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Red mud a byproduct of Aluminum production contains soluble vanadium that causes genotoxic and cytotoxic effects in higher plants

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ABSTRACT

Red mud (RM) is a byproduct of aluminum production, worldwide between 70 and 120 million tons are produced annually. We analyzed RM which was released in the course of the Kolonatar disaster in Hungary into the environment in acute and genotoxicity experiments with plants which are widely used for environmental monitoring. We detected induction of micronuclei which reflect chromosomal damage in tetrads of Tradescantia and in root cells of Allium as well as retardation of root growth with contaminated soils and leachates. Chemical analyses showed that RM contains metals, in particular high concentrations of vandadium. Follow up experiments indicated that vanadate causes the effects in the plants. This compound causes also in humans DNA damage and positive results were obtained in carcinogenicity studies. Since it was found also in RM from other production sites our findings indicate that its release in the environment is a global problem which should be studied in more detail.

Capsule abstract: Our findings indicate that the red mud causes genotoxic effect in plants probably due to the presence of vanadate which is contained at high concentrations in the residue.

Key words: red mud, Tradescantia, micronuclei, Allium, vanadium

1. Introduction

Red mud is a by-product of aluminum production with the Bayer process. Its global production is in the range between of 70 to 120 million tons per year (Mayes et al., 2011b). The material consists mainly of iron-, aluminum- and titanium- oxides and hydroxides (Burke et al., 2012; Mayes et al., 2011b). Chemical analyses showed that its contains also radionuclides (e.g. ^{226}RA, ^{230}Th and ^{40}K), as well as heavy metals including As, Cr, Co, Cd, Ni and V (Mayes et al., 2011a; Ruyters et al., 2011).

On Oct 4th 2010, approximately one million m^3 of the residue was released into the environment from the aluminum plant Ajkai Timfoldgyar Zrt in Western Hungary. According to the Hungarian Ministry of Interior the "Kolontar disaster" is the biggest environmental catastrophe which ever happened in this country (Ádám et al., 2011). Hundreds of houses were destroyed, 265 individuals were injured and ten died (Gundy et al., 2013).

After the accident, attempts were made to investigate the impact of the release of the material into the environment and to assess the health consequences in humans. Ecotoxicological studies were conducted with different plant species and bacteria concerning acute toxic effects (Klebercz et al., 2012; Ruyters et al., 2011); the motility and the concentrations of toxic trace elements were studied in physico-chemical measurements (Burke et al., 2012). Furthermore, studies were conducted to assess the consequences of inhalation of dust particles in humans and rodents (Czovek et al., 2012; Gelencser et al., 2011).

Radionuclides as well as certain heavy metals (found in red mud) cause damage of the genetic material (Knasmuller et al., 1998; Minouflet et al., 2005) which may lead to destabilization of ecosystems (Sarkar et al., 2006; Zvereva et al., 2008) and also causes adverse effects in humans such as cancer, ageing, infertility and birth defects in the offspring (Aitken and De Iuliis, 2007; Assem and Levy, 2009). Therefore it is of particular interest if

the release of this material into the environment induces chromosomal damage. This question has been addressed in a human study (Gundy et al., 2013) and in bacterial tests (Gelencser et al., 2011) but no firm conclusions can be drawn of these studies (for details see discussion).

The primary aim of the present study was the investigation of the genotoxic properties of red mud in two plant bioassays, namely in the micronucleus (MN) test with tetrads of Tradescantia (Trad-MN assay) and with root tip cells of Allium cepa (A-MN assay). The experiments were conducted with tetrads as they reflect damage in meiotic germ cells. Root tip cells were used as they enable the detection of effects in mitotic cells. It was postulated that differences exist in regard to sensitivity of these cell types towards DNA reactive compounds (Rodrigues et al., 1997). MN are formed as a consequence of chromosomal breakage or aneuploidy and can be monitored in a variety of organisms (Heddle et al., 2011). In addition acute toxic effects were studied in root cells of A. cepa by measuring the impact of the material on the root growth and by calculating the division rates of the cells. These bioassays are at present the most widely used genotoxicity tests with higher plants and have been employed in more than 300 investigations for the detection of DNA damaging properties of chemicals and complex mixtures (for reviews see Leme and Marin-Morales, 2009; Misik et al., 2011). We used these test systems since they provide information on environmental effects and it is known that they are, in contrast to other genotoxicity assays with bacterial indicators and mammalian cells (which are also used for environmental monitoring), highly sensitive towards radiation (Ma and Davies, 2009; Misik et al., 2011) and heavy metals (Knasmuller et al., 1998; Majer et al., 2002; Steinkellner et al., 1998).

We investigated the acute cytotoxic and genotoxic activities of red mud and of soils which were contaminated with the material collected from fields and gardens which were used for cultivation of crops and vegetables. Furthermore, we studied also the effects of waters leached from red mud and affected soils. In additional experiments, attempts were

made to identify the compound(s) which cause genotoxic effects. We determined the concentrations of trace elements in solid samples and in leachates and conducted additional Trad-MN assays with sodium metavanadate ($NaVO_3$) since vanadium was found to be the most abundant heavy metal in the samples.

2. Materials and methods

2.1 Sampling

Soil samples were collected from the Torna catchment on December 15th 2010. Table 1 contains a description of the sampling sites and specifications of their GPS locations. From each site, samples were collected from the 0-5; 5-15 and 15-25 cm horizons to assess if potentially toxic constituents in red mud contaminated the soils.

Total organic carbon (TOC) was determined in soil samples using a LECO SC-144DR elemental analyzer after removal of the inorganic carbon fraction using 20% HCl.

Table 1

Figure 1

2.2 Preparation of the leachates.

Soils were used as sampled (field moist). 50 g of each sample were suspended in 100 mL of deionised water in a 250 mL glass beaker for 2 hours using a magnetic stirrer. The extracts were filtered with filter paper before they were tested in stem absorption experiments. The pH values of the leachates were measured with a pH Meter 526 (WTW, Weilheim, Germany) and are listed in Table 1.

2.3 Measurements of the trace elements in the soil samples and leachates.

The detection of the trace elements is described in detail in a recent paper of Renforth et al. (2012). Prior to analysis by XRF the samples was prepared as follows. For major element analysis, samples were prepared as fused beads (after loss on ignition at 1050 °C) with lithium metaborate/tetraborate flux (Johnson - Matthey Spectroflux JM100B) (0.6g sample; 3 g Flux). For minor / trace element analysis approximately 10 g dried sample was prepared as a pressed pellet using ~10-20 drops of 6.6%w/v polyvinyl alcohol in a 1:6 mix of methanol and distilled deionised water as a binder (Moviol 88 solution). Elemental analysis of the soil composition was achieved using a PANalytical Axios Advanced X-ray Fluorescence (XRF) spectrometer (data corrected for loss on ignition; % weight loss after furnace treatment at 1050°C). The aqueous phase produced during water extractions was separated from solids by centrifugation (10 min; 2000 g) followed by membrane filtration (0.45 μm); the filtered samples were then acidified by addition of 2% v/v HNO_3. Aqueous elemental concentrations were then determined using a Perkin Elmer Optima 5300 DV ion-coupled plasma, optical emission spectrometer (ICP-OES).

2.4 Tradescantia micronucleus assays

Tradescantia clone #4430 was cultivated according to the protocol of Ma et al. (1994) For stem absorption experiments, 15 young inflorescences were treated in each group. The stems were cut to a length of 10 - 15 cm, transferred to plastic breakers (250 mL) and exposed to aqueous leachates (soil:water – 1:2) of the contaminated soils for 24 h. Subsequently, they were transferred to water for a 24 h recovery period. The flower buds were then collected and

fixed in acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol. Maleic hydrazide (20 mg L^{-1}; MH, Sigma-Aldrich, SL, US) was used as a positive control.

The protocol for root absorption assays is described in an article of Steinkellner et al. (1998). The soil which was used in the control cultures was biological, pesticide free (from Composana, Wien, Austria). Intact plants were removed from hydroponic culture, subsequently the roots were rinsed and individual plants with at least 15 inflorescences placed into plastic pots (250 ml, diameter 12 cm) which were filled with the different soil samples. The plants were exposed under standard conditions for 48 h (Ma et al., 1994). Subsequently, 15 inflorescences were collected from each soil sample in all experiments and fixed in a solution of acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol.

To find out if vanadium accounts for the effects which were found in the soils and in the leachetes, additional experiments were carried out with sodium metavanadate. The salt was dissolved in 0.1M NaOH (pH 13) at 1000 ppm (colorless solution) since earlier findings indicate that vanadium is present as V^{5+} in the soil (Burke et al., 2012). The stock solution was diluted with tap water and different concentrations (0.5-10.0 ppm) were used in experiments with Tradescantia.

Slides were prepared and evaluated as described in the protocol of Ma et al. (1994) The tetrads were stained with a 2% aceto-carmine solution. Early tetrads were analyzed under 400-fold magnification. For each experimental point a minimum of five buds with early tetrads was evaluated (300 tetrads were evaluated per slide, 1500 per sample).

2.5 Micronucleus assay with Allium cepa

The experiments were carried out according to the standard protocol published by Ma et al. (1995) with slight modifications. Young onion bulbs (diameter 12-21 mm, Schneeball

weis, Austrosaat, Wien, Austria) were placed in 13 ml glass tubes filled with tap water for 24 h in the dark. Subsequently, the roots (lengths ca. 0.5-1 cm) were exposed directly in the soils in the dark for 24 h and then transferred to fresh tap water for another 24 h. At the end of the recovery phase, the maximal root length of each onion was measured and the material was fixed in a mix of acetic acid and ethanol (1/3, v/v) for 24 h and stored in 70% ethanol. MH (10 mg L^{-1}; Sigma-Aldrich, SL, US) was used in all experiments as a positive control.

The root tips were hydrolyzed in a 1:1 mix of HCl (5.0 N) and ethanol (99%) for 3 min and washed in tap water before they were stained with 2% acetocarmine. MN were scored according to the criteria described by Ma et al. (1995). For each experimental point, the MN frequencies were determined in five plants. From each bulb, two slides were made and 500 cells were evaluated per slide (5000 cells per dose). Furthermore, also the mitotic indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The microscopic evaluation was carried out under a light microscope (Nikon YS200, Japan) with 400-fold magnification.

2.6 Statistical evaluation

The results of the MN experiments were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. The results of the experiments concerning the mitotic indices (MI) were analyzed with the Kruskal-Wallis test followed by Dunn´s comparison test. P-values ≤ 0.05 were considered as significant.

3. Results

3.1 TOC and pH values of the samples.

It can be seen in Table 1 that values between 8.0 and 9.3 were found in the soil leachates which were contaminated with red mud. Most of the samples had a total organic carbon content between 1.0-1.5%, which is typical for agricultural soils in the Ajka area (Lehoux et al., 2013). The sample from the upper horizon of the site 3 had a substantially lower TOC content (0.22%) and a higher pH (9.33) which is typical for red mud (Lehoux et al., 2013).

3.2 Micronucleus assays with Tradescantia (experiments with soils and leachates)

The results which were obtained in Trad-MN assays with red mud contaminated soil and with aqueous leachates from these samples are summarized in Fig. 2A and 2B. It can be seen that direct exposure of intact plants in material from the contaminated sites 2 (S2) and 3 (S3) caused a significant increase of the MN frequencies. In the case of S3, the strongest effect was seen with material from the upper layer, samples from deeper horizons caused only a statistically not significant increase. With the two samples from S4, clear cut negative results were obtained. As described above (Table 1), this material was collected after remediation (i.e. after removal of the upper layer).

Also with some leachates, positive results were obtained (Fig. 2B). Again the strongest effects were seen with extracts prepared with material from the upper layers (S2 and S3); soils from deeper horizons did not cause significant effects, also the sample which was collected from the cleanup site was devoid of activity.

Figures 2A and B

3.3 Micronucleus experiments with mitotic root tip cells of Allium cepa

The results of assays with A. cepa are shown in Figures 3A – C. The roots were exposed in these experiments directly in the samples. Since division delays may decrease the formation of MN (Fenech and Morley, 1985), we determined in all experiments also the mitotic indices (MI).

It can be seen in Figure 3A that a significant increase of the MN rates was observed with some samples. The most pronounced effect was detected with material collected from the middle layer of S2 while red mud enriched sample from surface of the same location did not cause a significant increase of the MN rates. This finding can be explained by inhibition of cell division, which was most pronounced with material collected from the upper horizon of this site (Figure 3B). As in the Tradescantia experiments, no increase was seen with samples from the remediation site (S4).

The impact of the material on root growth is shown in Figure 3C. It can be seen, that clear effects were obtained with samples from the surface of sites S2 and S3.

Figures 3A – C

3.4 Chemical analyses of the soil samples and of the leachates

The chemical composition of samples which were collected from S3 and S1 (Table 2A-B) and of the corresponding leachates (Table 2C) are shown in Table 2.

Table 2

The chemical composition of the topmost soil sample (S3 0-5 cm) is similar to that of other samples of red mud which were collected after the Ajka spill (Burke et al., 2012; Ruyters et al., 2011). Several potentially toxic metals including As, Cr, Ni, Pb and V were present at elevated concentrations.

The chemical composition of the control sample (S1) is consistent with unaffected soils found in the region (see Table 2c) (Mayes et al., 2011b).

The corresponding leachates from S3, contained elevated concentrations of potentially toxic elements, such as V, As, Cu, Al and Cr, notably, V concentrations were an order of magnitude higher than the levels of the other metals (except Al). Also chromium levels were relatively high.

3.5 Tradescantia micronucleus assays with vanadate

We found in earlier experiments with Tradescantia that the concentrations of Ni, Cr, Cd, Pb and As, which cause MN induction in tetrads are in the range of hundred to thousand ppm (Knasmuller et al., 1998; Steinkellner et al., 1998). We hypothesized that V, which was detected in higher levels than other metals, may account for the induction of MN, which was seen with the soils and the leachates. Therefore, we tested different concentrations of an aqueous vanadate solution in subsequent Trad MN assays. The results are summarized in Figure 4. It can be seen that exposure of the cuttings to concentrations ≥1.0 mg/L caused significant induction of the MN frequencies i.e. an approximately 6-fold increase over the background.

Figure 4

3.6 Impact of pH on MN formation in Tradescantia

Since the pH values of the soil extracts and of the vanadate solutions were quite high, i.e. in the range between 8.2 and 9.3 (for details see Table 1) we conducted an additional experiment in which plant cuttings were exposed in aqueous solutions (without metals) to different pH values. No indication of pH depended induction of MN by the pH itself was found in this experiment. The numbers of MN per 100 tetrads were 1.7±0.6; 2.1±0.6 and 1.6±0.6 for pH 8, 8.5 and 9.3 respectively (numbers are means ± SD of results obtained from 1500 tetrads).

4. Discussion

The results of this study show that red mud, which was released in large amounts into the environment, causes damage of the genetic material and also acute toxic effects in higher plants.

4.1 Acute toxic and genotoxic effects of red mud

Several ecotoxicological studies have been conducted with red mud from Ajka (Klebercz et al., 2012; Ruyters et al., 2011) and adverse effects were seen in experiments with higher plants; i.e. the material was found to inhibit the root of Sinapis alba and affect the shoot yield in barley (Klebercz et al., 2012; Ruyters et al., 2011). In the latter study, the authors monitored also the levels of trace elements which are contained in red mud such as Cu, Cr, Fe and Ni in exposed plants. They detected these metals in the shoots but stress that their levels did not exceed the toxic limits and hypothesize that Na is the prime cause which affected the growth of the indicator plants. Another factor which was made responsible for the toxic effects in Sinapis and in experiments with the Ostracod Heterocypris incongruens is

gypsum which may limit the nutrient supply and increase the availability of contaminants (Klebercz et al., 2012). The genotoxic effect of red mud has not been investigated in the invertebrates so far according to our knowledge. However, it is possible that several species may be affected due to the high metal concentration of the material. For example, it is know that earthworms are highly sensitive towards specific contaminants and specific metals. Genotoxic effects were detected with chromium, nickel and cadmium (Bigorgne et al., 2010; Bonnard et al., 2010; Manerikar et al., 2008).

The results of the present study show clearly that red mud inhibits the division and growth of root tip cells in A. cepa (Figures 3B and 3C) and causes induction of MN in this species. Furthermore, we showed that also aqueous leachates of red mud contaminated soils cause induction of MN in both plant bioassays. As shown in Figure 3 a stronger effect was obtained with sample S2 (5-15 cm) as with the sample from upper layer from the same site. This effect can be explained by reduction of MN formation due to inhibition of cell division. Furthermore, it is notable that the effects which were detected with soil exposure are more pronounced as those which were found with the leachetes. This observation is in agreement with the results obtained with heavy metals contaminated soils and can be explained by more efficient uptake via the roots by active transport mechanism (for detail see Steinkellner et al., 1998).

The only genotoxicity assay conducted so far with red mud is the SOS chromotest which is based on the detection of SOS responses in bacterial indicator cells (Gelencser et al., 2011). Consistently negative results were obtained with samples, which were prepared from fugitive dust from the area where the accident had happened. However, it is notable that it is known from earlier investigations that bacterial assays are insensitive towards heavy metals (Majer et al., 2002).

4.2 Identification of vanadate as the active principle of red mud

The results of the chemical analyses show that red mud contaminated soils and their aqueous leachates contain elevated concentrations of V (Table 2C). This observation is in agreement with the results of earlier investigations with material from Ajka (Mayes et al., 2011a; Mayes et al., 2011b; Renforth et al., 2012). In this context, it is notable that vanadium was also detected in red mud from several other aluminium production sites (Fontanier et al., 2012; Gawu et al., 2012; Rajeev et al., 1999; Samal et al., 2012).

Spectroscopic evidence showed that vanadium is present primarily as V^{5+} phases (Burke et al., 2012). Under alkaline conditions (high pH is induced in red mud leachate by dissolution of the NaOH present), V^{5+} is predicted to become readily solubilized in water as vanadate (Peacock and Sherman, 2004).

Vanadium is more abundant in red mud than other alkali-soluble trace metals (e.g. As), therefore its environmental mobility is enhanced relative to those of other carcinogenic heavy metals which are present in the material. Therefore, we conducted Trad-MN assays with sodium metavanadate solutions and compared the concentrations which cause positive results with those contained in the aqueous leachates. The extracts prepared from the upper layer of S3 contained 1.1 mg/L V (Table 2) and the results which were obtained with aqueous vanadate solutions (Figure 4) show, that levels $\geq$ 1.0 mg/L cause significant induction of the MN frequencies in Tradescantia indicating that vanadate accounts for the genotoxic effects which we detected in the leachate.

The solubility of vanadate in the environment is largely controlled by sorption reactions with mineral surfaces, with low solution concentrations expected at circumneutral pH (Peacock and Sherman, 2004). If the pH of red mud affected soils can be controlled to pH < 9, high aqueous V concentrations and their accompanying genotoxic effects may be avoided (Lehoux et al., 2013). In this context, it is notable that, several technologies have been

developed to recover vanadium from sludge (for review see Rajeev et al., 1999). As mentioned above, we found no impact of the pH on MN frequencies in experiments with waters to which NaOH was added in absence of metal ions. However, the situation may be different in soils which are contaminated with vanadium compounds.

4.3 Vanadate is an extremely potent mutagen in the Trad MN assay

As shown in Table 2A-C, a number of other genotoxic and carcinogenic heavy metals such as Ni, Cd, Cr and As were detected in the aqueous extracts. Results of earlier Trad-MN assays show that the concentrations of these metals, which are required to cause significant effects, are 10^2- to 10^3-fold higher as the levels which were detected in the leachates. The strongest activity was seen in these experiments with $Cr^{(VI)}O_3$ (129) followed by As_2O_3 (98.5), $Ni^{(II)}Cl_2$ (645), $CdCl_2$ (1006) and $Pb(NO_3)_2$ (1820); numbers in parenthesis indicate the LOELs in mg/L (Knasmueller et al., 2003; Knasmuller et al., 1998; Steinkellner et al., 1998). Furthermore, the results of the chemical analyses demonstrate that, these effects can be attributed to the presence of soluble vanadium. As mentioned above, we found in the present study significant induction of MN by vanadate in Tradescantia already at levels $\geq$ 1 mg/L. Comparisons with the effects seen with other metals indicate that vanadate is more potent in regard to induction of chromosomal damage. According to our knowledge, it has never been tested before in genotoxicity experiments with higher plants, but earlier findings with Allium roots showed that 25 mg/L cause stickiness of the chromosomes and a decrease of the MI (Marcano et al., 2006).

Results which were obtained in MN assays and other genotoxicity tests with mammalian cells and rodents with vanadium compounds are summarized in an IARC monograph (IARC, 2006) and in a review by Assem and Levy (2009) and Taylor et al.

(2012). The available data show that positive results were obtained in most in vitro experiments with mammalian cells and also in a number of in vivo studies with rodents.

4.4 Possible consequences of the release of DNA damaging toxins in red mud to the environment

It has been postulated that genotoxins may have an impact on the stability of ecosystems. In this context it is notable, that we found in an earlier study concerning urban air pollution, that the induction of MN in Tradescantia is paralleled by a decrease of the fertility of wildlife plants (Misik et al., 2007). It was also shown by other groups that DNA damaging toxins in the environment cause mutations which lead to transgenerational genomic instability in the offspring (Glen and Dubrova, 2012). The fact that we detected pronounced induction with leachates of red mud indicates that the compounds which induce genetic damage (presumably V^{5+}) contaminate surface and/or ground waters. Therefore, it is likely that they cause environmental damage.

In regard to potential effects in humans it is known that DNA damage is causally related to diseases such as cancer, infertility and heritable diseases (Shaugnessy and DeMarini, 2009) and leads to accelerated ageing. We found in an earlier occupational study that exposure of workers to V^{5+} causes induction of MN in peripheral lymphocytes (Ehrlich et al., 2008) which is a reliable biomarker for increased cancer risks (Bonassi et al., 2011). Furthermore, it was shown in a long term carcinogenicity study that inhalative exposure to this compound causes an increase of the tumor rates in the lungs of rats (Assem and Levy, 2009), as a consequence of those results V_2O_5 was classified as a possible (group 2B) human carcinogen by the IARC in 2006 (IARC, 2006).

It can be not excluded that humans living in the area where the accident happened are exposed to the genotoxic components which are contained in red mud. Gundy et al. (2013) analyzed chromosomal aberrations (CA) in lymphocytes of individuals who were either injured by the accident or exposed during cleanup activities. The authors conclude that the exposure does not pose an immediate short term genotoxic hazard but recommend further studies. In fact, no firm conclusions can be drawn from their investigation. The CA frequencies of the exposed group were almost 30% higher as those in an urban control group while no differences were seen with controls from a rural village. Furthermore, it is notable that the exposure periods of the individuals in the study group were quite heterogeneous (i.e. between 9 and 336 hrs) which enhardens the detection of effects.

Taken together, the results of the present investigation show for the first time that DNA damaging toxins were released in the course of the Kolontar disaster and that red mud contains vanadate which causes damage of the genetic material at low concentrations. As mentioned above, the metal was detected also in samples from other production sites and environmental pollution has been reported from other countries, for example from Brazil and India (2011; Gawu et al., 2012; Lima et al., 2009). The release of genotoxins into the environment may affect the stability of ecosystems and have a negative impact on human and environmental health; due to the worldwide production of the residue, the contamination of the material with DNA reactive and potentially carcinogenic vanadium compounds is a global problem which should be studied in more detail in the future.

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Red mud a byproduct of Aluminum production contains soluble vanadium that causes genotoxic and cytotoxic effects in higher plants

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ABSTRACT

Red mud (RM) is a byproduct of aluminum production, worldwide between 70 and 120 million tons are produced annually. We analyzed RM which was released in the course of the Kolonatar disaster in Hungary into the environment in acute and genotoxicity experiments with plants which are widely used for environmental monitoring. We detected induction of micronuclei which reflect chromosomal damage in tetrads of Tradescantia and in root cells of Allium as well as retardation of root growth with contaminated soils and leachates. Chemical analyses showed that RM contains metals, in particular high concentrations of vandadium. Follow up experiments indicated that vanadate causes the effects in the plants. This compound causes also in humans DNA damage and positive results were obtained in carcinogenicity studies. Since it was found also in RM from other production sites our findings indicate that its release in the environment is a global problem which should be studied in more detail.

Capsule abstract: Our findings indicate that the red mud causes genotoxic effect in plants probably due to the presence of vanadate which is contained at high concentrations in the residue.

Key words: red mud, Tradescantia, micronuclei, Allium, vanadium

1. Introduction

Red mud is a by-product of aluminum production with the Bayer process. Its global production is in the range between of 70 to 120 million tons per year (Mayes et al., 2011b). The material consists mainly of iron-, aluminum- and titanium- oxides and hydroxides (Burke et al., 2012; Mayes et al., 2011b). Chemical analyses showed that its contains also radionuclides (e.g. ^{226}RA, ^{230}Th and ^{40}K), as well as heavy metals including As, Cr, Co, Cd, Ni and V (Mayes et al., 2011a; Ruyters et al., 2011).

On Oct 4th 2010, approximately one million m^3 of the residue was released into the environment from the aluminum plant Ajkai Timfoldgyar Zrt in Western Hungary. According to the Hungarian Ministry of Interior the "Kolontar disaster" is the biggest environmental catastrophe which ever happened in this country (Ádám et al., 2011). Hundreds of houses were destroyed, 265 individuals were injured and ten died (Gundy et al., 2013).

After the accident, attempts were made to investigate the impact of the release of the material into the environment and to assess the health consequences in humans. Ecotoxicological studies were conducted with different plant species and bacteria concerning acute toxic effects (Klebercz et al., 2012; Ruyters et al., 2011); the motility and the concentrations of toxic trace elements were studied in physico-chemical measurements (Burke et al., 2012). Furthermore, studies were conducted to assess the consequences of inhalation of dust particles in humans and rodents (Czovek et al., 2012; Gelencser et al., 2011).

Radionuclides as well as certain heavy metals (found in red mud) cause damage of the genetic material (Knasmuller et al., 1998; Minouflet et al., 2005) which may lead to destabilization of ecosystems (Sarkar et al., 2006; Zvereva et al., 2008) and also causes adverse effects in humans such as cancer, ageing, infertility and birth defects in the offspring (Aitken and De Iuliis, 2007; Assem and Levy, 2009). Therefore it is of particular interest if

the release of this material into the environment induces chromosomal damage. This question has been addressed in a human study (Gundy et al., 2013) and in bacterial tests (Gelencser et al., 2011) but no firm conclusions can be drawn of these studies (for details see discussion).

The primary aim of the present study was the investigation of the genotoxic properties of red mud in two plant bioassays, namely in the micronucleus (MN) test with tetrads of Tradescantia (Trad-MN assay) and with root tip cells of Allium cepa (A-MN assay). The experiments were conducted with tetrads as they reflect damage in meiotic germ cells. Root tip cells were used as they enable the detection of effects in mitotic cells. It was postulated that differences exist in regard to sensitivity of these cell types towards DNA reactive compounds (Rodrigues et al., 1997). MN are formed as a consequence of chromosomal breakage or aneuploidy and can be monitored in a variety of organisms (Heddle et al., 2011). In addition acute toxic effects were studied in root cells of A. cepa by measuring the impact of the material on the root growth and by calculating the division rates of the cells. These bioassays are at present the most widely used genotoxicity tests with higher plants and have been employed in more ~~then~~than 300 investigations for the detection of DNA damaging properties of chemicals and complex mixtures (for reviews see Leme and Marin-Morales, 2009; Misik et al., 2011). We used these test systems since they provide information on environmental effects and it is known that they are, in contrast to other genotoxicity assays with bacterial indicators and mammalian cells (which are also used for environmental monitoring), highly sensitive towards radiation (Ma and Davies, 2009; Misik et al., 2011) and heavy metals (Knasmuller et al., 1998; Majer et al., 2002; Steinkellner et al., 1998).

We investigated the acute cytotoxic and genotoxic activities of red mud and of soils which were contaminated with the material collected from fields and gardens which were used for cultivation of crops and vegetables. Furthermore, we studied also the effects of waters leached from red mud and affected soils. In additional experiments, attempts were

made to identify the compound(s) which cause genotoxic effects. We determined the concentrations of trace elements in solid samples and in leachates and conducted additional Trad-MN assays with sodium metavanadate ($NaVO_3$) since vanadium was found to be the most abundant heavy metal in the samples.

2. Materials and methods

2.1 Sampling

Soil samples were collected from the Torna catchment on December 15th 2010. Table 1 contains a description of the sampling sites and specifications of their GPS locations. From each site, samples were collected from the 0-5; 5-15 and 15-25 cm horizons to assess if potentially toxic constituents in red mud contaminated the soils.

Total organic carbon (TOC) was determined in soil samples using a LECO SC-144DR elemental analyzer after removal of the inorganic carbon fraction using 20% HCl.

Table 1

Figure 1

2.2 Preparation of the leachates.

Soils were used as sampled (field moist). 50 g of each sample were suspended in 100 mL of deionised water in a 250 mL glass beaker for 2 hours using a magnetic stirrer. The extracts were filtered with filter paper before they were tested in stem absorption experiments. The pH values of the leachates were measured with a pH Meter 526 (WTW, Weilheim, Germany) and are listed in Table 1.

2.3 Measurements of the trace elements in the soil samples and leachates.

The detection of the trace elements is described in detail in a recent paper of Renforth et al. (2012). Prior to analysis by XRF the samples was prepared as follows. For major element analysis, samples were prepared as fused beads (after loss on ignition at 1050 °C) with lithium metaborate/tetraborate flux (Johnson - Matthey Spectroflux JM100B) (0.6g sample; 3 g Flux). For minor / trace element analysis approximately 10 g dried sample was prepared as a pressed pellet using ~10-20 drops of 6.6%w/v polyvinyl alcohol in a 1:6 mix of methanol and distilled deionised water as a binder (Moviol 88 solution). Elemental analysis of the soil composition was achieved using a PANalytical Axios Advanced X-ray Fluorescence (XRF) spectrometer (data corrected for loss on ignition; % weight loss after furnace treatment at 1050°C). The aqueous phase produced during water extractions was separated from solids by centrifugation (10 min; 2000 g) followed by membrane filtration (0.45 μm); the filtered samples were then acidified by addition of 2% v/v ~~c~~HNO_3. Aqueous elemental concentrations were then determined using a Perkin Elmer Optima 5300 DV ion-coupled plasma, optical emission spectrometer (ICP-OES).

2.4 Tradescantia micronucleus assays

Tradescantia clone #4430 was cultivated according to the protocol of Ma et al. (1994) For stem absorption experiments, 15 young inflorescences were treated in each group. The stems were cut to a length of 10 - 15 cm, transferred to plastic breakers (250 mL) and exposed to aqueous leachates (soil:water – 1:2) of the contaminated soils for 24 h. Subsequently, they were transferred to water for a 24 h recovery period. The flower buds were then collected and

fixed in acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol. Maleic hydrazide (20 mg L^{-1}; MH, Sigma-Aldrich, SL, US) was used as a positive control.

The protocol for root absorption assays is described in an article of Steinkellner et al. (1998). The soil which was used in the control cultures was biological, pesticide free (from Composana, Wien, Austria). Intact plants were removed from hydroponic culture, subsequently the roots were rinsed and individual plants with at least 15 inflorescences placed in to plastic pots (250 ml, diameter 12 cm) which were filled with the different soil samples. The plants were exposed under standard conditions for 48 h (Ma et al., 1994). Subsequently, 15 inflorescences were collected from each soil sample in all experiments and fixed in a solution of acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol.

To find out if vanadium accounts for the effects which were found in the soils and in the leachetes, additional experiments were carried out with sodium metavanadate. The salt was dissolved in 0.1M NaOH (pH 13) at 1000 ppm (colorless solution) since earlier findings indicate that vanadium is present as V^{5+} in the soil (Burke et al., 2012). The stock solution was diluted with tap water and different concentrations (0.5-10.0 ppm) were used in experiments with Tradescantia.

Slides were prepared and evaluated as described in the protocol of Ma et al. (1994) The tetrads were stained with a 2% aceto-carmine solution. Early tetrads were analyzed under 400-fold magnification. For each experimental point a minimum of five buds with early tetrads was evaluated (300 tetrads were evaluated per slide, 1500 per sample).

2.5 Micronucleus assay with Allium cepa

The experiments were carried out according to the standard protocol published by Ma et al. (1995) with slight modifications. Young onion bulbs (diameter 12-21 mm, Schneeball

weis, Austrosaat, Wien, Austria) were placed in 13 ml glass tubes filled with tap water for 24 h in the dark. Subsequently, the roots (lengths ca. 0.5-1 cm) were exposed directly in the soils in the dark for 24 h and then transferred to fresh tap water for another 24 h. At the end of the recovery phase, the maximal root length of each onion was measured and the material was fixed in a mix of acetic acid and ethanol (1/3, v/v) for 24 h and stored in 70% ethanol. MH (10 mg L^{-1}; Sigma-Aldrich, SL, US) was used in all experiments as a positive control.

The root tips were hydrolyzed in a 1:1 mix of HCl (5.0 N) and ethanol (99%) for 3 min and washed in tap water before they were stained with 2% acetocarmine. MN were scored according to the criteria described by Ma et al. (1995). For each experimental point, the MN frequencies were determined in five plants. From each bulb, two slides were made and 500 cells were evaluated per slide (5000 cells per dose). Furthermore, also the mitotic indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The microscopic evaluation was carried out under a light microscope (Nikon YS200, Japan) with 400-fold magnification.

2.6 Statistical evaluation

The results of the MN experiments were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. The results of the experiments concerning the mitotic indices (MI) were analyzed with the Kruskal-Wallis test followed by Dunn´s comparison test. P-values ≤ 0.05 were considered as significant.

3. Results

3.1 TOC and pH values of the samples.

It can be seen in Table 1 that values between 8.0 and 9.3 were found in the soil leachates which were contaminated with red mud. Most of the samples had a total organic carbon content between 1.0-1.5%, which is typical for agricultural soils in the Ajka area (Lehoux et al., 2013). The sample from the upper horizon of the site 3 had a substantially lower TOC content (0.22%) and a higher pH (9.33) which is typical for red mud (Lehoux et al., 2013).

3.2 Micronucleus assays with Tradescantia (experiments with soils and leachates)

The results which were obtained in Trad-MN assays with red mud contaminated soil and with aqueous leachates from these samples are summarized in Fig. 2A and 2B. It can be seen that direct exposure of intact plants in material from the contaminated sites 2 (S2) and 3 (S3) caused a significant increase of the MN frequencies. In the case of S3, the strongest effect was seen with material from the upper layer, samples from deeper horizons caused only a statistically not significant increase. With the two samples from S4, clear cut negative results were obtained. As described above (Table 1), this material was collected after remediation (i.e. after removal of the upper layer).

Also with some leachates, positive results were obtained (Fig. 2B). Again the strongest effects were seen with extracts prepared with material from the upper layers (S2 and S3); soils from deeper horizons did not cause significant effects, also the sample which was collected from the cleanup site was devoid of activity.

Figures 2A and B

3.3 Micronucleus experiments with mitotic root tip cells of Allium cepa

The results of assays with A. cepa are shown in Figures 3A – C. The roots were exposed in these experiments directly in the samples. Since division delays may decrease the formation of MN (Fenech and Morley, 1985), we determined in all experiments also the mitotic indices (MI).

It can be seen in Figure 3A that a significant increase of the MN rates was observed with some samples. The most pronounced effect was detected with material collected from the middle layer of S2 while red mud enriched sample from surface of the same location did not cause a significant increase of the MN rates. This finding can be explained by inhibition of cell division, which was most pronounced with material collected from the upper horizon of this site (Figure 3B). As in the Tradescantia experiments, no increase was seen with samples from the remediation site (S4).

The impact of the material on root growth is shown in Figure 3C. It can be seen, that clear effects were obtained with samples from the surface of sites S2 and S3.

Figures 3A – C

3.4 Chemical analyses of the soil samples and of the leachates

The chemical composition of samples which were collected from S3 and S1 (Table 2A-B) and of the corresponding leachates (Table 2C) are shown in Table 2.

Table 2

The chemical composition of the topmost soil sample (S3 0-5 cm) is similar to that of other samples of red mud which were collected after the Ajka spill (Burke et al., 2012; Ruyters et al., 2011). Several potentially toxic metals including As, Cr, Ni, Pb and V were present at elevated concentrations.

The chemical composition of the control sample (S1) is consistent with unaffected soils found in the region (see Table 2c) (Mayes et al., 2011b).

The corresponding leachates from S3, contained elevated concentrations of potentially toxic elements, such as V, As, Cu, Al and Cr, notably, V concentrations were an order of magnitude higher than the levels of the other metals (except Al). Also chromium levels were relatively high.

3.5 Tradescantia micronucleus assays with vanadate

We found in earlier experiments with Tradescantia that the concentrations of Ni, Cr, Cd, Pb and As, which cause MN induction in tetrads are in the range of hundred to thousand ppm (Knasmuller et al., 1998; Steinkellner et al., 1998). We hypothesized that V, which was detected in higher levels than other metals, may account for the induction of MN, which was seen with the soils and the leachates. Therefore, we tested different concentrations of an aqueous vanadate solution in subsequent Trad MN assays. The results are summarized in Figure 4. It can be seen that exposure of the cuttings to concentrations ≥1.0 ~~ppm~~ mg/L caused significant induction of the MN frequencies i.e. an approximately 6-fold increase over the background.

Figure 4

3.6 Impact of pH on MN formation in Tradescantia

Since the pH values of the soil extracts and of the vanadate solutions were quite high, i.e. in the range between 8.2 and 9.3 (for details see Table 1) we conducted an additional experiment in which plant cuttings were exposed in aqueous solutions (without metals) to different pH values. No indication of pH depended induction of MN by the pH itself was found in this experiment. The numbers of MN per 100 tetrads were 1.7±0.6; 2.1±0.6 and 1.6±0.6 for pH 8, 8.5 and 9.3 respectively (numbers are means ± SD of results obtained from 1500 tetrads).

4. Discussion

The results of this study show that red mud, which was released in large amounts into the environment, causes damage of the genetic material and also acute toxic effects in higher plants.

4.1 Acute toxic and genotoxic effects of red mud

Several ecotoxicological studies have been conducted with red mud from Ajka (Klebercz et al., 2012; Ruyters et al., 2011) and adverse effects were seen in experiments with higher plants; i.e. the material was found to inhibit the root of Sinapis alba and affect the shoot yield in barley (Klebercz et al., 2012; Ruyters et al., 2011). In the latter study, the authors monitored also the levels of trace elements which are contained in red mud such as Cu, Cr, Fe and Ni in exposed plants. They detected these metals in the shoots but stress that their levels did not exceed the toxic limits and hypothesize that Na is the prime cause which affected the growth of the indicator plants. Another factor which was made responsible for the toxic effects in Sinapis and in experiments with the Ostracod Heterocypris incongruens is

gypsum which may limit the nutrient supply and increase the availability of contaminants (Klebercz et al., 2012). The genotoxic effect of red mud has not been investigated in the invertebrates so far according to our knowledge. However, it is possible that several species may be affected due to the high metal concentration of the material. For example, it is know that earthworms are highly sensitive towards specific contaminants and specific metals. Genotoxic effects were detected with chromium, nickel and cadmium (Bigorgne et al., 2010; Bonnard et al., 2010; Manerikar et al., 2008).

The results of the present study show clearly that red mud inhibits the division and growth of root tip cells in A. cepa (Figures 3B and 3C) and causes induction of MN in this species. Furthermore, we showed that also aqueous leachates of red mud contaminated soils cause induction of MN in both plant bioassays. As shown in Figure 3 a stronger effect was obtained with sample S2 (5-15 cm) as with the sample from upper layer from the same site. This effect can be explained by reduction of MN formation due to inhibition of cell division. Furthermore, it is notable that the effects which were detected with soil exposure are more pronounced as those which were found with the leachetes. This observation is in agreement with the results obtained with heavy metals contaminated soils and can be explained by more efficient uptake via the roots by active transport mechanism (for detail see Steinkellner et al., 1998).

The only genotoxicity assay conducted so far with red mud is the SOS chromotest which is based on the detection of SOS responses in bacterial indicator cells (Gelencser et al., 2011). Consistently negative results were obtained with samples, which were prepared from fugitive dust from the area where the accident had happened. However, it is notable that it is known from earlier investigations that bacterial assays are insensitive towards heavy metals (Majer et al., 2002).

4.2 Identification of vanadate as the active principle of red mud

The results of the chemical analyses show that red mud contaminated soils and their aqueous leachates contain elevated concentrations of V (Table 2C). This observation is in agreement with the results of earlier investigations with material from Ajka (Mayes et al., 2011a; Mayes et al., 2011b; Renforth et al., 2012). In this context, it is notable that vanadium was also detected in red mud from several other aluminium production sites (Fontanier et al., 2012; Gawu et al., 2012; Rajeev et al., 1999; Samal et al., 2012).

Spectroscopic evidence showed that vanadium is present primarily as V^{5+} phases (Burke et al., 2012). Under alkaline conditions (high pH is induced in red mud leachate by dissolution of the NaOH present), V^{5+} is predicted to become readily solubilized in water as vanadate (Peacock and Sherman, 2004).

Vanadium is more abundant in red mud than other alkali-soluble trace metals (e.g. As), therefore its environmental mobility is enhanced relative to those of other carcinogenic heavy metals which are present in the material. Therefore, we conducted Trad-MN assays with sodium metavanadate solutions and compared the concentrations which cause positive results with those contained in the aqueous leachates. The extracts prepared from the upper layer of S3 contained 1.1 mg/L V (Table 2) and the results which were obtained with aqueous vanadate solutions (Figure 4) show, that levels ≥ 1.0 mg/L cause significant induction of the MN frequencies in Tradescantia indicating that vanadate accounts for the genotoxic effects which we detected in the leachate.

The solubility of vanadate in the environment is largely controlled by sorption reactions with mineral surfaces, with low solution concentrations expected at circumneutral pH (Peacock and Sherman, 2004). ~~Therefore, i~~If the pH of red mud affected soils can be controlled to pH < 9, high aqueous V concentrations and their accompanying genotoxic effects may be avoided (Lehoux et al., 2013). In this context, it is notable that, several

technologies have been developed to recover vanadium from sludge (for review see Rajeev et al., 1999). As mentioned above, we found no impact of the pH on MN frequencies in experiments with waters to which NaOH was added in absence of metal ions. However, the situation may be different in soils which are contaminated with vanadium compounds. ~~In this context, it is notable that, several technologies have been developed to recover vanadium from sludge (for review see Rajeev et al., 1999).~~

4.3 Vanadate is an extremely potent mutagen in the Trad MN assay

As shown in Table 2A-C, a number of other genotoxic and carcinogenic heavy metals such as Ni, Cd, Cr and As were detected in the aqueous extracts. Results of earlier Trad-MN assays show that the concentrations of these metals, which are required to cause significant effects, are 10^2- to 10^3-fold higher as the levels which were detected in the leachates. The strongest activity was seen in these experiments with $Cr^{(VI)}O_3$ (129) followed by As_2O_3 (98.5), $Ni^{(II)}Cl_2$ (645), $CdCl_2$ (1006) and $Pb(NO_3)_2$ (1820); numbers in parenthesis indicate the LOELs in mg/L (Knasmueller et al., 2003; Knasmuller et al., 1998; Steinkellner et al., 1998). Furthermore, the results of the chemical analyses demonstrate that, these effects can be attributed to the presence of soluble vanadium. As mentioned above, we found in the present study significant induction of MN by vanadate in Tradescantia already at levels $\geq$ 1 mg/L. Comparisons with the effects seen with other metals indicate that vanadate is more potent in regard to induction of chromosomal damage. According to our knowledge, it has never been tested before in genotoxicity experiments with higher plants, but earlier findings with Allium roots showed that 25 mg/L cause stickiness of the chromosomes and a decrease of the MI (Marcano et al., 2006).

Results which were obtained in MN assays and other genotoxicity tests with mammalian cells and rodents with vanadium compounds are summarized in an IARC monograph (IARC, 2006) and in a review by Assem and Levy~~et al.~~ (2009) and Taylor et al. (2012). The available data show that positive results were obtained in most in vitro experiments with mammalian cells and also in a number of in vivo studies with rodents.

4.4 Possible consequences of the release of DNA damaging toxins in red mud to the environment

It has been postulated that genotoxins may have an impact on the stability of ecosystems. In this context it is notable, that we found in an earlier study concerning urban air pollution, that the induction of MN in Tradescantia is paralleled by a decrease of the fertility of wildlife plants (Misik et al., 2007). It was also shown by other groups that DNA damaging toxins in the environment cause mutations which lead to transgenerational genomic instability in the offspring (Glen and Dubrova, 2012). The fact that we detected pronounced induction with leachates of red mud indicates that the compounds which induce genetic damage (presumably V^{5+}) contaminate surface and/or ground waters. Therefore, it is likely that they cause environmental damage.

In regard to potential effects in humans it is known that DNA damage is causally related to diseases such as cancer, infertility and heritable diseases (Shaugnessy and DeMarini, 2009) and leads to accelerated ageing. We found in an earlier occupational study that exposure of workers to V^{5+} causes induction of MN in peripheral lymphocytes (Ehrlich et al., 2008) which is a reliable biomarker for increased cancer risks (Bonassi et al., 2011). Furthermore, it was shown in a long term carcinogenicity study that inhalative exposure to this compound causes an increase of the tumor rates in the lungs of rats (Assem and Levy,

2009), as a consequence of those results V_2O_5 was classified as a possible (group 2B) human carcinogen by the IARC in 2006 (IARC, 2006).

It can be not excluded that humans living in the area where the accident happened are exposed to the genotoxic components which are contained in red mud. Gundy et al. (2013) analyzed chromosomal aberrations (CA) in lymphocytes of individuals who were either injured by the accident or exposed during cleanup activities. The authors conclude that the exposure does not pose an immediate short term genotoxic hazard but recommend further studies. In fact, no firm conclusions can be drawn from their investigation. The CA frequencies of the exposed group were almost 30% higher as those in an urban control group while no differences were seen with controls from a rural village. Furthermore, it is notable that the exposure periods of the individuals in the study group were quite heterogeneous (i.e. between 9 and 336 hrs) which enhardens the detection of effects.

Taken together, the results of the present investigation show for the first time that DNA damaging toxins were released in the course of the Kolontar disaster and that red mud contains vanadate which causes damage of the genetic material at low concentrations. As mentioned above, the metal was detected also in samples from other production sites and environmental pollution has been reported from other countries, for example from Brazil and India (2011; Gawu et al., 2012; Lima et al., 2009). The release of genotoxins into the environment may affect the stability of ecosystems and have a negative impact on human and environmental health; due to the worldwide production of the residue, the contamination of the material with DNA reactive and potentially carcinogenic vanadium compounds is a global problem which should be studied in more detail in the future.

Acknowledgement

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Table
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Table 1 Descriptions of the monitored sites (Kolontar – Devetsen, Hungary) with GPS position for more details see Fig 1.

Sampling site	Description	GPS location	pH of water extract	TOC (%)
Site 1 (S1)	Control site, agricultural area with soil of similar composition and structure as S2 and S3 but located on a hill (20 m above the site of red mud spill)	N47°05.400' EO17°28.159'	7.18 (0-5cm) 7.18 (5-15 cm)	1.36 (0-5cm) 1.23 (5-15 cm)
Site 2 (S2)	Agricultural field which was contaminated by red mud, 2.7 km northeast from the reservoir	N47°05.885' EO 17°27.947'	9.21 (0-5 cm) 9.19 (5-15 cm) 8.26 (15-25 cm)	0.99 (0-5 cm) 1.07 (5-15 cm) 1.52 (15-25 cm)
Site 3 (S3)	Agricultural field which was strongly polluted, 200 m from S1 and 4.8 km northeast from the red mud reservoir	N47°05.905' EO 17° 28.067'	9.33 (0-5 cm) 8.32 (5-15 cm) 8.24 (15-25 cm)	0.22 (0-5 cm) 1.05 (5-15 cm) 1.14 (15-25 cm)
Site 4 (S4)	Garden area located at a distance of 4.7 km from the reservoir which was flooded, red mud was removed from the surface by cleanup activities.	N47°05.410' EO17°28.253'	8.28 (0-5 cm) 8.9 (5-15cm)	

Table 2 (A – C). Major elements (2A) and trace elements in soils (2B) and in aqueous leachates (2C) prepared from these samples.[a]

Table 2A – Major elements in soil samples collected at sites 1 and 3

Site	**SiO_2**	**TiO_2**	**Al_2O_3**	**Fe_2O_3**	**MnO**	**MgO**	**CaO**	**Na_2O**	**K_2O**	**P_2O_5**	**SO_3**	**LOI**	**Total**
S1 (0-5 cm)	82.82	0.58	6.53	2.79	0.089	0.57	0.77	0.67	1.13	0.13	0.01	4.32	100.39
S3 (0-5 cm)	21.76	3.63	14.45	29.81	0.409	1.15	9.90	4.88	0.42	0.23	0.54	11.28	98.46
S3 (5-15 cm)	59.92	0.76	10.82	4.39	0.499	2.33	6.64	1.23	1.83	0.23	0.12	11.29	100.06
S3 (15-25 cm)	60.22	0.76	10.65	4.29	0.494	2.34	6.67	1.21	1.80	0.22	0.11	11.07	99.83

[a]all values are indicated as w/w%

Table 2B – Trace elements in soil samples collected at sites 1 and 3.[a]

Site	**As**	**Ba**	**Ce**	**Co**	**Cr**	**Cs**	**Cu**	**Ga**	**La**	**Mo**	**Nb**	**Nd**	**Ni**	**Pb**
S1 (0-5 cm)	17.8	266.0	44.4	7.3	40.0	1.5	54.1	7.1	24.7	0.9	9.0	21.5	12.5	22.2
S3 (0-5 cm)	201.1	120.4	437.8	54.6	703.3	12.3	112.1	27.0	192.6	9.3	76.9	153.2	283.1	143.0
S3 (5-15 cm)	22.3	414.0	69.7	17.8	76.5	1.7	36.8	12.1	32.3	3.5	13.5	31.3	31.7	23.8
S3 (15-25 cm)	21.7	417.5	70.8	18.5	125.1	3.6	26.0	12.3	34.2	2.4	13.1	31.2	27.7	22.3

Site	**Rb**	**Sb**	**Sc**	**Se**	**Sn**	**Sr**	**Th**	**U**	**V**	**W**	**Y**	**Zn**	**Zr**
S1 (0-5 cm)	56.4	0.9	7.7	0.5	30.1	232.3	4.8	1.4	57.9	0.8	18	42	180.2
S3 (0-5 cm)	26.8	15.9	94.4	1.1	20.1	357.6	64.6	16.0	898.8	9.1	125.2	123.4	920.5
S3 (5-15 cm)	80.1	1.0	14.4	0.5	12.9	166.1	9.2	3.6	97.4	1.6	32.2	57.8	271.1
S3 (15-25 cm)	76.9	1.0	15.5	1.0	6.9	161.2	8.9	3.3	100.0	1.7	30.5	56.8	273.7

[a]all values are indicated as ~~ppm~~mg per kg of dry weight

Table 2C – Trace elements in aqueous leachates prepared from soil samples collected at sites 1 and 3.[a]

Site	**Al**	**As**	**Ba**	**Ca**	**Ce**	**Co**	**Cr**	**Cu**	**Fe**	**Ga**	**K**	**Li**	**Mg**	**Mn**	**Mo**
S1 (0-5 cm)	0.010	0.001	0.104	100.178	0.038	0.002	0.002	0.005	0.008	0.013	24.601	0.003	9.574	0.008	0.011
S3 (0-5 cm)	0.951	0.123	0.005	5.253	0.019	0.002	0.068	0.041	0.123	0.02	45.979	0.01	1.703	0.005	0.364
S3 (5-15 cm)	0.000	0.002	0.061	127.421	0.001	0.002	0.0002	0.002	0.517	0.002	8.402	0.003	11.558	11.697	0.052
S3 (15-25 cm)	0.001	0.001	0.044	135.985	0.023	0.002	0.003	0.003	0.073	0.002	26.412	0.005	6.767	0.002	0.004

Site	**Na**	**Ni**	**P**	**Pb**	**S**	**Se**	**Si**	**Sr**	**Ti**	**V**	**Zn**	**Zr**	**Hg**
S1 (0-5 cm)	2.25	0.002	0.018	0.005	2.681	0.39	3.491	0.278	0.001	0.004	0.017	0.001	0.001
S3 (0-5 cm)	701.321	0.002	0.175	0.005	122.704	0.453	1.862	0.028	0.017	1.169	0.019	0.005	0.001
S3 (5-15 cm)	109.365	0.002	0.101	0.005	14.557	0.05	4.718	0.929	0.000	0.002	0.021	0.001	0.001
S3 (15-25 cm)	19.545	0.002	0.108	0.005	30.032	0.316	2.31	0.478	0.000	0.001	0.001	0.001	0.001

[a]all values are indicated as mg/L

~~ppm~~

Figure
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Figure 1

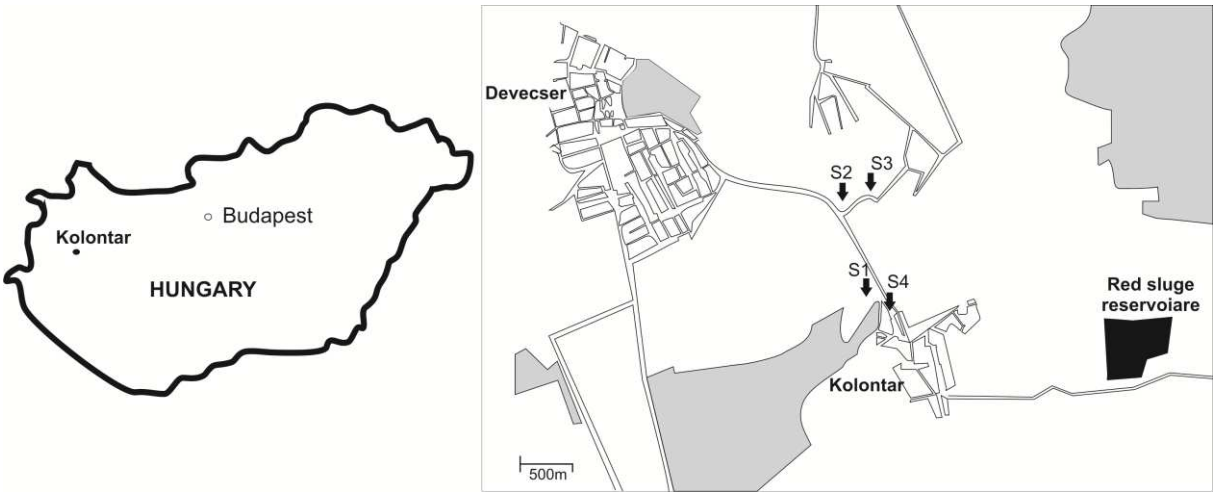


Figure 2A and B

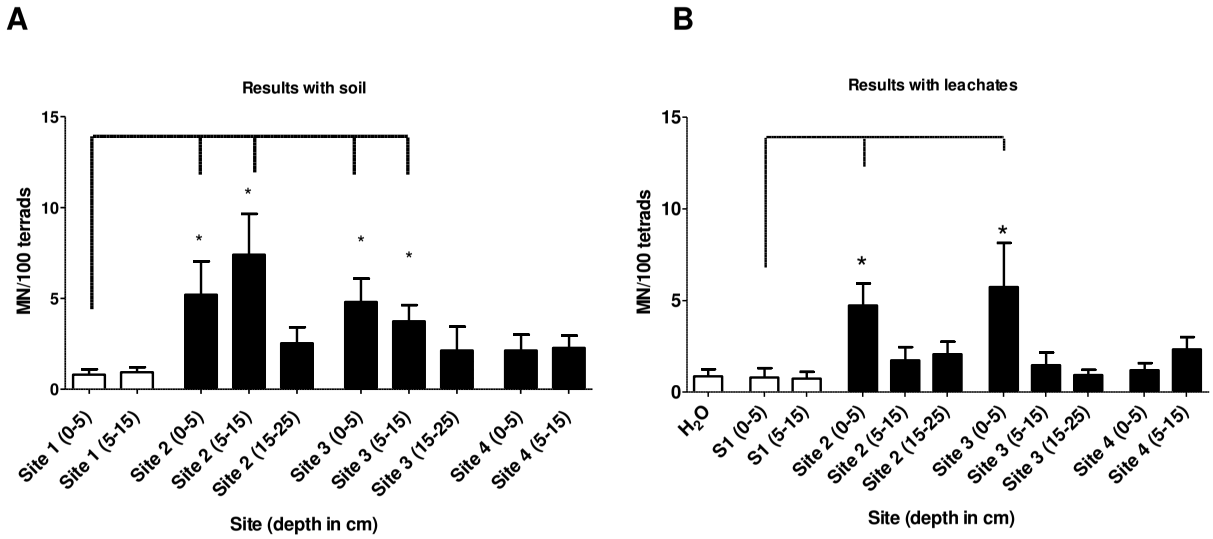
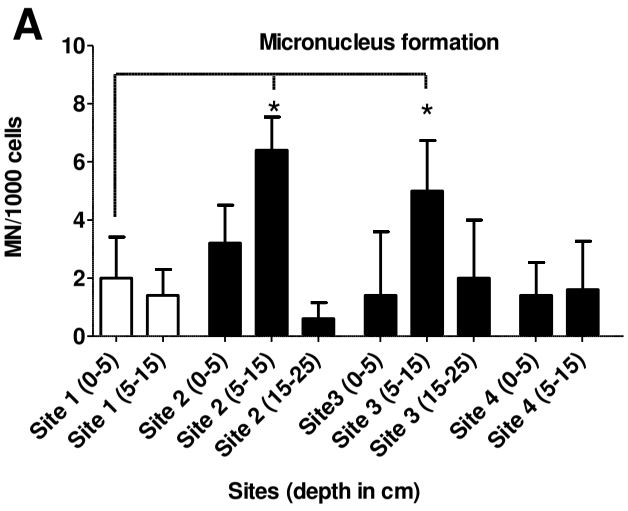


Figure 3A –C


A
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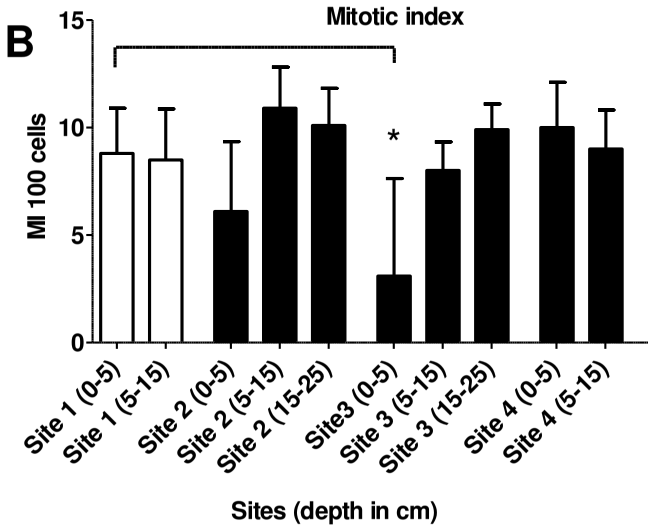
Site 1 (0-5)
Site 1 (5-15)
Site 2 (0-5)
Site 2 (5-15)
Site 2 (15-25)
Site3 (0-5)
Site 3 (5-15)
Site 3 (15-25)
Site 4 (0-5)
Site 4 (5-15)
Sites (depth in cm)


B
Mitotic index
MI 100 cells
15
10
5
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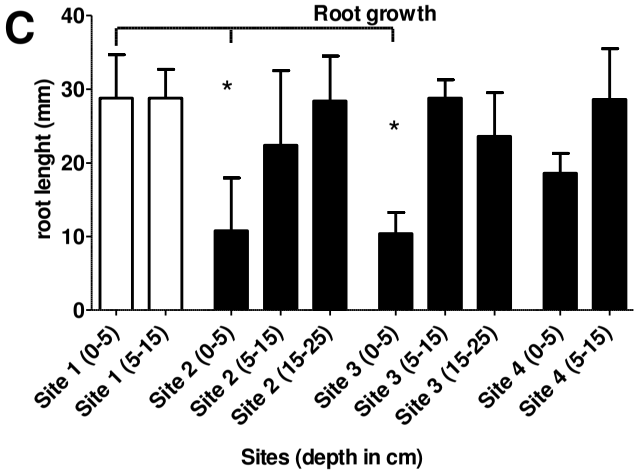
*
Site 1 (0-5)
Site 1 (5-15)
Site 2 (0-5)
Site 2 (5-15)
Site 2 (15-25)
Site3 (0-5)
Site 3 (5-15)
Site 3 (15-25)
Site 4 (0-5)
Site 4 (5-15)
Sites (depth in cm)


C
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root lenght (mm)
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0
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*
Site 1 (0-5)
Site 1 (5-15)
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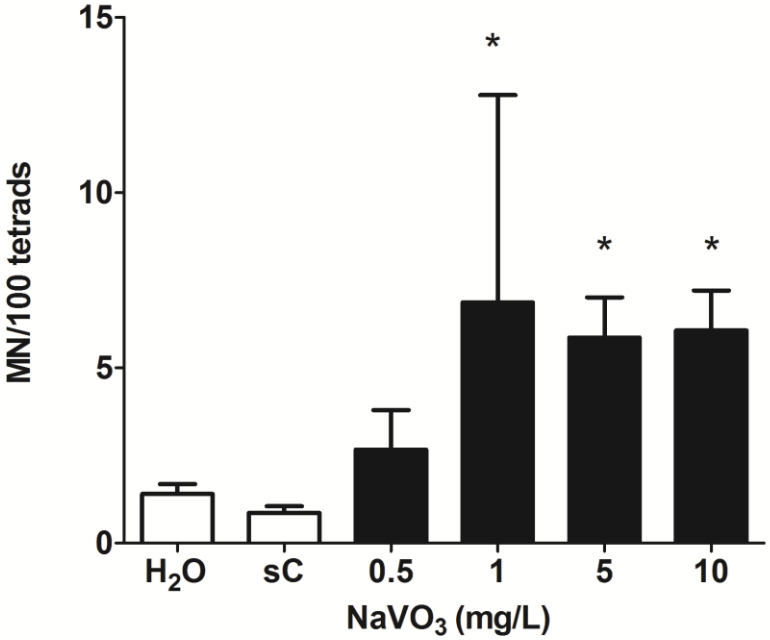
Site 2 (5-15)
Site 2 (15-25)
Site 3 (0-5)
Site 3 (5-15)
Site 3 (15-25)
Site 4 (0-5)
Site 4 (5-15)
Sites (depth in cm)

Figure 4



*Conflict of interest

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Dear Editor,

On behalf of all authors I declare that there is no conflict of interest and no financial support was received to carry out the study which is describe in the article.

Best personal greetings

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