial biosensor, bacteria act as the recognition element and providing the optimal environment for

Table 1
Heavy metals content (mg/kg) in ashes from olive cake (OK) and recycled wood (RW) combustion for renewable energy production.

Ashes samples	Pb	Cd	Cr
OK1	4 ± 1	<0.2	1 ± 5
OK2	67 ± 3[a]	<0.2	126 ± 20[a]
RW1	432 ± 23[a]	<0.2	323 ± 25[a]
RW2	6 ± 1	<0.2	28 ± 18

The origin of the ashes was as follows: OK1: Baena, Cordoba, Spain; OK2: La Loma, Jaen, Spain; RW1: Tradema, Linares, Jaen, Spain; RW2: Ence, Huelva, Spain. Values represent the mean of three replicates ± SD.
[a] $p < 0.05$ as compared with OK1.

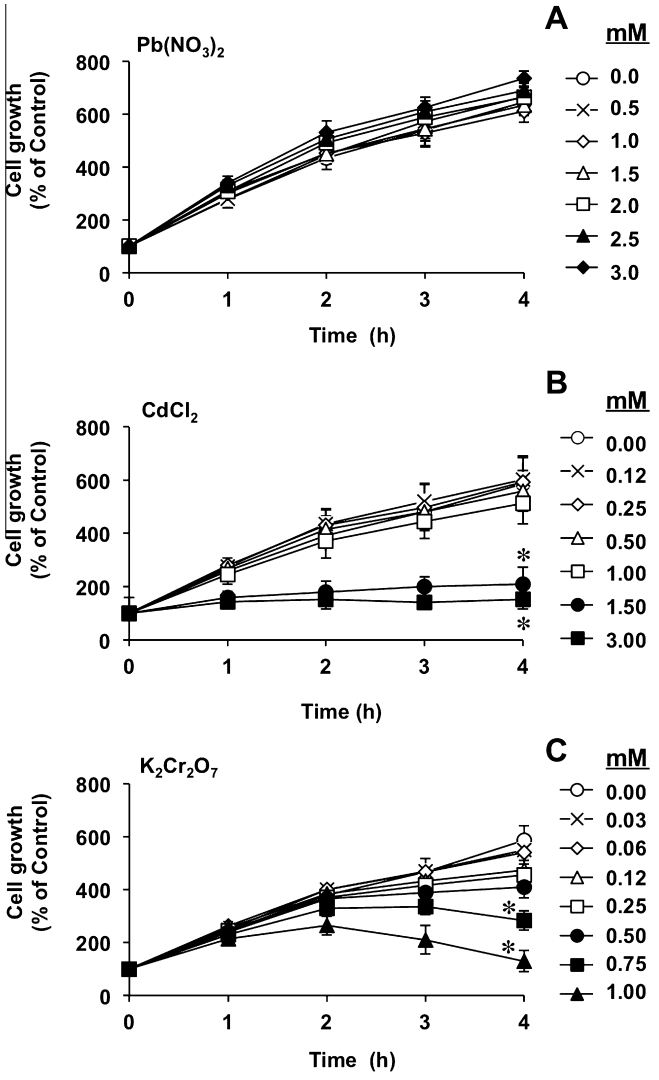

Fig. 1. Time- and concentration-dependent effects of $Pb(NO_3)_2$ (A), $CdCl_2$ (B) and $K_2Cr_2O_7$ (C) on BL21(DE3) bacterial growth. Aliquots of the bacterial culture were incubated with the indicated concentrations of each compound or vehicle alone with shaking at 37 °C. Culture growth was determined by optical density at 600 nm wavelength (OD_{600}) in samples collected at 1 h intervals. The results are expressed as a percentage of control (100%), which was OD_{600} (≈0.3) before adding any metal salt at time zero during the assay. The values are expressed as the means ± SD from nine samples per data point collected from three separate experiments. $^{*}p < 0.05$ compared with cells incubated with vehicle alone in a paired *t*-test.

endogenous RecA proteins to detect DNA damage. In response to DNA damage, the recApr–Luc2 construct, carried by the pcDNA6.2–recApr–Luc2 expression vector, acts as a transducer that converts activation of the SOS system into a measurable bioluminescent signal, which is proportional to the concentration of the genotoxic compound (Lozano et al., 2014). To determine the appropriateness of the bacterial strain for the recApr-based microbial biosensor and its performance, the effect of cisplatin at non-toxic concentrations on these bacteria (data not shown) was determined. Cisplatin was chosen as a positive control because this genotoxic agent has a well-established mechanism of action (Eastman, 1985) and is able to induce the SOS response (Lantzsch & Gebel, 1997).

To confirm the activation of the SOS response, the abundance of mRNA for four different SOS genes (*recA*, *lexA*, *dinI* and *umuC*) was measured in bacteria grown in the presence or absence of cisplatin (Fig. 2A). A cisplatin-induced up-regulation of *recA* (x2) and *lexA* (x1.5) was observed, whereas the abundance of *dinI* and *umuC* mRNA was not significantly ($p > 0.05$) affected by cisplatin.

When the exponential-phase BL21(DE3) and DH5α cultures containing the recApr–Luc2 microbial biosensor were incubated with cisplatin or vehicle alone (2.5 h), the luciferase activity due to recApr–Luc2 activation was measured (Fig. 2B). We observed that cisplatin enhanced (+286%) recApr–Luc2 activity in BL21 (DE3) but not in DH5α cells.

3.4. Genotoxicity bioassays

When the response of the recApr–Luc2 microbial biosensor to heavy metals was measured, neither $Pb(NO_3)_2$ nor $CdCl_2$ were able to induce an increase in luciferase activity. Values for luminescence found after exposing the bacteria to different concentrations of both compounds were similar to those found in untreated bacteria (Fig. 3A and B). In contrast, $K_2Cr_2O_7$ activated recApr–Luc2 in a concentration-dependent manner (Fig. 3C). The correlation between intensity of emitted light and $K_2Cr_2O_7$ concentration was linear ($y = 0.42x + 110.5$; $R^2 = 0.994$) (Fig. 3D). In separate assays, the direct effect of these heavy metals on the luciferase enzyme, which might have interfered with the bioluminescent measurements, was ruled out (data not shown).

The microbial biosensor recApr–Luc2 was exposed to aqueous extracts from RW1, RW2, OK1 and OK2 (Fig. 4). A significant increase in recApr–Luc2 activity was observed after exposure to extracts from RW1 and OK2. The activation of recApr–Luc2 was more marked in the case of RW1, in which the Cr content was highest (Table 1). The luminescence was not significantly ($p > 0.05$) enhanced using extracts from OK1 and RW2.

3.5. Cytotoxicity of Pb/Cd/Cr in liver and intestinal cells

To investigate the relationship between the results obtained, using the bacterial biosensor, with the toxic effects of Pb, Cd and Cr on mammalian cells at concentrations able to induce DNA damage (and hence activate the SOS system), concentration-dependent studies of cell viability were carried out using four different human cell lines (Supplementary Table 3). Two different experimental approaches were used: (i) acute toxicity due to rapid impairment of cell homeostasis after short-term interaction with the toxic compound was determined by incubating the cells with Pb, Cd or Cr for 6 h; and (ii) the cytostatic effect, which includes DNA damage and leads to impaired proliferation and enhanced cell death, and is proportional to the reduction in cell viability after incubation for 72 h. In cells treated with Pb, the concentration required to reduce cell viability by 50% (lethal concentration 50; LC_{50}) was above 1 mM for both acute toxicity and the cytostatic effect, except for Alexander cells, which were moderately more sensitive in both tests than the rest of the cells. Thus, statistically significant ($p < 0.05$) reduction in cell viability at lower concentrations was found (Fig. 5A and B).

The toxic effect of Cd was stronger than that of Pb. The Cd LC_{50} for acute toxicity was in the range 100–300 μM (Fig. 5C) whereas the cytostatic response was markedly different, depending on cell types, with Alexander cells ($LC_{50} = 1.5$ μM) being the most sensitive and LS174T ($LC_{50} = 11.5$ μM) the most resistant cell types (Fig. 5D).

The response to Cr acute toxicity was diverse, varying from a moderate reduction in cell viability (<20%) at concentrations as high as 1 mM in LS174T to greater sensitivity in Alexander cells (LC_{50} 3 μM, Fig. 5D). In contrast, the cytostatic response to Cr was similar and marked in all cell lines, with LC_{50} values approximately 1.5 μM.

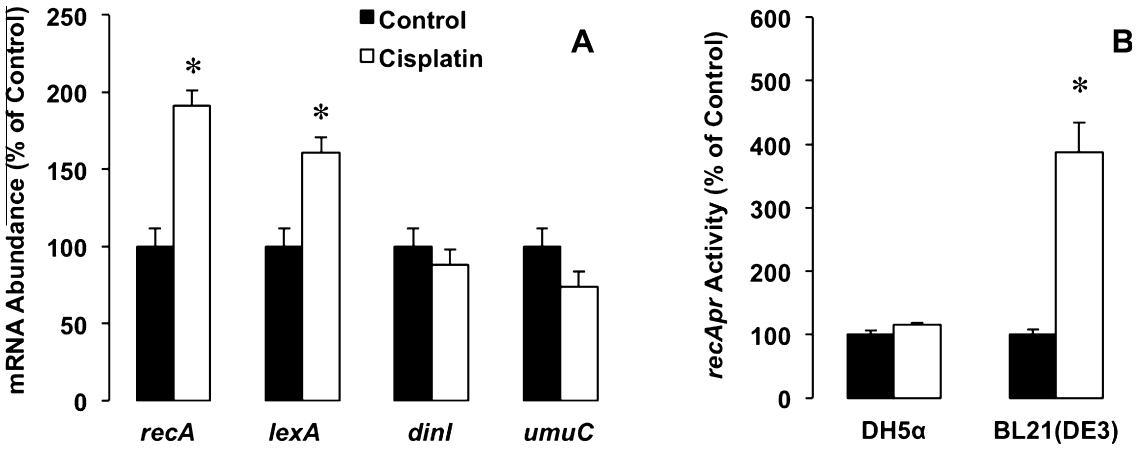

Fig. 2. (A) Effect of cisplatin-induced DNA damage on the expression of SOS genes in BL21(DE3) cells. Changes in mRNA abundance in response to bacterial incubation with 50 μM cisplatin or vehicle alone (Control) for 2.5 h with shaking at 37 °C were measured by RT-QPCR. (B) Effect of cisplatin-induced DNA damage on recApr activity in the microbial biosensor recApr–Luc2 using the DH5α or BL21(DE3) bacterial strains in response to incubation with 25 μM cisplatin or vehicle alone (Control) for 2.5 h with shaking at 37 °C. The values are expressed as the means ± SD from nine samples per data point collected from three separate cultures. $^{*}p < 0.05$ compared to controls in an unpaired *t*-test.

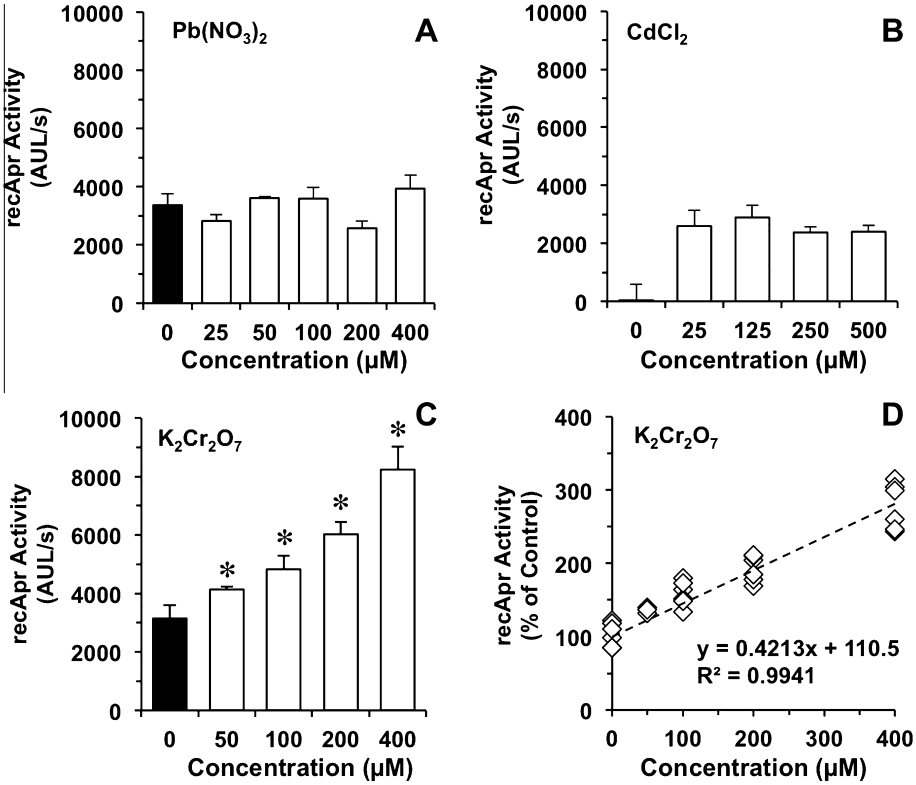

Fig. 3. Effect of $Pb(NO_3)_2$ (A), $CdCl_2$ (B) and $K_2Cr_2O_7$ (C and D) on the recApr–Luc2 bacterial biosensor. Recombinant BL21(DE3) recApr–Luc2 cultures at $OD_{600} = 0.3$ were incubated in the absence (Control) or presence of increasing concentrations of each compound for 2.5 h with shaking at 37 °C before luciferase activity was measured. Values are expressed as the means ± SD from nine samples per data point collected from three separate cultures. $^{*}p < 0.05$ compared to controls by the paired *t*-test. AUL, arbitrary units of light.

4. Discussion

Ash produced by the combustion of residual and recycled biomass can be exploited as a mineral source for livestock feed preparation. However, the presence of heavy metals might limit the usefulness of such residues due to the risk of human exposure via the food chain. After pyrolytic and combustion processes, these genotoxic agents remain within the solid end products (Kim et al., 2012; Nzihou & Stanmore, 2013). Thus, robust tests to detect these hazards are needed.

Different SOS-based microbial biosensors have been developed for the detection and quantification of genotoxicity. Genetically

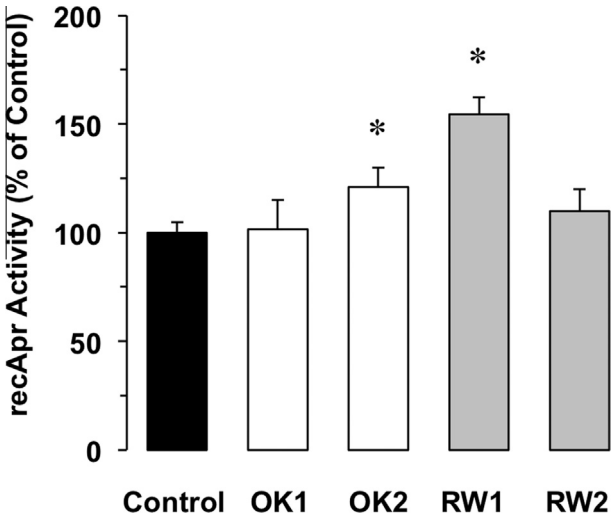

Fig. 4. Activation of the SOS system by aqueous extracts from ashes obtained from the combustion of olive cake (OK) or recycled wood (RW) from the following origins: OK1: Baena, Cordoba, Spain; OK2: La Loma, Jaen, Spain; RW1: Tradema, Linares, Jaen, Spain; and RW2: Ence, Huelva, Spain. A 20 μl aliquot of either water (Control) or one of the aqueous extracts (0.5 g/ml) of ashes was added to 5 ml recombinant BL21(DE3) recApr–Luc2 cultures at $OD_{600} = 0.3$. Incubation was carried out for 2.5 h with shaking at 37 °C before luciferase activity was measured. The values are expressed as the means ± SD from nine samples per data point collected from three separate cultures. $^{*}p < 0.05$ compared to controls according to the Bonferroni method of multiple-range testing.

modified bacteria with SOS gene promoter–reporter gene fusions that generate a measurable dose-dependent signal, which must be proportional to the genotoxic potential of the compounds tested, have been used previously (Biran et al., 2010). In response to DNA damage, the promoter is activated and triggers expression of the reporter gene responsible for production of the measurable signal. In the present study, a microbial biosensor based on the ability of RecA to recognize DNA damage was used to determine the genotoxic potential of heavy metals, such as Pb, Cd and Cr, which may be present in ashes obtained from residual/recycled wood and olive cake biomass combustion. This biosensor was developed to evaluate the safety of using these residues as a source of mineral complements in the preparation of livestock feeds.

Their greater resistance to toxicity induced by exposure to heavy metals compared with mammalian cells supported the choice of bacteria for the sensing elements in the recApr–Luc2 biosensor. This permitted the analysis of a wide range of heavy metal concentrations. In the transducer element *recApr* regulates expression of the gene encoding firefly luciferase, which can be expressed in *E. coli* despite being a eukaryotic gene, as previously reported (Shin, 2010). This exploited as a sensitive alternative to other frequently used reporter genes, such as *lacZ*, the *luxCDABE* cassette and *GFP* (Sorensen, Burmolle, & Hansen, 2006).

Selection of the *recA* promoter to build the recApr–Luc2 microbial biosensor was based on results from determination of cisplatin-induced changes in the expression of four SOS genes (*lexA*, *dinI*, *recA* and *umuC*) activated after DNA damage. Both *lexA* and *dinI* are among the most rapidly de-repressed genes in the SOS response. The expression of *recA* occurs later, but during the early phase of the SOS response, whereas *umuC* is expressed at the later stages of SOS induction (Janion, 2008). The results obtained using the *E. coli* strain BL21(DE3) incubated with cisplatin, used here as a model compound (Eastman, 1985), revealed that *recA*, followed by *lexA*, was up-regulated most. *recA* and *lexA* encode the inducer and repressor of the system, respectively. For the development of microbial biosensors, other authors have preferred to use different promoters, such as those of the *sfiA* and *umuC* genes, which are both expressed in the later phases of the SOS response (Biran et al., 2010). To check that the recApr–Luc2 microbial biosensor required functional RecA expression, as part of the SOS response (Radman, 1975), the bacterial strains DH5α and BL21(DE3), which possess or lack inactivating mutations in the *recA* gene, respectively, were genetically modified with the pcDNA6.2–recApr–Luc2 plasmid and compared. The DH5α strain was unable to up-regulate SOS genes in response to cisplatin, meaning the recApr–Luc2 microbial biosensor was not activated. In contrast, a marked activation of the recApr–Luc2 microbial biosensor was observed in BL21 (DE3) cells.

Although Pb, Cd and Cr are potential carcinogenic agents (Tchounwou et al., 2012), our results indicated they induce different responses in genotoxicity tests, suggesting their mechanisms of action might also be different. Previous studies have demonstrated that Pb and Cd do not elicit direct DNA damage but, instead, cause other deleterious events, such as DNA–protein crosslinking and sister-chromatid exchange (Lin, Lee, Chen, & Lin-Shiau, 1994; Misra, Smith, & Waalkes, 1998). Cr(VI) is reduced to Cr(III) in the cytoplasm, which unlike Cr(VI) can damage DNA directly through the formation of Cr-DNA adducts (Katz & Salem, 1993; Zhitkovich, 2005). Accordingly, our results suggest the recApr–Luc2 microbial biosensor was able to discriminate genotoxic agents, depending on their capacity to cause direct DNA damage. Our results are consistent with those obtained in studies carried out with cell lines derived from rat liver, in which Cr but not Cd was shown to up-regulate genes involved in DNA repair via the nucleotide excision repair (NER) mechanism. This is probably because Cr is able to induce formation of adducts and DNA strand breaks (Misra et al., 1998; Permenter, Lewis, & Jackson, 2011). Other studies using the SOS-Chromotest have reported that, apart from Cr and cisplatin, inorganic metal-based compounds have limited genotoxicity (Lantzsch & Gebel, 1997). Studies using the Ames test have found that Cr has a clear mutagenic capacity (Petrilli & De Flora, 1977), but the results of this test were not definitive for Pb and Cd (De Flora, Camoirano, Zanacchi, & Bennicelli, 1984; Maslat & Haas, 1989).

When the recApr–Luc2 microbial biosensor was used to determine genotoxic activity of ash samples their capacity to activate the biosensor matched the degree of pollution with the heavy metal in question; this was particularly true for Cr. Although the amount of Fe in the ash samples was high, and iron has been associated with DNA damage, the levels of this metal were similar in OK2 and RW2 despite their different ability for SOS response activation. Although the presence of other components contributing to the genotoxic effects cannot be ruled out, this should be considered a strength of the test, rather than a weakness, as the test detected genotoxicity regardless of whether causal agents had been identified.

In summary, at the concentrations assayed, the recApr–Luc2 microbial biosensor detected heavy metals induced cytostatic effects (Cr > Cd > Pb), including genotoxicity, *in vitro*. The test was able to detect genotoxic risk in ashes such as RW1, which contain high levels of DNA-damaging contaminants.

5. Conclusions

In conclusion, the recApr–Luc2 microbial biosensor could be used to evaluate the risk of DNA damage caused by pollutants that might be added to livestock feeds. This biosensor would eliminate the need for individual analysis of different potential genotoxic

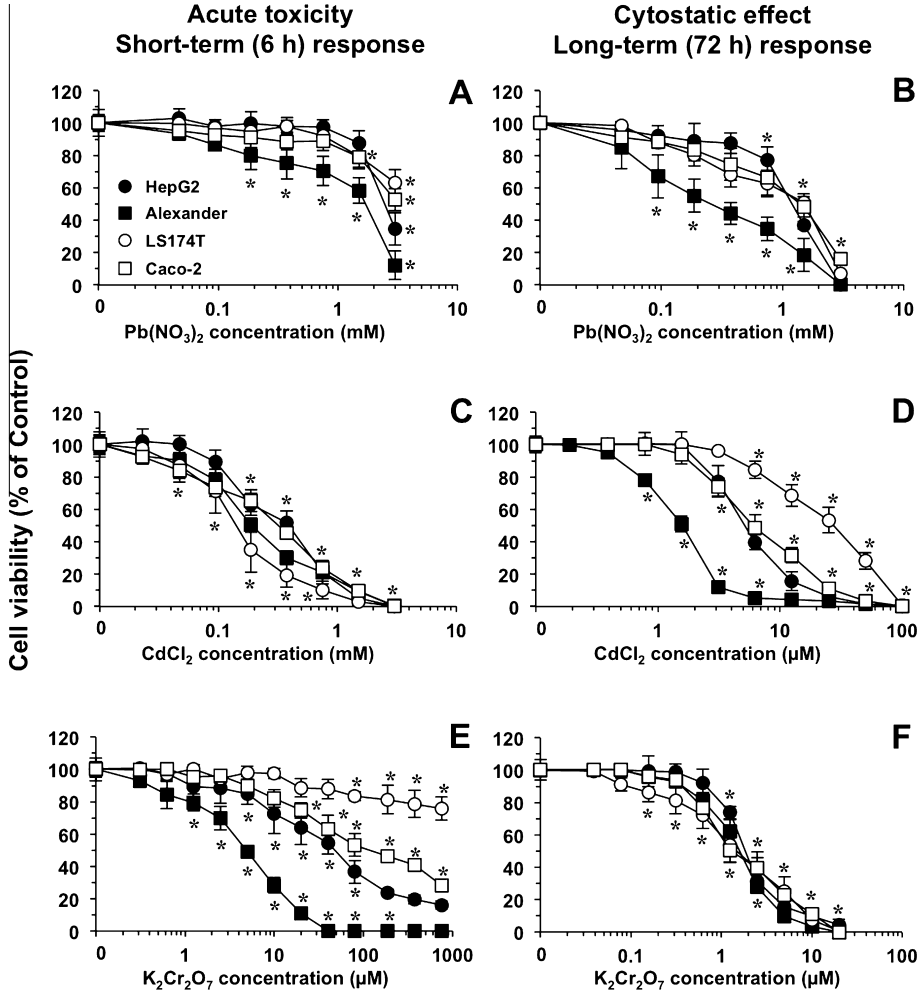

Fig. 5. Toxicity induced by $Pb(NO_3)_2$ (A and B), $CdCl_2$ (C and D) and $K_2Cr_2O_7$ (E and F) in human cell lines derived from liver (HepG2 and Alexander cells) and intestine (LS174T and Caco-2 cells), as determined by the MTT test. Cells were incubated with increasing concentrations of each compound for 6 h (acute toxicity) and 72 h (cytostatic effect). The values are expressed as the means ± SD from nine wells per data point collected from three separate experiments. $^{*}p < 0.05$ compared with untreated cells (Control) in a paired *t*-test.

agents, which might be concentrated in animal products, such as milk and meat, and could therefore enter the human food chain.

Financial support

This study was supported by the Spanish "Ministerio de Ciencia e Innovación" (SAF2013-40620-R), the "Junta de Castilla y León" (SA015U13 and BIO/SA52/15), the Excellence Programme of the "Junta de Andalucía" (P12-AGR-587), the "Fundación Mutua Madrileña" (Call 2015), and the Fundación Samuel Solórzano Barruso (FS/10-2014). The group is member of the Network for Cooperative Research on Membrane Transport Proteins (REIT) and CIBERehd. L.S.V. was supported by a PhD grant from the "Fondo Social Europeo/Junta de Castilla y León."

Conflict of interest statement

None.

Acknowledgments

The authors thank N. Skinner and Elsevier Language Editing Services for the revision of the English spelling, grammar and style of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.160.

References

Adetunji, V. O., Famakin, I. O., & Chen, J. (2014). Lead and cadmium levels in cattle muscle and edible tissues collected from a slaughter slab in Nigeria. *Food Additives & Contaminants: Part B: Surveillance, 7*(2), 79–83.

Ames, B. N., Durston, W. E., Yamasaki, E., & Lee, F. D. (1973). Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proceedings of the National Academy of Sciences of the United States of America, 70*(8), 2281–2285.

Arianejad, M., Alizadeh, M., Bahrami, A., & Arefhoseini, S. R. (2015). Levels of some heavy metals in raw cow's milk from selected milk production sites in Iran: Is there any health concern? *Health Promotion Perspectives, 5*(3), 176–182.

Bautista, A. C., Puschner, B., & Poppenga, R. H. (2014). Lead exposure from backyard chicken eggs: A public health risk? *Journal of Medical Toxicology, 10*(3), 311–315.

Beyersmann, D., & Hartwig, A. (2008). Carcinogenic metal compounds: Recent insight into molecular and cellular mechanisms. *Archives of Toxicology, 82*(8), 493–512.

Biran, A., Yagur-Kroll, S., Pedahzur, R., Buchinger, S., Reifferscheid, G., Ben-Yoav, H., ... Shacham-Diamand, Y. (2010). Bacterial genotoxicity bioreporters. *Microbial Biotechnology, 3*(4), 412–427.

Blazquez, A. G., Fernandez-Dolon, M., Sanchez-Vicente, L., Maestre, A. D., Gomez-San Miguel, A. B., Alvarez, M., ... Romero, M. R. (2013). Novel artemisinin

derivatives with potential usefulness against liver/colon cancer and viral hepatitis. *Bioorganic & Medicinal Chemistry, 21*(14), 4432–4441.
Briz, O., Macias, R. I., Vallejo, M., Silva, A., Serrano, M. A., & Marin, J. J. (2003). Usefulness of liposomes loaded with cytostatic bile acid derivatives to circumvent chemotherapy resistance of enterohepatic tumors. *Molecular Pharmacology, 63*(3), 742–750.
Chowdhury, B. A., & Chandra, R. K. (1987). Biological and health implications of toxic heavy metal and essential trace element interactions. *Progress in Food & Nutrition Science, 11*(1), 55–113.
De Flora, S., Camoirano, A., Zanacchi, P., & Bennicelli, C. (1984). Mutagenicity testing with TA97 and TA102 of 30 DNA-damaging compounds, negative with other Salmonella strains. *Mutation Research, 134*(2–3), 159–165.
Eastman, A. (1985). Interstrand cross-links and sequence specificity in the reaction of cis-dichloro(ethylenediamine)platinum(II) with DNA. *Biochemistry, 24*(19), 5027–5032.
El-Sadaawy, M. M., El-Said, G. F., & Sallam, N. A. (2013). Bioavailability of heavy metals in fresh water Tilapia nilotica (Oreachromis niloticus Linnaeus, 1758): Potential risk to fishermen and consumers. *Journal of Environmental Science and Health. Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 48*(5), 402–409.
Glombitza, F., & Reichel, S. (2014). Metal-containing residues from industry and in the environment: Geobiotechnological urban mining. *Advances in Biochemical Engineering/Biotechnology, 141*, 49–107.
Herraez, E., Gonzalez-Sanchez, E., Vaquero, J., Romero, M. R., Serrano, M. A., Marin, J. J., & Briz, O. (2012). Cisplatin-induced chemoresistance in colon cancer cells involves FXR-dependent and FXR-independent up-regulation of ABC proteins. *Molecular Pharmaceutics, 9*(9), 2565–2576.
Horii, T., Ogawa, T., Nakatani, T., Hase, T., Matsubara, H., & Ogawa, H. (1981). Regulation of SOS functions: Purification of E. coli LexA protein and determination of its specific site cleaved by the RecA protein. *Cell, 27*(32), 515–522.
Horii, T., Ogawa, T., & Ogawa, H. (1980). Organization of the recA gene of Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America, 77*(1), 313–317.
International Agency for Research on Cancer (IARC) (1990). Chromium, nickel and welding. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 49*, 115–208.
International Agency for Research on Cancer (IARC) (1993). Meeting of the IARC working group on beryllium, cadmium, mercury and exposures in the glass manufacturing industry. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 58*, 148–178.
International Agency for Research on Cancer (IARC) (2006). Inorganic and organic lead compounds. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 87*, 183–249.
Janion, C. (2008). Inducible SOS response system of DNA repair and mutagenesis in Escherichia coli. *International Journal of Biological Sciences, 4*(6), 338–344.
Jarup, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin, 68*, 167–182.
Kang, S. H., Kwon, J. Y., Lee, J. K., & Seo, Y. R. (2013). Recent advances in in vivo genotoxicity testing: Prediction of carcinogenic potential using comet and micronucleus assay in animal models. *Journal of Cancer Prevention, 18*(4), 277–288.
Katz, S. A., & Salem, H. (1993). The toxicology of chromium with respect to its chemical speciation: A review. *Journal of Applied Toxicology, 13*(3), 217–224.
Kim, J. Y., Kim, T. S., Eom, I. Y., Kang, S. M., Cho, T. S., Choi, I. G., & Choi, J. W. (2012). Characterization of pyrolytic products obtained from fast pyrolysis of chromated copper arsenate (CCA)- and alkaline copper quaternary compounds (ACQ)-treated wood biomasses. *Journal of Hazardous Materials, 227–228*, 445–452.
Lantzsch, H., & Gebel, T. (1997). Genotoxicity of selected metal compounds in the SOS chromotest. *Mutation Research, 389*(2–3), 191–197.
Lin, R. H., Lee, C. H., Chen, W. K., & Lin-Shiau, S. Y. (1994). Studies on cytotoxic and genotoxic effects of cadmium nitrate and lead nitrate in Chinese hamster ovary cells. *Environmental and Molecular Mutagenesis, 23*(2), 143–149.
Little, J. W. (1982). Control of the SOS regulatory system by the level of RecA protease. *Biochimie, 64*(8–9), 585–589.
Lozano, E., Sanchez-Vicente, L., Monte, M. J., Herraez, E., Briz, O., Banales, J. M., ... Macias, R. I. (2014). Cocarcinogenic effects of intrahepatic bile acid accumulation in cholangiocarcinoma development. *Molecular Cancer Research, 12*(1), 91–100.
Maslat, A. O., & Haas, H. J. (1989). Mutagenic effects of lead (II) bromide. *Journal of Trace Elements and Electrolytes in Health and Disease, 3*(4), 187–191.
McGrath, S. P., & Cunliffe, C. H. (1985). A simplified method for the extraction of the metals Fe, Zn, Cu, Ni, Cd, Pb, Cr, Co and Mn from soils and sewage sludges. *Journal of the Science of Food and Agriculture, 34*, 794–798.
Michel, B. (2005). After 30 years of study, the bacterial SOS response still surprises us. *PLoS Biology, 3*(7), e255.
Misra, R. R., Smith, G. T., & Waalkes, M. P. (1998). Evaluation of the direct genotoxic potential of cadmium in four different rodent cell lines. *Toxicology, 126*(2), 103–114.
Nriagu, J. O., & Pacyna, J. M. (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature, 333*(6169), 134–139.
Nzihou, A., & Stanmore, B. (2013). The fate of heavy metals during combustion and gasification of contaminated biomass – A brief review. *Journal of Hazardous Materials, 256–257*, 56–66.
Oda, Y., Nakamura, S., Oki, I., Kato, T., & Shinagawa, H. (1985). Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutation Research, 147*(5), 219–229.
Ostling, O., & Johanson, K. J. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications, 123*(1), 291–298.
Permenter, M. G., Lewis, J. A., & Jackson, D. A. (2011). Exposure to nickel, chromium, or cadmium causes distinct changes in the gene expression patterns of a rat liver derived cell line. *PLoS ONE, 6*(11), e27730.
Perry, P., & Evans, H. J. (1975). Cytological detection of mutagen–carcinogen exposure by sister chromatid exchange. *Nature, 258*(5531), 121–125.
Petrilli, F. L., & De Flora, S. (1977). Toxicity and mutagenicity of hexavalent chromium on Salmonella typhimurium. *Applied and Environment Microbiology, 33*(4), 805–809.
Quillardet, P., Huisman, O., D'Ari, R., & Hofnung, M. (1982). SOS chromotest, a direct assay of induction of an SOS function in Escherichia coli K-12 to measure genotoxicity. *Proceedings of the National Academy of Sciences of the United States of America, 79*(19), 5971–5975.
Radman, M. (1975). SOS repair hypothesis: Phenomenology of an inducible DNA repair which is accompanied by mutagenesis. *Basic Life Sciences, 5A*, 355–367.
Rychen, G., Jurjanz, S., Fournier, A., Toussaint, H., & Feidt, C. (2014). Exposure of ruminants to persistent organic pollutants and potential of decontamination. *Environmental Science and Pollution Research International, 21*(10), 6440–6447.
Shin, H. J. (2010). Development of highly-sensitive microbial biosensors by mutation of the nahR regulatory gene. *Journal of Biotechnology, 150*(2), 246–250.
Sorensen, S. J., Burmolle, M., & Hansen, L. H. (2006). Making bio-sense of toxicity: New developments in whole-cell biosensors. *Current Opinion in Biotechnology, 17*(1), 11–16.
Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. *EXS, 101*, 133–164.
Thliveris, A. T., Little, J. W., & Mount, D. W. (1991). Repression of the E coli recA gene requires at least two LexA protein monomers. *Biochimie, 73*(4), 449–456.
Wasi, S., Tabrez, S., & Ahmad, M. (2013). Toxicological effects of major environmental pollutants: An overview. *Environmental Monitoring and Assessment, 185*(3), 2585–2593.
Zhang, A. P., Pigli, Y. Z., & Rice, P. A. (2010). Structure of the LexA-DNA complex and implications for SOS box measurement. *Nature, 466*(7308), 883–886.
Zhitkovich, A. (2005). Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chemical Research in Toxicology, 18*(1), 3–11.