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Comparative study on elemental composition and DNA damage in leaves of a weedy plant species, *Cassia occidentalis*, growing wild on weathered fly ash and soil

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Abstract Open dumping of fly ash in fly ash basins has significant adverse environmental impacts due to its elevated trace element content. In situ biomonitoring of genotoxicity is of practical value in realistic hazard identification of fly ash. Genotoxicity of openly disposed fly ash to natural plant populations inhabiting fly ash basins has not been investigated. DNA damage, and concentrations of As, Co, Cr, Cu and Ni in the leaves of natural populations of *Cassia occidentalis* growing at two contrasting sites—one having weathered fly ash (fly ash basin) and the other having soil (reference site) as plant growth substrates—were assessed. The foliar concentrations of As, Ni and Cr were two to eight fold higher in plants growing on fly ash as compared to the plants growing on soil, whereas foliar concentrations of Cu and Co were similar. We report, for the first time, based upon comet assay results, higher levels of DNA damage in leaf tissues of *Cassia occidentalis* growing wild on fly ash basin compared to *C. occidentalis* growing on soil. Correlation analysis between foliar DNA damage and foliar concentrations of trace elements suggests that DNA damage may perhaps be associated with foliar concentrations of As and Ni. Our observations suggest that (1) fly ash triggers genotoxic responses in plants growing naturally on fly ash basins; and (2) plant comet assay is useful for in situ biomonitoring of genotoxicity of fly ash.

Keywords Weathered fly ash · Genotoxicity · Plant comet assay · Trace elements · *Cassia occidentalis* · In situ biomonitoring

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Introduction

Electricity production by coal-fired thermal power plants leads to the generation of large volumes of fly ash, a coal combustion by-product. In India, nearly 70% of the total electrical power is generated by 82 utility coal-fired thermal power plants, which consume about 260 million tonnes of coal per annum and generate around 108 million tonnes of fly ash annually (MoEF 2005). The large volumes of fly ash generated is openly disposed on land because it is classified as a nonhazardous waste product. The most common disposal method of fly ash is in the form of fly ash slurry which is disposed in fly ash settling basins (Kuzmick et al. 2007). Disposal and accumulation of fly ash over time in fly ash basins has led to concerns of its environmental safety (Twardowska and Szczepanska 2002) as it is a highly contaminating substrate. Elevated levels of trace elements (metals and mettalloids), polycyclic aromatic hydrocarbons (PAH) and radionuclides have been reported in fly ash as compared to parent coal (Kljajic et al. 1996; Wright et al. 1998; Rubin 1999; Celik et al. 2007).

Disposal of fly ash in ash basins has impact on the resident biota (plants and animals) and neighboring ecosystems due to elevated concentrations of trace elements found in it (Carlson and Adriano 1993; Rowe et al. 2002). Sample and Suter II (2002) also highlighted the ecological risks posed to terrestrial wildlife associated with coal ash

disposal sites. Of the different contaminants found in fly ash, trace elements are considered serious environmental contaminants due to: (1) their mobilization and bioaccumulation in food chain via plants, (2) contamination of waters due to leaching, and (3) their toxicity to biota (Carlson and Adriano 1993; Adriano et al. 2002; Brake et al. 2004; Reash et al. 2006). The issue that fly ash is nonhazardous, but some of the trace elements present in it are toxic is yet to be reconciled (Rubin 1999).

It is, therefore, important to carry out realistic hazard identification of fly ash dumped in ash disposal areas with particular reference to metal and metalloid elements found in it. One of the critical aspect in hazard identification of waste products is their genotoxic potential to living organisms. Studies on experimental model organisms such as *Paramecium tetraurelia* and *Salmonella typhimurium* have shown that the fly ash is genotoxic and mutagenic, and that both organic and inorganic contaminants (trace elements) found in fly ash are postulated to be the mutagenic agents (Chrisp et al. 1978; Fisher et al. 1979; Smith-Sonneborn et al. 1981). Genotoxicity of fly ash to plants and animals under controlled experimental conditions has also been reported (McMurphy and Rayburn 1993; Kuzmick et al. 2007). The genotoxic potential of openly disposed fly ash to plant populations inhabiting fly ash basins has not been assessed, although it is of practical value in hazard identification.

Of the different biomarkers used in assessing the genotoxicity of environmental contaminants, DNA single strand breaks have been considered as a sensitive indicator of genotoxicity and an effective biomarker in environmental biomonitoring (Mitchelmore and Chipman 1998; Shugart 2000). Comet Assay has been extensively used in the detection of DNA strand breaks in animals (including humans) and plants exposed to different chemicals, pollutants and xenobiotics (Cotelle and Ferard 1999; Hartmann et al. 2003; Collins 2004; Gichner et al. 2006). To our knowledge, the genotoxic effects of fly ash on populations of plants inhabiting fly ash basin have not been investigated. Here we report elevated foliar concentrations of selected trace elements and increased amount of DNA damage in leaves of *Cassia occidentalis*, a naturalized alien weedy species, growing on a fly ash basin.

Materials and methods

Location and characteristics of sampling sites

The experimental design of the study involved selection of two contrasting sites with different plant growth substrates—one having weathered fly ash and the other having soil. The two sites selected for the study were (1) fly ash basin of Badarpur Thermal Power Station (BTPS) which was the experimental site, and (2) the woodland of Garhi Mandu Van (GMV) as the reference site. Both the sites are located on the floodplains of river Yamuna in Delhi, India (28°12′–28°53′N latitude and 76°50′–77°23′E longitude). The GMV is located in the upstream of the river and the BTPS is located 25 km away from the GMV site on the downstream of the river (Fig. 1). Since the predominant wind direction in Delhi is west and northwest, the aerial fallout from the power plants at the reference site is negligible. It may be noted that GMV site is away from major point and non point sources of pollution such as major roads and is relatively less polluted site as compared to other areas in the National Capital Region of Delhi. Delhi has semiarid climate with mean annual rainfall of 75 cm, and mean temperature ranging from 6 to 41°C (IMD 2008).

The fly ash basin of BTPS site is spread over an area of 358 hectares. Fly ash generated at BTPS from the combustion of bituminous grade F coal is transported hydraulically from the power plant to the fly ash basins in the form of slurry (1:12, fly ash:water ratio). In fly ash basins, the fly ash is sedimented out of the slurry and settles down in the basin, whereas the supernatant water flows out through the drainage system. When fly ash basins are filled with fly ash, plants such as *Cassia occidentalis* (Leguminoseae–Caesalpinoideae), *Parthenium hysterophorus*

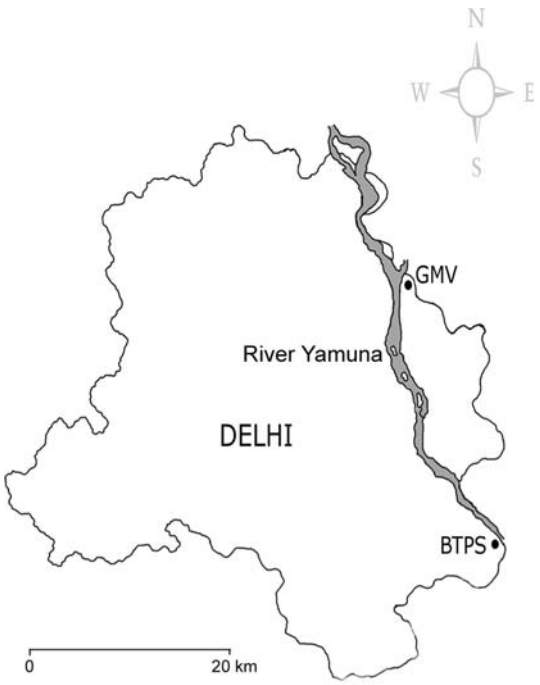


Fig. 1 A schematic map of Delhi showing the location of study sites: Garhi Mandu Van (GMV) and Badarpur Thermal Power Station (BTPS)

(Asteraceae), *Croton bonplandianum* (Euphorbiaceae), and *Cynodon dactylon* (Poaceae) establish and grow on the sedimented, old and weathered fly ash found along the edges and embankments of fly ash basins.

The GMV site spreads over an area of 350 acres. It is bounded by reserve forest on one side and river Yamuna on the other side. The woodland is composed of species such as *Pithecellobium dulce* (Leguminoseae–Mimosoideae), *Dalbergia sissoo* (Leguminoseae–Papilionoideae), *Pongamia pinnata* (Leguminoseae–Papilionoideae), *Bombax ceiba* (Bombacaceae) and *Alstonia scholaris* (Apocynaceae). Patches of *C. occidentalis* are found in the open areas amidst woodland trees, particularly along the tracks used by the local people. The soil of the GMV site is alluvial in origin and is predominantly composed of river borne sand.

Selection of plant species and collection of leaf samples

Cassia occidentalis was selected as the experimental species because it was abundant at both the contaminated (BTPS) and reference (GMV) sites and yields adequate amounts of leaves needed for trace element analysis and comet assay. It is a naturalized alien species of South American origin and is widely distributed throughout India and other tropical countries. It is annual to biennial suffruticose herb and grows to a height of about 1–1.5 m with even pinnate leaves having 4–6 pairs of foetid leaflets borne on a petiole bearing characteristic ovoid gland at the pulvinus base (Maheshwari 1963). The populations of *C. occidentalis* sampled from both the GMV and BTPS sites were morphologically similar, although ecological or physiological differentiation of the populations in response to differences in the growth substrates (soil in case of GMV and weathered fly ash in case of BTPS) of the two contrasting sites cannot be excluded. Since *C. occidentalis* is annual to biennial, the maximum duration of exposure of both the populations to weathered fly ash or soil varies from 1 to 2 years.

Natural populations of *C. occidentalis* of similar age and size group were selected from the BTPS and GMV sites. Plants, which had a height of 75–85 cm and basal stem diameter of 0.6–0.8 cm, were chosen. Leaves of the same size, texture and maturity, and free from visible sign of injury and disease were sampled to ensure uniformity in the leaves used for assay. The leaf material was harvested, separately, from ten randomly selected individuals from each site and stored in zip lock polybags at 4°C in an icebox and transported to laboratory for analysis. The leaf samples for trace element analysis and comet assay were collected from the same individual plant of *C. occidentalis*.

The sampling procedure is detailed below. Leaf samples of *C. occidentalis* were collected everyday consecutively over a period of 5 days from each site in such a way that the leaves sampled on any day (except Day 1) include leaf samples not only from additional randomly selected plants but also from any one plant which has already been sampled on the previous day. For example, on Day 1 leaf samples from three individual plants were collected and on the next day (Day 2), leaf samples were collected not only from two additional randomly selected plants but also from one plant sampled on Day 1 to serve as internal control to check the consistency in the performance of comet assay. This step was repeated till leaf samples from 10 randomly selected plants were collected. Staggered sampling schedule for collection of leaf samples was followed so that the comet assay could be carried out on the leaf samples collected on the same day of their collection so as to avoid effects of sample storage on the comet assay. Before processing the leaf samples, leaves were thoroughly washed in double distilled water to remove extraneous material. These washed leaf samples were blotted dry between layers of tissue paper and were used for further analysis. Some part of the leaf samples collected for comet assay were used for estimation of tissue concentrations of Cu, Cr, Co, Ni and As. The leaf samples used for estimation of trace elements were dried at 50°C for 24 h and stored till further analysis.

Collection of fly ash/soil samples

The total and phytoavailable concentrations of the trace elements estimated in the leaves of *Cassia occidentalis* were also estimated from fly ash and soil to find out the differences in the levels of trace elements between substrates. Fly ash/soil samples were collected, separately, from rhizospheres of randomly selected individual plants of *Cassia occidentalis* growing at BTPS and GMV sites. These samples were kept in sealed polythene bags and stored at room temperature till they were processed for analysis. About 10 g of air dried fly ash/soil was sieved using 2 mm mesh size sieve and the sieved sample was thoroughly ground and mixed before analysis.

Extraction procedure for fly ash/soil

Sequential extraction procedure outlined by Maiz et al. (1997, 2000) was followed to estimate mobile (exchangeable) and mobilizable (complexed, adsorbed and carbonate forms) concentrations of trace elements in fly ash and soils. The mobile fraction was extracted using $CaC1_2$ solution (0.01 M) and the mobilizable fraction was extracted using aqueous solution (pH 7.3) of 0.0005 M diethylene triamine pentaacetic acid (DTPA), 0.01 M $CaCl_2$, and 0.1 M Triethanolamine (TEA). Fly ash/soil (3 g) was used for the extraction of mobile and mobilizable fractions. The

extracts of both these fractions were, separately, used for the estimation of trace elements. Process blanks were also prepared and analyzed. The sum of the concentrations of a trace element in the mobile and mobilizable fractions was treated as phytoavailable concentration of that element (Kabata-Pendias 1993).

Digestion protocol

Dried and homogenized fly ash/soil (100 mg) was digested using 2 ml HNO_3 and 5 ml of 7:3 mixture of HCl:HF in "Parr" microwave acid digestion bomb (Lamothe et al. 1986). The digestion was carried out for 150 s at 900 W power setting in domestic microwave (Samsung CE 2977 M). The volume of the analyte was made up to 10 ml using analytical grade water.

The procedure followed by Bidar et al. (2007) was used for estimation of trace elements in leaf tissue. Dried and powdered leaf sample (200 mg) was digested with 3 ml HNO_3 in "Parr" microwave acid digestion bomb using a domestic microwave oven for 60 s at 900 W initially and continued for another 60 s at 600 W. The volume of the digests was made up to 6 ml using analytical grade water. Process blanks were prepared in the same way as that of test samples.

Estimation of trace elements

The digested analytes and the two sequential extracts (mobile and mobilizable fractions of fly ash/soil) were used for the estimation of trace elements. Cr, Co, Ni, Cu were analyzed by the flame atomic absorption technique, while As was estimated by the hydride generation technique using the Perkin Elmer Analyst-200 and MHS-15 system. Both the instruments were operated as per standard specifications mentioned in the instruments manual with slight modifications. Calibration standards for different elements were prepared from their respective stock standard solutions (1000 mg/l; E Merck, Germany) by carrying out appropriate dilutions. Precision and accuracy of analyses were ensured by replicate analyses of samples, process blanks and calibration standards. Standard Reference Material (SRM-1633 b) of the National Institute of Standard and Technology was also analyzed. The results were found to be in agreement with the certified values.

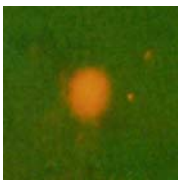
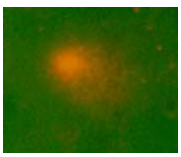
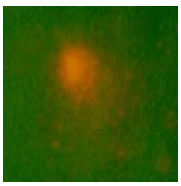
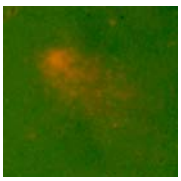
Comet assay

Comet assay was carried out as per the procedure outlined by Gichner et al. (1999). The terminal pair of leaflets of *C. occidentalis* were used for the assay. The leaflet was excised from the washed leaves and placed in a 60 mm Petri plate on an area layered with 100 µl of Sorensen buffer (50 mM Na_2HPO_4 (pH 6.8), 0.1 mM EDTA, 0.5% DMSO). Cold Sorensen buffer (400 µl) was then spread on the leaflet surface. The leaflet was gently sliced using a fresh razor blade and the Petri plate was kept tilted on ice so as that all the isolated nuclei were collected in the buffer. The whole procedure was performed under low light conditions.

Regular microscope slides were coated with 1% aqueous normal melting point agarose (NMA). For comet assay, 50 µl of nuclear suspension (isolated nuclei in Sorensen buffer) and equal volume of 1% low melting point agarose (LMA; prepared in phosphate buffered saline at 40°C) were placed on a NMA coated slide. Both the nuclear suspension and LMA were mixed gently, and layered on the slide. The slide was kept on an iced surface till the agarose and nuclear suspension mixture solidified. Final layer of 0.5% LMA (100 µl) was coated on the slide. The slides were dipped in freshly prepared ice-cold electrophoresis buffer (1 mM disodium EDTA and 300 mM NaOH, pH $>$ 13) in a horizontal gel electrophoresis tank (Bio-Rad) for 20 min. Subsequently, the electrophoresis was carried out at 0.72 V/cm (11 V, 300 mA) for 30 min at 4°C. After electrophoresis the slides were rinsed three times with neutralization buffer (400 mM Tris, pH 7.5) and processed for staining.

Slides were stained with 80 µl of ethidium bromide (20 µg/ml) for 5 min. Excess ethidium bromide was removed by dipping the slides in ice-cold water. The slides were scored at 200× magnification using an epifluorescence microscope (Nikon) with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm by visual analysis—a well validated and widely used method for evaluation of comets (Heuser et al. 2002; Avishai et al. 2004; da Silva et al. 2008). A total of 50 randomly chosen comets were scored per individual plant (2 slides per individual plant and 25 comets per slide). For each comet, image length (head diameter + tail length), head diameter and tail length were measured using a calibrated micrometer. Comets were visually classified into five classes from no visible tail (class 1) to maximally long tails (class 5) based upon comet characteristics and ratio of tail length to head diameter (Table 1). A cumulative damage score was calculated for 50 comets for each individual plant based upon the number of comets in different damage classes, and arbitrary scores allotted to each damage class (Table 1). The DNA damage score ranged from 50 (all comets with no tails; 50 × 1) to 250 (all comets with maximally long tails; 50 × 5). A mean DNA damage score for each site was calculated by taking an average of the scores of 10 individual plants.

Table 1 Scheme for classification of comets into different classes and the numerical scores allotted

Comet pictures	DNA damage		Tail/head ratio	Comet characteristics
	Class	Numerical score		
	1	1	No tail	Clear, round, dense head with intense fluorescence, and no visible tail
	2	2	0.2–0.79	Clear, round, dense head with intense fluorescence and very small diffuse tail
	3	3	0.8–1.74	Round, less dense, some what diffuse head, and a distinct tail
	4	4	1.75–3.9	Small and diffuse head, and relatively long prominent tail as compared to head
	5	5	>4	Very diffuse or no clear head and highly diffuse long tail having low fluorescence

Statistical analysis

Shapiro-Wilk's test was used to evaluate deviations from normal distribution in the variables estimated. The values of different variables were expressed as median ± inter-quartile range. Mann-Whitney *U* test was used as a test of statistical significance between samples of the two sites. Association between DNA damage (expressed as tail length of comet) and the foliar concentration of trace elements was assessed using Spearman's rank correlation coefficient. Statistical significance of the Spearman's rank correlation coefficient was tested using non parametric test of significance (Rees 1985). Single variable regression analysis was carried out between foliar concentrations of trace elements in plants growing on fly ash basin and phytoavailable concentration of trace elements in fly ash, after natural log transformation of the data. The critical level for rejection of null hypothesis was kept at $P < 0.05$. All statistical computations were carried out using SPSS 15.0.

Results

Fly ash showed significantly ($P < 0.05$) higher total and phytoavailable concentrations of all trace elements analyzed (except for phytoavailable concentration of Co) as compared to soil (Table 2). The foliar As, Ni and Cr contents were higher in plants growing on fly ash as compared to the plants growing on soil, whereas the foliar Co and Cu contents were similar between plants growing at both the sites (Table 3). As, Ni and Cr contents in leaf tissues of plants growing at BTPS were 2–8 fold higher as compared to those growing at GMV, and the differences in their median values between sites were statistically significant at $P < 0.05$ (Table 3). The regression analyses

Table 2 Total and phytoavailable concentrations of trace elements in the two plant growth substrates present at the BTPS (fly ash) and GMV (soil) sites

Element	Fly ash		Soil	
	Total[a]	Phytoavailable[b]	Total[a]	Phytoavailable[b]
As	3.06 ± 0.8	41 ± 9[c]	0.77 ± 0.45	5 ± 1[c]
Ni	37.05 ± 8.28	1.31 ± 0.37	7.01 ± 2.03	ND
Cr	86.19 ± 1.62	3.20 ± 1.62	52.37 ± 1.2	1.34 ± 0.23
Co	43.39 ± 1.22	1.22 ± 0.14	21.67 ± 2.25	1.42 ± 0.13
Cu	89.03 ± 2.15	5.06 ± 0.75	26.16 ± 0.99	1.73 ± 0.22

All values (except phytoavailable concentration of As) are expressed in ppm, $n = 10$

ND not detectable

[a] All values significantly different at $P < 0.05$

[b] All values significantly different at $P < 0.05$, except for Co

[c] Expressed in ppb

Table 3 Foliar concentrations of different trace elements (ppm, dry wt.) in *C. occidentalis* growing at GMV and BTPS sites

Element	Concentration of elements (median ± interquartile range)	
	GMV[a]	BTPS[a]
As	0.39 ± 0.11*	1.82 ± 0.38*
Cr	2.33 ± 0.54*	4.09 ± 0.64*
Co	4.09 ± 0.79	3.91 ± 0.43
Cu	21.15 ± 2.17	19.05 ± 2.33
Ni	0.34 ± 0.19*	2.33 ± 1.51*

* Significant at $P < 0.05$

[a] $n = 10$

between foliar concentrations of trace elements in plants growing on the fly ash basin and their phytoavailable concentrations in fly ash revealed statistically significant ($P < 0.05$) relationships between phytoavailable and foliar concentrations with respect to As, Cr and Cu (Fig. 2).

Figure 3 illustrates the percent of comets in each of the DNA damage classes for the plants inhabiting both the sites. For GMV population, the distribution pattern of the comets among different DNA damage classes followed distribution similar to normal distribution with high percentage of comets falling in intermediate DNA damage classes (DNA damage classes 2 and 3) and the number of comets rapidly declined in the higher damage classes 4 and 5; the percentage of comets in the least damaged class (DNA damage class 1) was more than two fold higher than the percentage of comets in the highest damage class (Fig. 3). The distribution pattern of comets, for BTPS population, was markedly different from that of the GMV population: the distribution was asymmetric and skewed towards the right; and there was an exponential increase in the percent of comets in higher DNA damage classes with the maximum percentage of comets (50 fold higher than that found in DNA damage class 1) at the highest DNA damage class (Fig. 3).

The average DNA damage score was higher for BTPS population in contrast to the DNA damage score of the GMV population (Table 4). The difference in the DNA damage score between sites was significant at $P < 0.05$. The median values of image and tail length of the comets of plants of BTPS site were higher than that of GMV plants (Table 4). On the other hand, the head diameter of comets of plants of the BTPS site was lower as compared to that observed for plants of GMV site (Table 4). The differences in all these comet parameters between the sites were significant at $P < 0.05$.

Plants inhabiting fly ash basin showed elevated levels of As, Ni and Cr contents and elevated levels of DNA damage in the leaf tissues, whereas the plants growing on the soil substratum had relatively low concentrations of As, Ni and Cr and low levels of DNA damage (Tables 3 and 4). DNA damage (expressed as tail length of comets) in the leaves of plants of both the populations growing at BTPS and GMV sites showed statistically significant positive correlations with the foliar As and Ni contents (Fig. 3). For Cr, statistically significant ($P < 0.05$) correlation was found only for GMV population and not for the BTPS population, although the foliar Cr content and DNA damage were higher in plants growing on the fly ash as compared to plants growing on the soil. Foliar Cu and Co content did not show significant relationships with DNA damage for both the populations (Fig. 4).

Discussion

Fly ash contains different classes of contaminants. In the present study we have focused on trace elements, an important class of contaminants. The weathered fly ash

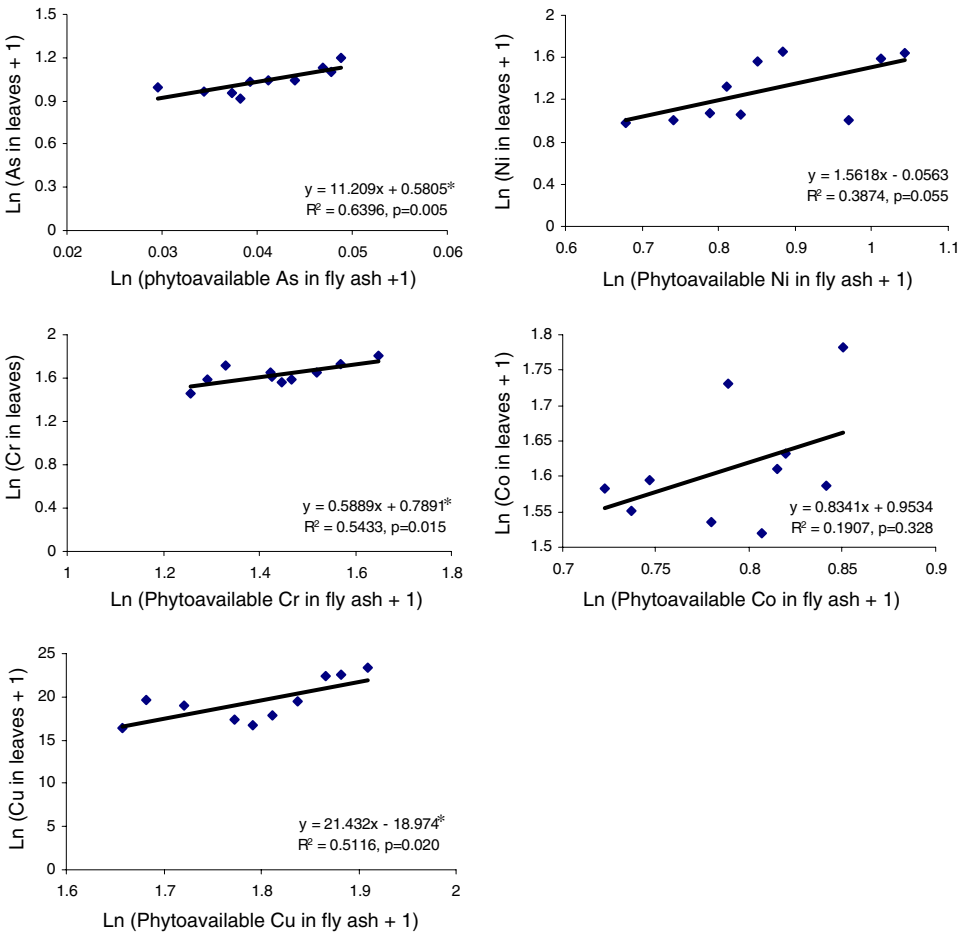


Fig. 2 Scatter plots with regression lines of '*y*' on '*x*' showing the relationship between phytoavailable concentrations of trace elements in fly ash and their foliar concentrations in *Cassia occidentalis* growing on the fly ash. * Significant at $P < 0.05$

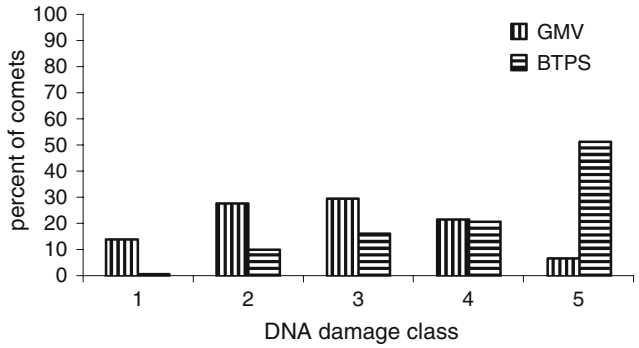


Fig. 3 Frequency distribution of comets among different DNA damage classes for natural populations of *C. occidentalis* inhabiting GMV (▥) and BTPS (▤) sites

present in the fly ash basin at BTPS site was enriched with higher concentrations of all the trace elements analyzed (except for phytoavailable Co) as compared to soil (Table 2). The concentrations of trace elements in fly ash is determined mainly by the physico-chemical properties of coal, the combustion process, the type of emission control devices and the degree of weathering of fly ash (Jankowski et al. 2006; Soco and Kalembkiewicz 2007). The most important

Table 4 Comet characteristics and DNA damage score among natural populations of *C. occidentalis* inhabiting GMV and BTPS sites

	GMV[a]	BTPS[a]
Image length (μm)	27.5 ± 6.71*	39.16 ± 1.9*
Tail length (μm)	13.69 ± 7.4*	29.42 ± 3.76*
Head diameter (μm)	13.44 ± 1.14*	10.6 ± 0.64*
Damage score[b]	141.18 ± 29.90*	206.22 ± 14.82*

* Significant at $P < 0.05$

[a] $n = 10$

[b] Arbitrary units

factor, from the point of view of risk assessment, is the levels of trace elements in the tissues of biota inhabiting areas in and around fly ash basins and their ability to take up these elements from the environment. *Cassia occidentalis* plants growing on the fly ash accumulated high concentrations of As, Ni and Cr in leaf tissues as compared to the plants growing on soil (Table 3). Carlson and Adriano (1991) reported statistically significant high foliar content of As and Ni in two tree species (*Platanus occidentalis* and *Liquidambar styraciflua*) growing on fly ash as compared to those growing on soils. Elevated foliar concentrations

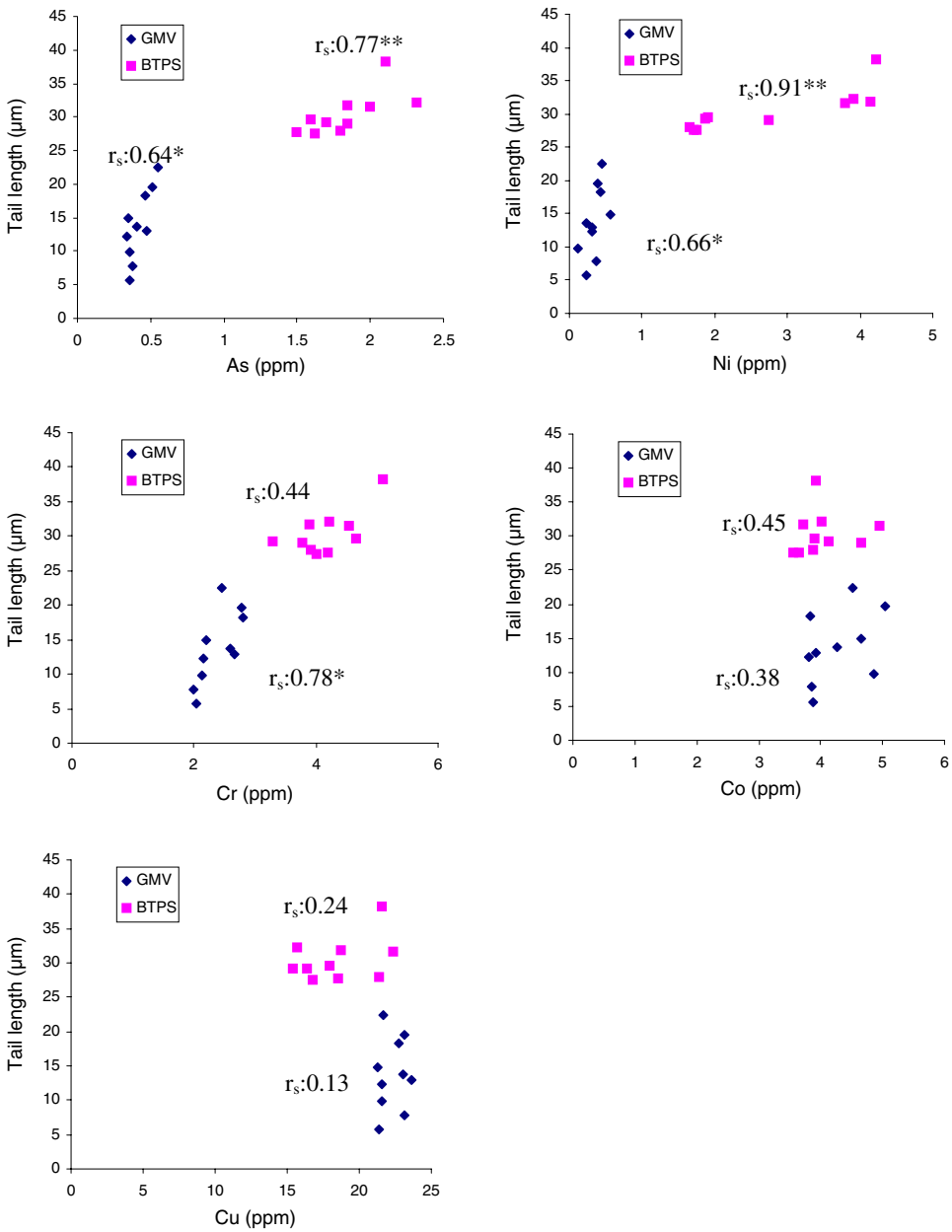


Fig. 4 Scatter plots of foliar DNA damage (expressed as tail length of comet) against foliar concentrations of different trace elements for *C.occidentalis* populations inhabiting GMV (◆) and BTPS (■) sites. r_s Spearman's rank correlation coefficient. * Significant at $P < 0.05$, ** Significant at $P < 0.001$

of As, Ni, and Cr have also been reported for other plant species growing on fly ash or on soil amended with fly ash (Adriano et al. 2002; Brake et al. 2004; Jensen et al. 2004; Maiti and Nandhini 2006).

The leaf tissue concentrations of trace elements observed in natural populations of *C. occidentalis* growing at two contrasting sites is the result of time-integrated uptake of these elements from the environment by the individual plants. In fact, plants are known to accumulate trace elements from the environment, and thus serve as an accumulative bioindicator for trace elements found in the environment (Weiss et al. 2003; Margui et al. 2007). The high levels of foliar toxic trace elements (As, Ni and Cr) in the plants growing on fly ash basin (Table 3) may be due to the uptake of these elements from fly ash, and as well as from the aerial depositions on the leaves. Both root and foliar absorption of trace elements by plants has been reported (Madejon et al. 2004). The results of regression analyses indicate that phytoavailable concentration of As, Cr and Cu in fly ash influence their foliar levels in plants growing on the fly ash (Fig. 2). These observations suggests that the elevated levels of trace elements observed in leaves of *C. occidentalis* growing on fly ash is, to some extent, related to their uptake from fly ash present in fly ash basins. The high concentrations of As, Ni and Cr in fly ash (Table 2) shows that these elements persist in fly ash disposed in fly ash basins, even after fly ash has undergone natural weathering.

We used the comet assay, for the first time, to assess the genotoxicity of fly ash to natural populations of *Cassia occidentalis* growing on the fly ash basin. The 50 fold increase in the number of comets falling in the highest DNA damage class as compared to DNA damage class 1 for the BTPS population (Fig. 3) and the high median values of image and tail length of comets, and high DNA damage score for the population growing on the fly ash as compared to that of the population growing on soil (Table 4) suggest that the fly ash is associated with higher DNA damage in natural plant populations growing on weathered fly ash present in fly ash basins. Based upon our experimental design, wherein, the populations of *C. occidentalis* growing both at reference site and fly ash basin having similar environmental settings but principally differing in the growth substrate of the plants only, it can be reasonably concluded that fly ash has a role in triggering genotoxic responses in plants of *C. occidentalis* growing naturally on it.

In fact, a number of workers also reported genotoxic effects of fly ash on biota under experimental conditions. McMurphy and Rayburn (1993) have reported alteration in the genome of maize seedlings grown on fly ash amended soils under experimental conditions. Kuzmick et al. (2007) showed that chronic exposure of coal combustion residues (fly ash) to grass shrimps elicit genotoxic responses in grass shrimps as detected by comet assay. They also mentioned that organisms inhabiting ash settling basins and its drainage systems for all or part of their lives may be exposed to and accumulate contaminants to the extent that toxic responses are induced. Van Maanen et al. (1999), and Prahalad et al. (2000) have also shown that coal fly ash induces DNA damage in vitro. The elevated level of DNA damage in natural populations of *C. occidentalis* growing on fly ash indicates towards exercising caution with respect to (1) the use of fly ash as a soil amendment in agriculture as suggested by many workers and (2) open disposal of fly ash in fly ash basins because of the potential adverse effects that the fly ash can have on neighbouring natural and managed ecosystems, till further detailed studies are carried out.

Even though high level of DNA damage was observed in the plants growing on weathered fly ash, they were morphologically similar to plants growing on the soils suggesting that plants growing on fly ash are able to repair the DNA damage detected by the comet assay. It has been shown that plants have the ability to regulate DNA repair pathways to cope up with enhanced DNA damage under severe and chronic periods of genotoxic exposure (Kovalchuk et al. 1998). Reinecke and Reinecke (2004) also suggested that the DNA repair process would be enhanced to compensate for high DNA damage (detected by comet assay) observed in earthworms exposed to high concentrations of $NiCl_2$ under experimental conditions. It may be noted that in the absence of effective functioning of DNA repair pathways, DNA damage detected by comet assay may be exacerbated into alterations in chromosomal structure. It needs further investigation whether the DNA damage detected by comet assay has any implications on the fitness of the plants growing on fly ash.

The DNA damage detected in plants growing at the reference site (Fig. 3; Table 4) might be the background level of DNA damage in plants of *C. occidentalis*. In plant cells, DNA single strand breaks are generated primarily due to reactive oxygen species as a result of normal oxidative cellular processes and also as an intermediate of DNA repair pathways (apurinic/apyrimidinic sites) even in the absence of any environmental stress (Bray and West 2005). These DNA single strand breaks generated in the plant cells would always be detected by comet assay. It is also likely that at least a part of the DNA damage observed in plants growing at the reference site might be associated with the some pollutants which may be present in the soil. It may be noted that the concentrations of trace elements such as As, Ni and Cr are significantly lower in the soil at GMV site in comparison to that present in the fly ash (Table 2). Further, in the absence of any study on the background level of DNA damage in natural populations of weeds it is difficult to compare DNA damage levels observed by us. The results of our in situ biomonitoring study on the assessment of DNA damage in leaf tissue of plants, using comet assay, have shown that the comet assay is a versatile technique which gives repeatable results, and thus it can be used to study genotoxicity in leaves of naturally growing plants in contaminated habitats.

Fly ash has a complex mixture of contaminants. Consequently, it is difficult, based on our in situ biomonitoring study, to establish a causal link between an individual contaminant/s and the DNA damage observed in the leaves of *C. occidentalis* growing on the fly ash basin. Though the patterns of variability observed between DNA damage and foliar concentrations of trace elements analyzed (Fig. 4) are indicative that there may be an association of foliar As and Ni contents with DNA damage but this statistically significant correlation does not alone establish a cause-effect relationship between them. Detailed experimental studies are required to establish any link between foliar As and Ni contents and DNA damage in the leaves of *C. occidentalis*. The interactive (synergistic and antagonistic) effects of other trace elements (Palit et al. 1994; Sinha et al. 2006) cannot also be ignored while analyzing genotoxic effects of complex substrates such as fly ash, which is also rich in many trace elements other than the ones examined in the study. Further, the role of other contaminants present in the fly ash such as PAH and radionuclides in DNA damage cannot be ruled out.

The elevated level of DNA damage observed in plants growing naturally on the fly ash basin indicates that in situ

biomonitoring (organismic responses and tissue contaminant levels in organisms) using natural populations of biota exposed to weathered fly ash present in fly ash basins is critical for the realistic risk assessment of fly ash disposal sites. In the case of fly ash, which contains a complex mixture of many contaminants, it is important to undertake more rigorously designed in situ biomonitoring studies to assess its long-term genotoxicity to biota. Since legal definitions exert a significant impact on waste management strategy (Twardowska and Szczepanska 2002), a critical assessment should be carried out on the methods of fly ash disposal and its environmental safety in the light of accumulating evidences about its toxicity so that safe methods of its disposal and utilization can be devised.

Conclusions

Our studies showed that: (1) high foliar levels of As, Ni and Cr are present in plants of *Cassia occidentalis* growing on the weathered fly ash; (2) *C. occidentalis* plants growing on the fly ash basin have elevated levels of DNA damage, and thus indiscriminate disposal of fly ash close to natural and managed ecosystems (terrestrial and aquatic) or use of fly ash as a soil amendment in agriculture may not be environmentally safe; (3) genotoxicity induced by fly ash may be perhaps associated with elevated levels of foliar toxic trace elements; and (4) comet assay is useful for in situ biomonitoring of genotoxicity of fly ash to plants.

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References

Adriano DC, Weber J, Bolan NS, Paramsivam S, Koo B-J, Sajwan KS (2002) Effects of high rates of coal fly ash on soil, turfgrass, and ground water quality. Water Air Soil Pollut 139:365–385. doi:10.1023/A:1015895922471

Avishai N, Rabinowitz C, Rinkevich B (2004) A 2.5-year genotoxicity profile for a partially restored polluted river. Environ Sci Technol 38:3482–3487. doi:10.1021/es035264a

Bidar G, Garcon G, Pruvot C, Dewaele D, Cazier F, Douay F, Shirali P (2007) Behavior of *Trifolium repens* and *Lolium perenne* growing in a heavy metal contaminated field: plant metal concentration and phytotoxicity. Environ Pollut 147:546–553

Brake SS, Jensen RR, Mattox JM (2004) Effects of coal fly ash amended soils on trace element uptake in plants. Environ Geol 45:680–689. doi:10.1007/s00254-003-0921-z

Bray CM, West CE (2005) DNA repair mechanisms in plants: crucial sensors and effectors for maintenance of genome integrity. New Phytol 168:511–528. doi:10.1111/j.1469-8137.2005.01548.x

Carlson CL, Adriano DC (1991) Growth and elemental content of two tree species growing on abandoned coal fly ash basins. J Environ Qual 20:518–587

Carlson CL, Adriano DC (1993) Environmental impacts of coal combustion residues. J Environ Qual 22:227–247

Celik M, Donbak L, Unal F, Yuzbasioglu D, Aksoy H, Yilmaz S (2007) Cytogenetic damage in workers from coal-fired power plant. Mutat Res 627:158–163

Chrisp CE, Fisher GL, Lammert JE (1978) Mutagenicity of filterates from respirable coal fly ash. Science 199:73–75. doi:10.1126/science.199.4324.73

Collins AR (2004) The comet assay for DNA damage and repair. Mol Biotechnol 26:249–261. doi:10.1385/MB:26:3:249

Cotelle S, Ferard JF (1999) The comet assay in genetic ecotoxicology–a review. Environ Mol Mutagen 34:246–255. doi:10.1002/(SICI)1098-2280(1999)34:4<246::AID-EM4>3.0.CO;2-V

da Silva J, Moraes CR, Heuser AD, Andrade VM, Silva FR, Kvitko K, Emmel V, Rohr P, Bordin DL, Andreazza AC, Salvador M, Henriques JAP, Erdtmann B (2008) Evaluation of genetic damage in a brazilian population occupationally exposed to pesticides and its correlation with polymorphism in metabolizing genes. Mutagenesis 23:415–422. doi:10.1093/mutage/gen031

Fisher GL, Chrisp CE, Raabe OG (1979) Physical factors affecting the mutagenicity of fly ash form a coal-fired power plant. Science 204:879–881. doi:10.1126/science.375394

Gichner T, Ptacek O, Stavreva DA, Plewa MJ (1999) Comparison of DNA damage in plants as measured by single cell gel electrophoresis and somatic leaf mutations induced by monofunctional alkylating agents. Environ Mol Mutagen 33:279–286. doi:10.1002/(SICI)1098-2280(1999)33:4<279::AID-EM4>3.0.CO;2-K

Gichner T, Patkova Z, Szakova J, Demnerova K (2006) Toxicity and DNA damage in tobacco and potato plants growing on soil polluted with heavy metals. Ecotoxicol Environ Saf 65:420–426. doi:10.1016/j.ecoenv.2005.08.006

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Spiet G, Thybaud V, Tice RR (2003) Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 18:45–51. doi:10.1093/mutage/18.1.45

Heuser VD, da Silva J, Moriske H-J, Dias JF, Yoneama ML, de Freitas TRO (2002) Genotoxicity biomonitoring in regions exposed to vehicle emissions using the comet assay and micronucleus test in native rodent *Ctenomys minutus*. Environ Mol Mutagen 40:227–235. doi:10.1002/em.10115

IMD 2008. India Meteorological Department. http://121.241.116.157/climatology/plm.htm as on 15 August 2008

Jankowski J, Ward CR, French D, Groves S (2006) Mobility of trace elements from selected Australian fly ashes and its potential impact on aquatic ecosystems. Fuel 85:243–256. doi:10.1016/j.fuel.2005.05.028

Jensen RR, Brake SS, Mattox JM (2004) Trace element uptake in plants grown on fly ash amended soils. Toxicol Environ Chem 86:219–230. doi:10.1080/02772240400007070

Kabata-Pendias A (1993) Behavioural properties of trace metals in soils. Appl Geochem 93:3–9. doi:10.1016/S0883-2927(09)80002-4

Kovalchuk I, Kovalchuk O, Arkhipov A, Hohn B (1998) Transgenic plants are sensitive bioindicators of nuclear pollution caused by Chernobyl accident. Nat Biotechnol 16:1054–1059. doi:10.1038/3505

Kljajic R, Masic Z, Zunic Z, Pavlovic S, Tosic M, Mandic M, Gordanic V, Polic P (1996) Natural radionuclide emission from a coal power plant and the population exposure to external radiation in its vicinity. Environ Int 22:S227–S235. doi:10.1016/S0160-4120(96)00112-2

Kuzmick DM, Mitchelmore CL, Hopkins WA, Rowe CL (2007) Effects of coal combustion residues on survival, antioxidant potential, and genotoxicity resulting from full-lifecycle exposure of grass shrimp (*Lalaemonetes pugio Holthius*). Sci Total Environ 373:420–430. doi:10.1016/j.scitotenv.2006.11.009

Lamothe PJ, Fries TL, Consul JJ (1986) Evaluation of a microwave oven system for the dissolution of geologic samples. Anal Chem 58:1881–1886

Madejon P, Maranon T, Murillo JM, Robinson B (2004) White poplar (*Populus alba*) as a biomonitor of trace elements in contaminated riparian forests. Environ Pollut 132:145–155

Maheshwari JK (1963) Flora of Delhi. Council for Scientific and Industrial Research, India

Maiti SK, Nandhini S (2006) Bioavailability of metals in fly ash and their accumulation in naturally occurring vegetation: a pilot scale study. Environ Monit Assess 116:263–273. doi:10.1007/s10661-006-7355-5

Maiz I, Esnaola MV, Millan E (1997) Evaluation of heavy metal availability in contaminated soils by a short sequential extraction procedure. Sci Total Environ 206:107–115

Maiz I, Arambarri I, Garcia R, Millan E (2000) Evaluation of heavy metal availability in polluted soils by two sequential extraction procedures using factor analysis. Environ Pollut 110:3–9

Margui E, Queralt I, Carvalho ML, Hidalgo M (2007) Assessment of metal availability to vegetation (*Betula pendula*) in Pb–Zn ore concentrate residues with different features. Environ Pollut 145:179–184. doi:10.1016/j.envpol.2006.03.028

McMurphy LM, Rayburn AL (1993) Nuclear alterations of maize plants grown in soil contaminated with fly ash. Arch Environ Contam Toxicol 25:520–524. doi:10.1007/BF00214342

Mitchelmore DL, Chipman JK (1998) Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. Aquat Toxicol 41:161–182. doi:10.1016/S0166-445X(97)00064-7

MoEF (2005) Ministry of Environment and Forests, Government of India, Annual report

Palit S, Sharma A, Talukdar G (1994) The effect of cobalt on plants. Bot Rev 60:149–181. doi:10.1007/BF02856575

Prahalad AK, Inmon J, Ghio AJ, Gallagher JE (2000) Enhancement of 2′-deoxyguanosine hydroxylation and DNA damage by coal and oil fly ash in relation to particulate metal content and availability. Chem Res Toxicol 13:1011–1019. doi:10.1021/tx000110j

Reash RJ, Lohner TW, Wood KV (2006) Selenium and other trace metals in fish inhabiting a fly ash stream: implications for regulatory tissue thresholds. Environ Pollut 142:397–408. doi:10.1016/j.envpol.2005.10.025

Rees DG (1985) Essential statistics. Chapman and Hall, London

Reinecke SA, Reinecke AJ (2004) The comet assay as biomarker of heavy metal genotoxicity in earthworms. Arch Environ Contam Toxicol 46:208–215

Rowe CL, Hopkins WA, Congdon JD (2002) Ecotoxicological implications of aquatic disposal of coal combustion residues in US: a review. Environ Monit Assess 80:207–216. doi:10.1023/A:1021127120575

Rubin ES (1999) Toxic releases from power plants. Environ Sci Technol 33:3062–3067. doi:10.1021/es990018d

Sample BE, Suter GWII (2002) Screening evaluation of the ecological risks to terrestrial wildlife associated with a coal ash disposal site. Hum Ecol Risk Assess 8:637–656. doi:10.1080/10807030290879880

Shugart LR (2000) DNA damage as a biomarker of exposure. Ecotoxicology 9:329–340. doi:10.1023/A:1026513009527

Sinha P, Dube BK, Chatterjee C (2006) Manganese stress alters phytotoxic effects of chromium in green gram physiology (*Vigna radiata* L.) cv. PU 19. Environ Exp Bot 57:131–138. doi:10.1016/j.envexpbot.2005.05.003

Smith-Sonneborn RA, Palizzi C, Fisher GL (1981) Mutagenicity of fly ash particles in *Paramecium*. Science 211:180–182. doi:10.1126/science.7444461

Soco E, Kalembkiewicz J (2007) Investigations of sequential leaching behaviour of Cu and Zn from coal fly ash and their mobility in environmental conditions. J Hazard Mater 145:482–487. doi:10.1016/j.jhazmat.2006.11.046

Twardowska I, Szczepanska J (2002) Solid waste: terminological and long-term environmental risk assessment problems exemplified in a power plant fly ash study. Sci Total Environ 285:29–51. doi:10.1016/S0048-9697(01)00893-2

Van Maanen JM, Borm PJ, Knaapen A, van Herwijnen M, Schilderman PA, Smith KR (1999) In vitro effects of coal fly ash ashes: hydroxyl radical generation, iron release, and DNA damage and toxicity in rat lung epithelial cells. Inhal Toxicol 11:1123–1141. doi:10.1080/089583799196628

Weiss P, Offenthaler I, Ohlinger R, Wimmer J (2003) Higher plants as accumulative bioindicators. In: Markert BA, Breure AM, Zechmeister HG (eds) Bioindicators & biomonitors: principles, concepts and applications. Elsevier Science Ltd., Oxford, UK, pp 465–500

Wright RJ, Codling EE, Stuczynski T, Siddaramappa R (1998) Influence of soil-applied coal combustion by-products on growth and elemental composition of annual ryegrass. Environ Geochem Health 20:11–18. doi:10.1023/A:1006571026303